The Function of Phosphatidylinositol 3-Kinase delta (PI3Kδ) Enzyme in Protective Immunity to *Trypanosoma congolense* Infection in Mice: Role of Regulatory B cells

by

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ABSTRACT

The PI3Kδ protein is an integral component of Phosphatidylinositol 3 kinase (PI3K) signaling in B lymphocytes necessary for their functions of proliferation and metabolism. *Trypanosoma* species utilize mechanisms targeted at evading host B cells and antibody responses which are critical for immunity. In this project, we sought to determine the impact of PI3Kδ in immunity to *Trypanosoma congolense* infection in mice.

Infection of p110δ^{D910A} mutant mice with *T.congolense* show a surprisingly enhanced control of parasitemia in early infection (7-9 days post-infection), when compared with the relatively resistant C57BL/6 (WT) mice. The mutant mice also showed a delay in B cell activation (CD86, CD80), germinal centre formation and generation of polyclonally-activated B cells (PCB).

Interestingly, drug treatment of C57BL/6 with a p110δ specific inhibitor Idelalisib, to generate partial inhibition of the PI3Kδ resulted similarly in improved control of parasitemia in early disease consistent with genetically-deficient p110δ^{D910A} mice. Idelalisib treatment also delayed B cell activation of CD80/86 and reduced PCB yet resulted in normal germinal centres.

Analysis of cytokine levels in the blood and peritoneal cavity showed a significantly higher proinflammatory (IFNγ) and lower anti-inflammatory (IL10) environment in the treated group was correlated with lower parasite load in the mice. Further analysis showed that the pattern for lower IL10 production was from specific B cells subsets with regulatory functions (B1) cells in the peritoneal cavity in the treated group compared to the WT. Similarly, we observed increased nitric oxide production which correlated with increased parasite killing in early infection in the treated group. Despite the improved early parasite control, there was a 100% mortality in the genetic mutants ($p110\delta^{D910A}$) and 25% mortality in the Idelalisib treated group presumably due to compromised generation of parasite-specific antibodies.

In conclusion, our findings suggest that PI3Kδ has both regulatory functions affecting the initial innate immune response necessary for the early control of trypanosomiasis and subsequently critical functions in generating protective antibodies necessary for long term parasite control and survival. These findings may provide insights into potential pathways that could be targeted to modulate immunity to trypanosome infections and may impact the ongoing search for an effective therapy for the disease.

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DEDICATION

This thesis is dedicated to God and my family; my parents, Mr Olayinka Adefemi and Mrs Funmilayo Adefemi and my siblings, Folakemi, Folamoluwa and Folarin for their unconditional love.

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LIST OF ABBREVIATIONS

µg (Microgram)

ADCC (Antibody-Dependent Cellular Cytotoxicity)

AID (Activation- induced cytidine deaminase enzyme)

APDS (Activated Phosphatidylinositol three kinase delta syndrome)

APOL-1 (Apolipoprotein L1)

ANOVA (Analysis of Variance)

BAFF (B-Cell Activating Factor)

BCR (B Cell Receptor)

BMM (Bone Marrow Derived Macrophages)

BSF (Blood stream form)

Bregs (Regulatory B cells)

BTK (Bruton's Tyrosine Kinase)

C3 (Complement Factor Three)

C5a (Complement Factor Five a)

C567(Complement Factor Five, Six, Seven Complex)

CD (Chagas Disease)

CD3 (Cluster of Differentiation three)

CD4 (Cluster of Differentiation four)

CD5 (Cluster of Differentiation five)

CD11b (Cluster of Differentiation eleven b)

CD14 (Cluster of Differentiation fourteen)

CD40 (Cluster of Differentiation forty)

CD44 (Cluster of Differentiation forty-four)

CSR- (Class Switch Recombination)

DC (Dendritic Cell)

DEAE (Diethylaminoethyl)

DMEM (Dulbecco's Modified Eagle's Medium)

DNA (Deoxyribonucleic Acid)

DTPa (Diphtheria, Tetanus, and *Bordetella pertussis*)

ELISA (Enzyme Linked Immunosorbent Assay)

FACS (Fluorescence Activated Cell Sorting)

FBS (Fetal Bovine Serum)

FDC (Follicular Dendritic Cell)

Fo (Follicular B cells)

Foxp3 (Fork head box p3)

Gal-1 (Galectin One)

GC (Germinal Centre)

GOF (Gain of function)

GM-CSF (Granulocyte Monocyte Colony Stimulating Factor)

GPI (Glycosylphosphatidylinositol)

HAT (Human African Trypanosomiasis)

Ig (Immunoglobulin)

IP (Intraperitoneal)

IFN-γ (Interferon gamma)

IL-1 (Interleukin One)

IL-4 (Interleukin Four)

IL-6 (Interleukin Six)

IL-8 (Interleukin Eight)

IL-10 (Interleukin Ten)

IL-12 (Interleukin Twelve)

IL-17 (Interleukin Seventeen)

iNOS (Inducible Nitric Oxide Synthase)

KI (Knock In)

KO (Knock Out)

LOF (Loss of Function)

MΦ' (Macrophages)

M1 (Classically Activated Macrophages)

M2 (Alternatively Activated Macrophages)

mAb (Monoclonal Antibody)

mTOR (mammalian target of rapamycin)

MAPK (Mitogen- Activated Protein Kinase)

MZ (Marginal Zone B cells)

NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells)

ml (Millilitre)

mM (Millimolar)

mVSG (membrane-form Variant Surface Glycoprotein)

ng (Nanogram)

NK (Natural Killer)

NKT (Natural Killer T Cells)

NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells)

nM (Nanomolar)

NO (Nitric Oxide)

NOD (None obese diabetic)

PBS (Phosphate Buffered Saline)

PCR (Polymerase Chain Reaction)

pg (Picogram)

ps6 (Phospho-s6 kinase)

p110δ (Phosphatidylinositol three kinase delta)

p110γ(Phosphatidylinositol three kinase gamma)

PI3K (Phosphatidylinositol three kinase)

PIP_2 (Phosphatidylinositol (4,5) bisphosphate)

PIP_3(Phosphatidylinositol (3,4,5) trisphosphate)

PRR (Pattern Recognition Receptors)

ROS (Reactive Oxygen species)

rpm (revolution per minute)

RPMI (Roswell Park Memorial Institute Medium)

SDF1 (Stromal Cell-Derived Factor One)

sVSG (Soluble Variant Surface Glycoprotein)

TC (*Trypanosoma congolense*)

Tfh (Follicular helper T cells)

Th1 (T Helper One)

Th2 (T Helper Two)

TLF (Trypanolytic Factors)

TLR (Toll-like Receptor)

TLR4 (Toll-like Receptor four)

TLR9 (Toll-like Receptor Nine)

TNF-α (Tumour Necrosis Factor Alpha)

TNF-α-R1 (Tumour Necrosis Factor Alpha Receptor One)

Tregs (Regulatory T Cells)

TSG (Tris-saline Glucose)

TSLP (Thymic Stromal Lymphopoietin)

USD (United States Dollars)

VSG (Variant Surface Glycoprotein)

WHO (World Health Organisation)

WT (Wild Type)

1. INTRODUCTION

Till date, the disease condition Trypanosomiasis has continued to successfully evade the immune system and the understanding of scientists worldwide[1]. This protozoan disease, documented for its chronicity and immune exhaustion mechanisms, is responsible for high morbidity and mortality rates of humans and animals in Africa (African Trypanosomiasis causing sleeping sickness) and the Americas (American trypanosomiasis causing Chagas disease)[1,2]. The disease causes a huge economic burden in these parts of the world, as an estimated 60 million people are at risk of the disease annually and a total of $4.5 billion USD is lost per annum due to food and domestic animal production losses incurred as a result of the infection (*Trypanosoma congolense*)[3].

Early immunologic insights into the protection from trypanosomiasis infection has shown that, unlike in some parasitic and trypanomastid infections that require a robust innate immune host response to control, an effective B lymphocyte response might be needed to successfully conquer this disease[4,]. While this organism can be controlled by humoral immunity involving binding of specific antibodies produced by B cells, it can specifically target and evade B cells by special decoy mechanisms such as antigenic variation and polyclonal activation of B cells, which ultimately hamper

effective long-term disease control[5,6]. The signaling mechanisms by which host B cells become paralyzed by this organism, are not known. Understanding these mechanisms in B cells with the current immunological advances at our disposal today might reveal novel signaling molecules as therapeutic targets for interventions that could lead to effective disease control and prevention.

1.1 Trypanosomiasis

Trypanosomiasis is a widespread protozoan disease caused globally by diverse species of the genus *Trypanosoma*. It is a vector-borne protozoan disease transmitted by different insect vectors depending on the geographical location. In the Old world, the vector; *Glossina spp* (Tsetse fly) is responsible for African Trypanosomiasis while in the New world Triatominae (kissing bug) is the vector for American Trypanosomiasis[1,3]. These insect vectors become infected following the consumption of a blood meal from an infected host (humans or animals). While some species of *Trypanosoma* are zoonotic (can be transmitted from animals to humans), most species are not and are specific to either humans or animals[7].

Human African Trypanosomiasis (HAT) which is responsible for sleeping sickness in man is endemic in thirty-six (36) sub-Saharan African

countries. It is a neglected disease currently estimated as affecting about 70 million people living in 1.15million km^2 in sub-Sahara Africa[8]. Despite several decades of control measures in these area, cases of this disease still remain prevalent in countries like the Democratic Republic of Congo and the Central African Republic which report high annual cases of the diseases. West African countries like Nigeria, Cameroon and Chad also report cases of the disease but in minimal numbers[1].

Another form of this disease is found in the Latin America. Referred to as American Trypanosomiasis, it causes cardiac and intestinal disease symptoms depending on the stage of the disease (acute or chronic). It is documented that over eight (8) million people in Mexico, Central America and South America are infected with this fatal disease[3].

1.2 The Vector

The vector for Human African Trypanosomiasis (HAT) is the *Glossina spp.* (Tsetse fly). They belong to the order *Diptera*, superfamily *Hippoboscoidea* and the family *Glossinidae.* There are about thirty-four (34) recognised specie and sub-specie of the Tsetse fly and currently, they are located exclusively south of the Sahara in the African continent. Of these species, only six (6) have been identified as vectors for HAT[9].

In New world trypanosomiasis (American Trypanosomiasis) however, Triatomine flies of the subfamily Triatominae are the vectors[5,9].

1.3 The Parasite and Life cycle

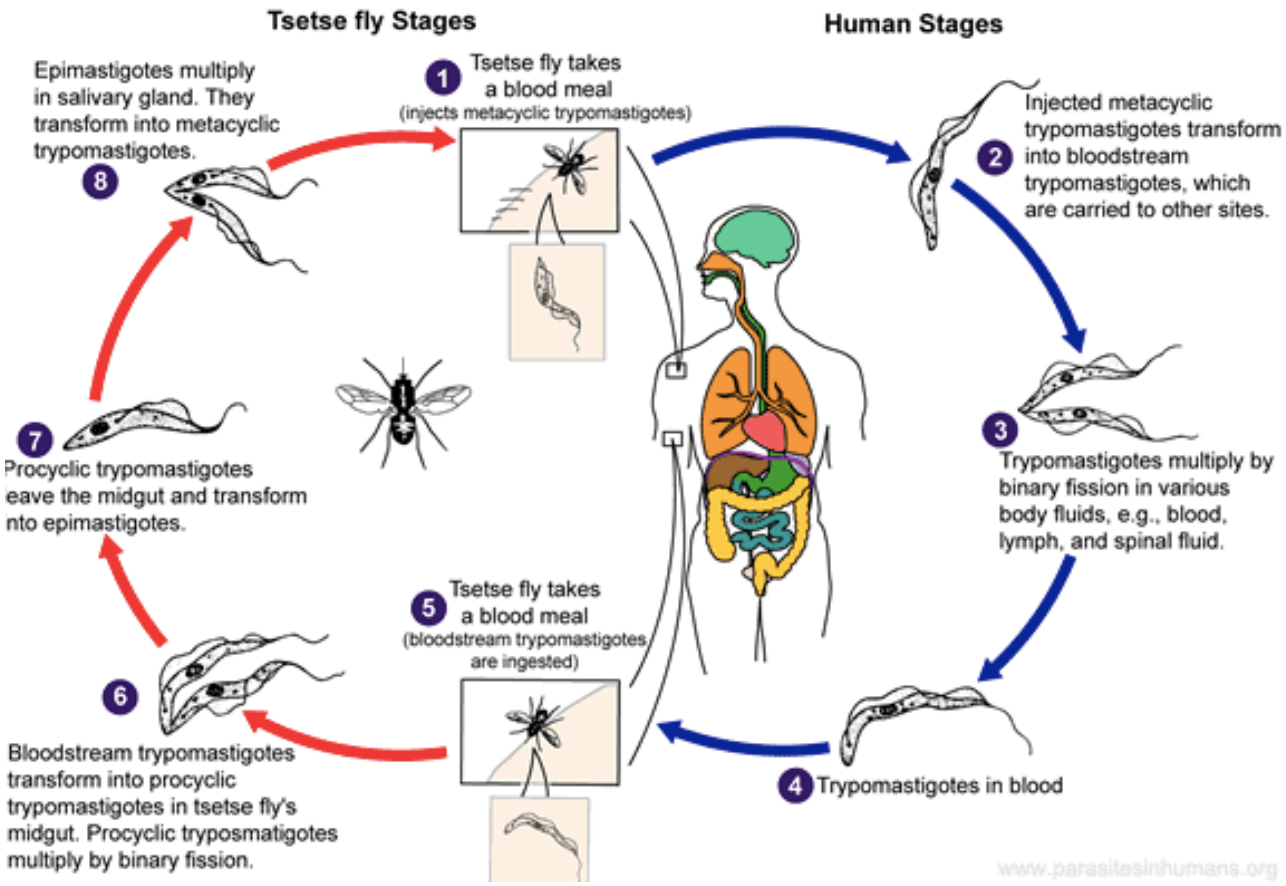
T. congolense like other *Trypanosoma* species is highly dependent on two hosts for survival and replication. Its complex life cycle begins when the Tsetse fly takes a blood meal containing the parasite from an infected animal after which the vector subsequently transmits the organism to another host animal following a bite to the skin[10]. The various forms of the organism exist in different developmental forms between the vector and the host. They are the procyclic trypomastigote, epimastigote, metacyclic trypomastigote and blood stream trypomastigote forms[3].

Inside the vector, the life cycle takes typically three (3) weeks. Ingested blood stream trypomastigote forms (BSF) get established in two locations in the fly: the midgut and proventriculus, although at different times[7,10] .

The BSF typically appear and get transformed into procyclic trypomastigotes in the naïve vector within two (2) days in the midgut, and via binary fission they multiply and expand. It takes about six (6) days to get established in the proventriculus of the fly, where they cease division and become uniform in size. They migrate through the foregut retaining a

trypomastigote morphology and in the proboscis, a trypomastigote - epimastigote transition occurs in the vector at about fourteen (14) days post ingestion. The epimastigote form proceeds into the salivary gland where they transform into the infective metacyclic trypomastigote forms in about twenty-one (21) days, where they further expand by binary fission[10].

The metacyclic trypomastigote is the form that gets deposited from the saliva of the fly into the skin of the host following a bite of the infected tsetse fly. The deposited parasites get into the blood stream and progress into other body fluids including spinal and lymphatic and blood fluids via the blood vessels and lymphatic channels and the cycle continues when another vector ingests the infected blood[1,3].



Typical life cycle of *Trypanosoma* species in the Tsetse fly and mammalian host[3].

1.4 The Disease

A wide range of disease and clinical symptoms are exhibited in trypanosomiasis and this is dependent on a variety of factors such as the environment, vector, parasite specie and specific host[11].

1.4.1 Chagas Disease (CD)

CD is a multisystemic disorder disease caused by the intracellular hemoflagellate, *Trypanosoma cruzi.* Also known as American Trypanosomiasis, its endemicity is reported to span from the geographical regions located between south of the United States and Argentina (latitude 40◦ north and latitude 45◦ south) including highly endemic areas of Bolivia and the lower prevalent zones of Latin America like Brazil, Mexico and Argentina[12]. Although CD is a vector-borne disease transmitted by the Triatomine bug, migratory patterns have further intensified the spread of the disease to other countries like the United States, Australia, Japan and major parts of Europe[8,13,14] .

In vector transmission, the Triatomine insect passes on the metacyclic trypanomsatids from its feces into the host via breaks in the skin during a blood meal. These parasites then invade host tissues like the liver, lymphatics, spleen, gut, central nervous system and cardiac muscles where they differentiate into the intracellular amastigote form of the parasite within

the tissues and then multiply. Subsequently, they transform back within these target organ cells into the flagellate form, which then lyse the cells and spread within the peripheral blood circulation and the lymphatics. The effects of these parasites on the host tissues is responsible for the severe clinical symptoms observed in the disease[15,16].

Other mechanisms of transmission besides vector transmission is transmission during blood transfusions and organ transplantations, transmissions due to contaminated fomites like syringes and needles, oral transmission from contaminated food such as meat, and maternal transmission to offspring. There are over 120 possible mammalian hosts for *T. cruzi* including humans, rodents and wild animals[16].

The clinical symptoms of CD are marked by abnormalities of the digestive, cardiovascular and central nervous systems often characterized by an acute febrile stage that progresses to severe chronic heart failure and sudden mortality[17,18] .

1.4.2 Human African Trypanosomiasis (HAT)

Human African Trypanosomiasis is caused by the extracellular forms of the parasite, *Trypanosoma brucei rhodesiense* (East African sleeping sickness) or *Trypanosoma brucei gambiense* (West-African sleeping sickness) sub-species of the *T. brucei* strain, with the latter implicated in majority of the diagnosed cases. HAT is endemic in these regions with the area reporting about 10,000 new cases annually to the World Health Organisation (WHO), still many cases are left undiagnosed and unreported. A third sub-specie, *T. brucei brucei* rarely infects humans[1,11,19].

This species of the parasite undergoes the vector- host transmission and life cycle that is typical of other members of the genus. Often times, the bite of the fly would result is skin eruptions and acute malaise conditions such as a febrile host state, headaches, joints as well as the muscles. Lymphadenopathy and splenomegaly are also features of the disease. The acute stage is further followed by the invasion of the central nervous system of the host by the parasites where they cause damage to the nerves and other organs of the nervous system. This damage is reflected in clinical manifestations in the host such as irritability and confusion, altered circadian rhythm and balance, as well as seizures. Sleeping sickness causes a high rate of mortality (100%) especially when left undiagnosed and untreated[3,20].

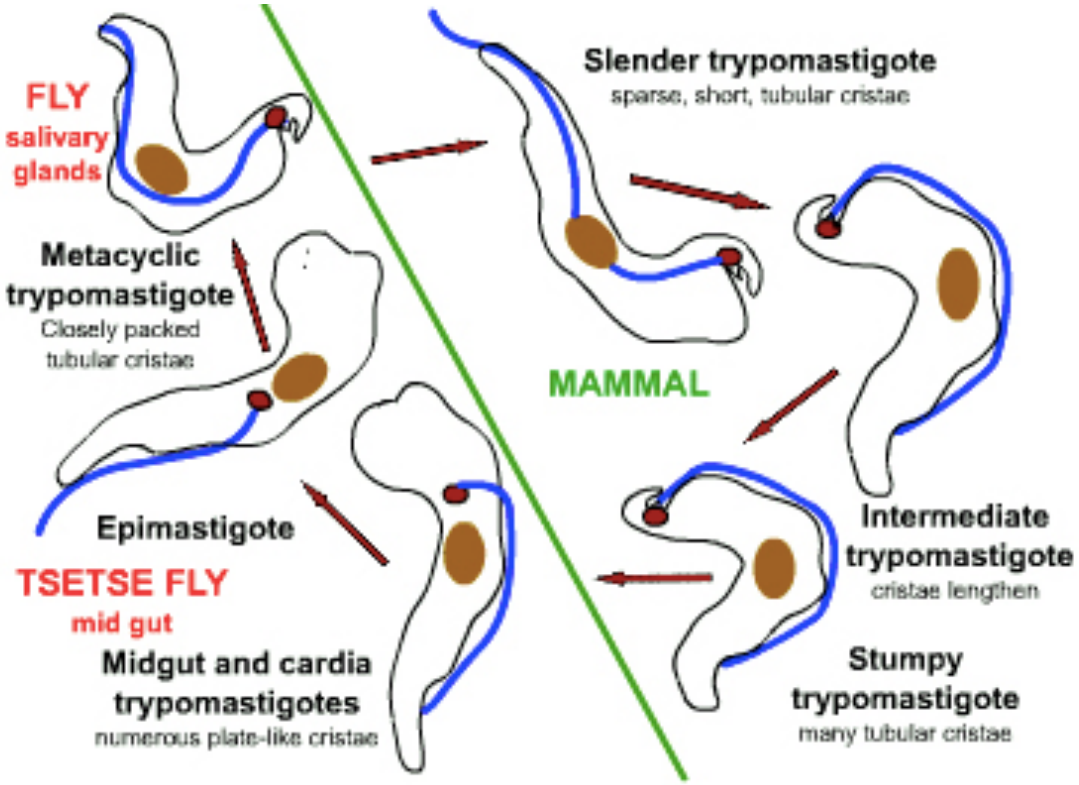
1.4.3 Nagana

Nagana is the name for trypanosomiasis in animals. It is caused by diverse specie of the genus *Trypanosoma* and different species are peculiar to the different animal hosts[21]. *T. congolense* is the major cause of Nagana in East and West Africa where it has been implicated in a variety of hosts ranging from domestic animals like dogs and horses, to livestock animals like cattle, sheep and goats. *T. congolense* which is extracellular, is the major etiologic cause of Nagana in murine hosts such as rats and mice[22]. Symptoms of the disease include fever, lethargy, nervous disorders, cachexia, anemia and mortality[7]. Other known trypanosome species that causes Nagana are *T. vivax,* and *T. brucei brucei* seen in cattle, camels and antelopes. For the majority of nagana disease, the vector is also the Tsetse fly, with the protozoan parasite going through the typical life cycle. However, *T. equinum* and *T. evansi* are transmitted by flies of the genus *Tabanidae* (Horsefly) and cause Mal-deras and Sura respectively in camels and deer. Sexual transmission of trypanosomiasis can occur in horses and donkeys, and the causative agent is *T. equiperdum*[23].

