Regulation of Immunity to Experimental *Trypanosoma congolense* Infection: Roles of Regulatory T Cells, Natural Killer Cells, and Myeloid-Derived Suppressor Cells

By

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ABSTRACT

African trypanosomiasis (also known as sleeping sickness) is a fatal disease that affects human and livestock. The disease puts the lives of millions of people at risk and poses massive agricultural and economic problems in the affected regions. There is currently no satisfactory treatment for the disease, and available treatments options are associated with severe side effects. Also, vaccination attempts have not been successful because of antigenic variation and profound immunosuppression observed in infected hosts. Therefore, continued research towards exploring various regulatory mechanisms that influence resistance and/or susceptibility to the disease could help identify novel targets for treatment and development of an effective vaccine against the disease. The overall focus of this thesis is to investigate the cellular mechanisms that regulate susceptibility and resistance to experimental *Trypanosoma congolense* infection. The first part shows the impact of regulatory T cells and how they enhance susceptibility to *T. congolense* following intradermal infection (which mimics the natural infection route). The second part reveals the requirement of Natural killer cells in maintaining optimal immunity during *T. congolense* infection, while the third part shows the function of myeloid-derived suppressor cells in the inhibition of $CD4^+$ T cell proliferation and function during *T. congolense* infection.

Repeated low dose intradermal infections predispose mice to enhanced susceptibility to an otherwise non-infectious dose challenge. I explored the mechanisms responsible for this low-dose-induced susceptibility to subsequent low dose challenge infection. I found that akin to intraperitoneal infection, low dose intradermal infection led to the production of IL-10, IL-6, IL-12, TNF-α, TGF-β and IFN-γ by spleen and draining lymph node cells.

Interestingly, despite the absence of parasitemia, low dose intradermal infection led to the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) in both the spleens and lymph nodes draining the infection site. Depletion of Tregs by anti-CD25 mAb treatment during primary or before challenge infection completely abolished low dose-induced enhanced susceptibility. In addition, Tregs depletion was associated with dramatic reduction in serum levels of TGF-β and IL-10.

NK cells are vital sources of IFN-γ and TNF-α; two key cytokines that are known to play important roles in the early control of African trypanosomiasis. I found that mice infected with *T. congolense* show increased levels of activated and functional NK cells in multiple tissue compartments. Depletion of NK cells with anti-NK1.1 monoclonal antibody led to increased parasitemia, which was accompanied by significant reduction in IFN-γ production by immune cells in the spleen and liver of infected mice, suggesting that NK cells may be contributing to optimal resistance following infection. Indeed, infected $NFIL3^{-/-}$ mice (which genetically lack NK cell development and function), on the normally resistant background were highly susceptible to *T. congolense* infection. These mice developed fulminating parasitemia and died significantly earlier (13 ± 1 days) than their wild-type (WT) control mice (106 ± 26 days). The enhanced susceptibility of $NFIL3^{-/-}$ mice to infection was accompanied by significantly impaired cytokine (IFN-γ and TNF-α) response by $CD3^+$ T cells in the spleens and liver. Adoptive transfer of WT NK cells into $NFIL3^{-/-}$ mice before infection rescued these mice from acute death in a perforin-dependent manner.

Given that immunosuppression is a hallmark of African trypanosomiasis, and MDSCs suppress T cell responses, I investigated the role of MDSCs in *T. congolense* infection. I

found increased numbers of MDSCs in the spleens and livers of infected mice, which correlated with increased parasitemia. Depletion of MDSCs during infection using anti-GR1 monoclonal antibody led to transient parasite control and prolonged survival of infected mice. This was associated with increased proliferation and IFN-γ production ability of $CD4^+$ T cells, suggesting that MDSCs could be suppressing $CD4^+$ T cells during *T. congolense* infection. Indeed, $CD4^+$ T cells from the spleens of anti-GR1-treated mice proliferated and produced more IFN-γ upon stimulation compared to those from untreated mice. Also, MDSCs from *T. congolense*-infected mice suppressed $CD4^+$ T cells in co-culture setting. This suppressive effect of MDSCs on $CD4^+$ T cells was abolished by the arginase-1 inhibitor, N(ω)-hydroxy-nor-l-arginine (nor-NOHA), indicating that MDSCs suppress $CD4^+$ T cell proliferation and function in an arginase-1-dependent manner.

Collectively, the results of my studies identified important roles played by Tregs, NK cells, and MDSCs during *T. congolense* infection. They revealed important mechanisms that regulate susceptibility and resistance to this disease, which could be novel therapeutic targets in African trypanosomiasis.

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DEDICATION

This thesis is dedicated to my late mother, Mrs. Veronica Onyilagha, for the unforgettable roles she played in providing me with a quality education.

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ABBREVIATIONS

µg (Microgram)

ANOVA (Analysis of Variance)

CD107a (Cluster of Differentiation one hundred and seven a)

CD11b (Cluster of Differentiation eleven b)

CD25 (Cluster of Differentiation twenty-five)

CD3 (Cluster of Differentiation three)

CD4 (Cluster of Differentiation four)

CFSE (Carboxyfluorescein Succinimidyl Ester)

CSF (Cerebrospinal Fluid)

DEAE (Diethylaminoethyl)

DMEM (Dulbecco's Modified Eagle's Medium)

DNA (Deoxyribonucleic Acid)

ELISA (Enzyme-Linked Immunosorbent Assay)

FACS (Fluorescence Activated Cell Sorting)

FBS (Fetal Bovine Serum)

Foxp3 (Forkhead box p3)

GPI (Glycosylphosphatidylinositol)

HBSS (Hanks Balanced Salt Solution)

HRP (Horseradish Peroxidase)

i.d. (Intradermal)

i.p. (Intraperitoneal)

i.v. (Intravenous)

IFN-γ (Interferon gamma)

IL-10 (Interleukin Ten)

IL-12 (Interleukin Twelve)

IL-15 (Interleukin Fifteen)

IL-6 (Interleukin Six)

iNOS (Inducible Nitric Oxide Synthase)

kDNA (Kinetoplast Deoxyribonucleic Acid)

KO (Knock Out)

mAb (Monoclonal Antibody)

MDSCs (Myeloid-Derived Suppressor Cells)

ml (Millilitre)

mM (Millimolar)

ng (Nanogram)

NK (Natural Killer)

nM (Nanomolar)

NO (Nitric Oxide)

PBS (Phosphate Buffered Saline)

PCR (Polymerase Chain Reaction)

pg (Picogram)

RPMI (Roswell Park Memorial Institute Medium)

STAT (Signal Transducer and Activator of Transcription Protein)

TC (*Trypanosoma congolense*)

TGF-β (Transforming Growth Factor Beta)

Th1 (T Helper One)

Th2 (T Helper Two)

TLR9 (Toll-like Receptor Nine)

TNF-α (Tumour Necrosis Factor Alpha)

Tregs (Regulatory T Cells)

TSG (Tris-saline Glucose)

TSLP (Thymic Stromal Lymphopoietin)

TSLPR (Thymic Stromal Lymphopoietin Receptor)

VSG (Variant Surface Glycoprotein)

WT (Wild Type)

1. CHAPTER ONE

INTRODUCTION

1.1. African trypanosomiasis

1.1.1. Parasite, Vector, and Forms of Disease

African trypanosomiasis is a disease caused by extracellular hemoprotozoan parasites that belong to the genus T*rypanosoma*. Trypanosomes are unicellular organisms that are equipped with flagella which helps with their movement [1]. The disease is associated with serious health and economic problems in the affected countries and can be fatal if not managed [2,3]. The human form of the disease is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* while the animal form of the disease is mostly caused by *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma brucei brucei, Trypanosoma evansi, and Trypanosoma equiperdum.*

The transmission of these parasites from one host to another is mostly through the bite of several species of tsetse flies belonging to the genus *Glossina* although other methods of transmission like mother-to-child, accidental laboratory pricks with contaminated needles, and sexual intercourse are possible [4-6]. Mechanical transmission by African tabanids has also been documented [7]. These flies are able to inject or pick-up the parasites during the course of their blood meals. Among the important *Glossina* species are *Glossina morsitans*, *Glossina fusca*, *Glossina palpalis, and Glossina tachinoides;* these tsetse flies are widely distributed in countries where the disease is endemic [8], and their presence is often used as a key predictor of the disease.

1.1.2. Epidemiology

African trypanosomiasis, a disease of public health importance, is still considered a global problem. Although it is estimated that over 50 million people living in 25 countries are at risk of contracting the disease, the number of reported cases per year has dramatically reduced (~10,000 new cases annually) due to increasing efforts to combat the disease [9-11]. Because the disease is mostly seen among rural dwellers in endemic areas, it has been difficult to get a true picture of the real situation. It has been estimated that only about 10% of the people living in these endemic areas are accounted for [12,13]. In other words, the majority of the cases remain undiagnosed and unreported, suggesting that the disease impact and statistics could be worse than currently known.

This disease affects both the young and old, especially those that engage in farm-related activities in the rural areas, although there has been a few reported cases in urban areas [14]. *Trypanosoma brucei rhodesiense* causes the disease in Eastern and Southern Africa and is often more fatal when compared to *Trypanosoma brucei gambiense* that causes the disease in Western and Central Africa [1]. The disease caused by *Trypanosoma brucei gambiense* makes up about 98% of all the cases while *Trypanosoma brucei rhodesiense* make up only about 2% of the cases [9,15].

Efforts towards eliminating the disease were almost successful in the 1960s, but because of poor surveillance related to political instabilities, the eradication process was disrupted, which allowed the disease re-emerge [16-19]. African trypanosomiasis usually occurs in areas where the disease vector is found and these geographical zones

are known as foci [20]. Interestingly, the threat posed by this disease to animals is much more than that posed to humans [21]. Hence, it is not surprising that this disease has caused severe food and economic loss in the affected regions [2,3]. The way forward would be to maintain constant surveillance, failure of which could lead to another epidemic that may attract severe consequences [8].

1.1.3. Transmission Cycle

The life cycle of African trypanosomes starts during the course of tsetse fly blood meal. Infected tsetse flies are able to inject or pick up the parasites when they feed on their mammalian hosts. The developmental stage in the tsetse fly starts with the multiplication of the parasites in the midgut, before eventually moving to the salivary gland. Further multiplication of the epimastigotes occurs in the salivary gland and is followed by transformation into metacyclic trypomastigotes, which are transmitted to the host during a blood meal [22]. Upon successful intradermal injection of metacyclic trypomastigotes into the skin by the tsetse fly, the parasites begin to adapt to life in their new host. The formation of chancre, due to the build-up of cell debris and metabolic wastes, is always associated with tsetse transmission of parasites. The trypomastigotes undergo further multiplication by binary fission in body fluids of the host and transform into full blood stream trypomastigotes.

In the blood stream, the parasites undergo morphological changes, transforming from slender to stumpy forms as their numbers increase in the blood [23]. This transformation prepares the parasites for life in the midgut of the tsetse fly [24]. When taken up by tsetse flies during a blood meal, the parasites transform into the procyclic form in the

midgut, and this is followed by rapid loss of surface coat [25,26]. The parasites then establish themselves in the midgut and later move to the salivary glands where they transform into epimastigotes and reacquire their variant surface glycoprotein (VSG) coat [22].

1.1.4. Classification

Trypanosomes are protozoans, belonging to the phylum Sarcomastigophora, class Zoomastigophorea, order Kinetoplastida, family Trypanosomatidae, and genus Trypanosoma. Trypanosomes have a kinetoplast, which is a self-replicating organelle containing DNA.

Trypanosomes can also be divided into groups based on their modes of transmission. The salivararian group has the development of the parasites occurring in the midgut of the vector followed by migration to the salivary gland. As a result, the parasites are present in the saliva of infected vectors and are injected into the vertebrate hosts during the course of a blood meal. Some examples of parasites belonging to this group are *T. brucei, T. congolense, and T. evansi.* In the Stercoraria group (using *T. cruzi* as an example), infection of the host occurs through faecal contamination of the site of the bite, as the parasite development occurs in the intestinal tract of the vectors [1,27].

1.1.5. Characterization

Trypanosomes are unicellular protozoans that possess several features that characterize them. They vary in size, usually between (20-30 μm x 1.5-3.5 μm) [1], and each trypanosome has a single flagellum which plays roles in the motility of the parasite, attachment to the surface of host cells, cell division and morphogenesis [1,28-

30]. They also have kinetoplast DNA (kDNA) which is simply a network made up of interconnecting DNA circles [1,31]. Antigenic variation is another key feature of trypanosomes; the parasites are able to switch their VSG coat during immune response against them by their host. This is considered an evasion strategy by the parasite in order to maintain their existence in their hosts. In response to the new VSG coat, the host mounts another immune response, a process that eventually leads to immune exhaustion [1].

1.1.6. Course of Infection and Pathology

Following inoculation of the infective metacyclics into the skin of the host by the tsetse fly, a local reaction characterized by swelling (commonly referred to as chancre) sometimes occur, and this is the first symptom of the disease in the infected host. It is estimated that about 19% of people infected with *T. brucei rhodesiense* develop chancre [32]. In animals, the disease usually takes a chronic course although acute cases are possible. In human, the disease can be chronic or acute depending on the species of parasite that caused the infection. Infections with *T. brucei gambiense* is usually chronic and lasting for about 3 years while infection caused by *T. brucei rhodesiense* is usually acute and if untreated, could result in death in a matter of weeks or months [33].

The disease comprises two stages, the early hemolymphatic stage and the late meningoencephalitic stage [34]. Intermittent fever attacks are the primary signs of the disease [35] and may be accompanied by a headache, lymphadenopathy, and hepatosplenomegaly [32]. In animals, the disease is associated with anemia, diarrhea,

lacrimation, loss of appetite, weight loss, splenomegaly, abortion, reduced milk production, oedema, ataxia, and lack of energy [36,37]. Disruption in the circadian rhythm of sleep/wake cycle is the hallmark of the late stage of the disease [38]. This is associated with other neurological symptoms such as tremor, limb paralysis, fasciculation, abnormal movements such as dyskinesia and general motor weakness [39,40]. In some cases, symptoms related to psychiatric disorders are observed [40,41].

1.1.7. Antigenic Variation and Variant Surface Glycoprotein (VSG)

Adaptation mechanisms within the host are known to exist among bacteria, parasites, and viruses. Antigenic variation is one of the hallmarks of African trypanosomes. The parasites use this as a major mechanism for evading their host immune response. In fact, it has been suggested that the inability to clearly understand the mechanisms regulating antigenic variation during infection with African trypanosomes is one of the major obstacles standing in the way of finding a vaccine for African trypanosomiasis [42].

Upon infection with African trypanosomes, the parasites grow and multiply in the bloodstream of the host. Since they are completely extracellular in nature, they are constantly exposed to the host's antibody attack. Antibodies have been shown to be effective at parasite clearance [43,44]. However, the parasites are naturally able to switch their VSG during the course of infection in an effort to remain unrecognizable by antibodies from their hosts. This process which is known as antigenic variation, allows the parasites to grow and multiply, requiring the host to start making new antibody responses.

Bloodstream form of trypanosomes is covered with a monolayer comprising of about 10^7 copies of VSG, which represents a major antigen of the parasites [1]. Trypanosomes contain hundreds of VSG genes with only about 7% being fully functional [45]. The transcription of VSG genes occurs at the telomere of the chromosomes containing the VSG expression sites [46]. Because only one expression sites can be active at a time, only one of the VSG molecules is expressed on the surface leading to the identical display of surface coat [1]. In addition, because antibody response is made to this particular antigenic type, a switch in VSG expression would lead to the initiation of new antibody response, a condition that could subsequently pave the way for immune exhaustion due to the continuous need to mount immune responses to new variants. Some of the mechanisms that control the ability of trypanosomes to switch their VSG are, turning off an active expression site (and turning on a previously silent expression site) and rearrangement of the VSG mostly by reciprocal recombination and gene conversion [47-49].

The VSG (usually 12-15nm thick) is dense and often forms a protective coat covering the plasma membrane of the parasites. It is made up of several millions of identical glycoprotein molecules which function to prevent complement-mediated lysis of the parasites [50,51]. The VSG, which exists in soluble and membrane forms, attaches to the surface of the plasma membrane through the glycosylphosphatidylinositol (GPI) that helps in VSG shedding [52]. Soluble forms of VSG is released into the circulation upon hydrolysis of the GPI anchor by endogenous phospholipase C (PLC) known as GPI-PLC [53]. This process has been reported to trigger some inflammatory responses during infection [54].

1.1.8. Diagnosis

Accurate diagnosis of any disease including African trypanosomiasis is critical for effective treatment measures. Parasites from blood or lymph can be viewed microscopically for proper identification, although it is usually a challenge due to the issue of low detection [55]. To this effect, methods geared towards concentrating the parasites, for instance, mini-anion exchange columns, quantitative buffy-coat analysis and microhematocrit centrifugation have proven to be helpful during detection by microscopy [13] although not satisfactory. Card agglutination test (CATT) for detection of anti-parasite (*T. brucei gambiense*) antibodies in serum has been widely used and is reported to have up to 98% sensitivity and up to 95% specificity [56-58].

In addition, other serological tests like enzyme-linked immunosorbent assay are used, although sensitivity remains an issue [57,59]. Polymerase chain reaction (PCR)-based assay has also been used, although controversial results have been reported when used as a diagnostic assay for the advanced stage of the disease using the CSF [60,61]. Field-designed latex agglutination test for IgM levels in CSF of patients appears to be a good test although it still needs standardization [62]. Furthermore, other advanced stage disease markers in the CSF which includes detection of autoantibodies during the disease course have been used in diagnosis [63,64]. Overall, confirmatory diagnostic assays are usually recommended, as no one diagnostic assay appears to be 100% reliable.

1.1.9. Treatment

Treatment of African trypanosomiasis is challenging, the treatment is stage dependent and available treatment options are old. The toxicity of the available drugs, parasite resistance and lack of new drug development also present bigger problems [65]. For the treatment of the first stage of the disease, suramin *(T. brucei rhodesiense)* and pentamidine *(T. brucei gambiense)* have been used although they come with adverse effects. Severe side effects such as anaphylactic shocks, neurotoxic signs, severe cutaneous reactions, renal failure, hypoglycaemia, and hypotension are associated with these drugs [65].

Melarsoprol, eflornithine, and nifurtimox are drugs of choice for treating the advanced stage of the disease. The use of these drugs is hampered by toxicity as well as severe side effects such as reactive encephalopathic syndrome, pancytopenia, diarrhoea, convulsions, and hallucinations [65-69]. The animal form of the disease is treated with trypanocidal agents like isometamidium, homidium, and diaminizne aceturate. These drugs have shown some level of efficacy against *T. vivax*, *T. congolense* and *T. brucei brucei* infections; however, the toxicity, high cost, drug resistance and disease relapse hamper their use [70-72]. More research is needed to discover safe and effective drugs against trypanosomes.

1.1.10. Control

The control of African trypanosomiasis is difficult. Because effective treatment and vaccines are not yet possible, it is reasonable that most control efforts are targeted towards measures aimed at tsetse fly control. To this effect, destruction of vegetation

and killing of wild animals that could act as potential reservoirs was previously practiced [73,74]. However, the use of this method has been discouraged. A more acceptable prevention strategy is achieved through the use of insecticides. This has yielded some degree of results although with some negative consequences on the environment [75]. Another widely accepted method is through sterilization, achieved by ionizing radiation of male tsetse fly [76]. This method involves creating sterile male tsetse flies and releasing them into the wild with the hope of having them mate unproductively with female tsetse flies. This usually leads to a reduction in tsetse fly population. In addition, the use of insect repellents, protective clothing and general education about the disease and its vector could be beneficial.

1.2. Mouse Models for African trypanosomiasis

Various mouse strains (from inbred to knock out mouse models) have been used to conduct studies aimed at advancing our understanding of the mechanisms that regulate immunity and pathogenesis of African trypanosomiasis.

Among the various inbred mice strains, C57BL/6 and BALB/c are popular and have mostly been used to study the factors that regulate resistance and susceptibility of the host to infection with African trypanosomes. BALB/c mice are highly susceptible to intraperitoneal infection with *T. congolense* and usually die within ten days after infection, whereas C57BL/6 mice are relatively resistant and are able to survive for up to 120 days [77,78]. Strains like C3H and A/J mice are less resistant than C57BL/6 mice as they usually have a survival time of around 25 days after *T. congolense* infection [77,78].

In addition to these strains, various knockout mice models have also been used to study this disease. For example, the combined effects of TNF-α and NO in controlling *T. congolense* infection was revealed by using iNOS$^{-/-}$, TNF$^{-/-}$, TNF-R1$^{-/-}$ and TNF-R2$^{-/-}$ mice [79]. Furthermore, data supporting the beneficial effects of antibodies and B cells during *T. congolense* infections were reported by the use of B cell deficient mice and IgM deficient mice [44]. Roles played by other cytokines such as IFN-γ and IL-10 in resistance and susceptibility to *T. congolense* infection have also been studied using IFN-γ and IL-10 deficient mice [44,80]. In fact, knock out mouse models are abundant, and this enables researchers to effectively reach their experimental conclusions.

1.3. Routes of Infection

Natural infection with African trypanosomes starts with an intradermal injection of parasites into the human and animal host by the infected tsetse fly. Following the bite of the fly, the injected parasites undergo several transformations before gaining access to the blood stream of the host. It is conceivable that making use of intradermal route in experimental African trypanosomiasis infection would capture the series of early events that occur during infection [81]. Today, most studies looking at infections with experimental African trypanosomes make use of intraperitoneal route of infection despite the fact that natural infection happens intradermally. Therefore, it is concerning that intraperitoneal route of infection may not clearly represent what happens in terms of early immune response that occurs during natural infection when the vector bites their human or animal hosts [81]. Knowing that intradermal infection of experimental animals is able to induce the activation of some immune mediators [81], utilizing both the intradermal and intraperitoneal infection routes of infection when conducting studies

involving experimental African trypanosomiasis would help us to compare immune responses due to different infection routes, and enable us to further understand the host-parasite interactions that regulate this process.

1.4. Immunity to African trypanosomiasis

1.4.1. Role of Macrophages

Macrophages are one of the most important components of innate immunity to African trypanosomiasis. They are capable of influencing the adaptive immune response as well as secreting many effector molecules including cytokines. During infection with African trypanosomes, classically-activated macrophages (M1) contribute to parasite clearance through phagocytosis [82], and release of proinflammatory cytokines and nitric oxide [83-87]. MacAskill and his colleagues conducted a study to specifically examine the roles played by antibodies, macrophage activation and complement in parasite clearance using trypanosomes labeled with [75Se]-methionine. They showed that clearance of parasites from the circulation was mostly dependent on antibody-mediated phagocytosis by hepatic macrophages (Kupffer cells), which was attributed to opsonization through complement in passively immunized animals [88]. This indicates the importance of macrophages during infection.

Classical and alternative macrophage activation phenotypes occur in African trypanosomiasis, and their effects are critically dependent on the timing of their activations. Survival of mice following infection has been associated with the ability to switch from classically-activated macrophages at the early stages of infection to alternatively-activated macrophages (M2) in the advanced stage of infection [89,90].

This is essential because alternatively-activated macrophages have been shown to regulate classically-activated macrophages during trypanosome infection [91,92] by preventing their over-activation, which could cause some damage to the host. Interestingly, trypanosomes are able to take advantage of the alternative macrophage activation to enhance their survival in the host. The parasites have been shown to preferentially induce alternative macrophage activation with the sole aim of upregulating host arginase which has been shown to reduce the synthesis of trypanocidal nitrosylated compounds as well as upregulate L-ornithine production; L-ornithine is critical in the synthesis of polyamine that is required for parasite growth [93,94]. Indeed, a recent study has shown that the inability of mice to upregulate alternative macrophage activation in the advanced stage of *T. congolense* infection and downregulate iNOS production by macrophages led to enhanced susceptibility to infection, and this was associated with excessive production of proinflammatory cytokines [95].

The roles played by macrophages following intradermal infection have not been well investigated. Wei *et al* demonstrated that mice deficient in inducible nitric oxide synthase (iNOS) were susceptible to intradermal low dose *T. congolense* infection [81], and proposed that macrophages are at the centre of innate control of primary intradermal infection. However, there is some evidence from our lab that suggest that depletion of macrophages before intradermal infection does not alter the resistance of mice to primary intradermal infection; this will be further investigated in this thesis. More studies need to be conducted in order to determine the roles played by macrophages during intradermal infection.

Activated macrophages also present trypanosomal antigens to $CD4^+$ T cells leading to activation and production of cytokines by $CD4^+$ T cells [96]. Because macrophages expand and continue to carry out their functions in the spleen and liver after infection, they often get over-activated, releasing excessive amounts of proinflammatory molecules that eventually contribute to disease severity and death of infected mice [83,97]. In contrast, because of their anti-inflammatory properties [98], alternatively-activated macrophages (M2) play a crucial role in dampening inflammatory responses during the advanced stage of infection with African trypanosomes [91,92,95].

