

**DETERMINATION OF THE ROLE OF CYTOKINES USING GENE
DEFICIENT MICE IN AFRICAN TRYPANOSOMIASIS INFECTION**

Mark Barkhuizen
B.Sc., B.Sc. (Hons.), M.Sc.

Thesis submitted to the University of Cape Town in fulfillment of the degree

Doctor of Philosophy

Division of Immunology

Faculty of Health Sciences

University of Cape Town

February 2008

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A handwritten signature in dark ink, appearing to read 'M. Barkhuizen', is written over a horizontal dotted line.

Mark Barkhuizen

DEDICATION

This thesis is dedicated to Snoekie and Dad.

The oxen are slow but the earth is patient

ACKNOWLEDGMENTS

I am grateful to my supervisor, Professor Frank Brombacher for his guidance and motivation during my PhD career. My co-supervisor, Dr. Stefan Magez, for sharing his vast knowledge on the subject of trypanosomes. A special thanks to Dr. Anita Schwegmann for her help with the critical reading of this dissertation.

Thank you to Lizette Fick, Marilyn Tyler and Zoë Lotz for their excellent histology services. To the University of Cape Town Animal Unit staff, Reagon Pieterse, Wendy Green and Erica Smit, thank you for breeding and genotyping the mice.

My deepest gratitude to Dr. Anita Schwegmann, Dr. Tony Cutler, Dr. Reto Guler, Dr. Reece Marillier who always made time to listen me and for their kind words of encouragement. Thank you to my colleagues in the lab for making the lab a vibrant and stimulating environment.

Again, my deepest thanks to Dr. Anita Schwegmann.

I would like to acknowledge and thank the Medical Research Council and the National Research Foundation for their financial support during the course of this study.

I would like to say a special thank you to my family, my mother, father and my sister for their unwavering love, support and belief in me.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Description
Ag	Antigen
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Antigen presenting cell
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
CD	Cluster of differentiation
cM	Centi-Morgans
CpG	Cytosine-Guanine Dinucleotide
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EAE	Experimental Autoimmune Encephalomyelitis
FITC	Fluorescein isothiocyanate
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1R α	Interleukin-1 receptor-related protein
iNOS	Inducible Nitric oxide Synthase
kDNA	Kinetoplast Deoxyribonucleic Acid
LPS	lipopolysaccharide
M1	Type I cytokine –associated myeloid cells

M2	Type II cytokine –associated myeloid cells
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
n	Number
NK	Natural killer cells
NO	Nitric oxide
PGDF	Platelet-derived growth factor
pi	Post infection
PLC ^{-/-}	Phospholipase C-deficient
SEA	Soluble egg antigen
<i>Sep</i>	Selenoprotein P
SIRS	Systemic Inflammatory Response Syndrome
SLE	Systemic lupus erythematosus
SPF	Specific Pathogen Free
SRBC	Sheep red blood cell
T	Trypanosoma
TCR	T cell receptor
TLR	Toll-like receptor
TLTF	Trypanosome Derived Lymphocyte Triggering Factor
TNF	Tumor necrosis factor
TNP	Trinitrophenyl
TREM-2	Triggering receptor expressed on myeloid cells 2
VSG	Variant-Specific Surface Glycoprotein

PUBLICATIONS

In addition to the work in this dissertation, the candidate has made significant contributions to the publications below:

TITLE: The Induction of a Type 1 Immune Response following a *Trypanosoma brucei* Infection Is MyD88 Dependent

AUTHORS: Michael B. Drennan, Benoît Stijlemans, Jan Van Den Abbeele, Valerie J. Quesniaux, Mark Barkhuizen, Frank Brombacher, Patrick De Baetselier, Bernhard Ryffel, and Stefan Mages.

JOURNAL: *The Journal of Immunology*, 2005, 175: 2501–2509.

TITLE: Interleukin-12p70-dependent interferon-gamma production is crucial for resistance in African trypanosomiasis

AUTHORS: Mark Barkhuizen, Stefan Mages, Robert A. Atkinson, Frank Brombacher

JOURNAL: *Journal of Infectious Diseases*, 2007, 196: 1253-1260.

TITLE: Interleukin-12p70 Deficiency Increases Survival and Diminishes Pathology in *Trypanosoma congolense* Infection.

AUTHORS: Mark Barkhuizen, Stefan Mages, Bernhard Ryffel, Frank Brombacher

JOURNAL: *Journal of infectious Diseases* in 2008 (accepted!)

ABSTRACT

African trypanosomiasis encompasses diseases caused by pathogenic trypanosomes, infecting both humans and animals alike. To determine the immunological role of IL-12 family members in *Trypanosoma brucei brucei*, *Trypanosoma evansi* and *Trypanosoma congolense* infections, IL-12p35^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} mice were used. While the two latter mouse strains lack all IL-12 homologues, IL-12p35^{-/-} mice still produce IL-12p80 homodimers and IL-23.

In infection with *T. b. brucei* and *T. evansi*; IL-12p35^{-/-}, IL-12p40^{-/-} or IL-12p35^{-/-}/p40^{-/-} mice were susceptible to both these pathogens, demonstrated by increased mortality compared to wild type C57BL/6 mice. The different IL-12 deficient mouse strains showed similar mortality kinetics, suggesting that IL-12p70 but not the IL-12p80 homodimer or IL-23 plays a crucial role in survival. Similarly, parasitemia control was reduced in the absence of IL-12p70. While plasma levels of IgM and IgG2c were similar between IL-12 deficient mice and wild type mice, IFN- γ production, especially during early infection, was severely impaired in all IL-12 deficient mouse strains, demonstrating an IL-12p70-dependent mechanism for IFN- γ production. As IFN- γ R^{-/-} mice were also highly susceptible to both *T. b. brucei* and *T. evansi*, IL-12p70-dependent IFN- γ production seems to be the important mechanism involved in resistance against both these pathogens.

Surprisingly, in infection with *T. congolense*, all IL-12 deficient mouse strains showed prolonged survival, while parasitemia levels were unaltered compared to wild type mice. Interferon- γ production in IL-12 deficient mice was strikingly reduced during the acute and chronic stages of infection, coinciding with significantly reduced chronic stage hepatocellular damage, demonstrated by histological analysis and plasma aspartate aminotransferase measurements. In contrast, IL-10 production was not affected by the absence of IL-12p70. Together, these results show that during *T. congolense* infections, the absence of IL-12p70, but not the IL-12p80 homodimer or IL-23, leads to a reduction

of IFN- γ production, which reduces hepatic pathology and improves host survival in conjunction with IL-10 without negatively affecting parasitemia control.

In summary, IL-12p70 is necessary for an optimal pro-inflammatory Type 1 response against *T. b. brucei* and *T. evansi* infections, however in *T. congolense* infection, IL-12 drives an over-shooting inflammatory response resulting in pathological manifestations leading to mortality.

University of Cape Town

CHAPTER I

LITERATURE REVIEW

The African Trypanosomes

1. Introduction

Trypanosomes are unicellular eukaryotic haemoflagellate parasites that replicate freely in the bloodstream of a mammalian host ultimately resulting in sleeping sickness in humans (*Trypanosoma brucei species*), Nagana (*T. congolense*) in cattle or Surra (*T. evansi*) in various animals. A common component of these two parasites' life cycle is their ability to invade both the tissue and vascular compartments of their host. Together these diseases result in a vast economic drain in Africa with the distribution of these diseases covering both Sub Saharan Africa (*T. b. brucei*) and Northern Africa (*T. evansi*) (Hoare 1972). Vaccination of man and domestic animals would be the most economical and effective method of controlling African trypanosomiasis infections, however due to the trypanosome's ability to constantly change its antigenic surface coat, the parasite effectively evades the host's immune response and vaccination strategies (Pino, Miller et al. 1977) (Cross 1979). The current treatment and control strategies are expensive and unsustainable (Taylor 1998) thus prompting new research into preventative or control mechanisms.

Although there have been great strides in modern medicine, pathogenic trypanosomes still maintain a stranglehold on humans and domestic animals spanning over most of tropical Africa. The lack of knowledge is attributed to delayed use of sophisticated experimental techniques; also the existence of diversity in pathogenesis amongst trypanosomes although they appear similar, further complicates analysis. Not only does the disease present differently amongst various infected host species but emergent trypanosome strains further complicates the disease phenotype (Losos and Ikede 1972). Another problematic factor in trypanosomiasis infections is the lack of obvious pathological lesions in either humans or domestic animals that may cause mortality. Also,

trypanosomes are known to release several biologically active compounds possibly inducing pathology during infection (Tizard, Nielsen et al. 1978).

2. Classification of trypanosomes

Kinetoplastids are protozoan flagellates exhibiting a unique organelle, the kinetoplast, containing the mitochondrial DNA at the base of the flagellum (Lumsden and Evans 1976). Kinetoplastids have been described to be an early branch of the eukaryote lineage after the assimilation of the mitochondrion (Sogin, Elwood et al. 1986) (Stevens, Noyes et al. 2001). The order Kinetoplastida is further subdivided into three families, namely; Trypanosomatidae, Bodonidae and Cryptobiidae (Lumsden and Evans 1976) (Levine, Corliss et al. 1980). Trypanosomatids are characterized as having a single flagellum and are all parasitic to invertebrates, vertebrates and flowering plants. Two forms of host-protozoan relationships exist within this group, namely monogenic and digenetic. Monogenetic trypanosomatids have a single host, mainly an invertebrate, and digenetic trypanosomatids parasitize two hosts, normally a vertebrate and an invertebrate. The genus *Trypanosoma* forms part of the digenetic trypanosomatids and contains a plethora of heterogeneous species (Alvarez, Cortinas et al. 1996) (See Table 1). The putative divergence of the genus *Trypanosoma* from the other trypanosomatids has been estimated to be more than 300 million years ago, predating the emergence of vertebrates onto the land and the evolution of insects (Fernandes, Nelson et al. 1993) (Haag, O'HUigin et al. 1998). This genus is further subdivided into salivarian and stercorarian sections, representing their mode of development in the vector. The salivarian trypanosomes are characterized by antigenic variation, mainly transmitted by tsetse flies and include (amongst others) the *Trypanozoon* and *Nannomonas* subgenera. Strains of the subgenus *Trypanozoon* include; *T. b. brucei* and *T. evansi*, and the subgenus *Nannomonas* includes *T. congolense* (See Table 1).

Table 1. Listed in the table below is the life cycle and host range of salivarian trypanosomes within the genus *Trypanosoma*

Subgenus	Species / Subspecies	Life Cycle	Host Range
<i>Trypanozoon</i>	<i>T. brucei brucei</i>	Digenetic	Cattle, sheep, etc.
	<i>T. brucei rhodesiense</i>	Digenetic	Humans & animals
	<i>T. brucei gambiense</i>	Digenetic	Humans & animals
	<i>T. evansi</i>	Monogenetic	Humans*, Cattle, sheep, etc.
	<i>T. equiperdum</i>	Monogenetic	Horses, Donkeys
<i>Nannomonas</i>	<i>T. congolense</i>	Digenetic	Cattle, sheep, etc.
	<i>T. simiae</i>	Digenetic	Pigs, camels

Table adapted from: (Lumsden and Evans 1976)

* Cases have been reported where humans have become infected (Joshi, Chaudhari et al. 2006) (Powar, Shegokar et al. 2006) (Joshi, Shegokar et al. 2005) (WHO 2005) (Truc, Gibson et al. 2007).

3. Geographic distribution of important infectious trypanosomes

The distribution of *T. brucei gambiense* occurs in tropical West and Central Africa and *T. brucei rhodesiense* is mainly limited to East and Southern Africa. Widespread incidences of both *T. congolense* and *T. b. brucei* occur in tropical Africa but are limited to the movement and distribution of their vectors (tsetse flies). *Trypanosoma evansi* has the largest geographical distribution as it is spread by a variety of vectors and thus encompasses many regions, including India, Far East, Near East, Philippines, North Africa, Central and South America (Gutierrez, Corbera et al. 2006) (Smith and Sherman 1994).

4. Karyotype and reproduction within salivarian trypanosomes

The chromosomal DNA from the *Trypanozoon* and *Nannomonas* subgenera varies in size from approximately 50 to 6000 kb. A further 100 minichromosomes of 50 to 100 kb have

also been identified and contain highly repetitive DNA elements or satellite DNA repeats of 177 to 500 base pairs (Stevens, Noyes et al. 2001). Minichromosomes are known to contain the genes which transcribe the variant surface glycoproteins necessary for immune evasion (Van der Ploeg, Cornelissen et al. 1984). Reproduction is primarily by binary fission; however *T. b. brucei* has been reported to undergo a form of sexual reproduction occurring only within its insect vector through nuclear fusion (Jenni, Marti et al. 1986) (Cibulskis 1988) (Paindavoine, Zampetti-Bosseler et al. 1986).

5. The different parasites causing African trypanosomiasis

5.1 Infection in humans

There are five different trypanosome species of pathogenic importance in Africa. Two subspecies cause disease in humans namely; *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* (Pepin and Meda 2001). Trypanosomes were first detected in humans causing sleeping sickness in 1902 although initial reports of the disease date to 1803 (Cross 1990).

5.1.1 Clinical signs:

Although trypanosomes are microscopically similar they have different disease characteristics; *T. b. rhodesiense* causes fever with a rapid course of disease resulting in death displaying little or no neurological symptoms. In *T. b. gambiense* a chronic slow progressing disease is characteristic together with neurological symptoms known as sleeping sickness (Tizard, Nielsen et al. 1978). These diseases are classed as stage 1 or stage 2 depending on the infiltration of the cerebrospinal fluid by the parasites. In stage 1 of the disease the parasites invade blood, tissue aspirates and lymph (Barrett, Burchmore et al. 2003); in stage 2 parasites can be detected in internal organs as well as the central nervous system (Pentreath 1995) (Enanga, Burchmore et al. 2002). In both stages, detection of the parasites is problematic and diagnosis may rely on the host's inflammatory response which results in major pathophysiological manifestations (Louis, Buscher et al. 2001) (WHO 1998).

5.1.2 Treatment:

Drugs, which have changed little since their discovery (Legros, Ollivier et al. 2002), induce adverse side effects with a substantial number of patients dying from drug therapy alone (Pepin and Milord 1991). Treatment failure rates are high and may be indicative of drug resistance (Legros, Evans et al. 1999) (Brun, Schumacher et al. 2001). Human African Trypanosomiasis was largely brought under control by the 1960s; however, an alarming increase in the disease prevalence has been reported and is now classed as a category 1 resurgent disease (Remme, Blas et al. 2002).

5.2 Infection in domestic animals

The first parasitic trypanosome was discovered in the blood of camels and equines in India in 1880, and named *T. evansi*, the causative agent of Surra. This disease has been identified in many animals and characterized in small ruminants as causing sub-clinical (Otieno and Gacanja 1976) (Mahmoud and Elmalik 1977), moderate or severe infections (Ngeranwa, Gathumbi et al. 1993). An example of severe infection occurs in horses, called “Mal de Cadeiras” in South America (Franke, Greiner et al. 1994) with rapid mortality occurring between 7 and 10 days (Seidl, Moraes et al. 2001). In 1894, *T. brucei* was found to cause Nagana in animals (Cross 1990). In domestic animals *T. congolense* and *T. vivax* are the principle trypanosomes which cause disease in sub-Saharan Africa. *Trypanosoma b. brucei* is considered less prevalent than *T. congolense* or *T. vivax* and not as pathogenic. Surveillance studies have found that it not uncommon for these different trypanosome species, which are widespread throughout tropical Africa, to simultaneously infect animals.

5.2.1 Clinical signs:

Trypanosomiasis in domestic animals is similar to the human form, however neurological symptoms are rare (Stephen 1970). The main features of infection include severe anemia, intermittent fevers, lymphatic enlargement as well as hepatomegaly and splenomegaly. Furthermore, during infection there is significant cachexia, found in both muscle tissue and body fat, which progresses together with the extent of the disease. The course of trypanosomiasis in domestic animals is highly variable, ranging from apparently

asymptomatic to a severe acute infection resulting in rapid onset of mortality (Tizard, Nielsen et al. 1978).

5.2.2 Distribution of trypanosomes within the host:

The distribution of trypanosomes within the hosts' tissues varies according to the parasitic species involved. *Trypanosoma evansi* and *T. b. brucei* have been found to cross the capillary endothelium and thus grow both within and outside the blood-vascular system (Losos and Ikede 1970) (Goodwin 1971) (Ngeranwa, Gathumbi et al. 1993). Regions where these trypanosomes have been identified include lymph, aqueous humor as well as connective tissue, cerebrospinal, synovial and peritoneal fluids (Hornby 1952) (Ngeranwa, Gathumbi et al. 1993) (Enanga, Burchmore et al. 2002). *Trypanosoma congolense*, however, remains localized within the bloodstream and attaches preferentially to the capillary vascular endothelium of the microcirculatory regions (Banks 1978) (Bungener and Muller 1976) (Losos and Ikede 1972). For this reason, the concentration of these parasites can be up to 10 times higher in capillary beds compared to larger vessels.

5.2.3 Histological lesions:

An important problem in determining a cause of death in infected animals is the absence of gross lesions in uncomplicated trypanosomiasis (Losos and Ikede 1972). In uncomplicated disease, findings may include enlarged spleen and lymph nodes, loss of body fat, and notably in chronic infections, atrophy occurring in the thymus, spleen and lymph nodes (Losos and Ikede 1972) (Sadun, Johnson et al. 1973) (Fiennes 1970).

6. Trypanosome life cycle

Salivarian trypanosomes are mostly transmitted by infected invertebrate vectors, such as tsetse flies, to their mammalian hosts. Both *T. congolense* and *T. b. brucei* rely on this mechanism of transmission; however *T. evansi* may be transmitted through the bites of other insects (Chansiri, Khuchareontaworn et al. 2002), transplacental infection (Losos 1986), vampire bats (Hoare 1965) and even orally (Raina, Kumar et al. 1985) thereby greatly expanding their host range. In a laboratory setting, clonal stocks are generally

used (trypanosomes initially expressing only one type of VSG) and infecting experimental animals by inoculation effectively bypasses the insect cycle.

Once a tsetse fly feeds from an infected host, the trypanosomes (such as *T. b. brucei* and *T. congolense*) go through their insect stage and develop into infective metacyclic forms. The cycle of development for *T. congolense* ranges from 7 (Nantulya, Doyle et al. 1978) to 53 (Hoare 1972) days. When the tsetse fly feeds again, these metacyclic forms, present in the insects' mouthparts, are transferred into the new mammalian host. In the host, after the prepatent period which varies depending on the number of introduced parasites and route of infection, the trypanosomes will become observable in the bloodstream. The characteristic waves of parasite growth and clearance can be observed in the hosts' bloodstream until death occurs (Gray and Luckins 1976). Salivarian trypanosomes exhibit differences as to their respective niches within the host; *T. evansi* (Ngeranwa, Gathumbi et al. 1993) and *T. b. brucei* (Enanga, Burchmore et al. 2002) are present within both tissue and intravascular compartments whereas *T. congolense* (Ojok, Kaeufer-Weiss et al. 2002) only occupies the intravascular regions. Furthermore, morphological changes occur in *T. brucei* subspecies known as pleomorphism where different physical states dominate depending on the cycle of parasitemia (Newton 1968).

7. Trypanosome immune evasion strategies

Antigenic variation by African trypanosomes, for the purpose of immune evasion, is a classical paradigm illustrating the mechanism of pathogens as a means of escaping the host's antibody-mediated immune response (Boothroyd and Komuniecki 1995). Initial experiments alluding to antigenic variation conducted in primates concluded that trypanosomes, during the course of infection, altered their biological characteristics rendering them resistant to the host's immune response (Franke 1905). The sequence of antigenic variation in trypanosomes has been found not to occur in a random manner; however a predetermined sequence is also not completely adhered too. Variant surface glycoproteins (VSGs) have been shown to be the major components of the trypanosomes coat during their bloodstream stage and are responsible for inducing variant-specific immunity (Vickerman 1969; Cross 1973; Cross 1975). Variant surface glycoproteins are

highly ordered monomolecular surface coat structures covering the plasma membrane of the parasite. Glycosylphosphatidylinositol (GPI) anchors the VSG molecules to the plasma membrane in a densely packed manner preventing Abs from binding to underlying epitopes or the plasma membrane (Kaufmann, Sher et al. 2002). In addition, the VSG molecules possess a highly conserved tertiary structure with many inter- and intramolecular disulfide linkages and internal α -helices posing a formidable barrier (Freyman, Down et al. 1990).

The VSG coat is initially expressed by metacyclic trypanosomes in the tsetse fly and protects these parasites from the newly infected host's immune response (Vickerman 1974). Salivarian trypanosomes, once transferred via its vector, grow in the host and appear in the blood, forming an initial parasitemia peak. The immunogenic VSG molecules trigger a potent humoral immune response which eliminates most of the trypanosomes (Figure 1). The small portion of surviving trypanosomes survives by expressing a different surface VSG and forms the next parasitemic peak. The host mounts a new immune response against the alternate VSG and the cycle of undulating parasitemia waves continues until, at some point, the host dies from the infection (Gray and Luckins 1976).

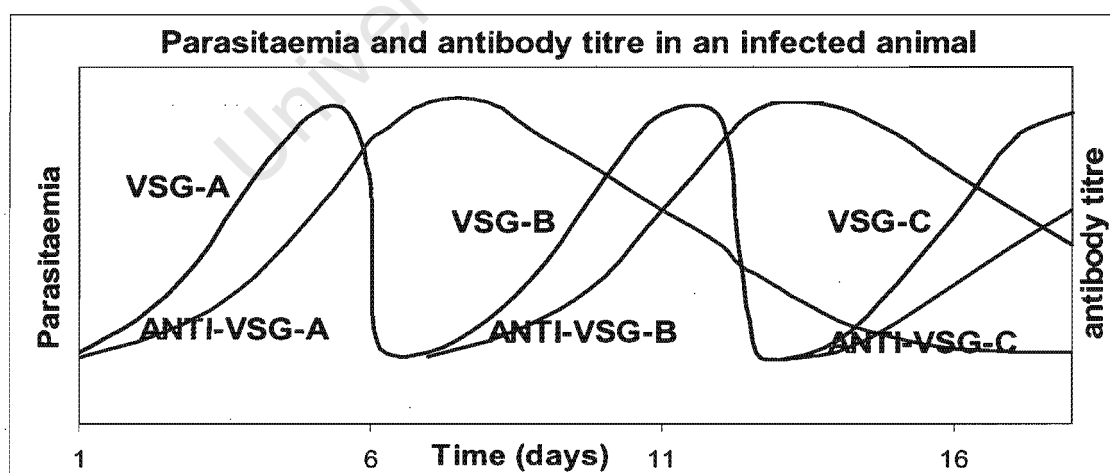


FIGURE 1. A diagrammatic representation illustrating the undulating waves of parasitemia formed by the interaction between the host's immune response and evasion strategies initiated by the invading trypanosome.

Other immune evasion strategies employed by pathogenic trypanosomes include the alteration of the hosts' immune response including the hyper activation of the complement system resulting in persistent hypocomplementaemia (Assoku, Tizard et al. 1977) (Rurangirwa, Tabel et al. 1980) (Tabel, Losos et al. 1980) (Malu and Tabel 1986). In addition, polyclonal B lymphocyte activation is induced (Luckins and Mehlitz 1976) (Morrison, Roelants et al. 1978) as well as immunosuppression (Rurangirwa, Tabel et al. 1978) (Yamamoto, Onodera et al. 1985) (Urquhart, Murray et al. 1973). The infected host thus becomes susceptible to other secondary infections (Rurangirwa, Tabel et al. 1978).

8. Immune responses to African trypanosomiasis infection

Once the trypanosomes are transferred to the host by the bite of the vector, the parasites multiply locally within the skin and induce a local immune response, identified as an indurated skin lesion called a chancre (Murray, Morrison et al. 1982) (Akol, Murray et al. 1986) (Emery and Moloo 1981) (Naessens, Mwangi et al. 2003). The parasites then spread via lymph vessels into the blood circulation and may, depending on the trypanosome species, move into the peripheral tissues (Tabel, Kaushik et al. 2000) (Mulligan and Potts 1970).

8.1 The B- and T-cellular responses

Due to the extracellular nature of trypanosomiasis infections, the humoral branch of the immune system dominates, and is characterized by polyclonal B-cell activation with elevated IgM levels in both bovine and murine infections (Hudson, Byner et al. 1976) (Luckins and Mehlitz 1976) (Morrison, Roelants et al. 1978) (Ellis, Scott et al. 1987). Trypanosomes have co-evolved mechanisms to regulate and escape the host's B- and T-cell responses. Foremost is antigenic variation whereby the surface coat of the trypanosome consisting of variant surface glycoprotein (VSG) is regularly replaced, thus continually evading the host's immune response (Pays, Vanhamme et al. 1994) (Cross, Wirtz et al. 1998) (Borst 2002) (Borst and Ulbert 2001) (Pays, Lips et al. 2001).

Upon infection the host responds with a rapid B-cell activation, to VSG and other factors, occurring largely in a T-cell independent manner (Mansfield 1994) (Reinitz and Mansfield 1990). The T-cell independent signals drive the IgM, IgG3 responses as well as part of the IgG1 response (Schopf, Filutowicz et al. 1998). The resultant VSG specific antibody response has been found to be important for clearance of parasites from within vascular regions (Dempsey and Mansfield 1983; Reinitz and Mansfield 1990).

The antibody responses against VSG molecules are functionally and genetically linked to the removal of trypanosomes from the bloodstream (Dempsey and Mansfield 1983). However these responses do not fully explain the overall resistance found in the host to infection with trypanosomes (DeGee and Mansfield 1984) (De Gee, Levine et al. 1988).

Together with VSG-specific B-cell responses, infected mice mount a Th1-cellular response to VSG (Mansfield, Craig et al. 1976) (Mansfield 1994) (Mansfield 1995). The activated T-cell response is mediated by $\alpha\beta$ TCR⁺ CD4⁺ cells which recognize peptides of the VSG molecules in a antigen-presenting cell- and MHC class II-dependent manner (Schleifer, Filutowicz et al. 1993). Despite the suppression of T-cell responses, T-cell dependent B-cell responses occur to VSG peptides (De Gee, Levine et al. 1988) (Dempsey and Mansfield 1983) (Levine and Mansfield 1981) (Reinitz and Mansfield 1990) where T helper-cell derived cytokines play a vital role in antibody class-switching (Schopf, Filutowicz et al. 1998). Here, IFN- γ secreted in response to VSG as well as other parasite antigens, induces a switch to VSG-specific IgG2a, but was not responsible for VSG-specific IgG3 switching (Schopf, Filutowicz et al. 1998). Thus, optimal resistance in the case of infection with African trypanosomiasis requires the action of both B- and Th1 cell responses during infection.

During infection with pathogenic trypanosomes, a predominant Th1-cell response occurs with no detectable Th2 response. The polarization of Th1 cells from Th0 was not found to be due to the VSG surface coat of the trypanosome (Schleifer, Filutowicz et al. 1993), but has been suggested to be driven by an early IFN- γ -independent stimulation of macrophage derived IL-12 production. During this response Th2-cell growth is inhibited.

8.2 Macrophages

Another critical mechanism of the hosts' immune response to trypanosome infections is the mononuclear phagocyte system. Here, Kupffer cells present within the liver clear parasites from the bloodstream in an antibody dependent manner (Macaskill, Holmes et al. 1980) (Macaskill, Holmes et al. 1981) (Holmes, MacAskill et al. 1979). Comparisons between peritoneal macrophages from resistant and susceptible mouse strains did not show any differences in the phagocytic activity of these macrophages (Kaushik, Uzonna et al. 1999), suggesting that the resistant or susceptible phenotype of the macrophages was due to mechanisms subsequent to this event (Tabel, Kaushik et al. 2000). Cytokine release from these macrophages co-cultured with *T. congolense* or *T. brucei* showed that susceptible mice produced more IL-10 and IL-6 but less TNF and IL-12 than did resistant mice (Kaushik, Uzonna et al. 1999) (Kaushik, Uzonna et al. 2000). In addition, macrophages from resistant C57BL/6 mice co-cultured together with *T. congolense* showed increased IFN- γ and nitric oxide (NO) production compared to cells from susceptible BALB/c mice (Kaushik, Uzonna et al. 1999). These findings indicated that cytokine gene expression, at the level of the macrophage, may play a significant role in resistance to Trypanosomiasis.

9. Cytokines in trypanosomiasis infection

A key mechanism of the immune response towards trypanosome infections in the host is the coordination of immune events as well as effective communication between the various immune compartments. Here cytokines play a significant role, including the synchronized production of pro-inflammatory as well as anti-inflammatory mediators.

9.1 Role of IL-12 in trypanosomiasis

Upon infection with trypanosomes, a rapid Th1 response is initiated (Magez, Stijlemans et al. 2002) (Schopf, Filutowicz et al. 1998) (Schleifer, Filutowicz et al. 1993) (Mansfield 1994) with the production of IL-12 and IFN- γ . Here, IFN- γ and IL-12 production occurs within 4hrs of infection in splenocytes and further suggests a partial regulatory role for the autonomic nervous system in this response (Liu, Ragaa et al. 1999). Studies have

suggested that the initiation of a polarized VSG-specific Th1 cellular response is dependant on the presence of IL-12 and IL-18 (Mansfield and Paulnock 2005), although this has not been confirmed using gene deficient mice.

Macrophages and dendritic cells that are stimulated by VSG and trypanosome DNA are the initial producers of IL-12 and IL-18 cytokines (Shoda, Kegerreis et al. 2001) (Drennan, Stijlemans et al. 2005). Indeed, the innate recognition of pathogen-associated molecular patterns via the Toll-like receptors plays a role in the initial production of IL-12 (Reiling, Holscher et al. 2002) (Medzhitov and Janeway 1997) (Ma and Trinchieri 2001).

In *T. congolense* infection, when comparing resistant to susceptible mice, splenocytes and macrophages from resistant mice produced more IL-12p40 (Kaushik, Uzonna et al. 2000) (Uzonna, Kaushik et al. 1999). This finding together with higher levels of IgG2a in resistant mice (Uzonna, Kaushik et al. 1999), it was suggested that IL-12 and IFN- γ synergize to up-regulate the switch of IgM to IgG2a (Metzger, McNutt et al. 1997) (Trinchieri 1995) (Trinchieri 1997). In resistant mice the increased IgG2a resulted in higher NO production compared to IgM, the production of which was found to be trypanostatic on *T. congolense* parasites (Kaushik, Uzonna et al. 1999).

9.1.1 Interleukin-12, structure and function

Interleukin-12 functions as a key initiator for an optimal Th1 immune response, which is necessary for the control and elimination of intracellular pathogens (Gately, Renzetti et al. 1998) (Germann, Gately et al. 1993) (Hsieh, Macatonia et al. 1993) (Trinchieri 1993). Activated inflammatory cells are the main sources of IL-12 including monocytes/macrophages, neutrophils, B cells and dendritic cells (Trinchieri 1994). Production of IL-12 induces IFN- γ secretion from T and NK cells and causes the proliferation of these cells once they are activated. Furthermore IL-12 increases the cytotoxicity of NK cells and promotes the differentiation of cytotoxic CD8⁺ T cells (Trinchieri 1994). The activity of IL-12 thus begins early after infection during the non-specific innate immune response and continues throughout the antigen-specific adaptive

immune response, thus encompassing a range of immunological functions leading to elimination of the infectious pathogen (Holscher 2004).

The IL-12 molecule is a heterodimeric protein comprised of two glycosylated subunits of p35 and p40 kDa which are covalently linked by a disulfide bond (Kobayashi, Fitz et al. 1989) (Stern, Podlaski et al. 1990). The two subunits of IL-12 are differently regulated; the p35 subunit is transcribed at low levels whereas expression of the p40 subunit is strongly enhanced in antigen presenting cells (APC) by viral and bacterial antigens (Ma, Aste-Amezaga et al. 1997) (D'Andrea, Rengaraju et al. 1992). The levels of bioactive IL-12 is therefore determined by the expression of the p35 subunit (Snijders, Hilkens et al. 1996). Secretion of the p35 subunit occurs as part of the IL-12p70 dimer whereas the p40 subunit can be secreted extracellularly (Snijders, Hilkens et al. 1996). Additionally the p40 subunits form homodimers (IL-12p80), and may act antagonistically to the functioning of IL-12 and IL-23 by blocking signal transduction via the receptor (Ling, Gately et al. 1995) (Mattner, Fischer et al. 1993) (Gillelsen, Carvajal et al. 1995) (Gately, Carvajal et al. 1996) (Mattner, Ozmen et al. 1997). However, IL-12p40 homodimer may also act in an agonistic manner to IL-12 in its ability to induce cell-mediated Th1 immune responses (Piccotti, Li et al. 1998) (Holscher, Atkinson et al. 2001).

Additionally, the p40 subunit has been found to bind to IL-23p19 forming bioactive IL-23. Because of the presence of a common subunit and thus the same receptor (IL-12R β -1) it was initially thought that IL-12 and IL-23 had similar roles in promoting Th1 responses (McKenzie, Kastelein et al. 2005). However, whereas IL-12 is known to drive a Th1 response with IFN- γ as the product, IL-23 promotes a T-cell population that is characterized by the production of IL-17-related cytokines, namely IL-17A and IL-17F (Aggarwal, Ghilardi et al. 2003) (Langrish, Chen et al. 2005) (Harrington, Hatton et al. 2005) (Park, Li et al. 2005). The ability of IL-23 in promoting a distinct T cell subset, namely Th17, has implicated this cytokine in playing a dominant role in the induction of chronic autoimmune inflammation of the central nervous system and joints (Langrish, Chen et al. 2005) (Cua, Sherlock et al. 2003) (Murphy, Langrish et al. 2003). Furthermore, recent studies have found that IL-12 and IFN- γ can suppress Th17

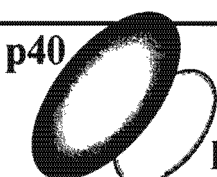
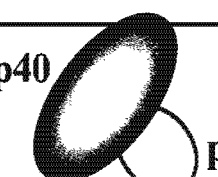
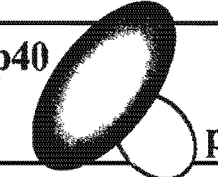
responses in that mice deficient in IFN- γ , IL-12R β 2 and IL-12p35 have an increased autoimmune predisposition (Cua, Sherlock et al. 2003) (Murphy, Langrish et al. 2003) (Zhang, Gran et al. 2003) (Ferber, Brocke et al. 1996).

Due to the similarities between IL-12p40 and Epstein-Barr virus-induced gene 3 (EBI-3) it has been hypothesized that EBI-3 can complex with IL-12p35 non-covalently and form a secreted heterodimer (Devergne, Hummel et al. 1996) (Holscher 2004) (Nieuwenhuis, Neurath et al. 2002). Although EBI-3 has been found to play a role in the induction of Th2 immune responses and as a growth factor for invariant NK T cells the EBI-3/IL-12p35 complex has as of yet no described biological function (Nieuwenhuis, Neurath et al. 2002) (Holscher 2004).

9.1.2 Interleukin-12 deficient mice in different disease models

In order to delineate the role of the different IL-12 family members, p40 (Magram, Connaughton et al. 1996) and p35 (Mattner, Magram et al. 1996) subunit gene deficient mice have been generated. For the generation of double deficient mice, i.e. IL-12p35^{-/-}/p40^{-/-} the single subunit deleted mice were intercrossed. Interleukin-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} deficient mouse strains lack all IL-12 homologues, while IL-12p35^{-/-} mice still produce IL-12p80 homodimers and IL-23 (Table 1).

TABLE 1 Interleukin-12 deficient mice used in infectious disease studies and their products

Mouse strain used:	Products		
	IL-12	IL-12p80 (homodimer)	IL-23
Wild type			
IL-12p35 ^{-/-} /p40 ^{-/-}	X	X	X
IL-12p40 ^{-/-}	X	X	X
IL-12p35 ^{-/-}	X		

Studies have found that mice deficient in IL-12 display increased susceptibility to infectious pathogens requiring a Th1 response for survival (Magram, Connaughton et al. 1996) (Holscher 2004). Interleukin-12 deficient mice have been shown to produce default Th2 responses to infectious pathogens such as in *Leishmania major* (Mattner, Magram et al. 1996), *Salmonella enterica* (Lehmann, Bellmann et al. 2001) and *Cryptococcus neoformans* (Decken, Kohler et al. 1998). In addition, IL-12 deficient mice have also been found to display residual resistance to infection. Here, in severe infectious disease models, such as toxoplasmosis (Ely, Kasper et al. 1999), listeriosis (Brombacher, Dorfmueller et al. 1999), leishmaniasis (Mattner, Magram et al. 1996), Chagas' disease (Muller, Kohler et al. 2001) and tuberculosis (Holscher, Atkinson et al. 2001), IL-12 independent IFN- γ can be produced resulting in residual resistance. Furthermore increased resistance to *Plasmodium berghei* infection has also been demonstrated in IL-

12 deficient mice compared to wild type control mice. In this model IL-12 was found to be responsible for liver injury via the activation of the TLR-MyD88 signaling pathway which resulted in hepatic lymphocytes lysing hepatocytes in a perforin dependant manner (Adachi, Tsutsui et al. 2001).

9.1.3 Interleukin-12 independent immunity

Although IL-12 has been described as the prime initiator for Th1 cellular differentiation, mice deficient in one or both subunits show evidence of a residual Th1 response (Mattner, Magram et al. 1996) (Magram, Connaughton et al. 1996) (Magram, Sfarra et al. 1996). In addition to IL-12p40 related molecules contributing to Th1 differentiation (Cooper, Kipnis et al. 2002) (Decken, Kohler et al. 1998) (Elkins, Cooper et al. 2002) (Holscher, Atkinson et al. 2001) (Lehmann, Bellmann et al. 2001), other ancillary factors are present to induce cell mediated immune responses. Interleukin-18, a member of the IL-1 family (Torigoe, Ushio et al. 1997), as well as other factors, have been found to have overlapping functions to that of IL-12 in its ability to similarly initiate the production of IFN- γ and promote a cell-mediated immune response (Takeda, Tsutsui et al. 1998) (Okamura, Tsutsi et al. 1995) (Freudenberg, Merlin et al. 2002).

9.2 Interferon- γ

Interferon gamma is essential for resistance to both *T. b. brucei* (Barkhuizen, Magez et al. 2007) (Hertz, Filutowicz et al. 1998) and *T. congolense* (Magez, Radwanska et al. 2006) infections, specifically for parasitemia control and survival. Interferon- γ is produced primarily by activated Th1 cells which in turn signals macrophages to release trypanocidal molecules including reactive oxygen intermediates, reactive nitrogen intermediates and TNF (Schleifer, Filutowicz et al. 1993) (Hertz, Filutowicz et al. 1998) (Schopf, Filutowicz et al. 1998) (Magez, Stijlemans et al. 1998). In addition, IFN- γ was also found to be necessary for the VSG-specific IgG2a switch response (Schopf, Filutowicz et al. 1998) (Hertz, Filutowicz et al. 1998). In contrast to *T. b. brucei* and *T. congolense* however, resistance to infection with *T. evansi* was found to be independent of IFN- γ signalling (Baral, De Baetselier et al. 2007).

An alternate signal for the production of IFN- γ may be the result of a specific triggering molecule produced by either *T. b. brucei* or *T. evansi*. Trypanosome derived lymphocyte triggering factor (TLTF) which is secreted by these trypanosomes has been found to induce the production of IFN- γ by binding to CD8+ cells in *T. b. brucei* (Olsson, Bakhiet et al. 1993) and *T. evansi* (Bakhiet, Buscher et al. 1996) infection models.

9.3 Tumor Necrosis Factor

The VSG molecules produced by *T. brucei* have been shown to trigger the production of TNF from macrophages in the infected host (Tachado and Schofield 1994) (Magez, Stijlemans et al. 1998) (Tachado, Mazhari-Tabrizi et al. 1999). Tumor necrosis factor has been found to have dual effects in *T. b. brucei* infections, causing immunopathological effects in the host (Magez, Radwanska et al. 1999) as well as trypanolytic effects (Lucas, Magez et al. 1994; Magez, Geuskens et al. 1997) reducing parasite burden in mice (Lucas, Magez et al. 1993; Magez, Lucas et al. 1993; Magez, Radwanska et al. 1999). In terms of immunopathology control in the host, the shedding of soluble p75 TNF-receptor at the peak of parasitemia was found to correlate with the inhibition of trypanosomiasis induced anemia (Magez, Truyens et al. 2004).

Trypanosoma b. brucei (Magez, Radwanska et al. 1999) as well as *T. congolense* (Kitani, Black et al. 2002) infected mice lacking TNF display reduced ability to control parasite load in the vascular compartment. In contrast to *T. b. brucei* where survival was similar between TNF deficient and wild type mice, *T. congolense* infected TNF gene deficient mice displayed reduced survival (Iraqi, Sekikawa et al. 2001). In *T. congolense* infections, macrophage and neutrophil derived soluble TNF, specifically TNFp55, are required for parasitemia control mediated by the trypanolytic activity of NO (Magez, Radwanska et al. 2007). In contrast to *T. b. brucei* and *T. congolense* however, resistance to *T. evansi* infection was independent of TNF, however TNF and TNFR2 was implicated in the induction of late stage anaemia (Baral, De Baetselier et al. 2007).

9.4 Nitric Oxide

The role of NO, using iNOS deficient mice has been determined in *T. b. rhodesiense* infections and was found to have no effect on survival or parasitemia control (Hertz and Mansfield 1999) suggesting that other downstream effects of IFN- γ signaling are necessary for survival. Similarly, *T. evansi* infection in iNOS deficient mice displayed no effect on mortality or parasitemia control (Baral, De Baetselier et al. 2007),.

Contrary to *T. b. brucei* and *T. evansi* infections, IFN- γ and TNF induced NO is essential for resistance to *T. congolense*. Here, NO deficient mice displayed decreased parasitemia control and survival compared to wild type mice (Magez, Radwanska et al. 2006). Immunoglobulins were suggested to play a substantial role in NO mediated resistance to *T. congolense* as the IgG isotypes induced significantly more NO production compared to IgM mediated phagocytosis by macrophages (Kaushik, Uzonna et al. 1999).

Together these studies indicate different immune mechanisms necessary for resistance to African trypanosomiasis.

9.5 Interleukin-10

Kemp *et al.*, 1997, identified three quantitative trait loci termed *Tir1*, *Tir2*, and *Tir3*, present on chromosomes 17, 5, and 1 respectively; that determined resistance in *T. congolense* infection (Kemp, Iraqi et al. 1997). Further mapping of *Tir3*, resolved this locus into three trypanosomiasis resistance quantitative trait loci, termed; *Tir3a*, *Tir3b* and *Tir3c* (Iraqi, Clapcott et al. 2000). Interleukin-10 present on chromosome 1 at 69.9 cM falls within the region of *Tir3b* (68-78 cM), thus indicating a strong putative role for this cytokine in resistance to *T. congolense* infection (Tabel, Kaushik et al. 2000).

This was confirmed using IL-10 gene deficient mice in both *T. b. brucei* (Namangala, Noel et al. 2001) and *T. congolense* (Guilliams, Oldenhove et al. 2007) infections, where this anti-inflammatory cytokine was shown to be essential for survival. Trypanosome infections in IL-10 deficient mice showed that this cytokine is vital during the acute

stages of infection to limit the pro-inflammatory response of IFN- γ and possibly TNF (Namangala, Noel et al. 2001). Furthermore in *T. congolense* infected mice treated with anti-IL-10 receptor, focal liver necrosis developed, associated with significantly enhanced plasma levels of IL-10, IL-6, IFN- γ , and IL-12p40 compared to controls (Shi, Pan et al. 2003). The role of IL-10 in *T. evansi* infection has not been elucidated.

9.6 Interleukin-4

Infections with various *T. brucei* subspecies using different mouse strains, have found the role of IL-4 to be either harmful (Bakhiet, Jansson et al. 1996), protective (Inoue, Inoue et al. 1999) or to have no effect (Schopf, Filutowicz et al. 1998) (Hertz, Filutowicz et al. 1998) in terms of mortality or parasitemia. In *T. congolense* infection, IL-4 and IL-4R α deficient mice displayed similar survival characteristics as wild type mice (Noel, Hassanzadeh et al. 2002). However, the study was performed using mice on a BALB/c background which are highly susceptible to infection. In the bovine model during *T. congolense* infection, increased IL-4 levels of mRNA was found in trypanotolerant cattle as compared to that found in trypanosusceptible cattle, and this was associated with increased survival (Mertens, Taylor et al. 1999). Therefore, the role of IL-4 in trypanosome infections may not be conclusive and with IL-13 having some overlapping activities to those of IL-4 (Brombacher 2000), this area of the immune response requires further investigation.

10. Toll like receptors

A recent study has begun to unravel the innate immune response to trypanosome infections. Here, using the *T. b. brucei* infection model, it was shown that a mammalian Toll-like receptor (TLR), or a combination of receptors, signaling via the MyD88 adaptor molecule are essential for survival and parasitemia control (Drennan, Stijlemans et al. 2005). Mice deficient in MyD88 had significantly reduced IFN- γ and TNF responses during the initial stage of infection indicating its importance in the initiation of the Type 1 immune response. Furthermore, TLR9 which is known to respond to its agonist CpG DNA, in this case trypanosome DNA, was identified to be necessary for parasitemia control in non-clonal *T. b. brucei* infections (Drennan, Stijlemans et al. 2005). However,

mice deficient for TLR9 were not as susceptible as MyD88 deficient mice indicating that other undetermined molecules signaling through MyD88 play a significant role in resistance to clonal *T. b. brucei* infection. In this study it was also necessary to delineate IL-1 and/or IL-18 signaling from MyD88 as the downstream signaling events rely on common components (Adachi, Kawai et al. 1998) (Akira, Takeda et al. 2001). However no role for these cytokines were found suggesting that MyD88 signaling via TLRs and possibly together with other surface proteins was crucial for the type 1 immune response to *T. b. brucei*.

11. Summary

Cumulatively, research has shown that a humoral and Th1 cellular response is critical for resistance to African trypanosomiasis. The Th1 response together with its associated Type 1 cytokines, are counter-balanced by their regulatory counterparts. Although these cytokines have been studied using a plethora of gene deficient mice, the prime initiator for Th1 cellular differentiation, namely IL-12 and its family members have not been fully explored. To this end, the roles of IL-12 and IL-23 together with the IL-12p80 homodimer are examined using three IL-12 gene deficient mouse strains namely; IL-12p35, IL-12p40 and IL-12p35/p40 in the development of resistance or susceptibility to *T. b. brucei*, *T. evansi* and *T. congolense* infections.

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CHAPTER II

The role of IL-12 and associated cytokines in resistance to T. b. brucei infection

ABSTRACT

African trypanosomiasis encompasses diseases caused by pathogenic trypanosomes, infecting both humans and animals. Here, we dissected the possible role of members of the IL-12 family during infection with *Trypanosoma brucei brucei* in gene deficient mice. Interleukin-12p35^{-/-}, IL-12p40^{-/-} or IL-12p35^{-/-}/p40^{-/-} mice were susceptible to this pathogen, demonstrated by increased mortality and decreased parasitemia control compared to wild type C57BL/6 mice. The different IL-12p70^{-/-} mouse strains showed similar mortality and parasitemia kinetics, suggesting that IL-12p70 but not the IL-12p80 homodimer or IL-23 plays a crucial role in resistance. While plasma levels of IgM and IgG2a were similar between IL-12 deficient mice and wild type C57BL/6 mice, IFN- γ production, especially during early infection, was severely impaired in all IL-12p70^{-/-} mouse strains, demonstrating an IL-12p70-dependent mechanism for IFN- γ production. As IFN- γ R^{-/-} mice were also highly susceptible to *T. b. brucei*, IL-12p70-dependent IFN- γ production seems to be the important mechanism involved in resistance against this pathogen.

Other related cytokines were also investigated in addition to IL-12, including IL-4/13 and IL-18. The findings in these deficient mice indicated that IL-4/13 plays no role in resistance or susceptibility and IL-12 can sufficiently provide host resistance to trypanosomiasis in the absence of IL-18 but not visa-versa.

INTRODUCTION

Trypanosomes are unicellular eukaryotic parasites that replicate freely in the bloodstream of a mammalian host, ultimately resulting in sleeping sickness in humans (*T. b. gambiense* and *T. b. rhodesiense*), Nagana in cattle (*Trypanosoma brucei brucei*) as well as infecting various wild animals and other domestic animals of economic value (Hoare 1972).