Animal trypanosomiasis although originally native to West Africa, has spread further into many parts of South America due to trade and importation

of animals[24]. This disease is of a huge economic burden in these parts of the world and an estimated amount of about $4.5 billion USD is lost annually due to food and domestic animal production losses incurred as a result of the infection [25].

Interestingly, some breeds of cattle in West-Africa like the N'dama are trypanotolerant to the disease, by mechanisms which remain unknown[26].



Developmental stages found in the bloodstream of the mammalian host, the midgut and the salivary glands of the tsetse fly[23]

1.5 Diagnosis

The clinical signs and symptoms associated with *Trypanosoma* diseases are non-specific and similar to other protozoan diseases[27]. The diagnosis of trypanosomiasis is often done via laboratory testing in endemic regions and though serological testing's for screening purposes are available, they are less diagnostic and specific as seroconversion can occur following the onset of the clinical symptoms therefore limiting the use of antibodies in the diagnosis[3].

A definitive diagnosis of the disease is the successful detection of the parasite in body fluids such as blood, lymph and tissue by microscopy.

In *T. rhodesiense* and *T. gambiense* HAT, cerebrospinal fluids must be tested to determine central nervous involvement and to determine the stage of infection as well as the choice of therapy[1].

1.6 Treatment

There are currently no vaccines to prevent trypanosomiasis in humans and animals, and the treatment of the disease is difficult due to the toxicity of the existing drugs to the host[1,3]. The currently available drugs for HAT are Pentamidine and Suramin for early stage disease, and Melarsoprol and Eflornithine for the late stages of the disease. Administration of these drugs

however, can be laborious and often require special expertise in their administration[28]

Similarly, in animals, there is the increasing challenge of parasite resistance to anti-trypanosome therapy. For example, the anti-protozoan drug dimenazene aceturate (Berenil™) effective against protozoan infections like Babesiosis and Cytauxzoonosis, is another drug often used in the treatment of Trypanosomiasis due to its anti-trypanocidal effects (*T. congolense*). However new studies show the occurrence of Berenil resistant *T. congolense* strains in some natural infections in cattle. In addition, the drug is reported as being highly toxic, especially in dogs, hence, the need for a more effective prophylactic therapy for the disease[27,28].

2. THE COMPONENTS OF IMMUNITY IN TRYPANOSOMIASIS

A wide range of immune responses are generated in *Trypanosoma* infections. They are;

i. Host specific responses by the innate and adaptive system, with the goal of combating the parasite and protecting the host from the invading pathogen[29,4].

ii. Pathogenic immune mechanisms employed by the parasite, with the aim of evading and paralyzing the defense mechanisms mounted by the host[2,30]

2.1 Protective mechanisms

The host immune system is capable of mounting a series of protective immune responses following the invasion of the protozoan pathogen[31]. They include;

2.1.1 The innate cellular responses

The host's innate immune responses to *Trypanosoma* species are originally mediated by cells of the myeloid lineage such as the monocytes, granulocytes, macrophages (MΦ') and some dendritic cell subsets. These cells are reported as being the first line of defence against the parasite and their components, following the invasion of the host. It is reported that to

trigger the innate immune response to the parasite, the glycosylphosphatidylinositol (GPI) that anchors the parasite's surface protein, the variant surface glycoprotein (VSG) needs to interact with pattern recognition receptors (PRR) such as CD14 on monocytic cells, to cause the activation of these cells and permit the simultaneous release of pro-inflammatory cytokines[29, 32].

Additionally, soluble VSG (sVSG) and membrane form VSG (mVSG) can also cause the activation of these innate cells. Although it remains unknown what specific receptors on macrophages they interact with, the induction and maintenance of host mediated innate response to *T. brucei* is thought to occur via Toll -Like receptor (TLR) families that are on cells that signal through MYD88 with a partial requirement for TLR9, to also cause their activation[29,33]. Furthermore, strong NF-kB signaling responses are generated when TLR9 on monocytes interact with the unmethylated CpG dinucleotides regions in the parasite deoxyribonucleic acid (DNA) following parasite lysis and the release of parasite components into the blood. This further strengthens the activation, amplifies type 1 immune responses and ultimately stimulate adaptive immune response through the generation of cytokines and enhanced antigen presentation functions[25, 29,33,34.]

The activation state of monocytes is important for parasite control. The IFNγ mediated activation of macrophages causes the release of pathogen-toxic molecules like reactive oxygen species (ROS), nitric oxide (NO) and TNFα . NO and ROS are thought to act directly on the extracellular parasite inhibiting their growth and multiplication, thereby causing oxidative damage directly to the parasite. In *T. congolense* infection, following the activation of classically activated macrophages (M1), a combination of these factors and the cytokine Interferon gamma (IFN γ) is needed for the destruction of the parasite[29,35].

M1 cells develop in a Type 1 cytokine environment (IL12 and IFNγ) which is needed in early or acute infection however, these responses must be regulated to permit the switch to a Type 2 cytokine environment (IL10, IL4) wherein the alternatively activated macrophages (M2) can be stimulated in late to chronic infection, to prevent the excessive damage to host tissue[29,]. Furthermore, dendritic cells perform antigen presentation functions to trigger the activation of adaptive (anti-trypanosomal) immune responses[36,37]. Innate cells such as macrophages also play the critical role of phagocytosis aside from the release of NO and ROS. The macrophages in the liver (Kupffer cells) , are effective for the phagocytosis and killing of the parasites at this site. They also release prostaglandins and inflammatory cytokines such as

TNFα, IL-1, IL-6, IL-8, and IL-12 to amplify host immune responses against the parasite[31].

There is paucity of information on the role of natural killer (NK) cells in trypanosomal infections. However, they are reported as important for parasitemia control and prolonged survival in *T. congolense* infection. In a study to determine the role of NK cells in *T. congolense* infection, it was observed that the number of NK cells increased in tissues like the spleen, liver and blood post-infection and so did the proportion of $CD4^+$ and CD3 cells which make IFNγ and TNFα. However, *T. congolense* infection of $NKFIL3^{-/-}$ (NK cell deficient mice) showed that the deficiency in NK cells was strongly correlated with increased parasite burden and acute death in the mice. Additionally, NK cell deficiency was associated with a reduction in the secretion of the mediators (IFNγ and TNFα) from T cells and CD3 cells. Invariably, the protective role of NK cells in *T. congolense* infection was concluded as mediated via direct perforin dependent cytotoxic mechanisms. This cytotoxic roles of NK cells has similarly been reported in *T. musculini* infection in mice[29,38].

2.1.2 Innate Trypanolytic components

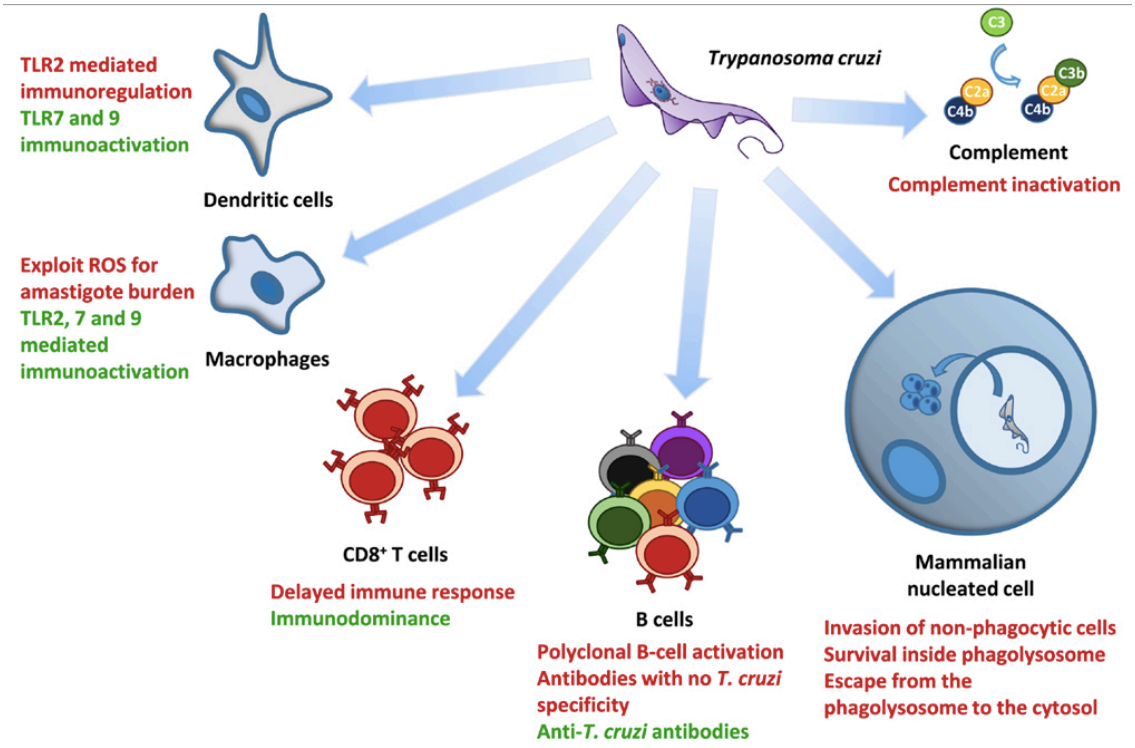
Innate trypanolytic factors (TLF) have been identified as being present in certain *Trypanosoma* resistant hosts. They are composed of apolipoproteins shown to have trypanolytic effects. They are thought to confer resistance to several zoonotic *Trypanosoma* species, as studies show that hosts having a variant form of this protein are more susceptible to the disease[29]. They have been identified in some humans and some subdivision of simian monkeys where they occur naturally in the serum[29,]. These TLF's rich in primate specific apolipoprotein L1 (APOL-1), cause trypanolysis via their N-terminal domains which generate ionic pores in the lysosomal membrane of the parasite, ultimately resulting in the death of the parasite. While certain species like *T. congolense, T. vivax, T. brucie* and *T. evansi* are susceptible to APOL-1, *T. brucie gambiense* and *T. brucei rhodesiense* are resistant to APOL-1[39].

2.1.3 Complement responses

The complement pathway generates several complement-mediated responses in trypanosomiasis infections. The complement factor C3 is necessary for the control of infection, as this protein is a potent opsonin that

enhances *T. congolense* clearance via phagocytosis, as shown by depletion studies of the C3 protein[40]. Furthermore, the relatively resistant strain, C57BL/6 mice are reported to have more plasma levels of C3, and complement factors H and B, when compared with the susceptible BALB/c and A/J mice strains, suggesting that these genetic differences in the plasma complement levels could be implicated in the resistance to disease[41]. Other breakdown components of complements such as C5a, C3a and the C567 complex are important chemo-attractants and contribute to the inflammatory responses seen in early infection[42].

In general, the classical complement pathway triggered by the trypanosome antigen interacting with antibodies is most effective in trypanosome lysis, phagocytosis and clearance, when compared to the alternative pathway. However, the mechanisms for protection via the mannose-binding lectin pathway is still yet to be understood[29.,43]



Components of immunity in *T. cruzi* trypanosomiasis[13] .
*Host protective mechanism = green, Parasite evasion mechanism =red

2.1.4 The role of B Lymphocytes

2.1.4.1 Types of B Cells

The immune system is comprised of different types of B lymphocytes. They can either be follicular (Fo) B cells, marginal zone (MZ) B cells, B1 cells, and regulatory B cells (Bregs) depending on their location, phenotype and function[44].

2.1.4.1.1 Follicular and Marginal Zone B Cells

In mice different subsets of B cells have been identified in the host immune response to a variety of diseases. In the spleen, B cells are mainly subdivided into two populations; the freely circulating Fo or conventional (B2) B cells and the non- circulating MZ B cells. Fo B cells identified by their higher surface expression of CD23 and IgD are the majority population and reside in lymphoid follicles. They depend on follicular helper $CD4^+$ cells (Tfh) help to differentiate into monoreactive plasma cells for specific antibodies and high affinity memory B cell generation[44,45]. The MZ B cells on the other hand, segregate around the marginal sinus of the spleen. They respond rapidly to thymus independent antigens and are often called ‘innate-like‘ B cells since their BCR’s are germ-line encoded with limited diversity and they respond to innate antigens. Additionally, they produce polyreactive and autoreactive

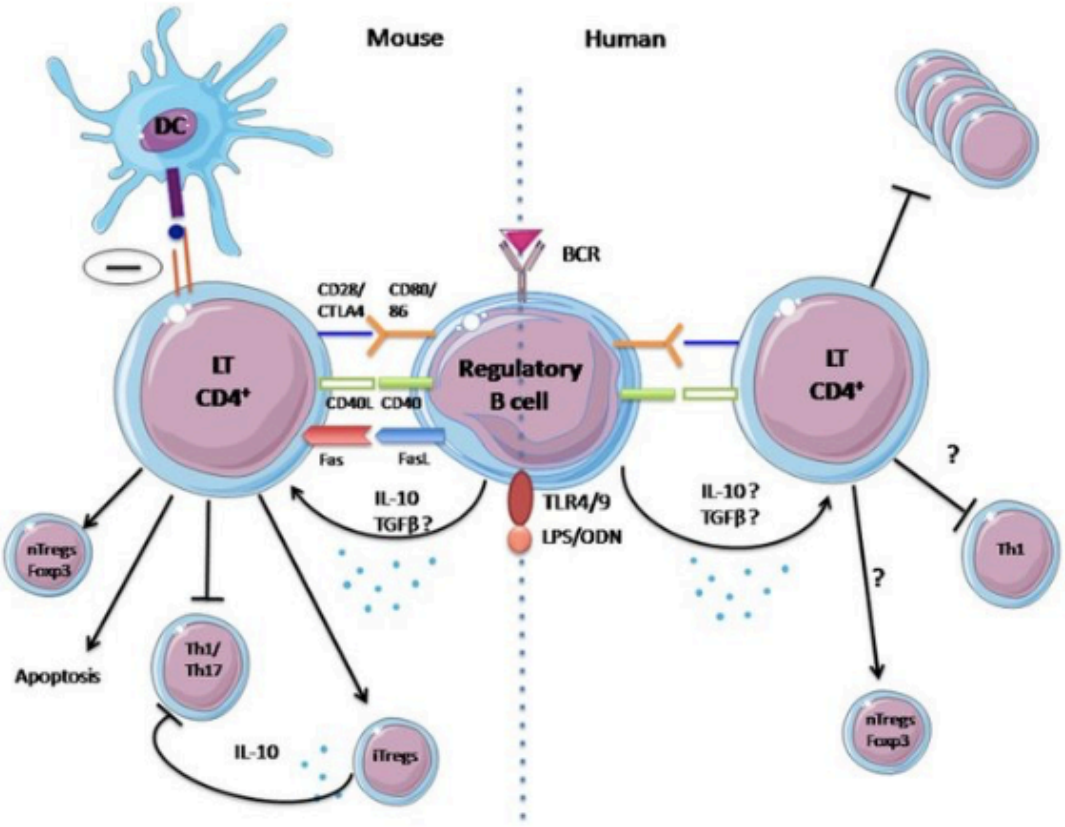
natural antibodies (mostly IgM) at steady state and as first line defence against infections. MZ cells express high levels of IgM and CD21[45].

2.1.4.1. 2 Regulatory B Cells

Another type of unconventional B cells that have been identified in mice are the regulatory B cells (Bregs). They are defined based on their functions, especially their capacity to secrete the regulatory cytokine IL10 which exerts a suppressive function on the host response and invariably regulates the disease outcome which could be protective or deleterious depending on the infection model. These cells are reported to play critical roles in inflammation, auto-immunity and infectious diseases[2,44,46]. Bregs are thought be activated via the BCR or through TLR signalling (TLR4 or 9) and their suppressive functions effected by the secretion of IL10, the activation of CD40-CD40L interactions and possibly via direct interactions with T cells[47]. In mice, their effects include the inhibition of Th1 cell differentiation, inhibition of antigen presentation by DC's and inducing natural regulatory cells. It remains unclear the exact mechanism of action of regulatory B cells in humans[47]. One example of Breg cells are the innate-like B1 cells found in large populations in the peritoneal cavity. MZ B cells have also been described as regulatory as they have been reported to also secrete the cytokine IL10[44,]. In addition,

B cells express Galectin-1 (Gal-1) following enhanced CD40 activation. Gal-1 is capable of inducing T cell apoptosis and consequently reduces IFNγ secretion by T cells[48].

Regulatory plasma cells are another type of Bregs that have recently been identified in the growing repertoire of immunosuppressive B cells. Regulatory plasma cells can arise at steady state or can be induced from other B cell subsets. At steady state they arise under the control of the BCR and develop into LAG-$3^{+}$$CD138^{hi}$ natural regulatory plasma cells. These cells are thought to be generated by self-proteins such as damaged red blood cells[49]. Similarly, via TLR stimulation these cells can also upregulate IL10. Regulatory plasma cells could also be induced from other B cells that have the propensity to upregulate IL10 or from other IL10 producing cells following the interplay of signals that could involve BCR,TLR, CD40 and cytokines like IL6, IL1 and CD21[49].



Immunosuppressive mechanisms of regulatory B cells in human and mice[47].

2.1.4.2 The Germinal Centre response

Germinal centres (GC's) are regions within the spleen and lymph nodes where B lymphocytes proliferate, differentiate, undergo somatic hypermutation and class switch recombination during a response to T-dependent antigens. The GC response is the hallmark of adaptive immunity and the premise for T-dependent humoral response to foreign antigen.

A network of cells collaborates in the development of the GC, they include, the proliferating antigen specific B cells, Tfh cells and Follicular dendritic cells (FDC). The process is thought to begin with the activation of Fo B cells who depend on Tfh help via CD40-CD40L to cause their differentiation into high affinity antibody producing plasma cells and memory B cells to combat foreign antigens. The GC reaction occurs in the presence of the activation-induced cytidine deaminase enzyme (AID) and cytokines[50,51].

2.1.4.3 B Cells in Trypanosomiasis

B lymphocytes are documented to be important for the host resistance to HAT, and murine studies using *T. congolense* show that B cell responses have a strong protective role in the control of the disease [2]. Studies utilizing B cell-deficient mice showed that B cells were necessary to periodically reduce circulating parasite levels and ultimately prolong mice survival via antibody production. As gathered from in-vitro studies using dead parasites, these antibodies are thought to bind to the parasite protein surfaces such as the VSG and other parts of the protozoan cell surface, where they are thought to cause their neutralization[52,53]. Antibodies from B cells such as the isotype, IgG2a, is critical for opsonizing trypanosomes to further augment NO-mediated trypanotoxicity and phagocytosis by macrophages. This

isotype has been described as being the most effective in parasite clearance when compared with other antibody isotypes and class[35].

However, the protective responses of B cells are limited by the VSG-specificity of the B cells. The intermittent changing of the VSG by the parasite is one of the evasion strategies employed by the parasite to evade the host immunity. Hence, to combat this disease, the immune system of the host must be capable of mounting an effective GC response. This ensures that improved trypanosome specific antibodies of the appropriate immunoglobulin class and isotype are constantly generated with each wave of parasitemia and change in VSG of the parasite. This allows the host humoral immunity to effectively recognize and neutralize the parasite[4,46].

The function of B cells in the context of trypanosomal disease, is however not restricted to antibody production and complement activation alone. The secretion of B cell derived cytokines is another mechanism by which B cells regulate immunity in *Trypanosoma* infections.

B cells are a potent source of the pro-inflammatory cytokine IL17 which is protective in acute *T. cruzi* disease. IL17 production by B cells is reported to cause the recruitment of IL10 producing neutrophils which in turn regulate IFNγ and limit collateral tissue damage during infection[2,54]

Preliminary studies show a potential for some memory response in *Trypanosoma* infection in mice (*T. rhodesiense*) in mice. In experimental infection with this specie (10^3 parasites), the mice normally would succumb to the disease five (5) days post infection. Interesting, when the mice were first immunized with a high dose (10^7 parasites) of irradiated *T. rhodesiense* parasites prior to infection, protection measured by survival was seen for up to thirty (30) days post infection[55]. Given that the functions of B cells are quite diverse, it would be interesting to understand the roles of B cells at different time points during the course of *T. congolense* infection as this would be crucial in better understanding immune responses in acute and chronic phases of the disease.