1.4.2. Role of Complements

The complement system is considered to be important in mediating parasite clearance, although trypanosomes have different mechanisms to avoid lysis by complement. There are classical and alternative pathways of complement activation, both of which occur during infection with African trypanosomes. The classical pathway is mediated by specific antibodies against the parasite and has been reported to contribute to parasite clearance via antibody-mediated lysis or opsonisation/phagocytosis [99]. The antibody-independent alternative pathway is usually activated during the early stages of infection when specific antibodies have not been formed. This pathway has also been shown to contribute to parasite clearance [100]. Because classical complement activation is critical in lysing VSG coated trypanosomes with the help of antibodies, trypanosomes in an effort to enhance their survival, are able to shed enormous VSG in the circulation which leads to the formation of immune complexes with antibodies. This often leads to a state of hypocomplementemia which is a hallmark of infection with African trypanosomiasis [101]. Also as an evasion mechanism by the parasites, the binding

sites present on VSG plasma membranes could be blocked, leading to the inability of the host to initiate alternative complement activation. This occurs as a result of the halt at the C3 convertase stage thereby preventing further activation of C5-C9 stages that usually initiate lysis of trypanosomes [102,103]. Furthermore, the anaphylatoxins, C3a and C5a, have been reported to help in the initiation of inflammatory responses during infection [104]. However, a more direct evidence showing the involvement of complements in immunity was demonstrated by studies conducted by Jarvinen and Dalmasso. They found that parasite control could not be attributed to the presence of C3, C5 or late-acting complement factors [105], suggesting that although complement activation may be required, it is not critical for overall parasite control.

1.4.3. Role of T cells

T cells are made up of two major subsets, $CD4^+$ T cells (aka helper T cells) and $CD8^+$ T cells (aka cytotoxic T cells). T cells mostly contribute to the resolution of infections by microbes by producing cytokines that regulate other innate and adaptive immune cells, and by providing help to B cells to ensure efficient class-switching and production of specific antibodies to antigens. $CD4^+$ T helper cells provide signals that regulate B cell survival and differentiation into antibody-producing cells [106]. In support of this, Shi *et al* showed that anti-parasite IgG2a production as well as IFN-γ and IL-10 levels were impaired in *T. congolense*-infected $CD4^{-/-}$ mice [96]. Following this observation, Tabel *et al* proposed that the future of vaccine against African trypanosomiasis should be targeted towards encouraging the generation of T helper 1 cells that would support B cells in class-switching from IgM to IgG2a during infection [107]. On the other hand, another report showed that $CD4^{-/-}$ mice infected with *T. congolense* did not show high

parasitemia or reduced survival compared with their WT counterpart mice [96], suggesting that the role of $CD4^+$ T cells in experimental African trypanosomiasis is still not clear.

The role of $CD8^+$ T cells in African trypanosomiasis has been controversial. A study by Wei *et al* showed that the beneficial effect of anti-CD25 treatment in mice during *T. congolense* infection was lost upon depletion of $CD8^+$ T cells [108], suggesting that $CD8^+$ T cells may be playing a protective role during infection. In another study conducted by Liu *et al* [109] to investigate the roles of $CD4^+$ and $CD8^+$ T cells in *T. brucei* infection, it was reported that IgG antibody synthesis was dependent on $CD4^+$ T cells and not $CD8^+$ T cells. In addition, they showed that infected $CD8^{-/-}$ mice had lower parasitemia and survived significantly longer than WT mice. However, the enhanced survival was lost upon depletion of $CD4^+$ T cells. Cytokine (IFN-γ and IL-10) production during infection was also attributed to $CD4^+$ but not $CD8^+$ T cells. Collectively, these observations indicate that $CD8^+$ T cells play a pathogenic role and mediate susceptibility while $CD4^+$ T cells mediate protection during infection with *T. brucei.*

Our understanding of the roles played by T cells has been simplified by the Th1 (proinflammatory properties) and Th2 (anti-inflammatory properties) paradigm. Overall, control of parasites during infection with African trypanosomes is believed to be associated with a Th1 response during the onset of infection and a switch to Th2 in the advanced stage of infection [89,90]. To support this, *T. congolense*-infected $TSLPR^{-/-}$ mice, which mounted impaired Th2 response, was highly susceptible to *T. congolense* infection and died significantly earlier than their WT controls; in addition, the $CD4^+$ T cells from these mice produced unregulated amount of proinflammatory cytokines,

which was reversed upon treatment of the infected TSLPR$^{-/-}$ mice with anti-IFN-γ monoclonal antibody [95].

The roles played by regulatory T cells have been well investigated in African trypanosomes. Independent *T. congolense studies* have shown that these cells contribute to susceptibility and prevent the control of parasites during infection by causing immunosuppression [108,110].

1.4.4. Humoral Immunity

1.4.4.1. Role of B cells and Germinal Centre Response

The general requirement of B cells during infection with African trypanosomiasis centres on optimal initial activation, efficient germinal centre (GC) formation and specific antibody responses to trypanosomes. The GC comprises of a large area of B cells where activities like B cell proliferation, somatic hypermutation, selection, and class switch recombination take place, resulting in the production of various antibody isotypes with high antigen binding affinity [106]. African trypanosomiasis is marked by excessive activation of B cells and increase in the levels of trypanosome-specific and non-specific immunoglobulins.

B cells get activated following an encounter with their cognate antigens, followed by the initiation of germinal centre response with the help of follicular CD4^{+} T helper cells (Tfh) [111]. This process usually results in the production of antibody-secreting plasma cells. A recent study using Bam32 deficient mice has further demonstrated the requirement of B cells response in infection with African trypanosomes [95]. Bam32 is a B cell adaptor protein that plays a critical role in B cell receptor cross-linking-mediated downstream

events [112], survival [113] and antigen presentation [114]. Bam32 deficient mice were more susceptible to *T. congolense* infection and showed impaired germinal centre response as well as parasite-specific IgG production. Although polyclonal B cell activation occurs during African trypanosomiasis (leading to polyspecific or autoreactive antibody response) [115,116], there is evidence that clearly shows that trypanosome-specific antibody responses are able to mediate parasite clearance during infection [43,117,118]. B cell-deficient mice have also been used to directly demonstrate the requirement of B cells during experimental African trypanosomiasis [119]. Interestingly, infection-induced depletion of B2 B cells in the spleen by Natural killer cells has been reported to contribute to early mortality of *T. brucei*-infected animals [120].

1.4.4.2. Antibodies

Trypanosomes are extracellular in nature and are continually exposed to antibodies in the blood stream of the host. During infection with African trypanosomes, specific antibody responses against variant and invariant VSG membranes, cytoplasmic, and nuclear parasite antigens are made via both T-dependent and T-independent processes, although the quality of the response is increased in the presence of T cells [121]. Several studies have investigated the relative contributions of different classes of antibodies in resistance to African trypanosomes. Although both parasite-specific IgM and IgG antibodies have been shown to be effective in protection, IgG and its subclasses have been associated with more efficient parasite clearance in both mice and cattle compared to IgM [43,122]. These antibodies are mostly involved in opsonisation of parasites, subsequent phagocytosis and clearance of the immune complexes by the liver macrophages (kupffer cells) [82].

To further demonstrate the requirement of antibodies during infection, reports have shown that specific antibodies generated after injecting irradiated parasites or the VSG in animals were able to confer protection during challenge infection with homologous parasite [123,124].

1.4.5. Role of Cytokines

Cytokine production during African trypanosomiasis is one of the key determinants of susceptibility and resistance. Although the roles of cytokines in immunity have been studied in great detail, and in different experimental settings using trypanosomes, determining the precise role of any cytokine is challenging because a change in disease course could drastically change the function of a cytokine [107]. The first set of cytokine response during infection with African trypanosomes usually consists of the release of proinflammatory molecules like TNF-α, IL-1, IL-6 and NO by classically activated macrophages, and these have been shown to play important roles in mediating early protection during infection [82,86,124-128]. Other cytokines like IL-12, MCP-1, IL-10, IFN-γ and IL-4 have also been shown to be associated with infection with African trypanosomes, some of which are pro-inflammatory or anti-inflammatory [80,82,96,97,129-131].

Although the initial outburst of inflammatory cytokines is essential, it has to be regulated to prevent collateral tissue damage. Modulation of classically activated macrophages by type II cytokines such as IL-10, IL-4, and IL-13 is critical. These cytokines help in maintaining a Th2 type of environment and the development of alternatively activated macrophages, which have anti-inflammatory properties [1,95]. In fact, protection during

infection with African trypanosomes is associated with the ability to switch from Th1 to Th2 response as well as from classically activated macrophages to alternatively activated macrophages in the later stages of infection [89,90,95,132]. One of these studies specifically showed that in the absence of TSLP signaling, which drives Th2 differentiation, *T. congolense*-infected mice were not able to control more than two parasitemia waves [95]. This was also associated with overproduction of proinflammatory cytokines and impaired induction of alternatively activated macrophages followed by death [95]. In addition, IFN-γ, TNF-α and NO are associated with protection during infection with African trypanosomes [44,133-135] although their production in excessive amounts could mediate susceptibility to infection [83,97,131].

Assessing the individual functions of cytokines is challenging and depends on dosage and timing [107]. For instance, studies using IFN-γ deficient mice in either T*. brucei* or *T. congolense* infection have shown the requirement of IFN-γ in mediating protection in the relatively resistant mice [44,134]. However, blockade of IFN-γ during *T. congolense* infection led to reduced parasitemia and increased survival of the highly susceptible mice [97]. In addition, the death of relatively resistant mice following treatment with anti-IL-10 receptor antibody could be reversed by the injection of anti-IFN-γ mAb [131], suggesting that IFN-γ is a key mediator of death in these mice.

Studies have shown that IL-10 is an anti-inflammatory cytokine that acts to downregulate the excessive effector function of both T cells and macrophages [131,136]. In *T. congolense*-infected cattle, reduced nitric oxide production and increased IL-10 and IL-4 mRNA levels were linked to protection [122,137]. Also, it has

been reported that TNF-α has cytotoxic properties which are capable of directly killing parasites in addition to its influence on cell (macrophage) activation [138]. In line with this, blockade of TNF-α during *T. brucei* infection in mice led to the loss of initial parasite control [138].

1.5. Immunosuppression

Immunosuppression is a key feature that complicates African trypanosomiasis. During infection, the infected host becomes immunosuppressed, leading to failure to effectively clear parasites and resolve the infection. This often leads to increased pathology. Antigenic variation (already discussed above), polyclonal B cell activation and induction of suppressor cells following infection are some of the key mechanisms through which immunosuppression is mediated.

1.5.1. Polyclonal B cell Activation

The roles played by B cells during infection with African trypanosomes are generally accepted since the parasites are extracellular and in constant contact with the host antibodies in the blood stream. As a way to suppress and evade the host-specific responses, trypanosomes are able to take advantage of the ability of B cells to produce antibodies and induce excessive activation of antibody-producing cells. This often leads to an increase in the amount of specific and non-specific immunoglobulins. Specific antibodies produced during infection are often effective at clearing parasites. These antibodies, mediated with or without the help of T cells, are made against parasite antigens like VSG epitopes, membrane, cytoplasmic and nuclear antigens [121]. However, induction of polyclonal B cell responses and the resulting non-specific

antibody production represent a strategy through which the parasites dilute the number of specific antibodies directed at them by their hosts [139]. Specific T-dependent B cell responses are often suppressed during infection whereas the T-independent responses remain at a higher level. The trypanosomal DNA are able to initiate TLR-9 signaling and could lead to non-specific B cell activation and production of poly-specific antibodies to VSG, leading to over-engagement of the Fcγ receptors on phagocytes, which would eventually contribute to impaired opsonisation and phagocytosis of parasites [139,140]. During infection, autoantibodies corresponding to non-specific B cell responses are not uncommon. Autoantibodies against red blood cells, cardiolipids, nucleic acids, rheumatoid factors and component of CNS myelin (the galactocerebrosides) have been documented [141-146]. In addition, an increased in a subpopulation of CD5-expressing B cells have also been documented, and these cells are believed to be responsible for the excessive production of immunoglobulins and autoantibodies during *T. congolense* infection in cattle [140]. These factors mediate immunosuppression and favour disease progression.

1.5.2. Suppressor Cells

Several cell types are involved in immunosuppression in African trypanosomiasis. Although suppressor macrophages and suppressor T cells have been implicated [147-149], more recent reports have clearly revealed additional roles played by regulatory T cells [108,110]. There is also evidence from our lab that shows that myeloid-derived suppressor cells contribute to immunosuppression and this constitutes one of the major topics investigated in this thesis. Schleifer and Mansfield [150] have shown that macrophages suppress T cell responses through the production of reactive nitrogen

intermediates and prostaglandins. Similarly, independent studies (using anti-CD25 mAb treatment) have shown that $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) play a role in suppression of immune response to *Trypanosoma congolense* [108,110]. In these studies, immunity to the parasites including control of parasitemia and survival was enhanced in the absence of Tregs. The cellular mechanisms that regulate immunosuppression are largely unknown and form a major aspect of this thesis. Increasing our knowledge of various factors mediating immunosuppression during infection with African trypanosomes could be beneficial in designing effective therapeutic strategies.

1.6. Vaccination Strategies in African trypanosomiasis

Effective therapy and vaccine against African trypanosomiasis are currently not available. In addition to being toxic and expensive, the use of available drugs is further hampered by the risk of drug resistance and disease relapse [70-72]. On the other hand, vaccination has been impossible mainly due to the difficulty in understanding the factors that regulate antigenic variation during infection [42]. Since attempts to develop a vaccine have not yielded the desired results [151], it is conceivable that current research strategies might be missing key information regarding effective immune response to these parasites. Therefore, a change in the current way of thinking is needed.

In agreement with Tabel and his colleagues [152], using the intraperitoneal route of infection, as is currently being practiced, may not correctly represent the activities that take place during natural intradermal infection. Therefore, a switch to a more natural

intradermal route of infection would be beneficial in understanding key immune responses to these parasites, thereby providing helpful information regarding vaccine development.

Also, the issue of polyclonal B cell activation needs to be further evaluated. The parasite's ability to induce non-specific B cell responses to enhance its survival in the host poses a great challenge in vaccine discovery. An understanding of the factors that regulate the parasite's ability to initiate this process could be beneficial in designing effective vaccines. Furthermore, vaccination strategies that involve the inhibition of various factors that mediate the suppression of effector cell proliferation and function during the disease could also be targeted [152].

2. CHAPTER TWO

RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1. Rationale

Efforts to control African trypanosomiasis have been hampered because of lack of understanding of the mechanisms that regulate disease pathogenesis and host protective immune response against the pathogen. In particular, the parasite's ability to undergo antigenic variation and our inadequate understanding of the molecular mechanisms that regulate the process contribute to failure to design an effective vaccine [42]. During infection, trypanosomes constantly modify their variant surface glycoprotein (VSG) following host response, resulting in the fluctuating waves of parasitemia that characterizes African trypanosomiasis [96,129,153]. In addition, infection with African trypanosomes is associated with profound immunosuppression, which increases the susceptibility of the host to the parasite and secondary (opportunistic) infections [43,147,154]. Understanding the mechanisms that regulate resistance and/or susceptibility to the disease could reveal novel interventions that might lead to effective disease control [155].

Overarching Aim: To understand how the optimal immune response to *T. congolense* infection is regulated by key immune cells by investigating the roles played by Tregs, MDSCs and NK cells.

Global hypothesis: Resistance to *T. congolense* infection is regulated by the activities of Regulatory T cells, Myeloid-derived suppressor cells, and Natural killer cells.

$CD4^+$ T cells that constitutively express CD25 and the transcription factor, Foxp3 (called regulatory T cells, Tregs), have been shown to play a major role in immune homeostasis by actively suppressing several pathological and physiological immune responses in the host [156-158]. Although their primary role is to prevent autoimmunity and suppress inflammatory responses, Tregs have also been implicated in the pathogenesis of several infectious diseases including those caused by parasites [108,159,160]. In particular, increased numbers of $CD4^+CD25^+Foxp3^+$ Tregs have been reported in experimental *T. congolense* infections [110,161] and these cells have been implicated in enhanced susceptibility to the infection [108,110], although the exact mechanisms remain unknown.

A previous study showed that BALB/c mice are relatively resistant to intradermal *T. congolense* infection [81]. Paradoxically, low dose i.d. *T. congolense* infection leads to enhanced susceptibility to reinfection [81]. However, the mechanisms underlying this low dose-induced enhanced susceptibility remains unknown. Since Tregs have been shown to mediate susceptibility to *T. congolense* infection, I propose that the expansion of Tregs following intradermal low dose infection is responsible for enhanced susceptibility to low dose reinfection.

The suppression of T cell responses during *T. congolense* infection remains a major issue affecting optimal immune response and effective control of the disease [43,147,154]. T cells, especially $CD4^+$ T cells, not only produce effector cytokines during *T. congolense* infection, they also help in the class-switching of B cells to produce optimal antibodies in response to pathogens [122]. Cytokines, specifically IFN-γ and TNF-α are associated with early protection during African trypanosome infection

[44,133-135]. Despite the importance of these key cytokines in early protection to the infection, studies looking at the cells producing them have been inadequately conducted. Natural killer cells belong to innate lymphocytes that regulate both the innate and adaptive immune response. These cells carry out their regulatory function through cytotoxicity or cytokine release [162,163]. They predominantly produce IFN-γ and TNF-α, key cytokines that are important in early protection during *T. congolense* infection.

Since NK cells are key producers of early IFN-γ and TNF-α, and because these cytokines have both been shown to mediate early protection in *T. congolense*-infected mice [44,133-135], it is conceivable that NK cells would be playing important roles in early immune response to this infection. Therefore, I propose that NK cells would be critical in mediating resistance in *T. congolense*-infected mice.

Myeloid-derived suppressor cells (MDSCs) are generally known to be immunosuppressive to T cell responses largely through the upregulation of arginase-1 and/or inducible nitric oxide synthase (iNOS) enzymes [164,165]. Although the suppressive roles of MDSCs have been well studied in tumor models [166,167], as well as bacteria and parasitic infections [168,169], no report exists so far about the roles of these cells in *T. congolense* infection.

Because T cell proliferation is impaired during experimental *T. congolense* infection [150,170,171], and because MDSCs are known to suppress T cell proliferation and function [164,166,168,172,173], it is conceivable that MDSCs would play a critical role during *T. congolense* infection. I propose that MDSCs would suppress $CD4^+$ T cells and contribute to susceptibility to *T. congolense* infection.

2.2. Hypothesis

a. Because Tregs mediate susceptibility to *T. congolense* infection, *I hypothesize that low dose intradermal infection with T. congolense induces the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells, which suppress the immune response leading to enhanced susceptibility to re-infection.*

b. Since NK cells are key producers of early IFN-γ and TNF-α, and because these cytokines have both been shown to mediate early protection in *T. congolense*-infected mice, *I hypothesized that NK cells expand after T. congolense infection and contribute to optimal resistance to the infection.*

c. Because T cell proliferation is impaired during experimental *T. congolense* infection, and because MDSCs are known to suppress T cell proliferation and function, *I hypothesise that MDSCs expand after T. congolense infection and promote susceptibility by suppressing the proliferation and effector function of $CD4^+$ T cells.*

2.3. Objectives

This thesis contains three key objectives:

a. To investigated the mechanism through which low dose intradermal *T. congolense* infection predisposes mice to enhanced susceptibility to subsequent reinfection

    i. Characterise immune response following low dose intradermal *T. congolense* infection.

ii. Assess the induction of Tregs after low dose intradermal *T. congolense* infection.

iii. Determine the effect of Tregs depletion on intradermal low dose *T. congolense* infection-induced susceptibility.

b. To determine the role of NK cells in resistance to *T. congolense* infection

i. Assess the expansion pattern of NK cells in multiple tissues after *T. congolense* infection.

ii. Determine the effect of NK cell depletion on general immune response during *T. congolense* infection.

iii. Determine the direct role of NK cells using adoptive transfer experiments.

iv. Investigate the mechanism through which NK cells function during *T. congolense* infection.

c. To determine the role played by MDSCs during *T. congolense* infection

i. Assess the levels of MDSCs in *T. congolense*-infected mice.

ii. Determine the effect of MDSCs on $CD4^+$ T cell proliferation and function.

iii. Investigate the mechanism through which MDSCs suppress $CD4^+$ T cells during *T. congolense* infection.

3. CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1. Mice

Six to eight weeks old female BALB/c, C57BL/6 and outbreed Swiss white (aka CD1) mice used in these experiments were purchased from Charles River, St. Constante, Quebec or Central Animal Care Services (CACS), University of Manitoba, Winnipeg, Canada. NFIL3$^{-/-}$ mice on the C57BL/6 background were bred by the CACS and supplied when needed. The origin and phenotype of NFIL3$^{-/-}$ mice have been previously described [174]. Perforin$^{-/-}$ and IFN-$\gamma^{-/-}$ mice on the relatively resistant C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, USA). Animals were housed at Central Animal Care Services (CACS), University of Manitoba, Winnipeg, Canada. Animal housing, handling, and feeding were done in accordance with the recommendations of the Canadian Council of Animal Care.

3.2. Parasite and mice infection

Trypanosoma congolense (Trans Mara strain) variant antigenic type TC13 was used in all experiments and the origin of this strain has been previously reported [175]. For expansion of frozen TC13 stabilates, CD1 mice were immunosuppressed by injecting cyclophosphamide (Cytoxan; 200 mg/kg) intraperitoneally, followed by infection with freshly thawed *T. congolense* stabilates from liquid nitrogen [175]. Three days after infection, mice were anaesthetized by isoflurane and blood was collected by cardiac puncture. Parasites for infection were purified from the blood using diethylaminoethyl (DEAE) cellulose anion exchange chromatography [176]. Eluted parasites were washed

in Tris-saline glucose (TSG), counted with the haemocytometer, resuspended in TSG containing 10% heat-inactivated FBS and diluted to the desired concentrations. Mice were infected by intraperitoneal injection of 100 µl TSG-FBS containing 10^2 or 10^3 parasites. Intradermal (hind foot pad) infections were made by injecting 50 µl TSG-FBS containing 10^2 or 10^3 parasites.

3.3. Estimation of parasitemia

A drop of blood taken from the tail vein of *T. congolense*-infected experimental mouse was placed on a microscopic slide, and estimation was done by counting the number of parasites present in at least 10 fields at 400x magnification of the light microscope. During the late stage of infections when parasite loads are usually high, estimation was done using a technique described previously [177].

3.4. Preparation of cells

At indicated days after infection, mice were anaesthetized by isoflurane and sacrificed by cervical dislocation. A single cell suspension of the spleens and lymph nodes were made and contaminating red blood cells in the spleen suspensions were lysed with red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM NaEDTA (Sigma). The cells were washed twice with PBS and resuspended in complete tissue culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, 100 U/mL Penicillin and 100 µg/ml streptomycin). Liver samples from the sacrificed mice were perfused (via the right ventricle) with 10 ml of ice-cold-PBS, digested with collagenase-D (125 µg/mL) for 30 minutes at 37 ºC. Processing of lung cells followed similar digestion method. The resulting tissue slurry was passed through

a 70 µm cell strainer (VWR, Mississauga, ON, Canada) and washed with 30 ml Hanks balanced salt solution (HBSS) (Invitrogen, ON, Canada) at 1200 rpm for 5 minutes. Contaminating red blood cells in the liver were lysed with red blood cell lysis buffer and the resulting cells were washed with HBSS. To separate lymphocytes from other cells, the cells were re-suspending in 4 ml of 40% percoll (Sigma) and layered on top of 70% percoll before spinning (without brakes) at 750 x g at 22°C for 20 min. The interface containing the lymphocytes was carefully removed from the rest of the cells. The cells were washed and re-suspended in complete tissue culture medium. Peritoneal wash cells from the peritoneal cavity were collected in PBS and washed with complete medium. All cells were counted with a haemocytometer and resuspended in appropriate numbers in complete medium for downstream experiments.

3.5. Cell culture for cytokine analysis

At sacrifice, spleens and lymph nodes (popliteal lymph nodes) draining the intradermal infection site were processed into single cell suspensions, treated with ACK lysis buffer and washed two times with phosphate buffered saline (PBS). The cells were counted, resuspended at a final concentration of 4 x 10^6/ml in complete tissue culture medium, and plated at 1 mL/well in 24-well tissue culture plates (Falcon; VWR Edmonton, AB, Canada) with or without whole trypanosome lysate (10^6/ml parasite equivalent). Cultured cell supernatant fluids were gently collected after 72 hours and stored at -20 ^oC until assayed for cytokines by ELISA.

3.6. Isolation of cell populations from the spleen

Mature natural killer ($NK1.1^+$) cells were isolated from the spleens of naïve WT, IFN-$\gamma^{-/-}$ and $Perforin^{-/-}$ mice by negative selection using EasySepTM Mouse NK Cell Isolation Kit (StemCell Technology, Vancouver, BC) according to the manufacturer's suggested protocol. The purity of the isolated NK cells was greater than 94% (as assessed by flow cytometry). Enriched NK cells were washed with complete medium followed by PBS. The cells were re-suspended in PBS for adoptive transfer experiments. EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (StemCell Technology, Vancouver, BC) was used to isolate $CD4^+$ cells (by negative selection) from the spleens of naive mice according to the manufacturer's recommended protocol. The purity of the isolated $CD4^+$ cells was greater than 95% (as assessed by flow cytometry). Isolated cells were washed and re-suspended in complete medium for use in cell culture experiments. $CD11b^+GR1^+$ cells were FACS-sorted (over 92% purity) from the spleen of infected mice (on day 7 post-infection) with BD FACSAria III cell sorter (BD Biosciences). After sorting, cells were washed and re-suspended in complete medium before being used for experiments.