Trypanosomes regulate and escape the host's B- and T-cell responses. Foremost is antigenic variation whereby the surface coat of the trypanosome consisting of variant surface glycoprotein (VSG) is regularly replaced, thus continually evading the host's immune response (Cross, Wirtz et al. 1998) (Pays, Lips et al. 2001). Upon infection, the host responds with a rapid B-cell activation which has been found to be important for clearance of parasites from within vascular regions (Dempsey and Mansfield 1983; Reinitz and Mansfield 1990). Furthermore a rapid Th1 response is initiated (Magez, Stijlemans et al. 2002) (Schopf, Filutowicz et al. 1998) (Schleifer, Filutowicz et al. 1993) (Mansfield 1994), with the characteristic production of IL-12 and IFN- γ (Liu, Ragaa et al. 1999). Results from J.M. Mansfield et al. have suggested that the initiation of a polarized VSG-specific Th1 cellular response is dependent on the presence of IL-12 and IL-18 (Mansfield and Paulnock 2005).

Interferon- γ , which plays a central role in resistance to trypanosomiasis, is produced primarily by activated Th1 cells which in turn induces macrophages to release trypanocidal molecules including reactive oxygen intermediates, reactive nitrogen intermediates and TNF (Schleifer, Filutowicz et al. 1993) (Hertz, Filutowicz et al. 1998) (Schopf, Filutowicz et al. 1998) (Magez, Stijlemans et al. 1998). Mice lacking TNF have displayed reduced ability to control parasite load in the vascular compartment (Magez, Radwanska et al. 1999). Although nitric oxide (NO) is induced downstream of TNF and IFN- γ signaling, it was not found to play a significant role in resistance or susceptibility to *T. brucei* infections (Hertz and Mansfield 1999). Furthermore, NO has been found to

be responsible for T-cell suppression (Beschlin, Brys et al. 1998) (Hertz and Mansfield 1999)..

The development of a Th1 response to infection with trypanosomiasis (Schleifer, Filutowicz et al. 1993) suggests a role for IL-12 in resistance as this cytokine is pivotal in this response (Brombacher, Kastelein et al. 2003) (Trinchieri 1993). Studies using IL-12 deficient mice in severe infectious disease models, such as toxoplasmosis (Ely, Kasper et al. 1999), listeriosis (Brombacher, Dorfmueller et al. 1999), leishmaniasis (Mattner, Magram et al. 1996), Chagas' disease (Muller, Kohler et al. 2001) and tuberculosis (Holscher, Atkinson et al. 2001), have found that IL-12 independent IFN- γ can be produced resulting in residual resistance.

AIMS

The establishment of a Th1 immune response during *T. b. brucei* infection has focused attention onto the role of IL-12 and IL-18 (Brombacher, Kastelein et al. 2003) (Trinchieri 1993). The aims of this chapter were to investigate the role of the IL-12 family, namely; IL-12p70, IL-12p80 homodimer and IL-23 during *T. b. brucei* infection using mice deficient in the IL-12p35, IL-12p40 and IL-12p35/p40 genes. Furthermore, the role of other genes involved in the induction of either a Th1 or Th2 immune response were investigated during *T. b. brucei* infections using the respective gene deficient mouse models.

MATERIALS & METHODS

Mice

All mice used in this study (Table 1) were bred in specific pathogen-free conditions at the University of Cape Town Animal Unit. Those mouse strains that were not on a C57BL/6 genetic background were backcrossed at least 5 times with C57BL/6 mice. Interleukin-12p35^{-/-} (Mattner, Magram et al. 1996) and IL-12p40^{-/-} (Magram, Connaughton et al. 1996) mice were on a 129Sv/Ev background and were backcrossed five times with C57BL/6 mice prior to interbreeding in order to obtain homozygous double-deficient IL-12p35^{-/-}/IL-12p40^{-/-} mice. Mice were housed in filter-cap cages in a biohazard level 2 physical containment facilities. In any given experiment, mice were matched for age (6–10 weeks) and sex. All experiments were performed in accordance with the guidelines of the Animal Research Ethics Board of the University of Cape Town (Cape Town, South Africa) (Animal ethics approval number: 005/041). Distress in the mice was monitored daily and scored using parameters adapted from (Wolfensohn and Lloyd 1994) (Appendix).

Genomic DNA extraction

1cm mouse tail cuttings were digested overnight in digestion buffer (50mM TRIS-HCL, pH8; 100mM EDTA; 100mM NaCl, 1% SDS, 0.5mg/ml Proteinase K) at 56°C with gentle shaking. The samples were centrifuged at 14000 rpm for 15 minutes and the clear supernatant transferred to a clean microcentrifuge tube. An equal volume isopropanol was added and thoroughly mixed. The genomic DNA was allowed to precipitate out of solution by incubating at room temperature for 30 minutes and centrifuged at 14000 rpm at 4°C for 30 minutes. The genomic DNA pellet was washed in 1ml 70% ethanol, air dried and resuspended in 500µl sterile water. The quantity and quality of the genomic DNA was checked by spectroscopy by reading the absorbance at A₂₆₀ and A₂₈₀.

Genotyping PCR

All mice used in this thesis were genotyped prior to experiments to confirm their genotype using gene specific primers (Appendix). 2µl of genomic DNA was added to 48µl of PCR cocktail (1 X Supertherm PCR Buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 0.125U/µl Supertherm Taq, 0.25µM forward primer and 0.25µM reverse primer). Control reactions included a (i) no template/water control, (ii) positive control and (iii) negative control. The PCR products were amplified on a MJ thermocycler (Biozym, Hessisch Oldendorf, Frankfurt, Germany) using the cycle parameters given in the Appendix.

Table 1. List of gene deficient mouse strains used in this study

Deleted Gene	Authors	Mouse Background
IL-12p35 ^{-/-}	(Mattner, Magram et al. 1996)	C57BL/6
IL-12p40 ^{-/-}	(Magram, Connaughton et al. 1996)	C57BL/6
IFN-γR ^{-/-}	(Andersson, Dai et al. 1998)	129Sv/Ev
IL-4 ^{-/-}	(Noben-Trauth, Kohler et al. 1996)	C57BL/6 & BALB/c
IL-4Rα ^{-/-}	(Mohrs, Holscher et al. 2000)	C57BL/6 & BALB/c
MyD88 ^{-/-}	(Adachi, Kawai et al. 1998)	C57BL/6
TNF ^{-/-}	(Marino, Dunn et al. 1997)	C57BL/6
TNF R1 ^{-/-}	(Rothe, Lesslauer et al. 1993)	129Sv/Ev
TNF R2 ^{-/-}	(Erickson, de Sauvage et al. 1994)	129Sv/Ev
iNOS ^{-/-}	(MacMicking, Nathan et al. 1995)	C57BL/6
IL-1Rrp ^{-/-}	(Takeda, Tsutsui et al. 1998)	C57BL/6
CD4 ^{-/-}	(Rahemtulla, Fung-Leung et al. 1991)	C57BL/6
TLR2	(Wooten, Ma et al. 2002)	129Sv/Ev

Parasites

The pleomorphic *T. b. brucei* AnTat 1.1E clone was kindly provided by Dr. N. Van Meirvenne and E. Magnus (Lab. Serology, Institute of Tropical Medicine (ITG), Antwerp, Belgium). The clonal Tc13 antigen type of *T. congolense* was kindly provided by Dr. H. Tabel from the University of Saskatchewan, Canada (Tabel 1982). *Trypanosoma evansi* was initially isolated from camels in Kenya (Gibson, Wilson et al. 1983) and was obtained from the collection of trypanosome stabilates maintained at the Institute of Tropical Medicine (Belgium) (Code: KETRI 2480, Stock no.: ITMAS 110297). Trypanosome stabilates were maintained at -80°C and thawed prior to infection.

Propagation of *T. brucei*, *T. evansi* and *T. congolense* stocks in mice

Cryopreserved *T. brucei*, *T. evansi* and *T. congolense* were expanded in mice to generate new stabilates. Stabilates were thawed and diluted in 1 X PBS. BALB/c mice were inoculated by intraperitoneal injection with 0.5 ml of the diluted parasites. The mice were monitored daily for the presence of parasitaemia by examining blood collected from tail snips. When high parasitaemia was detected ($>1 \times 10^7$ trypanosomes per ml), the mice were exsanguinated and blood was collected from the heart. To each blood sample an equal amount of 20% glycerol in phosphate saline glucose (PSG) buffer (9.5mM, Na₂HPO₄, 5mM NaH₂PO₄·2 H₂O, 72.6mM NaCl, 1% glucose) was added and thoroughly mixed. The parasite stock divided into 50µl aliquots and stored at -80°C.

Trypanosome Purification Protocol

Trypanosomes from infected blood were harvested from mice prior to the 1st peak of parasitemia. Trypanosomes were purified from blood using DEAE-Sephadex anion-exchange columns (DE52; Whatman, Maidstone, England) according to the procedure described by Lanham and Goodfrey (1970) (Lanham and Godfrey 1970). Infected blood was collected through cardiac puncture at peak parasitemia (usually day 5 after infection). Blood was centrifuged in heparinized tubes at 2200g for 10 min at 4°C and the buffy coat was removed and diluted 1:3 in phosphate saline glucose (PSG) buffer (9.5mM, Na₂HPO₄, 5mM NaH₂PO₄·2 H₂O, 72.6mM NaCl, 1% glucose). Briefly, DE52 columns

were washed and equilibrated with PSG buffer. Each DE52 column was loaded with 350ul of 1:3 diluted buffy coat and the parasite eluted in PSG buffer at pH 8.0 for *T. brucei* and pH 7.8 for *T. evansi* and *T. congolense*. The eluted fractions were centrifuged at 200rpm for 10 minutes at 4°C to concentrate the parasites. The parasite pellet was resuspended in PSG buffer and the parasite concentration determined by light microscopy (Nikon, Randburg, South Africa) using a hemocytometer. The trypanosome parasite samples were stored at -80°C.

Preparation of trypanosome antigen

Trypanosomes from infected blood were harvested from mice prior to the 1st peak of parasitemia and purified using DE52 cellulose column chromatography described above. The eluted parasites were washed once in 10ml 1 X PBS by centrifugation at 2000 rpm for 7 minutes at 4°C. The parasite pellet was resuspended in 1ml 950ul 1 X PBS and the number of alive and dead parasites determined by light microscopy (Nikon, Randburg, South Africa). 50ul of proteinase inhibitor cocktail (Sigma-Aldrich, Munich, Germany) was added to the sample and the parasites lysed by three sequential freeze-thaw cycles in liquid nitrogen and mechanical dispersion by passing 5 times through a 25-gauge needle. Cell debris was removed from the lysate by centrifugation at 13000 rpm for 5 minutes at 4°C. The supernatant was filtered through a 0.22um filter and the protein concentration determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) as per the manufacture's instructions. The protein lysates was divided into 100ug/ml aliquots and stored at -20°C.

Infection, Parasitemia and Anemia

For each experiment, a vial of frozen parasite stabilate was thawed and diluted in 1 x PSG before injection. Mice were injected into the peritoneal cavity with 100µl 1 x PSG containing either 5×10^3 *T. b. brucei* parasites, 1.0×10^3 *T. evansi* parasites or 1.0×10^3 *T. congolense* parasites per mouse. Parasitemia and anemia was measured by counting the number of parasites and red blood cells in the blood after infection. Approximately 3µl of blood was collected from tail snips and diluted 1/200 in RPMI (Gibco/Invitrogen, Paisley, UK) supplemented with 10% FCS (Gibco/Invitrogen, Paisley, UK). The number

of parasites and red blood cells was counted under a light microscope (Nikon, Randburg, South Africa) using a hemocytometer.

Cytokine determination by ELISA

The production of cytokines in plasma was measured by ELISA as described previously (Dai, Bartens et al. 1997) with modifications. Standards, coating and biotinylated detection antibodies for TNF- α and IFN- γ were purchased from BD PharMingen (San Diego, CA); and IL-10 from R&D Systems (Abingdon, UK). Maxisorb 96 well ELISA plates (Nalge Nunc International, Naperville, ILL, USA) were coated overnight at 4°C with 50 μ l of “coating antibody” diluted in coating buffer (1 X PBS, 0.02% sodium azide) at the recommended concentration. For TNF ELISAs, the plates were coated in carbonate coating buffer (15mM NaCO₃, 35mM NaHCO₃, 72mM NaCl). Plates were washed, blocked 2 hours at 37°C in 250 μ l blocking buffer (2% fat free milk powder, 0.1% sodium azide in 1xPBS) and then washed again. Blood plasma and cytokine standards were serially diluted three- and twofold, respectively in dilution buffer (4% BSA (fraction V), 0.02% sodium azide in 1 X PBS) and 50 μ l added to each well. The plates were incubated overnight at 4°C and washed. 50 μ l of the appropriate biotinylated antibody was diluted to the recommended concentration in dilution buffer (4% BSA (fraction V), 0.02% sodium azide in 1 X PBS) and 50 μ l was added to each well. The plates were incubated at 37°C for 3h, washed with and 50 μ l of streptavidin-horseradish-peroxidase (BD PharMingen) diluted 1:1000 in dilution buffer (4% BSA (fraction V), 0.02% sodium azide in 1 X PBS) was added to each well. The plates were incubated for 60 minutes at 37°C, washed and 50 μ l substrate solution containing 3,3',5,5-tetramethylbenzidine (KPL, Maryland, USA) added to each well. The enzymatic reactions were developed at room temperature and was stopped when the most concentrated standard reached 1.0 to 1.5 A_{405nm} OD units. The developing reaction was stopped by adding 50 μ l of 1M HCl to each well. The plates were read at an optical density of 450nm and 540nm using a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A). For the wash steps above, each well was washed 4 times with 300 μ l of azide-free wash buffer (2.68mM KCl, 1.47mM KH₂PO₄, 8.09mM Na₂HPO₄.2H₂O, 136mM NaCl, 0.05% Tween-20). The detection limit for IFN- γ and TNF was 16pg/mL, and IL-10 was 63pg/mL.

Quantification of anti-VSG plasma titres

Anti-trypanosome-derived antigen antibody titres were determined by ELISA as described previously (Radwanska, Magez et al. 2000) using the same methods described above. Briefly, Maxisorb 96 well ELISA plates (Nalge Nunc International, Naperville, ILL, USA) were coated overnight at 4°C with 50µl of trypanosome antigen diluted in 1 X PBS (at a concentration of 5µg/ml total soluble trypanosome protein extract). Plates were washed, blocked 2 hours at 37°C in 250µl blocking buffer (2% fat free milk powder, 0.1% sodium azide in 1xPBS) and then washed again. Blood plasma from infected mice was added in two-fold serial dilutions made in 1 x PBS. The plates were incubated overnight at 4°C and washed. 50µl of the alkaline phosphatase labeled anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology, Birmingham, AL) antibodies was added to each well. The plates were incubated at 37°C for 3h and washed and 50µl of substrate solution containing 4-nitrophenylphosphate, disodium salt hexahydrate (NBT) (Fluka, Switzerland) added to each well. The enzymatic reactions were developed at room temperature and was stopped when the most concentrated standard reached 1.0 to 1.5 A_{405nm} OD units. The developing reaction was stopped by adding 50µl of 1M NaOH to each well. The plates were read at an optical density of 405nm and 690nm using a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A). For the wash steps above, each well was washed 4 times with 300µl of azide-free wash buffer (2.68mM KCl, 1.47mM KH_2PO_4 , 8.09mM $Na_2HPO_4 \cdot 2H_2O$, 136mM NaCl, 0.05% Tween-20).

Here it is important to note that in C57BL/6 inbred mouse strains the IgG2a gene is absent. Although alkaline phosphatase labeled anti-mouse IgG2a was used to detect anti-VSG IgG2a responses in these mice, rather IgG2c was detected as the commercial antibody is non-specific (Martin, Brady et al. 1998).

Aspartate transaminase assay

Hepatocellular damage was determined by the aspartate transaminase assay (AST) (Sigma-Aldrich, Munich, Germany) as per the manufacturer's instructions.

Histopathological analysis

Mice were euthanized by CO₂ asphyxiation. Organs were fixed in 4 % phosphate-buffered formalin and paraffin-embedded. Sections (2 to 3 µm) were stained with hematoxylin and eosin and assessed by light microscopy (Nikon, Randburg, South Africa). Images were captured using the DXM1200 camera and ACT software (Nikon).

Immunohistochemistry

Frozen tissue sections were prepared using a cryostat (Leica CM 1850) and attached to 3-aminopropyltriethoxysilane-coated slides. Seven-micrometer sections were air-dried and fixed in 100% acetone for 5 min before storage at -80°C. Slides were washed in PBS and incubated with a primary antibody against iNOS (gift from Prof. B. Ryffel) and F4/80 (BMA Biomedicals, Switzerland) for 90 min at room temperature. After incubation with secondary antibody coupled with Texas Red (Abcam, Cambridge, UK) or FITC (BMA Biomedicals, Switzerland) for 30 min at room temperature, sections were mounted in Fluoromount (DAKO, Carpinteria, CA) and assessed by immunofluorescence microscopy (Nikon). Images were captured as per above.

Graph and statistical analysis

Data are expressed as the means of individual determinations and SD. Statistical analysis was performed using the unpaired Student's t-test for ELISA's and for mortality Kaplan-Meier with log rank test was used. Parasitemia data was analyzed using one-way ANOVA with Dunnett as the post test at each time point measured. Analysis between wild type groups of repeat experiments found that parasitemia was not significantly different and was thus pooled (data not shown).

RESULTS

*IL-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice infected with *T. b. brucei* are susceptible and display reduced survival.*

In order to determine the role of IL-12p70 (p35/p40), IL-23 (p19/p40) and IL-12p80-homodimer during infection with *T. b. brucei*, mice deficient in either or both p35 and p40 genes were infected with a clonal strain of *T. b. brucei* and monitored for survival (Table 2 and Figure 1). Mortality data generated in all three experiments were pooled. Survival time of *T. b. brucei* infected mice was significantly decreased in all IL-12 gene deficient mouse strains (IL-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}) compared to C57BL/6 wild type controls. As no difference was observed in the absence of either the single chains or both chains, these results suggest that IL-12p70 is the major host protective factor and that IL-23 or IL-12p80 homodimer has no crucial role for survival in *T. b. brucei*.

Table 2. Interleukin-12 gene deficient mice infected with *T. b. brucei* and *T. evansi* show increased mortality compared to wild type mice.

Parasite species ^a	Strain of mice	Mean survival (SD)	No. of mice/group ^b	P value compared to wild type mice ^c
<i>T. b. brucei</i>				
	Wild type	40.6 ± 8.8	29	
	IL-12p35 ^{-/-} /p40 ^{-/-}	35.1 ± 6.4	15	<i>P</i> < 0.05
	IL-12p40 ^{-/-}	32.0 ± 3.7	15	<i>P</i> < 0.0001
	IL-12p35 ^{-/-}	33.3 ± 4.2	15	<i>P</i> < 0.001

^a *Trypanosoma b. brucei* infected mice were injected with 5x10³ trypanosomes intraperitoneally and monitored daily for survival.

^b *Trypanosoma b. brucei* survival data were pooled from three independent experiments.

^c Tests for significance were performed using the Kaplan-Meier (Log rank) test for survival.

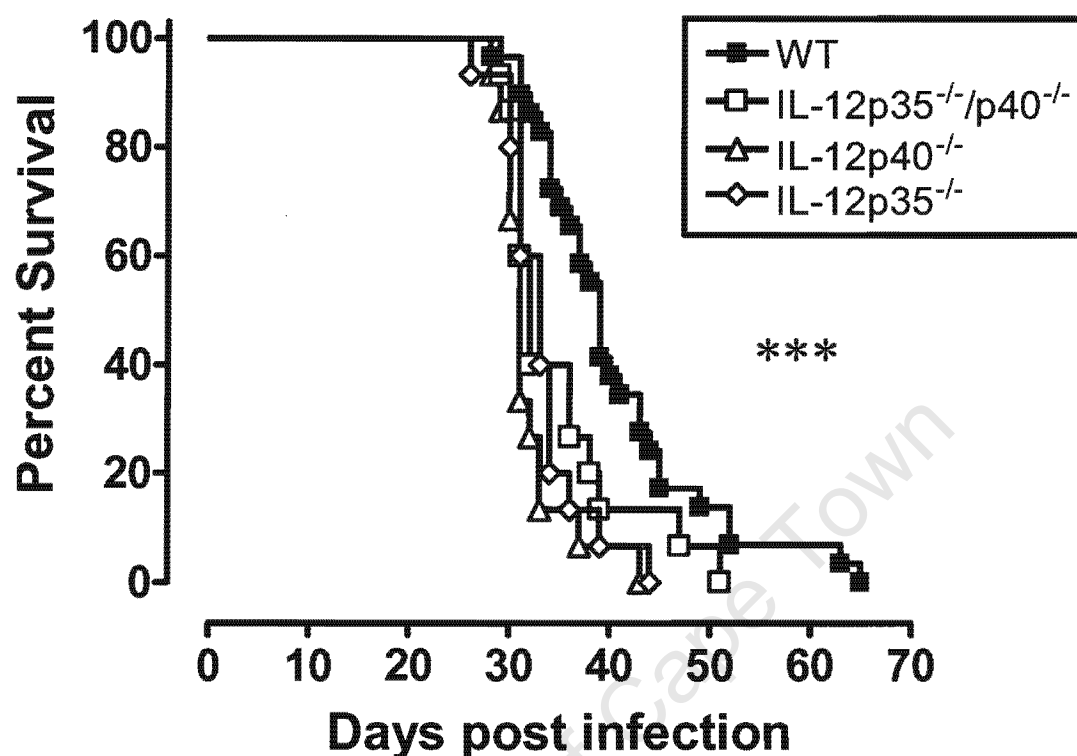


FIGURE 1. Interleukin-12 deficiency results in decreased resistance to infection with *T. b. brucei*. Mortality profiles of infected wild type (■), IL-12p35^{-/-}/p40^{-/-} (□) ($P < 0.0001$), IL-12p40^{-/-} (△) ($P < 0.0001$), and IL-12p35^{-/-} (◇) ($P < 0.0001$) mice are shown from 3 independent pooled experiments. Infectious dose was 5×10^3 trypanosomes per mouse and were subsequently monitored daily for survival. The mean survival time was; 40.6 days for wild type ($n=29$), 35.1 days for IL-12p35^{-/-}/p40^{-/-} ($n=15$), 32.0 days for IL-12p40^{-/-} ($n=15$) and 33.3 days for IL-12p35^{-/-} ($n=15$) mice. No significant differences were found between IL-12 gene deficient mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality and is indicated by; ***, $P < 0.001$.

Stage specific role of IL-12 in the control of parasitemia during *T. b. brucei* infection

Parasite burden during infection was measured to determine possible roles of IL-12 family members in the control of parasitemia caused by infection with *T. b. brucei*. No significant differences were found between the wild type mice ($P>0.05$) from the three experiments and were thus pooled as well as the IL-12 gene deficient mice (data not shown). The first parasitemia peak at day 5 post infection was similar in all three p35 and p40 mutant mice and wild type controls infected with *T. b. brucei* (Figure 2). These results showed that the absence of either or both p35 and p40 subunits had no influence on the first parasitemia peak control. However the rate of clearance of the 1st parasitemic peak was slightly reduced in IL-12 gene deficient mice compared to wild type mice by 4 to 8 fold at day 7 post infection ($P<0.05$).

Second peak and subsequent parasite control was clearly impaired during *T. b. brucei* infection with up to 100-fold increased parasite burden in IL-12 gene deficient mice compared to wild type controls. No significant difference in parasitemia control between the different IL-12 gene deficient mouse strains was observed. In addition, the loss of parasitemia control towards the end point of infection correlated with mortality in IL-12 gene deficient mice. Together, these results show that *T. b. brucei* blood parasite burden is controlled by IL-12p70 but not by IL-23 or the IL-12p80 homodimer in a stage specific manner.

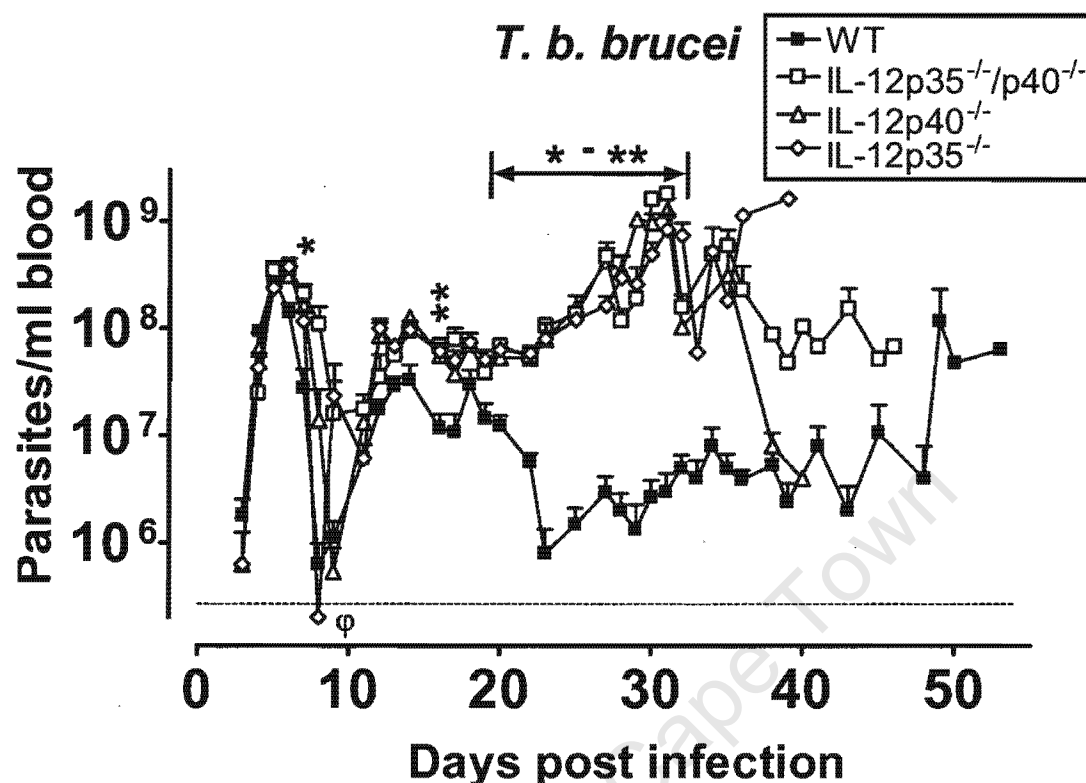


FIGURE 2. Interleukin-12 is necessary for optimal parasitemia control in *T. b. brucei* infected mice. Parasitemia profiles of *T. b. brucei* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (□), C57BL/6 IL-12p40^{-/-} (△), and C57BL/6 IL-12p35^{-/-} (◇) mice are shown. *Trypanosoma brucei brucei* infected mice were infected intraperitoneally with 5×10^3 trypanosomes, thereafter parasitemia was determined from peripheral blood obtained from tail snips with a lower detection limit of 2.7×10^5 parasites/ml (dotted line). Data points are the mean from 3 pooled experiments of 5-10 individual mice per group. Inferred data points appearing below the detection limit are denoted as; ϕ . Statistical significance between infected wild type and IL-12 gene deficient mice was determined by one-way ANOVA (Dunnett post test) and are indicated by; *, $P < 0.05$, and **, $P < 0.01$.

Interleukin-12 does not play a role in the development of anemia in *T. b. brucei* infected mice.

The development of anemia during *T. b. brucei* infection has been well documented and the role of cytokines in its development has been studied extensively. Using TNF gene deficient mice, it was found that TNF is involved parasite killing but was also responsible for the induction of anemia (Magez, Radwanska et al. 1999). Furthermore, the shedding of soluble p75 TNF-receptor at the peak of parasitemia was found to correlate with the inhibition of trypanosomiasis induced anemia (Magez, Truyens et al. 2004).

Interleukin-12 gene deficient mice infected with *T. b. brucei* have a similar anemia profile as that of the wild type controls (Figures 3A, B and C). At day 22 post infection IL-12 knock mice displayed significantly reduced anemia compared to wild type mice. However, throughout the infection the overall severity of anemia was similar for all mouse groups. A rapid drop in red blood cell number is evident after the 1st parasitemic peak and is later regained at a similar time as the emergence of the 2nd peak. As the infection enters the chronic stage, anemic conditions become more nominal. Interleukin-12 family members therefore play no role in the induction of anemia during *T. b. brucei* infections.

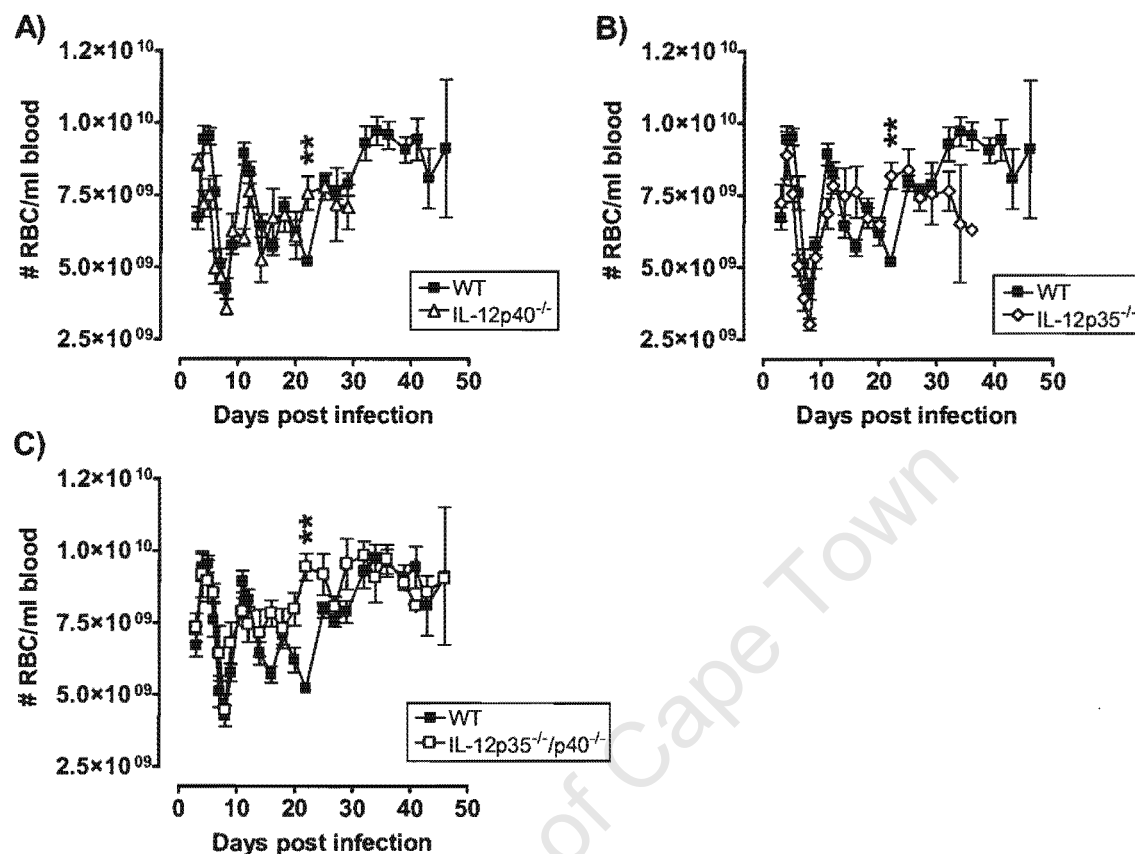


FIGURE 3. Interleukin-12 does not play a role in the induction of anemia in *T. b. brucei* infected mice. Red blood cell concentrations of *T. b. brucei* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (□), C57BL/6 IL-12p40^{-/-} (△), and C57BL/6 IL-12p35^{-/-} (◇) mice are shown. Separate comparisons of IL-12p40^{-/-} (A), IL-12p35^{-/-} (B) and IL-12p35^{-/-}/p40^{-/-} (C) mice are shown compared to wild type controls. *Trypanosoma brucei* infected mice were infected intraperitoneally with 5×10^3 trypanosomes. Thereafter red blood cell counts were determined from peripheral blood obtained from tail snips with a lower detection limit of 8.0×10^6 parasites/ml. Data points are the mean from of 5-10 individual mice per group. Statistical significance between infected wild type and IL-12 gene deficient mice was determined by one-way ANOVA (Dunnnett post test) and are indicated by; **, $P < 0.01$.

Interferon- γ and TNF are up regulated by IL-12p70 in *T. b. brucei* infection.

Interferon- γ and TNF are both known to play a role in resistance and parasitemia control (Hertz, Filutowicz et al. 1998) (Lucas, Magez et al. 1994) with IL-12 as the major initiator for IFN- γ production (Trinchieri 1994). To determine a possible relationship between these cytokines and IL-12 in trypanosomiasis infection, IFN- γ and TNF plasma cytokine levels during *T. b. brucei* infection were determined. Plasma was taken during the 1st parasitemia peak at day 6, and post 2nd parasitemia peak at day 20 (Figure 4). Levels of IFN- γ at day 4 were below the detection limit of the assay and the levels at day 5 were lower than at day 6 (data not shown). Infection in wild type mice induced strong systemic IFN- γ and TNF production, whereas IL-12 deficient mice displayed a marked reduction of IFN- γ ($p < 0.05$) and TNF ($p < 0.01$) production with similar residual low levels in all IL-12 mutant strains observed (Figure 4A and B). These results suggest that during *T. b. brucei* infection optimal IFN- γ and TNF production was dependent on IL-12p70 signaling. However, this data also indicates that low levels of IFN- γ and TNF production can be independently induced from signal transduction via p35 and p40 subunits.

Interleukin-4 production at the 1st parasitemic peak was undetectable in wild-type and IL-12 gene deficient mice (data not shown). At day 20 post infection IL-4 levels, although highly variable, were present in both wild type and IL-12 gene deficient mice (Figure 4C). Therefore, no shift to a Type 2 cytokine response in the absence of IL-12 was detected.

Levels of IL-12p40 and IL-18 were undetectable in plasma during the 1st parasitemic peak in wild-type and IL-12 gene deficient mice (data not shown).

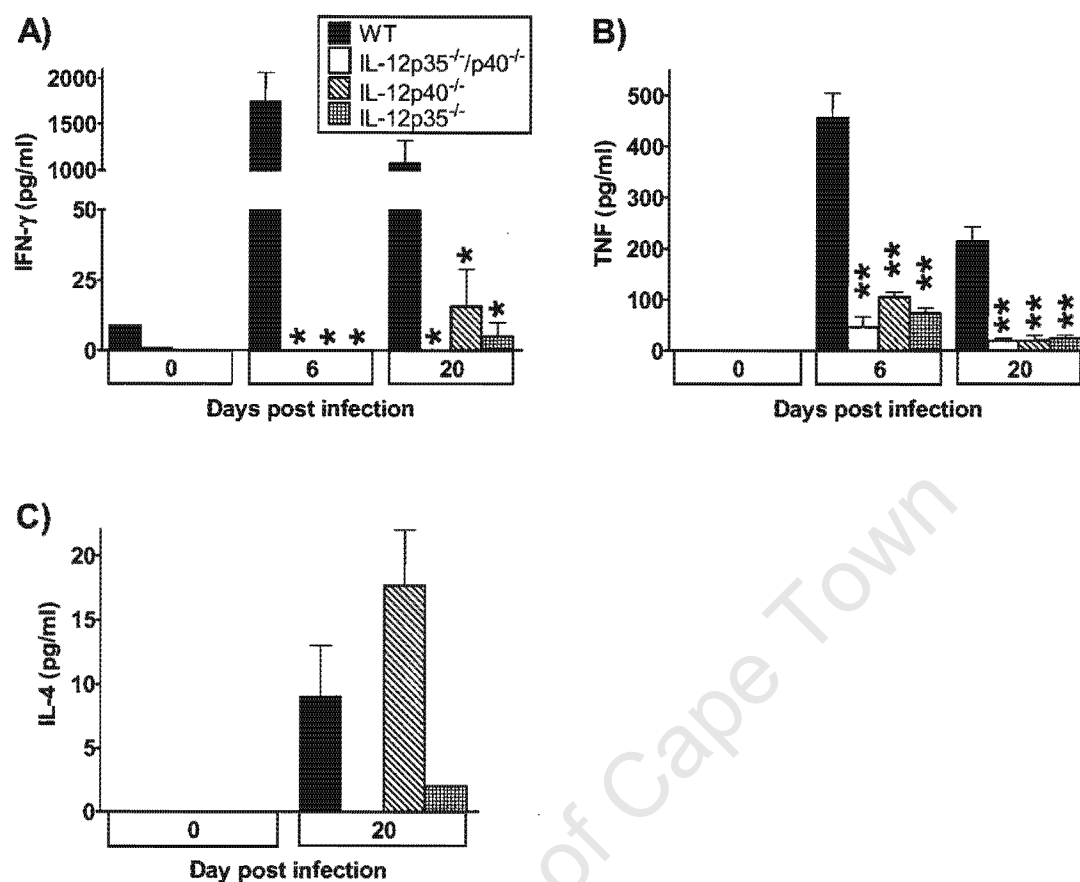


FIGURE 4. Interleukin-12p70 is necessary for an optimal IFN- γ and TNF responses to infection with *T. b. brucei*. Interferon- γ and TNF plasma levels were measured by ELISA in C57BL/6 wild type (Filled bars), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (Open bars), C57BL/6 IL-12p40^{-/-} (Striped bars) and C57BL/6 IL-12p35^{-/-} (Hatched bars) infected mice. Time points analyzed were at days 0 (controls), 6 (1st peak) and day 20 (post 2nd peak). Results shown are representative of two independent experiments. Data expressed as means ($\pm$ SEM) from 2-3 individual mice. Statistical significance between infected wild type and IL-12 gene deficient mice was determined by unpaired Student's t-test and are indicated by *, $P < 0.05$ or **, $P < 0.01$.

Interleukin-12 deficient mice have unimpaired IgG2c and IgM antibody titres compared to wild type mice in response to infection with T. b. brucei.

T-dependant as well as T-independent VSG-specific B-cell responses occur during *T. b. brucei* infection, which in turn induces immunoglobulin class switches (Reinitz and Mansfield 1990) (Mansfield 1994) (Schopf, Filutowicz et al. 1998). As anti-VSG antibody responses have been shown to play a key role in parasitemia control and host survival in trypanosome infections (Levine and Mansfield 1984). The anti-VSG specific antibody responses in wild type and IL-12 gene deficient mice were therefore measured to determine if increased mortality or parasitemia in IL-12 deficient mice correlated with decreased antibody responses. An IgM and high serum IgG2a antibody titres have been associated with increased resistance to *T. b. brucei* infection (S. Magez, paper in preparation) (Magez, Stijlemans et al. 2002) (Radwanska, Magez et al. 2000). Given the diverse strains of mice and commercial sources of antibody used in studies the relative proportions of either IgG2a and IgG2c measured in mouse serum is problematic. Both IgM and IgG2c levels (measured using anti-mouse IgG2a) were similar between wild type and IL-12 deficient mice throughout the course of infection (Figure 5A and B). Levels of IgG2b, which are induced by TGF- β (Roitt, Brostoff et al. 2001), are similar in IL-12 gene deficient and wild type mice (Figure 5C).

During *T. brucei* infections, the IgG1 class switch is dependant on IL-4. However this switch is not associated with resistance or susceptibility (Schopf, Filutowicz et al. 1998). The levels of IL-4 were similar in IL-12 deficient mice as compared to wild type mice (Figure 6A). The switch to IgG3 can be driven by IFN- γ production (Roitt, Brostoff et al. 2001); however in the case of trypanosome infections this switch occurs independent of this cytokine (Schopf, Filutowicz et al. 1998). Levels of IgG3 were slightly reduced in IL-12 gene deficient mice during the chronic stages of disease (Figure 6B).

In summary, plasma IgG2c and IgM anti-VSG antibody titres, were similar in IL-12 deficient and wild type mice throughout infection with *T. b. brucei* indicating that these responses were largely independent of IL-12.

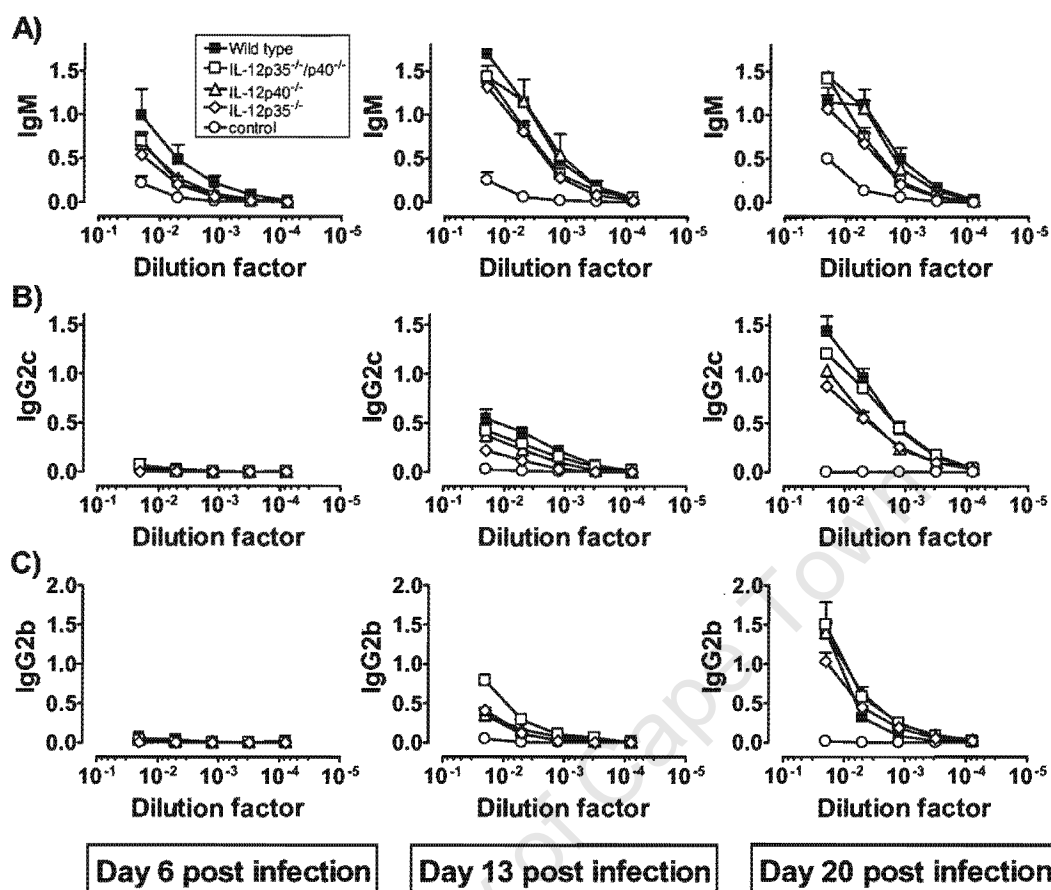


FIGURE 5. Immunoglobulin responses, namely IgM, IgG2c and IgG2b, are intact in IL-12 gene deficient mice compared to wild type control mice. A) IgM, B) IgG2c and C) IgG2b plasma antibody titres from *T. b. brucei* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice are shown. First peak (Day 6), 2nd peak (Day 13) and post 2nd parasitemia peak (Day 20) plasma antibody titers from infected mice are represented. Pre-immune serum was used as controls (○). Data points represent mean and $\pm$ SEM from plasma of 3 individual mice. Statistical significance tests between wild type and IL-12 deficient mouse groups was determined by Student's t-test.

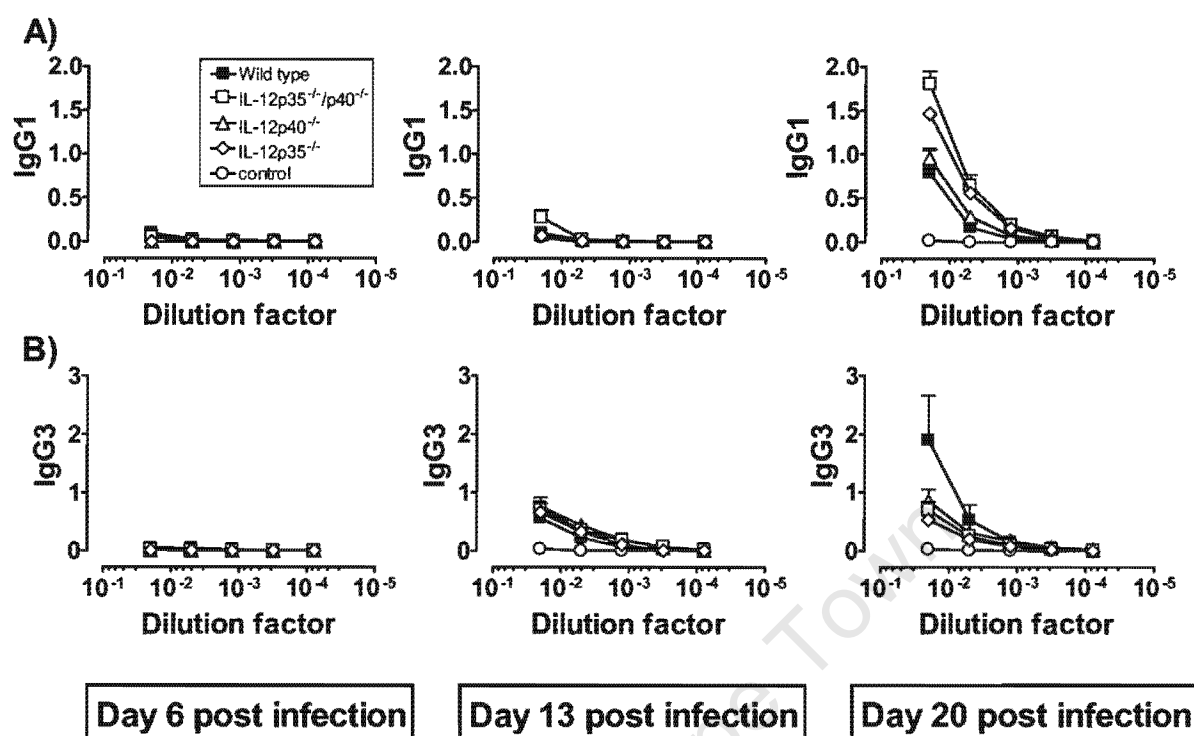


FIGURE 6. Immunoglobulin G3 response is slightly reduced in IL-12 gene deficient mice as compared to wild type control mice. A) IgG1 and B) IgG3 plasma antibody titers from *T. b. brucei* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice. Three analysis points are illustrated; namely, first peak (Day 6), 2nd peak (Day 13) and post 2nd parasitemia peak (Day 20) plasma antibody titers. Pre-immune serum was used as controls (○). Data points represent mean and ±SEM from plasma of 3 individual mice. Statistical significance tests between wild type and IL-12 deficient mouse groups was determined by Student's t-test.

Interferon- γ production is crucial for resistance to *T. b. brucei* infection.

As IFN- γ production was impaired in all IL-12 deficient mice infected with *T. b. brucei*, the role of IFN- γ signaling in the control of trypanosomiasis was investigated in mortality studies using IFN- γ receptor (IFN- γ R) gene deficient and wild type mice on a 129Sv/Ev background.

Reduced survival was clearly observed in IFN- γ R gene deficient mice as compared to wild type mice with mean mortalities of 16 vs. 41 days ($P < 0.001$), respectively (Figure 7A). Control of 1st parasitemia peak was independent of IFN- γ R responsiveness but mutant mice were unable to sufficiently clear the first parasitemia wave and died shortly thereafter. This decreased survival was associated with a greater than 10 fold increase in parasitemia from day 9 post infection until mortality ($P < 0.05$) as compared to wild type controls (Figure 7B).

These data demonstrated the importance of IFN- γ R responsiveness for survival in *T. b. brucei* infection and control of the parasite burden in *T. b. brucei* infected mice. The *T. b. brucei* results obtained are similar to those in *T. b. rhodesiense* infected C57BL/6 wild type and C57BL/6 IFN- γ deficient mice indicating no major differences in trypano-tolerance between 129Sv/Ev or C57BL/6 genetic background (Hertz, Filutowicz et al. 1998).