2.1.5 Role of Cytokines

Cytokines play a fundamental role in the immune response to trypanosomiasis. They are involved in the regulation of hematopoiesis, lymphopoiesis and affect the function of all cell types involved in the immune response. It is reported that resistance or susceptibility to African trypanosomiasis in mice is cytokine dependent. Two groups of mice C57BL/6 and B10.BR, described as relatively resistant to *T. brucei rhodesiense* and *T. congolense* develop strong Th1 cell cytokine response early on during

infection when compared to the susceptible C3H and Balb/c mice strains which develop Th2 cytokine responses with little to no Th1 responses[56,37].

The Th1 cytokine IFNγ is described as the master cytokine to confer resistance in trypanosomiasis. It is well established that innate or TLR signals first cause the release of the pro-inflammatory cytokine IL12 from activated macrophages which acts to polarize IFNγ producing T cells. The secreted IFNγ cause the further activation of macrophages which in turn produce NO and ROS needed for immediate control of the parasite as well as more IL12 to potentiate the immune response. This model has been proposed as the possible basis for resistance[57,].

In a study done to depict the protective role of IFNγ in trypanosomiasis, IFNγ-knockout (KO) mice were infected with *T. brucei rhodesiense* LouTat 1 strain and compared to IL4-KO mice. They were similarly compared to the corresponding relatively resistant C57BL/6 mice and the susceptible SCID mice. The IFNγ- KO mice were shown to have poor survival (19 days) similar to the SCID mice (20 days) while the IL4-KO mice had better survival (43 days) similar to the C57BL/6 (WT) mice. Additionally, while both the WT and IL4-KO mice were able to control parasitemia and mounted good VSG - specific antibody responses, the IFNγ- KO group had higher parasite burden and were unable to clear the second wave of infection before succumbing to

the disease. The antibody mediated response in these mice were also investigated and while the IFNγ- KO had similar levels of VSG specific IgM and IgG1 with the WT, their IgG3 levels was slightly lower and VSG-specific IgG2a completely abrogated. These findings suggest that IFNγ was critical for parasite control and specific isotype generation in their model[57,58,59].

A balance in the secretion of IFNγ is however needed in the host to avoid immunopathology. Over secretion of the cytokine has been correlated with to higher parasite load in the blood[36]. In addition, the overexpression of the IFNγ gene seen in trypanosomiasis is reportedly linked to fever, suppression of erythropoiesis, anemia, as well as neuropathology and dysfunction of the blood brain barrier[57].

TNFα is another pro-inflammatory cytokine made by macrophages that functions in innate immune response. TNFα is thought to be induced primarily by soluble VSG and is notably critical in targeting intracellular forms of the pathogens, such as *T. cruzi*[60]. Kupffer cell mediated phagocytosis of parasite and parasite debris as well as NO dependent trypanocidal functions are potentiated via TNFα -R1 signaling. Unlike other trypanomastids such as *Leishmania* known to proliferate in phagocytes, there is no evidence to support that this occurs with trypanosomes. The released NO can directly

alter major metabolic processes in extracellular (*T. congolense*) and intracellular forms (*T. cruzi*) of the parasite.

For example, cruzipain, a cysteine proteinase *in T. cruzi* is altered by NO. This promotes parasite killing and efficient parasite clearance in the liver, control infection induced pathology and extend mice survival in experimental trypanosomiasis. Conflicting studies in natural infection however, suggests that TNFα might be associated with rapid disease progression in sleeping sickness, as overproduction of this cytokine by macrophages promotes B cell activation, proliferation, differentiation and consequently the development of hypergammaglobulinemia, for reasons still yet to be fully understood. *T. congolense* specie of the parasite appear to be refractory to TNFα induced trypanolysis[29,60,61].

Other cytokines with pro-inflammatory functions in trypanosomal disease include IL12, IL6 and IL1β. Although their function in the context of the disease continues to be dissected, available studies suggest a role for protection in the acute phase of experimental trypanosomiasis in mice and HAT. IL1β and IL6 cause the recruitment of lymphocytes to the local inflammatory sites of the cardiac muscles in CD, by modulating the expression of adhesion molecules on the endothelium. However, their over production is linked with a variety of alterations in endothelial cells, reduction

in neuropathology function and consequent microvascular spasms often associated with CD myocardiopathy[36,56].

On the other hand, in experimental trypanosomiasis IL10, IL4 and transforming growth factor beta (TGF-β) are able to downregulate Th1 responses. They specifically downregulate the pro-inflammatory responses mediated by IFNγ-activated macrophages, inhibiting NO and IL12 release from these cells and ultimately regulating the activity of the Th1 subset of cells. In mice, IL10 production is critical for reducing pathology and prolonging survival. This was shown in a *T. congolense* infection study of IL10- KO mice where there was marked reduction in survival time in these mice which was associated with severe increases in the blood levels of the pro- inflammatory mediators; IFNγ and NO[59,57]. Similarly, the anti-inflammatory functions of IL10 is reported to curb the severity of anemias associated with trypanosomiasis[62,63].

Aside downregulating pro-inflammatory responses, IL10 further contributes to trypanotolerance by inhibiting pathogenic dendritic cell populations called Tip-DCs, known to cause the overproduction of NO and TNFα that cause collateral tissue damage especially in the liver[64].

IL10 inhibits their recruitment, differentiation and maturation, indirectly preserving the liver architecture and promoting the parasite clearance capacity of the organ[29].

In another study model of *T. congolense* infection, the source IL10 was found to be regulatory (Tregs), non-regulatory T cells, macrophage and Breg cells, with the latter cell types accounting for the bulk of IL10 being produced in the spleen of these mice[65]. Tregs were reported as critical for trypanotolerance only after the first wave of the infection had subsided[2,64] Taken together, these findings suggest a regulatory role for IL10 as a balance for trypanotolerance induced and pathogenic cellular immune responses in the context of this disease.

One of the hallmarks of African trypanosomiasis is a phenomenon referred to as *cytokine storm*. It is the dysregulation of the cytokine network characterized by the excessive release of inflammatory mediators and overproduction of cytokines intended as a defensive response by the host's immune system[66]. The excess release of these chemical mediators is suggested to promote collateral damage to the host tissues and likely account for *Trypanosoma* associated mortalities. Hence a state of equilibrium in quality, quantity and timing of release (early or late infection) of these chemical mediators is critical if an ideal immune response is to be

mounted, which would be detrimental to the parasite but yet favourable to the host[66,67].

2.2 Immune evasion mechanisms

Trypanosoma species equally employ an array of pathogenic mechanisms in a bid to evade the host immune responses[13]. These mechanisms would include;

2.2.1. Antigenic variation of the variable surface glycoprotein (VSG)

Antigenic variation is the critical virulence factor employed by trypanosomes in their evasion of the host humoral immunity. Extracellular *Trypanosoma* species like *T. brucei* and *T. congolense* are constantly exposed to the host effector cells and immune mediators. However, in a bid to evade these responses, they use an elaborate defence mechanism called *antigenic variation*. Antigenic variation describes the routine and speedy modification of the outer most surface protein of the parasite called the VSG, by the parasite as an evasion measure against the host immune responses[2]. Besides evading the humoral immune response, VSG's are highly immunogenic and cause the constant activation and stimulation of the immune system and cells, such as antibody producing B cells. This subsequently leads to hyperglobulinemia and immune exhaustion; hallmarks of African trypanosomiasis. These parasites are thought to have over two thousand (2000) VSG pseudogenes and genes in the gene repertoire and

can renew their entire surface protein coat within a short period of time. They are also capable of breaking down and recycling compromised VSG's, as well as internalizing VSG-specific antibodies[13,4]. Additionally, VSG's profoundly cause hyperactivation of the classically activated complement pathway but interestingly, inhibits the alternative complement pathway, consequently leading to auto-immune disease and host tissue pathology like glomerulitis. It is this pathogenic mechanism of antigenic variation by the parasite that makes the design of a potential trypanosome vaccine difficult[68].

2.2.2 Modulation of B cell compartments

Trypanosoma species are also capable of altering the B lymphocyte compartments of the host and manipulating B cell responses[6,14]. In the bone marrow, B cell precursor numbers are decreased following *T. brucei* infection in mice. This has been linked to the expression of the stromal cell-derived factor 1 (SDF1) which is increased following infection and correlates with loss of developing B cells. Similarly, *T. cruzi* is thought to cause a marked decrease of B cells precursors possibly via the cyclooxygenase pathway that can occur from myeloid cells, ultimately negatively impacting erythropoiesis and lymphopoiesis[53,69].

In the spleen, trypanosomes are capable of promoting B cell apoptosis through Fas receptor and Fas ligand (FasR/FasL) interactions, affecting both

transitional (T1 &T2) B cell populations. Furthermore, it is reported that perforins from NK cells lyse Fo and MZ B cells and ultimately distort their compartments in the spleen[2,70,]

2.2.3 Polyclonal activation of immune cells

It has been depicted using different experimental murine models of trypanosome, (*T. brucei*, *T. cruzi* and *T.congolense*) that as early as the first few days of infection, polyclonally activated B lymphocytes are present in the spleen[5]. While it is unclear if these non-specific activations are host mediated or parasite induced, available data supports the role of parasites as potent activators of B cells. This is thought to be another evasion strategy employed by the parasites in an attempt to dilute out VSG specific antibodies generated during the course of infection. Additionally, the polyclonally activated B cells secrete low affinity immunoglobulins, which consequently lead to high quantities of non-specific antibodies and auto antibodies in circulation[30, 6].

Not much is known on if the polyclonal activation of B cells by the parasites directly impact GC's or their responses. Still, several parasite derived metabolic molecules have been identified as polyclonal activators of B cells and some of these molecules aid the B cells survival possibly via the B cell activating factor (BAFF) signalling[72]. These parasite molecules include

transialidase and proline racemase found on *T. cruzi*. Transialidase induced B cell activation has also been linked to Bruton's Tyrosine Kinase (BTK) following BCR signalling. VSG and mitochondrial dehydrogenase are potent activators of B cells observed in *T. brucei* diseases. While no studies have clearly identified *T. congolense* specific proteins that cause the polyclonal activation of B cells, several of these parasite proteins are conserved among the different trypanosome strains. Polyclonally activated B cells are thought to be detrimental in the course of the disease and increased numbers of these cells are found in the susceptible Balb/C mice when compared to the relatively resistant C57BL/6 mice strain[2,54,73].

2.2.4 Depletion of memory cells.

Recent studies suggests the negative role of trypanosomes on the host's capacity to generate recall responses against non-related pathogens[74]. In a study to determine the impact of *T. brucei* infection on immune memory, mice were vaccinated with a non-related pathogen; *Bordetella pertussis*, using the established human diphtheria, tetanus, and *B. pertussis* (DTPa) vaccination model prior to *T. brucei* infection and then subsequently re-challenged with *B. pertussis.* It was observed that *T. brucei* infection caused the abrogation of vaccine induced protection. This

was attributed to the infection induced loss of IgM^+ B cells which coincided with the loss of variant-specific T-independent IgM antibody responses[75]. Additionally, the ongoing trypanosome infections was capable of altering B cell responsiveness and inhibiting the generation of protective memory responses[74].

Collectively, these evasion mechanisms cause the progression of the disease into an often chronic and debilitating state which hampers effective host immune responses, results in immune exhaustion and ultimately the death of the host that is eventually seen, even in the relatively resistant experimental mice models and natural infections in humans.

2.2.5 Immune suppression

Immunosuppression is another immune evasion mechanism observed in African trypanosomiasis. Although the exact cellular mechanisms that regulate immune suppression is not well understood, a number of suppressive cell types have been implicated to modulate the host immune response to the disease. These include M2 macrophages that can suppress T cells responses via prostaglandins and ROS intermediates[64]. Additionally, in *T. congolense* infection $CD4^+CD25^+$ $Foxp3^+$ Tregs are shown to suppress the immune system through their capacity to secrete the regulatory cytokine

IL10. More recently, myeloid derived suppressor cells (MSDC's) have been implicated in having immunosuppressive roles in trypanosomiasis via suppression of T cell proliferation and associated IFNγ production and this is thought to occur in an arginase -1- dependent meachanism[76].

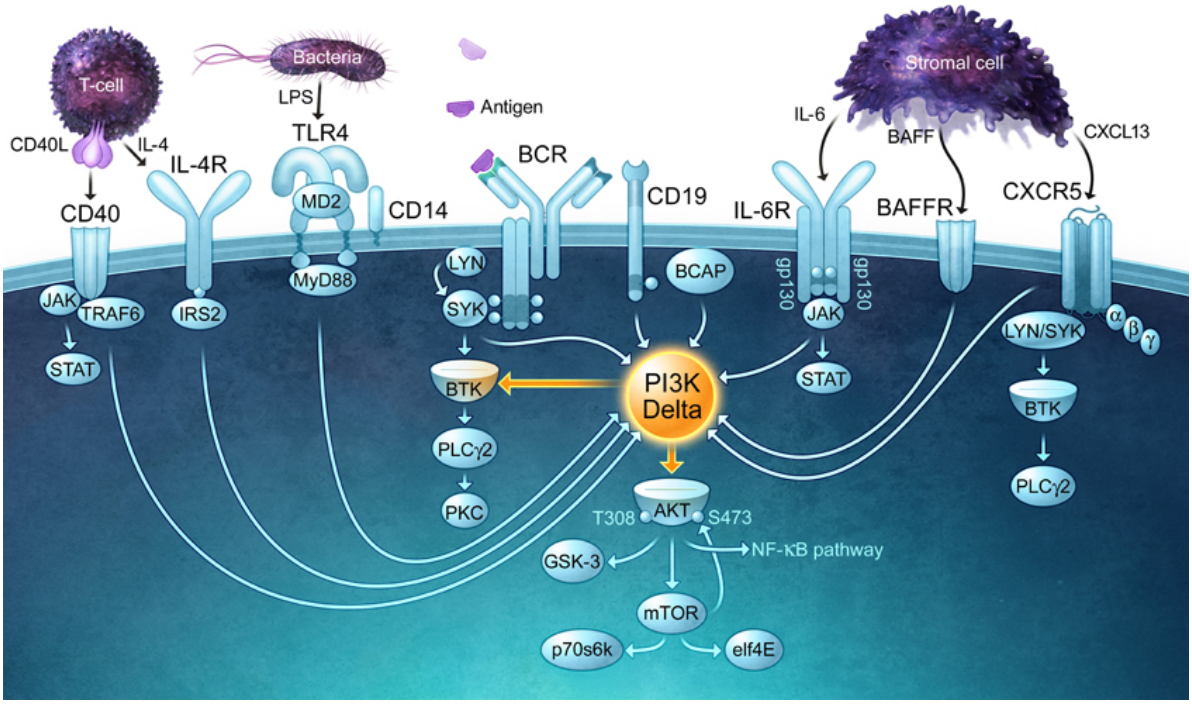
3. PHOSPHATIDYLINOSITOL-3-KINASES SIGNALING

The phosphatidylinositol 3-kinase (PI3K) proteins are a family of lipid kinases present in diverse cell types and integral for a variety of cellular functions such as cell survival, proliferation, differentiation and metabolism[77]. The PI3K's are grouped into different classes; class I, II and III based on their structure, regulation and lipid substrate specificities [78]. In-vivo, the class I PI3K's catalyze the conversion of the protein phosphatidylinositol (4,5) bisphosphate (PIP_2) to phosphatidylinositol (3,4,5) trisphosphate (PIP_3). The class I PI3K's are further subdivided into class IA and IB based on the similarity of their sequences. Class IA PI3K's are heterodimeric, composing of a p85 regulatory subunit and p110 catalytic subunits. There are 4 genes encoding distinct p110 catalytic subunit, which has four isoforms (α, β, δ, γ), however p110γ uses a distinct regulatory subunit p101[79,80]. Generally, each isotype of proteins is important for cellular functions depending on the distinct cell type.

3.1 Phosphatidylinositol 3-Kinase delta (PI3Kδ)

The p110δ protein is one of the catalytic subunit of the class IA heterodimer and is primarily expressed in leukocytes and myeloid cells[81]. It is found primarily to exist in association with the regulatory subunits

especially p85. The p85 subunit prevents its degradation, inhibits its catalytic activity and helps in its recruitment to tyrosine phosphorylated proteins at the plasma membrane[82]. The p110δ catalytic activity can be activated by variety of receptors depending on the specific cell types and receptor ligands in the surrounding environment. For example in B cells, BCR (antigen receptor), TLR's, costimulatory molecules like CD40 as well as cytokine and chemokine receptors are capable of activating p110δ, hence determining the different downstream effects of receptor engagement[83].



PI3Kδ activating receptors and signaling pathway on a B lymphocyte[83]

3.2 Immunoregulatory functions of PI3Kδ

In understanding of the role of p110δ protein in the regulation of immunity, loss of function (LOF) studies and gain of function (GOF) studies via different gene targeting techniques have been employed and over the last few decades the many functions of this protein have been dissected. They include roles of;

3.2.1. PI3Kδ in cell activation and signaling

PI3Kδ is critical for leukocyte activation and signaling. It has particularly been implicated as significant for B and T cell antigen receptor signaling. In LOF studies using the $p110\delta^{D910A}$ mice (a knock-in model expressing a catalytically inactive form of p110δ) the measure of downstream targets of activation such as Akt , the phosphorylation of the mitogen- activated protein kinase (MAPK) Erk and Ca^{2+} flux was severely compromised in their B and T cells following BCR and TCR stimulation with anti-IgM and anti CD3 respectively, hence loss or deficiency in p110δ reduces activation and compromises signalling in these cells[73,81,84].

Interestingly, bone marrow derived macrophages (BMM) from mice defective in p110δ showed enhanced TLR-induced MAPK activation which conflicts with that observed in B and T lymphocytes or BMM's from WT mice

[85]. Studies have shown that the activation of PI3K /Akt is a negative regulator of TLR and NF-kB in TLR stimulated macrophages [86]. The activation of this pathway leads to the suppression of several proteins needed for TLR4 signalling for example the inactivation of FOXO1, regulation of *let-7e* and the upregulation of IL1 receptor-associated kinase M (IRAK-M), which suppresses TLR4 signalling via TRAF6 inactivation. This consequently leads to reduced macrophage activation by LPS, restricts pro-inflammatory and promotes anti-inflammatory responses in TLR stimulated macrophages. Alternatively, the non-specific chemical inhibition of the PI3K signalling in TLR activated macrophages enhances NF-kB activation and iNOS expression favouring an M1 type macrophage response[86].

Similarly, gain of function (GOF) mutations have been identified in humans in the PIK3CD gene responsible for encoding the p110δ catalytic subunit. This results in a primary immunodeficiency disease called Activated PI3Kδ syndrome (APDS). The disease is characterized by complex immune phenotypes including perturbed humoral and cellular responses such as elevated natural IgM, IgG3 and baseline increases in plasma cells, peripheral innate B1a and MZ B cell compartments as well as altered class switching. APDS patients often times suffer from lymphopenia, recurrent respiratory infections and impaired vaccine responses[87].

Evidences from GOF studies in human as well as in mice supports the role for p110δ in lymphocyte activation. This mutation resulting in the hyperactivation of PI3K/Akt is reported to cause the hyperactivation of the mammalian target of rapamycin (mTOR) pathway[88]. B cells from Mb1-aPI3KCD mice, a GOF mice model with a B cell specific gain of function mutation, showed a hyperactivated state, marked elevation in phospho-S6 kinase (pS6) which is downstream of the PI3k/AKt/mTOR pathway and often time associated with increased cell growth and proliferation. These B cells also showed increased survival[89]. In another study with a different GOF mouse model, $Pik3cd^{E1020K}$ (germline GOF mutation), splenocytes and B cells from homozygous and heterozygous GOF mutants showed significant elevation in the basal levels of both phosphorylated Akt (T308 and S473) and pS6 and the phosphorylation was only reduced when these cells when pre-incubated with the p110δ specific inhibitor leniolisib[90]. These observations further support the role for PI3Kδ in activation and signaling in these cells.

3.2.2. PI3Kδ in cell development

The p110δ protein has been linked to developmental functions in immune cells. It is reported to be an important regulator of NK and NK T cell

development. In a study to compare the NK and NK T cellularity in three groups of mice; p110γ deficient, p110δ deficient mice and WT mice, no difference was observed in the p110γ deficient mice and WT groups, however, the p110δ- deficient mice showed significantly lower NK and NK T cells (40% and 60% respectively) than the WT. Additionally, NK cell surface markers Ly49G2, lY49C/I and the late stage differentiation markers CD11b/43 were abnormally expressed [91], suggesting that p110δ participates in NK development and differentiation.

Similarly, p110δ plays a role in T cell differentiation. A reduction in Tregs and Tfh cell populations were observed following LOF mutation of the protein and although the $CD4^+$ T cells from $p110KI\delta^{D910A}$ were not altered in number, they had altered expression of the activation marker CD44 which distinguishes naïve T cells ($CD44^{lo}$) from memory T cells ($CD44^{hi}$)[84,92,93]. GOF mutations are also characterized by defects in T cells in the spleen and lymph nodes of $Pik3cd^{E1020K}$ GOF mice. Significantly increased proportions of activated/memory T cells which are $CD44^{hi}$ $CD62L^{lo}$ expressing cells , as well as $Foxp3^+$ Tregs cells were observed in these mice which is the opposite of the characteristics seen in the LOF mutants[94].