3.7. Enzyme-linked immunosorbent assay (ELISA)

Cytokine (IL-6, IL-10, IL-12, IFN-γ, TNF-α and TGF-β) levels in the serum and cell culture supernatant fluids were determined by sandwich ELISA using antibody pairs purchased from BD Biosciences according to the manufacturer's suggested protocols. The sensitivities of the cytokine ELISAs range from 7.5 – 31 pg/ml. Briefly, ELISA plates purchased from Immulon® VWR (Mississauga, ON) were coated with primary antibodies (Biolegend, San Diego, CA) at a concentration of 1-1.2 µg/ml (in 50 µl/well)

in carbonate-bicarbonate coating buffer with a final pH of 9.6. Plates were incubated overnight at 4 ^{0}C followed by 5 times washing with ELISA wash buffer (1x PBS, 0.05 % Tween 20 (Sigma) pH 7.4). All washing was carried out using ELISA washing machine (BIOTEK ELX405, Biotek Instrument, Winooski, VT). All wells were blocked with blocking buffer (5% new calf serum in PBS, pH 7.4) at 200 µl/well and incubated at 37^{0}C for 2 hours. After washing the plates for 5 times as described above, recombinant cytokines and samples (cell supernatant or serum) were added to the plate and serial dilutions were made before incubating the plates overnight at 4^{0}C. Next, the plates were washed 5 times followed by the addition of 50 µl of biotinylated detection antibody purchased from Biolegend at 2 µg/ml in dilution buffer. Plates were incubated for 1-2 hours at 37^{0}C. Following 5 times washing, streptavidin-horseradish peroxidase (BD Pharmingen, San Jose, CA) in dilution buffer (1:3000) was added to the wells and incubated for 30 minutes at 37 ^{0}C. The final wash was done 10 times before the addition of two-component ABTS substrate (Mandel Scientific, Guelph, ON). All plates were read with Spectra Max at a wavelength of 405 nm.

3.8. Direct *ex vivo* staining and intracellular cytokine detection by flow cytometry

Cells were stained *ex vivo* for the expressions of CD3, CD4, CD25, Ki67, Foxp3, NK1.1, CD107a, CD11b, CD11c, B220, F4/80, NKp46, IFN-γ, TNF-α, GR1, Ly6G and Ly6C using flow cytometry. Briefly, cells were placed in flow cytometry tubes (BD Falcon) and washed with FACS buffer (0.1% new calf serum and 0.1% sodium azide in PBS). Cells were incubated on ice for 5 minutes with 100 µl of Fc receptor blocker (2.4G2 Hybridoma supernatant). Staining for surface markers was performed with fluorochrome-labeled antibodies (eBioscience) on ice for 25 minutes and washed with

FACS buffer. In some experiments involving cytokine assessment, the cells were stimulated with phorbol myristic acetate (PMA; 50 ng/mL), ionomycin (500 ng/mL), and brefeldin A (BFA; 10 µg/mL) for 4 hrs, stained for surface markers and fixed with 0.5 ml 2% paraformaldehyde (Sigma) on ice for 15 minutes. For membrane permeabilization, cells were further incubated with 0.1% saponin (Sigma) in FACS buffer for 10 minutes on ice. Fluorochrome-labeled antibodies (eBioscience; each at 0.5 ug per tube) in a final volume of 20 µl per tube was added to each tube and incubated for 30 minutes on ice. Stained cells were washed with 0.1% saponin in FACS buffer and then with FACS buffer for membrane closure. Foxp3 staining was done with Tregs staining kit (eBioscience) using Foxp3 fixation/permeabilization concentrate and diluent, as well as Foxp3 permeabilization buffer in accordance with the manufacturer's suggested protocols. At the end of staining procedure, all samples were washed and re-suspended in FACS buffer. The cells were acquired using BD FACS Canto II cytometer (BD Bioscience, San Diego CA), while FlowJo software (BD Bioscience) was used for analysis.

Table 1: List of fluorochrome-conjugated antibodies used in flow cytometry

Serial number	Antibody	Clone	Fluorochrome
1	CD3	145-2C11	FITC
2	CD4	GK1.5	Pacific blue
3	CD25	PC61	PE
4	NK1.1	PK136	FITC, APC, PE
5	NKp46	29A1.4	PE
6	B220	RA3-6B2	APC
7	CD11b	M1/70	APC, eFlour 450
8	CD11c	N418	Pacific blue
9	F4/80	BM8	FITC
10	GR1	RB6-8C5	PE, FITC
11	Ly6C	AL-21	FITC
12	Ly6G	1A8	Pacific blue
13	CD107a	ID4B	APC
14	Foxp3	FJK-16s	APC
15	IFN-γ	XMG1.2	APC, Pacific blue
16	TNF-α	MP6-XT22	APC, PE
17	Ki67	SolA15	APC, eFlour 450

3.9. Carboxyfluorescein succinimidyl ester (CFSE) assay

To assess proliferation and IFN-γ production by $CD4^+$ T cells in splenocytes or in MDSC co-culture experiments, *in vitro* CFSE dilution method, was used. Briefly, cells were washed with warm PBS and re-suspended in warm PBS to a concentration of 8 x 10^6/ml. Some cells were set aside unlabelled for compensation. Five millilitre of the suspension was labeled with 5 ml of 5 mM CFSE dye and gently rocked for 5 minutes. Warm FBS (Hyclone) was added to the tube and gently rocked before spinning at 4^0C for 5 minutes at 1200 rpm. The resulting cell pellet was resuspended in complete medium and counted to assess the number of viable cells. The viable cells were plated in 96-well plate (round bottom) at 2 × 10^5 per well in 200-µL and stimulated with or

without soluble anti-CD3 (1µg) and anti-CD28 (1µg). To assess the mechanism of MDSC suppression of $CD4^+$ T cells, an inhibitor of arginase-1 (N^{ω}-hydroxy-nor-l-arginine- nor-NOHA, 500 nM, Sigma) or iNOS (N^G-Methyl-L-arginine acetate salt- L-NMMA, 500 nM, Sigma) was added to the co-culture. Stimulated cells were incubated for 4 days at 37^0C in 5% CO_2. After 4 days, cells were taken off the plates for flow cytometry experiments.

3.10. Serum collection and measurement of trypanosome-specific antibodies

Mice were anaesthetized using isoflurane or by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (150 mg/kg). Blood was collected by cardiac puncture using a 1 ml syringe and 25G needle. Blood samples were kept at 4 degrees for 4 hr, spun at 2400 rpm for 10 min and serum was collected and stored at -20^0C until used for antibody determination. Serum levels of *Trypanosome*-specific IgM and IgG antibody classes in infected mice were determined by ELISA as previously described [43,178] with minor changes. Briefly, ELISA plate was coated with sonicated *T. congolense* lysate (10^5/well) in 100 µl of ELISA coating buffer overnight at 4^0C. The plate was washed twice with PBS and blocked with 5% skim milk in PBS (200 µl/well) for 2 hours at 37^0C. After washing twice with PBST, serum samples were added to the wells and serial dilution was made with 2.5% skim milk in PBST before incubating for 2 hours at 37^0C. This was followed by washing (4 times) with PBST and addition of 100 µl of HRP-conjugated goat anti-mouse IgM or IgG subtypes (Southern Biotech) in PBST for 1 hour at 37^0C. Plates were washed 4 times with PBST followed by the addition of 100 µl of the substrate (TMB) to the wells. Plates were incubated in the dark at room temperature. The reaction was stopped by the

addition of 50 µl of 1M H_2SO_4 followed by OD reading with Spectra Max at a wavelength of 405 nm.

3.11. DNA extraction and polymerase chain reaction

QIAamp DNA Mini Kit (Qiagen, Mississauga, ON Canada) was used to extract DNA from freshly collected whole blood samples (200 µl each) of infected and uninfected mice according to the manufacturer's suggested protocol. DNA concentration was estimated from 1µl of each sample using NanoVue Plus Spectrophotometer (GE Health Care Life Sciences, Mississauga, ON). PCRs were performed in a 25 µl reaction mixture using Platinum® PCR SuperMix (High Fidelity, Invitrogen™ Grand Island, NY) according to the manufacturer's suggested protocols. The amplification of *T. congolense* DNA was done using the GOL primers [179], which are highly specific for a 314 bp satellite DNA of *T. congolense*; Forward: 5'-GAGAACGGGCACTTTGCGATTTTC-3' and Reverse; 5'-GACAAACAAATCCCGCACAACCAT-3'. The amplified products were resolved by electrophoresis using a 2% agarose gel and visualized using Alphaimager® (Alpha Innotech, Fisher Scientific, Ottawa, ON, Canada).

3.12. *In vivo* depletion of cells

In experiments requiring Tregs depletion, each mouse received 100 µg of anti-CD25 monoclonal antibody (clone PC61) intraperitoneally. Previous studies from our lab have shown that this dose of antibody causes sustained depletion of $CD25^+Foxp3^+$ T cells (without affecting other cell populations) for up to 7 days in mice infected with *T. congolense* [110,180]. *In vivo* depletion of macrophages was done by intravenously

injecting 200 µl of chlodronate-loaded liposomes (5 mg/ml, Encapsular NanoSciences LLC, Nashville, TN) 24 hours before primary intradermal infection. The control group of mice was injected with liposomes. Macrophage depletion was confirmed by flow cytometric analysis of $F4/80^+$ cell populations. For experiments that required anti-NK1.1 treatment, mice were injected intraperitoneally with anti-mouse NK1.1 (PK136) monoclonal antibody or control Ig (BioXcell) at 500 µg/mouse. For experiments involving MDSC depletion, 200 µg of anti-GR1 (RB6-8C5) monoclonal antibody (BioXcell) was injected twice into mice, first on the day before infection and later on day 4 after infection.

3.13. Expansion, activation and adoptive transfer of purified NK cells

Purified NK cells were cultured in complete medium with IL-2 (1000 IU/ml) and IL-15 (50 ng/ml) for 4 days. Cells were harvested, washed with complete medium and counted. Three to four-fold increase in NK cell number was obtained after the 4-day culture. The viability and activation of the NK cells were assessed before adoptive transfer. For adoptive transfer experiment, cells were washed and re-suspended in PBS; approximately 9 million cells were injected into each mouse through the tail vein. Infection of the recipient mice was done 24 hours after cell transfer.

3.14. Statistical analysis

Data are presented as means and Standard Error of Mean (SEM). A Two-tailed Student's t-test or ANOVA were used to compare means and SEM between groups. GraphPad Prism software was used for data analysis and differences were considered

significant at $p < 0.05$. Experiments described in this thesis were repeated 2-3 times and there were 3-5 mice in each experimental group unless otherwise stated.

3.15. Ethics statement

The experiments described in this thesis were approved by the University of Manitoba Animal Care Committee and carried out in accordance with the regulation of the Canadian Council on Animal Care (Protocol Number 14-014).

4. CHAPTER FOUR

Effect of Low-dose Intradermal *T. congolense* Infection in the Enhanced Susceptibility of Mice to Subsequent Re-infection

4.1. Background and Rationale:

African trypanosomiasis (AT) is a disease that poses a serious threat to humans and livestock in sub-Saharan Africa. The disease is caused by several species of the extracellular hemoprotozoa belonging to the genus *Trypanosoma.* Although it is estimated that over 50 million people living in 25 countries are at risk of contracting the disease, the number of reported cases per year has dramatically reduced (~10000 new cases annually although the actual number of cases might be up to 30000-40000 per year) due to increasing efforts to combat the disease [9-11]. *Trypanosoma congolense* is one of the most important pathogens for cattle and it is estimated that 3 million head of cattle die annually from the associated disease [3], leading to an annual loss of about US $1.3 billion resulting directly from death, reduced meat and milk production and control costs [181].

Efforts to control African trypanosomiasis have been hampered because of lack of understanding of the mechanisms that regulate disease pathogenesis and host protective immune response against the pathogen. In particular, the parasite's ability to undergo antigenic variation and our lack of understanding of the molecular mechanisms that regulate the process contribute to failure to design an effective vaccine [42]. During infection, trypanosomes constantly modify their variant surface glycoprotein (VSG) during host antibody response, resulting in the fluctuating waves of parasitemia that

characterizes African trypanosomiasis [96,129,153]. Furthermore, infection with African trypanosomes is associated with profound immunosuppression, which increases the susceptibility of the host to the parasite and secondary infections [43,147,154]. Understanding the mechanisms that regulate resistance and/or susceptibility to the disease could reveal novel interventions that might lead to effective disease control [155].

The murine model of experimental African trypanosomiasis has provided insights into the immunopathogenesis of the disease. In particular, C57BL/6 and BALB/c mice have mostly been used to study resistance and susceptibility to *T. congolense* infection. BALB/c mice are highly susceptible to intraperitoneal infection with *T. congolense* and die within ten days after infection [77]. In contrast, C57BL/6 mice are relatively resistant and are able to control several waves of parasitemia and survive for more than four months after infection [77,78]. Most studies in this model utilize the intraperitoneal route of infection and have led to some interesting discoveries [43,147,154,155]. However, the fact that natural infection occurs naturally through the skin of animal suggests that observations made with the intraperitoneal route of infection may not correctly reflect the real events that occur following skin infection. For example, following the bite of an infected tsetse fly and deposition of parasites in the host skin, the parasites first induce a local cutaneous inflammatory response (known as chancre) before migrating from the skin to the blood through the lymphatic system [182,183]. Thus, the intraperitoneal route of infection bypasses these early but important host responses that may ultimately dictate the outcome of infection. Indeed, a recent intradermal infection model shows that the outcome of the infection is very different, with mice being relatively (about 1000

times) more resistant to intradermal than the intraperitoneal route [81]. Paradoxically, primary low dose intradermal infection predisposes to enhanced susceptibility following a challenge infection. However, the mechanisms of this low dose intradermal infection-induced enhanced susceptibility are unknown.

$CD4^+$ T cells that constitutively express CD25 and the transcription factor Foxp3 (called regulatory T cells, Tregs) have been shown to play a major role in immune homeostasis by actively suppressing several pathological and physiological immune responses in the host [156-158]. Although their primary role is to prevent autoimmunity and suppress inflammatory responses, Tregs have also been implicated in the pathogenesis of several infectious diseases including those caused by parasites [108,159,160]. In particular, increased numbers of $CD4^+CD25^+Foxp3^+$ Tregs have been reported in experimental *T. congolense* infections [110,161] and these cells have been implicated in enhanced susceptibility to the infection [108,110], although the exact mechanisms remain unknown.

In this aspect of my thesis, I investigated the mechanism through which low dose intradermal *T. congolense* infection predisposes mice to enhanced susceptibility to subsequent reinfection.

4.2. Results

4.2.1. Primary Low-Dose Intradermal *T. congolense* Infection and Susceptibility to Challenge Infection Due to Repeated Low-Dose Infection

Although BALB/c mice are highly susceptible to intraperitoneal infection with *Trypanosoma congolense* [77], a recent report showed that these mice are relatively

resistant to an intradermal infection route [81]. Paradoxically, primary intradermal low dose infection predisposes to enhanced susceptibility following re-challenge infection [81]. However, the mechanisms that regulate low dose intradermal infection-induced susceptibility are not known. In agreement with previous report [81], I found that intraperitoneal infection of BALB/c mice with 10^2 - 10^3 *T. congolense* leads to the development of fulminating parasitemia and death within 8 to 10 days (Fig. 1A and B). In contrast, mice infected intradermally with the same dose of parasites did not develop any parasitemia throughout the duration (21 days) of infection. Consistent with a previous report [81], a secondary low dose (10^3) i.d. infection of these mice (that normally does not lead to parasitemia in naïve mice) led to the development of fulminating parasitemia and death within 9-11 days (Fig. 1D and E). These results suggest that although BALB/c mice are resistance to primary low dose intradermal infection, such resistance is associated with immunoregulatory mechanisms that predispose to enhanced susceptibility following reinfection.

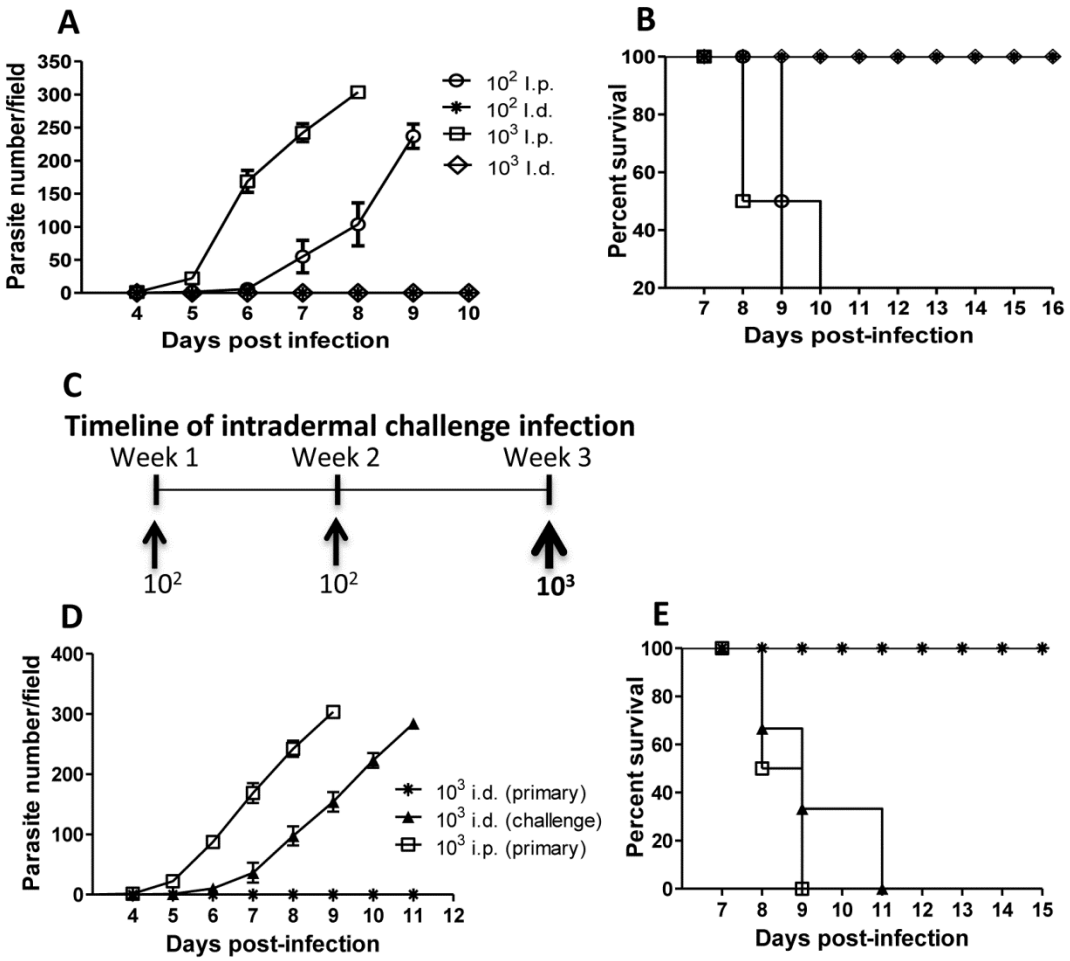

Figure 1: Low dose i.d. *T. congolense* infection and susceptibility to challenge infection. Groups of Balb/c mice were infected intraperitoneally or intradermally (4 mice per group) with 10^2 and 10^3 *Trypanosoma congolense* (clone TC13). At indicated time points, parasitemia (A) was monitored by counting the number of parasites in the blood taken from the tail vein of infected mice by microscopy. The survival of infected mice was also determined (B). Note that all mice infected i.d with 10^2 and 10^3 TC13 controlled the infection while all mice infected i.p. with 10^2 and 10^3 developed infections and died within 8-10 days post-infection. In another experiment, groups of mice were infected i.d. (see Fig. 1C) with 10^2 *T. congolense* once a week for 2 weeks (which does not lead to parasitemia). One week after the last parasite injection, mice were re-infected (challenged) intradermally with 10^3 *T. congolense*. Four naïve age-matched mice were also infected with 10^3 *T. congolense* i.d. or i.p. as controls. Parasitemia (D) and survival period (E) were determined. The results presented are representative of 4 different experiments with similar results. Bars show mean +/-SEM.

4.2.2. Induction of Proinflammatory Cytokines After Low-Dose Intradermal Infection with *T. congolense*

The enhanced susceptibility to experimental intraperitoneal *T. congolense* infection in mice has been associated with overproduction of proinflammatory cytokines, which leads to a cytokine storm and systemic inflammatory response syndrome [83]. Therefore, I compared the levels of proinflammatory cytokines after low dose i.d. and i.p. *T. congolense* infection in order to assess whether differences in the production of these cytokines could account for the apparent enhanced resistance to primary i.d. infection. As shown in Fig. 2A-L, intradermal low dose infection (akin to intraperitoneal infection) was associated with the production of IL-6, IL-10, IL-12, IFN-γ, TNF-α and TGF-β by spleen (2A-F) and draining lymph node (2G-L) cells. Interestingly, spleen cells from mice infected intradermally produced comparable levels of these cytokines (including TGF-β) with the exception of IFN-γ and TNF-α which were significantly higher in the culture supernatant fluids of cells from intraperitoneally infected mice (2D and E). In contrast, the draining lymph node cells from intradermally infected mice produced more IL-10 than those infected intraperitoneally (Fig. 2H).

A previous report showed that B cells and antibodies do not contribute to the enhanced resistance following intradermal *T. congolense* infection [81]. Consistent with this, I found that primary intradermal infection did not induce significant levels of parasite-specific antibodies (Appendix 1A and B). Interestingly, despite the absence of parasitemia, repeated low dose intradermal infection induced significant levels of parasite-specific antibodies that were comparable to those induced following primary intraperitoneal infection (Appendix 1A and B). Taken together, these results indicate

that despite the absence of parasitemia, low dose intradermal *T. congolense* infection results in some level of immune response (induction of antibodies and the production of proinflammatory cytokines by the spleen and lymph nodes cells). This is in agreement with a previous study that reported priming of adaptive immune response very early after intradermal infection [81] even in the absence of parasitemia.

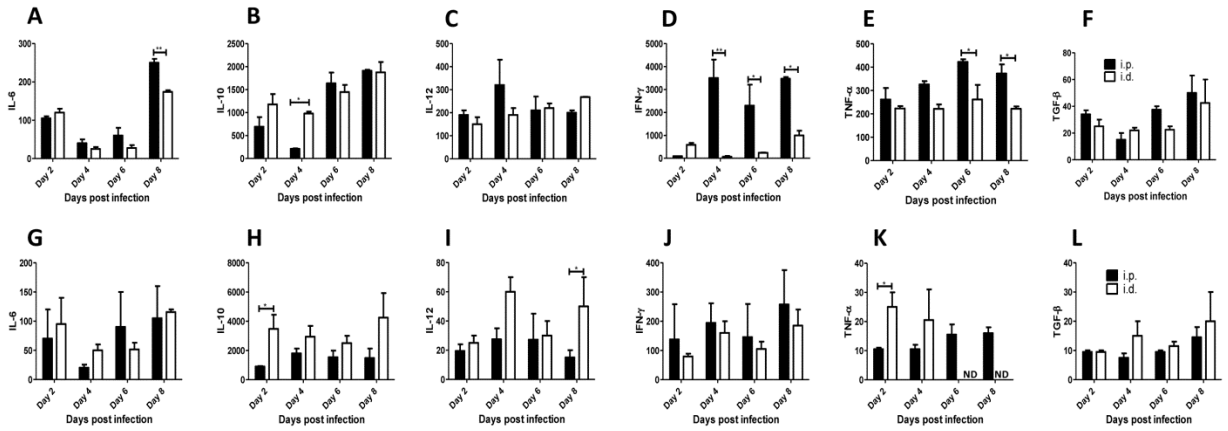

Figure 2: Cytokine profile in mice infected i.d. and i.p. with 10^3 *T. congolense*. Groups of mice (4-5 per group) were either infected i.d. or i.p. with 10^3 *T. congolense* and at indicated times, sacrificed and the spleen and draining lymph node cells were cultured in complete medium in the presence of freeze-thawed *T. congolense*. After 72hr, the levels of IL-6, IL-10, IL-12, IFN-γ, TNF-α and TGF-β in the cell culture supernatant fluids were determined by ELISA and expressed as change (pg/ml) over levels in cells from uninfected (naïve) animals. Figs. 2A-F represent cytokine level in the spleen and Figs. 2G-L represent cytokine levels in the lymph node. The data presented are representative of 3 different experiments with similar results. Bars show mean +/- SEM; *, $p < 0.05$; **, $p < 0.01$. ND; not detected i.e. below the ELISA sensitivity.

4.2.3. Primary Low-Dose Intradermal Infection Does Not Cause Latent Parasitemia but Induces Durable Susceptibility

Unlike an intraperitoneal infection, primary low dose (10^2 - 10^3 parasites) intradermal infection does not lead to parasitemia (Fig.1A). However, intradermal infection leads to robust cytokine response (Fig. 2A-L), comparable to intraperitoneal infection, suggesting that it might cause low level (latent) infection undetectable by the conventional method of assessing parasitemia. To investigate this, I treated low dose-infected mice with cyclophosphamide (3 injections given at three days interval) after 30 days of primary intradermal infection and monitored them for parasitemia for additional 4 weeks. Low dose i.d.-infected mice treated with cyclophosphamide did not develop parasitemia (Fig. 3A). Additionally, I assessed the blood of mice infected by the intradermal route for parasite DNA by PCR. As shown in Fig. 3B, while I could readily amplify parasite DNA in the blood of mice infected i.p., I was unable to amplify parasite DNA in mice infected by the intradermal route at different times after infection. Collectively, these results indicate that primary low dose i.d. infection is completely cleared by the immune system.