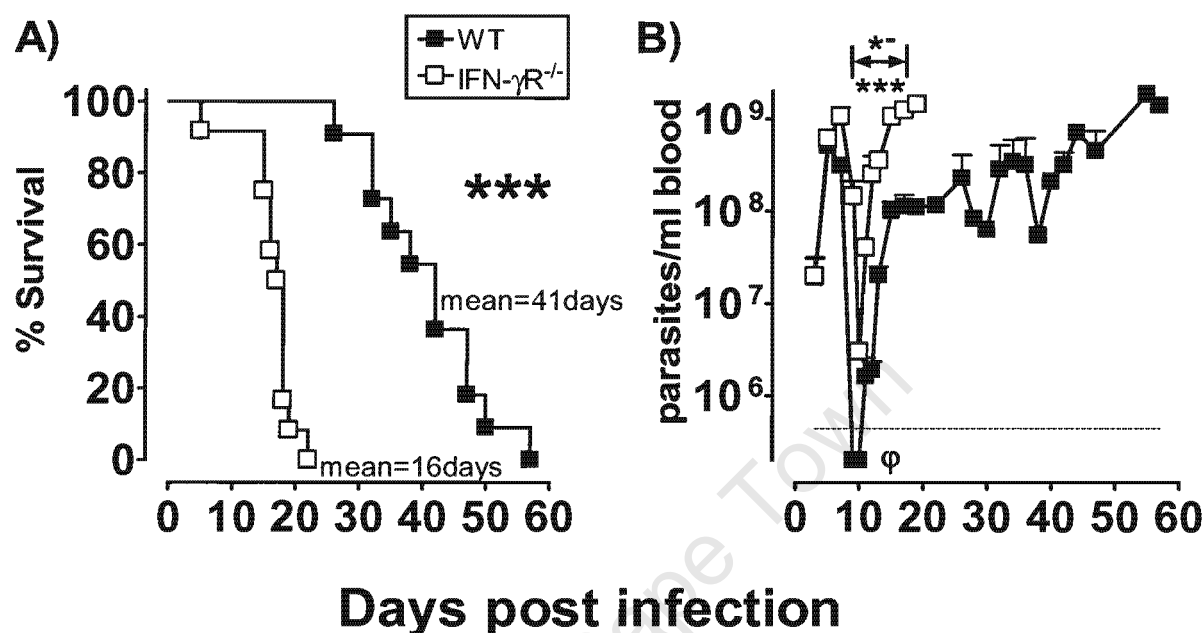


FIGURE 7. Interferon- γ signaling is necessary for resistance to infection with *T. b. brucei*. Sv/Ev129 wild type (■) and Sv/Ev129 IFN- γ R gene deficient (□) mice were infected intraperitoneally with 5×10^3 trypanosomes and monitored daily for survival (A). Mean survival times were 41 days for Sv/Ev129 wild type mice ($n = 11$) and 16 days for Sv/Ev129 IFN- γ R gene deficient mice ($n = 12$). Parasitemia profiles of infected Sv/Ev129 wild type (■) and Sv/Ev129 IFN- γ R gene deficient (□) mice (B) are shown. Parasitemia was determined from peripheral blood with a lower detection limit of 4.4×10^5 parasites/ml (dotted line). Mortality and parasitemia data were pooled from two independent experiments where 4-5 mice per group were used for parasitemia measurement. Inferred data points appearing below the detection limit are denoted as ϕ . Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia the unpaired Student's t-test was used; *, $P < 0.05$ and ***, $P < 0.001$.

Interleukin-18 signaling does not play a major role in survival during *T. b. brucei* infection.

Interleukin-18 forms part of the IL-1 family and signals via the IL-1 receptor-related protein (IL-1Rrp) (Torigoe, Ushio et al. 1997). The induction of a Th1 immune response is known to require IL-12 signaling, in addition IL-18 has overlapping functions with that of IL-12 and thus both act together in the induction of a Th1 immune response (Takeda, Tsutsui et al. 1998) (Holscher 2004). Given the synergistic activities of IL-18 and IL-12, the role of IL-18 in *T. b. brucei* infection was investigated.

The results of two independent pooled experiments showed that although all the IL-18 gene deficient mice survived the 1st peak of infection, IL-18 signaling is slightly detrimental to survival in *T. b. brucei* infection. The median survival of IL-18 gene deficient mice was 45.0 days as compared to 36.5 days in wild type mice (Figure 8). This result suggested that IL-18 plays a prominent during the chronic stage of disease (as does IL-12) and not in the establishment of the initial Th1 immune response. Therefore, IL-12 is sufficient for host resistance to *T. b. brucei* infection in the absence of IL-18 but not vice versa.

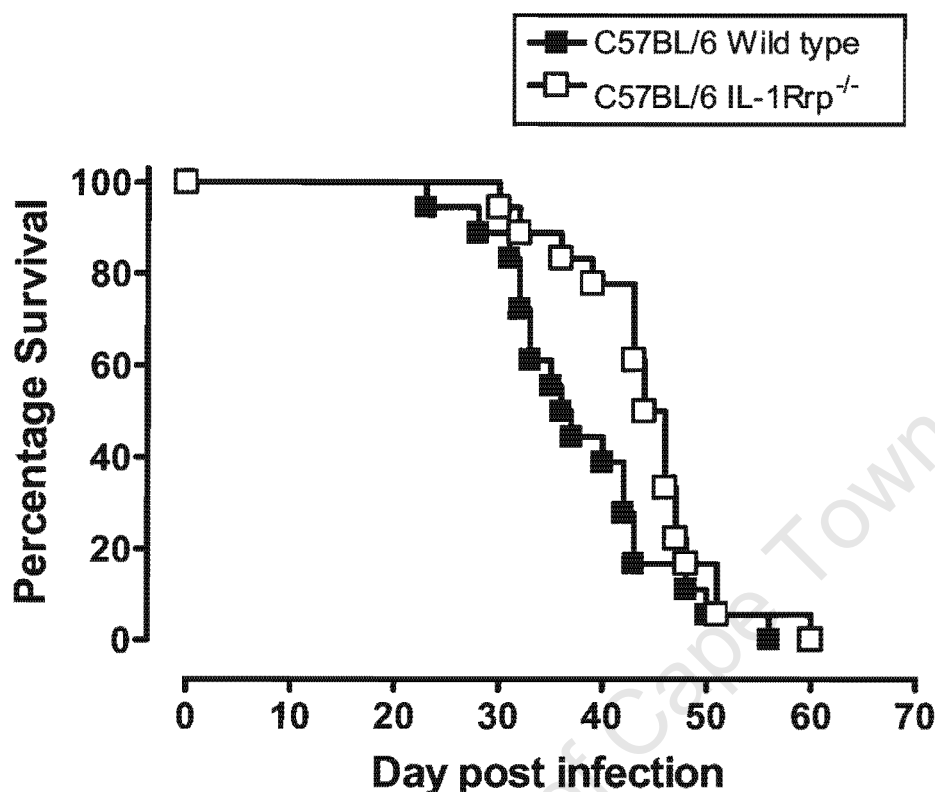


FIGURE 8. Interleukin-18 signaling has a slightly detrimental effect on survival during infection with *T. b. brucei*. Mortality profiles of infected C57BL/6 wild type (■) and C57BL/6 IL-1Rrp^{-/-} (□) mice, are shown from two pooled experiments. Infectious dose was 5×10^3 trypanosomes per mouse injected intraperitoneally and were subsequently monitored daily for survival. The median survival time was; 36.5 days for wild type (n=18) and 45.0 days for IL-1Rrp^{-/-} (n=18) mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality.

Interleukin-4 and -13 do not play a role in resistance during *T. b. brucei* infection.

There have been conflicting reports as to the role of IL-4 in resistance or susceptibility to different *Trypanosoma* species (Bakhiet, Jansson et al. 1996) (Inoue, Inoue et al. 1999) (Schopf, Filutowicz et al. 1998). Using an attenuated *T. b. brucei* parasite, a Type 2 cytokine response was measured in infected wild type mice which displayed increased survival compared to mice infected with wild type *T. b. brucei* parasites. This data suggested that a Th2 response decreased immunosuppression and was therefore protective (Namangala, de Baetselier et al. 2000). During infection with *T. b. brucei*, production of IL-4 was detected (Figure 4). The roles of IL-4 and IL-13, (IL-13 is known to have overlapping functions with IL-4) (Brombacher 2000) (Muller, Stenzel et al. 2007), were investigated using IL-4 and IL-4R α deficient mouse strains.

Although this was a pilot study performed once, IL-4 (Figure 9A) and IL-4/13 (Figure 9B) were not found to play a role in survival in *T. b. brucei* infection. Median survival times in BALB/c wild type, BALB/c IL-4^{-/-} and BALB/c IL-4R α ^{-/-} mice were 58, 58 and 64.5 days, respectively. No significant differences in survival were detected between BALB/c IL-4^{-/-} and BALB/c IL-4R α ^{-/-} mice.

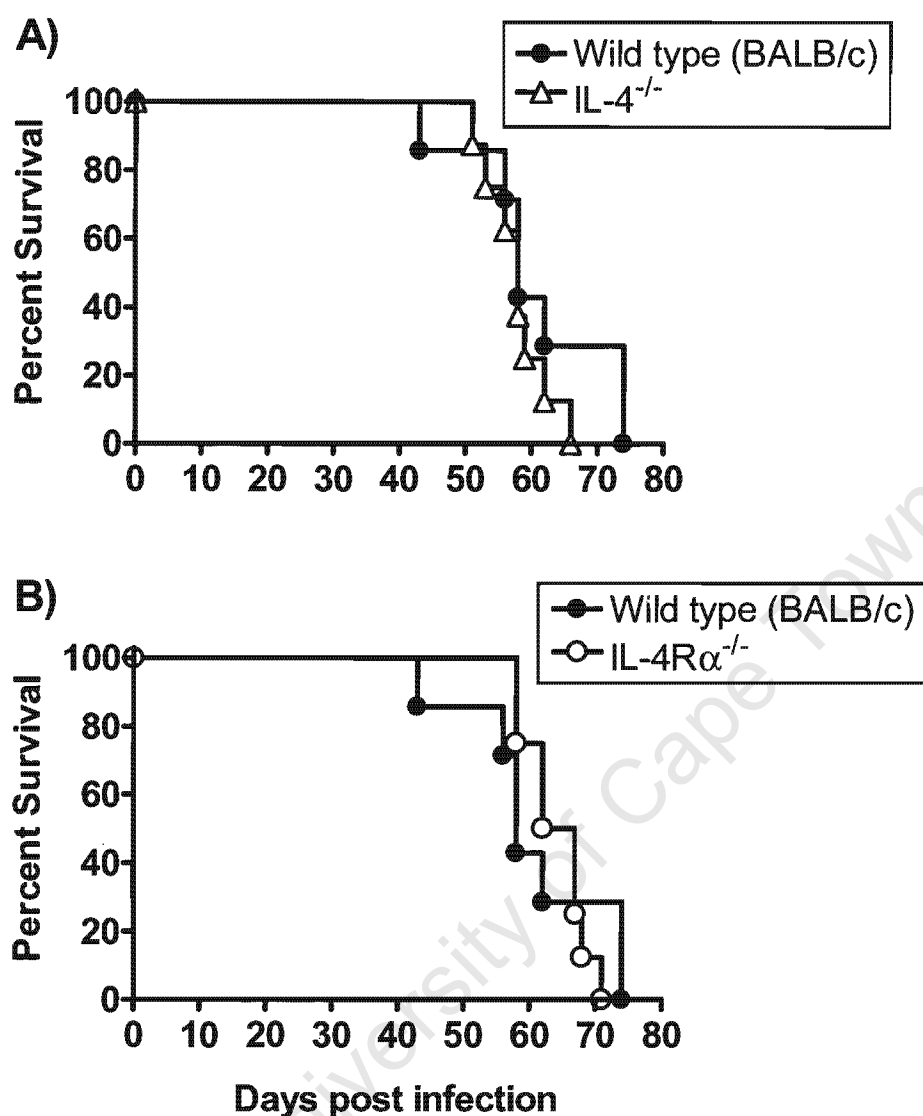


FIGURE 9. Interleukin-4 and -13 do not play a role in resistance to infection with *T. b. brucei*. Mortality profiles of infected BALB/c wild type (●), BALB/c IL-4^{-/-} (△) ($P > 0.05$) (A) and BALB/c IL-4Rα^{-/-} (○) ($P > 0.05$) (B) mice are shown. Infectious dose was 5×10^3 trypanosomes per mouse intraperitoneally and were subsequently monitored daily for survival. The median survival time was; 58 days for wild type ($n=7$), 58 days for IL-4^{-/-} ($n=8$) and 64.5 days for IL-4Rα^{-/-} ($n=8$) mice. No significant differences were found between the different gene deficient mouse groups or between gene deficient and wild type mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality.

DISCUSSION

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mouse strains showed significantly reduced survival compared to wild type controls in *T. b. brucei* infection. This outcome was in agreement with previous results showing an association with lower IL-12p70 levels in susceptible wild type mouse strains during the initial stages of infection compared to more resistant strains (Hertz, Filutowicz et al. 1998). Between the three IL-12 deficient mouse strains, no differences in survival rates were observed suggesting that IL-12p70 is the main protective factor for survival with no major contribution from other IL-12 family members, namely IL-23 or the IL-12p80 homodimer. Interleukin-12 deficient mice survived several trypanosome waves. Therefore residual protection occurred suggesting that IL-12 played a more striking role during the established disease. As IFN- γ is an important factor for survival in *T. b. brucei* infections (Hertz, Filutowicz et al. 1998), the plasma levels of IFN- γ was determined during infection. In wild type mice IFN- γ was present during the first parasite wave with striking reduced IFN- γ in all IL-12 deficient mouse strains. These results demonstrated that residual IL-12-independent IFN- γ production was induced during infection and that the observed limited Type 1 response allowed residual protective immunity.

Interleukin-12 independent induction of IFN- γ may also include a direct stimulus by the trypanosomes themselves. Trypanosome derived lymphocyte triggering factor (TLTF) has been found to induce the production of IFN- γ by binding to CD8⁺ cells in *T. b. brucei* (Olsson, Bakhiet et al. 1993) infections and therefore may have contributed to residual resistance in the absence of IL-12. In addition, work describing IL-12-independent IFN- γ production with residual Th1 responses have been demonstrated during *Listeria monocytogenes* (Brombacher, Dorfmueller et al. 1999), *Mycobacterium BCG* (Holscher, Atkinson et al. 2001) and *Leishmania major* (Mattner, Magram et al. 1996) infections. To evaluate the contribution of IFN- γ to resistance, IFN- γ R deficient mice were infected and were found to die shortly after the first parasite wave. Although the IFN- γ R deficient

mice used in this doctoral study were on a different genetic background compared to IL-12 deficient mice, these results support the conclusions of Hertz et al., 1998 which were based on experiments using IFN- γ deficient mice on a C57BL/6 background. These results clearly highlighted the crucial importance of IFN- γ responsiveness during acute infection with *T. b. brucei*.

Although IL-12 deficient mice did have significantly reduced rates of parasitemia clearance after the 1st peak compared to wild type mice, they were as effective in clearance of the 1st peak as control mice. Interferon- γ and TNF cytokine plasma levels in all groups of IL-12 deficient mice were significantly attenuated during the initial parasitemic peak. These data together with the drastically reduced capacity of IFN- γ R and TNF deficient mice to control parasitemia (Magez, Radwanska et al. 1999) during the first peak, suggested that IL-12 is important for optimal production of TNF and IFN- γ and but plays a more dominant role in parasitemia control after the 2nd peak. Optimal production of TNF and IFN- γ by IL-12 may be more important after the 2nd parasitemic peak. Tumor necrosis factor has been found to have dual effects in *T. b. brucei* infections, such as trypanolytic effects (Lucas, Magez et al. 1994; Magez, Geuskens et al. 1997) reducing parasite burden in case of *T. b. brucei* AnTat 1.1 infections (Lucas, Magez et al. 1993; Magez, Lucas et al. 1993; Magez, Radwanska et al. 1999) and causing immunopathological effects in the host (Magez, Radwanska et al. 1999). Although TNF was reduced in IL-12 deficient mice, no decrease in pathology was detected in spleen and liver sections in *T. b. brucei* (data not shown).

Additionally, B-cell responses are necessary for the elimination of vascular bound parasites. In particular IgM and IgG2a are quoted as the main antibodies necessary for clearance in *T. b. brucei* (Magez et al., manuscript in preparation) (Dempsey and Mansfield 1983). Both VSG specific antibodies in IL-12 deficient mice were present in plasma (mouse anti-VSG IgG2c measured by anti-mouse IgG2a) but at moderately reduced levels compared to wild type mice and thus may not be the overall mechanism of increased parasite burden. Thus the role of IL-12 in *T. b. brucei* seems to be limited to the

more chronic stage of disease and in promoting the production of TNF and IFN- γ necessary to control parasitemia.

Natural killer cells, which are activated by both IL-12 and IL-18 (Namangala, Sugimoto et al. 2007) , are thought to be necessary for resistance during the initial infection. IL-18 may therefore effectively drive the Th1 response in the absence of IL-12. In this regard, data from IL-1Rrp gene deficient mice suggested that IL-12 was the most important factor for resistance as in IL-1Rrp gene deficient mice since no major differences in survival were detected. In contrast to the aforementioned Type 1 cytokines, studies have found conflicting results concerning the role of IL-4 in resistance or susceptibility during infection with various *T. brucei* subspecies (Bakhiet, Jansson et al. 1996) (Inoue, Inoue et al. 1999) (Schopf, Filutowicz et al. 1998). Although plasma IL-4 was detected in all mouse strains during the chronic stage of infection, this cytokine as well as IL-13 did not seem to play a significant role in survival in *T. b. brucei* infection as demonstrated in IL-4 and IL-4R α gene deficient mice.

A recent paper by Drennan et al., 2005 found MyD88 signaling to be crucial for resistance, with a role for TLR9 in this immune pathway (Drennan, Stijlemans et al. 2005). The TLR9 and IL-12 deficient mice share many phenotypic characteristics in their response to *T. b. brucei* infection, including; effective 1st peak parasitemia control but increased parasitemia during the chronic stages, reduced IFN- γ production but normal IgG2c responses during the chronic stages of disease compared to wild type mice. Bone marrow-derived macrophages from TLR9 deficient mice also displayed severely reduced TNF and IL-12p40 in response to *T. b. brucei* genomic DNA. Therefore, IL-12 may be involved in the immunological pathway whereby TLR9 present on macrophages respond to *T. b. brucei* genomic DNA released following the 1st peak of parasitemia, and upon IL-12 secretion stimulate PBMCs to secrete IFN- γ .

With the presence of TLR9 on B cells, a role for IL-12 here may also be considered. However with only a slight reduction in IgG2c responses during the chronic stages as reported in this paper as well as in the paper by Drennan et al., 2005, it is more likely that

other factors play a more significant role in proliferation and differentiation of these cells. In contrast to IL-12 deficient mice, TLR 9 deficient mice produced more IFN- γ during the chronic stages of *T. b. brucei* infection. Reduced IFN- γ production may explain the increased susceptibility in IL-12 deficient mice compared to TLR9 deficient mice, which showed similar resistance as wild type mice. The function of IL-12 may thus be to link TLR9 signaling in macrophages during the chronic stage of *T. b. brucei* infection and the up-regulation of IFN- γ secretion in PBMCs necessary for effective parasitemia control and survival.

In conclusion the data presented in this chapter showed that IL-12p70, but not the IL-12p80 homodimer or IL-23, was necessary for optimal resistance to *T. b. brucei* infection in terms of mortality and parasitemia control. The resistance attributed to IL-12 was mediated through IFN- γ , the production of which was significantly decreased in the absence of IL-12. Furthermore a role for IL-12 in parasitemia control during the chronic stage of *T. b. brucei* infection was shown where the presence of IL-12 was needed for optimal TNF and IFN- γ production reducing parasitemia burden.

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CHAPTER III

The role of IL-12 and associated immunological mediators in resistance to *T. evansi* infection

ABSTRACT

African trypanosomiasis encompasses diseases caused by pathogenic trypanosomes, infecting both humans and animals. *Trypanosoma evansi* is an economically devastating pathogen present not only in Africa and South America but occurring in Asian countries as well. Recently the disease has been isolated in an infected human. Immunologically, not much is known about *T. evansi* infections in animals, however recent work has elucidated some of the mechanisms of trypanotolerance.

In this chapter, the possible role of members of the IL-12 family during infection with *T. evansi* in mice was dissected. Interleukin-12p35^{-/-}, IL-12p40^{-/-} or IL-12p35^{-/-}/p40^{-/-} mice were susceptible to *T. evansi*, as demonstrated by increased mortality compared to wild type C57BL/6 mice. The different IL-12 deficient mouse strains showed similar mortality kinetics, suggesting that IL-12p70 but not the IL-12p80 homodimer or IL-23 plays a crucial role in survival. While plasma levels of IgM and IgG2c were similar between IL-12 deficient mice and wild type mice, IFN- γ production, during early and late time points, was severely impaired in all IL-12 deficient mouse strains, demonstrating an IL-12p70-dependent mechanism for IFN- γ production. As IFN- γ R^{-/-} mice were also highly susceptible to *T. evansi*, as found in *T. b. brucei*, IL-12p70-dependent IFN- γ production seems to be the important mechanism involved in resistance against this pathogen. In addition, other Th1 related trypanotolerance factors were found to be important for host resistance to infection with *T. evansi*.

INTRODUCTION

General background on *T. evansi* infections

Trypanosoma evansi is a pathogenic haemoflagellate of significant economic importance. This parasite, which is present almost worldwide, occurs in Africa, Asia and South America, where it is known to cause epidemics. Blood-sucking insects of the genera *Stomoxys*, *Tabanus*, *Atylotus* and *Lyperosia* are known to mechanically transmit *T. evansi*. This pathogenic parasite, which has widest host distribution compared to other species of trypanosome, infects many animals, including; domestic, wild and laboratory mammals (Hoare 1972). The loss of maxicircle kinetoplast DNA (kDNA), which has only occurred in *T. evansi*, is suggested as the mechanism facilitating its range of potential vectors and consequently increasing the variety of hosts (Borst, Fase-Fowler et al. 1987) (Lun and Desser 1995). *Trypanosoma evansi* is considered to be a mutated form of *T. b. brucei*, as they are morphologically and biochemically similar and to have originated from the northern edge of the African tsetse fly belt (Hoare 1972). In contrast to *T. b. brucei*, *T. evansi* is thought to have only a limited capacity for antigenic variation which may increase the possibility for using immuno-prophylactics (Singh, Singh et al. 1995) (Jones and McKinnell 1985).

Host immune responses to infection with *T. evansi*

The immunological responses of the host to infection with *T. b. brucei* as well as *T. congolense* have been widely researched. In contrast, in *T. evansi* few studies have been performed and these did not tackle the immunologically-related mechanisms of host survival and parasitemia control (Onah, Hopkins et al. 1998) (Onah, Hopkins et al. 1998). Furthermore, these studies used different *T. evansi* isolates with diverse pathogenesis in animal models of varied genetic backgrounds thus making comparisons problematic (Luckins, Gray et al. 1978) (Luckins, Boid et al. 1979) (de Oliveira, Sogayar et al. 1989) (Aquino, Machado et al. 1999) (Queiroz, Legey et al. 2001) (Queiroz, Cabello et al. 2000). However, trypanosome mediated immuno-suppression, has been identified in *T.*

evansi as similarly found in *T. b. brucei* and *T. congolense* (Onah, Hopkins et al. 1998) (Holland, My et al. 2001).

Recently, using a compendium of different gene deficient mice, the humoral response was identified as being responsible for host trypanotolerance, with IgM found to be critical for parasitemia control and survival (Baral, De Baetselier et al. 2007). These authors also reported increased levels of IFN- γ , TNF and NO in wild type mice during infection, but infections in gene deficient mice demonstrated that these factors were dispensable in terms of survival and parasitemia control. However, as found in *T. b. brucei* infected mice, TNF was found to play a role in the induction of anemia in *T. evansi* infected mice.

AIMS

It has been previously demonstrated that Th1 immune responses are required for trypanotolerance (Schleifer, Filutowicz et al. 1993). Since IL-12 plays a pivotal role in skewing T-cell reactivity (Brombacher, Kastelein et al. 2003) (Trinchieri 1993), the aims of this chapter were to dissect the roles of IL-12, IL-23 and the IL-12p80 homodimer in *T. evansi* infection using IL-12p35, IL-12p40 and IL-12p35/p40 gene deficient mice. In addition, the role of other cytokines and immune related factors involved in trypanotolerance were investigated using gene deficient mice. However, it should be mentioned that this doctoral research was initiated prior to the publication of Baral et al. showing that IgM, but not Type 1 cytokine responses, were necessary for resistance to *T. evansi* infection (Baral, De Baetselier et al. 2007).

MATERIALS & METHODS

(Refer to Material & Methods section in Chapter II)

RESULTS

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice infected with *T. evansi* display reduced survival compared to wild type mice.

In order to determine the roles of IL-12p70 (p35/p40), IL-23 (p19/p40) and IL-12p80 homodimer during infection with trypanosomiasis, mice deficient in either one or both p35 and p40 genes were infected with *T. evansi*. Survival of *T. evansi* infected mice was significantly decreased in all deficient mice compared to infected wild type controls (Table 1 and Figure 1). No mortality differences were observed between the different IL-12 deficient mouse groups. From the mortality graph the role of IL-12 in resistance is more apparent during the chronic stage from day 25 post infection. From this point, wild type mice started showing a decreased rate of mortality compared to the IL-12 deficient mice. In contrast to infection with *T. b. brucei*, where mortality in both wild type and IL-12 deficient mice began from day 24 post infection, mortality in *T. evansi* infected mice began rapidly after infection. In summary, IL-12p70 is necessary for trypanotolerance to infection with *T. evansi* with no role for IL-23 or the IL-12p80 homodimer.

TABLE 1. Interleukin-12 deficient mice infected with *T. evansi* show increased mortality compared to wild type mice.

Parasite species ^a	Mouse Strain	Mean survival (SD)	No. of mice/group ^b	P value compared to Wild Type mice ^c
<i>T. evansi</i>	Wild Type	47.0 ± 24.1	33	
	IL-12p35 ^{-/-} /p40 ^{-/-}	31.4 ± 14.3	35	<i>P</i> < 0.001
	IL-12p40 ^{-/-}	31.1 ± 13.1	21	<i>P</i> < 0.001
	IL-12p35 ^{-/-}	30.3 ± 20.6	34	<i>P</i> < 0.01

^a Mice were infected with 1x10³ trypanosomes *T. evansi* intraperitoneally and monitored daily for survival.

^b Three independent experiments were combined.

^c Tests for significance were performed using the Kaplan-Meier test.

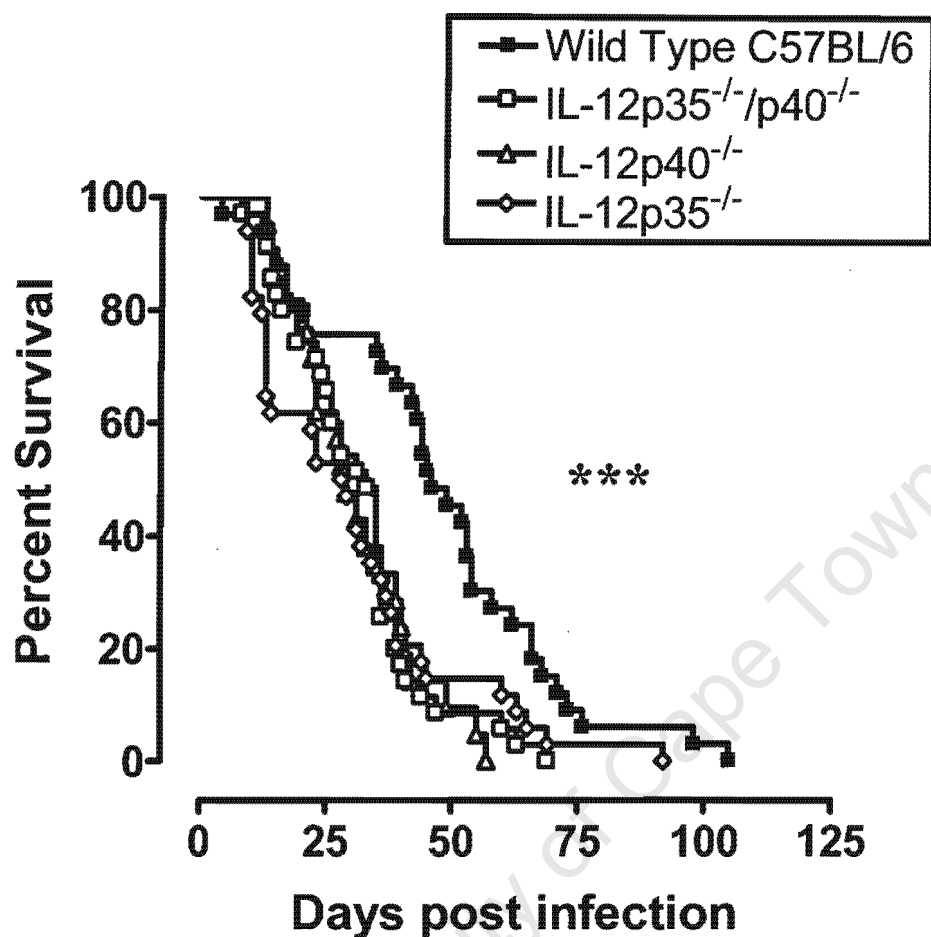


FIGURE 1. Interleukin-12 deficiency results in increased mortality to infection with *T. evansi*. Mortality profiles of infected C57BL/6 wild type (■), IL-12p35^{-/-}/p40^{-/-} (□) ($p < 0.0001$), IL-12p40^{-/-} (△) ($p < 0.0001$), and IL-12p35^{-/-} (◇) ($p < 0.0001$) mice are shown from 3 independent pooled experiments. Infectious dose was 1×10^3 trypanosomes per mouse and were subsequently monitored daily for survival. The mean survival time was; 47 days for wild type ($n=33$), 31.4 days for IL-12p35^{-/-}/p40^{-/-} ($n=35$), 31.1 days for IL-12p40^{-/-} ($n=21$) and 30.3 days for IL-12p35^{-/-} ($n=34$) mice. No significant differences were found between IL-12 gene deficient mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality and are indicated by; ***, $P < 0.001$.

Interleukin-12 is necessary for optimal clearance of parasitemia after each successive wave during T. evansi infection

Parasite burden during infection was measured to determine the roles of IL-12 family members in the control of parasitemia due to infection with *T. evansi*. The parasitemia profile of *T. evansi* was markedly different to that observed in *T. b. brucei* infected C57BL/6 wild type mice (Chapter II, Figure 2). In contrast to *T. b. brucei* infection, where loss of parasitemia control correlated with mortality in IL-12 deficient mice, both wild type and IL-12 deficient mice displayed high parasite burden during *T. evansi* infection. However, wild type mice significantly reduced their parasitemia at days 12 and 36 forming several waves of parasitemia as apposed to all IL-12 deficient mice which failed to reduce parasite burden (Figure 2). At day 23, following the second parasitemic wave, partial clearance in wild type mice was observed as compared to IL-12 deficient mice. However the reduced levels did not reach significance. Parasitemia clearance by wild type mice during the progression of the disease became less evident, most likely due to the erosion of the hosts' immune response over time. No significant differences in parasitemia clearance were observed between the IL-12 deficient strains. These results therefore suggested that blood parasite clearance after each successive wave of parasites is to a degree controlled by IL-12p70, but not by IL-23 or the IL-12p80 homodimer in *T. evansi* infection.

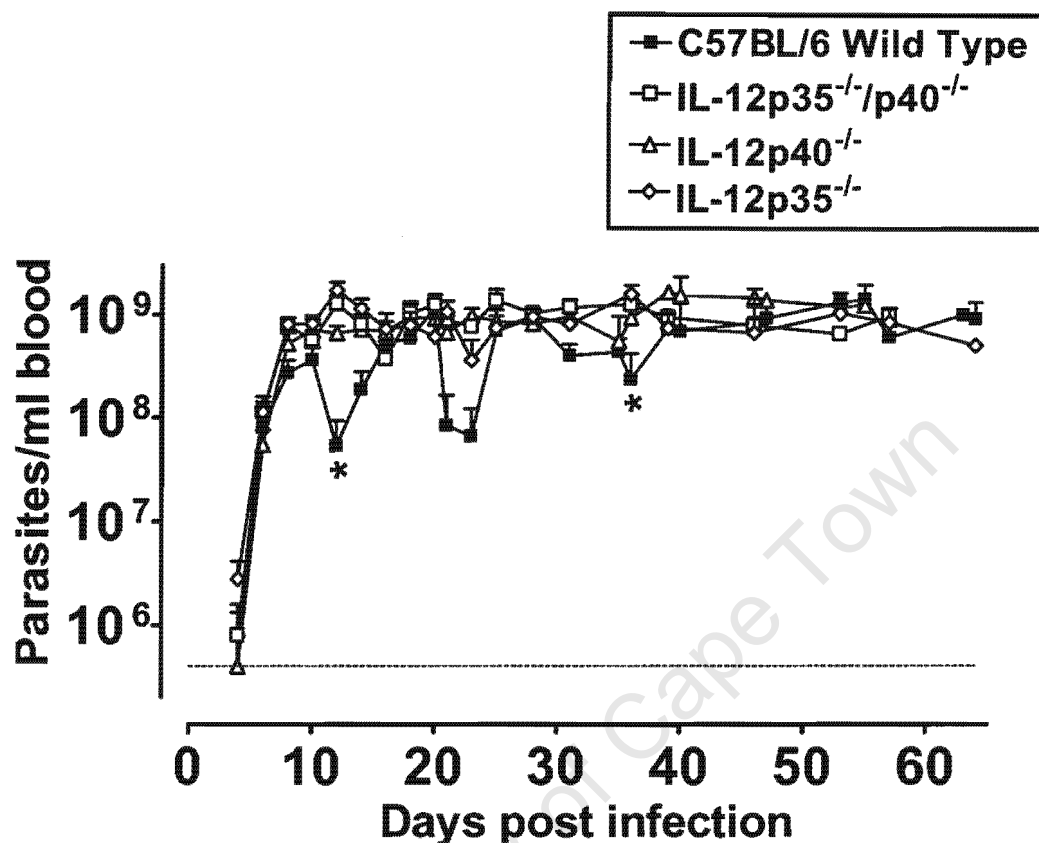


FIGURE 2. Interleukin-12 is necessary for optimal parasitemia control in *T. evansi* infected mice. Parasitemia profiles of *T. evansi* infected C57BL/6 wild type (■), IL-12p35^{-/-}/p40^{-/-} (□), IL-12p40^{-/-} (△), and IL-12p35^{-/-} (◇) mice are shown. *Trypanosoma evansi* infected mice were infected intraperitoneally with 1000 trypanosomes, thereafter parasitemia was determined from peripheral blood obtained from tail snips with a lower detection limit of 2.7×10^5 parasites/ml (dotted line). Data points are the mean from 3 pooled experiments of 5-10 individual mice per group. Inferred data points appearing below the detection limit are denoted as; ϕ . Statistical significance between infected wild type and IL-12 deficient mice was determined by one-way ANOVA (Dunnett post test) and are indicated by; *, $P < 0.05$.

Interleukin-12 does not play a role in anemia development in T. evansi infected mice.

The development of anemia during *T. evansi* infection in various animal models has been documented (Ngeranwa, Gathumbi et al. 1993) (Jatkar and Purohit 1971) (Stijlemans, Baral et al. 2007) (de Menezes, Queiroz et al. 2004) and the role of cytokines in its development has recently been determined. Using gene deficient mice, it was found that TNF and TNF receptor 2 were responsible for the induction of late-stage anemia (Baral, De Baetselier et al. 2007).

As shown in figure 3, a rapid decrease in red blood cell concentration occurred during the 1st parasitemic peak and later recovered at the emergence of the 2nd peak. As the infection entered the chronic stage, fluctuation in the extent of anemia became less. Overall, *T. evansi* infected IL-12 deficient mice displayed a similar anemia profile as that of wild type controls. Therefore, IL-12 family members did not appear to play a role in the induction of anemia in *T. evansi* infections.

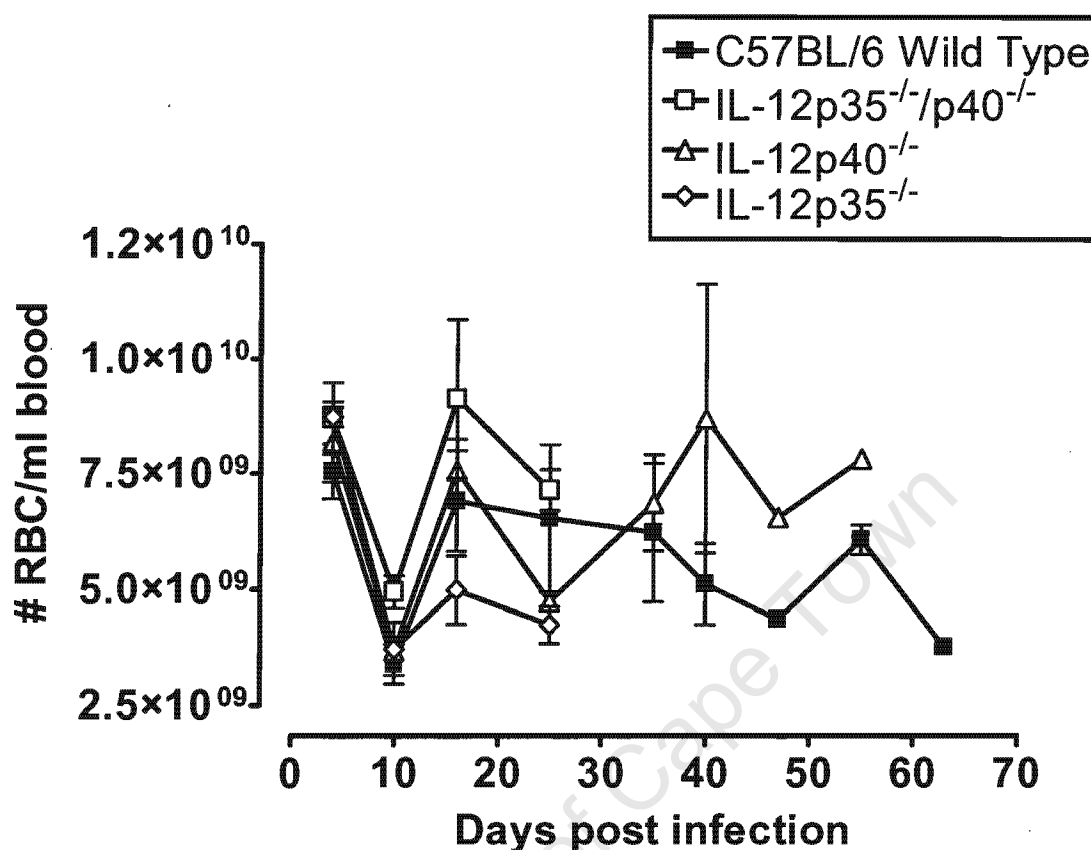


FIGURE 3. Interleukin-12 does not play a role in the induction of anemia in *T. evansi* infected mice. Red blood cell concentrations of *T. evansi* infected C57BL/6 wild type (■), IL-12p35^{-/-}/p40^{-/-} (□), IL-12p40^{-/-} (△), and IL-12p35^{-/-} (◇) mice are shown. *Trypanosoma evansi* infected mice were infected intraperitoneally with 1×10^3 trypanosomes. Thereafter red blood cell counts were determined from peripheral blood obtained from tail snips with a lower detection limit of 1.6×10^7 RBC/ml. Data points are the mean from of 5 individual mice per group. Statistical significance between infected wild type and IL-12 gene deficient mice was determined by one-way ANOVA (Dunnnett post test).

Interleukin-12 is necessary for optimal IFN- γ production during *T. evansi* infection.

Interferon- γ is critical for resistance and parasitemia control in African Trypanosomiasis infection (Hertz, Filutowicz et al. 1998) (Magez, Radwanska et al. 2006). Since IL-12 is known to initiate IFN- γ production (Trinchieri 1994), the plasma levels of IFN- γ during *T. evansi* infection were determined. Plasma was taken from all infected mouse groups during the 1st parasitemia peak at day 8, and post 2nd parasitemia peak at day 28 (Figure 4).

Trypanosoma evansi infection in wild type mice induced strong systemic IFN- γ and TNF production, as demonstrated in another study (Baral, De Baetselier et al. 2007). *Trypanosoma evansi* infection in IL-12 deficient mice resulted in impaired IFN- γ responses, as observed in *T. b. brucei* infection (Figure 4A). However, in contrast to *T. b. brucei* infection, *T. evansi* infected IL-12 deficient mouse strains produced substantial amounts of TNF with only a slight reduction observed (Figure 4B). These results suggested that during *T. evansi* infection, optimal IFN- γ production is dependent on IL-12p70, but that low levels of IFN- γ production can be independently induced from signal transduction via p35 and p40 subunits. In addition, plasma IL-4 was induced during infection with *T. evansi*, however levels were similar between IL-12 gene deficient and wild type mice (Figure 4C). Levels of IL-12 were undetectable in plasma at day 8 and 28 post infection in wild-type and IL-12 deficient mice (data not shown)

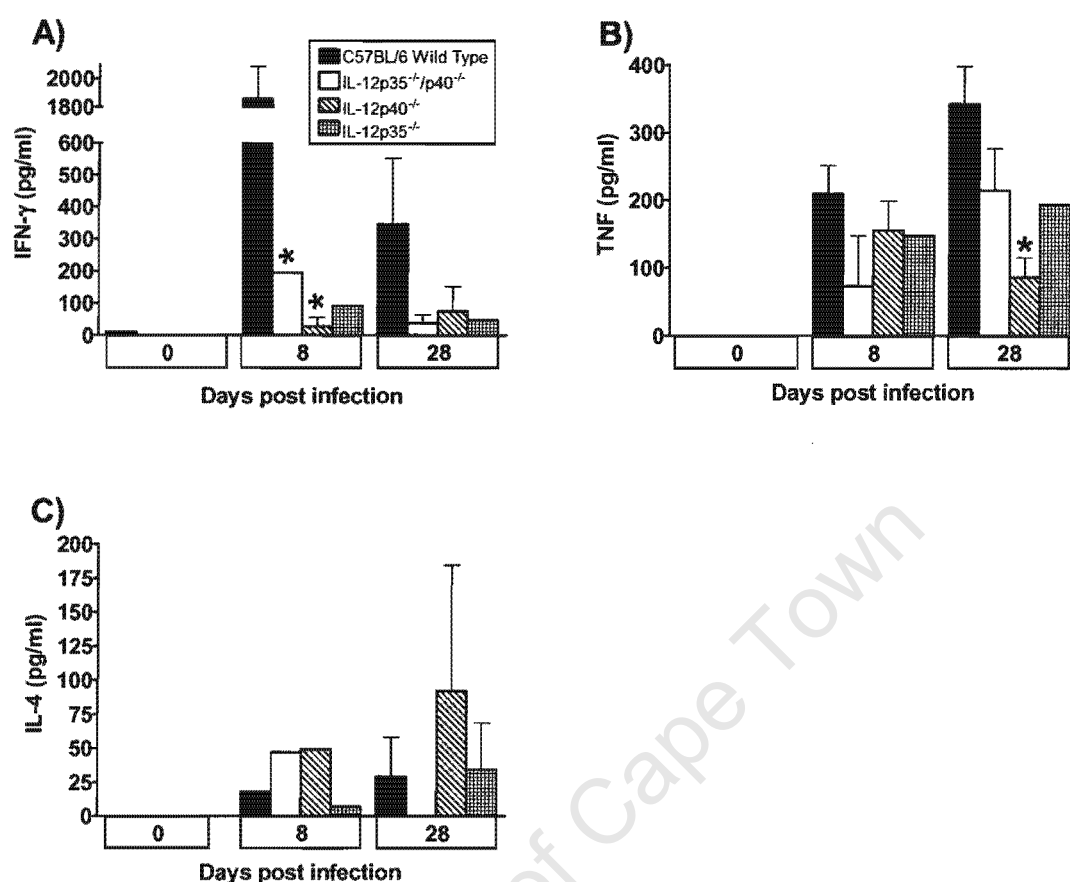


FIGURE 4. Interleukin-12 is necessary for optimal IFN- γ responses to infection with *T. evansi*. Interferon- γ (A), TNF (B) and IL-4 (C) plasma levels were measured by ELISA in C57BL/6 wild type (Filled bars), IL-12p35^{-/-}/p40^{-/-} (Open bars), IL-12p40^{-/-} (Striped bars) and IL-12p35^{-/-} (Hatched bars) mice infected with 1000 *T. evansi* trypanosomes. Time points analyzed were at days 0 (controls), 8 (1st peak) and day 28 (post 2nd peak). Results are representative of two independent experiments. However, TNF levels in IL-12p40 deficient mice were not consistently significantly reduced at day 28 post infection. Data expressed as means ($\pm$ SEM) from 2-3 individual mice. Statistical significance between infected wild type and IL-12 gene deficient mice was determined by unpaired Student's t-test are indicated by; *, $P < 0.05$.

Immunoglobulin M responses are normal in IL-12 deficient mice compared to wild type mice.

Anti-VSG antibody responses, namely IgM, have been shown to play a key role in parasitemia control and host survival in *T. evansi* infections (Baral, De Baetselier et al. 2007). The anti-VSG specific antibody responses in wild type and IL-12 deficient mice were therefore measured to determine if increased mortality or parasitemia in IL-12 deficient mice correlated with decreased antibody responses.

Immunoglobulin M levels at both day 7 or at day 28 post infection were similar in IL-12 gene deficient and wild type mice (Figure 5A). Both Ig2c (measured using anti-mouse IgG2a) and IgG2b levels were similar between wild type and IL-12 gene deficient mice, with considerably increased plasma levels present at day 28 compared to day 7 post infection in all mouse groups (Figure 5B and 5C).

Immunoglobulin-1 levels were slightly higher in all IL-12 mice compared to wild type mice at day 28 post infection (Figure 6A). Levels of IgG3 were slightly increased in wild type and IL-12 gene deficient mice at day 28 post infection compared to day 8 (Figure 6B).. However, high variation in IgG3 levels were found in IL-12p70 deficient mice at day 28 (Figure 6B).

In summary, IL-12 deficiency did not have a significant affect on anti-VSG antibody levels, the most important of which is IgM that is necessary for parasitemia control and survival (Baral, De Baetselier et al. 2007).

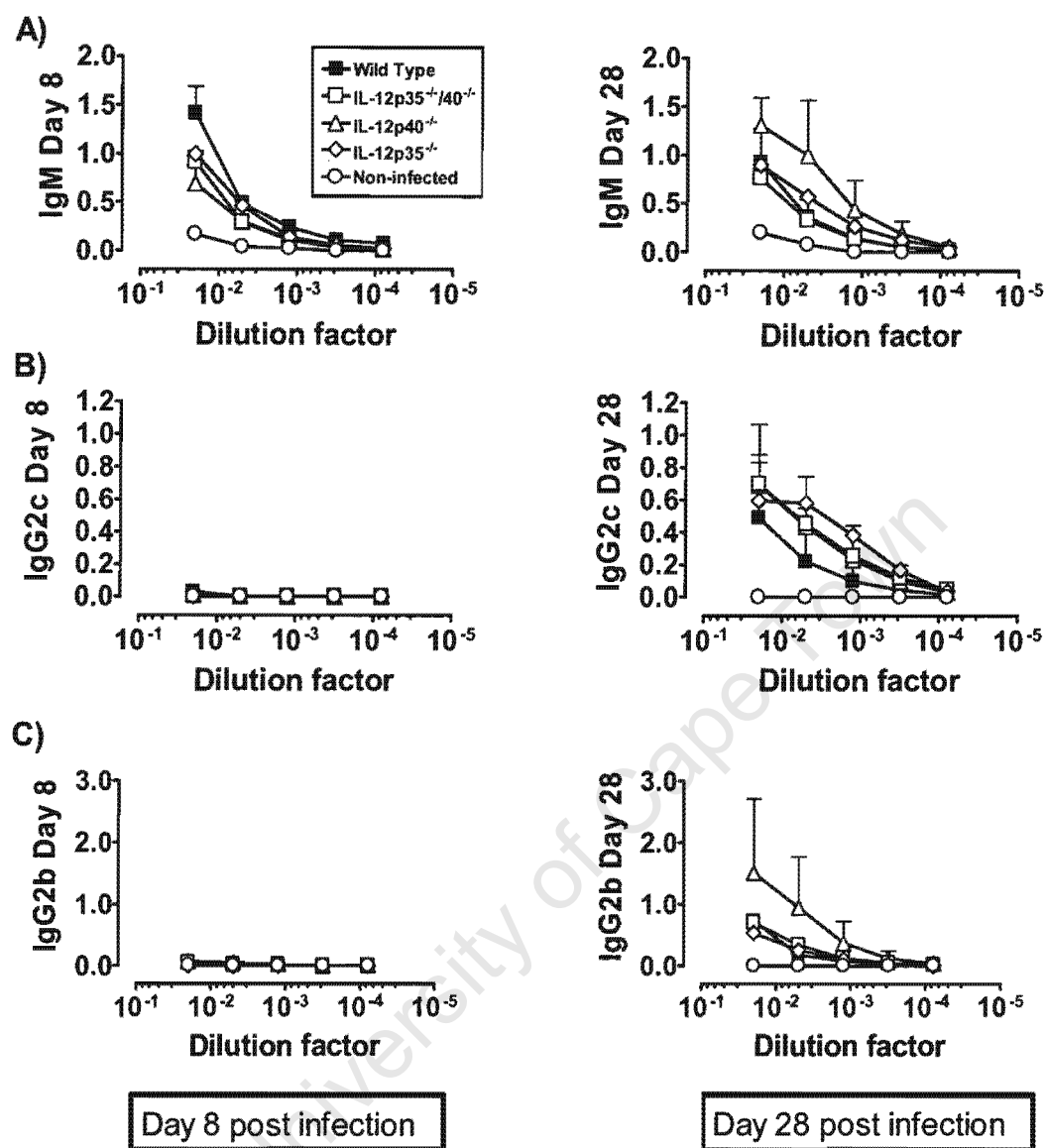


FIGURE 5. Immunoglobulin responses, namely IgM, IgG2c and IgG2b, are intact in IL-12 deficient mice compared to wild type mice. IgM (A), IgG2c (B) and IgG2b (C) plasma antibody titers from *T. evansi* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice are shown. Day 8 and 28 post infection plasma antibody titers from infected mice are represented. Pre-immune serum was used as controls (○). Data points represent mean and $\pm$ SEM from plasma of 2-3 mice. Statistical significance tests between wild type and IL-12 deficient mouse groups were determined by Student's t-test.