PI3Kδ is also critical for early B cell development in the bone marrow and spleen. LOF studies using the $p110KI\delta^{D910A}$ mice shows a marked

reduction in the $B220^{+}IgM^{+}$ B cell progenitors, specifically the ratio of pre-B cells to pro-B cells in the bone marrow were altered in the mutant mice. However, $Pik3cd^{E1020K}$ GOF mice show a contrasting pattern with a marked increase in pro-B cells but significant decrease in mature recirculating IgD^{+} cells similar to what is seen in the $p110K\delta^{D910A}$ LOF mice[94]. This suggests that a balance is needed as either too little or too much PI3Kδ activity can impairs mature IgD^{+} follicular B cell differentiation.

In the spleen, the $p110KI\delta^{D910A}$ mice had fewer cells (50%) than the WT mice, with B lymphocytes being more severely reduced than the T lymphocytes in the tissue. This pattern was however the reverse in the $Pik3cd^{E1020K}$ GOF mice as their splenic cellularity was observed as greater compared to the WT mice with B cell number being significantly increased in the spleen[81,94]. Additionally, innate-like B cells subsets (B1 cells in the peritoneum and splenic MZ cells) were observed as reduced in the heterozygous $p110\delta^{WT/D910A}$ mice and completely abrogated in the homozygous $p110\delta^{D910A/D910A}$. This is in contrast with what has been reported in the $Pik3cd^{E1020K}$ GOF mice which have markedly increased splenic B1 cells and MZ B cells. Similarly, there was an increase in total percentage of circulating B cells in the GOF mutants in comparison to the WT or LOF mutants[81,89], further demonstrating that p110δ is important in B cell

development and differentiation[81,89]. Ultimately, these reported findings emphasize the role of P110δ in leukocyte development, differentiation and survival.

3.2.3. PI3kδ in proliferation and migration

PI3kδ regulates the capacity of purified B cells to proliferate in response to exogenous ligands such as anti-IgM or anti-cd40, IL4 LPS. Cell proliferation in response to these stimulants were impaired following the genetic inhibition of this protein. This reduced capacity to proliferate was also observed when purified T cells from p110KIδ^{D910A} LOF mutants were stimulated with anti-CD3 only. CD8 T cell proliferation was also impaired in p110δ $^{-/-}$ knock-out mice[81,95].

Leukocyte migration has also been linked to the p110δ protein. It was reported in an in-vivo study model examining neutrophil migration, that the migration of neutrophils to chemo-attractants and their accumulation at sites of inflammation was reduced as a result of compromised PI3Kδ protein[96.97]. In a similar fashion, PI3Kδ is reported as indispensable for NK cell chemotaxis to S1P and CXCL10 , for their distribution in lymphoid and non-lymphoid tissues, as well as their extravasation to tumors[98].

T cells from p110KIδ^{D910A} LOF mice have similarly shown reduced chemotactic responses to the chemokines CXCL12, CCL19, CCL21.

Additionally, there was altered CD62L expression necessary for effector T cell homing to the lymph node, ultimately compromising their localization to antigenic tissues[99].

B cells migration is also compromised in PI3Kδ deficiency. B cells from p110KIδ^{D910A} mice show diminished chemotactic responses to the chemokine CXCL13 and adoptive transfer experiments with p110δ deficient B cells reveal diminished homing of B cells to Peyer's patches, mesenteric lymph nodes and splenic white pulp cords[100]. Taken together these reports suggests the importance of PI3Kδ in immune cell migration.

3.2.4. PI3Kδ in cytokine production

PI3Kδ has been incriminated in cytokine production in a variety of immune cells. The secretion of IFNγ, TNFα and GM-CSF from NK cells of p110δ deficient mice was remarkably decreased following stimulation with different stimulants for 48 hours in in-vitro cultures[91].

In an allergic model, p110δ was observed to regulate the production of TNFα and IL6 secretion from mast cells, as well as IL8 production from neutrophils[101]. In T cells , the pharmacological inhibition of PI3Kδ with the p110δ specific inhibitor (IC87114) in in-vitro cultures of $CD4^+$ T cells from NOD mice (non obese diabetic mice) following their stimulation, interestingly

resulted in impaired secretion of IFNγ when compared with the untreated group, even though T cell proliferation was not altered[102].

This finding is in tandem with a similar study that examined cytokine production from the different T helper cell lineages; Th17, Th1, Th2 following treatment with another p110δ specific inhibitor. There was significant reduction in specific cytokine production from all the T helper cell lineages examined[103].

PI3Kδ also regulates TLR-induced cytokine production from B cells. In-vitro culture of B cells from $p110\delta^{D910A}$ mice following stimulation with CpG oligodeoxynucleotide and LPS, resulted in significantly lower IL6 and IL10, elevated IL12 secretion and no significant cytokine production for IL4 further highlighting the role PI3Kδ in cytokine production[104].

PI3K similarly affect macrophage cytokine production. It has been reported that the activation of Akt promotes the induction of the suppressive cytokine IL10 from macrophages while the deletion of Akt allowed the upregulation of iNOS and IL12β enhancing bacteria clearance. Furthermore, macrophages deficient for p85a regulatory subunit had impaired IL12 production in response to LPS and IFNγ stimulation[86].

3.2.5. PI3Kδ in leucocyte function

The PI3Kδ protein also regulates leucocyte functions. In NK cells, in-vitro studies suggest the redundant roles of p110δ and P110γ in NK mediated cytotoxicity in host response[91]. In addition, the degranulation of mast cells upon IgE receptor cross-linking is compromised following the inhibition of p110δ, which is potentially of importance in allergic diseases[96]. In a study to examine the role of p110δ on $CD8^+$ T cell function using a $p110\delta^{-/-}$ knock out mice, it was observed that $CD8^+$ T cells from the p110δ deficient mice exhibited reduced perforin, granzyme A, and granzyme B mRNA expression when activated with anti-CD3 and were compromised in carrying out their cytotoxic roles[95]. Similarly, PI3Kδ deficiency not only altered Treg numbers at baseline but impaired their immunomodulatory functions[105]. Tregs pre-treated with the PI3Kδ inhibitor idelalisib were observed to lose their ability to suppress $CD3^+$ T cell proliferation[106]. Interestingly, in another study where oral PI3Kδ inhibitor IC87114 was administered in mice, the treatment had no effect on the percentage and absolute numbers of Tregs, or altered their proliferation in culture, however their secretion of IL10 significantly dropped[102].

P110δ and its regulatory subunits are the only PI3K molecules that contribute to the polarization of M1 macrophages, all other isoforms of the

protein polarize M2 macrophages. It has been reported that the activation of the PI3K /AKT pathway restricts pro-inflammatory responses and promotes anti-inflammatory responses[86]. However, macrophages show hyper-responsiveness to TLR signaling when p110δ activity is altered and they are stimulated with different TLR ligands; LPS (TLR4), TL9 (CpG), TLR5 (flagellin) and TLR2 (synthetic bacterial lipoprotein). Interestingly these p110δ deficient macrophages secreted more inflammatory cytokines and NO in response to the ligands when compared with WT BMM[85].

P110δ also regulates B cell functions especially the generation of T-independent and T-dependent humoral immune responses. Serum immunoglobulin levels and the generation of GC responses were severely compromised in p110KIδ^{D910A} mice. They were observed to have significantly reduced total IgM and IgA levels and were unable to generate sufficient TNP-specific antibodies (less than 5%) of that generated by WT mice, following immunization with TNP-KLH. The spleens of immunized p110KIδ^{D910A} mutants were absent of GC's when compared with WT mice, demonstrating that T cell dependent responses were impaired in these mice. Furthermore, T cell independent response were compromised when TNP-Ficoll was used to immunize the mutants[81,92,107].

This strongly opposes that which is observed in several GOF mouse models. In GOF studies, more GC B cells are observed in the LNs and the spleen of these mice and the GC cells showed enhanced survival. Similarly, splenic $CD138^{+}$ plasma cells and plasmablasts were increased. Despite the elevated numbers of GC B cells, the GC's of these mice were disorganised and there was poor demarcation of the light zones (LZ) from and dark zones (DZ) of the lymph node. In addition there was abnormal extensive infiltration of Tfh cells into the DZ [90] .

$Pik3cd^{E1020K}$ GOF mice also exhibit defective class switch recombination (CSR) due to reduced activation induce cytidine deaminase (AID) expression however, affinity maturation was unaffected[89]. Increased IgM antibody levels is a common feature of APDS patients and this was also observed in $Pik3cd^{E1020K}$ GOF mice but IgG1 and IgA levels were impaired, consistent with a defect in CSR. These antibody levels are in contrast with another GOF studies in which all IgG isotypes, IgA, IgE and auto antibodies were severely elevated[90,108]. The use of the PI3Kd inhibitor, leniolisib however, corrected most of the defects associated with the GOF mutation[94].

3.3 PI3Kδ in infectious disease

PI3Kδ has also been studied in the context of infectious diseases. In influenza viral infection, p110δ is reported as critical for $CD8^+$ T cells mediated protective response against the virus. It was observed that following the infection of $CD4^{-cre/p110\delta^{fl/fl}}$ mice (mice deficient in p110δ in their $CD4^+$ cells or cells that have expressed the CD4 in early development) with Influenza type A virus strain A PR/8/34 (H1N1), there was significant reduction in $CD8^+$ T cell expansion. Total and antigen specific CD8+ T cells numbers were markedly reduced in the lungs, mediastinal LN and Spleen at the peak of CD8 cell response (day 11 post infection). This defect impaired viral clearance, CD8+ cell TNFα production and the compromised memory CD8+ memory cell numbers[109].

Another study to investigate the function of PI3Kδ using a different infectious agent, *Streptococcus pneumoniae* has also been documented. In this study it was observed that hyperactivation of the PI3Kδ enhanced susceptibility of the respiratory disease caused by *S. pneumoniae*. This was shown when infected $PI3K\delta^{E1020K-GL}$ GOF mice, $PI3K\delta^{D910A}$ LOF mutants and WT mice where monitored for 10days post-infection. The $PI3K\delta^{E1020K-GL}$ showed accelerated onset of disease and increased mortally when compared to the WT and the $PI3K\delta^{D910A}$, with the $PI3K\delta^{D910A}$ LOF mice

surprisingly showing the least susceptibility to the disease. This increase in susceptibility in the GOF mice is attributed to elevated numbers of a population of Bregs ($CD19^{+}B220^{-}$ $IL10^{+}$) observed in the lungs of the hyperactivated $PI3K\delta^{E1020K\text{-}GL}$ mice. The use of the intranasal PI3Kδ inhibitor Nemiralisib 24hours prior to infection was able to reduce the mortality observed in this mice[108].

In a different disease model, using the intracellular protozoan *Leishmania major*, a parasite in which Th1 mediated responses are thought to be critical for control. It was discovered that despite impaired T cell responses characteristic of $PI3K\delta^{D910A}$ LOF mice, these deficient mice showed a more robust resistance to the disease. This was characterized by accelerated parasite clearance and significantly reduced lesion size. The resistance of these mice to the disease was ultimately linked to the impaired expansion and effector functions of Treg cells[110].

This report was further validated in a different study where pharmacologic inhibition of the enzyme with the PI3Kδ inhibitor Idelalisib (CAL-101) was carried out on C57BL/6 mice prior to infection with *Leishmania.* In the study the prophylactic and therapeutic administration of p110δ pharmacological inhibitors significantly reduced cutaneous lesion as well as parasite burdens in the spleens, livers and footpads of the infected mice and these findings

were attributed to a concomitant reduction in Treg numbers and cytokine production by visceral tissue cells[79,110]

4. RATIONAL AND HYPOTHESIS

Gaps in knowledge exist on the exact role played by PI3Kδ in the context of different infectious diseases. Trypanosomes are distinct in profoundly targeting B cells through a series of evasion mechanisms and ultimately crippling the humoral immune system. Much remains to be learned about B cell responses in trypanosome infection as most reported studies have focused on the innate mechanisms and T lymphocyte responses. Given the critical roles of PI3Kδ in B cell effector and regulatory functions and evidence that trypanosomes meddle with B cell responses, I propose to investigate the role of PI3Kδ in immunity following infection of mice with *T. congolense*. The overarching goal is to dissect and elucidate on the multiple roles of the p110δ protein in trypanosomiasis to gain insights into immunoregulatory functions of the PI3K signaling pathway, that could be targeted in order to modulate immunity to Trypanosoma infections.

Aim:

- To investigate the role of phosphatidylinositol 3-kinase (PI3K) signaling in experimental Trypanosomiasis using the p110δ ^{D910A} mutant mice and employing the pharmacological inhibition of p110δ in C57B/6 mice using the phosphoinositide 3-Kinase delta inhibitor; Idelalisib (CAL-101).

Hypotheses:

1. PI3Kδ modulates *T. congolense* infection by controlling innate B regulatory functions.
2. PI3Kδ is critical for effective adaptive humoral responses required to clear multiple waves of parasitemia.

Specific goals:

1. To investigate the impact of a defect in PI3Kδ protein on parasite burden in the blood of the host.
   a. Infection of the genetically inactivated PI3Kδ mutant mouse strain, p110δ ^{D910} mice.
   b. Infection of C57BL/6 mice, following partial inhibition of the PI3Kδ protein with Idelalisib.
2. To immunophenotype and characterize B cells functions in the two groups of PI3Kδ defective mice.

a. Identify and characterize B cell populations in the spleen, blood and peritoneal cavity.
b. Assess germinal center and antibody responses.
c. Compare B cell activation marker expressions.

3. To analyze cytokine responses in the p110δ ^{D910} mutant and Idelalisib treated mice with emphasis on IFNγ and IL10.
   a. Investigating blood and peritoneal fluid cytokine levels in both groups.
   b. Identify the immune cell subsets responsible for their production.
4. To investigate the impact of PI3Kδ defect on selected other non-B cell subsets populations, mainly T follicular helper lymphocytes.

5. MATERIALS AND METHOD

5.1 Mice

Female C57B/6 mice were purchased from the University of Manitoba Central Animal Care Services (CACS) or Charles River Laboratory, St Constant, Quebec. The p110δ^{D910A} mice and CD1 mice were obtained from the CACS breeding facility. They were aged 6-8 weeks and weighed approximately 20g. They were all kept in a specific pathogen free environment at the CAC and used according to the Canadian Council for Animal care guidelines.

5.2 Parasite and infection

Trypanosoma congolense of the Trans Mara strain and variant antigen type TC13, whose origin has earlier been reported were used for all conducted experiments[111]. CD1 mice were injected intraperitoneally (IP) with Cyclophosphamide (Cytoxan; 200mg/kg) and three days later, thawed TC13 stabilates originally stored in liquid nitrogen were used to infect the immunosuppressed mice. Isoflurane gas was used to anaesthetise the mice and blood containing the parasite retrieved from the mice via cardiac puncture. Anion exchange chromatography using by diethylaminoethyl (DEAE) cellulose was done to purify the parasites and the filtered parasites

washed in Tris-saline glucose (TSG) solution. The parasites were counted using the hemocytometer and further resuspended to the needed concentration. 100μl containing 10^3 parasites were injected IP per mice.

5.3 Parasite quantification

Parasite burden in infected mice was measured by transferring a drop of blood from the tail of the mice onto a slide and the number of parasites estimated by counting the number of blood form typanomastigotes present in at least 12 fields at 400x magnification on the light microscope[112].

5.4 Pharmacological inhibition of p110δ

The mice were administered CAL-101(GS-1101, Idelalisib) 0.1mg/mouse (Selleck Chemicals LLC, TX, USA) IP, prophylactically twice, 24hrs prior to infection and daily till the end of the experiment [79].The inhibition was confirmed by measuring phosphorylation of AKT, a downstream effector protein of p110δ, via western blot[81].

5.5 Cell preparation

At selected endpoints (day 4, 7 and 11) post-infection (pi) the mice were sacrificed using isoflurane gas anaesthesia and subsequent cervical dislocation. Spleen, blood and peritoneal aspirates were collected. Blood and single cell suspensions of spleen were lysed with ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1mM Na EDTA {Sigma}) and the cells subsequently washed with PBS and resuspended in RPMI (+2.05mM L-Glutamine). Peritoneal aspirates were collected in 100μL PBS, spun down at 1200 rpm for 5 minutes and supernatant stored at -80°C for further analysis. The cell pellets were resuspended in RPMI. All cells were counted with a haemocytometer and resuspended to the desired concentrations.

5.6 Measurement of antibody and cytokine levels

The blood collected at sacrifice was kept at 4°C for 4 hrs to clot the cells, and then spun at 3000rpm for 10minutes to retrieve the serum after which it was kept at -20°C until used for antibody and cytokine analyses. Total antibody levels for IgG1, IgG2a, IgG2b, IgG3, IgM and IgA were measured in the serum, while cytokine levels for IL10, IFNγ, IL4, IL5, IL12p450 and TNFα were measured in the serum and peritoneal cavity by Enzyme linked Immunosorbent Assay (Meso Scale Discovery ®U-PLEX

(ms) Assay). The assay was performed according to the manufacturer's instructions.

Serum and peritoneal levels of trypanosome-specific IgM, IgG1, IgG2a, gG2b, and IgG3 levels were measured by ELISA as described[113]. The ELISA plates were coated overnight at 4°C with sonicated *T. congolense* lysate at a concentration of 10^5 parasite /well in 100μl of ELISA coating buffer. The plate was washed twice with PBS and blocked with 5% skim milk in PBST (200μl/well) at 37°C for 2hrs. Then the plate was washed 4 times with ELISA wash buffer (1x PBS, 0.05% Tween 20 (Sigma) pH 7.4) using the ELISA washing machine (BIOTEL ELX405, Biotek Instrument, Winooski, VT). All subsequent wash steps were done on the machine. The samples were added to the well and two-fold serial dilution was made using 2.5% skim milk in PBST as diluent before incubating at 37°C for 2 hrs. This was followed by the wash step and the addition of 100μl Biotinylated Rat anti-mouse IgM, IgG, IgG2a, IgG2b, and IgG3 isotypes (Southern Biotech) at 37°C for 2hrs. Another wash step was repeated but followed by addition of 100μl of streptavidin alkaline phosphatase with a brief incubation at 37°C for 45mins. Finally, 100μl of dissolved 5mg phosphatase substrate tablet

(Sigma S0942-200TAB) were added to the well. The plates were incubated at room temperature in the dark and the optical density (OD) reading done with the Spectra Max at a wavelength of 405 nm.

5.7 Direct ex vivo staining and cytokine detection by flow cytometry

Ex-vivo staining of the cells were done for the expressions of B220, CD19, IgD, IgM, CD23, CD21, CD138, CD80, CD86, Sca1, GL7, CD95, CD4, CD3, CD8, PD1, ICOS, CXCR5, CD62L, CD44, Ly-6G/Ly-6C, CD43, CD5, CD11b, TIER 119, F4/80, GR1 (all extracellular staining) and Foxp3 (intracellular staining) by flow cytometry. First the cells were placed in flow cytometry tubes (BD Falcon), then washed with FACS buffer (0.1% Fetal bovine serum in PBS). The cells were then incubated on ice for 5 minutes with 100 μl of Fc receptor blocker (2.4G2 Hybridoma supernatant). The staining for surface markers was done using fluorochrome-labeled antibodies (eBioscience and Biolegend) on ice for 30 minutes and washed with FACS buffer. In some experiments spleen and peritoneal cells were stimulated with ionomycin (500ng/mL), phorbol myristic acetate (PMA; 50ng/mL) and brefeldin A (BFA; 10μg/mL) for 4hrs, fixed, surface stained for B220, CD3 and CD4, and subsequently stained for intracellular IFNγ, IL10 and Bcl6. The Foxp3 staining was done using Foxp3 staining kit

(eBioscience) in line with the manufacturer's instructions. All stained cells were finally washed with and resuspended in FACS buffer. They were then acquired and analysed using the BD FACS Canto II cytometer (BD Bioscience, San Diego CA) and FlowJo software (BD Bioscience) respectively.

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

Serial number	Antibody	Clone	Fluorochrome
1	B220	RA3-6B2	PerCP, PE-Cy7, Percp-Cy5.5
2	CD21	7G6	BV 421
3	CD23	B3B4	PE
4	IgM	II/41	FITC, APC
5	IgD	11-26c.2a	FITC
6	CD4	RM4-5	FITC, PE, APC-Cy7
7	CD8	53-6.7	APC-Cy7
8	TIER 119	TER-119	APC-Cy7
9	F4/80	BM8	APC-Cy7
10	Ly6G/Ly6C	RB6-8C5	BV510, Pacific Blue
11	GL7	GL7	Per-Cp
12	CD95 (Fas)	Jo2	PE-Cy7
13	CD80	16-10A1	APC
14	CD86	GL1	PE
15	IL10	JES5-16E3	PE
16	CD3	17A2	APC-Cy7
17	PD1	29F.1A12	PE-Cy7
18	ICOS	C398.4A	APC
19	CXCR5	2G8	BV 421
20	CD19	1D3	PE-Cy7
21	CD43	S7	PE
22	CD5	53-7.3	APC
23	CD11b	M1/70	APC-Cy7
24	FoxP3	FJK-16s	APC
25	IFNγ	XMG1.2	PerCp
26	CD138	281-2	PE, APC
27	CD44	IM7	PE
28	CD62L	MEL-14	PerCp
29	Sca1	D7	BV 421
30	Bcl6	7D1	APC
31	Aqua viability dye	****	Amcyan

5.8 Nitrite determination

Splenocytes were resuspended at a concentration of 4 x 10^6/ml in complete media (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100U/mL Penicillin and 100μg/ml streptomycin). The cell suspension (1ml) were seeded into 24 well tissue culture plate (Falcon: VWR Edmonton, AB, Canada) and stimulated for 72hrs with or without *T. congolense* lysate (10^5 parasite lysate/well). Nitrite concentration in the culture supernatant fluid was assayed using the standard Griess reaction[85].