Next, I investigated whether low-dose intradermal infection-induced susceptibility is durable. I infected mice with 10^2 *T. congolense* once a week for 2 weeks, waited for additional 40 days and re-challenged them with 10^3 *T congolense*. The results presented in Figure 3 show that low dose intradermal-infected mice were still susceptible to re-challenge infection after 40 days of primary infection as evidenced by the development of fulminating parasitemia (Fig. 3C) and death within 9-12 days post-

challenge (Fig. 3D). Taken together, these results show that low dose intradermal infection does not lead to latent infection and the susceptibility it induces is long lasting.

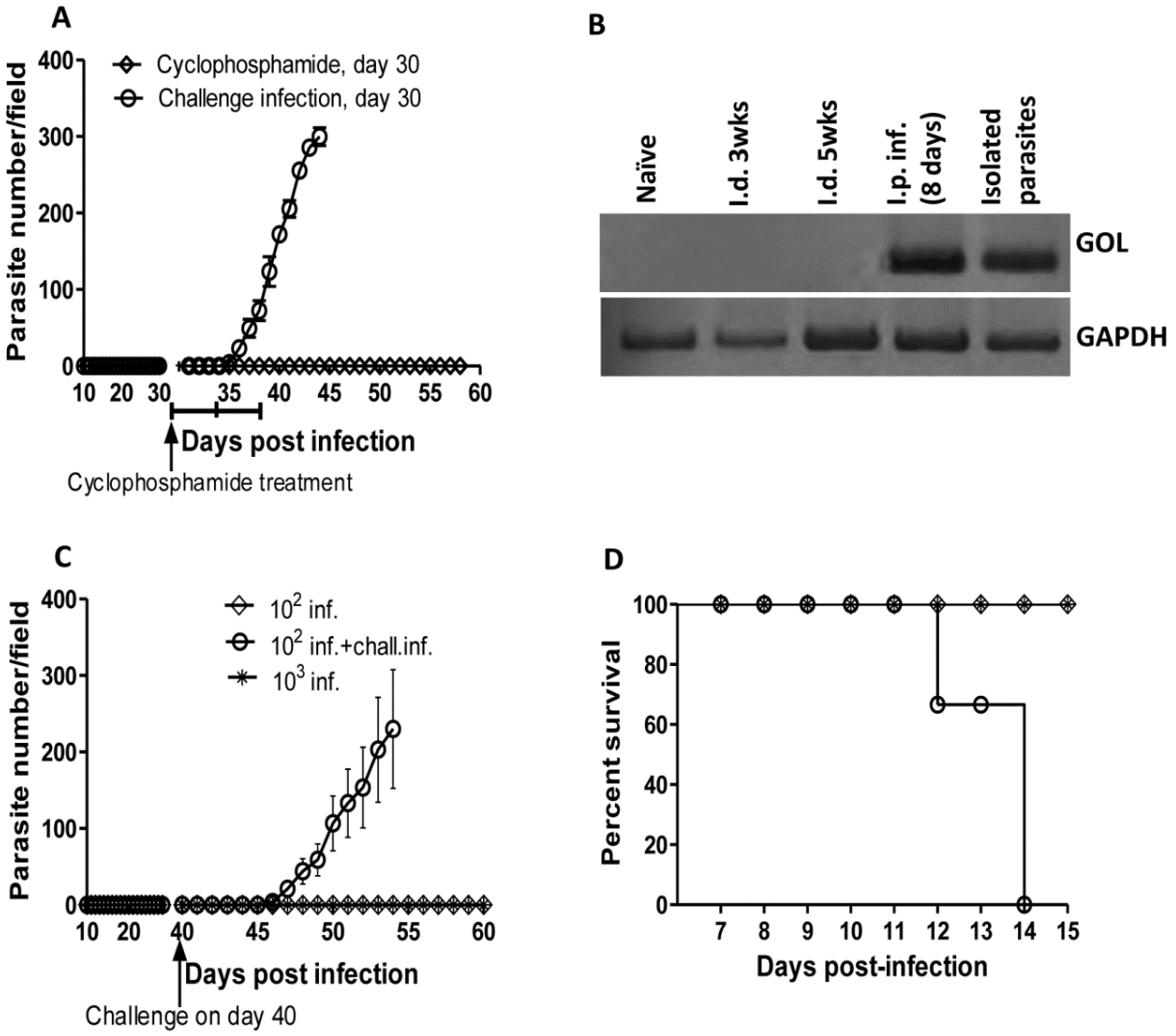

Figure 3. Primary low dose intradermal infection-induced susceptibility is durable. BALB/c mice (n = 6) were infected intradermally with 10^2 *T. congolense* weekly (for 2 consecutive weeks, see Fig. 1C), after 30 days of no detectable parasitemia, some mice (n = 3) were treated three times with cyclophosphamide (at 3 days interval) while others were challenged (i.d.) with 10^3 *T. congolense* and monitored for parasitemia (A). In addition, blood samples from uninfected (naïve) or infected mice (i.d. or i.p.) were assessed for the presence of *T. congolense* DNA by PCR. Parasites purified from the blood of i.p.-infected mice by DEAE-cellulose anion exchange chromatography were used as positive control (B). In another experiment, BALB/c mice (n = 8) were infected (i.d.) twice with 10^2 *T. congolense* (once a week for 2 weeks, see Fig. 1C). After 40 days of no detectable parasitemia, some mice (n = 4) and some age-matched controls (n = 3) were re-infected with 10^3 *T. congolense* and parasitemia (C) and survival (D) were monitored. The results presented are representative of 2 different experiments with similar results. Bars show mean ± SEM.

4.2.4. Induction of Regulatory T Cells Following Low-Dose Injection of Mice with *T. congolense*

Because previous studies (including those from our laboratory) have shown that regulatory T cells (Tregs) prevent the control of infection with *T. congolense* [108,110], I wondered whether low dose intradermal infection was associated with the expansion of these cells. Therefore, I infected mice intradermally with 10^3 *T. congolense* and on the indicated days assessed the percentages and absolute numbers of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the spleens and draining lymph nodes by flow cytometry. For comparison, I also included mice infected intraperitoneally in this study. My results show that despite the absence of parasitemia, the percentages and absolute numbers of Tregs in the spleens (Fig. 4A-D) and lymph node (Fig. 4E-H) significantly increased after low dose intradermal akin to intraperitoneal infection. This increased Tregs expansion was sustained such that after 3 weeks of primary intradermal infection, the number of Tregs was similar to that observed after 4 days of primary intradermal infection (Fig. 5 A and B). In addition, Tregs were further expanded following secondary low dose intradermal reinfection (Fig. 5A and B). Collectively, these results show a strong association between intradermal low dose *T. congolense* infection and increased numbers of Tregs in the spleen and draining lymph node. These data suggest that the expansion of Tregs in mice after low dose intradermal injection could play a role in the enhanced susceptibility following challenge infection.

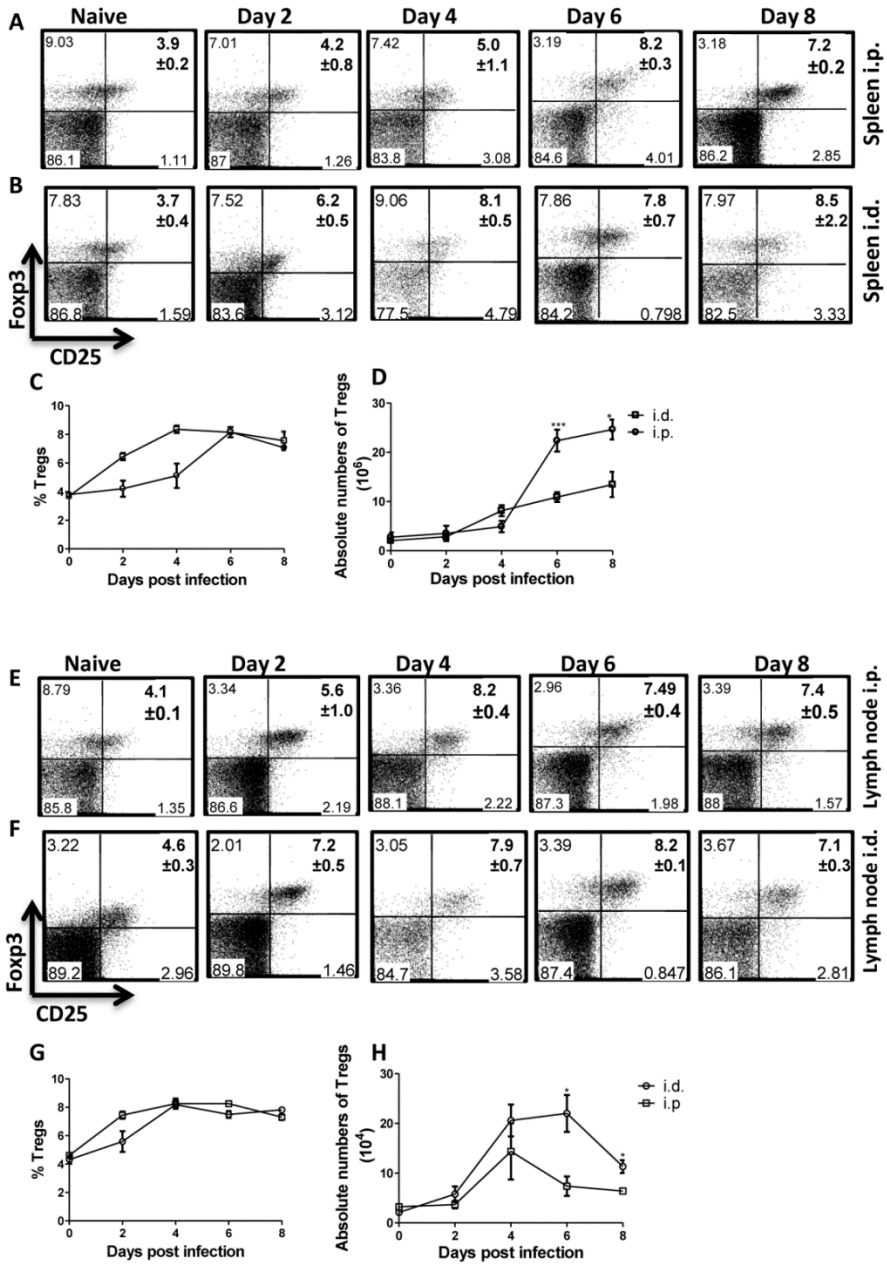

Figure 4. Low dose intradermal *T. congolense* infection leads to systemic expansion of $CD4^+CD25^+Foxp3^+$ (Tregs) cells. Groups of mice (4-5 mice per group) were infected either i.d. or i.p. with 10^3 *T. congolense*. At the indicated days, infected mice were sacrificed and single cell suspensions of the spleen and draining lymph nodes were stained with fluorochrome-conjugated antibodies against CD4, CD25, and Foxp3, and the percentages (A, B, C, E, F, and G) and absolute numbers (D and H) of Tregs were assessed by flow cytometry. A, B, E, and F are representative dot plots (percentages and SD) while C and G are line graphs of the means ± SE of 4-5 mice per group. The data presented are representative of 3 different experiments with similar results. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

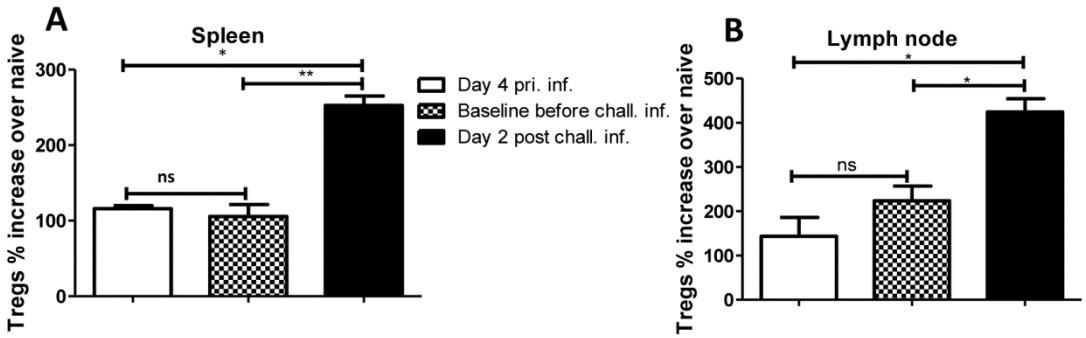

Figure 5. Expansion of Tregs following primary intradermal infection is sustained. Eight mice were infected intradermally with 10^2 *T .congolense* weekly (for 2 consecutive weeks). One week after the last infection, some mice (n = 4) were sacrificed to determine the baseline levels of Tregs. The remaining mice (n = 4) and some age-matched naïve controls were challenged intradermally with 10^3 *T. congolense* and sacrificed 4 days post challenge to determine the levels of Tregs. Shown are the percentages of $CD4^+CD25^+Foxp3^+$ cells (Tregs) in the spleens (A) and the lymph nodes (B) draining the infection site, presented as percent fold increase over the naïve (uninfected) mice. The data presented are representative of 2 different experiments with similar results. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$. ns; not significant.

4.2.5. Depletion of Tregs Abolished the Susceptibility Observed Following Intradermal Challenge Infection of Mice

I found that low dose intradermal *T. congolense* infection of mice led to the expansion of Tregs in the spleen and draining lymph node of mice infected intradermally (Figs 4B, C, F, and G; Fig. 5A and B). Because previous studies showed that Tregs negatively influence the outcome of *T. congolense* infection in mice [108,110], I hypothesized that depletion of Tregs during challenge infection will abrogate the enhanced susceptibility mediated by repeated low dose intradermal infection. I, therefore, injected mice repeatedly with 10^2 *T. congolense* (2 times with a one-week interval) and on the third week, injected them with the anti-CD25 monoclonal antibody (PC61) to deplete Tregs as our group has done previously [110,180]. Twenty-four hrs later, mice were challenged intradermally with 10^3 *T. congolense*. As shown in Fig. 6A and B, and consistent with my findings (Fig. 1D and E) repeated primary low dose intradermal infection led to enhanced susceptibility to 10^3 challenge infection, with all mice developing parasitemia and dying by day 12 post-challenge infection. In contrast, depletion of $CD25^+$ cells (by anti-CD25 monoclonal antibody treatment) prior to challenge infection completely abolished this susceptibility such that mice treated with anti-CD25 mAb (but not isotype control) did not develop any parasitemia and survived for over 21 days after challenge infection when the experiment was terminated (Fig. 6A and B). Furthermore, sera from mice treated with anti-CD25 produced significantly lower amounts of TGF-β (Fig. 6C) and IL-10 (Fig. 6D) when compared with the isotype control-treated group. However, the production of TNF-α and IL-6 by these cells did not change (data not shown). Collectively, these results indicate that Tregs expand after low

dose intradermal infection and mediate susceptibility to intradermal challenge infection of mice.

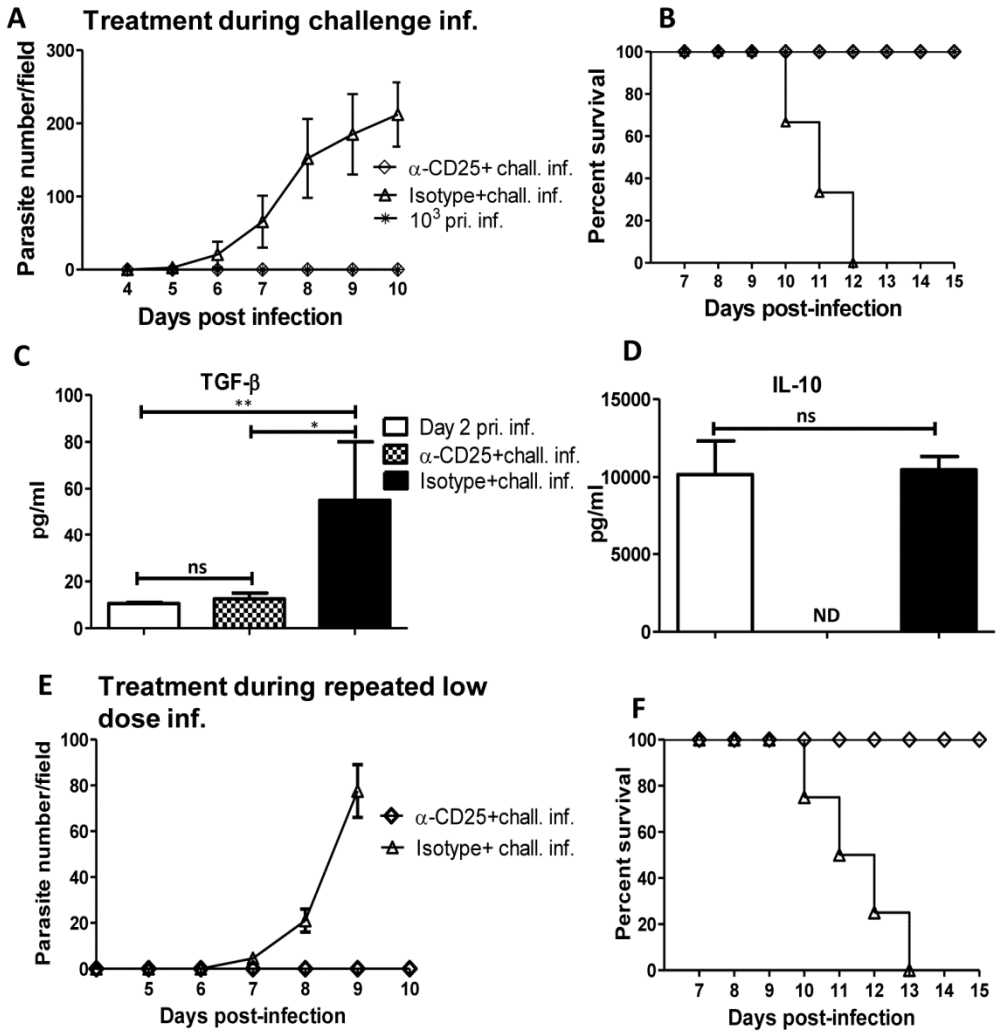

Figure 6. **Depletion of Tregs abolishes intradermal low dose-enhanced susceptibility to *T. congolense* infection**. Groups of mice (n = 4-5 mice) were injected weekly with 10^2 *T. congolense* or PBS (for 2 consecutive weeks). On the 3rd week, mice were treated with anti-CD25 mAb (PC61, 100 µg/mouse) or isotype-matched control mAb and re-challenged with 10^3 *T. congolense* the next day. Daily parasitemia (A) and survival period (B) were monitored as described in the materials and methods. At the humane endpoint, mice were sacrificed and sera were assessed for TGF-β (C) and IL-10 (D) by ELISA. In another experiment, groups of mice were injected with anti-CD25 or isotype control mAb (n = 3-4 mice/group) at each point of primary low dose (10^2) *T. congolense* infection (once weekly for 2 weeks). On the 3rd week, the mice were challenged with 10^3 *T. congolense* and parasitemia (E) and survival period (F) were determined. The data presented are representative of 3 (A-D) and 2 (E and F) different experiments with similar results. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$. ns; not significant, ND; not detected i.e. below the ELISA sensitivity.

4.2.6. Depletion of Tregs at Each Point of Repeated Low-Dose Infection Before Challenge also Abolished Susceptibility to Challenge Infection

I found that repeated low dose intradermal infection leads to expansion of Tregs and enhanced susceptibility to challenge infection and depletion of these cells at the time of challenge infection abolished this susceptibility (Figs. 4 and 6). Next, I wondered whether depletion of Tregs at the time of primary low dose infections would also abolish low dose-induced susceptibility to challenge infection. I, therefore, treated mice with anti-CD25 mAb 24 hours before each 10^2 low dose intradermal infection (once weekly for 2 weeks). On the 3rd week, I challenged the mice with 10^3 *T. congolense* to determine the outcome of infection following anti-CD25 mAb treatment. In contrast to isotype-treated controls, anti-CD25 mAb-treated mice remained disease free following challenge infection (Fig. 6E and F). These results strongly suggest that low dose intradermal infection leads to induction of Tregs that mediate susceptibility to *T. congolense* challenge infection.

4.2.7. Depletion of Macrophages Does Not Alter the Course of Primary Intradermal Infection

Because it was shown that iNOS deficient mice were susceptible to primary intradermal infection with *T. congolense*, macrophages were proposed as an important cell type in the innate control of primary intradermal infection [81]. Therefore, I investigated the role of macrophages during primary and challenge intradermal infection with *T. congolense*. Results presented in Fig. 7A shows that depletion of macrophages (using chlodronate liposomes) did not alter the resistance observed in primary intradermal infection as mice

depleted of macrophages were still resistant to primary low dose i.d. infection and survived until the end of the experiment (7B). Furthermore, when these mice were challenged after 3 weeks with 10^3 *T. congolense*, they developed parasitemia and succumbed to the infection within 12 days, akin to mice that were not depleted of macrophages. Collectively, these results suggest that macrophages are not primarily responsible for the relative resistance to low dose intradermal *T. congolense* infection.

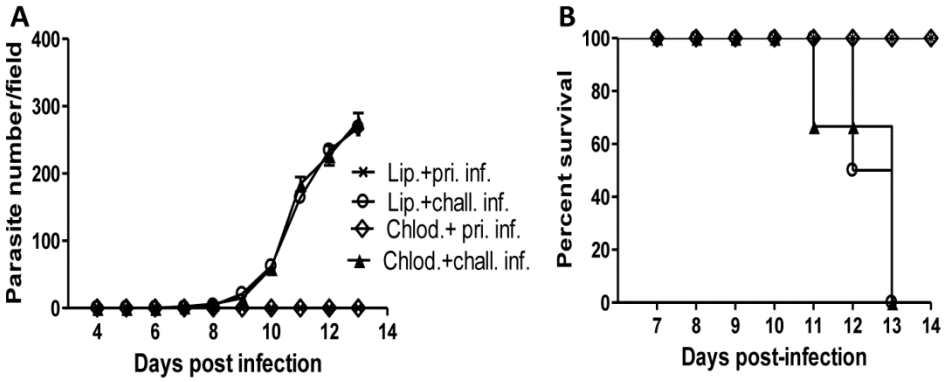

Figure 7. Depletion of macrophages does not abolish primary low-dose i.d. resistance and susceptibility to re-challenge infection. Groups of BALB/c mice (n = 4) were treated with liposomal chlodronate, (to deplete macrophages) or with control liposome. After 24 hrs, all mice were infected i.d. with 10^2 *T. congolense* and monitored for parasitemia (A). Three weeks after primary infection (without parasitemia), all mice were re-challenged with 10^3 *T. congolense* and parasitemia (A) and survival (B) were monitored. The results presented are representative of 2 different experiments with similar results. Bars show mean +/-SEM.

5. CHAPTER FIVE

Role of Natural Killer (NK) Cells in the Maintenance of Optimal Immunity to *Trypanosoma congolense* Infection

5.1. Background and Rationale:

Parasitic diseases including African trypanosomiasis still remain global health problem. African trypanosomiasis (aka sleeping sickness) is a disease that is caused by blood protozoan parasites that affect human and livestock [184]. Millions of people risk being infected with this disease and new cases continue to emerge annually [184]. The animal form of this disease has over a hundred fold prevalence than the human form [21], and both disease forms continuously pose agricultural and economic problems in the affected regions [185]. The disease is fatal if not treated, and the available treatment options are hampered by severe side effects [66,186] and lack of new drug designs [1]. Vaccination attempts have not been successful due in part to antigenic variation [42]. In addition, impaired lymphocyte proliferation during infection has also been reported to add to host's susceptibility [43,147,154]. More research towards exploring various regulatory mechanisms that influence resistance and /or susceptibility to the disease could help improve the search for effective vaccine [155].

Suppression of T cell responses during *T. congolense* infection is a hallmark of the disease and a major contributor to susceptibility [43,147,154]. T cells, especially $CD4^+$ T cells not only produce effector cytokines during *T. congolense* infection, they also help class-switching of B cells to produce optimal antibodies necessary for parasite clearance [122]. Antibody subtypes including IgG1, IgG2a, and IgG3 have all been

associated with resistance to *T. congolense*-infected animals [43,122] by enhancing opsonization and phagocytosis [82]. Cytokines, specifically IFN-γ and TNF-α are associated with early protection during African trypanosome infection [44,133-135], although their production in excessive amounts could contribute to susceptibility to infection [83,97,131]. Despite the importance of these key cytokines in early protection to the infection, studies looking at the cells making them have been inadequately conducted.

Natural killer cells belong to innate lymphocytes that regulate both the innate and adaptive immune response. They mediate their effector function through cytotoxicity or cytokine release [162,163]. NK cells predominantly produce IFN-γ and TNF-α, key cytokines that are important for protection during the early phase of *T. congolense* infection [44,133-135]. For NK cells to function optimally, adequate interaction with their surrounding cells (dendritic cells, macrophages, and T cells) and cytokines are critical [187]. IFN-γ and TNF-α produced by NK cells enhance the maturation and activation of dendritic cells, macrophages and T cells [162,188,189]. On the other hand, cytokines like IL-15 and IL-2 are important for NK cell activation and function by inducing their proliferation as well as cytotoxicity and cytokine production [188,190,191]. Most cytotoxic functions of NK cells occur through the release of perforin and granzymes which mediate the killing of target cells [192].

Regulatory T cells (Tregs) are known to promote disease during *T. congolense* infection [108,110]. Interestingly, these cells have been reported to regulate the activities of NK cells in a TGF-β-dependent manner [193,194].

Since NK cells are key producers of early IFN-γ and TNF-α and because these cytokines have both been shown to mediate early protection in *T. congolense*-infected mice [44,133-135], it is conceivable that NK cells play important roles in early immune response and protection to this infection. Therefore, I hypothesized that NK cells would be critical in mediating resistance in *T. congolense*-infected mice.