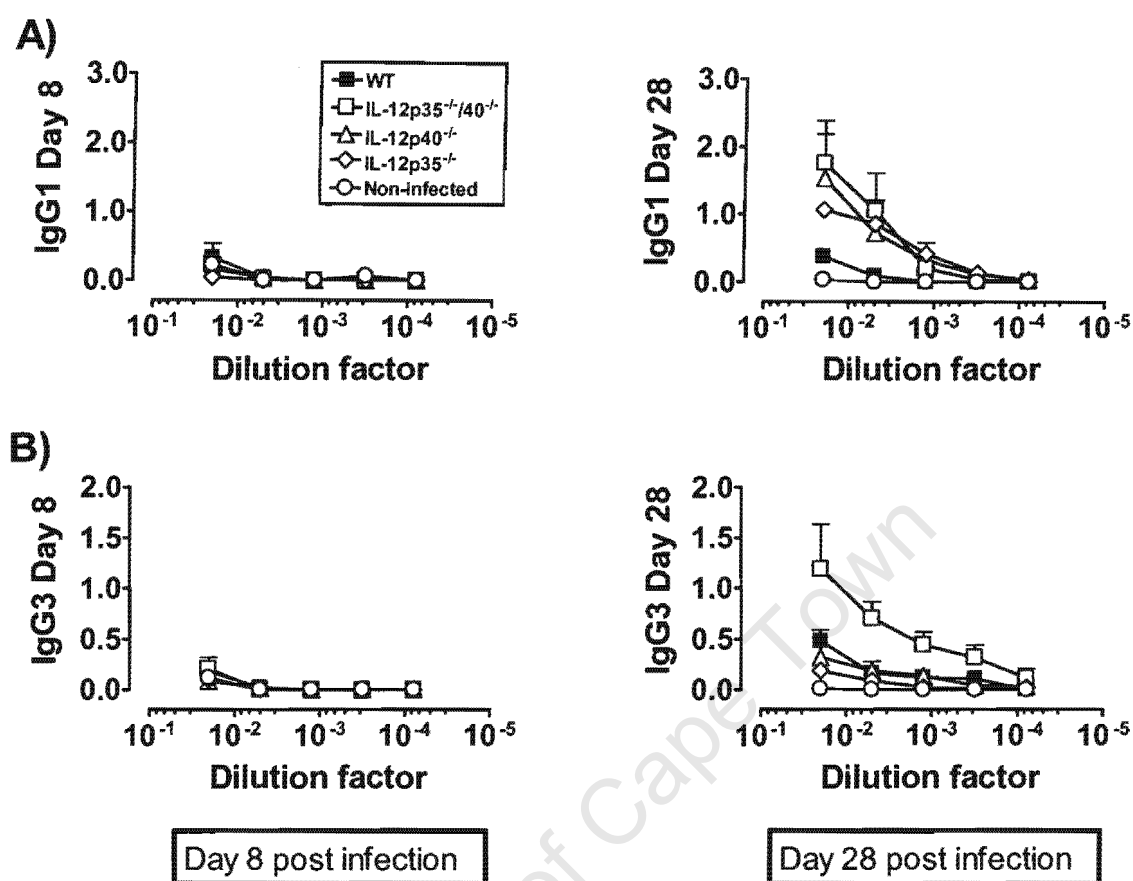


FIGURE 6. A slightly increased IgG1 response in IL-12 deficient mice compared to wild type control mice. IgG1 (A) and IgG3 (B) plasma antibody titers from *T. evansi* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice. Day 8 and 28 post infection plasma antibody titers from infected mice are represented. Pre-immune serum was used as controls (○). Data points represent mean and $\pm$ SEM from plasma of 3 mice. Statistical significance tests between wild type and IL-12 deficient mouse groups were determined by Student's t-test.

Interleukin-12 plays no major role in the development of pathological lesions in the liver during *T. evansi* infection.

Histological analysis of spleen and liver sections was performed using light microscopy to determine the extent of pathology due to a lack of IL-12, IL-23 and IL-12p80 homodimer functioning in *T. evansi* infection. In *T. b. brucei* infection both the liver (Holmes, MacAskill et al. 1979) (Macaskill, Holmes et al. 1980) and spleen are known to be involved in the elimination of blood stream parasites, with a further role for the spleen in survival (Anosa 1980). However, in *T. evansi* infection, similar evaluations of these organs have not been performed in published data. Studies have concentrated on characterizing pathological lesions caused by infection with *T. evansi* but have been carried out in diverse animal models (Dargantes, Campbell et al. 2005) (Biswas, Choudhury et al. 2001) (Aquino, Machado et al. 1999) (Gutierrez, Corbera et al. 2006).

No differences in the extent of hepatomegaly were found between wild type and IL-12 deficient mouse groups infected with *T. evansi* (data not shown). At days 8 (data not shown) and 28 post infection pathological lesions in the liver were noted in all groups of mice studied increasing together with the progression of disease. However, at days 8 and 28 post infection, no differences in pathological lesions were observed between wild type and IL-12 deficient mouse groups. At day 28 post infection, wild type and IL-12 deficient mouse groups displayed dispersed mononuclear cellular infiltrates and many focal mononuclear cellular infiltrates and in the sinusoidal spaces of the liver parenchyma (Figure 7). Both IL-12 deficient and wild type mice had similar cellular compositions of focal mononuclear cell infiltrates, consisting of activated macrophages with scattered lymphocytes and neutrophils. These results therefore suggested that IL-12 does not play a role in the induction of pathological lesions in the liver during *T. evansi* infection. However, some, but not all, IL-12p40 gene deficient mice, displayed severely enlarged sinusoidal spaces together with greatly disrupted liver structure compared to the other IL-12 deficient mouse groups and wild type controls. An analysis of a larger group of IL-12p40 deficient mice would be needed to determine if this trend is significant.

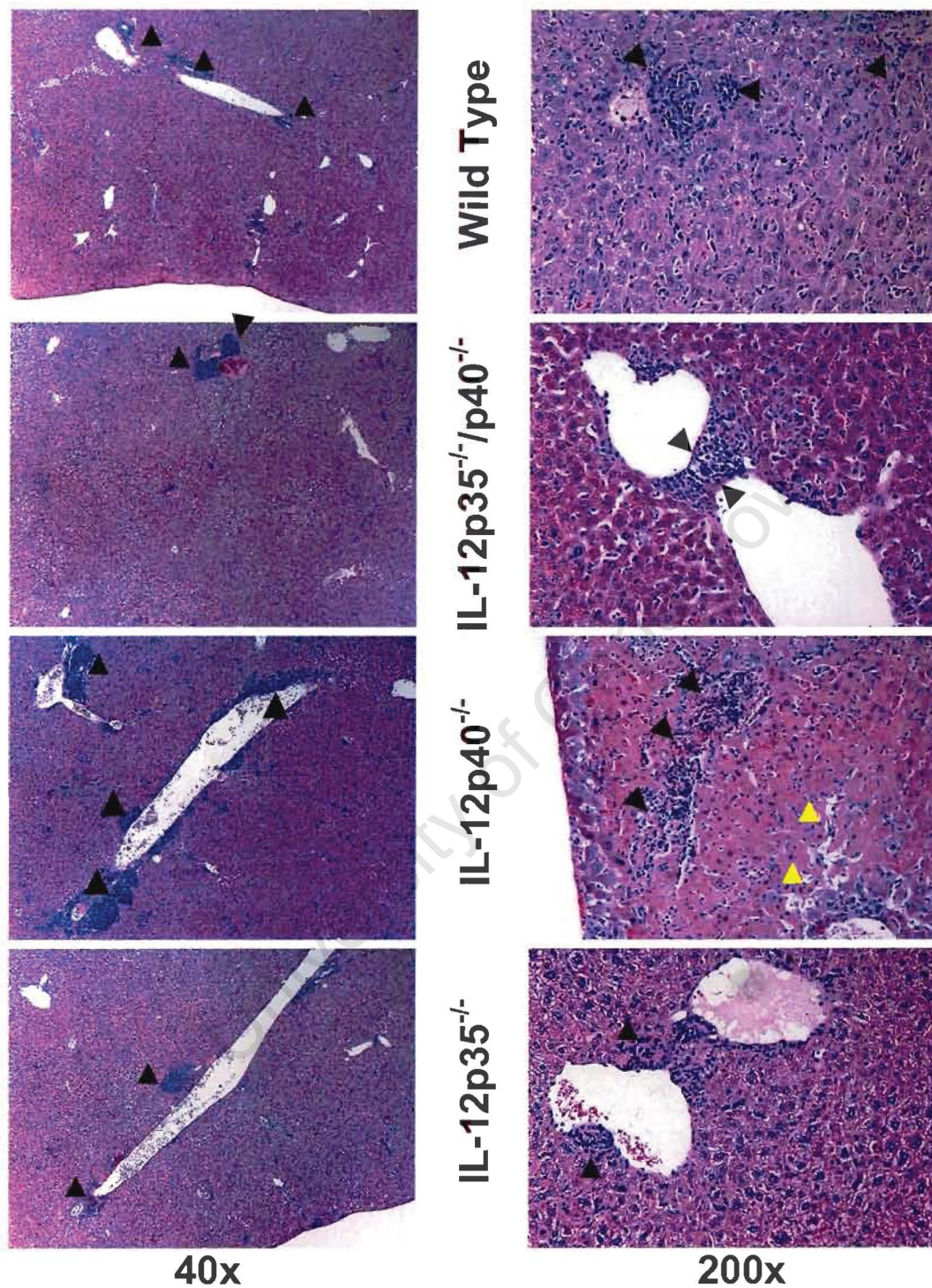


FIGURE 7. Deletion of IL-12p40 results in slightly increased tissue disruption in liver of *T. evansi* infected mice compared to wild type and other IL-12 deficient mouse strains. Liver sections were mounted and stained with H&E for visualization. Representative images shown are of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 28 post infection. Black arrowheads indicate regions of inflammatory cell foci and yellow arrowheads indicate enlarged capillary beds. (Magnification of images: 40× and 200×).

Both IL-12 gene deficient and wild type mouse groups display a similar extent of pathology in the spleen during the course of T. evansi infection

During *T. evansi* infection, both wild type and IL-12 deficient mouse groups displayed splenomegaly (data not shown). At day 8 (data not shown) and 28 post infection, pathological lesions were found in both wild type and IL-12 deficient mice, and together with the course of disease the extent became more pronounced. At both day 8 and 28 post infection, no differences in the extent of pathological lesions was found between IL-12 deficient mouse groups or between the mutant and wild type mice. The absence of discrete germinal centers was also observed throughout infection in all mouse groups.

At day 28 post infection (Figure 8) the extent of the pathological lesions was the greatest as compared to day 8 post infection. Overall splenic structure was disrupted in all mouse groups studied. The spleen white pulp follicles from infected wild type and IL-12 deficient mice exhibited lymphocyte depletion and disorganization. Similar observations have been found in intravenously infected goats (Ngeranwa, Gathumbi et al. 1993). Mononuclear cell infiltrate and tissue structure disruption of the red pulp region was found to be similar in wild type and IL-12 gene deficient mice. Thus, in *T. evansi* infection, IL-12 does not play a role in the induction of pathological lesions in the spleen.

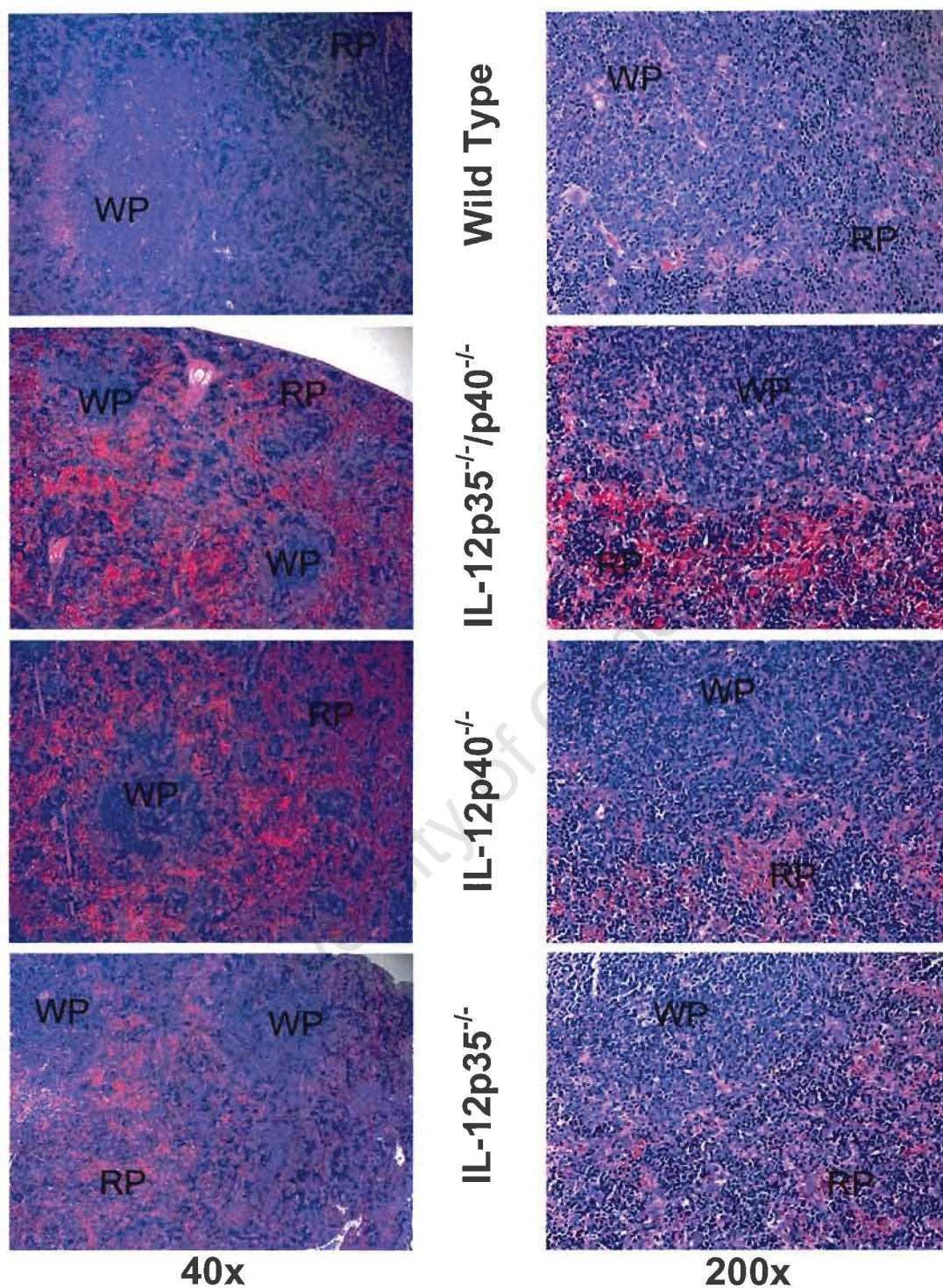


FIGURE 8. Interleukin-12 does not play a role in the induction of pathological lesions in splenic tissue in *T. evansi* infected mice. Spleen sections were mounted and stained with H&E for visualization. Representative images of spleen sections stained with hematoxylin and eosin (H&E) are shown of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 28 post infection. WP and RP denote white pulp and red pulp, respectively. (Magnification of images: 40× and 200×).

Interferon- γ production is crucial for resistance to *T. evansi* infection.

As IFN- γ plasma levels were impaired in all IL-12 deficient mice infected with *T. evansi*, the role of IFN- γ signaling in the control of trypanosomiasis was investigated using IFN- γ receptor (IFN- γ R) gene deficient and wild type mice on 129Sv/Ev background. Although another study showed no role for IFN- γ R in survival in *T. evansi* infections (Baral, De Baetselier et al. 2007), reduced survival was observed in IFN- γ R (129Sv/Ev) deficient mice compared to wild type (129Sv/Ev) mice, with mean mortalities of 15 vs. 27 days ($P < 0.001$), respectively (Figure 9A). Interferon- γ R deficient mice were unable to clear the first parasitemia wave, with mortality occurring concurrently (Figure 9B), as compared to wild type control. Although wild type mice displayed high parasitemia, they still survived longer than IFN- γ R deficient mice. These data demonstrated the importance of IFN- γ R responsiveness for survival in *T. evansi* infection as found in *T. b. brucei* infection. A role for IFN- γ in parasitemia control may not be ruled out, since 129Sv/Ev wild type mice are highly susceptible and are unable to clear parasitemia as effectively as C57BL/6 mice.

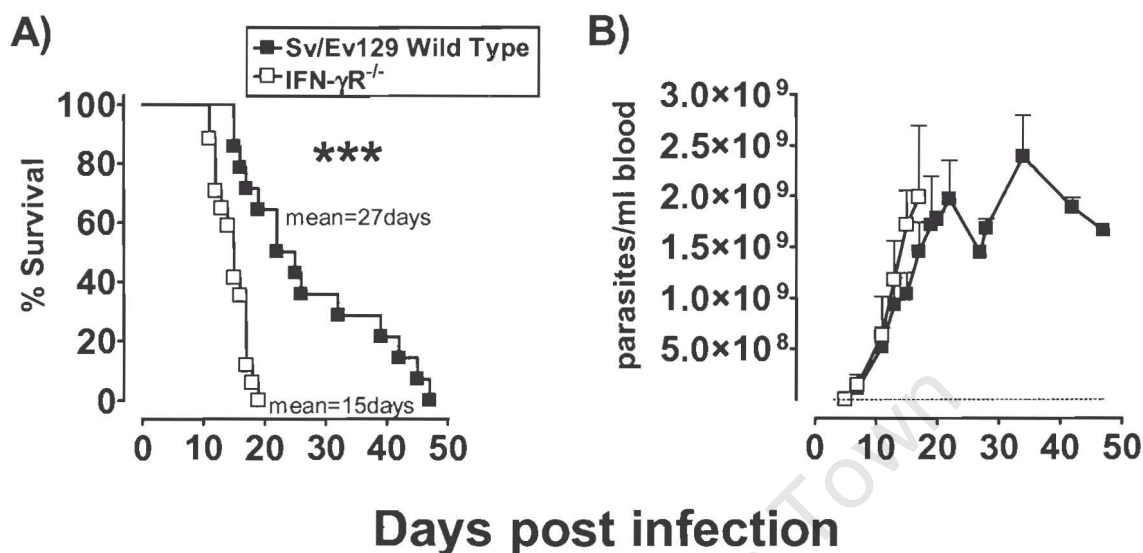


FIGURE 9. Interferon- γ signaling is necessary for survival to infection with *T. evansi*. Survival of 129Sv/Ev wild type (■) and IFN- γ R deficient (□) mice are shown. Mice were infected intraperitoneally with 1×10^3 trypanosomes and monitored daily for survival. Mean survival times were 27 days for wild type mice ($n = 14$) and 15 days for IFN- γ R gene deficient ($n = 17$) mice ($P < 0.001$) (A). Parasitemia profiles of infected 129Sv/Ev wild type (■) and IFN- γ R gene deficient (□) mice (B) were similar. Parasitemia was determined from peripheral blood with a lower detection limit of 1.0×10^6 parasites/ml (dotted line). Mortality and parasitemia data were pooled from two independent experiments where 4-5 mice per group were used for parasitemia measurement. Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia the unpaired Student's t-test was used; ***, $P < 0.001$.

Tumor necrosis factor plays a role in parasitemia control but not survival or induction of anemia in *T. evansi* infection

Since *T. evansi* infected IL-12 deficient mice displayed slightly reduced plasma levels of TNF (Figure 4), the role of TNF in resistance to *T. evansi* infection was investigated. Although a recent report found no role for TNF in parasitemia control or survival (Baral, De Baetselier et al. 2007), the results from this single pilot experiment do not concur completely with those published.

Survival periods between wild type (32.0 days) and TNF deficient mice (36.0 days) were similar (Figure 10A). However, throughout infection, peaks of significantly increased parasitemia were found in the TNF deficient mice as compared to the wild type mice (Figure 10B). Similar findings have been reported in TNF deficient mice infected with *T. b. brucei* (Magez, Geuskens et al. 1997). The levels of anemia were similar for all mice (Figure 10C). However, at day 5 post infection anemia was significantly decreased in TNF gene deficient, suggesting that the induction of anemia by TNF may be restricted to the acute stage of infection. These results were confirmed in TNF-R1 and TNF-R2 gene deficient mice (on a Sv/Ev129 background), where similar survival, parasitemia and anemia results were found (data not shown). The differences in anemia found in this study may be due to the SPF conditions that these mice are kept in, whereas the mice from the published work are not kept under such stringent conditions. Unpublished data may indicate a dietary component playing a role in anemia in infected mice (Magez, personal communication).

In summary, this data is consistent with the requirement of a Type 1 cytokine response for optimal trypanotolerance to infection with *T. evansi*.

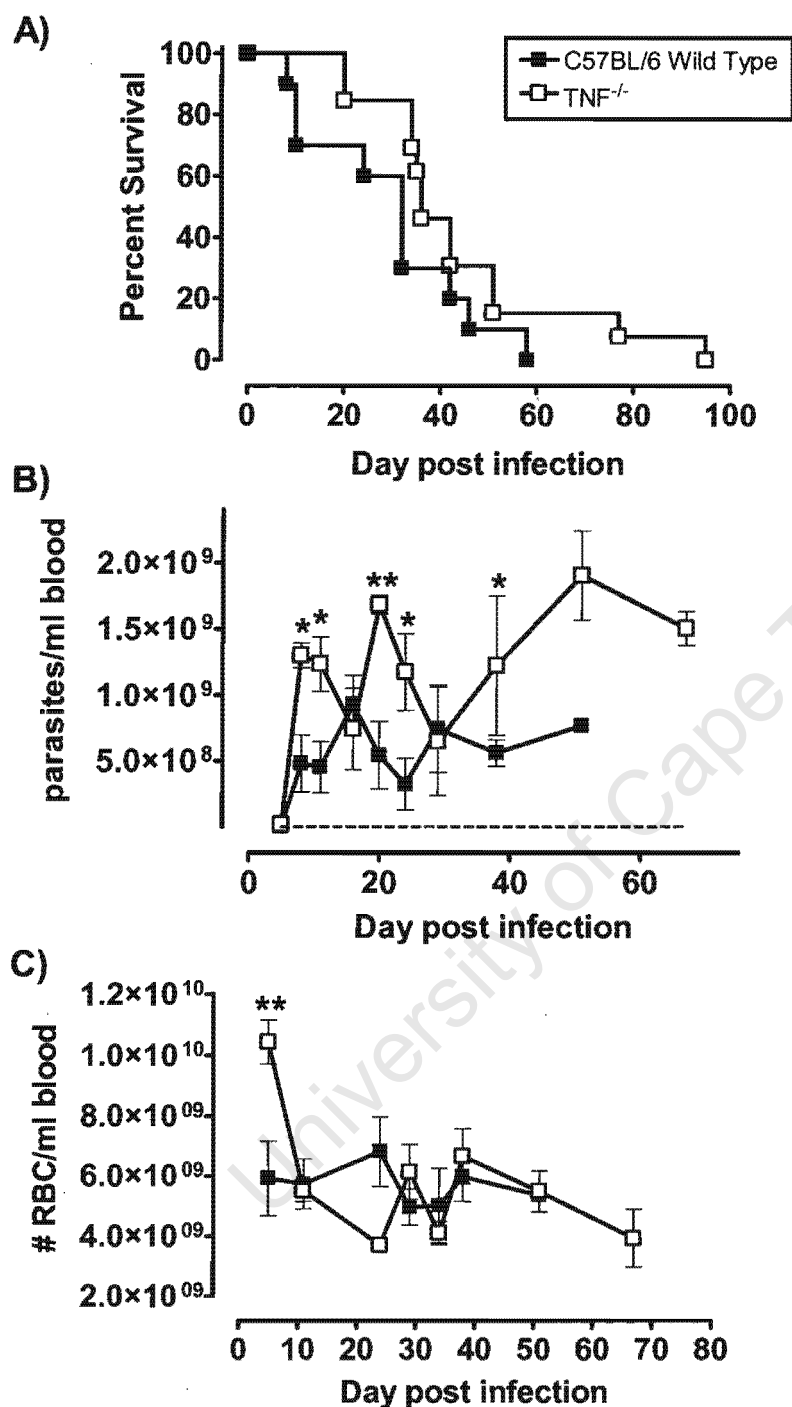


FIGURE 10. Tumor necrosis factor is required for parasitemia control in infection with *T. evansi*. Survival (A), parasitemia (B) and RBC concentration (C) profiles of *T. evansi* infected C57BL/6 wild type (■) and C57BL/6 TNF^{-/-} (□) mice. Infectious dose was 5×10^3 trypanosomes per mouse intraperitoneally and mice were subsequently monitored daily for survival. The median survival time was; 32.0 days for wild type ($n=10$) and 36.0 days for TNF^{-/-} ($n=13$) mice. Parasitemia was determined from peripheral blood with a lower detection limit of 4.0×10^6 parasites/ml (dotted line) from 5 mice per group. Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia and RBC concentration the unpaired Student's t-test was used and are indicated by; *, $P < 0.05$ or **, $P < 0.01$.

Inducible nitric oxide is not required for host resistance in *T. evansi* infection

In *T. congolense* infection, NO was identified as crucial for host resistance (Magez, Radwanska et al. 2006). However, in both *T. brucei rhodesiense* (Hertz and Mansfield 1999) and *T. evansi* (Baral, De Baetselier et al. 2007) no role for this molecule was found in host resistance. Infections with the latter two pathogens demonstrated that NO played role in immune suppression, although in both instances the trypanosome-induced suppression had no detrimental effect on survival or parasitemia control.

Since data presented in this chapter using various Type 1 cytokine deficient mice infected with *T. evansi* contradicted those published (Baral, De Baetselier et al. 2007), the role of NO in host resistance to infection was investigated. Results from the iNOS deficient mice demonstrated that there was no role for this molecule in host resistance as shown by data measuring survival (Figure 11A), parasitemia control (Figure 11B) and anemia (Figure 11C). Therefore other molecules, downstream of IFN- γ , may be involved in mediating resistance to *T. evansi* infection.

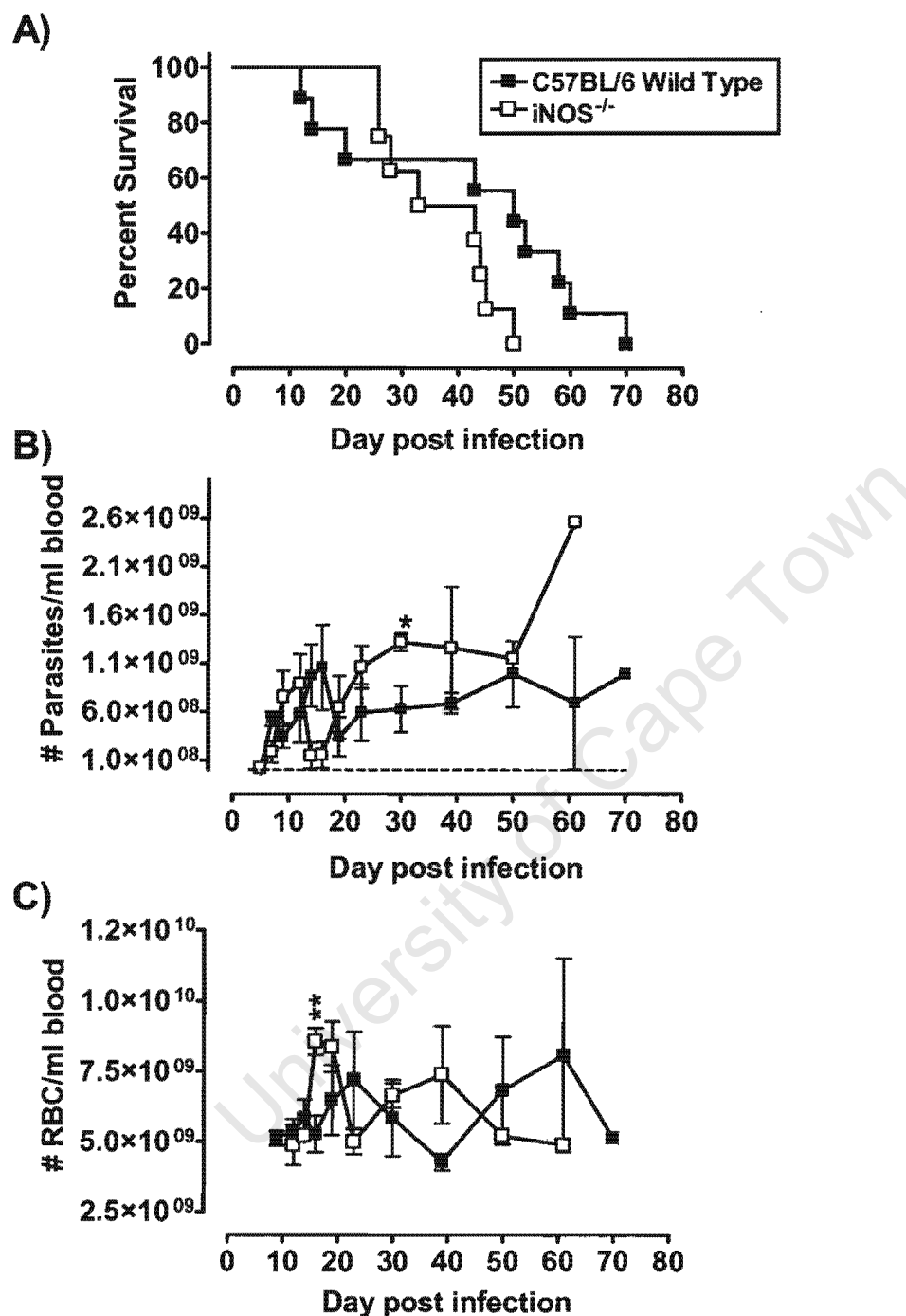


FIGURE 11. Inducible nitric oxide is not required for host resistance in *T. evansi* infection. Survival (A), parasitemia (B) and RBC concentration (C) profiles of *T. evansi* infected C57BL/6 wild type (■) and C57BL/6 iNOS^{-/-} (□) mice. Infectious dose was 1×10^3 trypanosomes per mouse intraperitoneally. Mice were subsequently monitored daily for survival. The median survival time was; 50.0 days for wild type ($n=9$) and 38.0 days for iNOS^{-/-} ($n=8$) mice. Parasitemia was determined from peripheral blood with a lower detection limit of 4.0×10^6 parasites/ml (dotted line) from 5 mice per group. Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia and RBC concentration the unpaired Student's t-test was used and are indicated by; *, $P < 0.05$ or **, $P < 0.01$.

Interleukin-18 signaling does not play a role in resistance during T. evansi infection.

The induction of a Th1 immune response requires the activities of IL-12 and IL-18 (as well as other molecules), with IL-18 having overlapping functions with that of IL-12. Interleukin-12 and IL18 therefore synergistically act together in mediating the early events of a Th1 immune response (Takeda, Tsutsui et al. 1998) (Holscher 2004). Interleukin-18 is a member of the IL-1 family and signals via the IL-1 receptor-related protein (IL-1Rrp) (Torigoe, Ushio et al. 1997). Given the complementary roles of IL-18 and IL-12, the role of IL-18 during *T. evansi* infection was investigated using IL-1Rrp deficient mice. Although this pilot experiment was performed only once, no role for IL-18 signaling in survival (Figure 12A), parasitemia control (Figure 12B) and anemia (Figure 12C) during *T. evansi* infection was found.

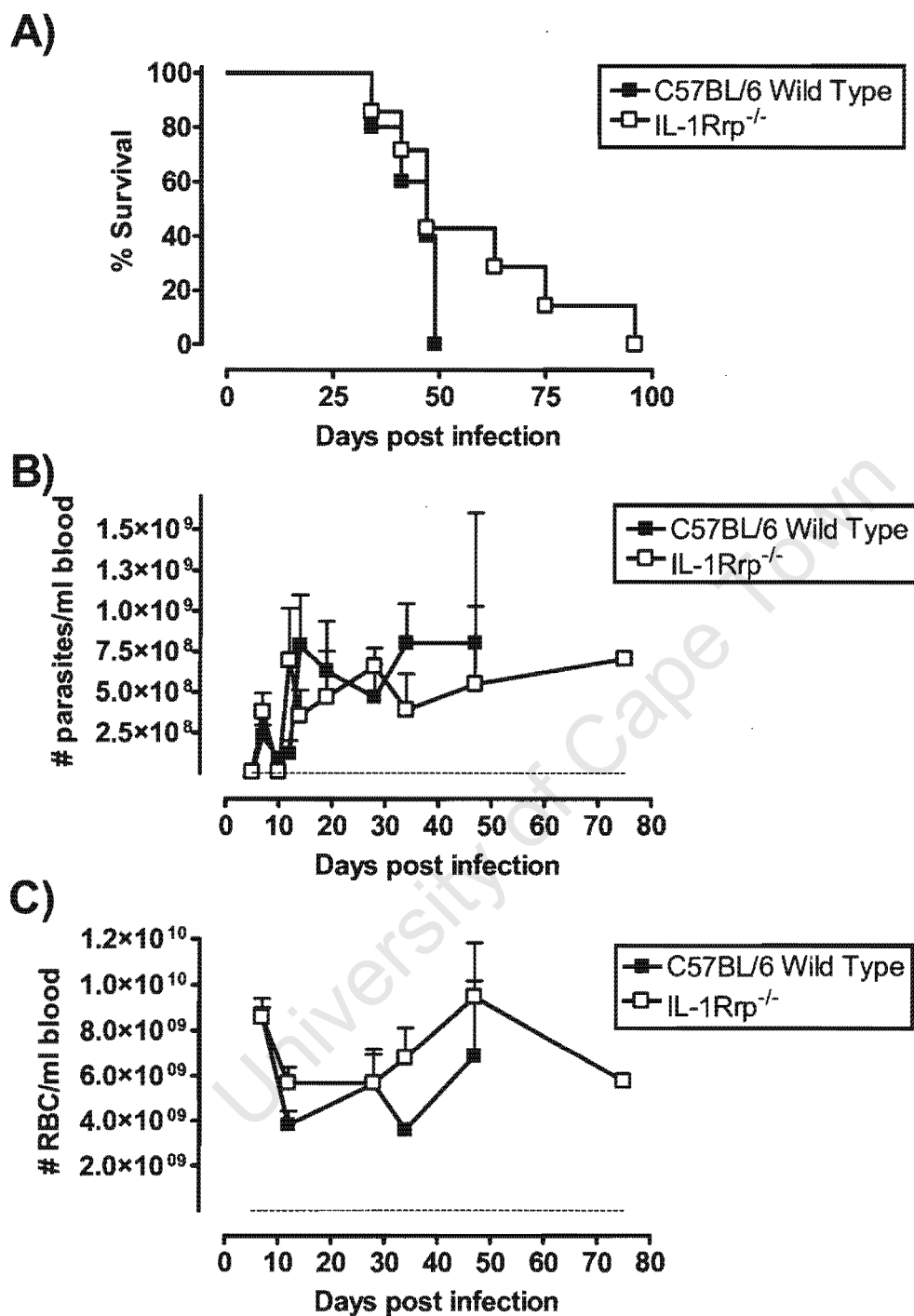


FIGURE 12. Interleukin-18 signaling plays no role in resistance to infection with *T. evansi*. Survival (A), parasitemia (B) and RBC concentration (C) profiles of *T. evansi* infected C57BL/6 wild type (■) and C57BL/6 IL-1Rrp^{-/-} (□) mice. Infectious dose was 1×10^3 trypanosomes per mouse intraperitoneally and were subsequently monitored daily for survival. The median survival time was; 47.0 days for wild type (n=5) and 47.0 days for IL-1Rrp^{-/-} (n=7) mice. Parasitemia was determined from peripheral blood with a lower detection limit of 4.0×10^6 parasites/ml (dotted line) from 5 mice per group. Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia the unpaired Student's t-test was used.

Interleukin-4 and -13 do not play a role in resistance or susceptibility during *T. evansi* infection.

Conflicting reports as to the role of IL-4 in resistance to different *Trypanosoma* species have been published (Bakhiet, Jansson et al. 1996) (Inoue, Inoue et al. 1999) (Schopf, Filutowicz et al. 1998). During infection with *T. evansi* production of IL-4 was found to be present in blood plasma. Therefore the roles of Th2 cytokines namely; IL-4 and IL-13, which are known to have overlapping functions (Brombacher 2000) (Muller, Stenzel et al. 2007), were investigated. Although this was a pilot study performed once, no role for IL-4 (Figure 13A) or IL-13 (Figure 13B) in survival during *T. evansi* infection was found. The median survival times in BALB/c wild type, IL-4^{-/-} and IL-4R α ^{-/-} mice were; 47, 40.5 and 39.5 days, respectively. Furthermore, no differences in survival between *T. evansi* infected IL-4^{-/-} and IL-4R α ^{-/-} mice were found.

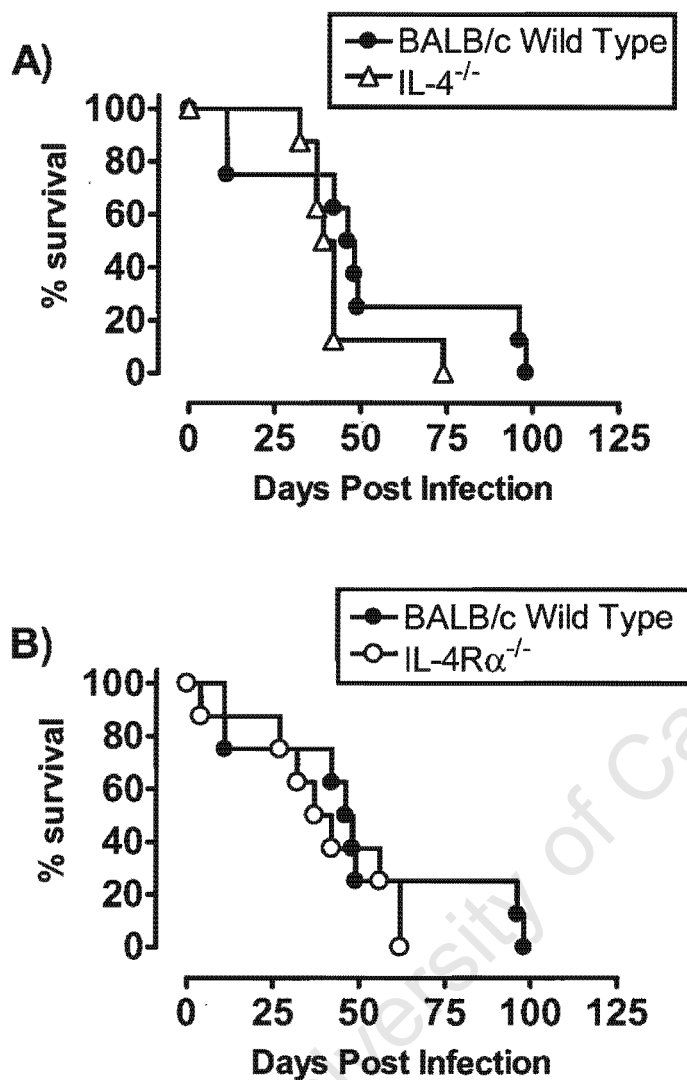


FIGURE 13. Interleukin-4 and -13 do not play a role in survival during infection with *T. evansi*. Mortality profiles of infected BALB/c wild type (●), BALB/c IL-4^{-/-} (Δ) ($P>0.05$) (A) and BALB/c IL-4Rα^{-/-} (○) ($P>0.05$) (B) mice, are shown from 1 experiment. Infectious dose was 1×10^3 trypanosomes per mouse intraperitoneally and were subsequently monitored daily for survival. The median survival time was; 47 days for wild type ($n=8$), 40.5 days for IL-4^{-/-} ($n=8$) and 39.5 days for IL-4Rα^{-/-} ($n=8$) mice. No significant differences were found between gene deficient mice or between gene deficient and wild type mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality.

The intracellular adapter molecule MyD88 is crucial for host resistance in *T. evansi* infection

During the innate immune response during *T. b. brucei* infection, the intracellular adapter molecule MyD88 was found to be crucial for the initial production of IFN- γ and TNF (Drennan, Stijlemans et al. 2005). Both these cytokines are essential for host protection (Hertz, Filutowicz et al. 1998) (Magez, Radwanska et al. 1999) (Namangala, Noel et al. 2001). Thus the initiation of a Type 1 immune response was dependant on MyD88 in *T. b. brucei* infection. The increased susceptibility found in the MyD88 deficient mice was not attributed to the interruption of IL-1 α , IL-1 β or IL-18 mediated signaling, but was suggested to rely on one or several TLR. In part, TLR 9 was identified as being necessary for host survival, but did not explain the severe susceptibility found in MyD88 deficient mice.

Data presented so far in this chapter has suggested that a Type 1 cytokine response was necessary for host resistance against *T. evansi* infection. Thus the role of MyD88 which plays a role in the induction of cytokines during *T. evansi* infection was investigated using MyD88 deficient mice. Although this pilot study was performed only once, the results obtained fitted the paradigm that in *T. evansi* infection Type 1 cytokines are crucial for host resistance. MyD88 deficient mice were highly susceptible to *T. evansi* (Figure 14), most likely due to decreased production of IFN- γ and TNF as found in *T. b. brucei* infection (Drennan, Stijlemans et al. 2005). Similarly, as found in *T. b. brucei* infection, IL-18 (Figure 12A) and TLR2 (data not shown) were excluded as resistance factors dependant on the MyD88 signaling pathway during *T. evansi* infection.

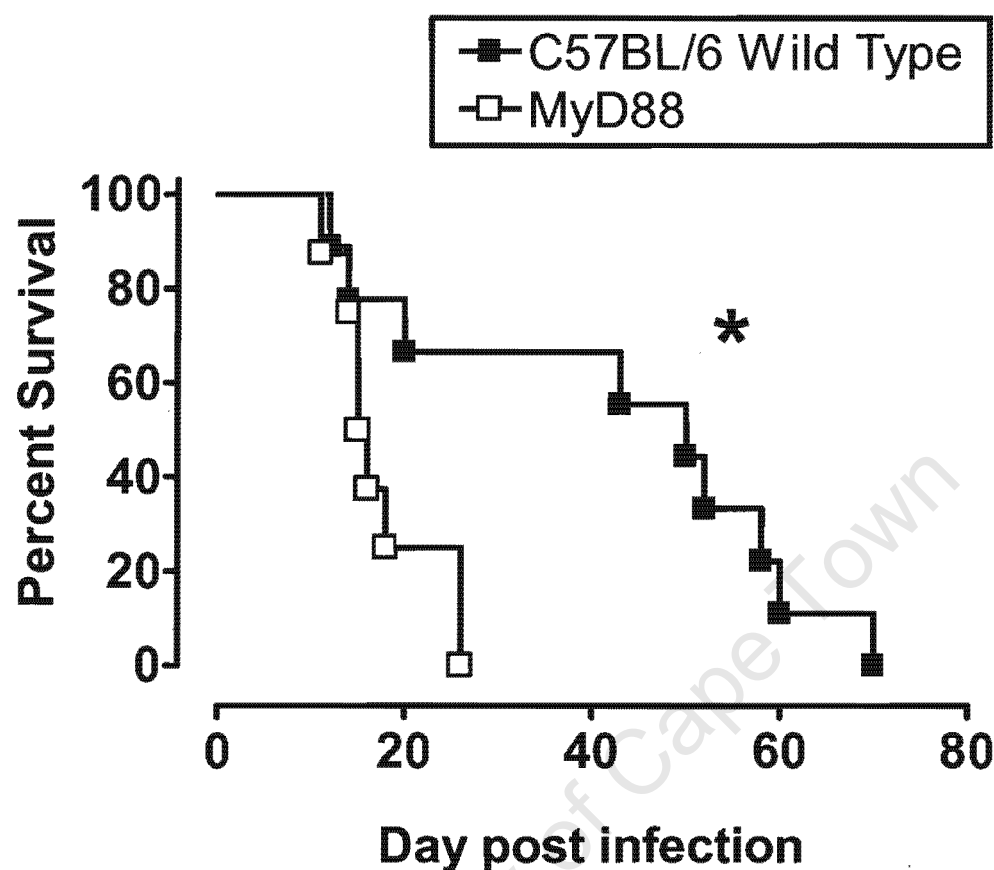


FIGURE 14. Myeloid differentiation factor 88 is essential for host resistance in *T. evansi* infection. Survival profile is shown of *T. evansi* infected C57BL/6 wild type (■) and C57BL/6 MyD88^{-/-} (□) mice. Infectious dose was 1×10^3 trypanosomes per mouse intraperitoneally. Mice were subsequently monitored daily for survival. The median survival time was; 50.0 days for wild type (n=9) and 15.5 days for MyD88^{-/-} (n=8) mice. Statistical significance was determined by the Kaplan-Meier test for mortality and is indicated by; *, $P < 0.05$.

CD4 T cells are required for parasitemia control and survival during T. evansi infection

T-cell independent as well as T-cell dependent B-cell responses to VSG epitopes occurs in trypanosome infected mice (Reinitz and Mansfield 1990). Variant surface glycoprotein specific T-cells were found to be principally localized in the peritoneal cavity. These T-cells, identified as CD4-positive, do not proliferate in response to VSG but produce substantial amounts of cytokines such as IFN- γ when stimulated. Thus, VSG molecules stimulate a classical Th1 cellular subset during infection, together with the associated cytokine responses (Schleifer, Filutowicz et al. 1993). As Type 1 cytokines, such as IL-12 and IFN- γ , were found to be necessary for trypanotolerance in *T. evansi* infection (Barkhuizen, Magez et al. 2007), the role of CD4-positive cells in resistance to *T. evansi* was investigated. .

Although this pilot experiment was performed only once, the data suggests a crucial role for CD4-positive T-cells in survival ($P<0.05$) (Figure 15A) and post 1st peak parasitemia control ($P<0.01$) (Figure 15B). A similar role for CD4-positive T-cells in parasitemia control was found in *T. b. gambiense* infection (Inoue, Inoue et al. 1999). The role of these CD4-positive T-cells in the induction of anemia displayed two extremes and may correlate with the level of parasitemia (Figure 15C). In the first extreme, low levels of parasitemia and reduced anemia were found in the CD4 gene deficient mice. However, as parasitemia rapidly increased and mortality began, the level of anemia increased.

These preliminary data suggests that CD4-positive T-cells may be required for survival and the control of parasitemia after the initial peak of parasitemia during *T. evansi* infection.