5.9 Statistical analysis

Data are presented as means and standard error of mean (SEM). Student's t-test were used for comparisons of means and SEM between two groups, and non- parametric analysis of variance (one-way or two-way ANOVA) were used to compare means and standard deviations of groups greater than two. Data were analysed using GraphPad prism program (GraphPad Software Inc., CA, USA). Turkey's or Mann-Whitney post tests were used where there were significant differences in the ANOVA. The error bars are indicative of ± SEM and the differences taken as significant at

$p<0.05$. The experiments were repeated 2-3 times with 3-4 mice per experimental group.

5.10. Ethics

The experiments described above were approved by the University of Manitoba Animal Care Committee and all experimental procedures carried out according to the guidelines of the Canadian Council on Animal care (Protocol number 18-033).

5.11 Flow cytometry gating strategy

Cells were first gated of total lymphocytes, single cells separated from the doublets based on forward scatter width and live cells gated away from the dead cells using Aqua viability stain. We then proceeded to gate on the cells of our interest.

6. RESULTS

6.1 *p110δ inhibition results in enhanced parasite control in early T. congolense infection but poor survival outcome.*

T. congolense is highly immunogenic, rapidly replicates in the blood of the host and it takes several days for an immune competent host to control the parasites at the peak of infection. We therefore hypothesized that immunocompromised p110δ^{D910} mice lacking an active PI3Kδ enzyme would have elevated parasite burden early on after infection and succumb to infection within the first week. To test this, we infected C57BL/6 (WT) and p110δ^{D910A} mice with 10^3 *T. congolense* parasites IP and monitored parasitemia as well as survival period after infection. Surprisingly, we observed a significant decrease in parasite levels in the p110δ^{D910A} mutants in early infection when compared with the WT (Fig. 1A) however, they fail to control the first wave of parasitemia and succumb (Fig. 1B) to the infection (mean survival time 11± 2days). These findings suggest that p110δ^{D910A} mutant mice can control early *T. congolense* induced parasitemia better than WT mice.

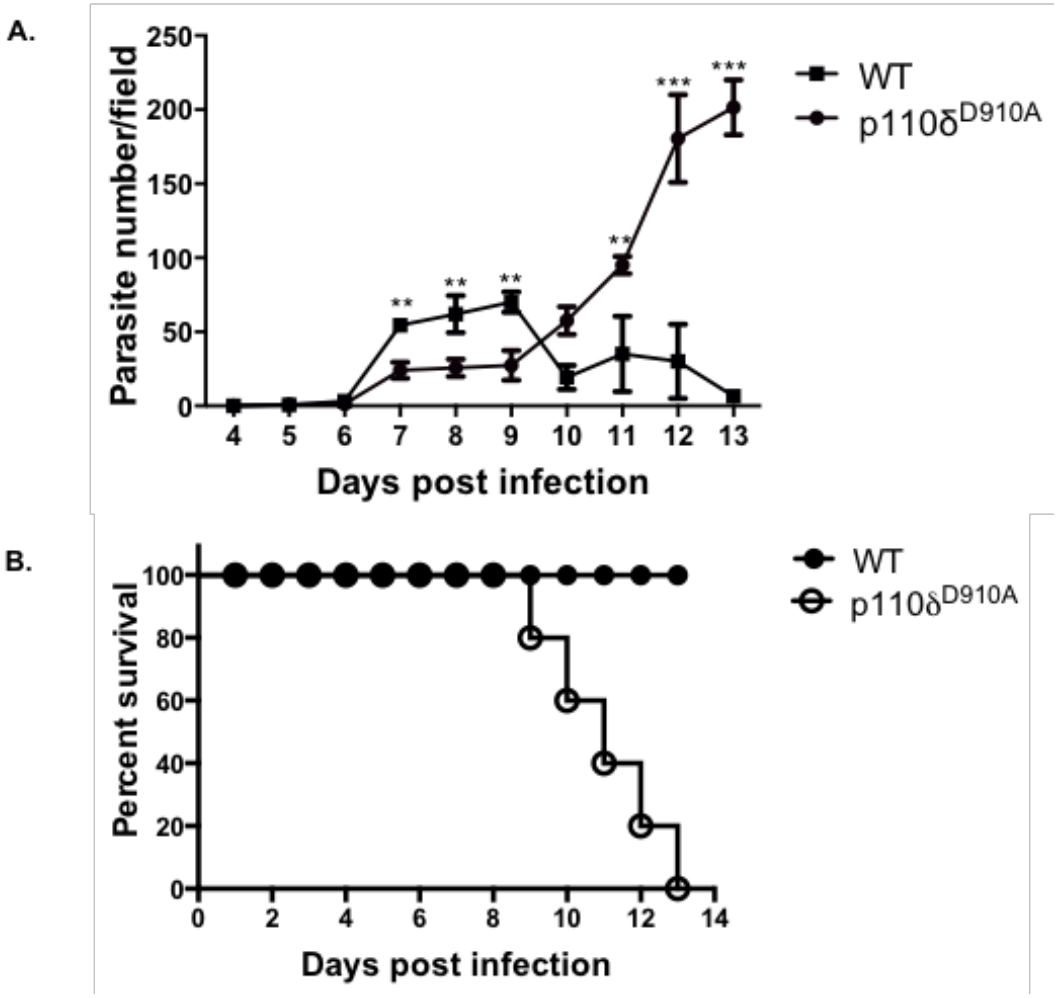


Figure 1: Impaired p110δ signaling enhances parasite control in early infection but increases susceptibility to infection. (A) Mice were infected IP with 10^3 *T. congolense* (TC13). At indicated timepoints, parasitemia was monitored by counting the number of parasites in the blood taken from the tail vain by microscopy. (B) Survival curve following infection. Results are representative of 3 independent experiment (n= 3 per group per experiment) with similar result. Error bars, ± SEM (**, P< 0.01, ***, P<0.001).

6.2 *Pharmacological inhibition of p110δ recapitulates enhanced parasite control in early infection and without compromising mice survival.*

One significant caveat with the p110δ^{D910A} mouse model is their known defects in development of immune cell lineages such as innate-like B cells populations. To determine the effect of acutely inhibiting PI3Kδ in mice with a normal immune system, we examined whether pharmacological inhibition of p110δ would recapitulate the enhanced *T. congolense* control parasitemia previously observed in the genetically impaired p110δ^{D910A} mutants. To do this, we administered a p110δ specific inhibitor, Idelalisib (CAL-101) IP to C57BL/6 (WT) mice 24 hours prior to infection, and daily till we terminated the experiment (day 11). We monitored parasitemia as well as survival during infection. We found a pattern of lower parasite burden during early infection in CAL-101 treated mice (Fig. 2A), which was similarly observed in the p110δ^{D910A} mutants. However, in contrast to p110δ^{D910A} mice, the parasite burden in the CAL-101 treated WT mice stay significantly low and they control and survive the first wave of infection (Fig. 2B). Taken together, these findings suggest that an acute partial inhibition of p110δ signalling might be favourable for the control of *T. congolense* in mice.

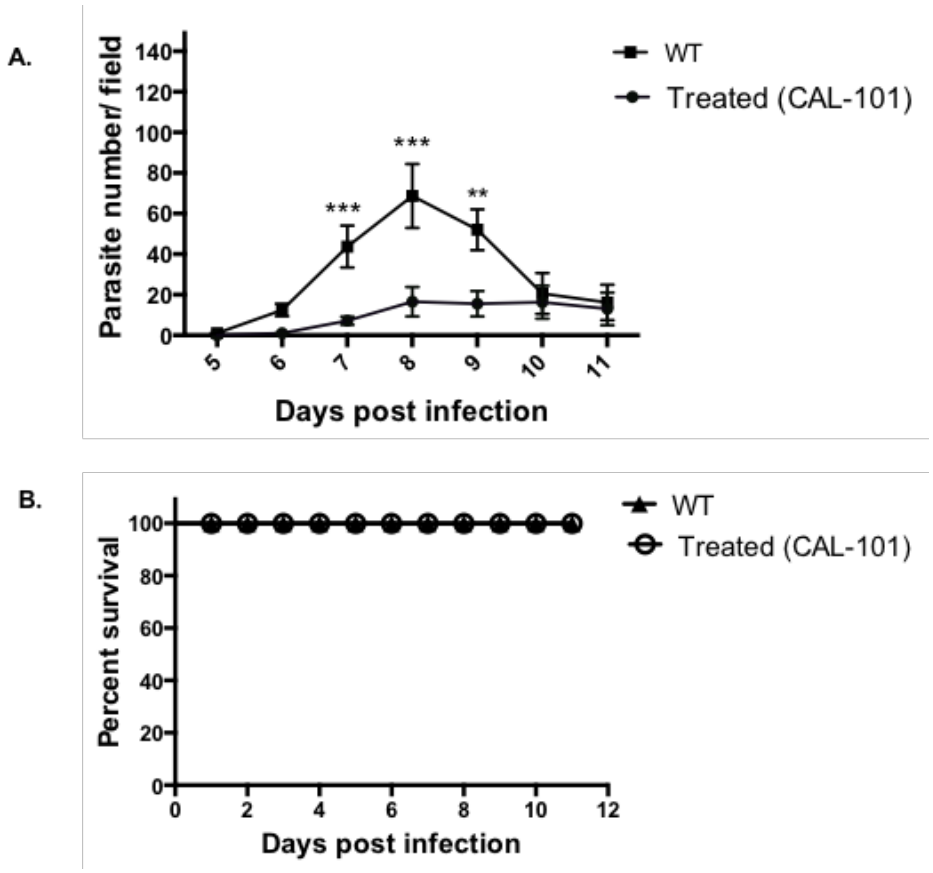


Figure 2: Acute inhibition of p110δ with CAL-101 significantly controls *T. congolense* infection without compromising mice survival at endpoint. (A) Mice were treated with 0.1mg/mouse CAL-101 IP, 24hrs prior to infection with 10^3 *T. congolense* (TC13) IP, and daily till endpoint. At indicated timepoints, parasitemia was estimated by daily counting of number of parasites in the blood taken from the tail vain by microscopy. (B) Survival of treated mice. Results are representative of 2 independent experiments with similar results (n= 4 pe r group per experiment) (mortality of 3 out of 12 mice recorded in a separate experiment). Error bars, ± SEM (**, P< 0.01, ***, P<0.001).

6.3 *p110δ inhibited mice show delayed activation of B cells in T. congolense infection.*

Next, we wanted to examine the immune status of p110δ inhibited mice following infection with *T. congolense*, so as to understand the enhanced parasite control observed in these mice. We began by examining three types of activated B cells; polyclonally activated B cells (PcB), $CD86^{+}$ expressing B cells and $CD80^{+}$ B lymphocytes. To do this we evaluated the expression of CD21, CD23, CD86 and CD80 on B cells during the course of the infection and analysed by flow cytometry. Our results show significantly reduced generation of PcB's ($CD21^{-}CD23^{-}$ $B220^{+}$) in both the genetically impaired $p110\delta^{D910A}$ and CAL-101 treated WT mice at day 7, a time close to the peak of parasitemia in the WT and coinciding with lower parasite burden in the mutants (Fig. 3A & B). However, by day 11, when the first wave of infection is normally resolved in WT C57BL6 mice, we see a reverse trend, as PcB's begin to increase in the mutants but decrease in the WT. Similarly, CD86 expression was lower (Fig. 3C) at day 7 in both groups of PI3Kδ-inhibited mice, while CD80 expression was no different at this time. However, while that genetic mutants maintain a low CD86 in contrast to high PcB at day 11, CD80 expression becomes significantly elevated in the genetic mutants at this time (Fig.3D). These results indicate that inhibiting p110δ

delays the activation of polyclonal B cells and lowers CD86 activation on B cells during early *T. congolense* infection.

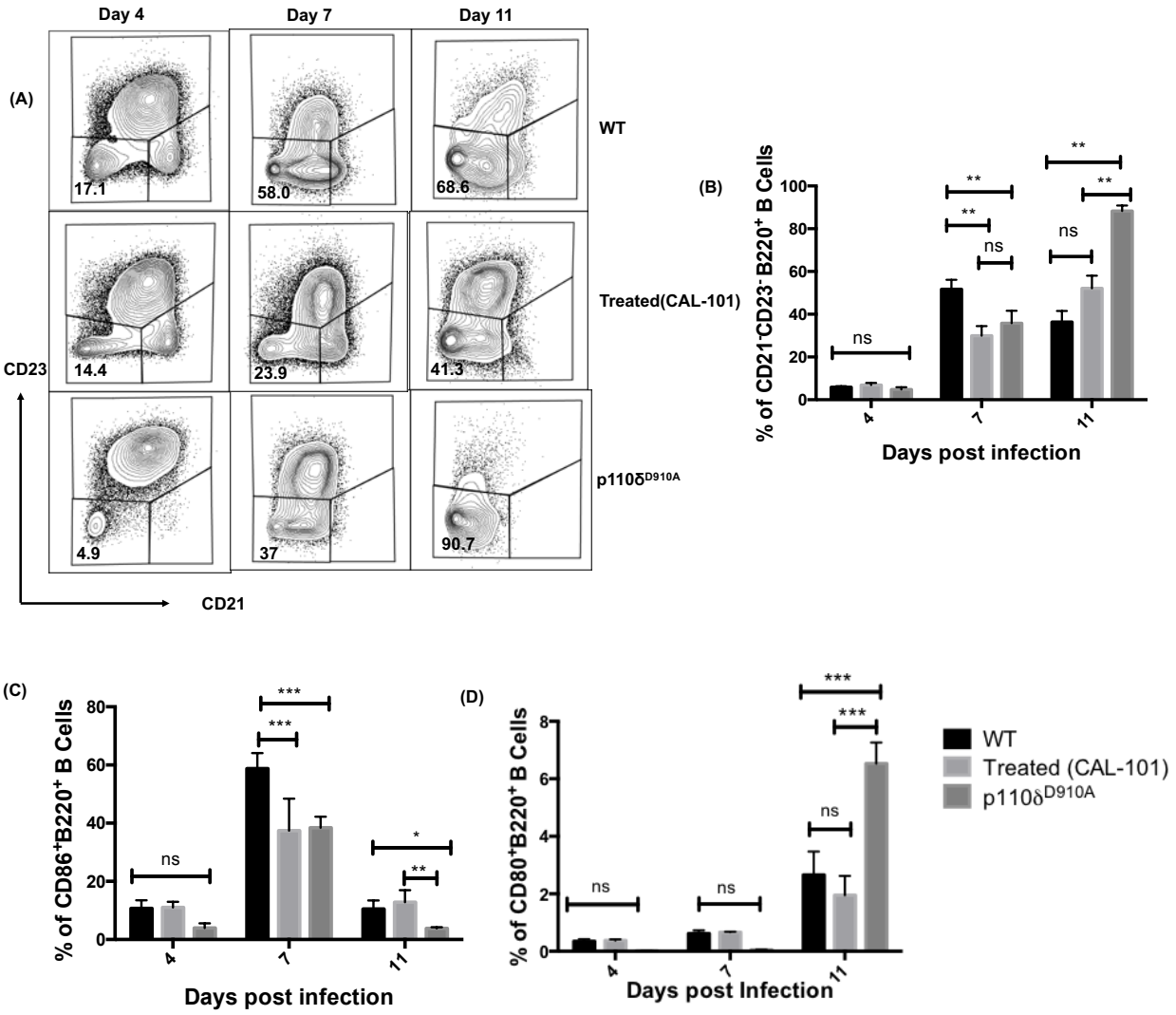


Figure 3: B cell activation is delayed in p110δ compromised mice in early *T. congolense* infection. Splenocytes from infected p110δ^{D910A} and CAL-101 treated mice were analysed for (A & B) CD21^{-}CD23^{-} expressing B cells (polyclonally activated B cells) by flow cytometry done by gating on live B220^{+} B cells. (C) graphical representation of CD86^{+} B cells (D) CD80. Results are representative of 3 independent experiment (n= 3-4 per group per experiment) with similar result. Error bars, ± SEM ((*, p< 0.05, **, P< 0.01, ***, P<0.001).

6.4 *Pharmacologic but not genetic inhibition of p110δ permits sufficient germinal centre (GC) formation in T.congolense infection.*

The generation of sufficient GC responses is the hallmark of adaptive immune response which allows the production of antigen specific immunoglobulins needed for *T. congolense* clearance. We therefore accessed the effect of the varying degrees of p110δ inhibition on GC formation during infection. This was done by checking for GC B cell ($GL7^+Fas^+B220^+$) and Follicular helper ($PD1^+ICOS^+CD4^+$) T cell markers on splenocytes from WT, $p110\delta^{D910A}$ and CAL-101-treated WT mice, by flow cytometry. As expected, GC response was generated after the first week of infection. We observed, interestingly, that the CAL-101 treated group generated GC B cells sufficient enough to clear the first wave of parasites, although slightly reduced in comparison to the WT (Fig. 4A & B). This was in contrast to the genetically defective $p110\delta^{D910A}$ that were significantly compromised and failed to control parasitemia. This same trend was observed in the generation of Follicular helper T cells (Fig 5A & B).

Together, these results indicate that the capacity to generate an effective GC response is dependent on a functional p110δ.

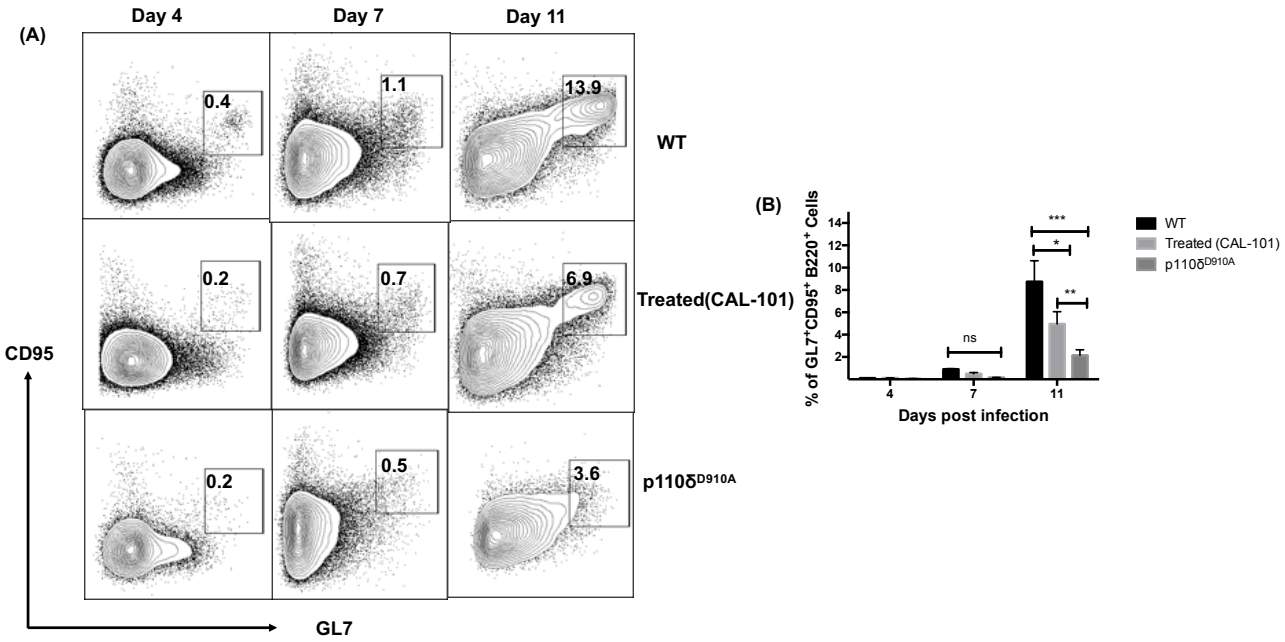


Figure 4: Germinal centre B cell development in response *T. congolense* is dependent on p110δ PI3K. Spleen cells from infected p110δ^{D910A} and CAL-101 treated mice were analysed by flow cytometry using the markers for germinal centre B cells (A) GL7 and CD95 (Fas) double staining (B) Average percent GL7^{+}CD95^{+} cells and SEM are representative of 3 independent experiment (n= 3-4 per group per experiment) with similar result. *, p< 0.05, **, P< 0.01, ***, P<0.001 as compared to the WT.