In this section, I investigated the role of NK cells in immunity to experimental *T. congolense* infection.

5.2. Results

5.2.1. Increased NK Cell Numbers, Activating Receptor, Degranulation, and Cytokine Production After Infection

Early protection from *T. congolense* infection in mice has been associated with the production of IFN-γ and TNF-α [44,133-135]. Because NK cells are considered to be key innate producers of IFN-γ and TNF-α [163,190,191], I hypothesized that they would be vital in mediating protection against *T. congolense* infection in mice. First, I assessed the numbers of NK cells in various tissue compartments (spleen, liver, blood, lung and peritoneal cavity) after infection with *T. congolense*. I found that NK cell numbers significantly increased in these tissues after infection (Fig. 8A), suggesting that they could be playing a role during infection with *T. congolense*.

Next, I wanted to assess activation and function of NK cells following infection. I found that in addition to increasing in numbers, NK cells also displayed increased expression of the NKP46 activating receptor (Fig. 8B) as well as increased production of IFN-γ (Fig. 8C) and TNF-α (Fig. 8D) in the various tissue compartments assessed. In addition, they

also showed increased degranulation after infection as assessed by CD107a expression (Fig. 8E). These results clearly show expansion and effector activities of NK cells shortly after infection with *T. congolense*, suggesting a possible role for this cell population in protection during infection.

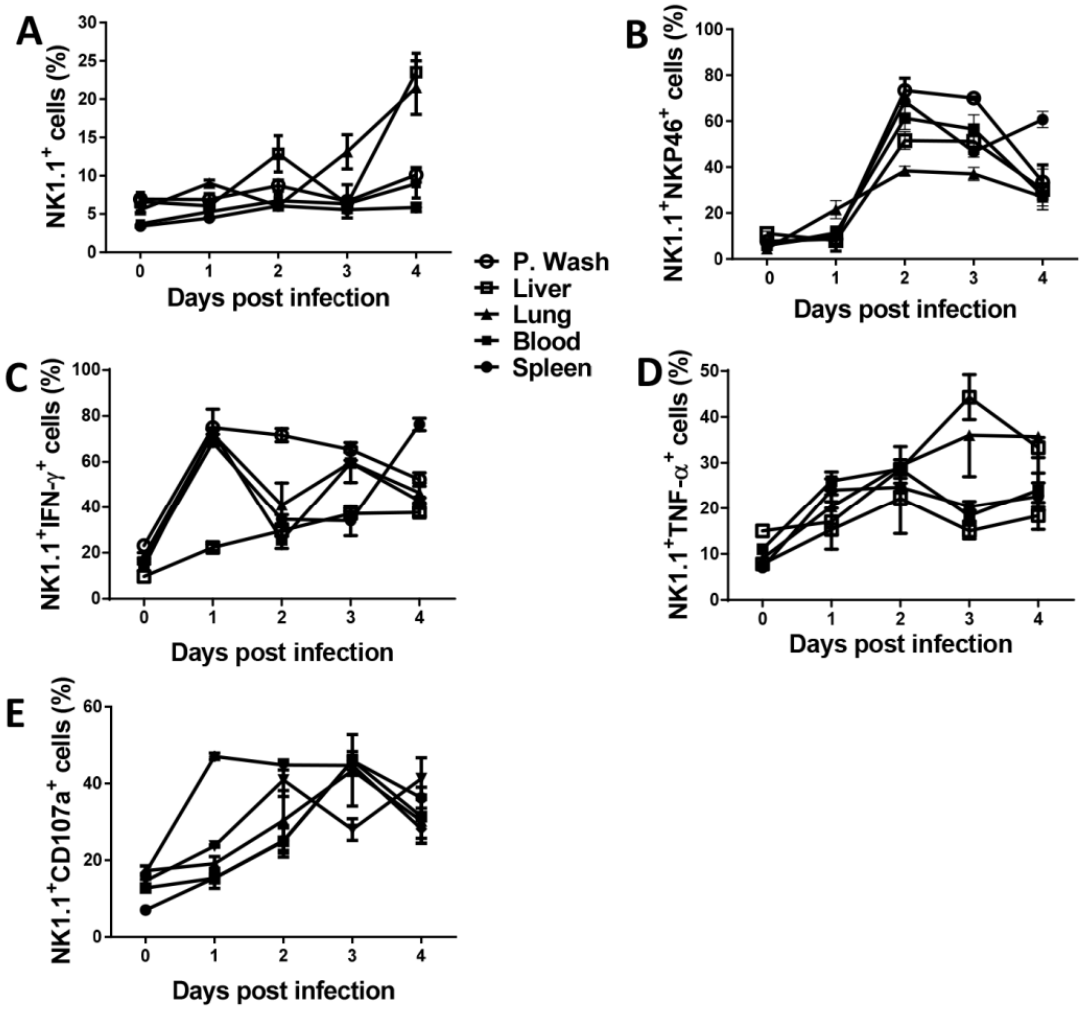

Figure 8. Numbers of NK cells and their expression of NK1.1, NKp46, IFN-γ, TNF-α and CD107a in various tissues from *T. congolense*-infected mice. Mice were infected intraperitoneally with 10^3 *T. congolense.* At indicated times, infected mice were sacrificed, cells from the spleen, blood, peritoneal wash, liver, and lung were harvested and processed into single cell suspensions. The cells were assessed for NK1.1 (A), NKp46 (B), IFN-γ (C), TNF-α (D) and CD107a (E) expressions by flow cytometry and presented as line graphs (A-E). Cells were first gated for NK1.1 expression and assessed for NKp46, IFN-γ, TNF-α and CD107a expressions. Results are representative of 2 different experiments (n = 4-5 mice) with similar outcomes. Line graphs show mean +/-SEM.

5.2.2. Depletion of NK Cells Leads to Increased Parasitemia and Reduced IFN-γ Production by Immune Cells from Infected Mice.

Because I observed increased numbers of NK cells in multiple tissue compartments after infection (see Fig. 8A), I wanted to further assess the importance of these cells during *T. congolense* infection. I hypothesized that depletion of NK cells would make the relatively resistant mice more susceptible to *T. congolense* infection. To address this, I depleted NK cells in mice with anti-NK1.1 monoclonal antibody before infection with *T. congolense* and assessed daily parasitemia. As shown in Fig 9A, mice treated with anti-NK1.1 showed increased parasitemia when compared with their isotype-matched antibody treated control group. This increased parasitemia following NK cell depletion was also associated with impaired ability of $CD3^-$ (Fig. 9B, C, E & F) and $CD3^+$ (Fig. 9B, D, E & G) T cell population to produce IFN-γ in both the spleens (Fig. 9B-D) and liver (Fig. 9E-G) of *T. congolense*-infected mice. Together, these results indicate that depletion of NK cells led to increased parasitemia and reduced IFN-γ production by immune cells during *T. congolense* infection.

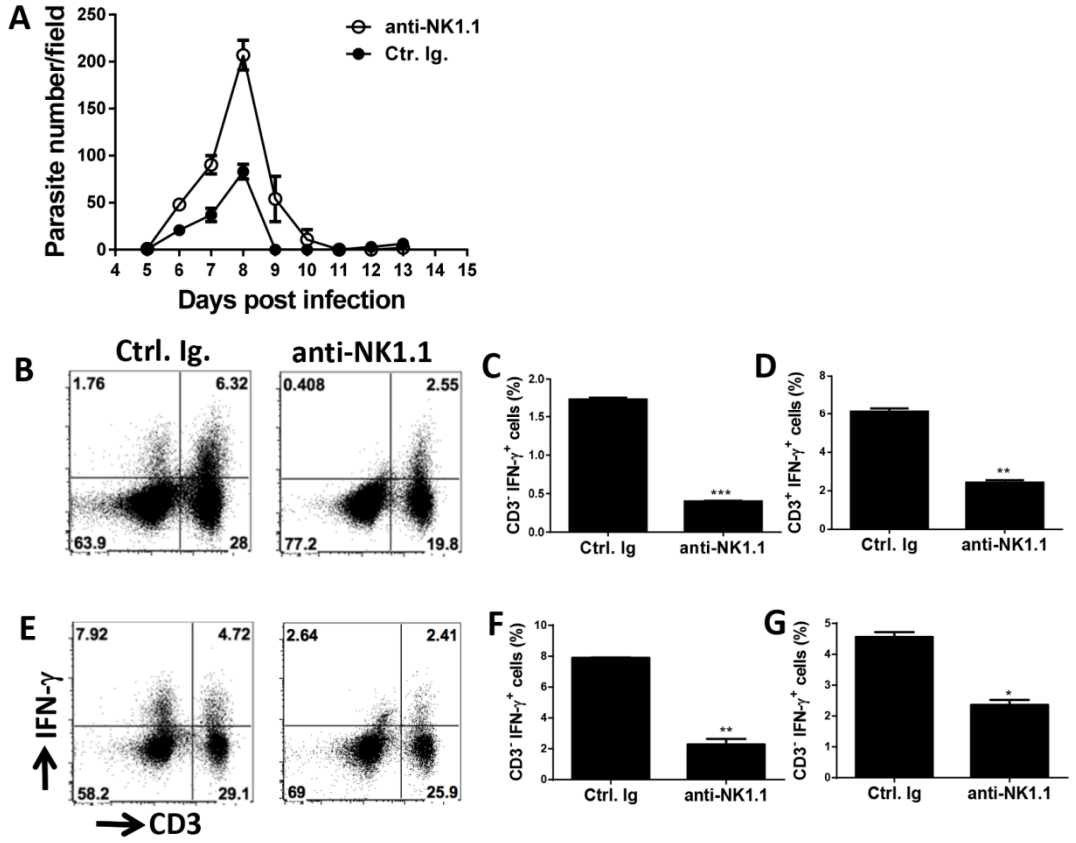

Figure 9. Increased parasitemia and reduced IFN-γ production by immune cells after NK cell depletion. Groups of mice (n = 4-5 mice per group) were injected intraperitoneally with anti-NK1.1 mAb (PK136, 500 µg/mouse) or isotype-matched control mAb a day before intraperitoneal infection with 10^3 *T. congolense* and on day 4 after infection. Daily parasitemia (A) was monitored. At sacrifice, splenic (B-D) and liver (E-G) cells were assessed for IFN-γ production by $CD3^+$ and $CD3^-$ populations. The data presented are representative of 3 different experiments with similar results. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

5.2.3. Deficiency of NK Cells Leads to Enhanced Susceptibility to *T. congolense* Infection

Because of the observed increase in parasitemia (Fig. 9A) and impaired ability of immune cells to produce IFN-γ (Fig. 9B-G) in NK cell-depleted mice, I hypothesized that genetic ablation of NK cells would increase the susceptibility of mice during *T. congolense* infection. To directly assess this, I utilized NFIL3$^{-/-}$ mice, which lack NK cell development, maturation, and function [174,195]. I first confirmed that NK cell numbers were dramatically absent in NFIL3$^{-/-}$ mice without any significant reduction in other lymphocyte populations including T cells, B cells, macrophages, total CD11b- and CD11c-expressing cells (Fig. 10 A-G).

Next, I infected these mice together with their WT counterpart control mice and assessed parasitemia and survival period. NFIL3$^{-/-}$ mice on the usually resistant background developed fulminating parasitemia (Fig. 10H) and succumbed to infection (mean survival time 13±2 days, Fig. 10I), unlike the WT control mice that had low parasitemia and survived till day 20 after infection when the experiment was terminated. The enhanced susceptibility of *T. congolense*-infected NFIL3$^{-/-}$ mice was associated with significant reduction in the levels of IFN-γ (Fig. 11A, B, E & F) and TNF-α (Fig. 11C, D, G & H) production in both the spleen (Fig. 11A-D) and liver (Fig. 11E-H) CD3^{+} T cells. Collectively, these results show that NK cells are critical in parasite control and cytokine production in *T. congolense*-infected mice.

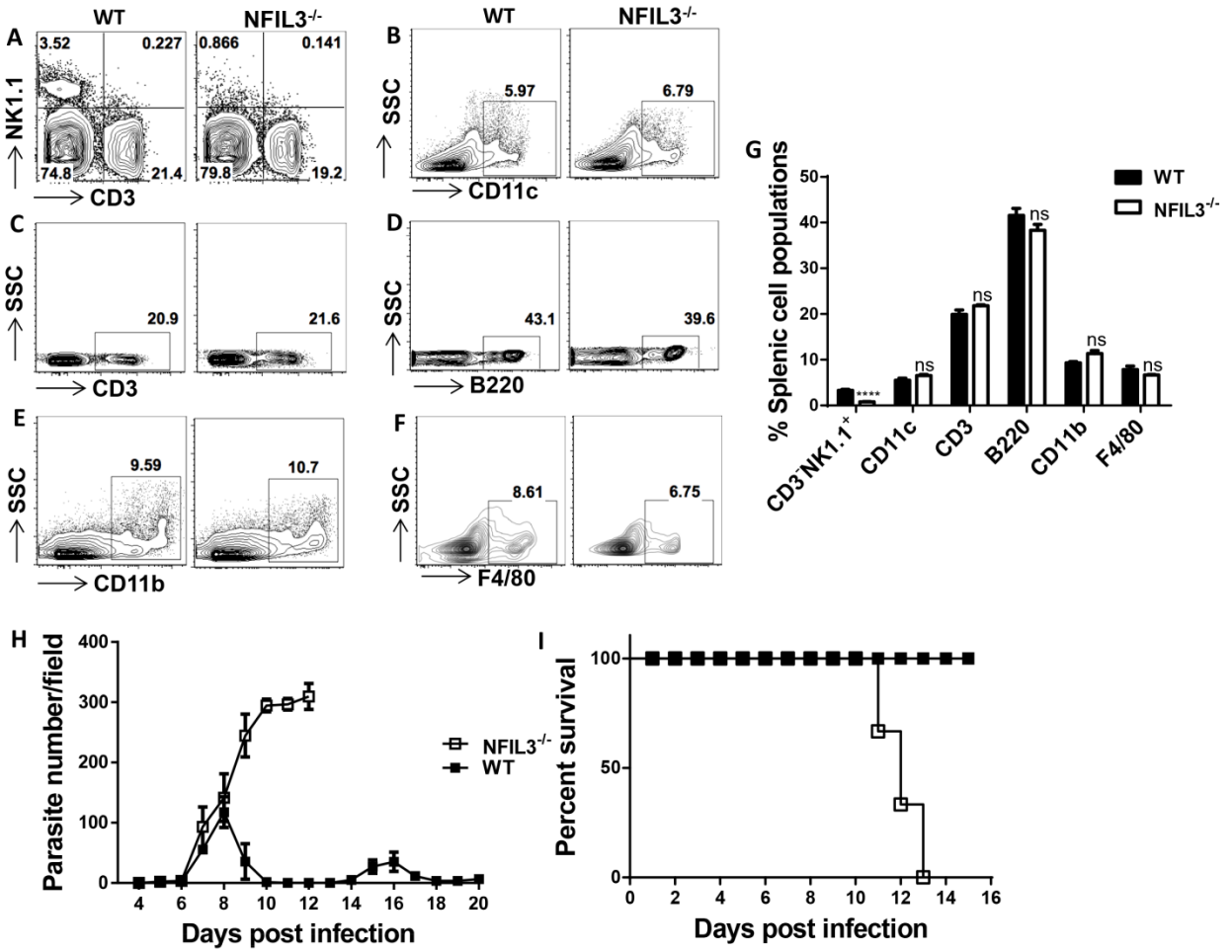

Figure 10. Enhanced susceptibility of NFIL3$^{-/-}$ mice to *T. congolense* infection. Groups of uninfected WT and NFIL3$^{-/-}$ mice were sacrificed and the levels of CD3^{-}NK1^{+} (A), CD11c (B), CD3 (C), B220 (D), CD11b (E) and F4/80 (F) -expressing cells were assessed in the spleens. In another experiment, groups of WT and NFIL3$^{-/-}$ mice (n = 5 mice per group) were infected intraperitoneally with 10^3 *T. congolense.* At indicated time points, parasitemia (H) was monitored by counting the number of parasites in the blood taken from the tail vein of infected mice by microscopy. The survival of infected mice was also determined (I). The data presented as contour plots (A-F) and bar graphs (G) are representative of 2 different experiments with similar outcomes. The results presented as line graphs (H & I) are representative of 3 different experiments with similar results. Bars and line graphs show mean +/-SEM; ns, not significant.

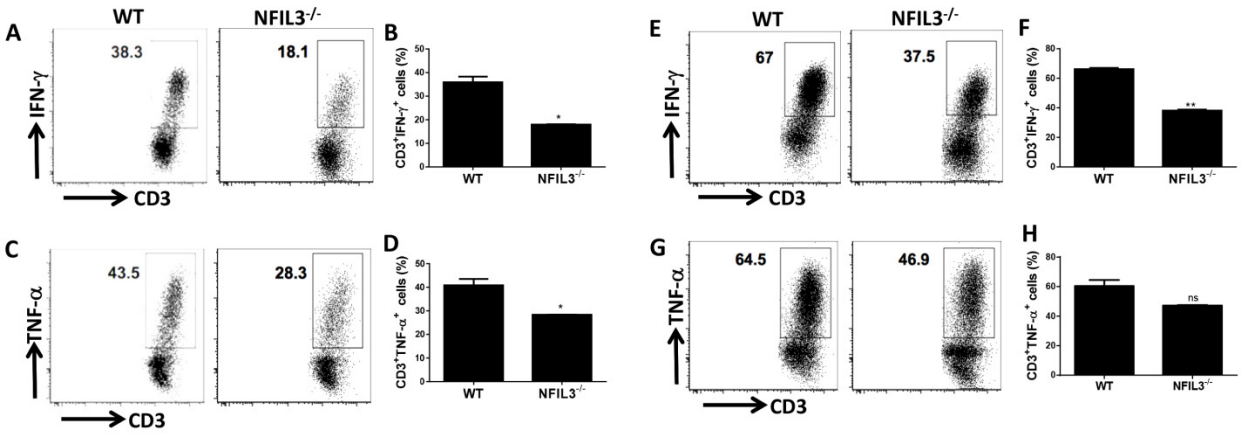

Figure 11. Reduced IFN-γ and TNF-α production by $CD3^+$ cells in infected $NFIL3^{-/-}$ mice. Groups of WT and $NFIL3^{-/-}$ mice (n = 5 mice per group) were infected intraperitoneally with 10^3 *T. congolense.* On day 11 after infection, mice were sacrificed and the percentages of IFN-γ (A, B, E & F) and TNF-α (C, D, G & H) production by $CD3^+$ cells were assesses in the spleen (A-D) and liver (E-H) by flow cytometry. The data presented as dot plots (A, C, E & G) and bar graphs (B, D, F & H) are representative of 2 different experiments with similar outcomes. Bars show mean +/- SEM; *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

5.2.4. Adoptive Transfer of Activated WT NK Cells to NFIL3$^{-/-}$ Mice Led to Control of Parasitemia and Prolonged Survival After Infection

In addition to NK cell development and function, NFIL3 has also been shown to regulate some aspects of Th2 cytokine gene expression and IgE class switching [196]. Therefore, I wanted to determine whether the impaired resistance to *T. congolense* infection in NFIL3-deficient mice is strictly related to impaired NK cell numbers and function in these mice. Therefore, I assessed whether adoptive transfer of NK cells from NK cell competent mice into NFIL3-deficient mice would rescue them from acute death following *T. congolense* infection. I isolated splenic NK cells from WT mice, activated them *in vitro* with recombinant IL-2 and IL-15 cytokine cocktail, and adoptively transferred (i.v.) them into NFIL3$^{-/-}$ mice before *T. congolense* infection. Parasitemia and survival were assessed. Results presented in (Fig. 12A & B) show that adoptive transfer of WT NK cells into NFIL3$^{-/-}$ mice before infection led to parasite control (Fig. 12A) and prolonged the survival of infected mice (Fig. 12B), similar to their infected WT counterpart mice. NFIL3$^{-/-}$ mice treated with only PBS developed uncontrolled parasitemia and succumbed to infection (Fig. 12A & B). These results directly confirm that the enhanced susceptibility of NFIL3$^{-/-}$ mice to *T. congolense* infection is related to functional impairment in their NK cells.

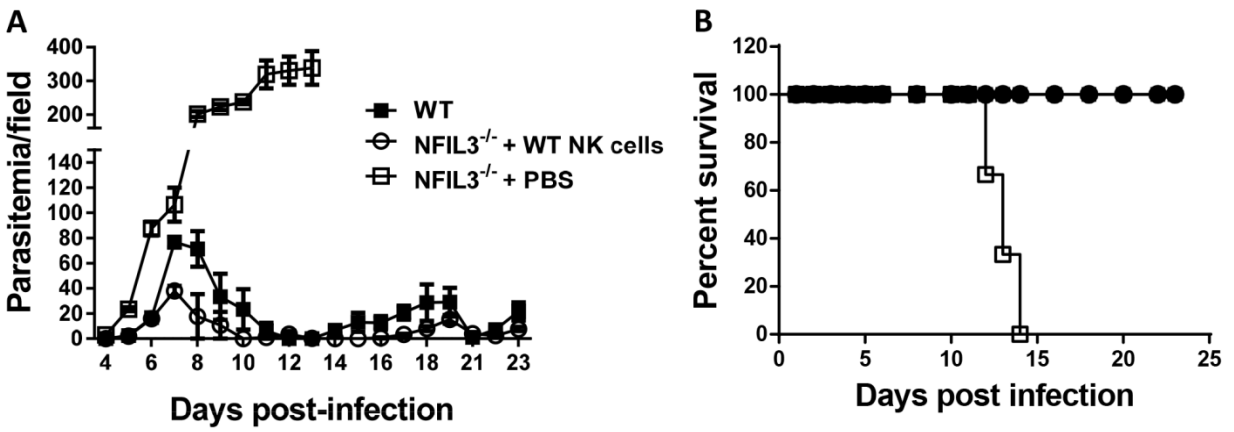

Figure 12. Adoptive transfer of activated NK cells into NFIL3$^{-/-}$ mice. Activated WT NK cells were transferred intravenously into NFIL3$^{-/-}$ mice a day before infection with 10^3 *Trypanosoma congolense.* At indicated time points, parasitemia (A) was monitored by counting the number of parasites in the blood taken from the tail vein of infected mice by microscopy. The survival of infected mice was also determined (B). The results presented are representative of 2 different experiments (n = 4-5 mice per group) with similar results. Graph show mean +/-SEM.

5.2.5. NK Cells Mediate their Protection to *T. congolense* via Perforin-Dependent Mechanism

Since the adoptive transfer of NK cells into NFIL3$^{-/-}$ mice before *T. congolense* infection rescued these mice from death (Fig. 12A & B), I wanted to determine the mechanism through which they mediate this protection. NK cells mediate their effector functions either by direct cytotoxicity via perforins and granzymes or by IFN-γ production [162,163]. Therefore, I isolated NK cells from WT, perforin-deficient (killing) and IFN-γ deficient mice and adoptively transferred them into NFIL3$^{-/-}$ mice before infection with *T. congolense*. Results show that NFIL3$^{-/-}$ mice that received perforin-deficient NK cells failed to control parasitemia (Fig. 13A) and died like the control NFIL3$^{-/-}$ mice treated with PBS (Fig. 13B). Interestingly, NK cell-deficient mice that received IFN-γ deficient NK cells controlled parasitemia and survived longer, similar to the WT control mice and NFIL3 deficient mice that received WT NK cells (Fig. 13A & B). Taken together, these results indicate that NK cells mediate protection to *T. congolense* infection via a perforin-dependent mechanism.

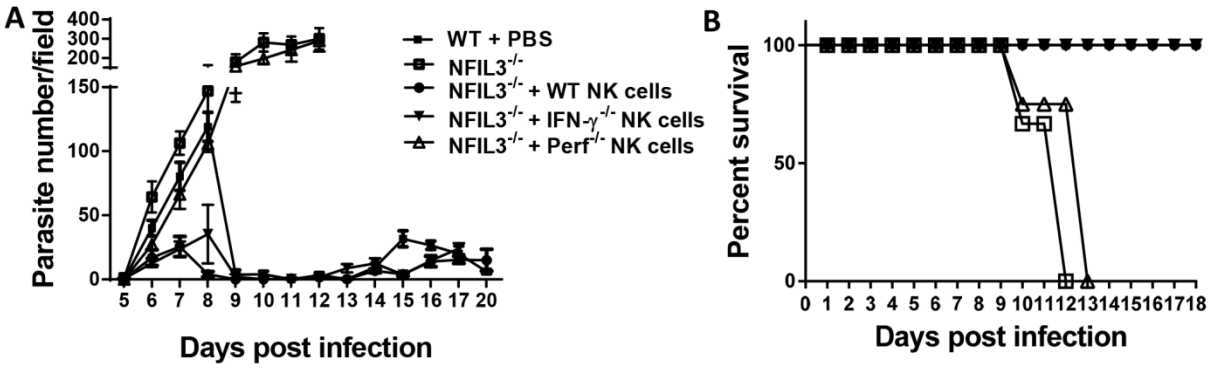

Figure 13. NK cell action after adoptive transfer of WT NK cells into NFIL3$^{-/-}$ mice. NK cells isolated from uninfected WT, IFN-$\gamma^{-/-}$ and Perforin$^{-/-}$ mice were activated in vitro and transferred intravenously into NFIL3$^{-/-}$ mice a day before infection with 10^3 *Trypanosoma congolense.* At indicated time points, parasitemia (A) and survival (B) of infected mice were determined. The results presented are representative of 2 different experiments (n = 4 mice per group) with similar results. Graph show means +/-SEM.