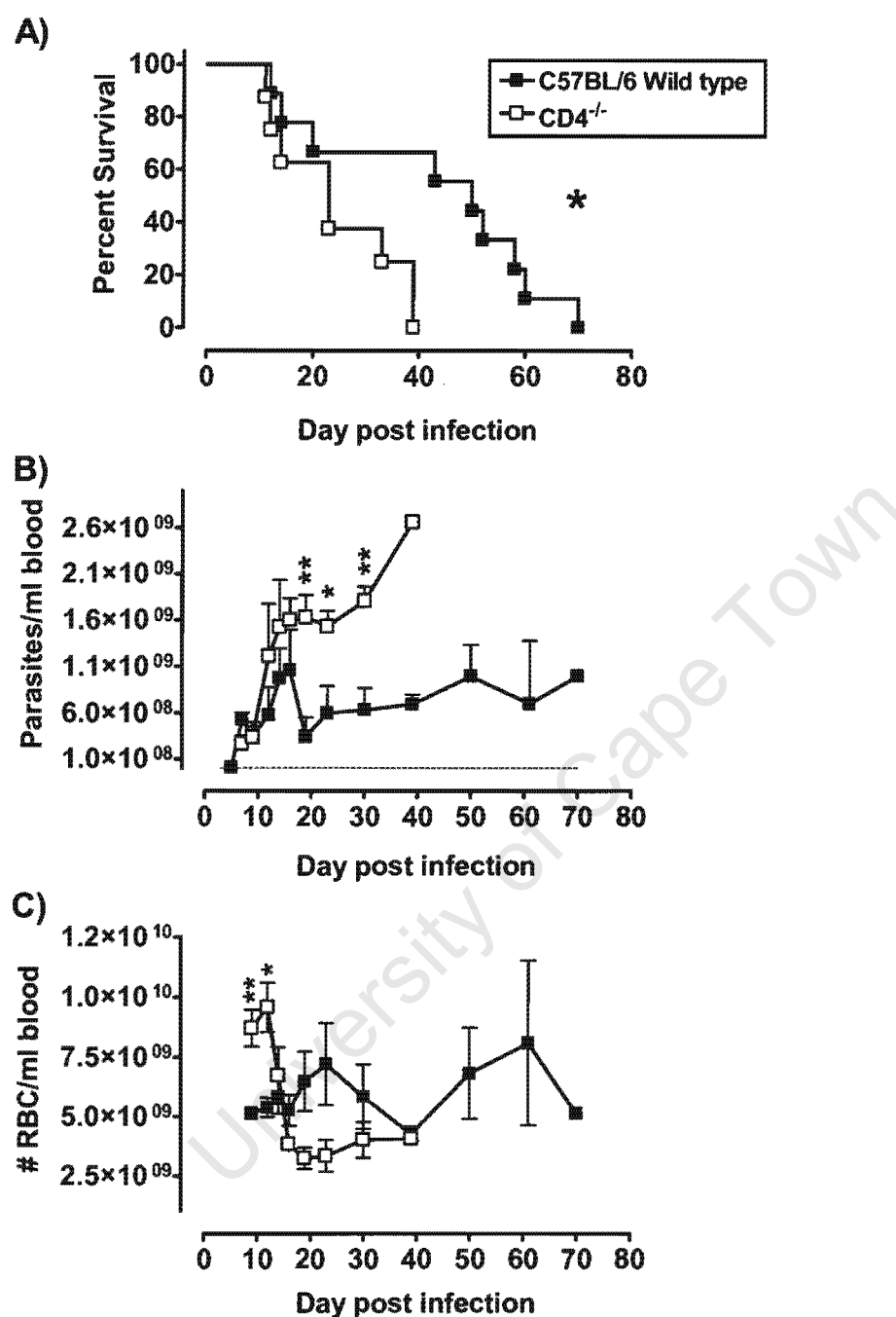


FIGURE 15. CD4-positive T-cells are responsible for parasitemia control and survival in *T. evansi* infection. Survival (A), parasitemia (B) and RBC concentration (C) profiles of *T. evansi* infected C57BL/6 wild type (■) and C57BL/6 CD4^{-/-} (□) mice. Infectious dose was 1×10^3 trypanosomes per mouse intraperitoneally. Mice were subsequently monitored daily for survival. The median survival time was; 50.0 days for wild type ($n=9$) and 23.0 days for CD4^{-/-} ($n=8$) mice. Parasitemia was determined from peripheral blood with a lower detection limit of 4.0×10^6 parasites/ml (dotted line) from 5 mice per group. Statistical significance was determined by the Kaplan-Meier test for mortality and for parasitemia and RBC concentration the unpaired Student's t-test was used and are indicated by; *, $P < 0.05$ or **, $P < 0.01$.

DISCUSSION

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mouse strains displayed significantly reduced survival compared to wild type mice during *T. evansi* infection. This outcome contradicted previously published work which indicated that only a humoral response was required for host resistance, and not a Type 1 inflammatory response (Baral, De Baetselier et al. 2007). The three IL-12 deficient mouse strains showed no differences in survival rates, suggesting that IL-12p70 is the main protective factor for survival, with no major contribution from other IL-12 family members. Interleukin-12 deficient mice survived through several waves of parasitemia demonstrating that residual protection occurs in these deficient mice.

The parasitemia of wild type mice infected with *T. evansi* differed to that of *T. b. brucei*, with high variation found in the former pathogen. This is similar to what has been described in other animal models (Ngeranwa, Gathumbi et al. 1993). *Trypanosoma evansi* infection in wild type mice showed severe inability to efficiently clear parasitemia waves, deteriorating after each successive wave, suggesting a progressive erosion of immune control. Interleukin-12 deficient mice infected with *T. evansi* were unable to reduce parasitemia compared to wild type mice. However, later during infection, no differences between IL-12 deficient and wild type mice were found. This suggests a role for IL-12p70 signaling for early parasitemia control.

During the initial and late time points examined, plasma levels of IFN- γ were severely reduced but, in contrast to *T. b. brucei*, only moderately lower for TNF. *Trypanosoma evansi* infected IFN- γ R deficient mice were highly susceptible and were unable to clear the 1st parasitemic peak and rapidly died thereafter. This clearly demonstrated the importance of IFN- γ , although a direct comparison with IL-12 deficient mice was hampered by the difference in the genetic background of the deficient strains and mortality of wild type controls in *T. evansi* infection. Tumor necrosis factor plasma levels were slightly reduced in IL-12 deficient mice during the chronic stages, but work from

another laboratory found TNF not to be important in survival or parasitemia control (Baral, De Baetselier et al. 2007). However, the results reported here suggested that TNF was necessary for parasitemia control in *T. evansi* infection. In terms of anti-VSG B-cell responses, it has been recently shown that IgM is crucial for parasitemia control and survival (Baral, De Baetselier et al. 2007). However no obvious differences between wild type and IL-12 deficient mouse strains were found in this doctoral study. Therefore the mechanism of impaired parasitemia control during *T. evansi* infection is dependent on IL-12, with a possible role for IFN- γ and TNF (or a combination of both) as found in the *T. b. brucei* infection. These results also indicated that the increased mortality found in IL-12 deficient mice was due to severely decreased IFN- γ responses.

Interleukin-12 independent induction of IFN- γ may also include a direct stimulus via the release of stimulatory molecules by *T. evansi*. Trypanosome derived lymphocyte triggering factor (TLTF) has been shown to induce the production of IFN- γ by binding to CD8+ cells in *T. evansi* infections (Bakhiet, Buscher et al. 1996), and therefore may have contributed to residual levels and thus partial resistance in the absence of IL-12. Furthermore, IL-12-independent IFN- γ production with residual Th1 responses have been demonstrated during *Listeria monocytogenes* (Brombacher, Dorfmueller et al. 1999), *Mycobacterium BCG* (Holscher, Atkinson et al. 2001) and *Leishmania major* (Mattner, Magram et al. 1996) infections. In *T. b. brucei* infection, natural killer cells are thought to be necessary for resistance during the initial infection which are activated by both IL-12 and IL-18 (Namangala, Sugimoto et al. 2007). Similarly in *T. evansi* infection, in the absence of IL-12, IL-18 may drive the Th1 response, thus providing residual host protection. Indeed, in the absence of IL-18, IL-12 was found to be effective in providing host resistance to *T. evansi* infection.

In terms of the induction of pathology during infection, TNF has been found to have dual effects in *T. b. brucei* infections, such as trypanolytic effects (Lucas, Magez et al. 1994; Magez, Geuskens et al. 1997) reducing parasite burden in case of *T. b. brucei* AnTat 1.1 infections (Lucas, Magez et al. 1993; Magez, Lucas et al. 1993; Magez, Radwanska et al. 1999) and causing immunopathological effects in the host (Magez, Radwanska et al.

1999). In *T. congolense* infection, although IFN- γ was required for host resistance (Magez, Radwanska et al. 2006), this cytokine was also responsible for causing pathological lesions in the liver from infected mice (Shi, Pan et al. 2003) (Guilliams, Oldenhove et al. 2007). In *T. evansi* infection there was no overall difference in pathology in the liver and spleen of IL-12 deficient mice as compared to wild type mice. However, plasma levels of IFN- γ were drastically reduced and a slight reduction in TNF during the chronic stage of infection was observed. These findings demonstrated that the induction of pathology was independent of IL-12 and may also be independent of IFN- γ production.

Work by Drennan et al., 2005 found MyD88 signaling to be crucial for resistance in *T. b. brucei* infection, with a partial role for TLR9 in parasitemia control (Drennan, Stijlemans et al. 2005). Interleukin-12 may be involved in this immunological pathway whereby TLR9 present on macrophages respond to *T. b. brucei* genomic DNA released following the 1st peak of parasitemia, and upon IL-12 induction stimulates the secretion of IFN- γ and TNF (Barkhuizen, Magez et al. 2007). As MyD88 deficient mice were severely susceptible in *T. evansi* infection, a similar mechanism may play a role and may thus explain the reduced parasitemia control and survival found in IL-12 deficient mice compared to wild type mice.

A comparison of the mean survival in wild type mice infected with *T. evansi* or *T. b. brucei* shows a slight increase in *T. evansi* (47 ± 24.1 days) as compared to *T. b. brucei* (40.6 ± 8.8 days). However, the onset of mortality in *T. evansi* started from day 12 post infection whereas in *T. b. brucei* onset began only at day 28 post infection. Furthermore parasitemia profiles were strikingly different between the two pathogens. A study by Baral et al., 2007 found that levels of parasitemia in wild type mice infected with *T. evansi* was low during the chronic stages of disease as found in *T. b. brucei* by others. In contrast, *T. evansi* infections in this doctoral thesis displayed a high burden from the initial parasitemic peak, suggesting that a more virulent strain of *T. evansi*, in terms of increased parasitemia, has emerged in the laboratory.

Furthermore, the differences in the results from the 129Sv/Ev IFN- γ R and C57BL/6 TNF deficient mice in this study, as compared to those of Baral et al., 2007, may also be due to the emergence of a more virulent *T. evansi* strain. It is possible that passaging this parasite for the purpose of maintenance of stock may have resulted in increased virulence. Researchers have found that strain selection occurring through this process in mice has resulted in various changes, such as a more rapid onset of parasitemia (Davila, Ramirez et al. 1998) and increased virulence (Murray, Morrison et al. 1982). Also, various *T. evansi* isolates demonstrate significant differences in morbidity patterns indicating possible heterogeneity in terms of virulence (de Menezes, Queiroz et al. 2004). In addition the SPF conditions at our facilities may exclude concurrent infections that may independently initiate an IL-12 driven Th1 response necessary for optimal resistance. These effects, although untested, adds impetus for further investigation into the immunology of *T. evansi*.

Studies using different isolates of *T. evansi* concluded that a humoral response to infection was not sufficient to account for the observed host resistance (Queiroz, Legey et al. 2001). This doctoral thesis provides evidence which suggests that a Th1 response is necessary for host resistance. Interferon- γ deficient mice were found to have increased mortality compared to wild type mice. Tumor necrosis deficient mice displayed reduced parasitemia control. Myeloid adapter molecule 88, which is known to play a role in Th1 initiation by inducing Type 1 cytokines in trypanosome infections, displayed severely increased susceptibility to *T. evansi* infection. Furthermore, CD4 deficient mice displayed decreased host resistance in terms of parasitemia control and survival. Additionally, CD4 deficient mice readily undergo immunoglobulin isotype switching from IgM to IgG during infection (Rahemtulla, Kundig et al. 1994). Again, indicting that immune factors other than immunoglobulins are required for resistance.

Furthermore, IL-4 production was also noted in *T. evansi* infected wild type mice, however in IL-4 and IL-13 gene deficient mice a role for Th2 inducing cytokines can be excluded as playing a role in resistance to infection. In conclusion, IL-12p70 but not the IL-12p80 homodimer or IL-23 was found to be necessary for optimal resistance to *T.*

evansi infection in terms of mortality and parasitemia control. The resistance attributed to IL-12 was mediated through IFN- γ , the production of which was significantly decreased in the absence of IL-12.

University of Cape Town

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CHAPTER IV

The role of IL-12 and associated cytokines in resistance to *T. congolense* infection

ABSTRACT

To determine the immunological role of IL-12 family members in *T. congolense* infections, IL-12p35^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} mice were used. While the two latter strains lack all IL-12 homologues, IL-12p35^{-/-} mice still produce IL-12p80 homodimers and IL-23. Compared to wild type mice, all infected IL-12 deficient mouse strains showed prolonged survival, while parasitemia levels were unaltered. Interferon- γ production in IL-12 deficient mice was strikingly reduced during the acute and chronic infection stages, coinciding with significantly reduced chronic stage hepatocellular damage, demonstrated by histological analysis and plasma AST measurements. In contrast, IL-10 production was not affected by the absence of IL-12. Together, these results show that during *T. congolense* infections, the absence of IL-12, but not the IL-12p80 homodimer or IL-23, leads to a reduction of IFN- γ production, which reduces hepatic pathology and improves host survival in conjunction with IL-10 without negatively affecting parasitemia control.

Default Th2 responses were not found in the absence of IL-12, IL-12p80 homodimer or IL-23 and the deletion of these cytokines, namely IL-4 and IL-13, did not affect survival. The adapter molecule MyD88 was found to be essential for resistance to *T. congolense*, but was independent of IL-18, which shares the same intracellular signaling pathway. In conclusion this study demonstrates that an over-shooting pro-inflammatory response, mediated in part by IL-12, results in liver pathology leading to premature death in *T. congolense* infections.

INTRODUCTION

General background on *T. congolense* infections

Trypanosomes are unicellular eukaryotic parasites that replicate freely in the bloodstream of the mammalian host resulting in disease in both humans (sleeping sickness) caused by *T. brucei* species and animals (Nagana) of which *T. congolense* is most important. In contrast to *T. b. brucei* and *T. evansi*, *T. congolense* is strictly an intravascular blood parasite (Ojok, Kaeufer-Weiss et al. 2002). Thus, due to the continued exposure to the host's innate and adaptive defenses, trypanosomes have co-evolved mechanisms to regulate and escape the host's immune responses. Several mechanisms exist of which most significantly is antigenic variation whereby the surface coat of the trypanosome consisting of variant surface glycoprotein (VSG) (Rovis, Barbet et al. 1978) is regularly replaced, thus continually evading the host's immune response (Richards, Rosen et al. 1981). Cumulative evidence to date shows that in *T. congolense* infection both an antibody and a cellular mediated immune response are necessary for an optimal immune response. Due to the extracellular nature of *T. congolense* parasites, the humoral branch of the immune system is dominant, characterized by polyclonal B-cell activation with elevated IgM levels in both bovine and murine infections (Hudson, Byner et al. 1976) (Luckins and Mehlitz 1976) (Morrison, Roelants et al. 1978) (Ellis, Scott et al. 1987).

Tolerance to infection with *T. congolense*

Trypanosoma congolense, a parasite exclusively occurring in sub Saharan Africa causes severe wasting in cattle and draft animals thus causing an economic burden in already poor areas. Immunology of *T. congolense* has been extensively studied in cattle and laboratory mouse models. Of interest is the ability of certain bovine species to display little or no symptoms of infection (Murray, Morrison et al. 1982). Diverse mortality rates have been found in different murine inbred models with trypanosusceptible BALB/c mice surviving only a few days compared to trypanotolerant C57BL/6 mice which are able to

survive for several months (Baetselier, Namangala et al. 2001). The rapid mortality of BALB/c mice has been attributed to systemic inflammatory response syndrome (SIRS) which is mediated by pro-inflammatory Th1 cytokines resulting in death after 7-9 days post infection (Shi, Pan et al. 2003).

Cytokine responses in inbred mouse strains infected with *T. congolense*

Using a compendium of different gene deficient C57BL/6 mice Magez *et al.*, 2006 illustrated the requirement for a Th1 response for effective resistance to *T. congolense* infection. Major histocompatibility complex class-II deficient *T. congolense* infected mice were deficient in IFN- γ production indicating the necessity for this molecule (Magez, Radwanska et al. 2006). IFN- γ and TNF signaling was necessary for upregulation of iNOS, which is required for survival and parasitemia control (Magez, Radwanska et al. 2006). Using *T. congolense* infected IFN- γ R deficient mice, demonstrated the role for IFN- γ signaling in resistance in C57BL/6 mice (Magez, Radwanska et al. 2006). However, in BALB/c mice IFN- γ was a susceptibility factor as overproduction occurred during the 1st parasitemia peak resulting in mortality (Uzonna, Kaushik et al. 1998).

Infections using the IL1180 *T. congolense* clone showed that TNF was necessary for survival (Iraqi, Sekikawa et al. 2001) and parasitemia control (Kitani, Black et al. 2002). Furthermore, macrophage and neutrophil derived soluble TNF, specifically TNFp55, was required for parasitemia control mediated by the trypanolytic activity of NO present in the liver (Magez, Radwanska et al. 2007).

The role of IL-10 in *T. congolense* infected mice has been shown to be necessary to limit the proinflammatory IFN- γ response (Guilliams, Oldenhove et al. 2007) which was shown to induce focal liver necrosis when mice are treated with anti-IL-10 receptor (Shi, Pan et al. 2003). In these treated mice, acute death was associated with significantly enhanced plasma levels of IL-10, IL-6, IFN- γ , and IL-12p40 compared to controls.

Antibody responses in inbred mouse strains infected with *T. congolense*

Phagocytosis of opsonized trypanosomes by macrophages and Kupffer cells of the liver was mediated by both IgM and IgG antibodies during the 1st parasitemic peak, followed by the rapid release of cytokines including IL-12p40 (Shi, Wei et al. 2004). However, anti-VSG IgG responses were determined as the key mediators in resistance and not those of IgM (Magez, Radwanska et al. 2006). A possible resistance mechanism may be due to the finding that the IgG isotype induced significantly more NO production compared to IgM mediated phagocytosis (Kaushik, Uzonna et al. 1999). Studies comparing trypanotolerant and trypanosusceptible mouse strains suggested that the higher IgG2a responses in trypanotolerant mice correlated with the increased IL-12 production, which induces the associated B-cell switch (Uzonna, Kaushik et al. 1999) (Finkelman, Holmes et al. 1990).

AIMS

Since a Th1 immune response, driven by IL-12, was found to be important for resistance during *T. b. brucei* and *T. evansi* infections, the aims of this chapter were to investigate the role of IL-12 in *T. congolense* infections. Furthermore, the role of other cytokines and immune related factors which may act in concert with IL-12, were investigated using gene deficient mice.

MATERIALS & METHODS

(Refer to Material & Methods section in Chapter II)

RESULTS

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice infected with *T. congolense* display enhanced survival compared to wild type mice.

Survival was measured in wild type and IL-12 gene deficient mice infected with *T. congolense* to determine if the IL-12 family members play a role in resistance. Surprisingly, survival of infected mice was significantly increased in all IL-12 gene deficient mice compared to wild type controls ($P < 0.0001$) (Figure 1). Mean survival times of 97, 163, 155 and 150 days were observed for wild type, IL-12p40, IL-12p35/p40 and IL-12p35 gene deficient mice, respectively. Onset of mortality began at similar time points in both wild type and IL-12 deficient mice. However, the rate of death in IL-12 deficient mice was less compared to wild type controls. Furthermore, no differences were observed in the absence of either the single chain or both chains in IL-12 deficient mice. These results therefore suggest that IL-12p70 is the major host susceptibility factor and that IL-23 and IL-12p80 homodimer does not play a crucial role in *T. congolense* infection.

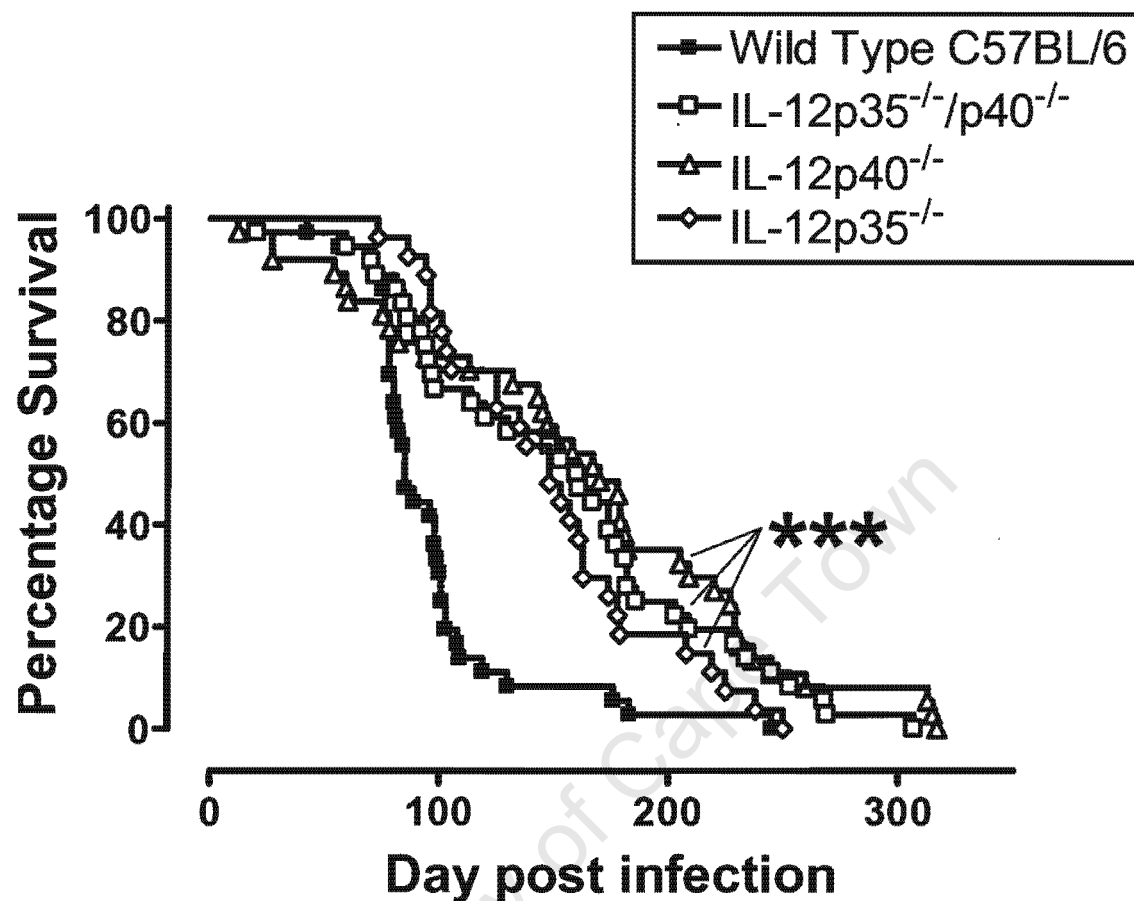


FIGURE 1. Interleukin-12 deficiency results in increased resistance to infection with *T. congolense*. Mortality profiles of *T. congolense* infected wild type C57BL/6 (■), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (□), C57BL/6 IL-12p40^{-/-} (△), and C57BL/6 IL-12p35^{-/-} (◇) mice are shown from three independent pooled experiments. Infectious dose was 1×10^3 trypanosomes per mouse delivered intraperitoneally. Mice were subsequently monitored for survival. The mean survival time was; 97 days for wild type (n=36), 155 days for IL-12p35^{-/-}/p40^{-/-} (n=36) ($P < 0.0001$), 163 days for IL-12p40^{-/-} (n=37) ($P < 0.0001$) and 150 days for IL-12p35^{-/-} (n=27) ($P < 0.0001$) mice. Statistical significance was determined by the Kaplan-Meier together with log rank test for mortality and are presented as ***, $P < 0.001$.

Parasitemia control and the development of anemia are independent of IL-12p70, IL-23 and IL-12p80 homodimer in *T. congolense* infection.

In order to determine a possible role of IL-12p70 (p35/p40), IL-23 (p19/p40) and IL-12p80 homodimer during infection with *T. congolense*, C57BL/6 mice deficient in either or both p35 and p40 genes were infected and subsequent parasite burden and anemia monitored. No significant differences were found between the different strains of IL-12 deficient and wild type mice during the 1st and 2nd parasitemia peak as well as during the subsequent waves of parasitemia until death (Figure 2A). It should also be noted that as *T. congolense* is an intra-vascular parasite and binds to the endovascular endothelium, the concentration of the parasites in the micro-vasculature may be up to one log₁₀ higher than found in the major blood vessels (Morrison, Roelants et al. 1978). The onset of anemia occurred rapidly during the 1st week of infection, coinciding with the initial parasitemic peak, followed by a lower rate of decrease thereafter (Figure 2B). No significant differences in the rate of onset or extent of anemia between wild type and IL-12 deficient mice were found. Lastly, no differences in red blood cell concentrations were found between uninfected wild type and IL-12 deficient mice (Figure 2B inset). Together, these results demonstrated that control of *T. congolense* blood parasite burden, as well as the development of anemia, was not dependent on IL-12p70, IL-23 or the IL-12p80 homodimer during the acute and chronic stages of disease.

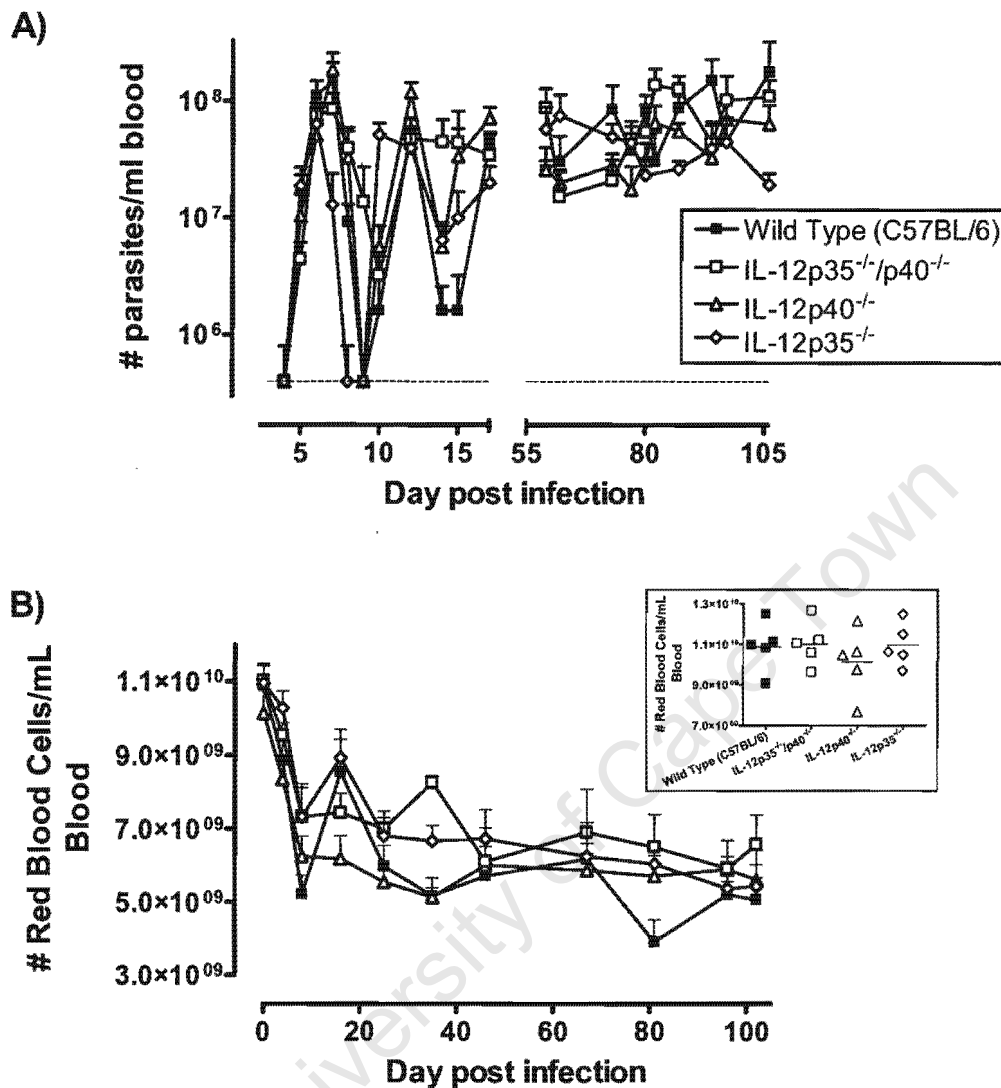


FIGURE 2. Deletion of IL-12 has no significant effect on the extent of parasitemia or anemia in *T. congolense* infection. Parasitemia (A) and anemia (B) profiles of *T. congolense* infected wild type (■), IL-12p35^{-/-}/p40^{-/-} (□), IL-12p40^{-/-} (△), and IL-12p35^{-/-} (◇) mice are shown from two independent pooled experiments. Infectious dose was 1×10^3 trypanosomes per mouse. Mice were monitored for parasite burden and anemia development from peripheral blood obtained from tail snips (10 individual mice per strain) with a lower detection limit of: 4.0×10^5 parasites/ml (dotted line) and 1.6×10^7 RBC/ml (not indicated), respectively. Portions of the entire parasitemia and anemia profiles are shown; including days 2 to 17 and 55 to 106 for parasitemia and 0 to 106 for anemia. Inset in (B) indicates no significant differences in red blood cell concentrations between uninfected wild type, IL-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-}, and IL-12p35^{-/-} mice. Statistical significance tests between wild type and IL-12 deficient mice for both parasitemia and anemia were determined at individual time points ($\pm$ SEM) by one-way ANOVA with Dunnett as the post test and presented as; *, $P < 0.05$ **, $P < 0.01$ ***, $P < 0.001$.

Interleukin-12p70 is necessary for optimal production of IFN- γ during the initial parasitemic peak and during the chronic stage of *T. congolense* infection.

In both *T. b. brucei* and *T. evansi* infection of IL-12 deficient mice, a reduction in IFN- γ responses were found compared to those of wild type mice. Tumor necrosis factor was moderately lower in *T. evansi* infected IL-12 mutant mice but significantly decreased in *T. b. brucei* compared to their respective controls. Therefore, with survival and parasitemia control dependant on the expression of both TNF and IFN- γ (Magez, Radwanska et al. 2006) (Iraqi, Sekikawa et al. 2001) in *T. congolense* infections; plasma cytokine levels were determined at the 1st parasitemia peak at day 7, at day 28, and during the chronic stage, at day 105 post infection (Figure 3). Infection induced strong systemic IFN- γ and TNF production in wild type mice at the 1st peak and at day 105 post infection (Figure 3A and B). However, *Trypanosoma congolense* infection in IL-12 mutant mice resulted in severely impaired IFN- γ responses during 1st peak ($P < 0.05$) and at day 105 post infection ($P < 0.05$) (Figure 3A). Tumor necrosis factor responses were found to be variable between the different IL-12 deficient mouse strains and tended to be slightly lower than wild type mice at all time points investigated (Figure 3B). At day 28 post infection IFN- γ plasma levels were markedly reduced and similar between wild type control and IL-12 mutant strains. These results suggested that during *T. congolense* infection, optimal IFN- γ and to a moderate extent TNF production, during the 1st parasitemic peak was dependent on IL-12p70. However, decreased levels of IFN- γ and TNF production could be independently induced from signal transduction via both or either p35 or p40 subunits. Similarly IL-12p70 dependant IFN- γ production was shown at day 105 post infection. However, TNF production was clearly independent of p35 and p40 at this stage of infection. Furthermore a strong suppression of inflammatory cytokines occurred at day 28 post infection that was independent of IL-12p70, IL-23 or the IL-12p80 homodimer expression.

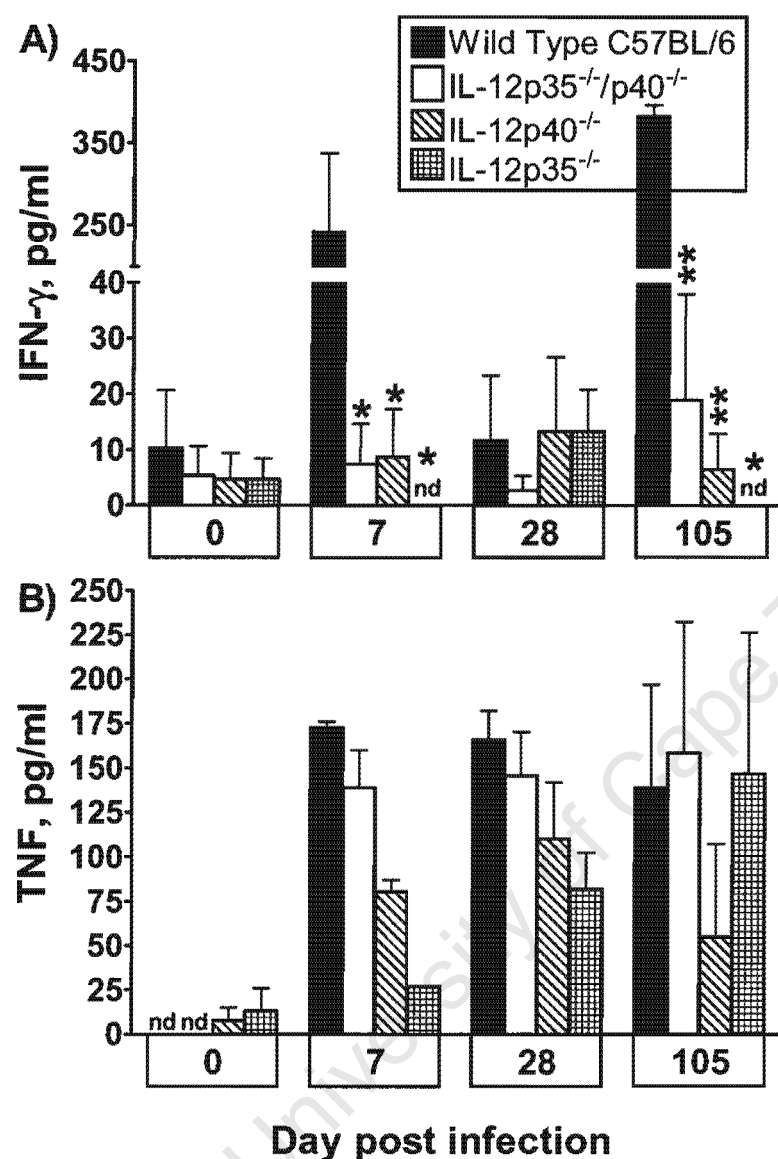


FIGURE 3. Interleukin-12 is necessary for an optimal IFN- γ response during the 1st parasitemic peak as well as during the chronic stages in mice infected with *T. congolense*. Interferon- γ (A) and TNF (B) plasma levels were measured by ELISA in C57BL/6 wild type (Black bars), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (White bars), C57BL/6 IL-12p40^{-/-} (Diagonally striped bars) and C57BL/6 IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense*. Time points analyzed were; day 7 (1st parasitemic peak), day 28 and day 105 post infection. Data expressed as means ($\pm$ SEM) from 2-3 individual mice, showing one representative experiment of two independent experiments. Detection limit for both IFN- γ and TNF assays was 16pg/ml. Statistical significance between *T. congolense* infected C57BL/6 wild type and IL-12 deficient mice was determined by unpaired Student's t-test and are indicated by; *, $P < 0.05$; **, $P < 0.01$. nd: not detected.

Interleukin-12 deficient mice display similar responses to wild type mice in terms of IL-10 cytokine production during *T. congolense* infection.

Studies have shown that IL-10 is a key protective factor in *T. congolense* infection (Guilliams, Oldenhove et al. 2007). Plasma levels of IL-10, although variable, were similar in both wild type and IL-12 deficient mice at day 28 and day 105 post infection (Figure 4). These results parallel other studies that showed that IL-10 was strongly up-regulated during infection, suggesting that production remains sustained during the late chronic stage. Since IL-12 deficient mice were able to survive and clear the initial parasitemic peak, IL-10 levels were not measured at day 7 post infection. The results in combination with the IFN- γ data demonstrated a reduced pro-inflammatory response during the chronic stages of infection in IL-12 deficient mice as compared to wild type mice infected with *T. congolense*.

Interleukin-12 plasma levels were also measured at all three time-points in wild type and IL-12 deficient mice, however no detectable levels were found (data not shown).

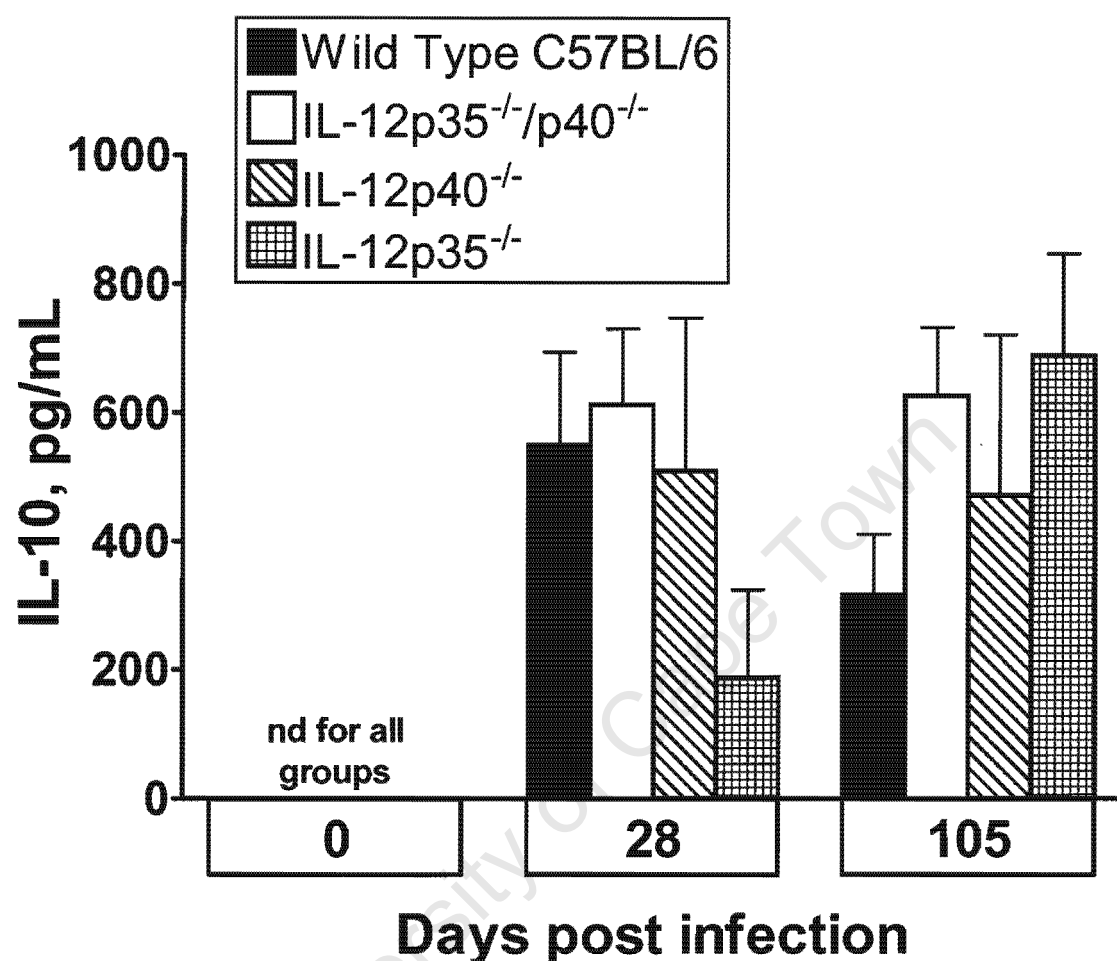


FIGURE 4. Interleukin-12 deficient mice display comparable IL-10 responses compared to wild type mice infected with *T. congolense*. Interleukin-10 plasma levels were measured by ELISA in C57BL/6 wild type (Black bars), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (White bars), C57BL/6 IL-12p40^{-/-} (Diagonally striped bars) and C57BL/6 IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense*. Time points analyzed were; day 28 and day 105 post infection. For plasma IL-10 levels, data were expressed as means ($\pm$ SEM) from 2-3 individual mice, showing one representative experiment of two independent experiments. Detection limit for IL-10 was 63pg/ml. Statistical significance between *T. congolense* infected C57BL/6 wild type and IL-12 deficient mice was determined by unpaired Student's t-test (nd: not detected).

Interleukin-12 deficient mice do not display a default Th2 cytokine response during *T. congolense* infection.

Interleukin-12 deficient mice, in certain infectious disease models, have been found to exhibit a default Th2 cytokine response (Mattner, Magram et al. 1996) (Lehmann, Bellmann et al. 2001) (Decken, Kohler et al. 1998). Together, with conflicting reports as to the role of IL-4 in infection with various *T. brucei* subspecies (Bakhiet, Jansson et al. 1996) (Inoue, Inoue et al. 1999) (Schopf, Filutowicz et al. 1998), it was necessary to investigate the role of this cytokine in *T. congolense* infection. Plasma IL-4 levels, found in all IL-12 deficient mouse groups at each time point, were highly variable between different experiments but did not indicate a trend of increased or decreased levels compared to wild type mice (data not shown).

The IL-4 data was pooled in order to make out a general trend by increasing the number of mice per group (Figure 5). The pooled data more convincingly showed that IL-4 responses were not increased in IL-12 deficient mice. However, IL-4 levels in IL-12 deficient mice showed an interesting decrease at day 28 post infection as compared to wild type mice, which was not seen in the individual experiments. The pooled data shows similar results to the individual experiments with IL-4 plasma levels up-regulated during infection to similar levels at all three time points investigated. Therefore, enhanced survival of IL-12 deficient mice cannot be attributed to increased IL-4 production potentially driving a Th2 response. Moreover, IL-12 was not required to suppress IL-4 production in *T. congolense* infection.

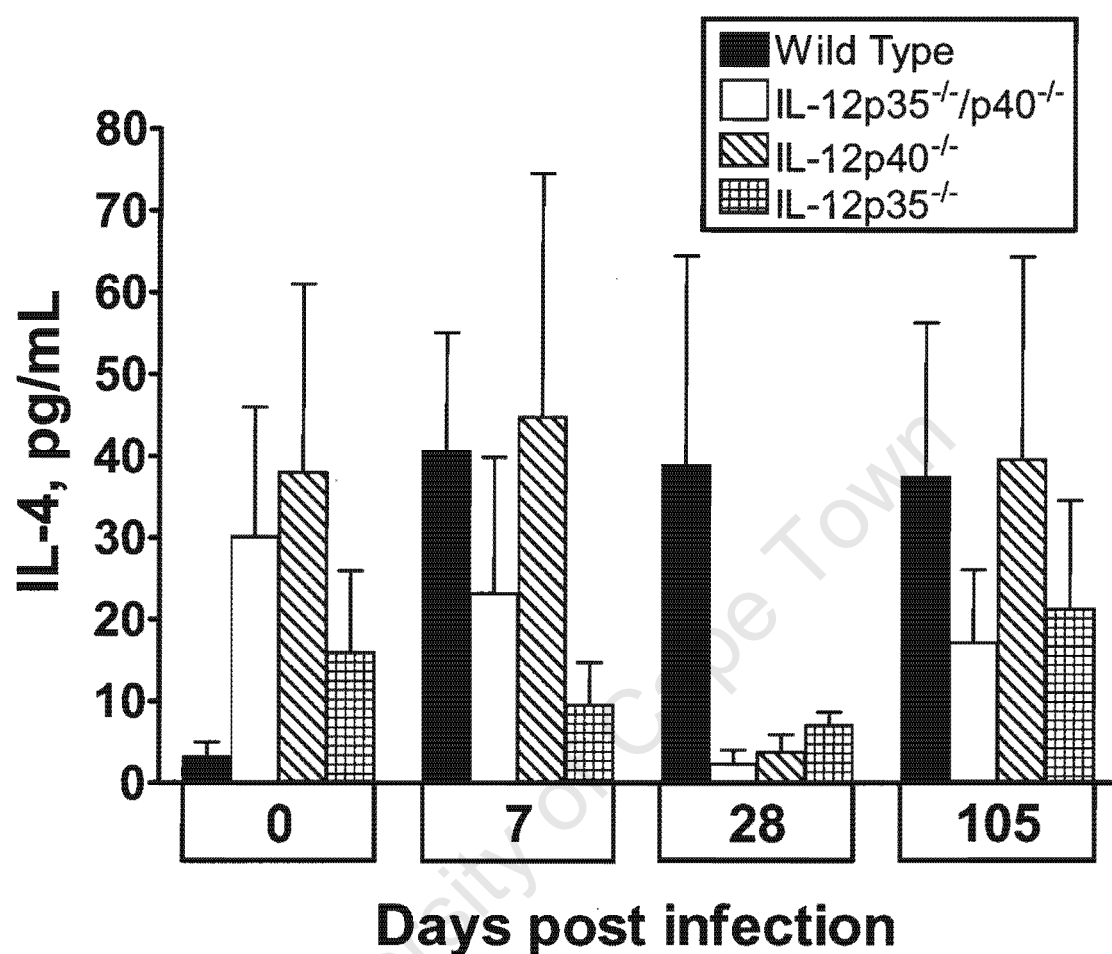


FIGURE 5. Interleukin-12 deficiency does not result in a default Th2 cytokine response in mice infected with *T. congolense* compared to wild type mice. Interleukin-4 plasma levels were measured by ELISA in C57BL/6 wild type (Black bars), C57BL/6 IL-12p35^{-/-}/p40^{-/-} (White bars), C57BL/6 IL-12p40^{-/-} (Diagonally striped bars) and C57BL/6 IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense*. Time points analyzed were; day 7 (1st parasitemic peak), day 28 and day 105 post infection. Graphed IL-4 data was pooled from three independent experiments, resulting in wild type or IL-12 deficient groups of between 4 and 8 mice. Detection limit for IL-4 was 16pg/ml. Statistical significance between *T. congolense* infected C57BL/6 wild type and IL-12 deficient mice was determined by unpaired Student's t-test.

Interleukin-12 deficiency does not influence antibody responses during the acute and chronic stages of *T. congolense* infection.

Humoral responses against infection with *T. congolense* has been shown to be necessary for effective resistance (Magez, Radwanska et al. 2006). However, the exact antibody or combination of antibodies mediating resistance has yet to be determined. Immunoglobulin-M responses were highest during the 1st parasitemic peak compared to the other measured antibodies (Figure 6A). Throughout the acute and chronic stages of infection no overt differences between wild type control and IL-12 deficient mice were found. Although splenectomized mice of different genetic backgrounds showed varying degrees of susceptibility to infection with this parasite (Morrison, Roelants et al. 1978), in C57BL/6 mice impaired IgG2a/3 responses were found and were unable to control *T. congolense* infection (Magez, Radwanska et al. 2006). Immunoglobulin G2a was found to play a role in opsonization of parasites by macrophages (Shi, Wei et al. 2004) and in overall resistance in C57BL/6 mice compared to BALB/c mice (Uzonna, Kaushik et al. 1999). Levels of IgG2c (measured using anti-mouse IgG2a) in IL-12 deficient mice was similar as compared to wild type mice during the acute and chronic stages of infection (Figure 6B). Immunoglobulin G3 plasma levels were found to be increased at day 28 post infection in both IL-12 deficient and wild type mice, Immunoglobulin G2b responses were similar between wild type and IL-12 gene deficient mice at all three time points studied (Figure 6C). The IgG1 antibody responses were highest at days 28 and 105 compared to day 7 post infection (Figure 7A). The slightly increased IgG1 responses in IL-12 deficient mice were variable amongst the different gene deficient groups, and may be attributed to parasite growth and clearance cycles within the hosts. Reduced levels of IgG3 at day 105 post infection were found to be similar to those found at day 7 (Figure 7B). Surprisingly, the pattern of the IgG3 response followed an inverse response to the pro-inflammatory cytokine production.

Overall, these results suggest that in the absence of IL-12 compensatory mechanisms exist which enable effective antibody responses against *T. congolense*.