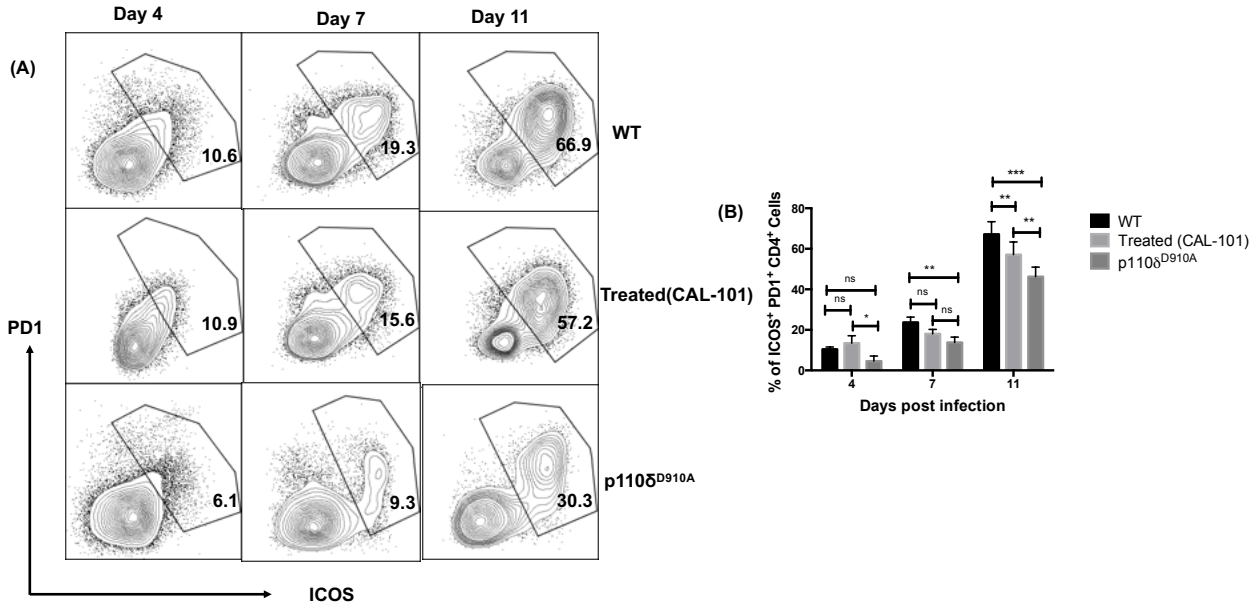


Figure 5: p110δ regulates Follicular helper T cell (Tfh) cells differentiation in the germinal centre formation in response *T. congolense* infection. Flow cytometry of splenocytes from infected p110δ^{D910A} and CAL-101 treated mice. (A) ICOS and PD1 double positive T cells (B) Average percent ICOS^{+}PD1^{+} T cells. Cells were gated from CD3^{+}CD4^{+} live cells. SEM represents of 3 independent experiment (n= 3-4 per group per experiment) with similar result. (*, p< 0.05, **, P< 0.01, ***, P<0.001).

6.5 *p110δ activity is required for antibody production in T. congolense infection in mice*

Next, we quantified antibody production from p110δ^{D910A} and CAL-101 treated mice and compared with WT, using total and antigen specific antibodies as measures of functional B cells and GC activity respectively. We assessed the 3 groups of mice for serum levels of IgG and IgM at day 4, 7 and 11. As expected based on previous studies, total and antigen specific antibodies were significantly reduced in p110δ^{D910A} mutants (Fig. 6A, B, C & D). Interestingly, the total and antigen specific antibody production of the CAL-101 inhibited mice were comparable with the WT (Fig. 7A, B, C & D). In fact, the CAL-101 mice show a slight increase in parasite-specific IgG antibodies at day 7. Interestingly, Trypanosome-specific IgG2a levels showed a slight decrease in the treated group (Fig.7E), suggesting generation of different IgG subclasses may be altered by this treatment. Taken together, this data indicates that a developmental defect in p110δ alters B and plasma cells antibody production functions much more severely than an acute inhibition with CAL-101 would and suggests that the lack of anti-trypanosome antibodies may account for the inability of p110δ mutant mice to survive the first wave of parasitemia.

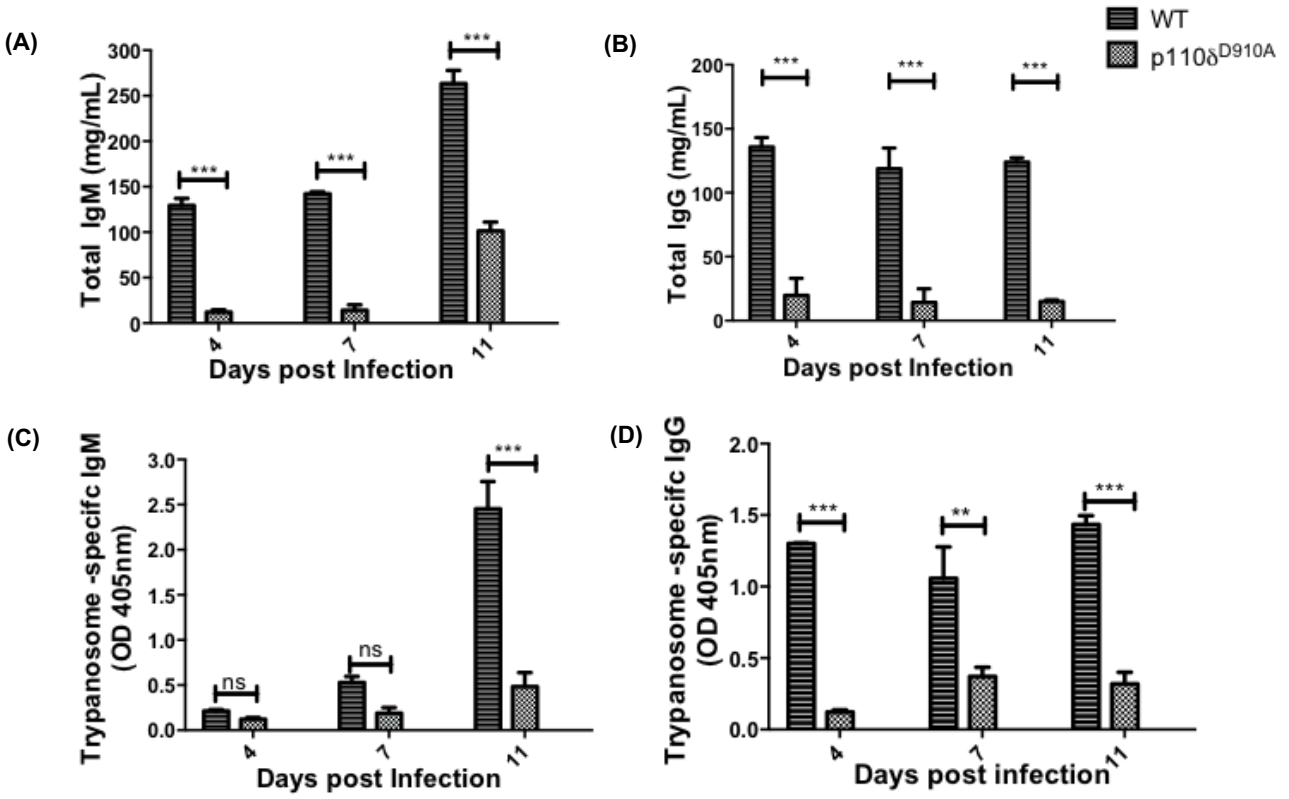


Figure 6: Compromised antibodies cells in p110δ^{D910A} mice. Serum levels of total and trypanosome specific IgM and IgG were measured in p110δ^{D910A} and WT mice by ELISA. p110δ^{D910A} mutants are severely compromised in total (A) IgM and (B) IgG. (C) Trypanosome specific IgM and (D) IgG (**, P< 0.01, ***, P<0.001)

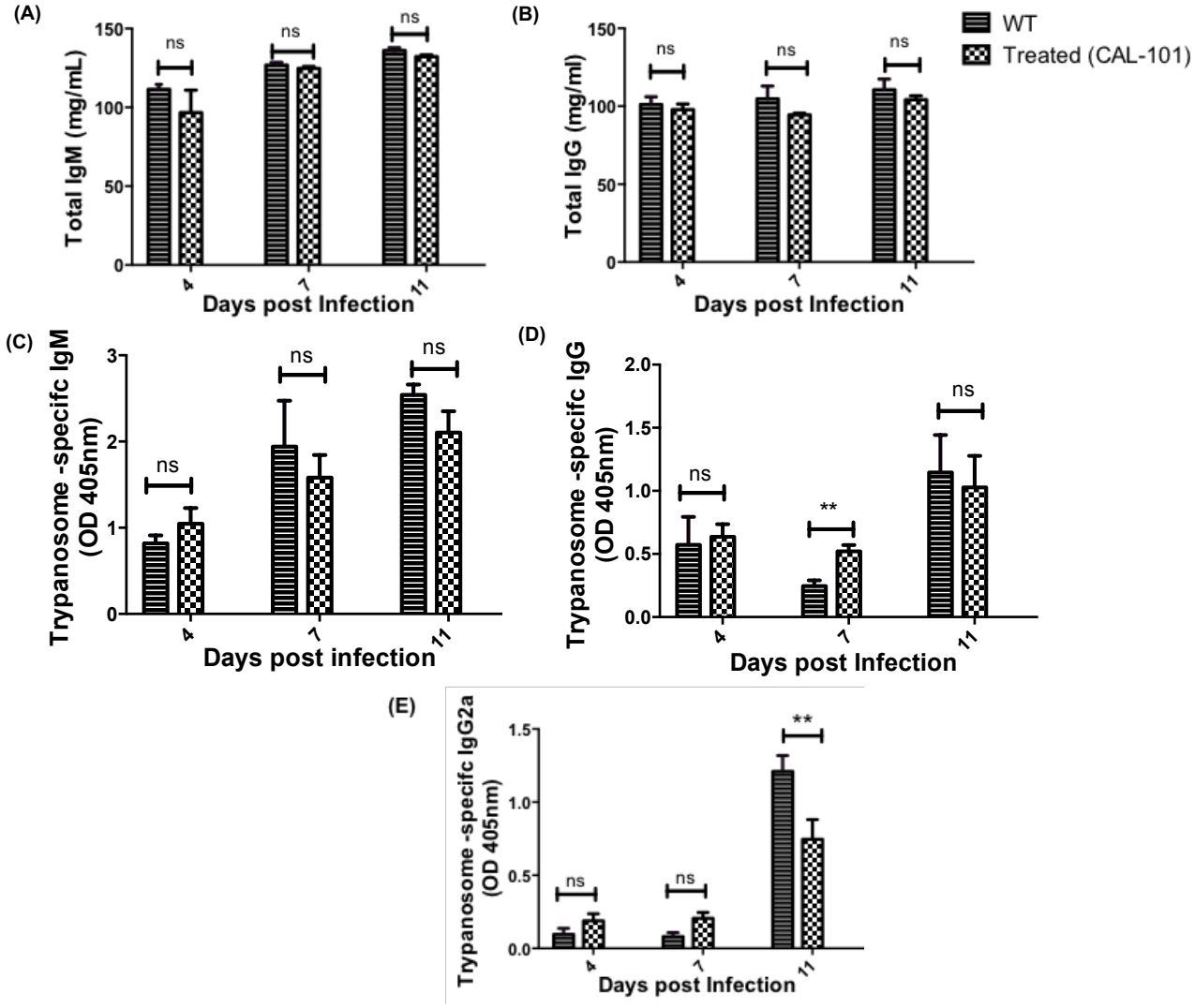


Figure 7: Serum antibody levels of CAL-101 treated mice following infection. Serum IgM and IgG were analysed by ELISA and compared in the CAL-101 treated and untreated WT group. Comparable quantities of (A) Total IgM and (B) IgG. (C) Trypanosome specific IgM and (D) IgG and (E) IgG2a. (ns, not significant, **, P< 0.01)

6.6 *p110δ inhibition results in altered cytokine responses in early T. congolense infection.*

Cytokines play a major role in the pathogenesis of trypanosomiasis and some studies indicate that an IFNγ cytokine response is required for a favourable disease outcome[58]. We measured the impact of p110δ mutation or inhibition on levels of IFNγ and the regulatory cytokine IL10. While all mouse groups show large increases in serum cytokine levels upon infection, we did observe a trend of elevated IFNγ and reduced IL10 in the $p110\delta^{D910A}$ mutants during early infection (Fig. 8A & B). This observation was buttressed when we examined the IFNγ : IL10 ratio for each mouse which revealed a significantly higher ratio of IFNγ : IL10 in the serum of the $p110\delta^{D910A}$ mutants (Fig. 8C). Interestingly, this trend was recapitulated in the serum and peritoneal cavity of CAL-101 treated mice, both of which show significantly higher IFNγ : IL10 ratios at day 4 (Fig 9). This data suggests that p110δ inhibition alters the regulatory cytokine environment in early *T. congolense* infection in mice leading to enhanced production of IFNγ and reduced dampening of inflammation by IL10.

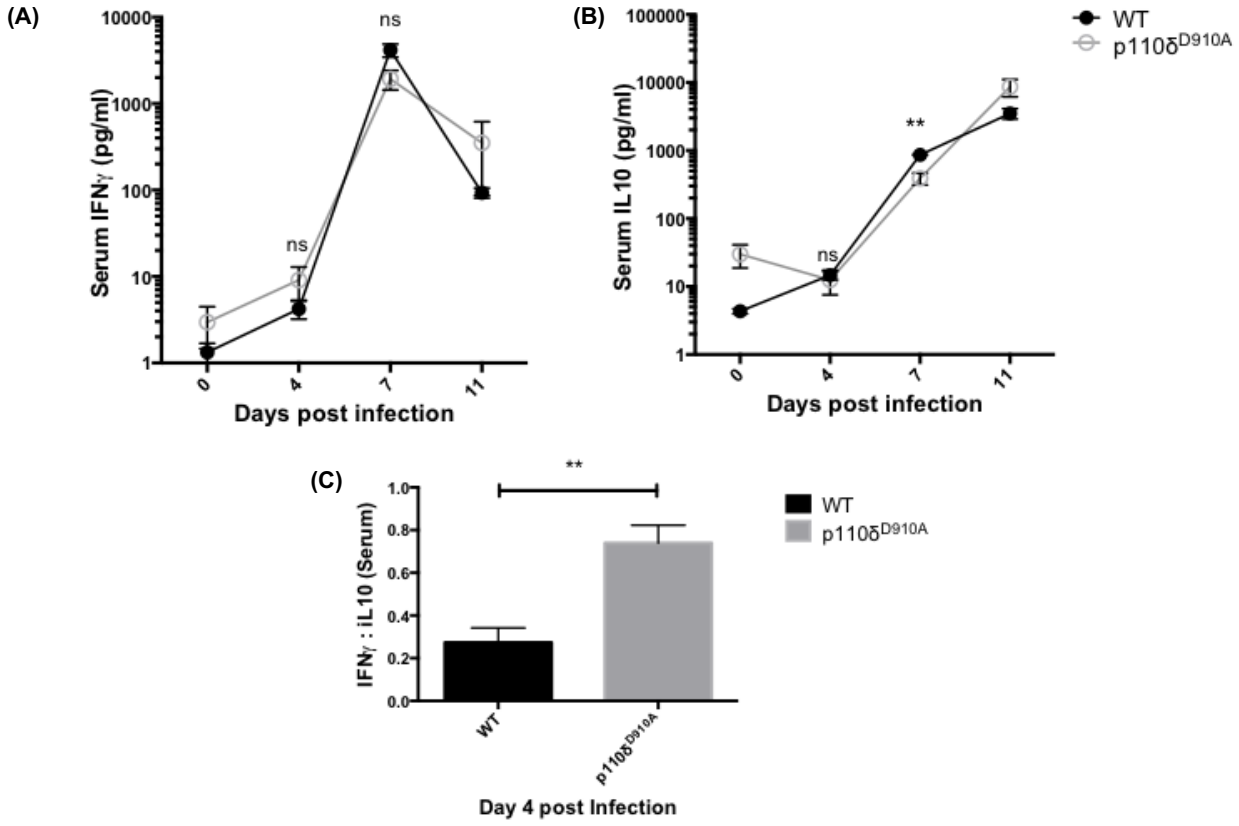


Figure 8: Serum IFNγ and IL10 levels of p110KIδ^{D910A} mutants following *T. congolense* infection. Cytokines were analysed by ELISA. (A) IFNγ (B) IL10 (C) IFNγ : IL10 ratio, and SEM from pooled data of 2 independent experiments. (ns, not significant, **, P< 0.01)

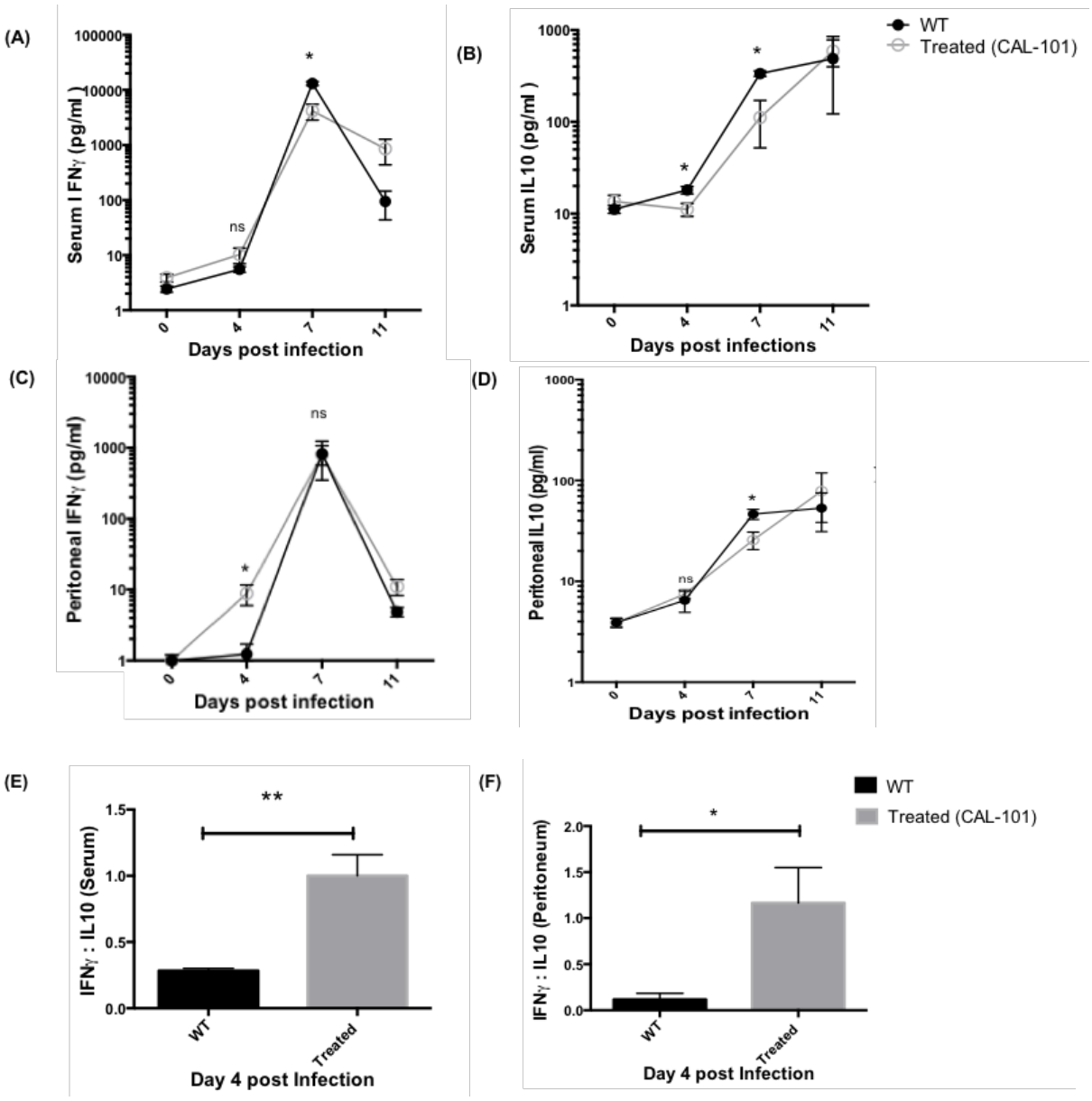


Figure 9: Serum and peritoneal IFNγ and IL10 levels in CAL-101 inhibited mice. Cytokines were analysed by ELISA (A, B) Serum IFNγ and IL10, (C, D) peritoneal IFNγ and IL10 (E, F) IFNγ : IL10 ratio in serum and peritoneal fluid, and SEM from pooled data of 2 independent experiments. (ns, not significant, *, p< 0.05, **, p< 0.01)

6.7 *CD19+ B cells produce IL10 in early T. congolense infection.*

We hypothesized that p110δ may control IL10 produced by specific regulatory cells. We performed intracellular cytokine staining to detect IL10 producing cell populations by flow cytometry in the spleen and peritoneal cavity of the CAL-101 inhibited and WT mice. After gating on total $IL10^+$ cells, we determined the percentage of $CD19^+$ to $CD4^+$ IL10 producing cells. We observed that in both groups of mice most of the $IL10^+$ cells are $CD19^+$ B cells in the first week of infection, but by day 11 there was a shift in IL10 production to the CD4+ cells (Fig. 10A, B & C).