6. CHAPTER SIX

Role of Myeloid-Derived Suppressor Cells (MDSCs) in *T. congolense* Infection

6.1. Background and Rationale:

African trypanosomiasis is a disease caused by various species of extracellular blood parasites affecting both human and livestock. The disease can lead to death as well as huge economic loss in the endemic regions [2,3]. Although annual reported cases of the disease have reduced over the past years due to several interventions, only about 10% of the people living in the endemic areas are accounted for [12,13]. In other words, the majority of the cases remain undiagnosed, suggesting that the disease impact could be worse than reported. The threats caused by these parasites towards livestock is much more than that caused towards human, and among all species that cause the diseases, *Trypanosoma congolense* is well studied experimentally and is perceived as one of the most important pathogens for livestock [21,197]. Drugs targeted towards this disease have been unsatisfactory [66]. Vaccination attempts have also failed because of inadequate understanding of the disease pathogenesis including antigenic variation and immunosuppression [42,129,147].

Suppression of lymphocyte proliferation has been reported to be one of the key problems that hamper parasite control during infection [170,171,198]. T cells, especially $CD4^+$ T cells, play an important role during experimental trypanosome infection; from cytokine production to helping B cell class-switching [96,122]. Considering the importance of $CD4^+$ T cells in resistance, understanding the mechanisms through which

they are suppressed following infection could be beneficial towards improving available vaccination strategies.

Myeloid-derived suppressor cells (MDSCs) are a mixed population of cells that are characterized by the co-expression of CD11b and GR-1 molecules. They are made up of immature myeloid cells that show similar morphology as granulocytes and monocytes but do not express markers specific for identifying macrophages, dendritic cells or monocytes [199,200]. The anti-GR1 antibody is capable of binding two epitopes, Ly6C and Ly6G, which has led to the discovery of two MDSCs subsets; the $CD11b^{+}LY6G^{+}LY6C^{low}$ (granulocytic) and the $CD11b^{+}LY6G^{-}LY6C^{hi}$ (monocytic) MDSCs [200,201]. The anti-GR1 monoclonal antibody (mAb), RB6-8C5, appears to be very specific and advantageous when depleting MDSCs, as it binds to both the Ly6C and Ly6C subsets [202]. In contrast, the anti-Ly6G mAb, 1A8, selectively depletes neutrophils [202] and may not be suitable for depleting MDSCs.

MDSCs mediate suppression of T cells mostly through the upregulation of the arginase-1 and/or inducible nitric oxide synthase (iNOS) [164,165]. Both arginase-1 and iNOS (that generates nitric oxide-NO) use L-arginine as substrate. Increased expression of these enzymes leads to L-arginine catabolism, which eventually leads to a reduction in the availability of L-arginine. This insufficient L-arginine level eventually leads to suppression of T cell proliferation through reduced CD3 zeta-chain and cell cycle regulators on the cells [172,173]. On the other hand, enhanced nitric oxide generation also contribute to suppression of T cell function by inhibiting JAK3 and STAT5 pathways in T cells, and induction of apoptosis in T cells [203-205].

Although the suppressive roles of MDSCs have been well studied in in tumor model [166,167], as well as bacteria and parasitic infections [168,169], no report exists so far about the roles of these cells in *T. congolense* infection.

Because T cell proliferation is impaired during experimental *T. congolense* infection [150,170,171] and because MDSCs are known to suppress T cell proliferation and function [164,166,168,172,173], it is conceivable that MDSCs would play a critical role during *T. congolense* infection. I hypothesised that MDSCs suppress proliferation and function of $CD4^+$ T cells and promote susceptibility during *T. congolense* infection.

In this section of the thesis, I investigated the role of MDSCs in the pathogenesis of experimental *T. congolense* infection in mice.

6.2. Results

6.2.1. MDSCs expand in the spleens and liver of *T. congolense*-infected mice

Suppression of lymphocyte proliferation occurs during infection with experimental African trypanosomes and is thought to be responsible for impaired T cell responses and increased susceptibility of infected animals [150,170,171]. Given that MDSCs are known to suppress T cell proliferation and function [164,165,172,173], I hypothesized that they would play important role in the pathogenesis of *T. congolense* infection. To test this, I infected mice with *T. congolense* and assessed the pattern and kinetics of expansion of MDSCs in both the spleens and liver. There was an increase in the percentages (Fig. 14A, B, D & E) and absolute numbers (Fig. 14C & F) of MDSCs in the spleens (Fig. 14A-C) and liver (Fig. 14D-F) of infected mice. I also assessed the

expansion pattern of monocytic and granulocytic MDSCs (M-MDSCs and G-MDSCs, respectively) and found that after infection, M-MDSCs numbers increased in both the spleens (Fig. 15A & B) and liver (Fig. 15D & E) of infected mice. In contrast, G-MDSCs numbers decreased in both the spleens (Fig. 15A & C) and liver (Fig. 15D & F), with more profound reduction observed in the liver. Collectively, these results indicate that *T. congolense* infection leads to the expansion of MDSCs in the spleen and liver of mice.

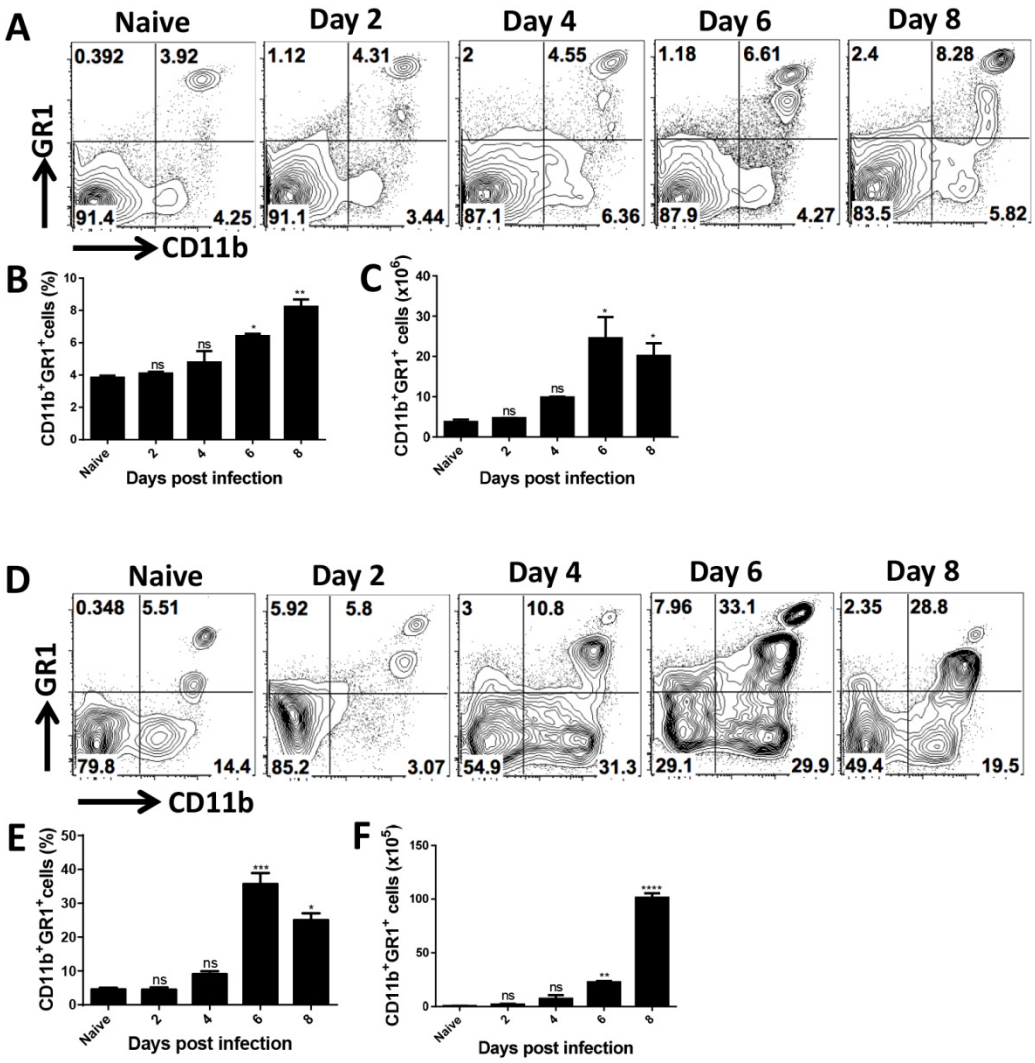

Figure 14. Expansion of MDSCs in mice after *T. congolense* infection. Mice were infected i.p. with 10^3 *T. congolense* and at indicated times, sacrificed and their spleen and liver cells were directly stained *ex vivo* for surface expressions of CD11b and GR1 myeloid-derived suppressor cells (MDSCs). Shown are contour plots (A & D) and bar graphs (B, C, E & F) of the percentages (A, B, D, & E) and absolute numbers (C & F) of $CD11b^+$ $GR1^+$ myeloid-derived suppressor cells in the spleen (A-C) and liver (D-F) at different times after infection. The results presented are representative of three different experiments (n = 4-5 mice) with similar outcomes. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

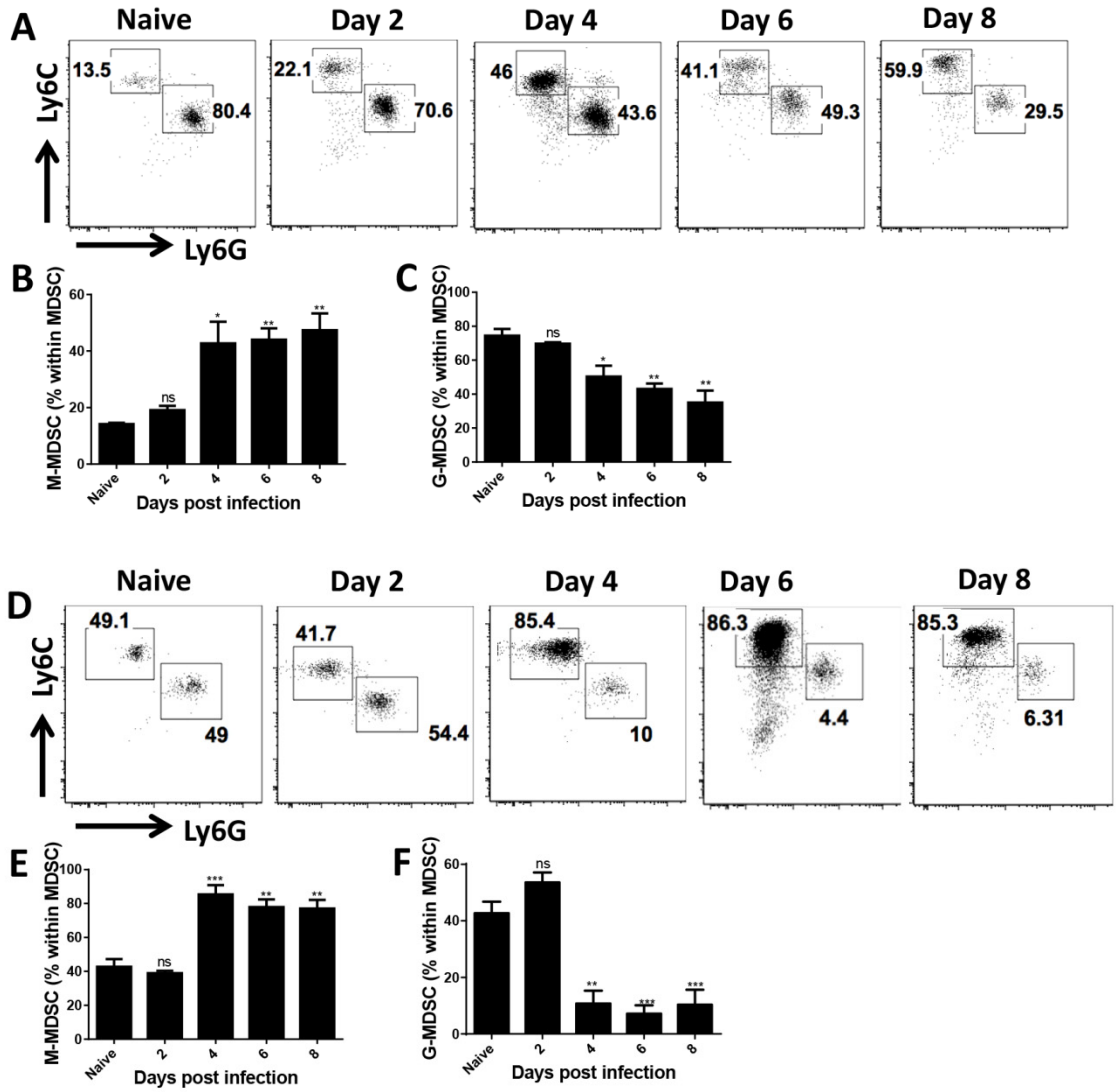

Figure 15. Levels of monocytic and granulocytic subsets of MDSCs in *T. congolense-infected mice*. Mice were infected i.p. with 10^3 *T. congolense* and at indicated times, sacrificed and their spleen and liver cells were directly stained *ex vivo* for surface expressions of CD11b, Ly6C, and Ly6G. Shown are contour plots (A & D) and bar graphs (B, C, E & F) of percentages (A-F) of $CD11b^{+}LY6G^{-}LY6C^{hi}$ monocytic-M-MDSCs (A, B, D & E) and $CD11b^{+}LY6G^{+}LY6C^{low}$ granulocytic-G-MDSCs (A, C, D & F) in the spleen (A-C) and liver (D-F) at different times after infection. The results presented are representative of three different experiments (n = 4-5 mice) with similar outcomes. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

6.2.2. MDSC Depletion has Marginal Effect on Parasitemia but Prolongs the Survival of Infected Mice

I found that *T. congolense* infection led to the expansion of MDSCs in the spleens and liver of mice (see Fig. 14A-F). Because of their suppressive nature, I hypothesized that depletion of MDSCs following *T. congolense* infection would make mice less susceptible to the parasites. I, therefore, injected mice with anti-GR1 (RB6-8C5) monoclonal antibody a day before, and on day 4 after 10^3 *T. congolense* infection and monitored parasitemia and survival. Depletion of MDSCs led to transient parasite control of the first wave of parasitemia and prolonged survival when compared to the control Ig-treated group (Fig.16A and B). These results suggest that MDSCs could be contributing to susceptibility of mice to experimental *T. congolense* infection.

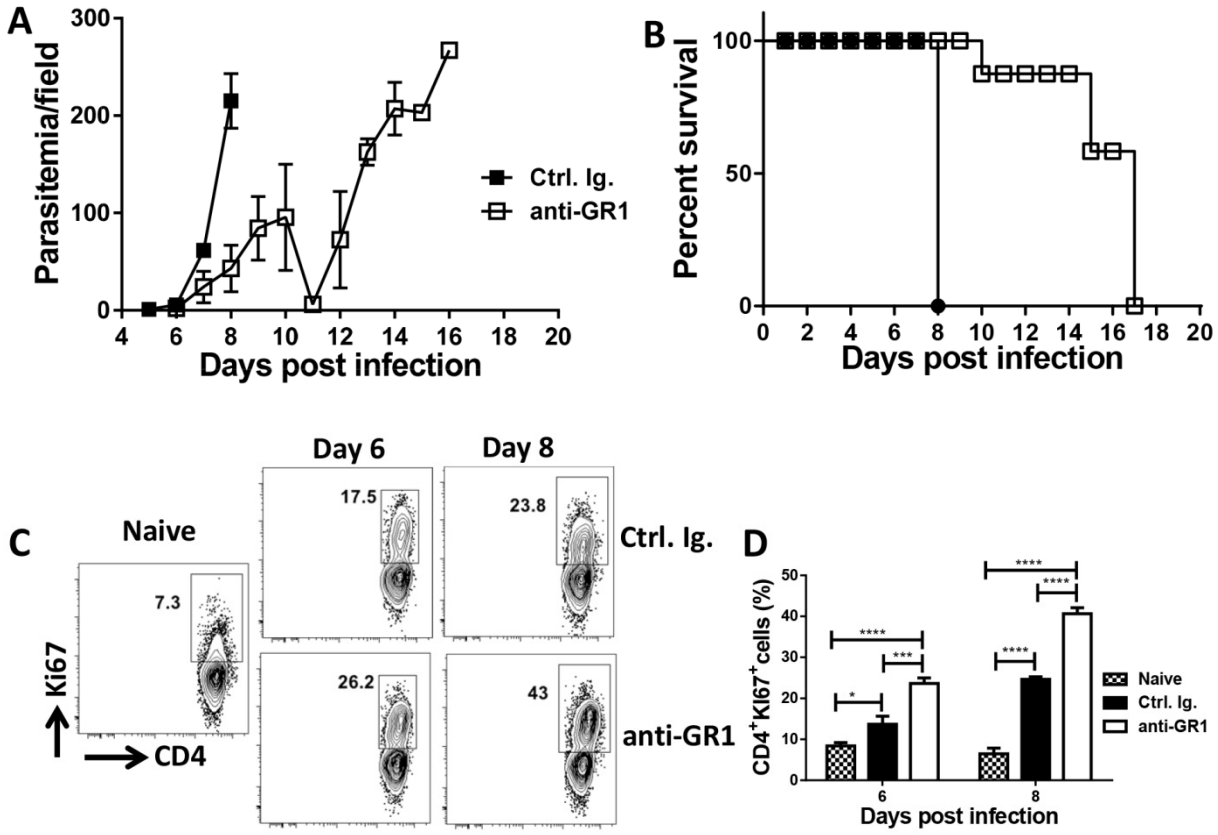

Figure 16. Enhanced resistance and proliferation of $CD4^+$ T cells after anti-GR1 treatment following *T. congolense* infection. Groups of mice (n = 4-5 mice per group) were injected intraperitoneally with anti-GR1 mAb (RB6-8C5), 200 µg/mouse) or isotype-matched control mAb a day before intraperitoneal infection with 10^3 *T. congolense.* Anti-GR1 mAb treatment was repeated on day 4 after infection. Daily parasitemia (A) and survival (B) were determined. At indicated times, mice were sacrificed and $CD4^+$ T cells from the spleens were assessed directly *ex vivo* for Ki67 expression (C & D). The data presented are representative of 2 different experiments with similar results. Bars show mean +/-SEM; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

6.2.3. MDSCs suppress CD4$^+$ T cell proliferation during T*. congolense* infection in Arginase-1-dependent manner

Because CD4$^+$ T cells have been shown to be important in experimental trypanosome infection [96,122], and because MDSCs have been shown to suppress CD4$^+$ T cell proliferation [164,165], I hypothesized that the depletion of MDSCs would lead to enhanced CD4$^+$ T cell proliferation during *T. congolense* infection. To test this, I treated mice with anti-GR1 mAb and infected them with *T. congolense*. On days 6 and 8 after infection, mice were sacrificed and the level of proliferating CD4$^+$Ki67$^+$ T cells was assessed *ex vivo*. Results showed that depletion of MDSCs led to the increased Ki67 expression on T cells (Fig. 16C & D).

To more clearly assess the role of MDSCs in the suppression of CD4$^+$ T cell proliferation, I labeled splenocytes from infected mice treated with anti-GR1 mAb or control Ig with CFSE dye, stimulated them *in vitro* with anti-CD3 and anti-CD28 for 4 days and assessed cell proliferation by flow cytometry. Results show that CD4$^+$ cells from anti-GR1-treated mice significantly proliferated more than those treated with control Ig. (Fig. 17A-C). Not surprisingly, CD4$^+$ cells from uninfected mice proliferated significantly more than those from the infected Ig-treated group (Fig. 17A-C), an observation that is consistent with the fact that *T. congolense* infection results in suppression of T cell proliferation. Addition of arginase (but not iNOS) inhibitor to the cell culture restored the proliferation of CD4$^+$ T cells (Fig. 17A-C). Taken together, these results show that during *T. congolense* infection, MDSCs suppress CD4$^+$ T cell proliferation through arginase-1 dependent pathway.

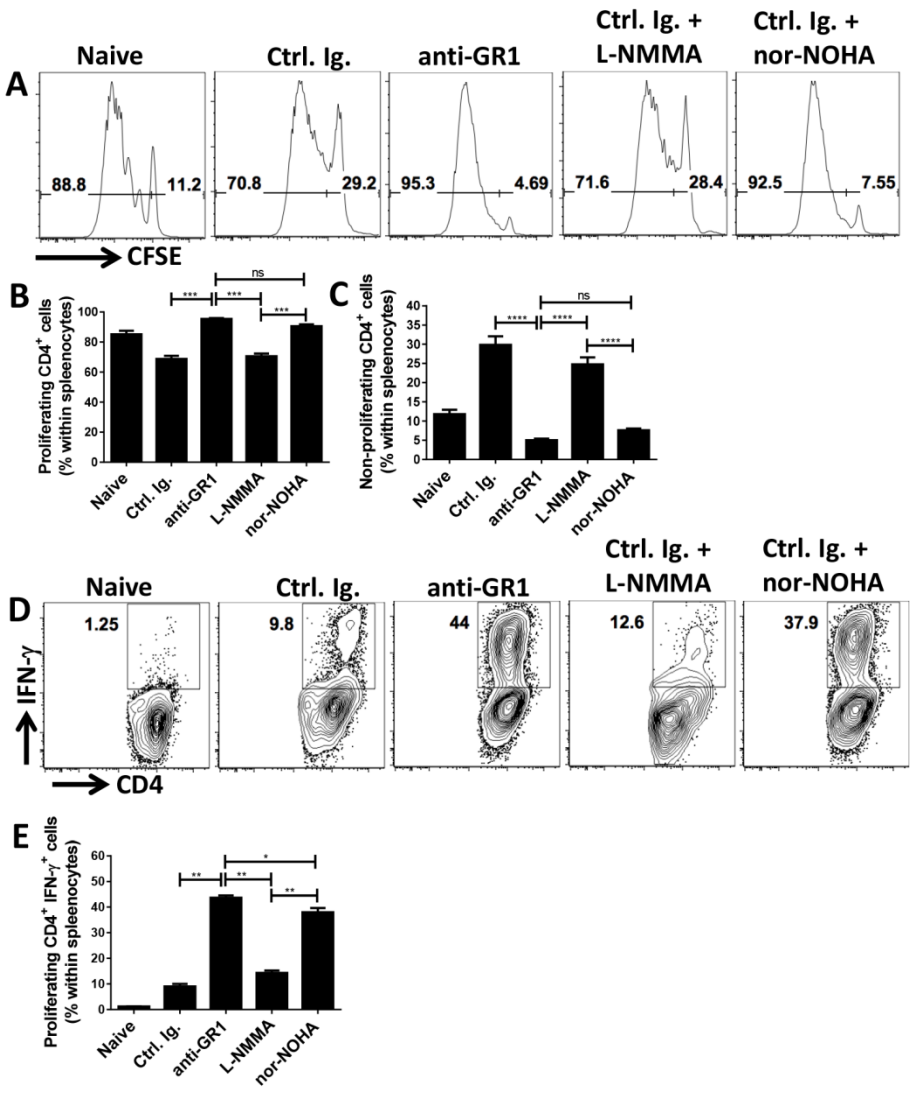

Figure 17. Inhibition of CD4$^+$ T cell responses by MDSCs from the splenocytes of *T. congolense*-infected. Groups of mice (n = 4-5 per group) were injected intraperitoneally with anti-GR1 mAb (RB6-8C5, 200 µg/mouse) or isotype-matched control mAb a day before and 4 days after intraperitoneal infection with 10^3 *T. congolense*. Mice were sacrificed on day 7 post-infection and splenocytes were labeled with CFSE and stimulated with soluble anti-CD3 and anti-CD28 mAb in the presence or absence of L-NMMA or nor-NOHA for 4 days and assessed for proliferation and IFN-γ production by flow cytometry. Shown are histograms (A) and representative bar graphs (B & C) of the percentages of proliferating (A & B) and non-proliferating (A & C) CD4$^+$ T cells. Contour plots (D) and bar graph (E) show the percentages of CD4$^+$ T cells IFN-γ-producing CD4$^+$ T cells in the cultures. The data presented are representative of 2 different experiments with similar outcomes. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

6.2.4. $CD4^+$ T Cell Function during *T. congolense* Infection is Suppressed by MDSC in Arginase-1-dependent Mechanism

Because $CD4^+$ T cell proliferation was suppressed by MDSC (see Fig. 17A-C), I wanted to check if they could also suppress $CD4^+$ cell function, notably the production of IFN-γ which is critical for resistance to *T. congolense* infection [44,134]. Splenocytes from infected mice treated with or without anti-GR1 mAb were labeled with CFSE dye and stimulated with soluble anti-CD3 and anti-CD28 mAbs. After 4 days, cells were briefly stimulated with PMA, BFA, and ionomycin and assessed for intracellular expression of IFN-γ by flow cytometry. Results show that anti-GR1 mAb treatment dramatically increased the numbers of proliferating $CD4^+$ cells producing IFN-γ compared to those from control Ig-treated group (Fig. 17D & E). Addition of arginase (but not iNOS) inhibitor to the cell culture restored IFN-γ production ability of $CD4^+$ T cells in control Ig-treated mice (Fig. 17D & E). Collectively, these results show that MDSCs suppress $CD4^+$ T cell function during *T. congolense* infection in an arginase-1-dependent manner.