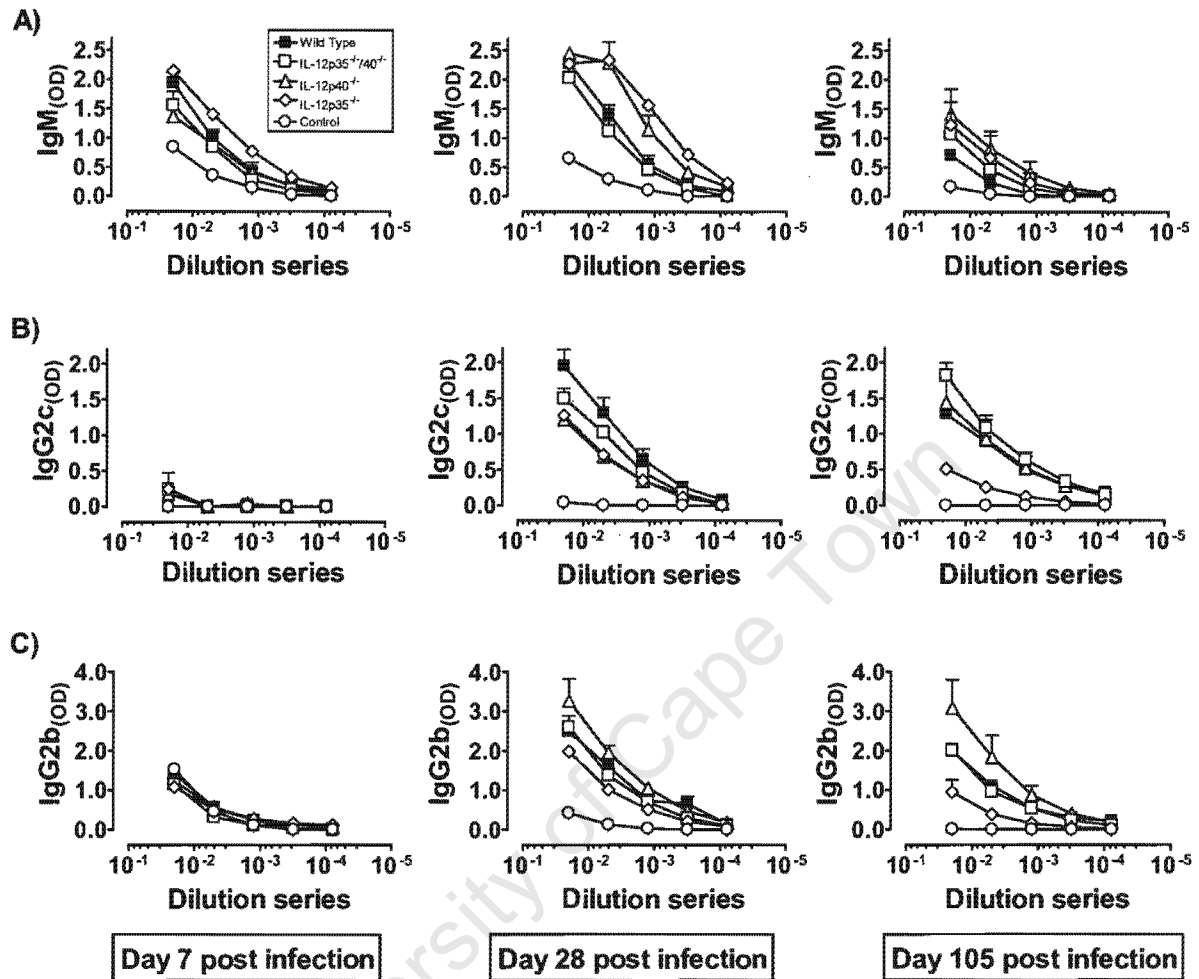


FIGURE 6. Immunoglobulin-M, IgG2c and IgG2b responses are similar in IL-12 gene deficient mice compared to wild type control mice. A) IgM, B) IgG2c and C) IgG2b plasma antibody titers from *T. congolense* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice are shown. First peak (Day 7), post 2nd peak (Day 28) and chronic stage (Day 105) plasma antibody titers from infected mice are represented. Pre-immune serum was used as controls (○). Data points represent mean and $\pm$ SEM from plasma of 3 mice, and are representative of two independent experiments. Statistical significance tests between wild type and IL-12 deficient mouse groups was determined by Student's t-test.

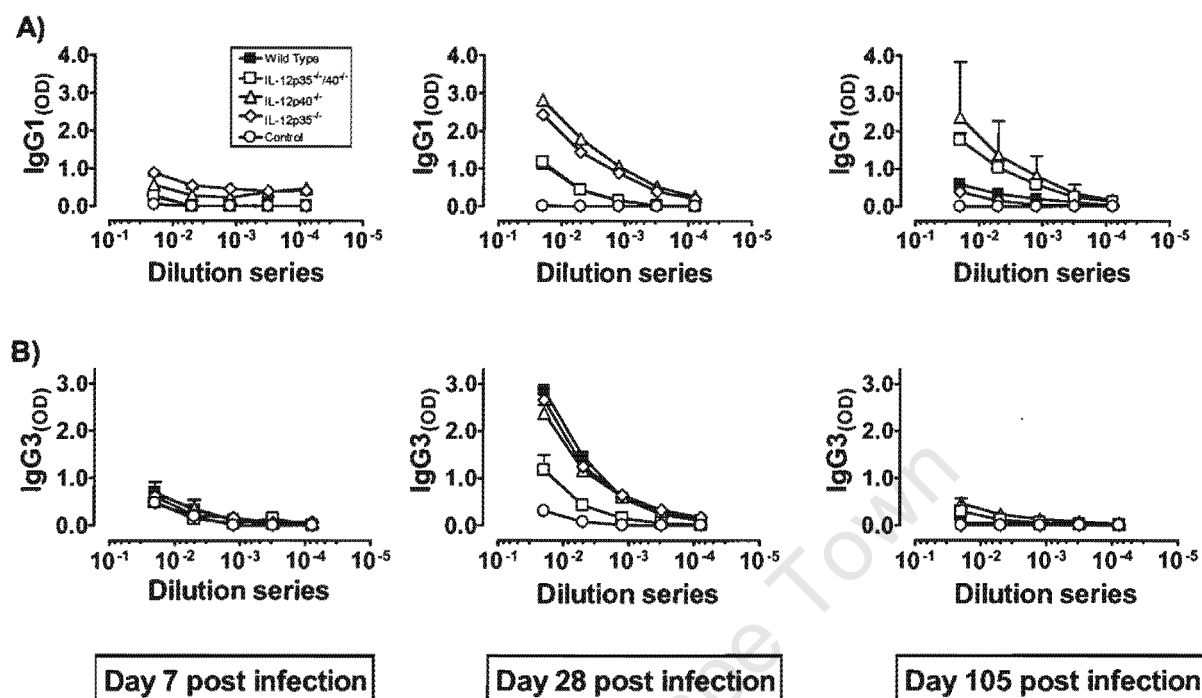


FIGURE 7. The immunoglobulin; IgG3, shows a trend of an inverse response when compared to pro-inflammatory cytokine production. A) IgG1 and B) IgG3 plasma antibody titers from *T. congolense* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}p40^{-/-} (□) mice. Three analysis points are illustrated; namely, first peak (Day 7), post 2nd peak (Day 28) and chronic stage (Day 105) plasma antibody titers. Pre-immune serum was used as controls (○). Data points represent mean and $\pm$ SEM from plasma of 3 mice, and are representative of two independent experiments. Statistical significance tests between wild type and IL-12 deficient mouse groups was determined by Student's t-test.

Inducible nitric oxide expression is present in spleen but is absent in liver sections of IL-12 gene deficient mice in the acute response.

Nitric oxide, which is catalyzed by IFN- γ activated iNOS (Brombacher, Kastelein et al. 2003), is an essential component of the hosts' immune response against *T. congolense* infection (Kaushik, Uzonna et al. 1999) (Magez, Radwanska et al. 2006) (Magez, Radwanska et al. 2007). The role of the spleen is essential in survival and parasitemia control as shown in infected splenectomized mice (Magez, Radwanska et al. 2006). Furthermore Kupffer cells present in the liver have been shown to actively phagocytose trypanosomes in an antibody dependant manner during the 1st parasitemic peak (Shi, Wei et al. 2004). As IL-12 gene deficient mice displayed unimpaired resistance during the 1st parasitemic peak, production of iNOS in both spleen and liver was evaluated, given its role in survival and parasitemia control.

The three IL-12 deficient mouse groups all displayed decreased iNOS production compared to wild type mice at day 105 post infection (Figure 10), but most notably at day 7 post infection in liver sections (Figure 8). At day 28 post infection, however both wild type and IL-12 deficient groups displayed similarly decreased iNOS production (Figure 9). Inducible nitric oxide production in wild type mice (days 7 and 105 post infection) and IL-12 deficient mice (day 105 post infection) was associated with isolated infiltrating inflammatory cells, but was also concentrated in localized areas associated mostly with inflammatory cell foci which were smaller in the case of IL-12 gene deficient mice (data not shown).

Increased concentrations of cd11b stained cells in liver sections were found in wild type mice with lower amounts found in IL-12 deficient mice at days 7 and 105 post infection (Figures 8 and 10). At day 28 post infection the expression of cd11b was mostly absent from liver tissue in both wild type and IL-12 deficient mice (Figure 9). The large patches of iNOS in wild type mice co-localized mainly with large conglomerates of cd11b positive cells at day 7 post infection (Figure 8). Whereas in IL-12 deficient mice, the

fewer and smaller clusters of cd11b cells were mainly negative for iNOS production at day 7 post infection.

In spleen tissue sections, slightly lower but variable iNOS production was detected in IL-12 gene deficient mice compared to wild type mice at both days 7 and 105 post infection (Figures 11 and 12). Inducible nitric oxide synthase production in wild type and IL-12 deficient mice was distributed in localized patches within the red pulp, but with the greatest concentration occurring at the interface of the white and red pulp. Much lower production was present within the white pulp compared to the red pulp in all mouse groups studied.

Macrophages pulsed with *T. congolense* parasites *in vitro* were found to produce little NO, but was markedly up-regulated in the presence of IFN- γ (Kaushik, Uzonna et al. 1999). The number of cd11b stained cells, which include macrophages, in spleen sections were found at similar levels in both wild type and IL-12 deficient mouse groups at days 7 and 105 post infection (Figures 11 and 12). The distribution pattern of cd11b stained cells was the same as for iNOS with localized patches within the red pulp but with the greatest concentration occurring at the interface of the white and red pulp. Very low numbers of cd11b positive cells were present within the white pulp compared to the red pulp in all mouse groups studied. Data for iNOS production and cd11b infiltrating cells were not obtained for spleen tissue at day 28 post infection.

These results demonstrated that IL-12p70 was necessary for the up-regulation of iNOS production in liver tissue during the acute stages of disease, but that IL-12p70 independent iNOS production occurred later during the chronic stages of *T. congolense* infection. In contrast, iNOS production in spleen sections was largely independent of IL-12p70 signaling, and during the acute stage of the disease, may ensure survival in IL-12 deficient mice.

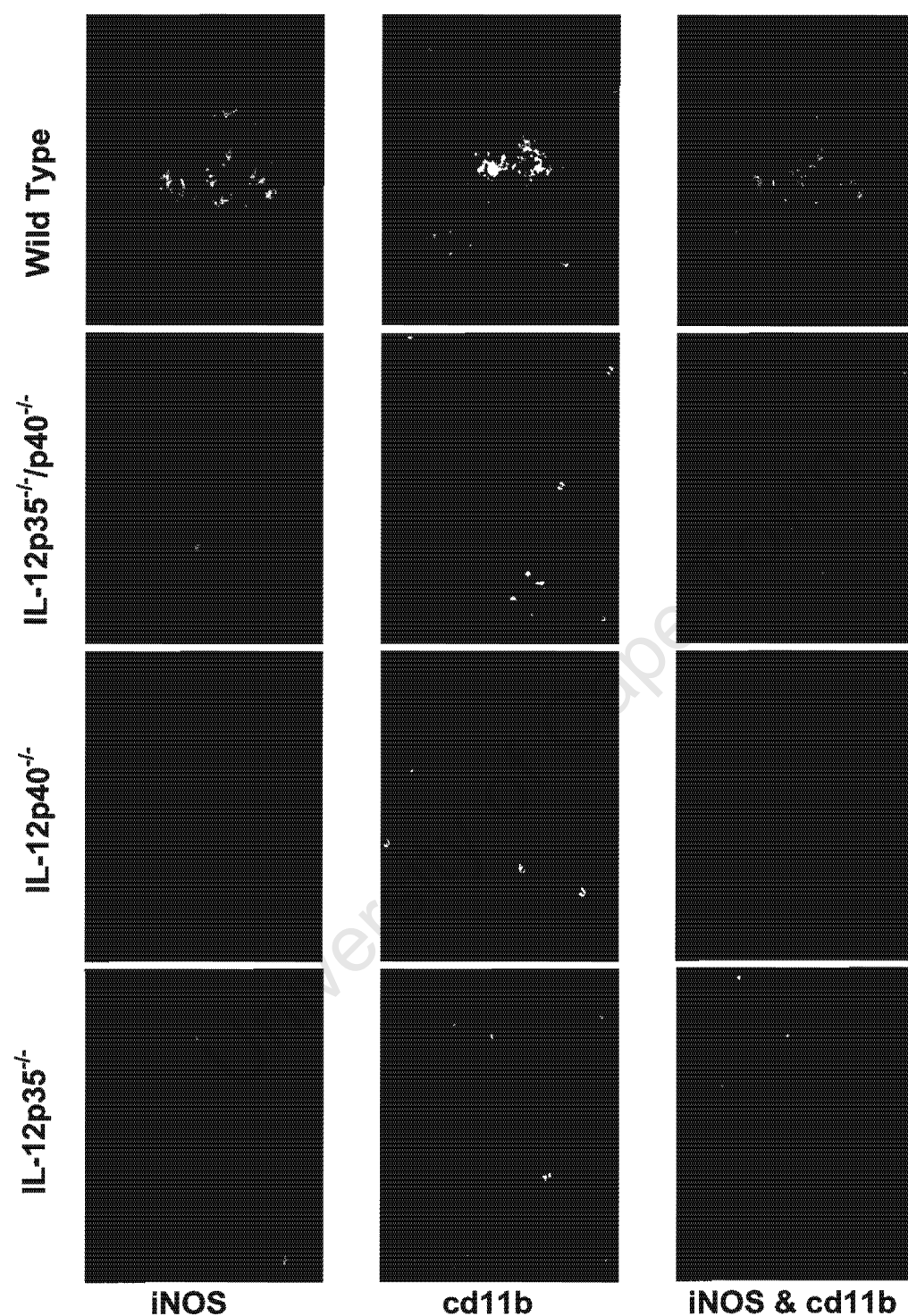


FIGURE 8. Inducible nitric oxide production in liver sections is dependent on IL-12 production during the acute stage of *T. congolense* infection. Representative images of frozen liver sections double stained with mAb against iNOS (green) and cd11b (red) are shown, together with an overlay of both stains. Infected; C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 7 post infection are shown. Areas of inflammatory cell infiltration together with iNOS production are depicted. (Magnification of images: 400×).

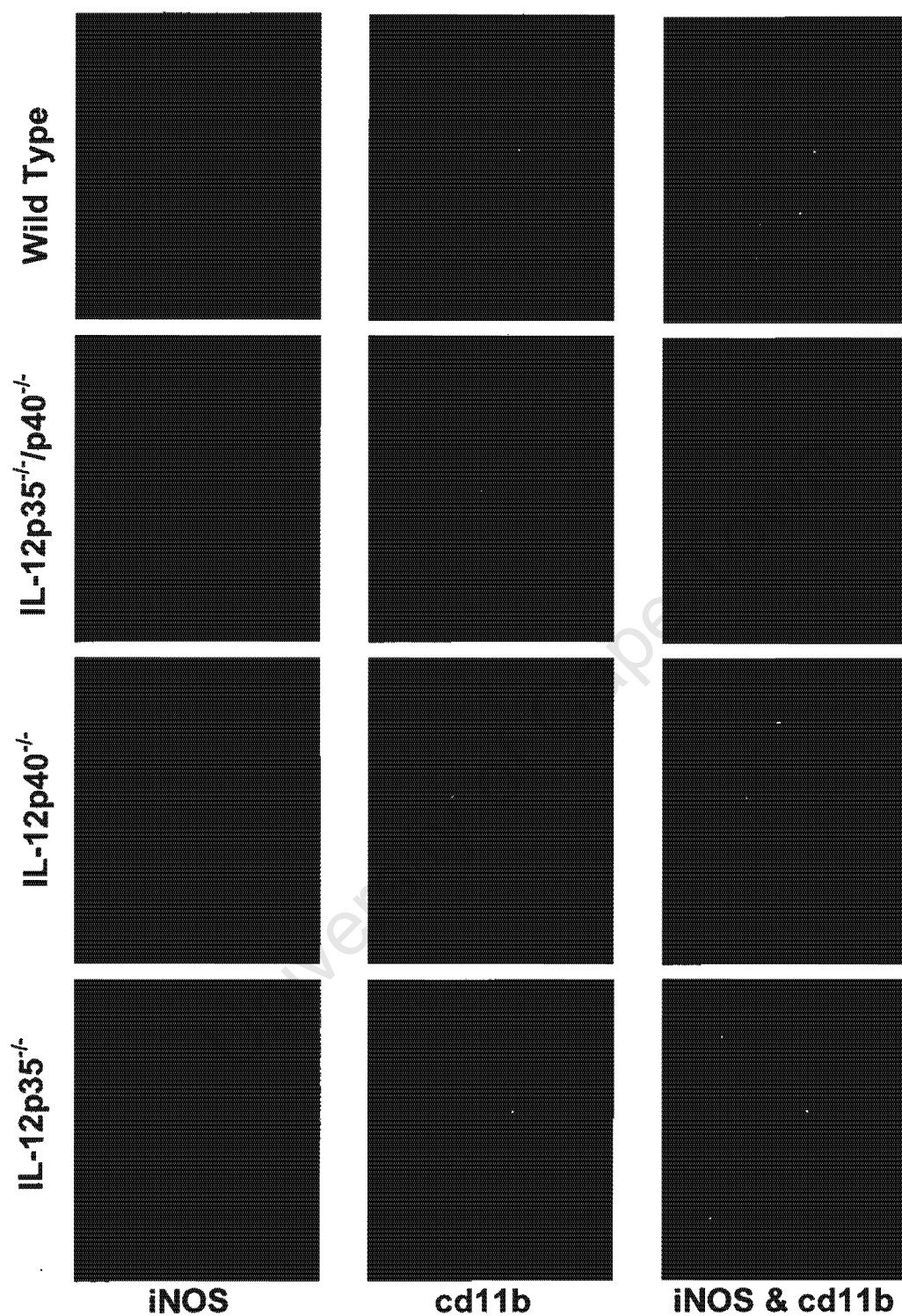


FIGURE 9. Severely decreased iNOS production in liver sections at day 28 post infection in both wild type and IL-12 deficient mice during *T. congolense* infection. Representative images of frozen liver sections double stained with mAb against iNOS (green) and cd11b (red) are shown, together with an overlay of both stains. Infected; C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 28 post infection are shown. Areas of inflammatory cell infiltration together with iNOS production are depicted. (Magnification of images: 200×).

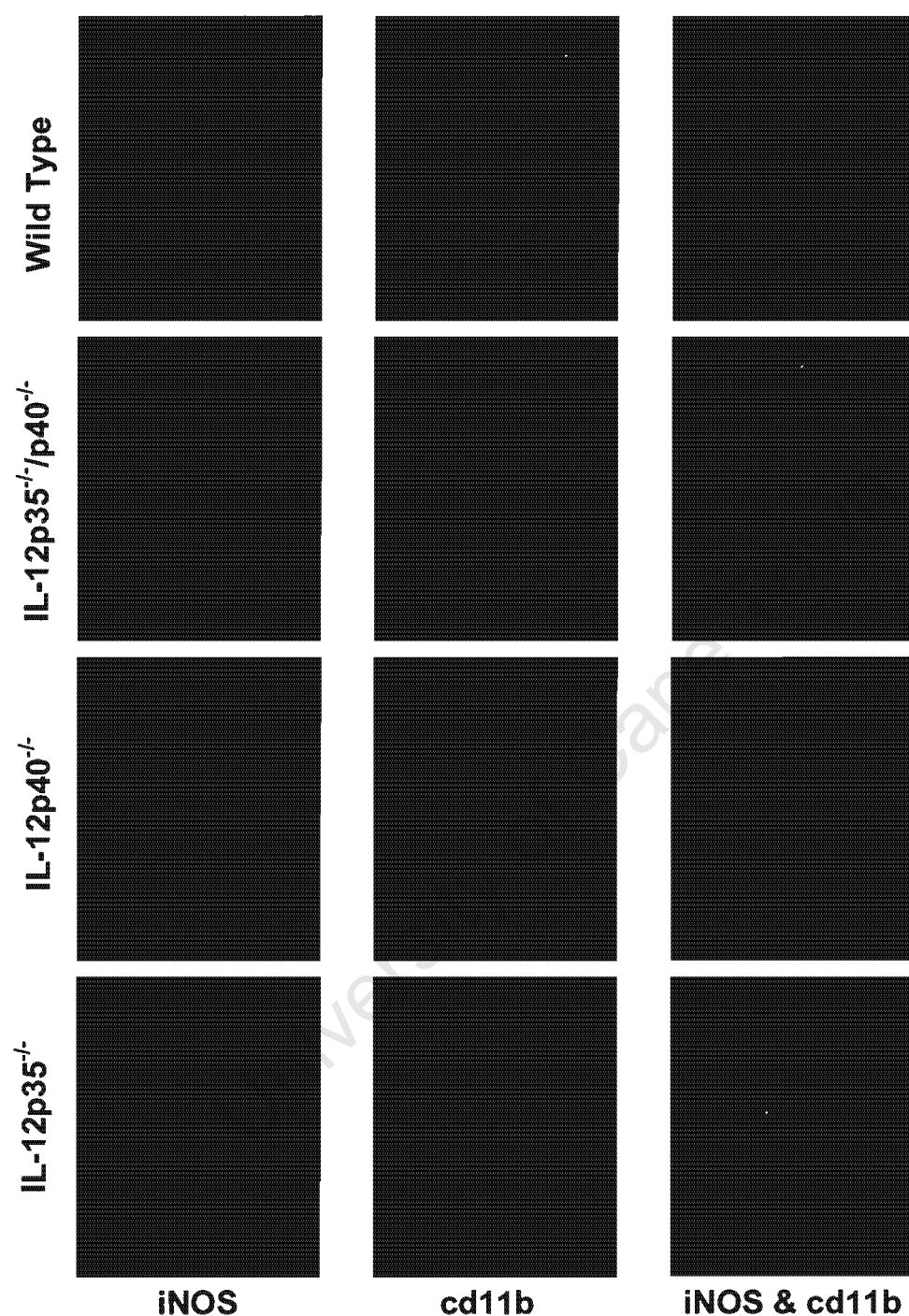


FIGURE 10. Inducible nitric oxide production in liver is slightly reduced in IL-12 deficient and wild type mice during the chronic stage of *T. congolense* infection. Representative images of frozen liver sections double stained with mAb against iNOS (green) and cd11b (red) are shown, together with an overlay of both stains. Infected; C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 105 post infection are shown. Areas of inflammatory cell infiltration together with iNOS production are depicted. (Magnification of images: 200×).

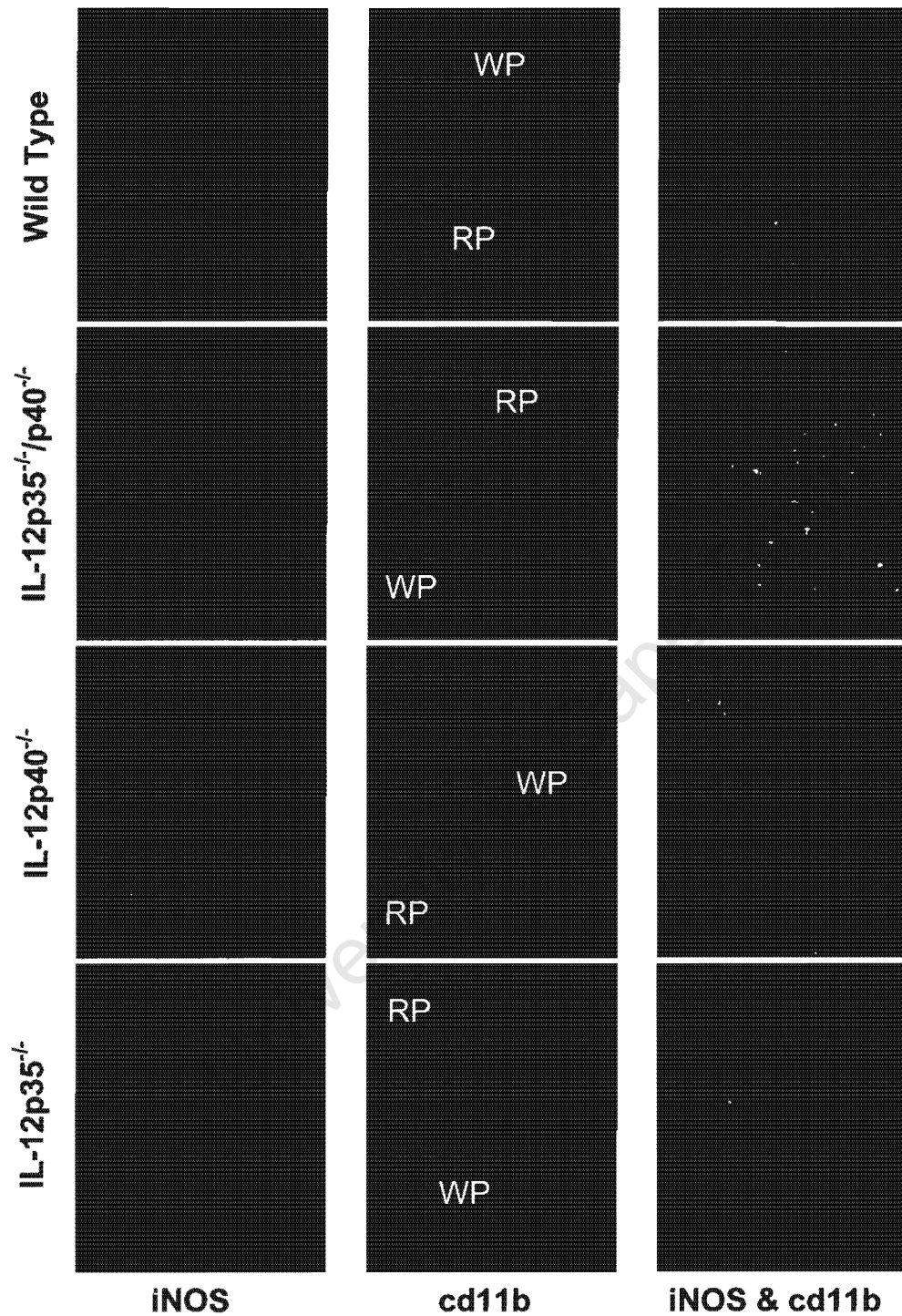


FIGURE 11. Inducible nitric oxide (iNOS) production in spleen is independent of IL-12 during the acute stage of *T. congolense* infection. Representative images of frozen spleen sections double stained with mAb against iNOS (green) and cd11b (red) are shown, together with an overlay of both stains. Infected; C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 7 post infection are shown. White pulp (WP) and red pulp (RP) are illustrated. Areas of inflammatory cell infiltration together with iNOS production are depicted. (Magnification of images: 200×).

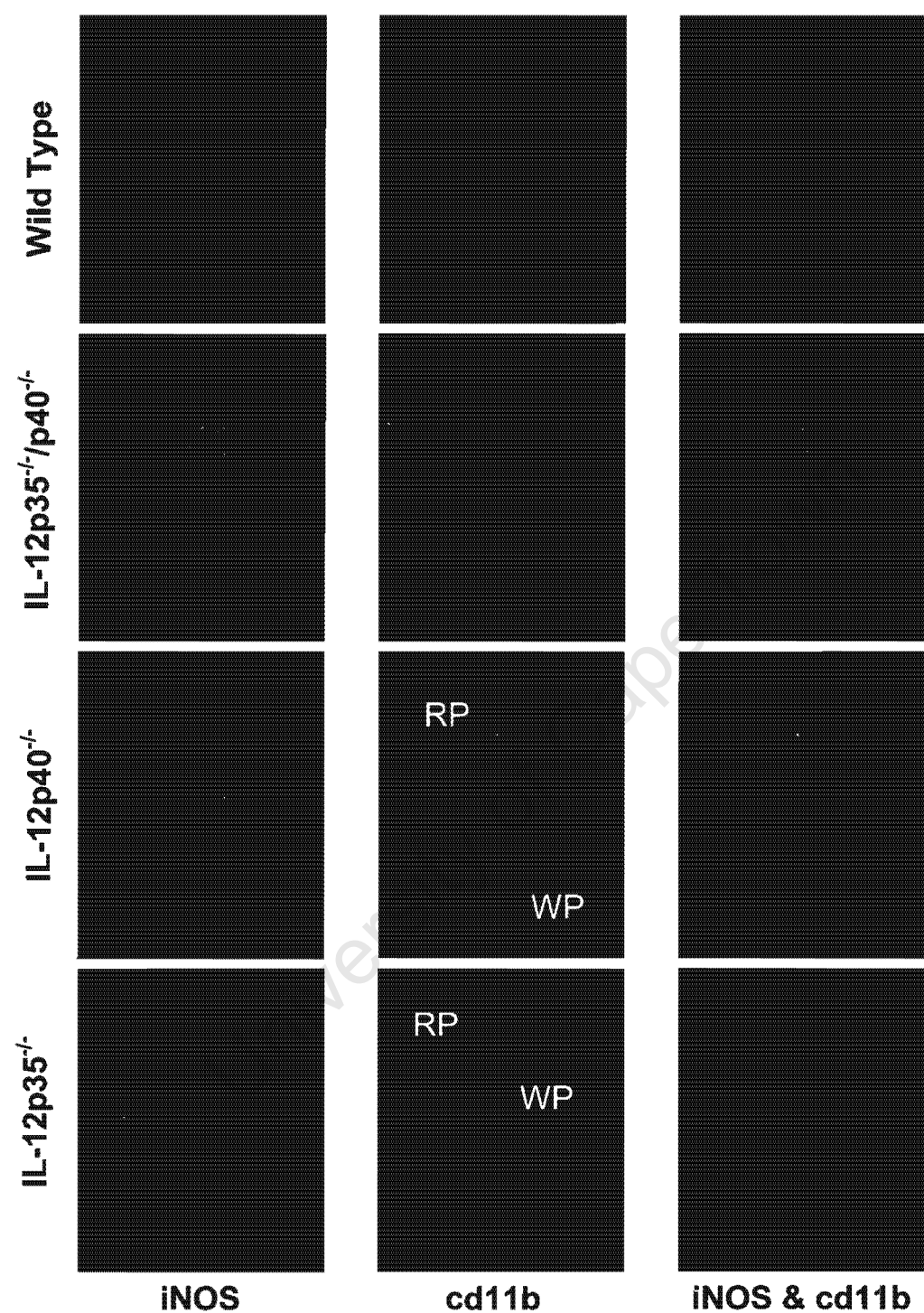


FIGURE 12. Inducible nitric oxide production in spleen is present in both IL-12 deficient and wild type mice during the chronic stage of *T. congolense* infection. Representative images of frozen spleen sections double stained with mAb against iNOS (green) and cd11b (red) are shown, together with an overlay of both stains. Infected; C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 105 post infection are shown. White pulp (WP) and red pulp (RP) are illustrated. Areas of inflammatory cell infiltration together with iNOS production are depicted. (Magnification of images: 200×).

Interleukin-12 gene deficient mice display reduced liver pathology compared to wild type controls during the chronic stage of *T. congolense* infection.

Liver inflammation and hepatomegaly have previously been shown to be a hallmark of *T. congolense* infections in wild type laboratory mice (Morrison, Murray et al. 1982), rats (Igbokwe and Nwosu 1997) and cattle (Naylor 1971) (Valli, Forsberg et al. 1978). Attention was focused onto the liver as this organ is considered to be a major site of trypanosome clearance (Holmes, MacAskill et al. 1979) (Shi, Wei et al. 2005) (Shi, Wei et al. 2004) in a NO dependant manner (Magez, Radwanska et al. 2007). It has been demonstrated that trypanosomes are phagocytosed by Kupffer cells resident in the liver mediated by both IgM and IgG2a monoclonal antibodies specific for VSG with a corresponding increase in production of IL-12p40 and other pro-inflammatory cytokines (Shi, Wei et al. 2004).

In addition, the release of cytokines localized in the liver are implicated in the induction of infection-associated immunopathology (Shi, Pan et al. 2003) (Stijlemans, Baral et al. 2007) with apoptosis of Kupffer cells occurring at the acute stage of infection in BALB/c mice (Shi, Wei et al. 2005) and eventually late stage necrosis in C57BL/6 mice (Kitani, Yagi et al. 2004) (Guilliams, Oldenhove et al. 2007). As IL-12 gene deficient mice displayed enhanced survival compared to wild type mice the extent of the pathological lesions was assessed in liver during the course of infection.

At days 7, 28 and 105 post infection pathological lesions in the liver were noted in all groups of mice studied increasing together with the progression of disease. During the acute stage of *T. congolense* infection (Day 7 post infection) no differences in pathology was observed between wild type and IL-12 deficient mouse groups (data not shown). At day 28 post infection, a slight decrease in overall liver pathology in IL-12 deficient mouse groups compared to wild type groups was beginning to become apparent, but no differences between IL-12 deficient mouse groups were found (Figure 13). Here, IL-12 deficient mouse groups displayed slightly smaller and fewer focal mononuclear cellular

infiltrates compared to wild type mice. Furthermore, fewer mononuclear cellular infiltrates were observed in the sinusoidal spaces of IL-12 deficient mice compared to wild type mice.

In contrast, during the chronic stage of infection (Day 105 post infection), liver sections from IL-12 deficient mice displayed strikingly reduced pathology compared to wild type mice (Figure 14). Liver sections from IL-12 deficient mice displayed significantly smaller and fewer focal mononuclear cellular infiltrates compared to wild type mice. Both IL-12 deficient and wild type mice had similar cellular compositions of focal mononuclear cell infiltrates consisting of activated macrophages with scattered lymphocytes and neutrophils. Subcapsular hemorrhagic infarctions present in the liver were also found in certain wild type mice but not in IL-12 deficient mice. Enlarged sinusoidal spaces were found in IL-12 deficient mice as well as in wild type mice; however mononuclear cell infiltration in these areas was distinctly reduced in IL-12 deficient mice compared to wild type mice. All IL-12 deficient mice displayed a similar reduction of pathological lesions compared to wild type mice regardless of subunit deletion as well as less severely disrupted liver structure compared to wild type mice.

Interleukin-12p70 was therefore implicated in driving pathology in the liver, with the effect more significant during the chronic stages of *T. congolense* infection.

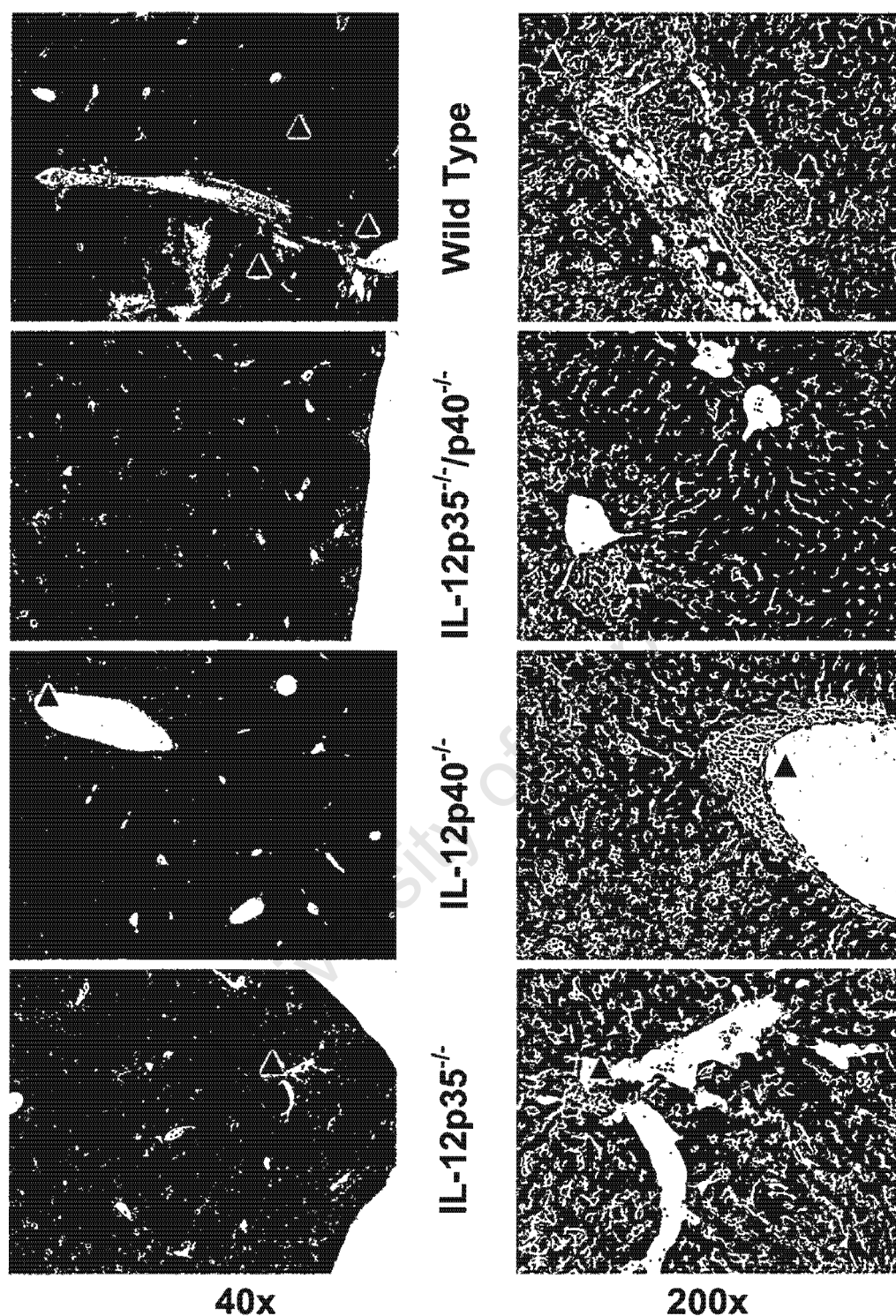


FIGURE 13. Interleukin-12 deficiency results in slightly decreased liver pathology compared to wild type mice at day 28 post infection. Representative images of liver sections stained with hematoxylin and eosin (H&E) are shown of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 28 post infection. Arrowheads indicate regions of inflammatory cell foci. (Magnification of images: 40× and 200×).

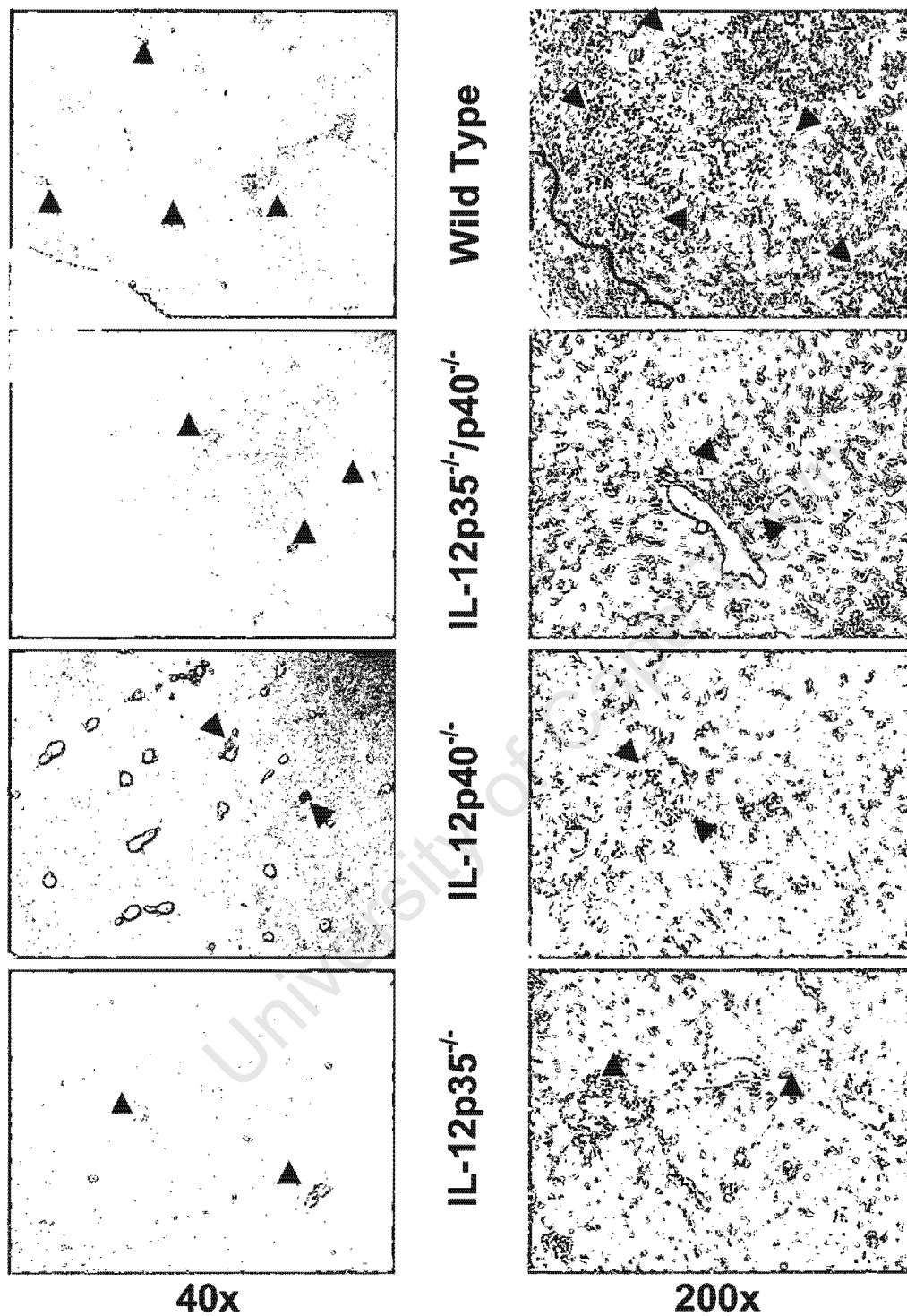


FIGURE 14. Interleukin-12 deficiency results in decreased liver pathology compared to wild type mice at day 105 post infection. Representative images of liver sections stained with hematoxylin and eosin (H&E) are shown of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 105 post infection. Arrowheads indicate regions of inflammatory cell foci and the line denotes a region of an infarct at the bottom left. (Magnification of images: 40× and 200×).

Interleukin-12 gene deficient mice display decreased infection related hepatocellular damage as measured by liver transaminases compared to wild type mice at the chronic stage of infection.

Interleukin-12 deficient mice displayed decreased liver histopathology compared to wild type controls at day 105 post infection with a slight trend emerging at day 28 post infection. Studies have found that in the 1st month of *T. congolense* infection, liver damage has been reported to be marginal in wild type C57BL/6 mice with little liver transaminases detected in serum (Guilliams, Oldenhove et al. 2007), however late stage or chronic levels have not been quantified. The liver transaminase; aspartate aminotransferase (AST) is present in liver parenchyma and catalyzes the transfer of α -amino groups from aspartic acid to α -ketoglutaric acid to yield glutamic acid and oxalacetic acid. Raised levels of AST in blood plasma is a marker for acute liver damage (De Ritis, Coltorti et al. 1956). Therefore, to quantify liver damage in wild type compared to IL-12 deficient mice, levels of AST were determined to address the extent of hepatocellular damage. All IL-12 deficient strains had significantly reduced AST levels compared to wild type mice ($P < 0.05$) at day 105 post infection (Figure 15). These results therefore correlate with the pathology results found in the liver (Figure 14) at day 105 post infection. No significant differences were noted between uninfected IL-12 deficient and wild type mice and similarly in infected mice at day 28 post infection. Taken together these results give evidence that IL-12 drives a strong pro-inflammatory response in *T. congolense* infection that towards the end point of infection results in increased pathology that in turn may cause mortality.

Another liver transaminase namely; alanine aminotransferase (ALT) levels were also measured, however possibly due to processing difficulties the results were inconclusive (data not shown). Levels of lipopolysaccharides (LPS) reported to be increased during trypanosome infections and possibly involved in liver damage (Alafiatayo, Crawley et al. 1993) (Pentreath 1994) were also measured. However levels not higher than the uninfected controls were measured in plasma in both infected IL-12 deficient and wild type mice (data not shown).

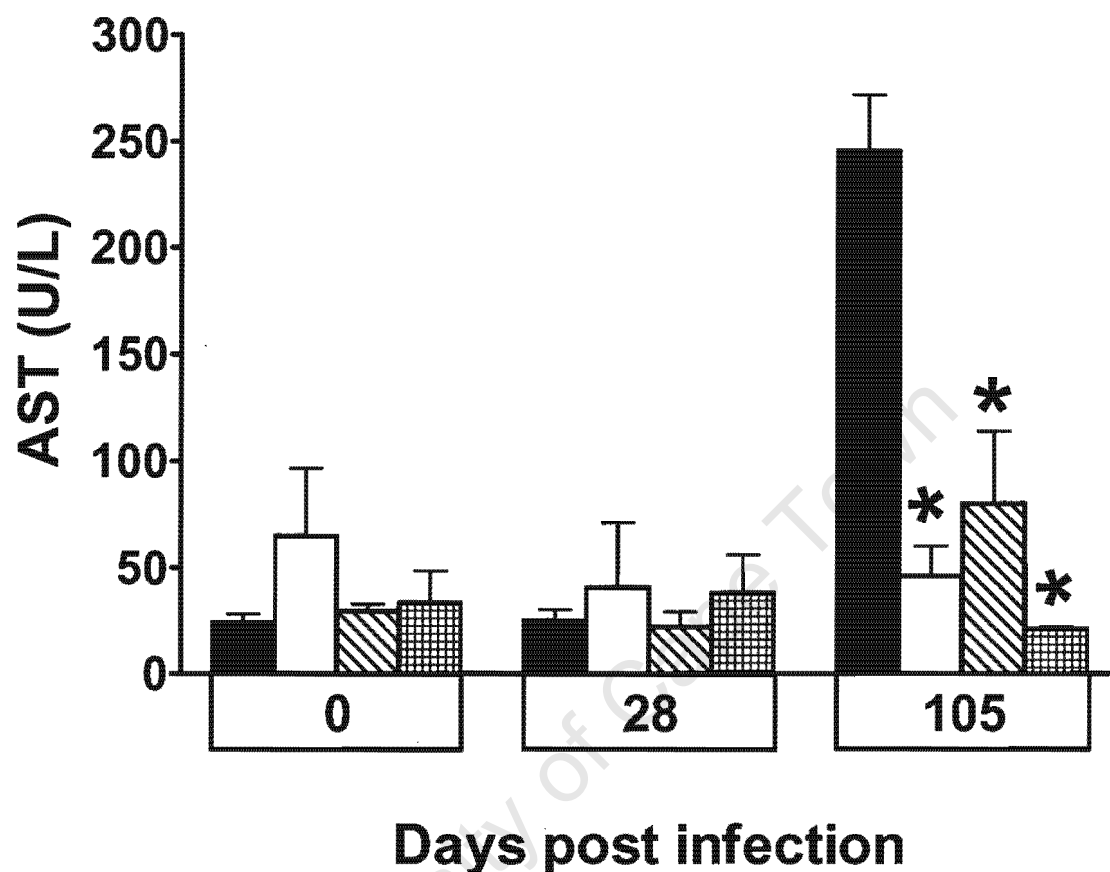


FIGURE 15. Mice deficient in IL-12 have significantly decreased aspartate aminotransferase (AST) levels during infection with *T. congolense* compared to wild type mice during the chronic stage of infection. Aspartate aminotransferase plasma levels were measured in wild type (Filled bars), IL-12p35^{-/-}/p40^{-/-} (Open bars), IL-12p40^{-/-} (Striped bars) and IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense*. Aspartate aminotransferase, an indicator of hepatocellular damage, were determined by the colorimetric reaction resulting from its catalytic activity. Non-infected controls and infected mice at days 28 and 105 post infection were measured for AST plasma levels. Data are from two independent experiments and expressed as means ($\pm$ SEM) from 2-3 individual mice. Detection limit for AST was 6 U/L. Statistical significance between infected wild type and IL-12 deficient mice was determined by unpaired Student's t-test and are indicated by *, $P < 0.05$. nd: not detected.