The data above supports a previously reported finding that $CD19^+$ B cells are important for IL10 production in early *T. congolense* infection[65].

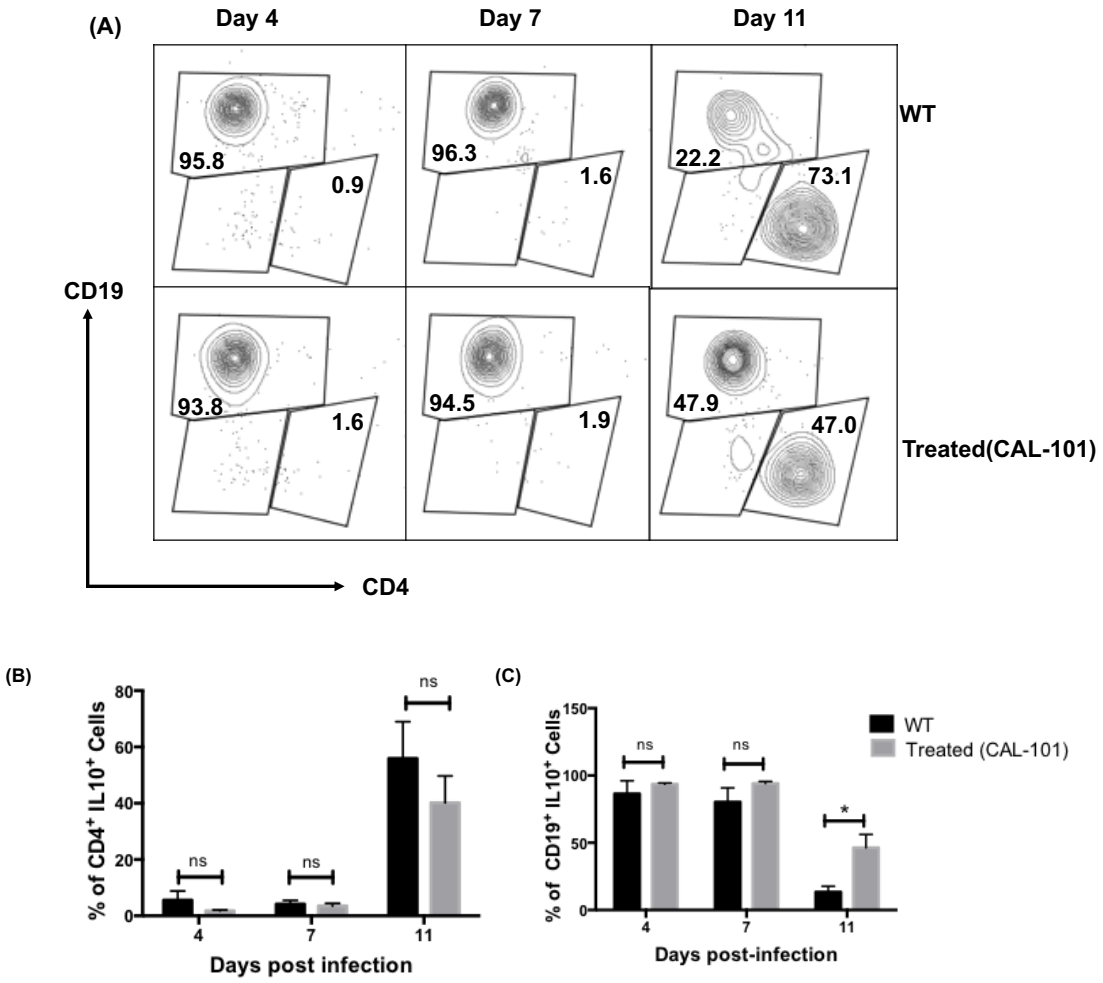


Figure 10: Shift in IL10 production from CD19+ B cells to CD4+ T cells in early infection. IL10 was analysed by Intracellular cytokine flow cytometry staining of peritoneal cells. (A) Percentage of $CD19^+$ to $CD4^+$ of total $IL10^+$ cells, graph plots of (B) $CD4^+$ $IL10^+$ and (C) $CD19^+IL10^+$ cells. SEM represents of 2 independent experiment (n= 3-4 per group per experiment) with similar result. (ns, not significant, *, $p< 0.05$)

6.8 *p110δ inhibition results in impaired IL10 production from peritoneal B1 cells during T. congolense infection.*

Since we established that B cells produce IL10 in very early infection, next we thought to identify the specific B cell subsets with the capacity to make IL10 by intracellular flow cytometry. On examination of spleen and peritoneal cells, we identified that the B cell subsets with the propensity to make significantly measurable IL10 were the innate B1 cells, which resided largely in the peritoneum (Fig.11B). This is consistent with previous studies in the literature[114,115.] When we stained for B1 cells further using the markers CD11b, CD5 and IgM, we observed that specifically B1a cells (CD5+) were completely abrogated in the p110δ^{D910A} mutants (Fig. 12A), consistent with previous reports[81]. Interestingly, the B1a cell frequencies were unaffected in the CAL-101 inhibited mice (Fig. 13A) however, when we quantified the IL10 production from these cells, we observed a significant reduction in the secretion of the cytokine in early infection from the $CD5^{+}CD11b^{+}$ B1a (Fig. 13B) and $CD5^{-}$ $CD11^{+}$B1b populations (Fig. 13C).

This trend is reversed at day 11, as the mutants begin to make more IL10 in comparison to the WT at this later time.

Taken together, the data above suggests that mutation of p110δ ablates B1 cell development while the acute inhibition of p110δ compromises B1

cytokine production, hence altering their regulatory functions in early *T. congolense* infection.

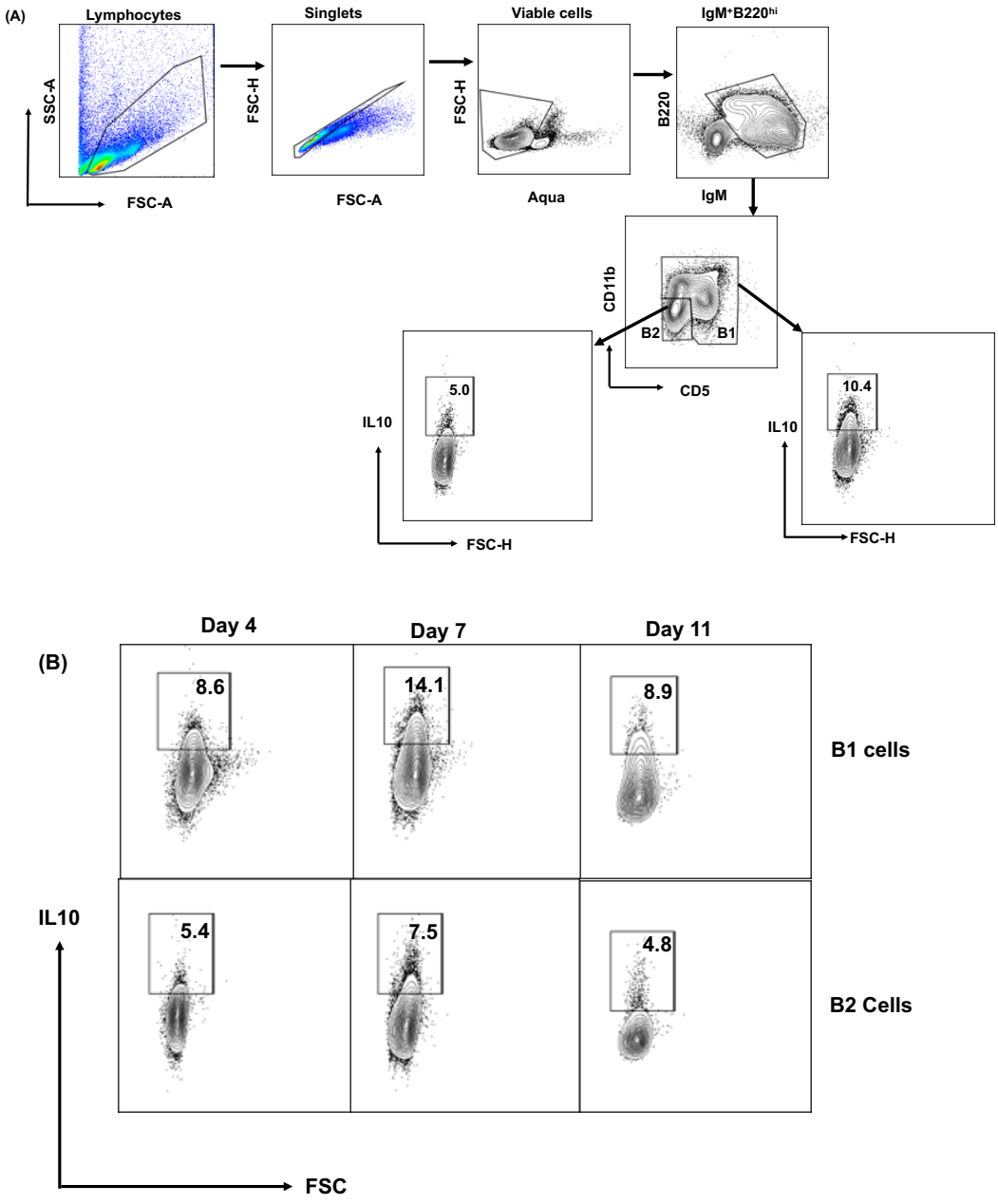


Figure 11: IL10 producing B1 cells in *T. congolense* infected WT mice. Flow cytometry of peritoneal cells from infected WT mice, (A) Gating Strategy for B1 and conventional B2 Cells, (B) IL10 producing B1 and B2 Cells. Flow plots is representative of 2 independent experiment (n= 3-4 per group per experiment) with similar results.

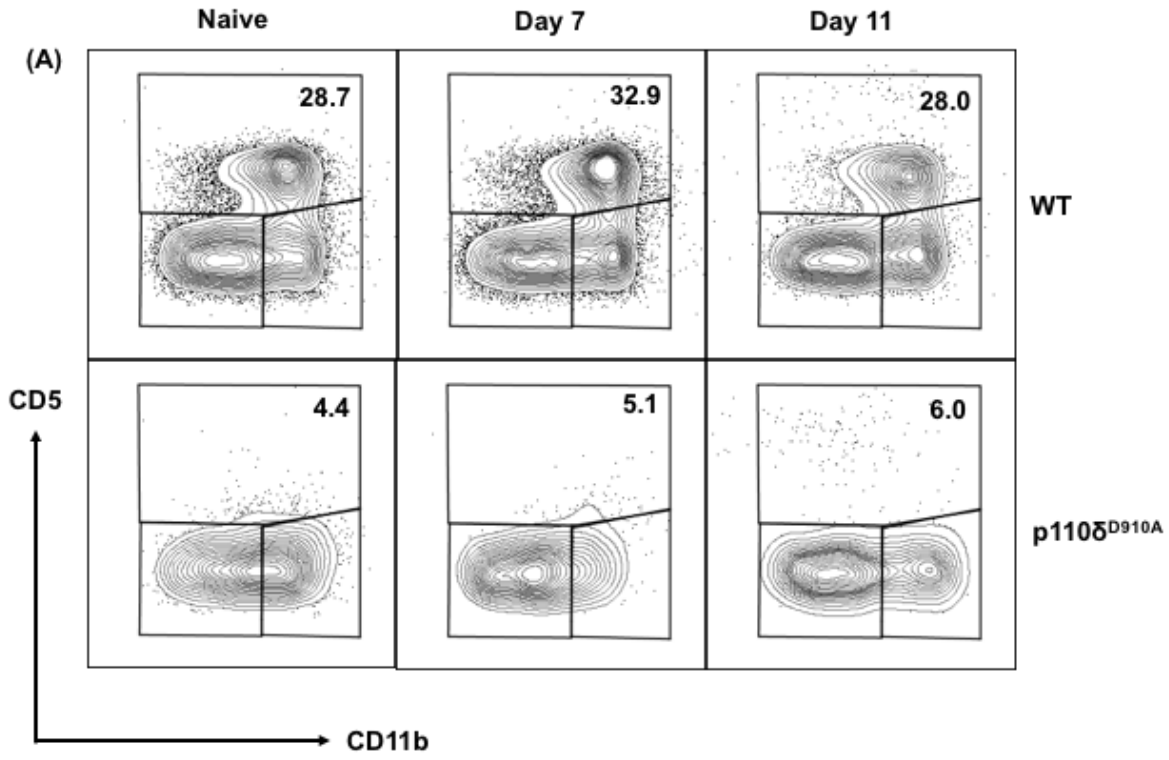


Figure 12: Absence of B1 cells in p110δ^{D910A} mutants. Flow cytometry of peritoneal cells from infected p110δ^{D910A} mutant mice, (A) Percentage of CD5+CD11b+ IgM+ B1a cells. Flow plots is representative of 2 independent experiment (n= 3-4 per group per experiment) with similar results.

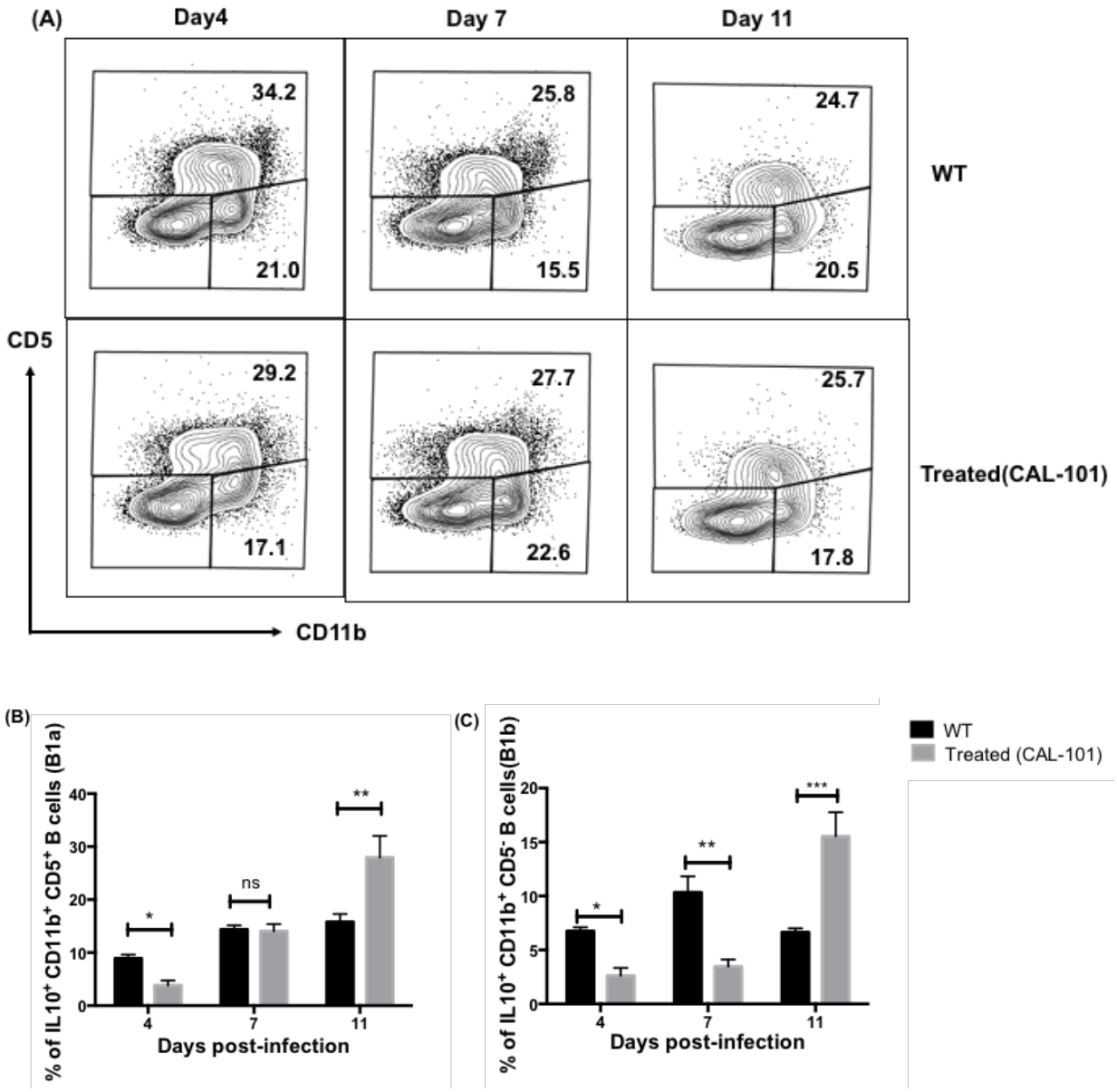


Figure 13: Acute inhibition of p110δ with CAL-101 alters IL10 production in B1 cells. IL10 was analysed by Intracellular flow cytometry staining of peritoneal cells. (A) Percentage of $CD5^{+}CD11b^{+}$ IgM^{+} B1a and $CD5^{-}CD11b^{+}$ IgM^{+} B1b cells, (B) graph plots IL10+B1a and (C) $IL10^{+}$ B1b cells. SEM represents of 2 independent experiment (n= 3-4 per group per experiment) with similar result. (ns, not significant, *, p< 0.05, **, P< 0.01, ***, P<0.001).

6.9 *CD4$^+$ T cells make IFNγ in early T. congolense infection following CAL101 treatment.*

Next, we sought to determine the cellular source of the elevated IFNγ observed in the p110δ inhibited group in early infection. We obtained spleen and peritoneal cells from the CAL-101 inhibited group and performed intracellular flow cytometry staining to assess IFNγ production by CD4 T cells. While there was no significant difference in the spleen we observed an increase in IFNγ production by the CD4+ cells in the peritoneal cavity at day 4 (Fig. 14A, B & C), suggesting that a Th1 environment good for disease control was already established prior to the peak of infection. We proceeded to examine IFNγ production by non-CD4 T cells to ascertain if there was a difference in the level cytokine production by these cells. We observed no difference in their secretion of IFNγ (Fig 15 A & B). These results suggest that the CD4+ T cells in the CAL-101 inhibited group are the key IFNγ producing cells at this time.

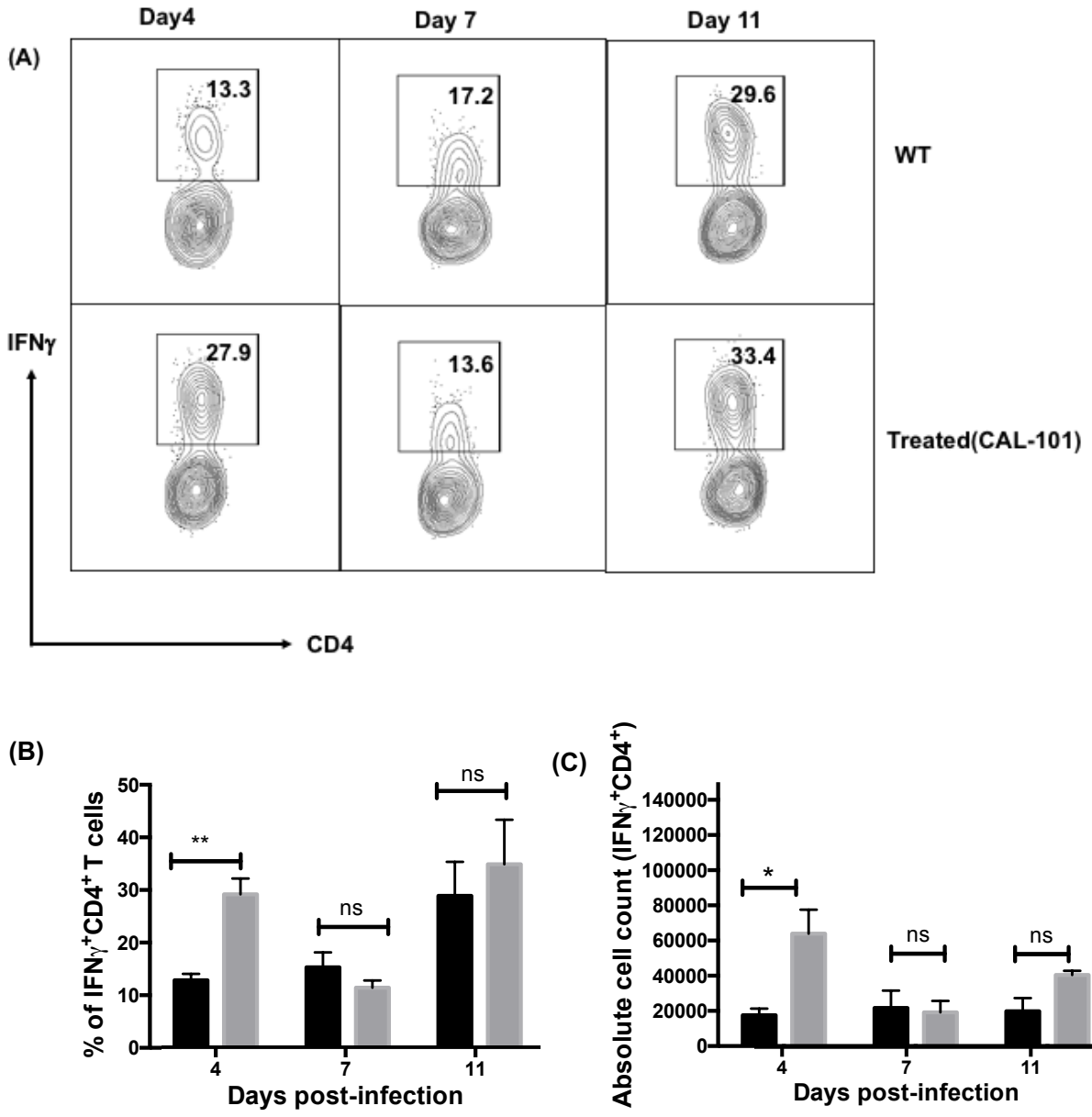


Figure 14: Elevated IFNγ producing cells in CAL-101 treated mice in early *T. congolense* infection. IFNγ was assessed by Intracellular flow cytometry staining of peritoneal cells. (A) Percentage of IFNγ producing CD4+ T cells, (B) graphical representation of $IFN\gamma^{+}CD4^{+}$ cells (C)Absolute cell count of $IFN\gamma^{+}CD4^{+}$ cells. SEM represents of 2 independent experiment (n= 3-4 per group per experiment) with similar result. (ns, not significant, *, $p< 0.05$, **, $P< 0.01$).