6.2.5. MDSC suppress $CD4^+$ T cell proliferation and function in a direct co-culture setting

I found that depletion of MDSCs led to increased proliferation and function of $CD4^+$ T cells. Because splenocytes were used in those experiments, and because arginase-1 could have been coming from cells other than MDSCs, I wanted to directly assess the suppressive ability of MDSCs in a co-culture setting with $CD4^+$ T cells. I co-cultured purified and CFSE dye-labeled $CD4^+$ T cells from naïve mice and MDSCs from infected (day 7) mice with or without soluble anti-CD3 and anti CD28 mAbs. After 4 days, I

assessed $CD4^+$ T cell proliferation and function (IFN-γ production) by flow cytometry. Results show that MDSC from *T. congolense*-infected animals were able to suppress naïve $CD4^+$ T cell proliferation (Fig. 18A-C) and IFN-γ production (Fig. 18D-F). This suppression was also dependent on arginase-1, as the addition of arginase-1 inhibitor into the cell culture restored $CD4^+$ cell proliferation and IFN-γ production (Fig. 18A-F).

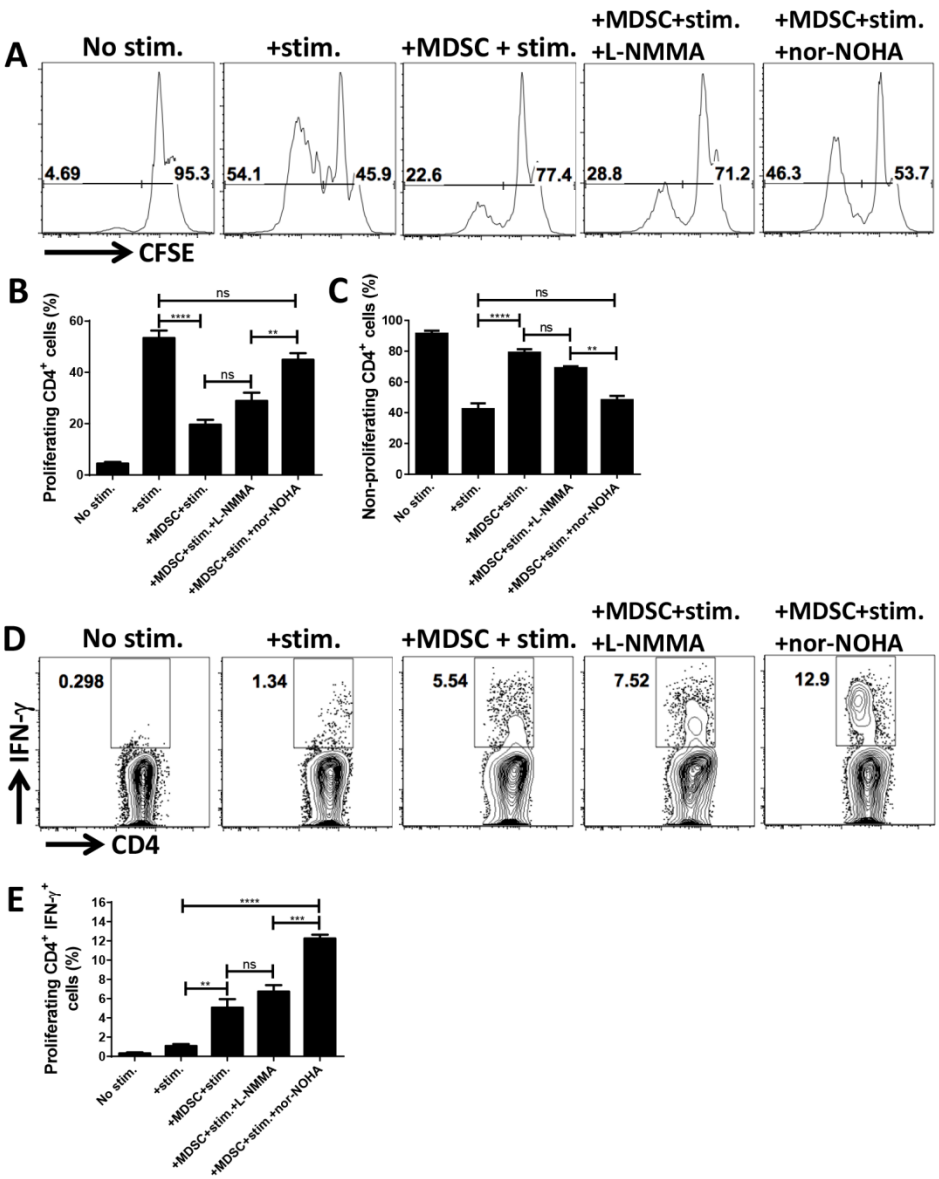

Figure 18. Direct suppression of purified naïve CD4$^+$ T cell responses by isolated MDSCs. CD4$^+$ T cells were purified from naïve mice, labeled with CFSE and co-cultured with FACS-sorted MDSC (1:1). The cells were stimulated with soluble anti-CD3 and anti-CD28 mAb for 4 days in the presence or absence of L-NMMA or nor-NOHA. Shown are histograms (A) and bar graphs (B & C) of flow cytometric data representing the percentages of proliferating (A & B) and non-proliferating (A & C) CD4$^+$ T cells. Contour plots (D) and bar graph (E) show the percentages of IFN-γ-producing CD4$^+$ T cells cultured in the presence or absence of L-NMMA or nor-NOHA (D & E). Cells were first gated for CD4 expression before assessing proliferation and IFN-γ expression. The data presented are representative of 2 different experiments with similar outcomes. Bars show mean +/-SEM; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

7. CHAPTER SEVEN

DISCUSSION

7.1. Effect of Low-Dose Intradermal *T. congolense* infection in the Enhanced Susceptibility of Mice to Subsequent Re-infection

The primary aim of this study was to investigate the mechanism through which repeated intradermal low dose infection enhances susceptibility to *T. congolense* infection of mice. Wei et al reported that infection of mice with a low number of *T. congolense* enhanced susceptibility to reinfection [81]. However, the mechanism(s) behind this important observation was not addressed. Here, I focused on determining the role of regulatory T cells in this process. I found that despite the absence of parasitemia and disease, primary infection of mice with low dose *T. congolense* led to the expansion of regulatory T cells in the spleens and lymph nodes draining the infection sites. Depletion of these cells by anti-CD25 mAb treatment during the time of repeated low dose infection or at the time of secondary reinfection abolished the low dose-induced susceptibility to *T. congolense* infection. This was associated with dramatic reduction in serum levels of IL-10 and TGF-β, key cytokines that mediate Tregs-induced dampening of immune responses. Collectively, these findings show that intradermal low dose *T. congolense* infection leads to expansion of Tregs that mediate the enhanced susceptibility to subsequent low dose reinfection.

Immunosuppression is a hallmark of African trypanosomiasis. Trypanosome infected animals (including humans, cattle, and mice) show marked suppression of immune response to vaccines as well as to T cell mitogens including Con A and PHA

[97,130,206-213]. In addition, infected mice also show reduced antibody response to sheep red blood cells (SRBC) following immunization [115,170,214-216]. Several mechanisms have been proposed as the cause of Trypanosome-induced immunosuppression, including macrophages [217], suppressor T cells [218], nitric oxide [150] and Tregs [108,110]. Recent reports (including those from our laboratory) have shown that conventional and naturally occurring Tregs (characterized as $CD4^+CD25^+Foxp3^+$) contribute to enhanced susceptibility to experimental *T. congolense* infection in mice [108,110]. Depletion of these cells prior to infection significantly enhances the resistance of the highly susceptible BALB/c mice. I found here that intradermal low dose infection leads to systemic expansion of $CD4^+CD25^+Foxp3^+$ cells and this was associated with enhanced susceptibility following reinfection. Depletion of these cells completely abolished this susceptibility, directly confirming the role of the expanded population of Tregs in intradermal low dose infection-induced susceptibility.

How does low dose intradermal infection lead to induction of Tregs? A previous report shows that low dose intradermal infection is controlled primarily by NO and TNF-α produced presumably by activated macrophages (innate immunity) [81]. However, our results clearly rule out macrophages as the primary mediator of either increased resistance to primary i.d. *T. congolense* infection or enhanced susceptibility to re-challenge infection following repeated low dose i.d. infection. There is evidence that NKT cells play a major role in suppressing immunity following intradermal infection [81]. It has been proposed that the presentation of trypanosomal GPI-derived antigens by CD1d molecules to a subpopulation of NKT cells leads to alternative activation of macrophages and a concomitant production of high amounts of TGF-β [219]. This

microenvironment would favor the preferential development of Tregs from naïve T cells, which in turn, further promotes the alternative activation of macrophages leading to more IL-10 and TGF-β production. It is also conceivable that the expansion of Tregs after low dose intradermal infection is associated with the production of TGF-β and IL-10, which contribute to enhanced susceptibility during challenge infection. In line with this, we found that depletion of Tregs by anti-CD25 mAb treatment was associated with a reduction in TGF-β and IL-10 production in infected mice (Fig. 6C and D).

Previous studies [110,180] show that anti-CD25 mAb is effective at depleting mostly $CD25^{+}Foxp3^{+}$ Treg cells. Another study found that while this antibody effectively depletes over 80% $Foxp3^{+}$ cells, only about 45-50% $CD25^{+}$ (mostly $CD25^{high}$) cells were depleted, suggesting that the antibody is highly selective and does not globally deplete $CD25^{+}$ T cells. This is very significant because CD25 is the high-affinity IL-2R alpha chain, which is universally expressed and critically important for T cell responses. Following anti-CD25 mAb treatment, Treg depletion lasts for 7 days in both the spleens and draining lymph nodes, after which repopulation of $CD25^{+}$ cells is initiated. This suggests that the dose of antibody used in this study is incapable of causing permanent depletion of $CD25^{+}$ cells, which might inhibit subsequent T cell activation and contribute to suppressed immunity.

B cells (via the production of antibodies) are important in immunity to experimental African trypanosome infection. B cell-deficient mice are highly susceptible to various strains of African trypanosomes, and this susceptibility is reversed by passive transfer of VSG-specific antibody or primed B cells [220]. Both IgM and IgG antibody subclasses are important in mediating anti-trypanosome clearance [221], although IgG antibodies

may be qualitatively more important [43,178]. Hence, I investigated whether low dose intradermal infection elicits a strong antibody response that could account for the enhanced resistance following primary infection. In contrast to a previous report [81], repeated primary intradermal infection elicited significant antibody (IgM and IgG) responses. Despite this antibody response, these mice became susceptible following low dose re-challenge, suggesting that the antibodies were ineffective at mediating resistance to intradermal *T. congolense* infection.

In conclusion, I have shown that low dose intradermal *T. congolense* infection leads to systemic and sustained expansion of Tregs, which is responsible for mediating enhanced susceptibility to repeated low dose *T. congolense* reinfection. Depletion of Tregs by anti-CD25 mAb treatment completely abolished the low dose intradermal infection-induced susceptibility to secondary reinfection. I speculate that low dose intradermal induction of Tregs and subsequently enhanced susceptibility may be an evasion strategy by the parasite to enhance its survival and transmission in the host. Thus, it will be interesting to determine when the low dose intradermal infection-induced expansion of regulatory T cells occurs in other parasitic diseases like malaria, leishmaniasis, and babesiosis that are usually transmitted by low dose injection of the infective stage into the dermis of mammalian hosts by their respective vectors.

7.2. Role of Natural Killer (NK) Cells in in the Maintenance of Optimal Immunity to *Trypanosoma congolense* Infection

Natural killer cells are a critical population of the innate immune cells that have the ability to regulate both innate and adaptive immunity through cytokine production and

cytotoxicity [188,189,222-224]. Studies on NK cells and *T. congolense* infection are lacking. This is surprising, knowing that these cells are key producers of IFN-γ and TNF-α which are key protective cytokines during the early stages of infection [44,133-135]. The primary aim of this aspect of the study was to determine the functional role of NK cells in regulating immunity to *T. congolense* infection. I observed that after *T. congolense* infection, NK cells expanded in various tissues showed upregulation of activation (NKp46) and degranulation (CD107a) markers, and produced key effector cytokines (IFN-γ and TNF-α). Although temporary depletion of NK cells did not lead to the death of infected animals, there was an increase in parasitemia and impairment in IFN-γ production by immune cells from infected NK cell-depleted mice. This may be due to the inability of the antibody to completely deplete NK cells in various tissue compartments or rapid restoration of the cells from the bone marrow. Indeed, NFIL3$^{-/-}$ mice (that lack NK cell development and function) on a usually resistant background died acutely when infected with *T. congolense*, highlighting the requirement of NK cells in mediating resistance to infection. In addition to this, the CD3^{+} cells showed reduced ability to produce cytokines in NFIL3$^{-/-}$ mice, suggesting that NK cells are important for priming T cells for efficient cytokine response following *T. congolense* infection as has been reported in other models [222,225]. As a proof of concept, I adoptively transferred NK cells into NFIL3$^{-/-}$ deficient animals before infecting them with *T. congolense*. These otherwise highly susceptible mice controlled parasitemia as efficiently as the infected WT animals and survived until the termination of the experiment.

Because B cell, NKT cell and T cell populations and functions are normal in NFIL3$^{-/-}$ mice [174,195], this mouse strain emerged as a very good model for studying the role of

NK cells during infection. Several knockout mice models have been used to study NK cell development [226,227], but these models are usually not preferred due to the impact of gene deletion on other cell lineages [174]. Therefore, my results unequivocally show that NK cells are required for protection during *T. congolense* infection.

I used activated NK cells in all the adoptive transfer experiments reported here. This might raise the question regarding the need to activate these cells before adoptive transfer. In our hands, adoptive transfer of unactivated NK cells into NFIL3$^{-/-}$ mice did not rescue these mice from death after *T. congolense* infection. This observation could be accounted for by the unfavourable microenvironment of the transferred unactivated NK cells in NFIL3$^{-/-}$ mice. It is conceivable that in NFIL3$^{-/-}$ mice, the necessary activation factors needed to trigger NK cell activation and maintenance *in vivo* could be absent. In addition, activating NK cells *in vitro* yielded more cell number, as the cells were expanded by more than 3 folds without affecting their viability.

Interestingly, I found that the ability of activated NK cells to mediate protection to *T. congolense* in NFIL3$^{-/-}$ mice was dependent on perforin. This observation is surprising considering the fact that no interaction between perforin and *T. congolense* has been reported, probably because of the extracellular nature of these parasites. Direct coculture of NK cells and *T. congolense* did not lead to parasite killing (not shown), suggesting that this may not be a direct interaction. A recent study with *T. brucei* attributed the depletion of splenic B cells and early mortality after infection to NK cells in a perforin-dependent manner [120]. These observations differ from my findings and beg for reconciliation. First, my study employed the use of NFIL3$^{-/-}$ mice that lack NK cell development, maturation, and function [174,195], whereas they made use of perforin-

deficient mice to confirm their findings. Assessment of B2 B cell subsets in *T. congolense*-infected NFIL3$^{-/-}$ mice in the presence or absence of adoptively transferred activated WT NK cells showed no difference in B2 B cell subsets (Not shown). Furthermore, I used a protocol that involved the activation and expansion of NK cells *in vitro* prior to adoptive transfer, whereas they used unactivated NK cells or NK cells from *T. brucei*-infected mice. These differences could impact on the function of the cells after adoptive transfer. Therefore, it is conceivable that the differences in mouse strains, state of NK cells used for adoptive transfer, the timing of the adoptive transfer as well as the difference in parasite species could account for the different outcomes. More study is therefore needed in order to address these interesting, but opposing observations.

Although it remains to be tested, I speculate that NK cells could be regulating (probably through cytotoxicity) some of the rapidly expanding cell populations that are associated with immune suppression during *T. congolense* infection, specifically Tregs [108,110] and myeloid-derived suppressor cells (discussed later). In support of this speculation, I observed increased numbers of Tregs (Appendix 2A & B) and MDSCs (Appendix 2C & D) in *T. congolense*-infected NFIL3$^{-/-}$ mice. In addition, it is also conceivable that NK cell-mediated cytotoxicity might be targeting macrophages. Macrophages are key immune cells that produce proinflammatory cytokines and have been reported to mediate immunosuppression following infection with African trypanosomes [147,148]. Because overproduction of inflammatory cytokines and immunosuppression are key mechanisms that mediate pathology and death, reduction in the numbers of these cells (via NK cell cytotoxicity) would enhance survival of infected mice.

In conclusion, I have shown in this section that NK cells are required for mediating protection in *T. congolense*-infected mice in a perforin-dependent manner. Deficiency of these cells leads to acute death in infected NFIL3$^{-/-}$ mice on a normally resistant background. Thus, these results show that targeting NK cell activation could be useful in designing effective therapy against African trypanosomiasis.

7.3. Role of Myeloid-Derived Suppressor Cells (MDSCs) in *T. congolense* Infection

The main focus of this section of my study was to assess the role of myeloid-derived suppressor cells in the pathogenesis of experimental *T. congolense* infection in mice. First, I investigated whether there is an expansion of MDSCs and if they mediate suppression of CD4$^+$ T cells during *T. congolense* infection in mice. Although suppression of CD4$^+$ T cells by MDSCs is known, it remains to be elucidated in *T. congolense* infection model. I wanted to specifically address this and find out the mechanisms through which the suppression occurs. I found that MDSCs expanded in the spleens and liver of *T. congolense*-infected mice, and their depletion during infection led to transient control of the first wave of parasitemia and prolonged survival of infected highly susceptible mice. This was associated with increased proliferation and IFN-γ production by CD4$^+$ T cells when assessed directly *ex vivo*, suggesting that MDSCs may be suppressing CD4$^+$ T cells responses in infected mice. Indeed, I demonstrated that MDSCs directly suppressed the proliferation and IFN-γ production by anti-CD3 and CD28 mAb-stimulated CD4$^+$ T cells from naïve mice in a co-culture setting. I further found that the suppression of CD4$^+$ T cells by MDSCs was mediated via arginase-1. Collectively, these results show that MDSCs suppress CD4$^+$ T cell responses during *T. congolense* infection, which adversely affects optimal immunity to the parasites.

Immunosuppression in infected hosts remains a major hurdle in the race towards finding a vaccine against African trypanosomiasis. Animals infected with African trypanosomes show immune suppression to vaccines and T cell mitogens including concanavalin A (Con A) and phytohemagglutinin (PHA) [97,213]. Several mechanisms have been proposed to mediate this process including suppressive macrophages [217], suppressor T cells [218] and nitric oxide [150]. Our group and others have also shown that Tregs prevent the control of *T. congolense* infection [108,110]. Interestingly, it has been reported that MDSCs help in the induction of Tregs [228,229]. It is conceivable that MDSCs and Tregs could work together to mediate suppression during *T. congolense* infection. However, I did not observe any significant change in the numbers of Tregs after MDSC depletion during infection (Appendix 3A & B), suggesting that MDSCs may not influence Treg numbers in experimental *T. congolense* infection.

Based on the differential epitope bindings of anti-GR1 antibody, MDSCs have been classified into two functional subsets; the $CD11b^{+}LY6G^{+}LY6C^{low}$ (granulocytic, G-MDSCs) and the $CD11b^{+}LY6G^{-}LY6C^{hi}$ (monocytic, M-MDSCs) MDSCs [200,201]. I found that the numbers of M-MDSCs increased in both the spleens and liver of infected mice. In contrast, G-MDSC numbers decreased in these organs. This suggests that the M-MDSCs could be playing a major role in the suppression of $CD4^{+}$ T cells in my study. In line with this, M-MDSCs have been reported to use the high expression of arginase-1 and iNOS to mediate their suppressive functions, whereas the G-MDSCs mostly use ROS to mediate their suppressive functions [230,231]. Indeed, I found that MDSCs used arginase-1 to mediate the suppression of $CD4^{+}$ T cells in my study, supporting the inference that M-MDSCs are the major cells involved in immunosuppression in this

model of infection. Because both arginase-1 and iNOS use L-arginine as their substrates, the high expression of arginase-1 could lead to enhanced usage of L-arginine by this metabolic pathway, reducing its availability to iNOS [232]. This could explain why iNOS was found not to be playing a key role in MDSC-mediated suppression of $CD4^+$ T cells in this study.

My previous findings showed that resistance of *T. congolense*-infected mice was associated with the activation of classically activated macrophages and expression of iNOS during the early phase of infection, and a switch to alternatively activated macrophages and arginase-1 expression during the chronic stage of the infection [95]. It is conceivable that in addition to suppression of $CD4^+$ T cells by MDSCs (as observed in this study), the availability of arginase-1 in the microenvironment could also favour the early induction of alternatively activated macrophages. This could be one of the strategies used by the parasites to induce early immunosuppression during African trypanosome infection [93].

In conclusion, I have demonstrated that suppression of $CD4^+$ T cells during *T. congolense* infection is, in part, mediated by MDSC. Using co-culture studies, I showed that blockade of arginase-1 completely abolished MDSCs-mediated suppression of $CD4^+$ T cell proliferation and IFN-γ production, indicating that suppression is mediated through arginase-1. I speculate that targeting arginase-1 during infection could be beneficial and may restore optimal T cell responses during *T. congolense* infection. Given that immunosuppression is a hallmark of African trypanosomiasis, it would be interesting to investigate whether MDSCs play similar roles following infections with other species of African trypanosomes.

7.4. Thesis summary

Taken together, my entire thesis has been able to show that at the onset of experimental African trypanosomiasis, NK cell activation is critical. These cells are required to produce IFN-γ and TNF-α that help in the early phase of parasite control as well as help in optimal T cell activation. However, TGF-β produced by Tregs has been reported to inhibit the activity of NK cells [193,194]. In line with this, I found that Tregs was preferentially induced by *T. congolense* infection, and contributed to enhanced susceptibility to reinfection. Although not tested, it is conceivable that in addition to T cell suppression, Tregs could also be suppressing NK cell activities in *T. congolense* infection. It has also been reported that MDSCs help in the induction of Tregs [228,229]. Therefore, I speculate that MDSCs could be working in tandem with Tregs in mediating immunosuppression during *T. congolense* infection and African trypanosomiasis in general. Conclusively, the crosstalk between Tregs and MDSCs would contribute to susceptibility, while NK cell activation contributes to resistance during *T. congolense* infection.

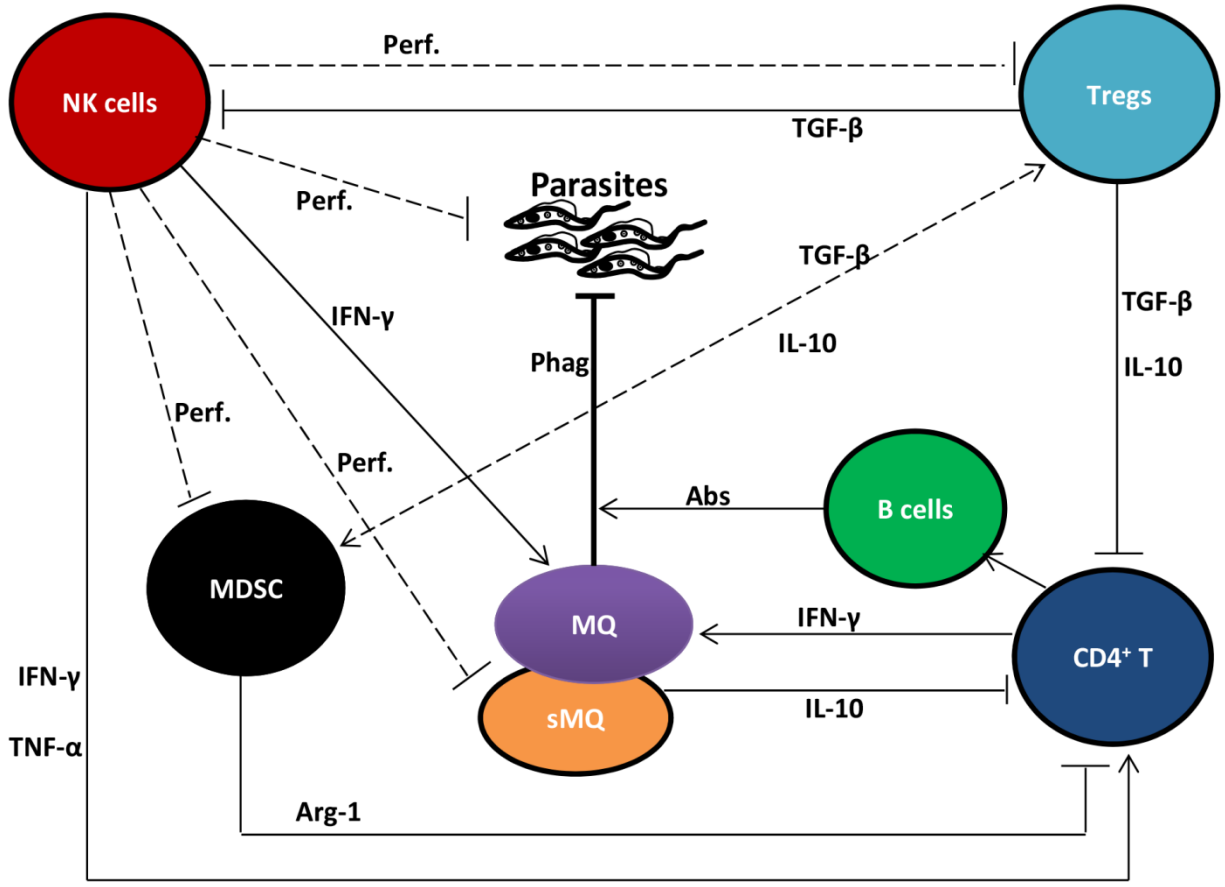

Figure 19. Summary of thesis work and proposed model of events during *T. congolense* infection. Infection with *T. congolense* leads to activation of NK cells. These cells produce cytokines including IFN-γ, and activate macrophages and $CD4^+$ T cells. Activated $CD4^+$ T cells further activate macrophages as well as provide help to B cells (for production of trypanosome-specific antibodies (Abs)) to mediate enhanced phagocytosis (phag). On the other hand, Tregs (via TGF-β and IL-10), suppressor macrophages (sMQ, via IL-10) and MDSCs via Arginase-1 (Arg-1) are able to suppress $CD4^+$ T cell function, which in turn impact negatively on parasite clearance. I speculate that NK cells are also able to indirectly contribute to parasite clearance by killing Tregs, MDSCs and suppressor macrophages during infection via perforin (perf.)-dependent mechanism. Although unlikely, due to the extracellular nature of the parasites, direct killing of parasites by NK cells could be possible.