Both IL-12 gene deficient and wild type mouse groups display a similar extent of pathology in the spleen during the course of *T. congolense* infection.

A critical role for the spleen in resistance to *T. congolense* infection has been reported, with uncontrolled parasitemia and rapid mortality occurring in splenectomized mice (Magez, Radwanska et al. 2006). The spleen was identified as a major source of anti-trypanosome IgGs as well as IFN- γ , thus the exclusive role of anti-trypanosome IgGs was not fully examined. A common finding in trypanosome infections, including *T. congolense*, is polyclonal B cell activation occurring in the spleen coinciding with increased immunoglobulin levels. Interestingly, it has been postulated that the polyclonal lymphocyte activation is induced by trypanosome parasites themselves (Urquhart, Murray et al. 1973) (Greenwood 1974). In *T. congolense* infection the spleen has also been suggested to be the site of intravascular trypanosome phagocytosis as well as erythrophagocytosis (Brown and Losos 1977) (Omeke and Ugwu 1991). The role of the spleen in resistance was also examined in different inbred mouse strains and found to have a more detrimental effect in susceptible BALB/c/A mice compared to more resistant C57BL/6J mice (Morrison, Roelants et al. 1978). Thus, given the essential role of the spleen in *T. congolense* infection, the function of IL-12 was investigated in terms of the induction of pathology.

During the progression of *T. congolense* infection, in both wild type and IL-12 deficient mouse groups examined, splenomegaly was also found (data not shown). This was also demonstrated in C3H inbred mouse strains where a 20-fold increase in splenic weight was recorded (Morrison, Murray et al. 1981). At day 7 and 28 post infection pathological lesions were noted in both wild type and IL-12 deficient mice but together with the progression of disease the extent became more marked. At both day 7 and 28 post infection no differences in the extent of pathological lesions were found between IL-12 deficient mouse groups as well as between these and wild type mice. A lack of discrete germinal centers was also observed throughout infection in all mouse groups observed.

At day 105 post infection (Figure 17) the extent of the pathological lesions were greatest compared to days 7 and 28 post infection (Figure 16) but with similar characteristics. Overall splenic structure was disrupted in all mouse groups studied. The spleen white pulp follicles from infected wild type and IL-12 deficient mice showed marked lymphocyte depletion and disorganization. Wild type mice displayed only a moderately increased mononuclear cell infiltrate and tissue structure disruption of the red pulp region compared to IL-12 gene deficient mice. Therefore IL-12 seems not to play a major role in the induction of pathological lesions in the spleen.

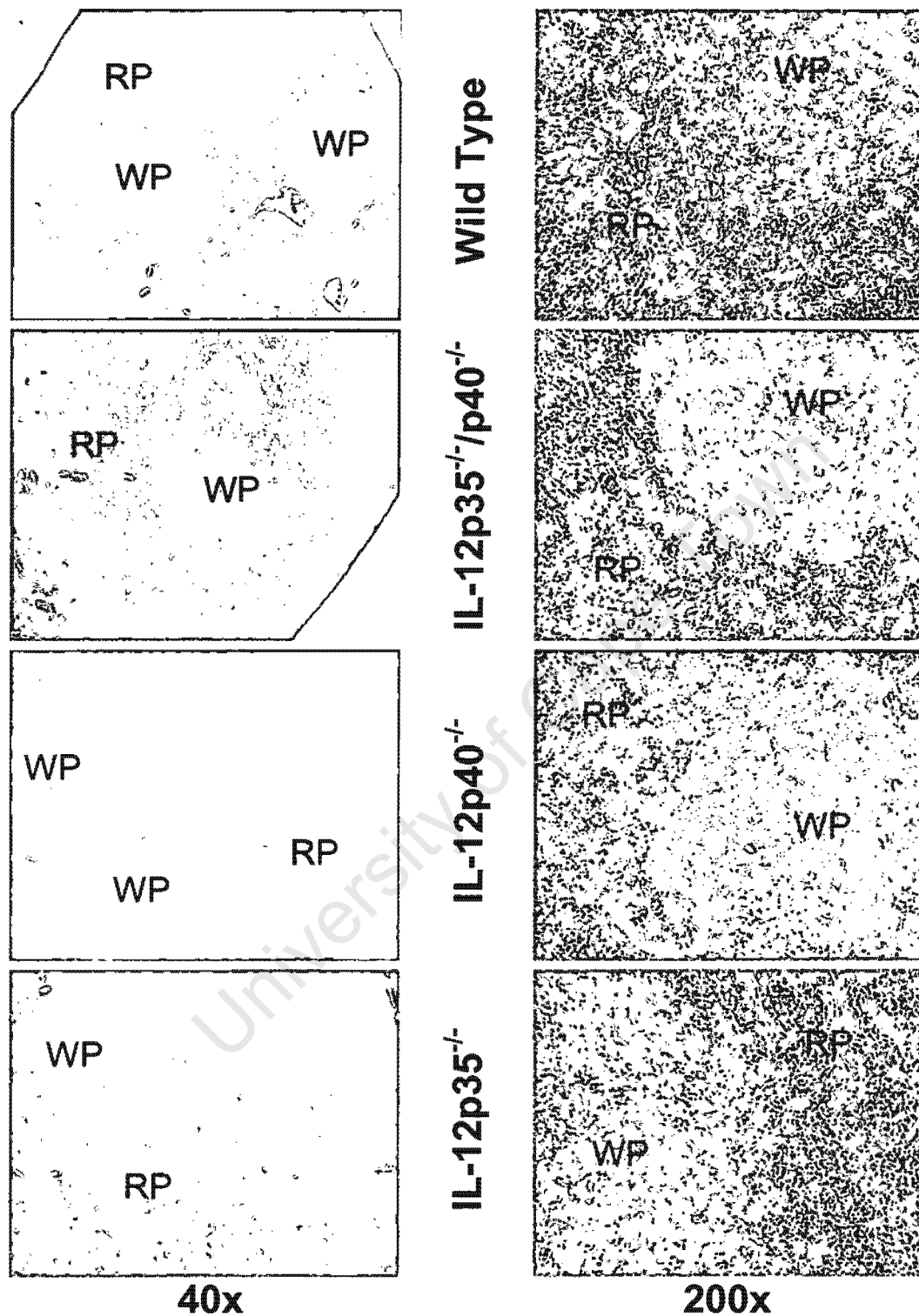


FIGURE 16. Both IL-12 deficient and wild type mice display similar splenic pathology at day 28 post infection with *T. congolense*. Representative images of spleen sections stained with hematoxylin and eosin (H&E) are shown of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 28 post infection. WP and RP denote white pulp and red pulp, respectively. (Magnification of images: 40× and 200×).

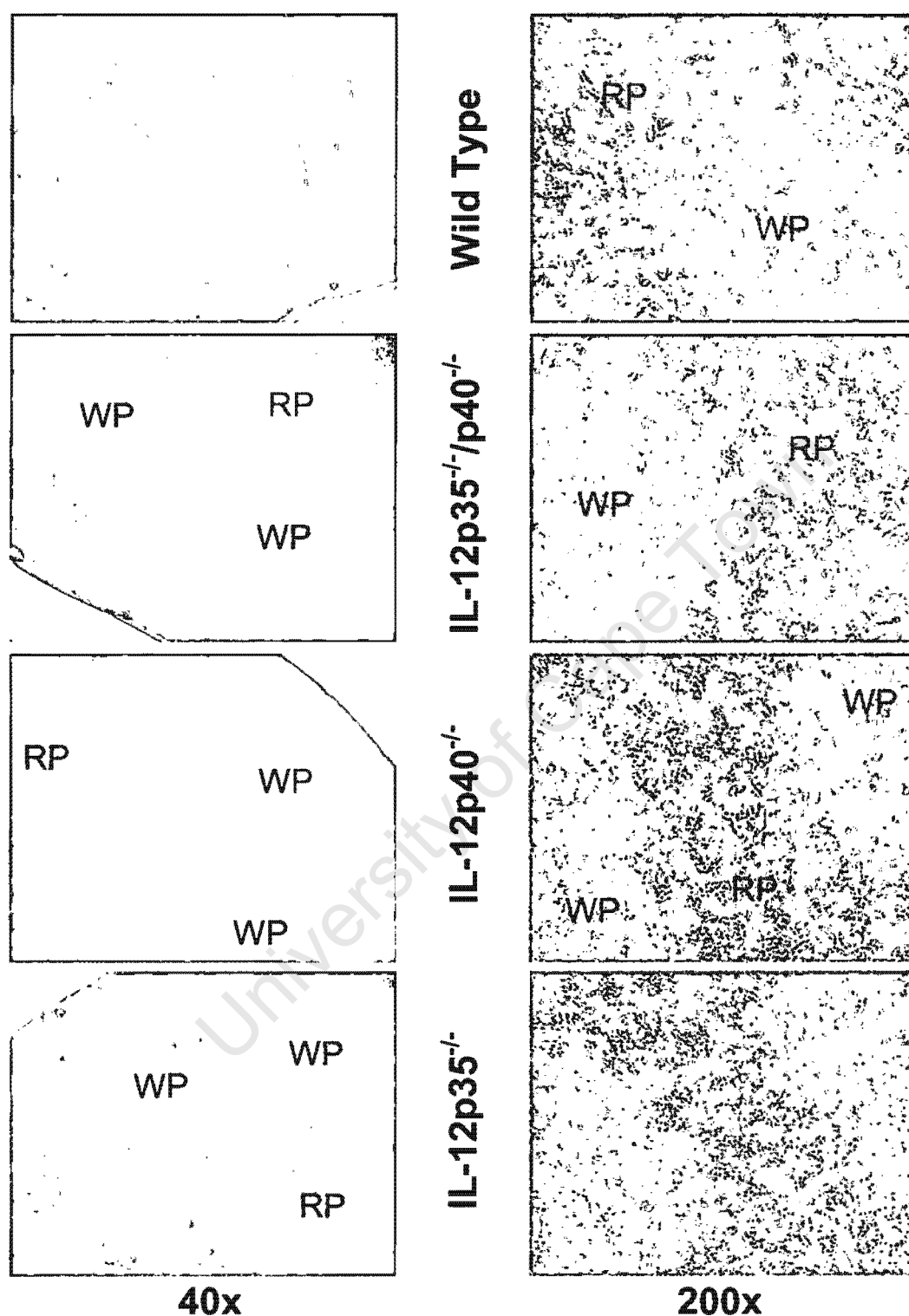


FIGURE 17. Interleukin-12 deficiency results in slightly decreased splenic pathology compared to wild type mice at day 105 post infection. Representative images of spleen sections stained with hematoxylin and eosin (H&E) are shown of infected C57BL/6 wild type, C57BL/6 IL-12p35^{-/-}/p40^{-/-}, C57BL/6 IL-12p40^{-/-} and C57BL/6 IL-12p35^{-/-} mice at day 105 post infection. WP and RP denote white pulp and red pulp, respectively. (Magnification of images: 40× and 200×).

Interleukin-4 and IL-4R α do not play a role in survival in *T. congolense* infections.

The role of IL-4 and IL-4R α have been previously investigated in BALB/c mice and found not to play a role in survival in these mice (Noel, Hassanzadeh et al. 2002). However in BALB/c mice survival time is limited to the 1st parasitemic peak and thus omits the chronic stage of disease. Therefore the role of IL-4 and IL-13 was investigated in C57BL/6 mice to determine, if in the absence of IL-12, that IL-4/13 may mediate protection. Furthermore the switch over from a principally type 1 cytokine environment in the early stages of infection to a type 2 cytokine environment in the chronic stages of infection is associated with resistance to *T. congolense* (Baetselier, Namangala et al. 2001). In addition, conflicting reports on the role of IL-4 in resistance to different *Trypanosoma* species have been reported (Bakhiet, Jansson et al. 1996) (Inoue, Inoue et al. 1999) (Schopf, Filutowicz et al. 1998). Therefore the role of IL-4 as well as IL-13, which is able to partially compensate for certain functions of IL-4 (Brombacher 2000) (Muller, Stenzel et al. 2007), was investigated. Although this was a pilot study performed once, these data indicate that IL-4 and IL-13 do not play a role in survival in *T. congolense* infection with mean survival time of wild type, IL-4^{-/-} and IL-4R α ^{-/-} mice; 137, 140 and 140 days respectively.

The increased mean survival time in wild type mice in this experiment (137 days) (Figure 18) was longer than that of the wild type mice in the IL-12 experiment (97 days) (Figure 1). This discrepancy was attributed to the time taken to infect the mice housed at the animal facility as the parasite rapidly loses its infectivity after thawing. Therefore, control and experimental mouse groups were either infected at the same time or rapidly one after the other.

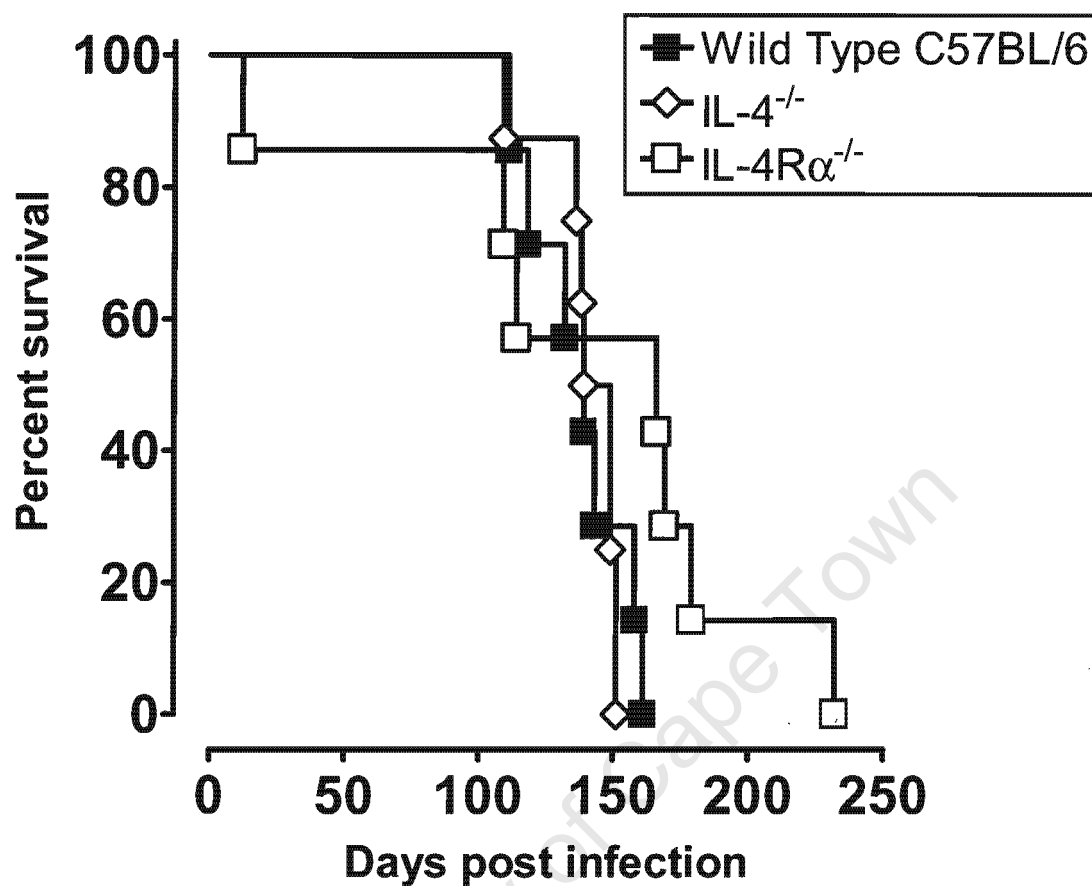


FIGURE 18. Interleukin-4 and IL-4Rα (IL-4 and IL-13) do not play a pivotal role in survival during *T. congolense* infection. Mortality of *T. congolense* infected C57BL/6 wild type (■), C57BL/6 IL-4^{-/-} (◇) and C57BL/6 IL-4Rα^{-/-} (□) mice are shown. Mean survival time of wild type, IL-4^{-/-} and IL-4Rα^{-/-} mice are; 137, 140 and 140 days respectively. Data are of 7-8 mice per group from one experiment. Statistical analysis of mortality was done using the Kaplan Meier Test.

Myeloid differentiation factor 88 is essential for survival in *T. congolense* infections.

To address the involvement of possible upstream signaling molecules relative to IL-12 in the activation of the immune response against trypanosomiasis, the role of MyD88 was investigated (Seki, Tsutsui et al. 2001) (Adachi, Tsutsui et al. 2001). Myeloid differentiation factor 88 dependent and independent activation pathways have been found, with the former regulating genes encoding inflammatory cytokines and the latter regulating a subset of LPS-inducible genes which are also known to be IFN inducible (Kawai, Adachi et al. 1999) (Kawai, Takeuchi et al. 2001). Myeloid differentiation factor 88 deficient mice have been investigated in *T. b. brucei* infection and found to be susceptible, displaying increased parasitemia burden and decreased survival (Drennan, Stijlemans et al. 2005). The significantly reduced survival of MyD88 deficient mice compared to wild type mice (Figure 19) ($P < 0.001$) are similar to those of IL-12 deficient mice (Figure 1), in that they were able to survive sometime after the 2nd parasitemic peak. The results found in the *T. congolense* study are unlike *T. b. brucei* in that survival of MyD88 deficient mice is limited to the onset of the 2nd parasitemic peak with a mean survival of 14 days compared to 134 for the wild type mice. Myeloid differentiation factor 88 deficient mice in the *T. congolense* and *T. evansi* (Chapter III, Figure 12A) studies however share a similar mortality phenotype. Although parasitemia data was not obtained a blood smear of the MyD88 deficient mice prior to mortality showed greatly elevated parasite burden compared to controls (data not shown).

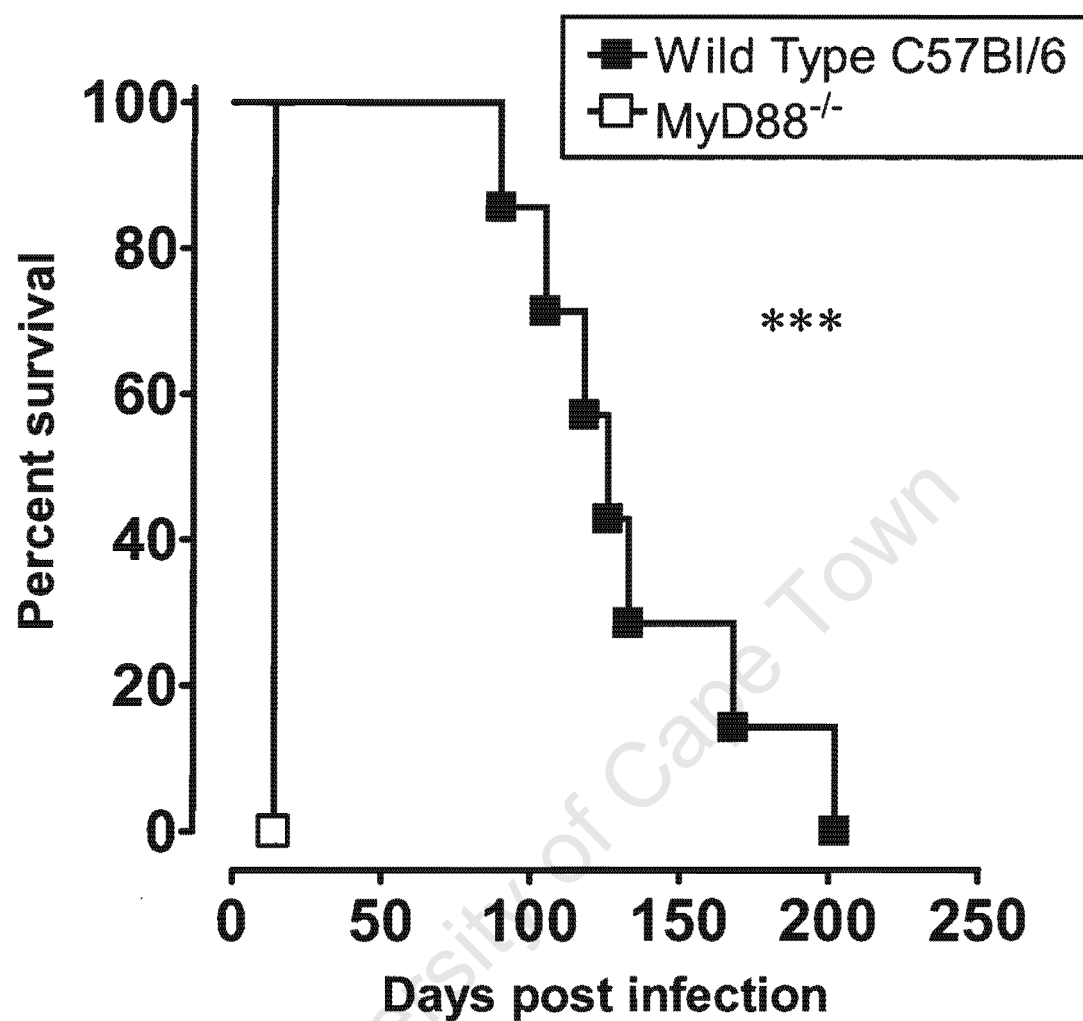


FIGURE 19. MyD88 plays a pivotal role in survival during *T. congolense* infection. Mortality of *T. congolense* infected C57BL/6 wild type (■) and C57BL/6 MyD88^{-/-} (□) mice are shown. Mean survival time of wild type and MyD88^{-/-} mice are; 134 and 14 days respectively. Data are of 7 mice per group from one experiment. Statistical analysis of mortality was done using the Kaplan Meier Test and are presented as; ***, $P < 0.001$.

Preliminary results indicate that IL-18 does not play a pivotal role in survival, parasitemia and the induction of anemia during *T. congolense* infection.

These preliminary results are from a pilot study assessing the impact of the loss of IL-18 in *T. congolense* infection, as the role of this cytokine in initiating Th1 responses in trypanosomiasis has not been investigated. This study was performed as IL-18 shares an intra-cellular signaling pathway with MyD88 (Adachi, Kawai et al. 1998) (Akira, Takeda et al. 2001) and may also initiate a Th1 response in the absence of IL-12 (Mansfield and Paulnock 2005) (Namangala, Sugimoto et al. 2007) (Muller, Kohler et al. 2001). This study was performed once and due to unforeseen circumstances was terminated prior to the endpoint of infection. However, these results do suggest that IL-18, using the IL-1 receptor related protein deficient (IL-1Rrp) mice, does not influence the onset of mortality (Figure 20A). Also, parasitemia was not significantly affected in IL-18 deficient mice during the recorded infection period (Figure 20B). The development of anemia in IL-18 gene deficient mice, although largely not significant, tended to be less severe compared to wild type control mice (Figure 20C). A study with greater resolution and more time points may clarify the involvement of IL-18 in this aspect of pathology in *T. congolense* infection. Nevertheless, as IL-18 deficient mice survive acute infection whereas MyD88 deficient mice die, it suggests that TLR(s) signaling is necessary for survival in *T. congolense* infection.

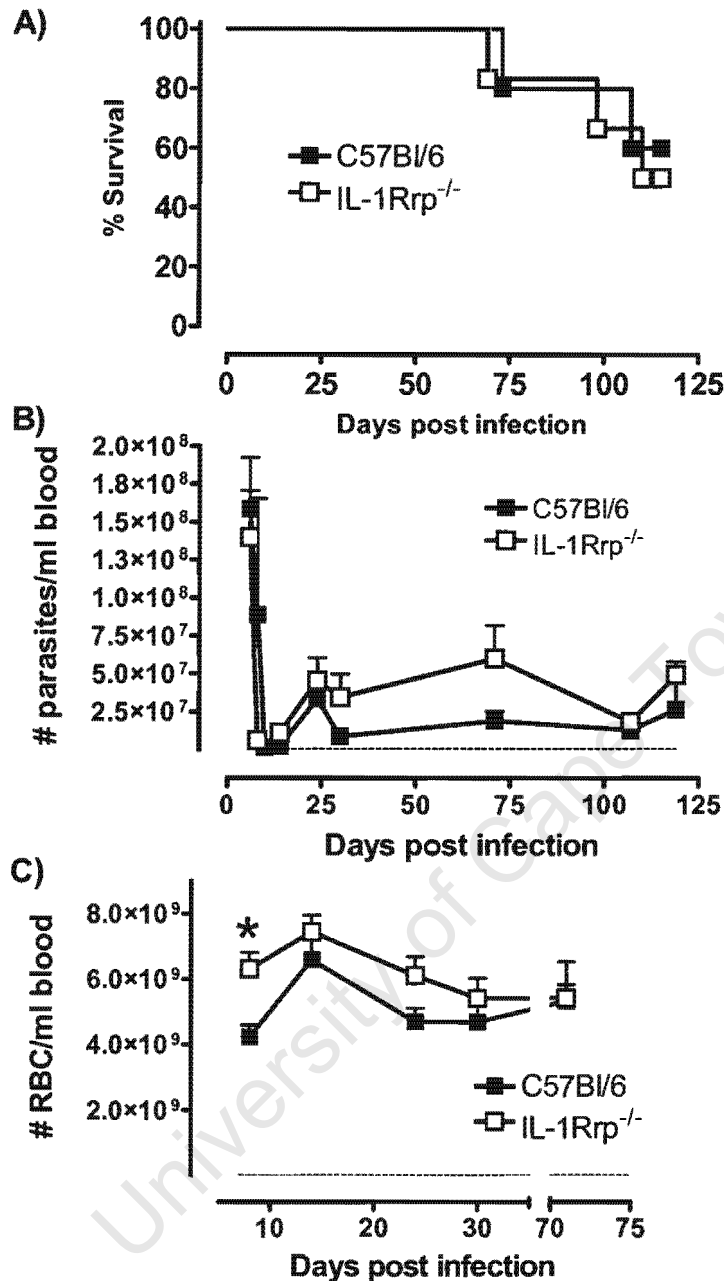


FIGURE 20. Interleukin-18 does not play a pivotal role in survival, parasitemia and the induction of anemia during *T. congolense* infection. Mortality (A), parasitemia (B) and anemia profiles (C) of *T. congolense* infected wild type (C57BL/6) (■) and IL-1Rrp^{-/-} (□) mice are shown. Parasitemia and RBC concentration was determined from peripheral blood obtained from tail snips with a lower detection limit (dotted line) of; 8.0×10^5 parasites/ml and 1.6×10^7 RBC/ml, respectively. Data points are the mean ($\pm$ SEM) from 5 individual mice from one experiment. Statistical significance tests on parasitemia and anemia between wild type and IL-1Rrp deficient mice was determined by Student's t-test and are presented as; *, $P < 0.05$. Statistical analysis of mortality was done using the Kaplan Meier Test.

DISCUSSION

The finding of increased survival with similar parasitemia burden in IL-12p35^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} mice in comparison to wild type mice shows evidence that IL-12p70 but not IL-23 or the IL-12p80 homodimer is detrimental during experimental murine *T. congolense* infection. We may eliminate a role for the IL-12p80 homodimer and IL-23 in *T. congolense* infection as no phenotypic differences were found between IL-12p35^{-/-} deficient mice, which produce both IL-12p80 homodimer and IL-23, and IL-12p35^{-/-}/p40^{-/-} mice which lack the IL-12p40 related products (Brombacher, Kastelein et al. 2003).

Interleukin-12 is important for a rapid activation of IFN- γ -producing cells in order to promote protective Type 1 responses, including T helper 1 differentiation and classical macrophage activation. The latter induces both IFN- γ and TNF production necessary for optimal iNOS synthesis (Brombacher, Kastelein et al. 2003). Although global deletion of either TNF (Iraqi, Sekikawa et al. 2001) (Naessens, Kitani et al. 2004) or IFN- γ (Magez, Radwanska et al. 2006) resulted in early mortality and uncontrolled parasitemia in *T. congolense* infections, suboptimal production of IFN- γ due to IL-12-independent pathways (Magram, Sfarra et al. 1996) during early and late time points had no deleterious effects on parasitemia control or survival in *T. congolense* infected IL-12 deficient mice. Thus, IL-12 dependent IFN- γ production is dispensable during *T. congolense* infection.

Nitric oxide catalyzed by iNOS and essential for parasitemia control and survival in *T. congolense* infection, is a highly effective killing effector radical for both intra- and extracellular pathogens (Magez, Radwanska et al. 2006). Interleukin-12 deficient mouse strains infected with *T. congolense* produced residual iNOS, localized in the spleen, probably due to the presence of IL-12-independent IFN- γ . Spleen derived iNOS seemed

to be sufficient for the control of parasitemia and survival, but the low levels did not induce drastic histopathology, especially in the liver.

Nitric oxide production in *T. congolense* infection is induced by IFN- γ and has an in vitro trypanostatic activity as demonstrated on parasites (Kaushik, Uzonna et al. 1999). NO deficient mice displayed increased susceptibility to *T. congolense* infection in terms of parasitemia and mortality (Magez, Radwanska et al. 2006). During the initial parasitemic peak, NO was severely depleted in liver sections of IL-12 deficient mice but with comparable expression in the spleen as seen in wild type mice. It has been suggested by Shi et al., 2003 that IFN- γ produced in the spleen is disseminated via the circulation and may in turn activate Kupffer cells present in the liver. Possibly, in IL-12 deficient mice the reduced circulating spleen derived IFN- γ during the 1st peak of infection may be insufficient to adequately stimulate Kupffer cells resident in the liver to produce NO as seen in wild type mice.

In contrast to the acute stage of infection, at day 105 post infection, NO was detected in liver sections in all IL-12 deficient mice but not to the same extent as found in wild type mice. Although high NO levels are associated with resistance in *T. congolense* infection (Kaushik, Uzonna et al. 1999) it could be postulated that persistent NO production in the liver as seen in wild type mice results in the observed increased pathological lesions by its induction of apoptosis and the formation of toxic radicals (Brune, Messmer et al. 1995). Similarly, slightly reduced extent of pathological lesions was seen in the spleen of IL-12 deficient mice compared to wild type mice, coinciding with a similar slightly decreased production of NO in these mouse groups. Hepatocyte necrosis and macrophage apoptosis has been demonstrated in liver of *T. congolense* infected BALB/c mice and was attributed to high levels of IFN- γ which in turn is responsible for NO up-regulation (Magez, Radwanska et al. 2006). It could be speculated that the decreased IFN- γ plasma levels found in IL-12 deficient mice resulted in reduced NO expression in liver and in turn results in reduced hepatopathology.

The detrimental role of IL-12 was seen to be mainly manifesting in liver pathology in accordance with studies associating liver pathology with mortality in *T. congolense* infection (Guilliams, Oldenhove et al. 2007) (Shi, Pan et al. 2003). The presence of immunosuppressive IL-10 in infected IL-12 deficient mice in combination with reduced IFN- γ production may explain the reduced liver histopathology and increased survival as apposed to wild type mice (Shi, Pan et al. 2003) (Guilliams, Oldenhove et al. 2007). Together, these results indicate that a qualitative rather than quantitative cytokine response to *T. congolense* infection would translate into effective parasite control and survival of the host.

In the BALB/c and C57BL/6 mouse models the neutralization of IL-10 induced the formation of enlarged capillaries and necrotic lesions in the liver together with significantly increased plasma levels of IL-6, IL-12p40 and IFN- γ (Shi, Pan et al. 2003). Infected IL-10 deficient mice display significantly increased IFN- γ and TNF production from cultured spleen and liver cells as well as AST/ALT serum levels (Guilliams, Oldenhove et al. 2007). Early mortality was shown in IL-10 deficient (Guilliams, Oldenhove et al. 2007) and in IL-10 neutralized mice, with IFN- γ implicated in the induction of mortality (Shi, Pan et al. 2003) (Shi, Wei et al. 2007). The results of this study parallels those of Shi et al., 2003 in that IL-12 deficient mice with severely low levels of IFN- γ throughout the infection period would have cumulative effects resulting in reduced lesions in the liver, together with the presence of similar levels of IL-10 in all mouse groups. Indeed, this hypothesis would also explain the enhanced survival time of IL-12 deficient mice compared to wild type control mice.

Similar findings have been reported implicating IL-12 in promoting hepatic immunopathology in *Leishmania donovani* (Satoskar, Rodig et al. 2000), *Plasmodium berghei* (Adachi, Tsutsui et al. 2001) and in *Schistosoma mansoni* infection models (Araujo, Bliss et al. 2001). Together, these results suggest that IL-12 can play a role in pathogenesis during infection by its induction of pro-inflammatory immune responses, as demonstrated in *T. congolense* infection.

The results found in the present study are in striking contrast to the known beneficial role of IL-12 observed in *T. b. brucei* infection, where IL-12 is found to be essential for parasite control and survival (Barkhuizen, Magez et al. 2007). A reason for these differences may be that, as *T. congolense* and *T. b. brucei* belong to different subgenus's, the host may rely on different immune effector molecules for resistance. Downstream of the IFN- γ signaling pathway, NO is induced which is essential for resistance to *T. congolense* (Magez, Radwanska et al. 2006) and was detected in infected IL-12 deficient mice. In contrast to *T. congolense* infection the downstream mechanisms of IFN- γ in *T. b. brucei* (Hertz and Mansfield 1999) remains to be elucidated and, as our previous results demonstrated, the mechanism is also dependent on IL-12 signaling (Barkhuizen, Magez et al. 2007).

Furthermore, the induction of TNF in *T. b. brucei* infection is largely dependant on IL-12 signaling (Barkhuizen, Magez et al. 2007), however in *T. congolense*, production was only slightly impaired. As TNF is necessary for parasitemia control in both *T. b. brucei* and *T. congolense* infections, this may also explain the differences in terms of parasitemia control in IL-12 deficient mice infected with either parasite.

A surprising finding in terms of increased survival in IL-12 deficient mice was that this was independent of parasitemia control as no differences were detected between wild type and IL-12 deficient mice. A genetic analysis has illustrated a positive correlation between the efficiency of clearance of the 1st parasitemic peak and survival in *T. congolense* infections (Ogunremi and Tabel 1995). As no differences in parasitemia were found between IL-12 deficient and wild type control mice, this may suggest that IL-12 plays a role during the more chronic infection period as apposed to very early in infection where this study focused. This may be supported by the long-term bovine *T. congolense* infection model where upregulation of IL-12p40 mRNA transcripts in unstimulated PBMCs was found from day 21 post infection (Mertens, Taylor et al. 1999).

Residual IFN- γ responses seem also to be sufficient for optimal type 1 responses, as IL-12 deficient mice produced similar levels of VSG-specific IgG2c antibodies during *T.*

congolense infection as wild type mice, a result also found in *T. b. brucei* and *T. evansi* infection (Barkhuizen, Magez et al. 2007). Contrary to a previous hypothesis, these results suggest that IL-12 together with high IFN- γ responses are not necessary to drive this B-cell switch in *T. congolense* infections (Kaushik, Uzonna et al. 1999) (Uzonna, Kaushik et al. 1999). These studies measured IgG2a in C57BL/6 mice which likely detected IgG2c as C57BL/6 inbred mice do not have the gene (Martin, Brady et al. 1998).

Induction of IFN- γ has been shown in both *T. evansi* and *T. b. brucei* by a direct stimulus by TLTF released by the parasite. (Bakhiet, Buscher et al. 1996), and could have partially induced residual levels of IFN- γ in IL-12 deficient mice. However in *T. congolense* this molecule has not been studied and may or may not exist. Interleukin-12-independent IFN- γ production has been demonstrated during *Listeria monocytogenes* (Brombacher, Dorfmueller et al. 1999), *Mycobacterium BCG* (Holscher, Atkinson et al. 2001) and *Leishmania major* (Mattner, Magram et al. 1996) infections. In *T. b. brucei* infection, natural killer cells are thought to be necessary for resistance during the initial infection which are activated by both IL-12 and IL-18 (Namangala, Sugimoto et al. 2007). Similarly in *T. congolense* infection, in the absence of IL-12, IL-18 may drive the Th1 response, thus providing residual host protection. Moreover, in the absence of IL-18, IL-12 was found to be effective in providing host resistance to infection.

Following *T. congolense* infection the majority of IFN- γ is produced by MHC-II restricted CD4+ T cells with a contribution of 5% from CD8+ T cells (Shi, Wang et al. 2006) (Shi, Wei et al. 2007). The importance of MHC class II molecules in C57BL/6 mice has been shown as gene deficient mice display decreased survival (Magez, Radwanska et al. 2006). In addition, expression of IFN- γ in CD4+ cells was found at the area of cell to cell contact with CD11b+ cells (Shi, Wei et al. 2006). Together, these results may suggest a role for IL-12 production by antigen presenting cells signaling via MHC-II molecules initiating CD4+ T cell IFN- γ hyper-responses leading to long-term pathogenesis in wild type mice.

Increased survival has also been shown experimentally in *T. congolense* infected BALB/c mice, with a similar phenotype as found in infected IL-12 deficient mice. Here, deletion of CD4⁺ T cells in BALB/c mice displayed no benefit for host survival, although IFN- γ was found to be markedly reduced in these mice, protective factors, namely; IL-10 (Shi, Pan et al. 2003) and IgG2a (Uzonna, Kaushik et al. 1999) (Magez, Radwanska et al. 2006) were similarly reduced. However, partial depletion of either CD4⁺ T cells or MHC class II positive cells in infected BALB/c mice resulted in significantly reduced IFN- γ production together with increased survival, normal IL-10 and parasite specific IgG2a antibody responses (Shi, Wei et al. 2006). These data parallel the results reported in this present study using IL-12 deficient mice, in that the attenuation of IFN- γ production together with anti-inflammatory IL-10 increases host survival.

Interleukin-12 deficient mice have been found to produce default Th2 responses to infectious pathogens such as in *Leishmania major* (Mattner, Magram et al. 1996), *Salmonella enterica* (Lehmann, Bellmann et al. 2001) and *Cryptococcus neoformans* (Decken, Kohler et al. 1998). In the bovine model the increased IL-4 mRNA present in trypanotolerant cattle compared to that found in trypanosusceptible cattle was associated with increased survival in *T. congolense* infection (Mertens, Taylor et al. 1999). In the mouse model using different mouse strains, the role of IL-4 in infection with various *T. brucei* subspecies has been found to be harmful (Bakhiet, Jansson et al. 1996), protective (Inoue, Inoue et al. 1999) or to have no effect (Schopf, Filutowicz et al. 1998) in terms of mortality or parasitemia. Plasma IL-4 levels found in all IL-12 deficient mouse groups at the time points investigated were not significantly increased compared to wild type mice. Furthermore, no significant differences in mortality were found between C57BL/6 wild type and IL-4 or IL-4R α deficient mice, as similarly shown using these deficient mice in the BALB/c mouse background (Noel, Hassanzadeh et al. 2002). The enhanced survival of IL-12 deficient mice cannot be attributed to increased IL-4 production, which would potentially drive a Th2 response. Moreover IL-12 was not required to suppress IL-4 production in *T. congolense* infection.

The findings from the IL-12 deficient mice parallel those of other studies describing characteristics linked to trypanotolerance in *T. congolense* infection. Recently, regulatory T cells have been shown to be necessary to limit the sustained type I immune response resulting in pathology in *T. congolense* infected mice (Guilliams, Oldenhove et al. 2007). In addition, the emergence of an alternatively activated macrophage profile has been documented during the chronic stages of disease exhibiting properties such as the down-regulation of pro-inflammatory responses (Ghassabeh, De Baetselier et al. 2006). The findings here, illustrating decreased pro-inflammatory responses together with decreased immunopathology in IL-12 deficient mice, parallel the above-mentioned studies and indicate that immune responses in wild type mice are ultimately detrimental to the host.

Work by Baetselier et al., 2001, led to the proposed model where a type II cytokine switch from a type I response may facilitate survival into the chronic stage of disease in *T. congolense* infection with the concurrent induction of alternate macrophage activation (Baetselier, Namangala et al. 2001) (Ghassabeh, De Baetselier et al. 2006). However, deletion of IL-4 and/or IL-13 cytokines that may drive a type II response revealed a similar susceptibility to *T. congolense* infection as wild type mice. The postulated switch from type I to type II cytokine response involving IL-4/IL-13 driving protective alternate macrophage activation during chronic *T. congolense* infection alluded to by Baetselier et al., 2001, may thus be induced by other factors.

Factors, such as IL-10, are able to induce phenotypes similar to that induced by IL-4 and IL-13 (Mantovani, Sica et al. 2004). Interleukin-10 has down-regulating anti-inflammatory activities and has been shown to be essential for decreasing pathology during *T. congolense* infection and is found to be up-regulated during chronic infection (Shi, Pan et al. 2003) (Baetselier, Namangala et al. 2001). However, Gordon, 2003 suggests that IL-10, in the absence of IL-4/IL-13, is not able to compensate for alternative macrophage activation (Gordon 2003). Work which supports this contention was done by Herbert et al., 2004, where IL-10 was unable to stimulate Arginase activity in macrophages stimulated with IL-4 and IL-13 (Herbert, Holscher et al. 2004). Also, using suppression subtractive hybridization it was found that IL-10 and IL-4 induce

substantially different gene expression patterns in macrophages (Stumpo, Kauer et al. 2003). The work presented here, although incomplete in terms of all potential effector immune molecules, does suggest that factors other than IL-4/IL-13 induces the proposed alternative macrophage activation necessary for survival into the chronic stage of *T. congolense* infection. Similarly, Noel et al., 2002 found that in the BALB/c infection model, IL-4 and IL-13 deficient mice were not impaired in their ability to induce alternatively activated macrophages (Noel, Hassanzadeh et al. 2002). Furthermore, work by Ghassabeh et al., 2006, found that Type II cytokine-associated myeloid cell signature genes induced by *T. Congolense* as well as other parasitic and cancer murine models were not induced by IL-4/IL-13 or IL-10 under *in vitro* conditions and may thus be induced by other factors.

Macrophages from IL-12 deficient mice have been shown to exhibit a M2 profile (Bastos, Alvarez et al. 2002) that may induce a more protective immune response towards infection with *T. congolense* compared to that of wild type mice. Ghassabeh et al., 2006 using *T. congolense* infection model (as well as others) in mice characterized the M2 profile during the chronic stages of disease (Ghassabeh, De Baetselier et al. 2006). The genetic profile of these M2 cells included properties such as down-regulation of inflammation, immunomodulation, protection from oxidative damage, increased phagocytosis and tissue repair. The results of this paper using IL-12 deficient mice indeed shows dramatic down-regulation of inflammation and perhaps enhanced tissue repair and/or protection from oxidative damage as shown in the liver compared to wild type mice. The emergence of the M2 profile during the chronic stages of disease may be enhanced in the case of IL-12 deficient mice as these mice lack a strong counter type I cytokine response. Furthermore the establishment of a type II cytokine response from a type I cytokine response is considered beneficial for survival of mice (Baetselier, Namangala et al. 2001), and in the case of IL-12 deficient mice it suggests an optimal balance could have been established.

In the study by Ghassabeh *et al.*, 2006 the gene *trem2* (triggering receptor expressed on myeloid cells 2) was identified and up-regulated (87 ± 4 fold induction) after 3-4 months

of infection (chronic stage) compared to 2 weeks post infection (early stage) in the *T. congolense* infection model (Ghassabeh, De Baetselier et al. 2006). This gene expresses a protein found on macrophages infiltrating tissues from the circulation and is inducible by alternative activation stimulated by IL-4. Studies using TREM-2 gene deficient mice found that it functions to reduce cytokine production in response to TLR ligands LPS, CpG and zymosan stimulation (Turnbull, Gilfillan et al. 2006) (Hamerman, Jarjoura et al. 2006). A study by Drennan *et al.*, 2006 has found TLR9 (responsible for CpG recognition) to play a role in resistance in non-clonal *T. b. brucei* infections and may thus indicate the connection between TREM-2 and the recognition of trypanosome antigens by the immune system (Drennan, Stijlemans et al. 2005). The up-regulation of the anti-inflammatory *trem2* gene towards the chronic stages of infection are in line with the results in this study which show that an anti-inflammatory condition existing in IL-12 deficient mice resulting in decreased pathology and enhanced survival is beneficial.

Another M2 signature gene, namely Selenoprotein P (Sep), involved in enhancing host-protective mechanisms present during the chronic stages of *T. congolense* infection was identified by Ghassabeh *et al.*, 2006 in infected mice. Selenoprotein P was found to be up regulated during the chronic stage of disease compared to the acute stage (Ghassabeh, De Baetselier et al. 2006). Sep is an extracellular glycoprotein involved in selenium homeostasis, but also plays a role in antioxidant defense, it has been suggested that this molecule binds to cell membranes in inflamed areas and scavenges free radicals (Burk, Hill et al. 1997) (Arteel, Mostert et al. 1998) (Saito, Hayashi et al. 1999). Moreover, in the diquat-induced liver necrosis model using selenium deficient rats, it was found that Sep plasma levels correlated with protection against diquat injury (Burk, Hill et al. 1995) (Atkinson, Hill et al. 2001). Research on the mechanisms involved in Gemcitabine resistance, a standard chemotherapeutic agent used to treat pancreatic cancer, have found that the resistance was due to a reduction of intracellular reactive oxygen species by Sep (Maehara, Tanaka et al. 2004). Of interest in the present IL-12 study is that Maehara *et al.*, 2004 showed that IFN- γ suppressed the expression of Sep mRNA with a resultant increase in intracellular reactive oxygen species. The protective properties of Sep may thus offer an explanation for the decreased liver pathology in IL-12 deficient mice in that

these mice show reduced chronic stage IFN- γ production which may suggest increased protective activity by Sep expression, inhibiting the activity of reactive oxygen species. An infection study using the *T. congolense* model illustrated that, *in vitro*, IL-10 induced Sep expression but not IL-4 or IL-13 (Ghassabeh, De Baetselier et al. 2006). This may validate the results here, as IL-4 or IL-13 deletion had no effect on survival. However, in IL-12 deficient mice IL-10 was present, but with reduced IFN- γ , which may drive Sep expression allowing for protection. Of further interest is that selenium binding protein 2 was found to be up-regulated in resistant C57BL/6 mice compared to susceptible A/J mice at days 4, 7, 10 and 17 post infection (Kierstein, Noyes et al. 2006).

Further interactions between down-regulating molecules such as monocyte chemoattractant protein-1 (MCP-1) and IL-12 may also account for enhanced resistance in *T. congolense* infected mice deficient in IL-12 compared to wild type mice. The activity of MCP-1 has been shown to be involved in the regulation of Th1/Th2 lymphocyte differentiation, by inducing Th2 development via IL-4 up-regulation and inhibiting Th1 development by reducing IL-12 production (Chensue, Warmington et al. 1996) (Karpus and Kennedy 1997) (Lukacs, Chensue et al. 1997) (Karpus and Kennedy 1997). In addition to its chemotactic properties, MCP-1 has been implicated in playing a role in balancing pro- and anti-inflammatory cytokines during infection (Zisman, Kunkel et al. 1997). Monocyte chemoattractant protein-1 may have protective characteristics, down regulating IL-12 and TNF- α and up-regulating IL-10 with the result of increased survival (Zisman, Kunkel et al. 1997) (Matsukawa, Hogaboam et al. 2000) (Gomes, Figueiredo et al. 2006). Although not determined in this study, levels of MCP-1 are found to be highly increased in wild type mice towards the late stage of infection in *T. congolense* infection (Noel, Hassanzadeh et al. 2002). It could be speculated that in the absence of IL-12, MCP-1 may more effectively drive the anti-inflammatory response (in the form of IL-10) resulting in the observed increased survival presenting in IL-12 deficient mice. Furthermore, as well as being an immune modulator together with chemotactic activities, MCP-1 is also able to activate monocytes to release lysosomal enzymes as well as the production of H₂O₂ which may have trypanolytic/static activities

(Matsushima, Larsen et al. 1989) (Rollins, Walz et al. 1991) (Mansfield and Paulnock 2005).

Several genetic mapping studies, comparing trypanosresistant and trypanosusceptible inbred strains of mice, have highlighted alternate immunological pathways utilized during infection with different *T. congolense* strains. For example, genome-wide mapping scans identified quantitative trait loci which contained putative resistance genes (Iraqi, Clapcott et al. 2000) (Clapcott, Teale et al. 2000). Furthermore a large scale gene microarray study (Kierstein, Noyes et al. 2006) compared the gene expression profiles of trypanosresistant and trypanosusceptible infected cattle (O'Gorman G, Park et al. 2006) (Yoshihara, Morris et al. 2007). However, these studies and the the work by Namangala et al., 2007 did not identify IL-12 as playing a significant role in *T. congolense* infections. Instead these studies broadly identified that a proinflammatory cytokine response was necessary for survival. The common strain of *T. congolense* used in these studies was the IL1180 clone. However, research in this thesis using the *T. congolense* Tc13 clone identified IL-12 as a susceptibility factor. The work presented here may indicate strain differences within *T. congolense* which may have immunological ramifications.