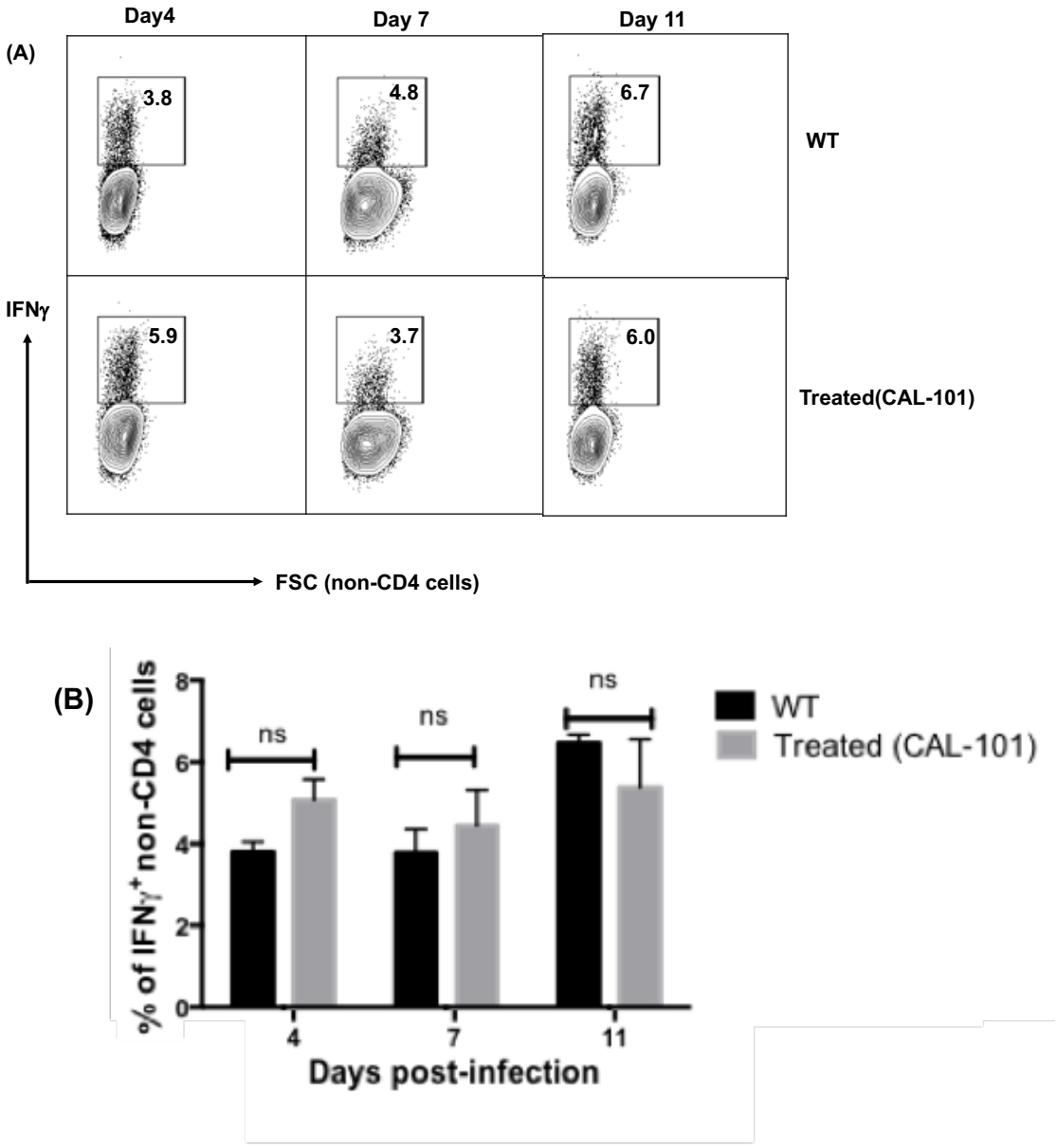


Figure 15: Comparable IFNγ production by non-CD4 cells in early *T. congolense* infection. IFNγ was assessed by Intracellular flow cytometry staining of peritoneal cells. (A) Percentage of IFNγ producing non-CD4 T cells, (B) graphical representation of IFNγ$^+$ non-CD4 cells SEM represents of 2 independent experiment (n= 3-4 per group per experiment) with similar result. (ns, not significant).

6.10 *Increased Nitric Oxide production in early T. congolense infection following CAL101 inhibition*

Finally, we assayed for the release of NO from both group of mice following infection. The production of NO is reported to be induced by IFNγ stimulation of activated macrophages and is needed for effective parasite clearance[29]. We carried out in-vitro culture experiments of single cell suspension from the spleen of Tc13 infected mice at different timepoints to measure NO production. The cells were cultured for 3 days following re-stimulation with Tc13 lysates and the supernatants quantified for nitrite.

We observed that NO was more elevated at peak of infection as well as after parasite clearance in the CAL-101 treated group when compared to the WT controls (Fig. 16A). This data therefore suggests that increased skewing of the early immune response to a Th1 cytokine environment following inhibition of p110δ results in increase production of NO.

(A)

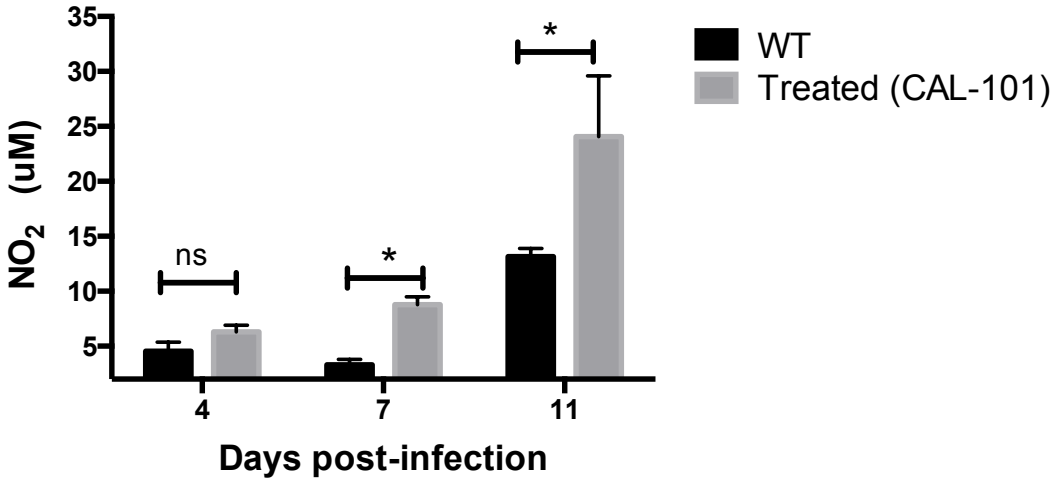


Figure 16: Increased NO production in CAL-101 treated mice in early *T. congolense* infection. (A) Assessment of Nitric oxide production by Griess assay. SEM is representative of only 1 experiment (n= 3-4 per group) (ns, not significant, *, p< 0.05, **, P< 0.01).

7. DISCUSSION

Phosphatidylinositol 3-kinases have been established as important proteins in immune cells. Several isoforms of the protein are identified in a variety of cell types where they perform unique functions[80].

Recently there has been a growing interest in the specific impact of the different isoforms of this protein which have also been implicated in influencing a wide range of disease such as inflammatory, autoimmune and most recently infectious diseases[90,94,79.]

The findings from this study have identified discriminatory immunomodulatory roles for the p110δ isoform of PI3K in regulatory B cell functions, early innate cell responses and antibody production in experimental African trypanosomiasis caused by *T. congolense* in mice. I investigated the protective role of p110δ signalling in B cells in this disease by employing the use of both genetic and pharmacological inhibition to avoid the limitations often encountered using either of the study approaches alone. I have shown evidence that p110δ impacts IL10 production from B cells in a timely manner as the disease progresses and favours the generation of a Th1- like milieu from T Lymphocytes which is needed for host protection in the acute phase of this disease.

The first interesting finding from my data was the reduced parasite burden early during infection in both genetic p110δ deficiency and drug inhibited group. When the physical appearance and behavioural patterns of these deficient mice were observed and compared to the WT at the peak of infection, they appeared to be physically more active than the WT and had shiny hair coat unlike the ruffled coats of the WT. Behaviorally the deficient mice moved individually in the cages while the WT were crowded together and appeared to be stressed. These observations suggest that the lowered parasitemia in the deficient mice at this time might have alleviated the clinical symptoms associated with acute trypanosomiasis. Hence, it could be ascertained from the data that inactivating this enzyme might be favourable in controlling the peak of infection.

This enzyme was previously observed to play a role in the response to a different infectious agent *Leishmania major,* where a similar pattern in reduced parasite load and improved mouse survival was seen following the inhibition of this enzyme[79]. However, *L. major* is different from *T. congolense* as there are differences in their predilection sites in the host (intracellular versus extracellular for *L. ma*jor and *T. congolense respectively*) hence the nature of immune responses thought to be essential for their clearance are different (cell-mediated versus humoral respectively)[110,113].

Studies have shown the link between p110δ signalling and IL10 production via TLR 4 and 9 receptors, and in the studies with *L. major*, the control of parasite burden was attributed to the altered immunomodulatory effect of p110δ signalling which results in the lowering of IL10 production from regulatory T cells, hence by-passing the immunosuppressive effect of the cytokine on host immune responses targeted against the parasite[79,110]. This effect earlier reported in Tregs was not consistent with the observations I made in the *T. congolense* model, at least not in the early phase of the disease. Similarly, I saw a pattern of reduced IL10 in the blood and locally in the peritoneal cavity, however my data did not indicate that the reduced IL10 was due to the effects on Tregs. Findings garnered from this and data from other groups show that within the first week of *T. congolense* infection in WT mice, B cells are the major producers of IL10 (over 90%) and IL10 production from Tregs become more evident around 10 days post infection. In line with this finding my data showed that the enzyme, p110δ was exerting its immunomodulatory effects in our model on a type of regulatory B cells in the peritoneal cavity (B1 cells). These innate-like B1 cells have the inherent capacity to produce polyreactive antibodies and IL10 via TLR signalling as well as migrate to other tissues when activated [116]. In a previous study investigating *T. cruzi* infection in Balb.Xid mice, a phenotype which lacks B1

cells and an associated block in B2 cell development, the lack of B1 cells and their production of polyreactive antibodies was associated with less severe disease[117]. My studies suggest that the lack of production of the immunosuppressive cytokine IL10 cytokine in Balb.Xid mice could also be contributing to the poor prognosis of the disease in their model and gives room for further investigation.

In addition to the altered p110δ immunomodulation of B1 cells in the peritoneal cavity, I observed an increased Th1 environment in our model which may originate with the hyper-responsiveness of macrophages due to p110δ inactivation. Several studies have shown that PI3K signalling actively inhibits TLR signalling in macrophages (M1) needed to prime Th1 responses from T cells, and this alters macrophage activation, compromises their function of NO production and phagocytosis as well as their cytokine release of IL12 for Th1 priming. Conversely, inhibiting this pathway increased macrophage activation and function like iNOS generation[86,85]. My data validates these previous studies in the context of *T. congolense* infection as my data shows increased IFNγ and NO in p110δ inhibited mice. NO is known to be trypanocidal, and IFNγ from macrophage-activated Th1 cells can further potentiate macrophage responses. I observed these increases at a time that correlates with decreased parasitemia in the p110δ inhibited mice.

While I cannot conclusively determine from this data, that the effect on parasite burden observed originates with the immune modulation of IL10 producing B cells as opposed to enhanced innate responses, I would like to speculate that a fusion of these two events might be occurring concurrently and could be responsible.

Earlier studies using protein in adjuvant vaccinations of p110δ^{D910A} mutant mice found that germinal centre responses and antibody production were highly impaired[81]. My data suggest that antibody responses to *T. congolense* infection are also dependent on this enzyme as the genetically inactivated group had compromised germinal centre responses hence failed to generate adequate levels of total and antigen specific IgM or class switched IgG antibodies. My data however suggests a dose dependent activation of p110δ in the development of germinal centre responses, as the pharmacologically inhibited group in our model were able to mount humoral responses sufficient to clear the first wave of infection, unlike the genetic mutants. Although I observed a slight decrease in the amount of GC B cells and Tfh cells in the drug inhibited group in comparison to the WT, this reduced GC response does appear sufficient to generate specific IgG antibodies. I believe these reductions could either be as a result of once daily treatments with the drug which only partially inhibit the enzyme activity or

from reduced stimulation of GC responses due to significantly lowered parasite numbers in the drug inhibited group when compared with the WT.

p110δ signalling has also been linked to B cell activation. Studies have reported compromised B cell activation in p110δ inhibited mice[118]. I examined three types of activated B cells in our model (CD80, CD86, PcB's) and as expected CD86 and PcB B cell activation was compromised in early infection, in both inhibited mouse groups, with no difference in CD80 at this time. It is unclear if the compromised polyclonally activated B cells observed in the inhibited mice serve a protective role in the early control of infection observed. Several studies have expatiated on the mechanisms of trypanosome induced polyclonal activation of cells and the detrimental role of polyclonal activation in host immune evasion[110]. One could assume from our data that since parasite load was significantly reduced in the p110δ inhibited mice at a time that coincided with reduced PcB's, the compromised activation of the cells was in favour of the host at this time (day 7). The genetically inhibited mice eventually lose control of parasitemia and their PcB become severely elevated at humane endpoint (day 11) unlike the WT and drug inhibited group, showing that a lot of poorly functional non- specific B cells were activated in the genetic mutants. My findings thus further

support the detrimental effects of polyclonally activated B cells that have previously be reported in infectious diseases [5]

Furthermore, an early lymphoma study in mice associated the expression of CD86 on B cells as p110δ and STAT3 signal dependent mechanisms and that while CD86 expression was linked to proliferation and production of class switch IgG antibodies like IgG2a, CD80 expression was correlated with altered IgG production[119,120]. While I cannot conclusively validate this report in our own study, I however observe an interesting trend that could support this in our model. $CD86^{+}$ B cells were significantly reduced and CD80+ cells increased only in the p110δ genetic mutants at endpoint which surprisingly correlates with severely compromised total and class switched antibodies. My data indicates that CD86-CD80 expression could be somewhat linked to IgG production.

A pertinent question however continues to hover in the field of trypanosomal studies: what is the limiting factor for parasite control and host survival? Is it cytokines or antibodies? Although I did observe elevated levels in seven cytokines checked in naïve p110δ genetically inhibited mice (not shown) which confirms the reports from several studies that p110δ enzyme modulates cytokine production in different cell types, I hypothesize that the lack of survival in 100% of our genetically inhibited mice and 25% of the

pharmacologically inhibited mice is likely due to broadly compromised antibody production in the genetic mutants and probably defects in trypanosome specific IgG2a in the pharmacologic inhibition model. Antibodies have been documented as needed for clearing the first wave of infection and trypanosome specific IgG2a is the critical isotype specific for parasite clearance in mice[58]. My findings from this study further supports the critical role played by antibodies in trypanomastid diseases.

In all, my perspective is that an effective and lasting immune response to *T. congolense,* would be one that comprises of an amalgamation of targeted cytokine responses and quality antibody production in the host, and the enzyme p110δ could be used as a potential target.

7.1 Conclusion

In conclusion, p110δ may act as a double edge sword necessary for protective roles in experimental *T. congolense* infection; first acting in early parasite control via its function on regulatory B cells, innate cell mediated control and subsequently in adaptive humoral response via its role in germinal centre formation.

7.2 Significance of Study

This study further provides insights into the immunoregulatory functions of the PI3K pathway that could be targeted in modulation of immunity to Trypanosoma infection and possibly other infectious agents. This study elucidates on the role of PI3K in aspects of cytokine production and expatiates on its importance in the humoral immunity system which could be harnessed in the ongoing search for an effective treatment to *Trypanosoma* diseases.

7.3 Limitations of Study

One of the limitations of our study is the continuously expanding phenotypes of regulatory B cells in the field and cell markers to identify the array of cells are constantly been developed. For now, we speculate on the phenotype of regulatory B cells in our models based on their production of IL10, the available cell surface markers which are sometimes shared by different B cell subsets, and most especially their location in the host. But it has come to light that normal B cells can be induced to make IL10, and additionally the different Bregs in tissues have been known to migrate into other tissues. Hence, we have speculated from our study that the Bregs in our model are the peritoneal B1 cells based on their cell surface markers and their location. We cannot ascertain if they are other Bregs that could have migrated such as IL10 producing regulatory plasma cells, MZ cells, other innate like B cells and even B2 cells with the capacity to produce IL10.

Additionally, in our study the concentration of Idelalisib administered per mouse was about 8μM which is comparable to the peak serum levels in humans treated with Idelalisib[121,122]. However, there is the possibility of sub-optimal levels of the drug being absorbed into the blood and other tissues due to variations in host (human versus mouse), as well as the complication

of an ongoing infection which may have altered the pharmacokinetics of the drug with each daily administration. Therefore, we cannot ascertain the exact extent of Idelalisib mediated inhibition of p110δ throughout the duration of our study.

7.4 Future Directions

7.4.1 *Determine longevity of pharmacologically inhibited mice group.*

We have shown that acute inhibition of C57BL/6 mice with CAL-101 prior to infection with *T. congolense* resulted in lower parasite burden in acute infection and favoured the control of the first wave of parasitemia. However, it is unclear how the long-term treatment with CAL-101 would impact subsequent waves of infection, the immune milieu, humoral response and ultimately mice survival. We would like to explore this by doing a longer time point studies and survival experiments in the drug inhibited group.

7.4.2 Determine the effects of targeted inhibition of p110δ on disease outcome.

The data from our study indicates that the genetic or prophylactic inhibition with a pharmacological agent establishes a Th1 environment prior to the appearance of parasites in the blood, which subsequently is favorable for parasite control at the peak of the infection. However, we were unsure if this effect on parasite burden would be recapitulated if the inhibition of the enzyme commences after infection has already been established. We could ascertain this by employing the use of the Cre-Lox system to turn off the

p110δ gene after the disease is established or commence CAL-101 treatment after the appearance of parasites in the blood.

7.4.3 Determine the role of antibodies in p110δ^{D910A} mutant's survival

In our study, we observed that unlike the WT and CAL-101 inhibited mice, the p110δ^{D910A} mutants failed to control the first wave of parasitemia and die within 11-13 days of the infection. We however speculate that the severe mortality observed is due to compromised humoral immune responses. To definitely determine the role that antibodies play in the control of the infection, we would like to do targeted serum transfer experiments from infected WT mice into p110δ^{D910A} mutants that are infected with *T. congolense.*

7.4.4 Immuno-phenotype the majority of Regulatory B cells in our model and ascertain the mechanism for regulation of Breg functions by p110δ

Our study show that Bregs play major role in the progression of *T. congolense* induced trypanosomiasis. However, we have only teased for and identified one subset of Bregs in our model, it would be interesting to expand our cell markers and ascertain the possibility that other regulatory B cells such as the CD138^{+} LAG3^{+} regulatory plasma cells might be involved, this

we can be done by extensive immunophenotyping for different regulatory B cells in the spleen, blood and peritoneal cavity. Additionally, we would like to use in-vitro studies to determine the exact mechanistic pathway by which p110δ signalling affect aspects of their function such as cytokine production, antibody response, proliferation and possibly migration.

7.4.5 *Determine the function of p110δ and the cytokine IL10 in protection to T. congolense by doing PI3Kδ Gain of function (GOF) studies.*

Our studies have shown us the effects of p110δ in our infectious model from the perspective on a Loss of function (LOF) study. Incorporating a GOF studies into our experiment will further validate, refute or shed new light into the exact function of p110δ and the cytokine IL10 in trypanosomal disease. We would like to do this with the use of Mb1aPI3KCD mice which has a B cells specific hyperactivation of the PI3Kδ and IL10 reporter mice in our infectious disease model. We would predict that hyperactivated PI3Kδ would lead to more Breg activity and impaired control of *T. congolense,* opposite of the results observed from this study.

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