8. CHAPTER EIGHT

GENERAL DISCUSSION

8.1. Missed Opportunities and Limitations

8.1.1. Effect of Low-Dose Intradermal *T. congolense* infection in the Enhanced Susceptibility of Mice to Subsequent Re-infection

The data presented here have shown that regulatory T cells expand after primary intradermal infection of mice with low dose *T. congolense* and in turn, suppress immune response to challenge infection. Depletion of Tregs abolished the suppression and this was associated with a reduction in the levels of IL-10 and TGF-β, which are conceivably the suppressive mechanisms of Tregs in this process. However, some questions remain unanswered. Since Tregs were promoting susceptibility, and depletion of Tregs with anti-CD25 mAb abolished this susceptibility, what would be the outcome of a similar experiment using CD25 knock out mice (hereafter, CD25 KO mice) that have been shown to lack functional Tregs? What is the implication of the reduced level of IL-10 and TGF-β after depletion of Tregs? Are these cytokines directly or indirectly involved in Treg-mediated susceptibility? In other words, will Treg-mediated susceptibility disappear in the absence of these cytokines? I believe that the data shown here would have been strengthened if the following experiments were conducted.

Therefore, it would have been interesting to demonstrate the effect of Tregs by using CD25 KO mice that are deficient in Tregs [233]. The hypothesis to be tested is that CD25 KO mice will be resistant to *T. congolense* challenge infection after repeated low dose exposure. Should this hypothesis hold, it would support and confirm my findings

that Tregs mediate susceptibility during intradermal challenge infection of mice with *T. congolense*. If the result is different, concerns would be raised as to whether there are other pathways that compensate for Tregs function in CD25 KO mice.

Also, one of the major ways that Tregs mediate their effect is through TGF-β [234]. Indeed, I found that depletion of Tregs using anti-CD25 mAb (which abolishes low dose intradermal infection-induced susceptibility to reinfection) led to a reduction in the level of this cytokine, suggesting that Tregs might be mediating the enhanced susceptibility through TGF-β. I would hypothesise that treatment of mice with anti-TGF-β mAb before challenge infection will abrogate the function of Tregs thereby abolishing the enhanced susceptibility to *T. congolense* challenge. This would confirm whether the function of Tregs is TGF-β dependent or not. If however, the hypothesis does not hold (i.e. anti-TGF-β mAb-treated mice still develop enhanced susceptibility to re-infection), it will suggest that the function of Tregs is not TGF-β dependent. In that case, it would be necessary to repeat the same experiment with anti-IL-10 mAb treatment or IL-10R KO mice to see if the function of Tregs is IL-10 dependent [235]. In addition, isolation of Tregs from WT, TGF-β KO or IL-10R KO mice, and adoptively transferring these cells into CD25 KO mice during the described experiments would have further helped to determine the mechanism through which Tregs mediate their activity in this model.

8.1.2. Role of Natural Killer (NK) Cells in in the Maintenance of Optimal Immunity to *Trypanosoma congolense* Infection

The results described here clearly show that adoptive transfer of NK cells from WT or IFN-γ deficient mice (but not perforin-deficient mice) into NFIL3$^{-/-}$ mice before infection

rescued these mice from death and prolonged their survival, suggesting that NK cells mediate protection in *T. congolense* infection in a perforin-dependent manner. However, a major limitation in these experiments is the missed opportunity of assessing the immune response of these mice after adoptive transfer, and before death in the susceptible groups. Assessing immune response such as trypanosome-specific antibody levels, proinflammatory cytokine levels, extensive T and B cell status as well as germinal centre responses would have helped to identify the factors that correlate positively with protection and/or susceptibility.

Although I was able to generate interesting and clear results using NFIL3$^{-/-}$ mice, a confirmatory experiment using Beige mice would have added validity to the data obtained here.

8.1.3. Role of Myeloid-Derived Suppressor Cells (MDSC) in *T. congolense* Infection

Although I clearly showed that MDSCs suppress CD4^{+} T cells through arginase-1; data showing the level of arginase-1 and iNOS expressions on MDSC are missing. The data would have been very helpful in the discussion regarding the arginase-1-dependent mechanism of suppression by MDSCs. Therefore, I missed the opportunity of assessing the levels of iNOS and arginase-1 expressions on MDSCs using flow cytometry and/or real-time PCR.

Also, two times injection of anti-GR1 mAb was able to mediate transient parasite control and increased CD4^{+} T cell proliferation; it remains unknown whether subsequent anti-GR1 treatment would have further improved parasite control. Further injection of the

mAb between day 7 and 9 after infection would have helped me to address this question. Finally, since *T. congolense* is extracellular and lives in the blood stream of its host, it is conceivable that MDSCs might be playing suppressive roles in the blood during the course of infection; assessing the frequency of these cells in the blood of mice during infection would have been beneficial.

8.2. Key Findings and Conclusions

8.2.1. Effect of Low-Dose Intradermal *T. congolense* infection in the Enhanced Susceptibility of Mice to Subsequent Re-infection

This part of my thesis focused on determining the impact of infection-induced Regulatory T cells and how these cells enhance susceptibility to *T. congolense* in the context of intradermal (mimicking natural infection route) infection.

I found that low dose intradermal infection led to the production of cytokines as well as the expansion of Tregs in both the spleens and lymph nodes draining the infection site; these Tregs enhanced susceptibility of mice to challenge infection. Furthermore, depletion of Tregs by during primary or before challenge infection following repeated low dose infection completely abolished the low dose-induced enhanced susceptibility and dramatically reduced serum levels of TGF-β and IL-10. The key finding here is that low-dose intradermal infection of mice (that did not cause parasitemia) led to the expansion of Tregs in both the spleen and lymph nodes. Interestingly, these Tregs mediated suppression during a challenge infection. Collectively, I believe that the induction of Tregs following low dose intradermal infection could be an evasion strategy adopted by the parasite to enhance its survival and completion of the lifecycle, thereby

maintaining its transmission from one host to another. These findings have been published in a peer review journal: **Onyilagha C**, Okwor I, Kuriakose S, Singh R, Uzonna J. *Low-dose intradermal infection with Trypanosoma congolense leads to expansion of regulatory T cells and enhanced susceptibility to reinfection*. Infect Immun. 2014 Mar;82(3):1074-83. doi: 10.1128/IAI.01028-13. Epub 2013 Dec 16. PubMed PMID: 24343657; PubMed Central PMCID: PMC3957988.

8.2.2. Role of Natural Killer (NK) Cells in in the Maintenance of Optimal Immunity to *Trypanosoma congolense* Infection

In this section, I investigated the importance of NK cells in *T. congolense* infection and found for the first time that mice infected with *T. congolense* show increased levels of NK cells in multiple tissue compartments. Depletion of these cells during infection led to increased parasitemia and a significant reduction in IFN-γ production by immune cells. When NFIL3 deficient mice (which genetically lack functional NK cells) were infected with *T. congolense*; they developed fulminating parasitemia and died significantly earlier than their wild-type controls (could not control the first wave of parasitemia). In addition, the enhanced susceptibility in these mice was accompanied by significantly impaired cytokine production by $CD3^+$ T cells. Adoptive transfer of WT NK cells into NFIL-3 deficient mice before infection rescued them from acute death in a perforin-dependent mechanism. The major/novel finding here is the perforin-dependent mechanism of parasite control, as the direct interaction between *T. congolense* and perforin has not been reported. This opens the door for future research to look at the possible direct trypanolytic effect of perforin on African trypanosomes. I believe that NK cells are

required for optimal resistance to *T. congolense* including the activation of T cells. Findings from this part of my work have been put together in a manuscript to be submitted to PLoS Pathogens: **Chukwunonso Onyilagha**, Ping Jia, and Jude Uzonna. *Myeloid-derived suppressor cells inhibit CD4$^+$ T cell proliferation and interferon gamma production during T. congolense infection*.

8.2.3. Role of Myeloid-Derived Suppressor Cells (MDSCs) in *T. congolense* Infection

This part of my thesis specifically investigated the suppressive ability of MDSCs on CD4$^+$ T cells during *T. congolense* infection and for the first time, I showed that the numbers of MDSCs increased in the liver and spleen of infected mice as parasitemia increased. Depletion of these cells during infection led to increased proliferation and IFN-γ production by CD4$^+$ T cells. Suppression of CD4$^+$ T cells by MDSCs was found to be dependent on arginase-1. The key finding here is that MDSCs not only suppressed proliferation of CD4$^+$ T cells, they also inhibited their ability to produce IFN-γ during infection. These findings contribute significantly towards understanding the factors that mediate lymphocyte suppression during infection with African trypanosomes. I believe that the information provided here could help the development of effective therapy by targeting arginase-1 during infection. Findings from this part of my work have been put together in a manuscript to be submitted soon to a peer review journal: **Chukwunonso Onyilagha**, Ping Jia and Jude Uzonna. *Myeloid-derived suppressor cells inhibit CD4$^+$ T cell proliferation and interferon gamma production during T. congolense infection.*

8.3. Overall Significance and Future Directions

8.3.1. Significance

The main focus of my thesis was to investigate various cellular mechanisms that influence the outcome of infection with *T. congolense,* with a view to understanding the role of various immune cells in susceptibility and resistance. To achieve this goal, I have worked on (i) determining the impact of infection-induced regulatory T cells and how these cells enhance susceptibility to *T. congolense* following intradermal infection, (ii) understanding the role played by Natural killer cells in maintaining optimal activation of effector cells during *T. congolense* infection, and (iii) unravelling the role of myeloid-derived suppressor cells in the inhibition of $CD4^+$ T cell proliferation and function during *T. congolense* infection. One of the major concerns that remains unaddressed in African trypanosomiasis is how to effectively prevent the induction of immunosuppression in infected animals. My studies showed that Tregs play a role in the induction of immunosuppression following Trypanosoma *congolense* infection. If this can be translated into the human form of the disease, then targeting $CD4^+CD25^+Foxp3^+$ Tregs may be a step in the right direction when considering vaccination strategies against infections by African trypanosomes including effective therapy. I believe the low dose mediated susceptibility to be an evasion strategy by the parasite to enhance its survival and transmission. Therefore, I speculate that this pathway may be of importance in most infections that are transmitted via low dose injection of parasites into the dermis of the mammalian host by arthropod vectors. Hence, these studies may have broader implications for the control of other arthropod-borne diseases including malaria, leishmaniasis, and babesiosis.

In addition, my study was able to show that NK cells are required for optimal resistance and effector T cell functions during *T. congolense* infection and that this resistance is perforin dependent. I believe that these finding would be of great importance in designing effective vaccination and therapeutic strategies, as vaccines and therapies against African trypanosomiasis could be designed to induce NK cell activation thereby helping in the optimal activation and maintenance of optimal immunity.

Furthermore, I identified arginase-1 as being the key suppression mechanism used by MDSCs on $CD4^+$ T cells during *T. congolense* infection; this could also be beneficial when considering vaccination strategies. Vaccines and therapies that work to prevent excessive expression of arginase-1 or interfere with arginase pathway, thereby limiting the suppression of $CD4^+$ T cells, could improve disease outcome.

8.3.2. Future Directions

8.3.2.1. Effect of Low-Dose Intradermal *T. congolense* infection in the Enhanced Susceptibility of Mice to Subsequent Re-infection

Depletion of Tregs using anti-CD25 mAb may not be the best way of studying the effect of Tregs, as some other immune cells do express CD25 and not just Tregs. As a consequence, CD25 KO mice may also have some limitations. Although anti-CD25 has been widely used to study Tregs function, it poses a problem because the antibody could be depleting some other cells that express CD25. In future, it would be important to reconcile this by using an alternative approach of depleting $Foxp3^+$ Tregs using the DEREG mice (DEpletion of REGulatory T cells). These mice can be completely depleted of only $Foxp3^+$ cells. They have diphtheria toxin receptor upstream of an additional Foxp3 promoter such that the injection of diphtheria toxin into the mice will specifically wipe out all Foxp3 expressing cells and allows specific depletion of Tregs at desired time points during experiments [236]. Since the experiments described here did not address if Treg-mediated suppression of cells occurs through contact-dependent (via receptors) or contact-independent (secretions) mechanisms, it will be important to conduct Trans-well experiments in order to get answers to these questions. Collectively, these studies would clearly reveal the contribution of Tregs in low dose intradermal infection-enhanced susceptibility to reinfection.

8.3.2.2. Role of Natural Killer (NK) Cells in the Maintenance of Optimal Immunity to *Trypanosoma congolense* Infection

I successfully expanded and activated NK cells *in vitro* and used them in adoptive transfer experiments before infection. This experimental approach mimics a prophylactic measure against this disease. Future studies should consider the option of using these activated NK cells as therapeutic intervention by (i.) activating and expanding NK cells *in vitro* as already described in the methods section; (ii.) infecting NFIL3 deficient mice with *T. congolense* and allowing infection to establish in the blood of these mice; and (iii.) adoptively transferring *in vitro*-activated NK cells into these mice to see if these cells would be able to rescue the NFIL3 deficient mice from acute death. This experiment, if positive, would validate the importance of NK cells in disease control (both preventative and therapeutic). However, this might be questionable when applied to the human form of the disease, as impaired NK cell function in infected patients has not been documented.

It would also be necessary repeat the experiments described here using *T. brucei* instead of *T. congolense.* This would help determine whether the role of NK cells is parasite strain-specific or a generalized phenomenon observed in African trypanosomiasis. This is critically important because a recent study with *T. brucei* shows that NK cells maybe promoting disease through depletion of B cells leading to the suppression of humoral immunity [120]. It will also be important to focus on understanding the relationship between perforin and trypanosomes; particularly how perforin plays a role in resistance to *T. congolense* infection.

8.3.2.3. Role of Myeloid-Derived Suppressor Cells (MDSCs) in *T. congolense* Infection

Future work in this section should focus on finding the translational benefit of the observed results. I showed that MDSCs suppressed $CD4^+$ T cell proliferation and IFN-γ production in arginase-1-dependent manner. It would be critical and interesting to conduct studies aimed at understanding the role of this enzyme during infection by specifically inhibiting arginase-1 *in vivio* during an ongoing infection with *T. congolense*. I hypothesize that blockade of arginase-1 during infection would restore $CD4^+$ T cell proliferation and ability to produce cytokines. This, if successful, would have a significant implication not just in animal infection, but in the human form of the disease.

Although I observed accumulation of MDSCs in spleens and liver during infection, it remains unknown if their expansion occurs locally in these organs or the cells are being recruited from other tissue compartments. This question could be addressed by assessing Ki67 (marker of proliferation) expression on MDSCs in different tissues compartments in order to see if their increase is due to proliferation or emigration from other tissues.

9. CHAPTER NINE

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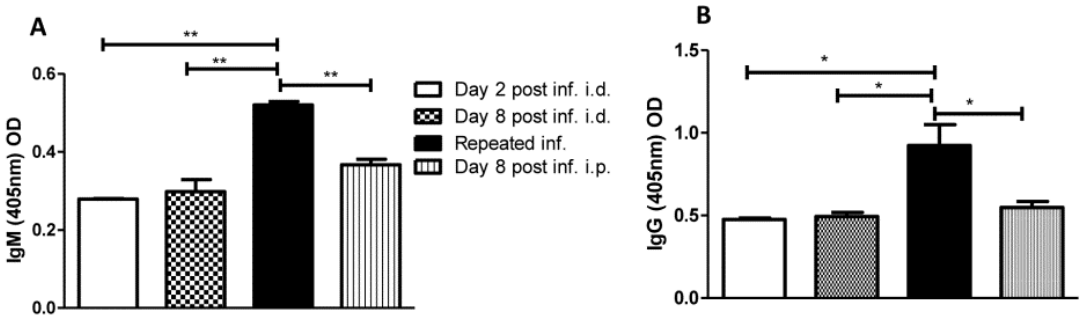
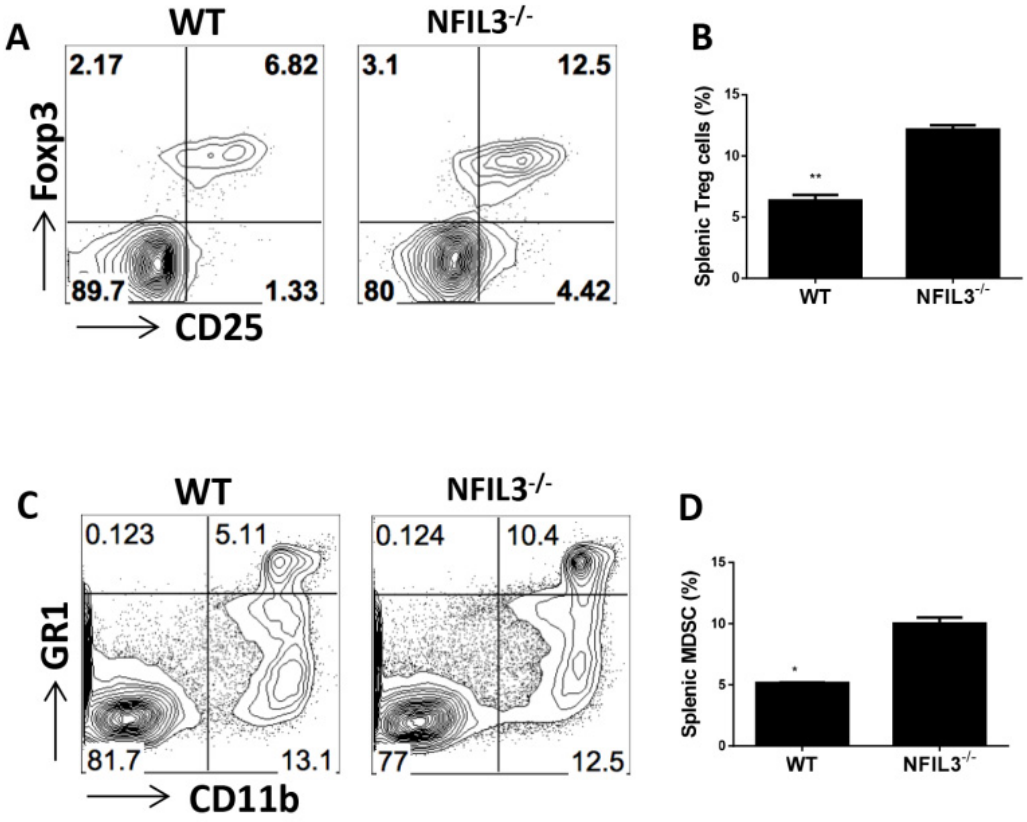
Appendix 1



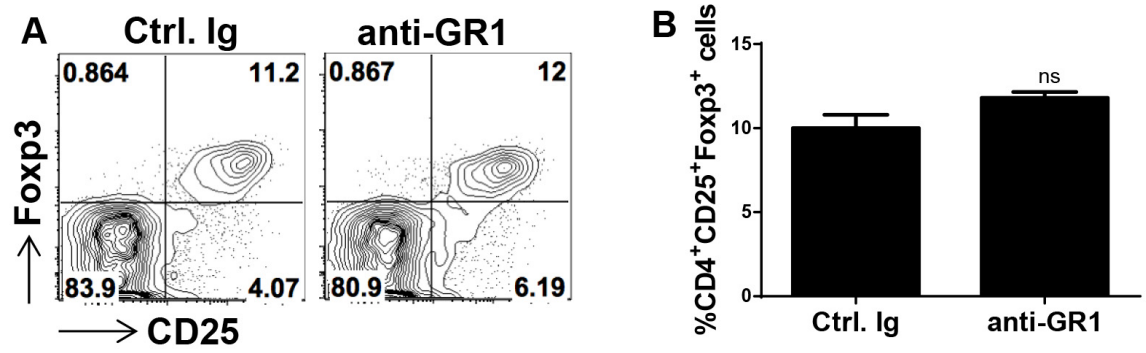
Trypanosome-specific antibody responses in mice infected intradermally with *T. congolense*. Groups of BALB/c mice (n = 3-4) were infected intradermally with a low dose (10^2) *T. congolense* given twice (once weekly for 2 weeks). After 3 weeks, mice were sacrificed and serum levels of parasite-specific IgM (A) and IgG (B) antibodies were determined by ELISA. Mice infected i.d. for 2 and 8 days or i.p for 8 days only were also included. Results are representative of 2 different experiments with a similar outcome. *, $p < 0.05$, **, $p < 0.01$; . ns; not significant.

Appendix 2



Enhanced susceptibility of NFIL3$^{-/-}$ mice to *T. congolense* infection is associated with increased Tregs and MDSC levels. Groups of WT and NFIL3$^{-/-}$ mice (n = 5 mice per group) were infected intraperitoneally with 10^3 *T. congolense.* On day 11 after infection, mice were sacrificed and the percentages of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells-Tregs (A & B) and CD11b$^+$ GR1$^+$ myeloid-derived suppressor cells (C & D) were assessed in the spleen by flow cytometry. Cells were first gated for CD4 expression before assessing CD25 and Foxp3 expressions. The data presented as dot plots (A & C) and bar graphs (B & D) are representative of 2 different experiments with similar outcomes. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$.

Appendix 3



Level of Tregs in anti-GR1-treated *T. congolense*-infected mice. Groups of mice (n = 4 mice per group) were injected intraperitoneally with anti-GR1 mAb (RB6-8C5), 200 µg/mouse) or isotype-matched control mAb a day before intraperitoneal infection with 10^3 *T. congolense.* Anti-GR1 mAb treatment was repeated on day 4 after infection. On day 8 after infection, mice were sacrificed and percentage of $CD4^+CD25^+Foxp3^+$ cells from the spleens were assessed directly *ex vivo* (A & B). Cells were first gated for CD4 expression before assessing CD25 and Foxp3 expressions. The data presented are representative of 2 different experiments with similar results. Bars show mean +/-SEM; ns, not significant.

List of Publications

1. **Onyilagha C**, Singh R, Gounni AS, Uzonna JE. *Thymic Stromal Lymphopoietin Is Critical for Regulation of Proinflammatory Cytokine Response and Resistance to Experimental Trypanosoma congolense Infection*. Front Immunol. 2017 Jul 14;8:803. doi: 10.3389/fimmu.2017.00803. eCollection 2017. PubMed PMID: 28769924; PubMed Central PMCID: PMC5509795.
2. **Onyilagha C**, Jia P, Jayachandran N, Hou S, Okwor I, Kuriakose S, Marshall A, Uzonna JE. *The B cell adaptor molecule Bam32 is critically important for optimal antibody response and resistance to Trypanosoma congolense infection in mice*. PLoS Negl Trop Dis. 2015 Apr 13;9(4):e0003716. doi: 10.1371/journal.pntd.0003716.eCollection 2015 Apr. PubMed PMID: 25875604; PubMed Central PMCID: PMC4395458.
3. **Onyilagha C**, Okwor I, Kuriakose S, Singh R, Uzonna J. *Low-dose intradermal infection with Trypanosoma congolense leads to expansion of regulatory T cells and enhanced susceptibility to reinfection*. Infect Immun. 2014 Mar;82(3):1074-83. doi: 10.1128/IAI.01028-13. Epub 2013 Dec 16. PubMed PMID: 24343657; PubMed Central PMCID: PMC3957988.
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5. Khadem F, Gao X, Mou Z, Jia P, Movassagh H, **Onyilagha C**, Gounni AS, Wright MC, Uzonna JE. *Hepatic stellate cells regulate liver immunity to visceral leishmaniasis through P110δ-dependent induction and expansion of regulatory T cells in mice. Hepatology.* 2016 Feb;63(2):620-32. doi: 10.1002/hep.28130. Epub 2015 Oct 10. PubMed PMID: 26289140.

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8. Okwor IB, Jia P, Mou Z, **Onyilagha C**, Uzonna JE. *CD8+ T cells are preferentially activated during primary low dose leishmania major infection but are completely dispensable during secondary anti-Leishmania immunity.* PLoS Negl Trop Dis. 2014 Nov 20;8(11):e3300. doi: 10.1371/journal.pntd.0003300. eCollection 2014. PubMed PMID: 25412267; PubMed Central PMCID: PMC4238992.

9. Okeke EB, Mou Z, **Onyilagha N**, Jia P, Gounni AS, Uzonna JE. *Deficiency of Phosphatidylinositol 3-Kinase δ Signaling Leads to Diminished Numbers of*

Regulatory T Cells and Increased Neutrophil Activity Resulting in Mortality Due to Endotoxic Shock. J Immunol. 2017 Aug 1;199(3):1086-1095. doi: 10.4049/jimmunol.1600954. Epub 2017 Jun 28. PubMed PMID: 28659355.