Recent work by Namangala et al., 2007 has shown that exogenously administrated TGF- β had protective activity against the IL1180 strain of *T. congolense*. Enhanced 1st parasitemic peak control and survival was reported as well as reduced infection related pathology, namely; anemia and splenomegaly. The effect of administered TGF-beta in this model was an enhanced parasite-antigen specific Th1 cellular response associated with high levels of type-1 cytokines which correlated with increased resistance against *T. congolense* (Namangala, Sugimoto et al. 2007). However, an enhanced type-1 cytokine response has been shown to be detrimental in C57BL/6 and BALB/c inbred strains as well as in IL-12 deficient mice infected with the Tc13 strain of *T. congolense* (Shi, Pan et al. 2003). The disparity between these results may highlight differences between strains of this parasite and caution future studies using *T. congolense*.

Infection with *T. congolense* requires IL-10 to counter the effects of pro-inflammatory cytokines (Shi, Pan et al. 2003) (Guilliams, Oldenhove et al. 2007) (Shi, Wei et al. 2007). Recent findings have demonstrated that T regulatory cells produce a minor portion of the total IL-10 detected in spleen and liver samples. Conventional Th1 cells as well as CD4 negative cells, such as macrophages (Kaushik, Uzonna et al. 1999) (Kaushik, Uzonna et al. 2000) and/or regulatory B cells (Lund, Garvy et al. 2005), were suggested to be the major source(s) of IL-10 during infection. Of interest are Ly-1 (B1 or CD5) B cells as these cells are known to readily produce IL-10 (O'Garra, Chang et al. 1992) and upon expansion form a large part of circulating B cells during *T. congolense* infection (Naessens and Williams 1992). Further evidence for this connection comes from infected cattle where an increase in IL-10 mRNA expression occurred concurrently with expansion of CD5+ B cells (Taylor, Mertens et al. 1998).

In the murine model, activation of CD5 B cells is thought to occur via T cell independent processes (Naessens and Williams 1992). In terms of cytokine regulation, IL-12 or IFN- γ has been shown to inhibit the expansion of CD5+ B cells which also express the receptor for IL-12 in murine cells (Velupillai, Sypek et al. 1996) (Vogel, Showe et al. 1996) (Vogel, Lester et al. 1996). Studies have shown that in murine schistosomiasis the expansion of B-1 cells (CD5) was elicited by injection of saline soluble egg antigens (SEA) or the polylactosamine sugar lacto-N-fucopentaose III and resulted in the production of Th1-down-regulating molecules namely; IL-10 and prostaglandin E₂ (Velupillai and Harn 1994). In this study it was found that B-1 cell outgrowth could be completely inhibited by IL-12, IFN- γ or anti-IL-10. In this thesis, the lack of IL-12 signaling may have allowed for a greater expansion of CD5+ cells and IL-10 production. This may have added to the anti-inflammatory condition in the IL-12 gene deficient mice, leading to their prolonged survival compared to wild type mice. In contrast however, these B cells are implicated in several autoimmune diseases and may play a role in autoimmunity in *T. congolense* infection (Duan and Morel 2006) (Naessens and Williams 1992).

Although infection with fatal strains *Plasmodium berghei* represents a very different mouse model compared to that of *T. congolense* it may provide further clues as to the role of IL-12 in the formation of lesions in the liver. In this model IL-12 was found to induce liver injury but was excluded in having a direct toxic effect on hepatocytes (Adachi, Tsutsui et al. 2001). Lymphocytes present in the liver expressed high levels of IL-12R and killed normal hepatocytes in a perforin-dependant and MHC unrestricted manner. Perforin has been found to be highly up-regulated in T cells infiltrating lesions caused by autoimmune diseases such as rheumatoid arthritis, myocarditis and non obese diabetic mice (Lowin, Beermann et al. 1994). Interleukin-12 has been found to play a role in the induction of autoimmunity in EAE (Conant and Swanborg 2004) (Ichikawa, Williams et al. 2002), it is possible that IL-12 in chronic *T. congolense* infection may similarly induce autoimmunity.

The production of IFN- γ in *T. congolense* infection is necessary to prevent mortality in C57BL/6 mice (Magez, Radwanska et al. 2006). However, in a chronic setting with continuously up-regulated IFN- γ production, it could be possible that autoimmune pathology is subsequently induced (Schroder, Hertzog et al. 2004). Studies have found that long term IFN- γ exposure may contribute to conditions such as autoimmune nephritis (Heremans, Billiau et al. 1978) (Jacob, van der Meide et al. 1987) and insulin-dependent mellitus (Sarvetnick, Liggitt et al. 1988). The enhanced resistance in IL-12 deficient mice to *T. congolense* may be due to the attenuation of IFN- γ production and thus its autoimmune promoting activity.

The results of *T. congolense* shown here and by others using *T. b. brucei* infections (Drennan, Stijlemans et al. 2005) shows the requirement of the adaptor molecule MyD88 for survival and here, was also demonstrated to exclude the overlapping IL-18 signaling pathway in *T. congolense* (Adachi, Kawai et al. 1998). In the *T. b. brucei* infection model, MyD88 was shown to be necessary for IFN- γ and TNF production (Drennan, Stijlemans et al. 2005), in *T. congolense* this may be the case as well as both these cytokines are necessary for survival (Magez, Radwanska et al. 2006) (Magez, Radwanska et al. 2007) and may thus explain early mortality in these gene deficient mice. Toll-like

receptor 9 is known to respond to its agonist CpG DNA and was identified to be necessary for resistance to non-clonal infections in the *T. b. brucei* infection model (Drennan, Stijlemans et al. 2005). As suggested previously in the *T. b. brucei* infection model, TLR9 signaling induces IL-12 production, the deletion of either resulted in a similar disease outcome of increased susceptibility (Barkhuizen, Magez et al. 2007). However, in *T. congolense* infection, IL-12 is a susceptibility factor, and not a resistance factor as found in *T. b. brucei*, suggesting different TLR molecules play a role in resistance to *T. congolense*.

CONCLUSIONS

The data presented in this thesis demonstrated that IL-12p70, but not the IL-12p80 homodimer nor IL-23, was necessary for optimal resistance to *T. b. brucei* infection in terms of mortality and parasitemia control. The resistance attributed to IL-12 was mediated through IFN- γ , the production of which was significantly decreased in the absence of IL-12. Moreover, IL-12 was found to be important for parasitemia control during the chronic stage of *T. b. brucei* infection, where it was needed for optimal TNF and IFN- γ production. The protective role of IFN- γ was validated in IFN- γ R deficient mice infected with *T. b. brucei*. Similar results were obtained in IL-12 deficient mice infected with *T. evansi*. However, these results differed to published data which reported that IgM was necessary for control of infection rather than a Th1 type immune response (Baral, De Baetselier et al. 2007). This was most likely due to the laboratory's *T. evansi* strain evolving into a highly virulent strain that required a robust Type I response for effective host resistance.

In contrast to the results obtained from *T. b. brucei* and *T. evansi* infections, IL-12 was shown to play a detrimental role during *T. congolense* infection. Here, IL-12 was found to drive the production of IFN- γ and iNOS, the over production of which resulted in liver damage during the chronic stages of the disease leading to mortality. Furthermore, in contrast to *T. b. brucei* and *T. evansi* infections, the attenuated cytokine response in *T. congolense* infected IL-12 deficient mice had no negative effect on parasitemia control. Another Th1 inducing cytokine, IL-18 did not play a role in resistance in any of the *Trypanosoma* strains suggesting that IL-12 can sufficiently provide host resistance to trypanosomiasis in the absence of IL-18, but not visa-versa. Interleukin-12 independent IFN- γ production was also detected in all *Trypanosoma* infected mice possibly driven by IL-18 or TLTF produced by the parasites.

In addition, MyD88 was shown to play a significant role in survival in *T. evansi* and *T. congolense* infections as has been already found in *T. b. brucei*. Although the production of the T helper 2 inducing cytokine, namely; IL-4 was detected during infection, there

was no significant role in the outcome of the disease in mice deficient for IL-4 and IL-13 for any of the *Trypanosoma* strains used. The results of this thesis also highlight the major immunological differences between *T. congolense*, *T. b. brucei* and *T. evansi* strains and that this should be taken into consideration in future studies.

FUTURE WORK

Currently the immunology of *T. evansi* infection is largely unknown compared to that of *T. b. brucei*, which has been extensively studied. Recent work has begun to shed light onto the immune mechanisms necessary for resistance against *T. evansi*, as well as those causing pathology. For example, Baral et al (2007) reported that IgM was necessary for control of infection rather than a Th1 type immune response, which is characteristic of a resistant phenotype against trypanosomiasis infection.(Baral, De Baetselier et al. 2007) However, preliminary data presented in this thesis has demonstrated that resistance to *T. evansi* is mediated through MyD88, IFN- γ R, IL-12 and TNF signaling pathways. Furthermore, CD4 cells were found to be necessary for resistance to *T. evansi*. These differing results may be due to the continual passaging of the parasite in the laboratory, resulting a more virulent, non-clonal strain of *T. evansi*. It is therefore necessary to repeat this experiment and in parallel, draw on the original strain as a comparison.

The role of the adaptor molecule MyD88 in *T. b. brucei* infection has already been investigated, although the exact signaling pathway remains unknown as only a partial role for TLR9 was found (Drennan, Stijlemans et al. 2005). It was suggested that trypanosomal DNA, when released from lysing cells, triggered TLR9 via MyD88 and consequently IL-12 production during the chronic stages of disease (Barkhuizen, Magez et al. 2007) (Bastos, Barboza et al. 2007). In this thesis, MyD88 was found to be necessary for resistance to *T. congolense*. Given the increased resistance of IL-12 deficient mice in *T. congolense* infection, as apposed to *T. b. brucei* where IL-12 deficient mice are susceptible, it is possible that other TLR molecules besides TLR9 may be involved in resistance or susceptibility in *T. congolense* infection. A further study using gene deficient mice for TLR(s), would therefore identify those TLR(s) signaling through MyD88 that required for resistance to *T. congolense*.

Recently, regulatory T cells in combination with IL-10, were shown to be for essential limiting liver damage induced by type I inflammatory cytokines in *T. congolense* infected

mice (Guilliams, Oldenhove et al. 2007). Extended survival in IL-12 deficient mice as compared to controls presented in this thesis may therefore be due to the enhanced activity of regulatory T cells. Since a negative association between IL-12 and regulatory T cells have been found in other models (Kilinc, Aulakh et al. 2006), a further study will be performed to determine whether IL-12 inhibits the expansion of regulatory T cells that contribute to host protective responses and thus survival.

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APPENDIX

DISTRESS SCORING SHEET (Adapted from Wolfensohn & Lloyd, 1998)

APPEARANCE	Score:
Normal	0
General lack of grooming	1
Coat staring. Ocular & nasal discharges	2
Piloerection, hunched up	3
CLINICAL SIGNS	
Normal Colour & Movement	0
Slight Changes: pallor, diminished activity, increased water intake (polydipsia)	1
Moderate Changes: weight loss, wet or blood on bedding (i.e. polyuria, diarrhoea, or gastrointestinal bleeding)	2
Severe Changes: immobility, abnormal gait, lameness, paralysis, fitting	3
NATURAL BEHAVIOUR	
Normal	0
Minor Changes	1
Less mobile and alert, isolated	2
Vocalisation, self mutilation, restless or very still	3
PROVOKED BEHAVIOUR	
Normal	0
Minor depression or exaggerated response	1
Moderate Change in expected behaviour	2
Reacts violently, or very weak and precomatose	3
SCORE ADJUSTMENT	
If you have scored a 3 more than once, score an extra point for each 3	2-4
TOTAL	0-16

JUDGEMENT

0-4 Normal

5-10 Monitor Carefully

11 or over Termination

Genotyping Primer Sequences and Product Sizes

Genotyping PCR	Primer sequences
IFN γ R	IFN γ R P1: 5'-CCC ATT TAG ATC CTA CAT ACG AAA CAT ACG G-3' IFN γ R P2: 5'-TTT CTG TCA TCA TGG AAA GGA GGG ATA CAG -3'
IL-12p35	IL-12p35 P1: 5'-AGC TCC TCT CAG TGC CGG TC-3' IL-12p35 P2: 5'-GGT CTT CAG CAG GTT TCG GG-3' IL-12p35 P3: 5'-GGC TCT GGA CTC ACC TGG AT-3' IL-12p35 P4: 5'-GCA TCG CAT TGT CTG AGT AGG-3'
IL-12p40	IL-12p40 P1: 5'-AGT GAA CCT CAC CTG TGA CAC G-3' IL-12p40 P2: 5'-TCT TTG CAC CAG CCA TGA GC-3' IL-12p40 P3: 5'-GCG GGA AGG GAC TGG CTG CTAT-3' IL-12p40 P4: 5'-CAC CCG TGC GTT TTA TTC TGT CTT-3'
IL-1Rrp ^{-/-}	IL-18 P1: 5'-ATC TGC ACA CTT GTG AAG CAG AAA CAC-3' IL-18 P2: 5'- ATC ACT ATA CAC ACT GCT GCC ACA GAG-3' IL-18 P3: 5'- ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'
IL-4	IL-4 P1: 5'-GTG AGC AGA TGA CAT GGG GC-3' IL-4 P2: 5'-CTT CAA GCA TGG AGT TTT CCC-3' IL-4 P3: 5'-GCG CAT CGC CTT CTA TCG CCT TC-3'
IL-4R α	IL-4Ra P1: 5'-TGA CCT ACA AGG AAC CCA GGC-3' IL-4Ra P2: 5'-CTC GGC GCA CTG ACC CAT CT-3' IL-4Ra P3: 5'-GGC TGC TGA CCT GGA ATA ACC-3' IL-4Ra P4: 5'-CCT TTG AGA ACT GCG GGC T-3'
iNOS	iNOS P1: 5'-ATC AGC CTT TCT CTG TCT CC-3' iNOS P2: 5'-GGC TTT CTG CTG TTC TCT C-3' iNOS P3: 5'-GAGCAA TGT GAC AAA GCT CCT TCA GAC TAG -3' iNOS P4: 5'-GCC TGA AGA ACG AGA TCA GCA GCC TC -3'
MyD88	Myd88 P1: 5'-AGA CAG GCT GAG TGC AAA CTT GTG CTG-3' Myd88 P2: 5'-AGC CTC TAC ACC CTT CTC TTC TCC ACA-3' Myd88 P3: 5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'
TLR2	TLR2 P1: 5'-CTT CCT GAA TTT GTC CAG TAC AGG-3' TLR2 P2: 5'-TCG ACC TCG ATC AAC AGG AGA AG-3' TLR2 P3: 5'-GGG CCA GCT CAT TCC TCC CAC TCA-3'
TNF	TNF P1: 5'- AGA TAG CAA ATC GGC TGA CGG-3' TNF P2: 5'-CCT TCT ATC GCC TTC TTG ACG-3' TNF P3: 5'-ATC AGT TCT ATG GCC CAG ACC-3'
TNFR1	TNFR1 P1: 5'-CTC TCT TGT GAT CAG CAC TG-3' TNFR1 P2: 5'-AGA AAT CTC AAG ACA ATT CTC TGC-3' TNFR1 P3: 5'-TCC CGC TTC AGT GAC AAC GTC-3'
TNFR2	TNFR2 P1: 5'-CCT CTC ATG CTG TCC CGG AAT-3' TNFR2 P2: 5'-AGC TCC AGG CAC AAG GGC GGG-3' TNFR2 P3: 5'CGG TTC TTT TTG TCA AGA C-3' TNFR2 P4: 5'-ATC CTC GCC GTC GGG CAT GC-3'

Appendix

Genotyping PCR Cycle Parameters

Genotyping Reaction	PCR Cycle Parameters
IFN γ R-/-	Step1: 94°C for 2 min. Step 2: 94°C for 20 sec. Step3: 58°C for 20 sec. Step4: 72 ° C for 2 min. 30 sec. Step5: repeat steps 2-4 5X Step6: 94°C for 20 sec. Step7: 58°C for 20 sec. Step8: 72 ° C for 2 min. Step9: repeat steps 6-8 35X Step10: 72 ° C for 3 min. Step11: hold at 4°C
IL-12p35	Wildtype PCR (primers P1 and P2): Step1: 94°C for 2 min. Step 2: 94°C for 20 sec. Step3: 58°C for 20 sec. Step4: 72 ° C for 2 min. 30 sec. Step5: repeat steps 2-4 5X Step6: 94°C for 20 sec. Step7: 58°C for 20 sec. Step8: 72 ° C for 2 min. Step9: repeat steps 6-8 28X Step10: 72 ° C for 3 min. Step11: hold at 4°C Knock-out PCR (primers P3 and P4):: Step1: 94°C for 3 min. Step 2: 94°C for 45 sec. Step3: 58°C for 45 sec. Step4: 72 ° C for 2 min Step5: repeat steps 2-4 35X Step6: 72 ° C for 3 min. Step7: hold at 4°C
IL-12p40	Step1: 94°C for 3 min. Step 2: 94°C for 45 sec. Step3: 60°C for 45 sec. Step4: 72 ° C for 1 min. 30 sec. Step5: repeat steps 2-4 35X Step6: 72 ° C for 3 min. Step7: hold at 4°C

Appendix

Genotyping PCR Cycle Parameters (*continued*)

Genotyping Reaction	PCR Cycle Parameters
IL-1Rrp ^{-/-}	Step1: 94°C for 10 min. Step 2: 94°C for 30 sec. Step3: increase temp. at 0.8°C/sec until 60°C Step4: 60°C for 1 min. Step5: 74°C for 1min. Step6: repeat steps 2-4 40X Step7: 72°C for 5min. Step8: hold at 4°C
IL-4	Step1: 94°C for 2 min. Step 2: 94°C for 30 sec. Step3: 58°C for 45 sec. Step4: 72°C for 2 min. 30 sec. Step5: repeat steps 2-4 5X Step6: 94°C for 30 sec. Step7: 58°C for 45 sec. Step8: 72°C for 30 sec. Step9: repeat steps 6-8 35X Step10: 72°C for 3 min. Step11: hold at 4°C
IL-4Rα	Step1: 94°C for 1 min. Step 2: 94°C for 30 sec. Step3: 58°C for 20 sec. Step4: 72°C for 1 min.. Step5: repeat steps 2-4 35X Step6: 72°C for 5min. Step7: hold at 4°C
iNOS	Step1: 94°C for 1 min. Step 2: 94°C for 30 sec. Step3: 55°C for 45 sec. Step4: 72°C for 3 min.. Step5: repeat steps 2-4 40X Step6: 72°C for 5min. Step7: hold at 4°C
MyD88	Step1: 94°C for 5 min. Step 2: 94°C for 40 sec. Step3: 55°C for 40 sec. Step4: 72°C for 1 min. 30 sec. Step5: repeat steps 2-4 40X Step6: 72°C for 5min. Step7: hold at 4°C

Appendix

Genotyping PCR Cycle Parameters (*continued*)

Genotyping Reaction	PCR Cycle Parameters
TLR2	Step1: 94°C for 5 min. Step 2: 94°C for 1 min. Step3: 55°C for 1 min. Step4: 72 °C for 2 min.. Step5: repeat steps 2-4 35X Step6: 72°C for 5min. Step7: hold at 4°C
TNF	Step1: 94°C for 5 min. Step 2: 94°C for 1 min. Step3: 60°C for 1 min. Step4: 72 °C for 2 min.. Step5: repeat steps 2-4 35X Step6: 72°C for 5min. Step7: hold at 4°C
TNFR1	Step1: 94°C for 11 min. Step 2: 94°C for 60 sec. Step3: 57°C for 1 min. Step4: 72 °C for 2 min.. Step5: repeat steps 2-4 35X Step6: 72°C for 5min. Step7: hold at 4°C
TNFR2	Step1: 94°C for 11 min. Step 2: 94°C for 60 sec. Step3: 57°C for 1 min. Step4: 72 °C for 2 min.. Step5: repeat steps 2-4 35X Step6: 72°C for 3min. Step7: hold at 4°C

RECIPES

General Lab Solutions

Mouse Tail Digestion Buffer

12.5ml 1M TRIS-HCL, pH8

50ml 500mM EDTA, pH8.0

12.5ml 1M NaCl

25ml 10% SDS

2.5ml 10mg/ml Proteinase K

Add 12.5ml of 1M TRIS-HCL (pH8), 50ml of 500mM EDTA (pH8.0), 12.5ml of 1M NaCl and 25ml of 10% SDS to 140ml of ddH₂O. Bring up the final volume to 250ml and filter sterilize with a 0.22μM filter (Millipore Corporation, Bedford, USA). Aliquot into sterile tubes and store at -20°C. Thaw buffer and add 10mg/ml Proteinase K at a 1:100 dilution.

10mg/ml Proteinase K

1g Proteinase K

Weigh out 1g of Proteinase K and dissolve in 100ml of ddH₂O. Filter sterilize with a 0.22μM filter (Millipore Corporation, Bedford, USA) and aliquot into sterile tubes. Store at -20°C and use at a 1:100 dilution in Mouse Tail Digestion Buffer.

0.5M EDTA, pH 8.0

93.06g EDTA

Weigh out 93.06g of EDTA and dissolve in 450ml ddH₂O. Adjust pH to 8.0 and bring final volume to 500ml. Autoclave and store at room temperature.

2M NaCl

11.688g NaCl

Weigh out 11.688g of NaCl and dissolve in 90ml of ddH₂O. Bring final volume to 100ml and autoclave. Store at room temperature.

10% SDS

10g SDS

Weigh out 10g of SDS and dissolve in 90ml of ddH₂O. Bring final volume to 100ml and autoclave. Store at room temperature.

1M TRIS-HCl, pH8.0

60.55g TRIS-HCl

Weigh out 60.55g of TRIS-HCl and dissolve in 450ml ddH₂O. Adjust pH to 8.0 and bring final volume to 500ml. Autoclave and store at room temperature.

1 X Phosphate Buffered Saline (PBS)

8g NaCl

0.2g KCl

1.44g Na₂HPO₄

0.24g KH₂PO₄

Weigh out chemicals and dissolve in 900ml of ddH₂O and adjust pH to 7.4. Bring final volume to 1000ml and filter sterilize with a 0.22μM filter (Millipore Corporation, Bedford, USA). Store at 4°C or room temperature.

phosphate saline glucose (PSG) buffer

20ml 100% glycerol

10g SDS

Add 10g of SDS to 90ml of ddH₂O and dissolve. Bring final volume to 100ml and autoclave. Store at room temperature.

4 % phosphate-buffered formalin

52.63ml 38% formaldehyde

500ml 1 X PBS

Mix together 52.63ml of 38% formaldehyde and 400ml of 1 X PBS. Bring up volume to 500ml and filter sterilize with a 0.22μM filter (Millipore Corporation, Bedford, USA). Aliquot into sterile tubes and store at 4°C.

10% glycerol

10ml 100% Glycerol

90ml 1 X PBS

Mix together 10ml of 100% of Glycerol and 90ml of 1 X PBS. Autoclave and store at temperature.

0.2% Triton-X100

2ml 10% Triton-X100

98ml 1 X PBS

Mix together 2ml of 10% Triton-X100 and 98ml of 1 X PBS. Autoclave and store at temperature.

1% BSA (Fraction V)

1g BSA (fraction V)

100ml 1 X PBS

Weigh out 1g of BSA (fraction V) and dissolve in 95ml of 1 X PBS. Bring up the final volume to 100ml and filter sterilize with a 0.22 μ M filter (Millipore Corporation, Bedford, USA). Aliquot into sterile tubes and store at -20°C.

Cell Culture Solutions

Fetal Calf Serum (FCS)

500ml FCS

Thaw one 500ml bottle of FCS at 37°C and heat inactivate at 56°C for 30 minutes with intermittent agitation. Make 25ml aliquots and store at -20°C.

RPMI Medium 1640

17.66g RPMI powder

2.0g of sodium bicarbonate

100ml Fetal calf serum

10ml 100X Penicillin G and Streptomycin Solution

Weigh out 17.66g of RPMI powder and 2.0g of sodium bicarbonate and dissolve in 800ml ddH₂O. Adjust pH to between 7.35 with 1M NaOH. Add 100ml heat decomplexed fetal calf serum and 10ml of 100X Penicillin G and Streptomycin Solution. Bring the final volume to 1000ml and filter sterilize with a 0.22µM filter (Millipore Corporation, Bedford, USA). Store at 4°C.

Immunology Solutions

ELISA Coating Buffer

0.2g NaN_3

Weigh out NaN_3 and dissolve in 900ml of 1 X PBS, bring the final volume to 1000ml and store at room temperature.

ELISA Carbonate Coating Buffer

1.6g Na_2CO_3

2.9g NaHCO_3

4.2g NaCl

Weigh out chemicals and dissolve in 900ml of ddH₂O. Adjust pH to 9.5 with 1M Citric Acid. Bring the final volume to 1000ml and filter sterilize with a 0.22 μM filter (Millipore Corporation, Bedford, USA) and store at 4°C.

ELISA Blocking Buffer

20g Powder Milk

0.2g NaN_3

Weigh out chemicals and dissolve in 900ml of 1 X PBS, bring the final volume to 1000ml and store at 4°C.

ELISA Dilution Buffer

10g BSA

0.2g NaN_3

Weigh out chemicals and dissolve in 900ml of 1 X PBS, bring the final volume to 1000ml and store at 4°C.

ELISA Washing Buffer (20 X)

20g KCL

20g $\text{KH}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

800g NaCl

50ml Tween-20

100ml 10% NaN_3

Weigh out chemicals and dissolve in 4500ml double distilled water (ddH₂O). Add 50ml of Tween-20 and 100ml of 10% NaN₃. Bring final volume to 5000ml and store at room temperature. Dilute 1:20 in ddH₂O for a 1 X working concentration buffer.

ELISA Substrate Buffer (for alkaline phosphatase conjugates)

0.2g NaN₃

97ml diethanolamine

0.8g MgCl₂·6H₂O

Weigh out chemicals and dissolve in 700ml of ddH₂O. Add 97ml of liquefied diethanolamine and adjust pH to 9.8. Bring the final volume to 1000ml and store at 4°C.

ELISA Substrate Buffer (for horseradish peroxidase conjugates)

TMB Peroxidase Substrate Solution A ((KPL, Maryland, USA)

Peroxidase Substrate Solution B (KPL, Maryland, USA)

1M H₃PO₄

Just before use, mix equal volumes of TMB Peroxidase Substrate (Solution A) with Peroxidase Substrate Solution B. Add 50µl per well and let the reaction develop at room temperature for 5 minutes. Stop the reaction by adding 50µl of 1M H₃PO₄.

REAGENTS AND SUPPLIERS:

Reagent or Kit	Supplier
3,3',5,5'-tetramethylbenzidine (TMB)	KPL, Maryland, USA
alkaline phosphatase labeled anti-mouse IgG1	Southern Biotechnology, Birmingham, AL
alkaline phosphatase labeled anti-mouse IgG2a	Southern Biotechnology, Birmingham, AL
alkaline phosphatase labeled anti-mouse IgG2b	Southern Biotechnology, Birmingham, AL
alkaline phosphatase labeled anti-mouse IgG3	Southern Biotechnology, Birmingham, AL
alkaline phosphatase labeled anti-mouse IgM,	Southern Biotechnology, Birmingham, AL
Bovine Serum Albumin (fraction V)	Roche Diagnostics GmbH, Mannheim, Germany
calcium chloride	BDH Chemicals Ltd., Poole, England
diethanolamine	Merck Laboratory Supplies, South Africa
dNTPs (100 μ M)	Promega, Madison, WI, USA
EDTA	BDH Chemicals Ltd., Poole, England
eosin stain	Sigma-Aldrich, Munich, Germany
Fat free milk powder (Spar House Brand)	Spar Grocery Store Franchise, South Africa
Fetal Calf Serum (FCS)	Gibco, Invitrogen Corporation, Carlsbad, CA, USA
Formaldehyde	BDH Chemicals Ltd., Poole, England
Glucose	BDH Chemicals Ltd., Poole, England
Glycerol	BDH Chemicals Ltd., Poole, England
hematoxylin stain	Sigma-Aldrich, Munich, Germany
IFN- γ Biotinylated antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IFN- γ Coating antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IFN- γ Recombinant Protein Standard	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-10 Biotinylated antibody	IL-10 from R&D Systems (Abingdon, UK)
IL-10 Coating antibody	IL-10 from R&D Systems (Abingdon, UK)
IL-10 Recombinant Protein Standard	IL-10 from R&D Systems (Abingdon, UK)
IL-12p35 Biotinylated antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-12p35 Coating antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-12p35 Recombinant Protein Standard	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL12p40 Biotinylated antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL12p40 Coating antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL12p40 Recombinant Protein Standard	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-18 Biotinylated antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-18 Coating antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-18 Recombinant Protein Standard	BD Pharmigen, BD Biosciences, San Jose, CA, USA

Reagent or Kit	Supplier
4-nitrophenylphosphate, disodium salt hexahydrate	Fluka, Geneva, Switzerland
Aspartate Transaminase Assay (AST)	Sigma-Aldrich, Munich, Germany
Bicinchoninic Acid (BCA) protein assay kit	Pierce, Rockford, IL, USA
DEAE-Sephadex anion-exchange DE52 columns	Whatman, Maidstone, England
F4/80 biotinylated antibody	(BMA Biomedicals, Switzerland)
Fluoromount	DAKO, Carpinteria, CA
IL-TNF Biotinylated antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-TNF Coating antibody	BD Pharmigen, BD Biosciences, San Jose, CA, USA
IL-TNF Recombinant Protein Standard	BD Pharmigen, BD Biosciences, San Jose, CA, USA
iNOS biotinylated antibody	gift from Prof. B. Ryffel, University of Orleans, Centre National de la Recherche Scientifique UMR6218, Molecular Immunology and Embryology, France.
magnesium chloride	BDH Chemicals Ltd., Poole, England
<i>n</i> -1-naphthylethylenediamide	Sigma-Aldrich, Munich, Germany
normal rat serum	BD Pharmigen, BD Biosciences, San Jose, CA, USA
Oligonucleotide Primers	DNA Synthesis Laboratory, Dept. Molecular and Cellular Biology, University of Cape Town , South Africa
Peroxidase Substrate (Solution B)	KPL, Maryland, USA
potassium chloride	BDH Chemicals Ltd., Poole, England
potassium dihydrogen phosphate	BDH Chemicals Ltd., Poole, England
Proteinase K	Roche Diagnostics GmbH, Mannheim, Germany
RPMI Medium 1640 for Cell Culture	Gibco, Invitrogen Corporation, Carlsbad, CA, USA
sodium acetate	BDH Chemicals Ltd., Poole, England
Sodium Azide	BDH Chemicals Ltd., Poole, England
sodium bicarbonate	BDH Chemicals Ltd., Poole, England
Sodium Chloride	BDH Chemicals Ltd., Poole, England
sodium citrate	BDH Chemicals Ltd., Poole, England
Sodium dehydrogenate phosphate	BDH Chemicals Ltd., Poole, England
Sodium Dodecyl Sulfate (SDS), 10%	BDH Chemicals Ltd., Poole, England
Sodium hydrogen phosphate	BDH Chemicals Ltd., Poole, England
stepavidin-alkaline-phosphatase	BD Pharmigen, BD Biosciences, San Jose, CA, USA
Texas Red	Abcam, Cambridge, UK
TMB Peroxidase Substrate (Solution A)	KPL, Maryland, USA
TRIS-HCl	BDH Chemicals Ltd., Poole, England
β-mercaptoethanol	Sigma-Aldrich, Munich, Germany

PUBLICATIONS

Interleukin-12p70-Dependent IFN- γ Production is Crucial for Resistance in African Trypanosomiasis.

Mark Barkhuizen¹, Stefan Magez^{1,2}, Robert A. Atkinson¹ and Frank Brombacher^{1*}

¹The Division of Immunology, Institute of Infectious Diseases and Molecular Medicine,
Faculty of Health Sciences, University of Cape Town (UCT), Cape Town, South Africa.

²Laboratory of Cellular and Molecular Immunology, Department of Molecular and
Cellular Recognition, Flanders Interuniversity Institute for Biotechnology, Vrije
Universiteit Brussels (VUB), Brussels, Belgium.

Word count: Abstract: 145, Article text: 3500

Number of text pages: 24; Tables: 1; Figures: 4.

Running title: Role of IL-12 in trypanosomiasis.

FOOTNOTES:

Conflict of Interest: The authors have no commercial or other association that may cause a conflict of interest.

Grant numbers and sources of support: This work was supported by the Flanders-South African Research Cooperation Program as well as the MRC, NRF and NHLS, South Africa. S.M. was a Professor for the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen and F.B. was a holder of a Wellcome Trust Senior Fellowship for Medical Science. (Grant no. 056708/Z/99).

* **Address for correspondence and reprint requests:** Prof. Frank Brombacher, University of Cape Town, IIDMM, Wernher Beit South, S27.1, Anzio road, Observatory, 7925, Cape Town, South Africa; Phone: +27-21-406-6616, Fax: +27-21-406-6029, e-mail: fbrombac@uctgsh1.uct.ac.za

ABSTRACT

African trypanosomiasis encompasses diseases caused by pathogenic trypanosomes, infecting both humans and animals. Here, we dissected the possible role of members of the IL-12 family during infection with *Trypanosoma brucei brucei* and *Trypanosoma evansi* in mice. Interleukin-12p35^{-/-}, IL-12p40^{-/-} or IL-12p35^{-/-}/p40^{-/-} mice were susceptible to both pathogens, demonstrated by increased mortality compared to wild type C57/Bl/6 mice. The different IL-12p70^{-/-} mouse strains showed similar mortality kinetics, suggesting that IL-12p70 but not the IL-12p80 homodimer or IL-23 plays a crucial role in survival. While plasma levels of IgM and IgG2a were similar between IL-12 deficient mice and wild type mice, IFN- γ production, especially during early infection, was severely impaired in all IL-12p70^{-/-} mouse strains, demonstrating an IL-12p70-dependent mechanism for IFN- γ production. As IFN- γ R^{-/-} mice were also highly susceptible to both *trypanosoma* species, IL-12p70-dependent IFN- γ production seems to be the important mechanism involved in resistance against both pathogens.

Keywords: Trypanosomiasis, Immunology, Knock-out mice, Cytokines, Antibodies.

INTRODUCTION

Trypanosomes are unicellular eukaryotic parasites that replicate freely in the bloodstream of a mammalian host, ultimately resulting in sleeping sickness in humans, Nagana in cattle (*Trypanosoma brucei brucei*) or Surra (*T. evansi*) in various other animals [1].

Trypanosomes regulate and escape the host's B- and T-cell responses. Foremost is antigenic variation whereby the surface coat of the trypanosome consisting of variant surface glycoprotein (VSG) is regularly replaced, thus continually evading the host's immune response [2] [3]. Upon infection, the host responds with a rapid B-cell activation which has been found to be important for clearance of parasites from within vascular regions [4, 5]. Furthermore a rapid Th1 response is initiated [6] [7] [8] [9], with the characteristic production of IL-12 and IFN- γ [10]. Results from J.M. Mansfield et al. have suggested that the initiation of a polarized VSG-specific Th1 cellular response is dependent on the presence of IL-12 and IL-18 [11].

The IL-12p70 molecule is a heterodimeric protein comprised of two glycosylated subunits of p35 and p40 kDa [12] [13]. The p40 subunits also form homodimers (IL-12p80), and may act antagonistically to the functioning of IL-12 by blocking signal transduction via the receptor [14]. However, IL-12p80 homodimer may also act in an agonistic manner to IL-12 in its ability to induce cell-mediated Th1 immune responses [15] [16]. In addition to forming p35/p40 and p80 dimers, the p40 subunit also binds to p19, forming IL-23 [17]. The IL-12p80 homodimer also acts as an antagonist in IL-23 signaling [14].

Studies using IL-12 deficient mice in severe infectious disease models, such as toxoplasmosis [18], listeriosis [19], leishmaniasis [20], Chagas' disease [21] and tuberculosis [16], have found that IL-12 independent IFN- γ can be produced resulting in residual resistance. Interferon- γ which plays a central role in resistance to trypanosomiasis is produced primarily by activated Th1 cells which in turn induces macrophages to release trypanocidal molecules including reactive oxygen intermediates, reactive nitrogen intermediates and TNF [8] [22] [7] [23]. Mice lacking TNF have displayed reduced ability to control parasite load in the vascular compartment [24].

In this comparative infection study using *T. b. brucei* and *T. evansi* infection models, the IL-12 family, namely; IL-12p70, IL-12p80 homodimer and IL-23 were examined using IL-12p35, IL-12p40 and IL-12p35/p40 deficient mice. The data obtained suggests that IL-12p70 but not IL-12p80 homodimer or IL-23 was necessary for resistance against both *T. brucei* and *T. evansi* parasites. Interleukin-12 deletion resulted in impaired IFN- γ production necessary for parasitemia control and survival.

MATERIALS AND METHODS

Mice.

Interleukin-12p35^{-/-} [20] and IL-12p40^{-/-} [25] on a 129Sv/Ev background, backcrossed five times to C57BL/6; IFN- γ R^{-/-} 129Sv/Ev [26], C57BL/6 and 129Sv/Ev mice were maintained under SPF conditions. For the generation of homozygous double-deficient mice, IL-12p35^{-/-} and IL-12p40^{-/-} mice were intercrossed and genotyped by PCR [20] [25]. Experiments were performed within the guidelines of the Animal Research Ethics Board of the University of Cape Town (South Africa) (Animal ethics approval number: 005/041).

Parasites.

The pleomorphic *T. b. brucei* AnTat 1.1E clone and *T. evansi* (Code: KETRI 2480, Stock no.: ITMAS 110297) was obtained from the Institute of Tropical Medicine (ITG), Antwerp, Belgium.

Infection & parasitemia.

Mice were infected intraperitoneally with 5×10^3 parasites for *T. b. brucei*, and 1.0×10^3 parasites for *T. evansi*. Blood for parasite enumeration was obtained, diluted 1/200 in RPMI (10% FCS) (Highveld Biological, JHB, South Africa) and counted under a light microscope (Nikon, Randburg, South Africa).

Cytokine ELISAs.

Cytokines in plasma was measured by ELISA as described previously [27] with modifications. Plasma and standards were serially diluted three- and twofold respectively. Standards, coating and biotinylated antibodies for TNF- α and IFN- γ (BD PharMingen, CA) were used. Streptavidin labeled horseradish peroxidase (BD PharMingen) and 3,3',5,5-tetramethylbenzidine (KPL, Maryland, USA) were used for detection and development, respectively. The detection limit for both IFN- γ and TNF was 16pg/ml.

Quantification of anti-VSG plasma titres.

Anti-trypanosome-derived antigen antibody titres were determined as described previously [28].

Graph and statistical analysis.

Data are expressed as the means of individual determinations and SD with statistical analysis performed using the unpaired Student's t-test for ELISA's. Mortality data showed at least one IL-12 deficient group to be significantly increased compared to wild types using Kaplan-Meier with log rank test per experiment and were thus pooled increasing the number of mice per group. Analysis between wild type groups of repeat experiments using one-way ANOVA with Dunnett as the post test found that parasitemia was not significantly different and was thus pooled. Parasitemia at each time point was analyzed using one-way ANOVA with Dunnett as post test comparing deficient mice to wild type controls.

RESULTS

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice infected with T. b. brucei and T. evansi are susceptible with reduced survival.

In order to determine the role of IL-12p70 (p35/p40), IL-23 (p19/p40) and IL-12p80 homodimer during infection with trypanosomiasis, mice deficient in either one or both p35 and p40 genes were infected with *T. b. brucei* and *T. evansi* and monitored for survival (Table 1). Survival time of *T. b. brucei* and *T. evansi*-infected mice was significantly decreased in all deficient mice compared to infected wild type controls. No mortality differences were observed between the different deficient groups.

A specific role for IL-12 in the control of parasitemia in T. b. brucei during the chronic stages of disease.

The first parasitemia peak (day's 5-8 post infection, depending on the *Trypanosoma* strain) was similar in all three p35 and p40 deficient mice infected with *T. b. brucei* or *T. evansi* and wild type controls (Figure 1; A and B). However the rate of clearance of the

1st parasitemic peak was slightly reduced in IL-12 deficient mice compared to wild type mice infected with *T. b. brucei* by 4 to 8 fold at day 7 post infection ($p<0.05$) (Figure 1A).

Second peak and subsequent parasite control was clearly impaired during *T. b. brucei* infection with up to 100-fold increased parasite burden in IL-12 deficient mice compared to wild types. No significant differences in parasitemia between the different IL-12 deficient strains were observed.

In *T. evansi* infection wild type mice reduced their parasitemia at days 12, 23, and 36 forming several waves of parasitemia as apposed to all IL-12 deficient mice which failed to reduce parasite burden (Figure 1B). No significant differences were observed between the IL-12 deficient strains. In contrast to *T. b. brucei* infection, where loss of parasitemia control correlated with mortality in IL-12 deficient mice, *T. evansi* infections showed a high parasite burden during infection in both wild type and IL-12 deficient strains.

Interferon- γ and TNF are differentially regulated by IL-12p70, depending on the Trypanosoma species.

Interferon- γ and TNF play a role in resistance and parasitemia control [22] [29] with IL-12 a known promoter of IFN- γ production [30]. Plasma was taken during the 1st parasitemia peak at days 6 and 8, and post 2nd parasitemia peak at days 20 and day 28 for *T. b. brucei* and *T. evansi* (Figure 2). Both *Trypanosoma sp.* induced systemic IFN- γ and TNF production in wild type mice, with a few days delay in *T. evansi* infection. *Trypanosoma b. brucei* infection in IL-12 deficient mice resulted in a marked reduction

of IFN- γ ($p<0.05$) and TNF ($p<0.01$) production (Figure 2; A and B) with similar residual low levels in all IL-12 deficient strains observed.

Trypanosoma evansi infection in IL-12 deficient mice also resulted in impaired IFN- γ responses as seen in *T. b. brucei* infection (Figure 2C). In contrast to *T. b. brucei* infection, *T. evansi* infected IL-12 deficient mouse strains produced substantial amounts of TNF with only a slight reduction observed (Figure 2D).

Interleukin-12 deficient mice have unimpaired IgG2a and IgM antibody titres compared to wild type mice in response to infection with trypanosomiasis.

Anti-VSG antibody responses are important in parasitemia control and survival; and were thus tested in IL-12 deficient mice (Figure 3). In *T. b. brucei*, IgG2a levels were similar between wild type and IL-12 deficient mice at day 6 (Figure 3A) and 20 (Figure 3B) post infection. In *T. evansi*, IgM levels at either the 1st peak (Figure 3C) or during the chronic stages (Figure 3D) were similar in IL-12 deficient and wild type mice.

Interferon- γ production is crucial for resistance to trypanosomiasis.

Reduced survival was observed in IFN- γ R (129Sv/Ev) deficient mice compared to wild type (129Sv/Ev) mice in both *Trypanosoma* strains with mean mortalities of 16 vs. 41 days ($p<0.001$) in *T. b. brucei* (Figure 4A), 15 vs. 27 days ($p<0.001$) in *T. evansi* (Figure 4C), respectively. Interferon- γ R deficient mice in *T. b. brucei* infection were unable to sufficiently clear the first parasitemia wave and died shortly thereafter (Figure 4B), associated with a greater than 10 fold increase in parasitemia from day 9 post infection until mortality ($p<0.05$) compared to wild type controls. The *T. b. brucei* results obtained are similar to those in *T. b. rhodesiense* infected C57Bl/6 wild type and C57Bl/6 IFN- γ

deficient mice indicating no major differences between 129Sv/Ev or C57Bl/6 genetic background [22]. Interestingly, *T. evansi* infection resulted in high parasitemia in both wild type and IFN- γ R deficient strains (Figure 4D).

DISCUSSION

Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mouse strains showed significantly reduced survival compared to wild type controls in both *T. b. brucei* and *T. evansi* infections. This outcome is in agreement with previous results showing an association with lower IL-12p70 levels in susceptible wild type mouse strains during the initial stages of infection compared to more resistant strains [22]. Between the three IL-12 deficient mouse strains no differences in survival rates were observed suggesting that IL-12p70 is the main protective factor for survival with no major contribution from other IL-12 family members, namely IL-23 or the IL-12p80 homodimer. Interleukin-12 deficient mice survived several trypanosome waves; therefore residual protection occurs and may imply that IL-12 plays a more striking role during the established disease. As IFN- γ is an important factor for survival, shown in *T. b. brucei* [22], we determined IFN- γ plasma levels during infection. In wild type mice IFN- γ was present during the first parasite wave with striking reduced IFN- γ in all IL-12 deficient mouse strains. These results also demonstrate that residual IL-12-independent IFN- γ production is induced during infection by both trypanosome strains. Therefore, the observed limited Type 1 response allowed residual protective immunity. Interleukin-12 independent induction of IFN- γ may also include a direct stimulus by either trypanosome species. Trypanosome derived lymphocyte triggering factor (TLTF) has been found to induce the production of IFN- γ by binding to CD8⁺ cells in *T. b. brucei* [31] and *T. evansi* [32] infections and therefore may have contributed to residual resistance in the absence of IL-12. In addition, work

describing IL-12-independent IFN- γ production with residual Th1 responses have been demonstrated by us and others during *Listeria monocytogenes* [19], *Mycobacterium BCG* [16] and *Leishmania major* [20] infections. Furthermore, to evaluate the possible contribution of IFN- γ to resistance, IFN- γ R deficient mice were infected, which died shortly after the first parasite wave. Although the IFN- γ R deficient mice used here were on a different genetic background compared to IL-12 deficient mice these results are in line with the conclusions of Hertz et al., 1998 using IFN- γ deficient mice on a C57Bl/6 background. These results clearly highlight the crucial importance of IFN- γ responsiveness during acute infection with *T. b. brucei* or *T. evansi*.

Although IL-12 deficient mice did have significantly reduced rates of parasitemia clearance after the 1st peak compared to wild type mice, ultimately the IL-12 deficient mice were as effective in clearance of the 1st peak as control mice. Interferon- γ and TNF cytokine levels in all groups of IL-12 deficient mice were significantly attenuated during the initial parasitemic peak. Together, with the observation of a drastically reduced capacity to control parasitemia in infected IFN- γ R and TNF deficient mice [24] during this period, the low plasma concentrations of these two cytokines in IL-12 deficient mice are still effective for initial parasitemia control. These results further suggest that IL-12 plays a more dominant role in parasitemia control after the 2nd peak for optimal promotion of TNF and IFN- γ production. Tumor necrosis factor has been found to have dual effects in *T. b. brucei* infections, such as trypanolytic effects [29, 33] reducing parasite burden in case of *T. b. brucei* AnTat 1.1 infections [24, 34, 35] and causing immunopathological effects in the host [24]. Although TNF was reduced in IL-12 deficient mice no decrease in pathology was detected in spleen and liver sections in *T. b. brucei* or *T. evansi* (data not shown). Additionally, B-cell responses are necessary for the

elimination of vascular bound parasites, in particular IgM and IgG2a are quoted as the main antibodies necessary for clearance in *T. b. brucei* (Magez et al., manuscript in preparation) [4]. Both VSG specific antibodies in IL-12 deficient mice were present in plasma but at moderately reduced levels compared to wild type mice and thus may not be the overall mechanism of increased parasite burden. Thus the role of IL-12 in *T. b. brucei* seems to be limited to the more chronic stage of disease and in promoting the production of TNF and IFN- γ necessary to control parasitemia.

Natural killer cells are thought to be necessary for resistance during the initial infection which are activated by both IL-12 and IL-18 [36] and thus IL-18 may effectively drive the Th1 response in the absence of IL-12. Furthermore a recent paper by Drennan et al., 2005 found MyD88 signaling to be crucial for resistance, with a role for TLR9 in this immune pathway [37]. The TLR9 and IL-12 deficient mice share many phenotypic characteristics in their response to *T. b. brucei* infection, including; effective 1st peak parasitemia control but increased parasitemia during the chronic stages, reduced IFN- γ production but normal IgG2a responses during the chronic stages of disease compared to wild type mice. Bone marrow-derived macrophages from TLR9 deficient mice also displayed severely reduced TNF and IL-12p40 in response to *T. b. brucei* genomic DNA. Therefore, IL-12 may be involved in the immunological pathway whereby TLR9 present on macrophages respond to *T. b. brucei* genomic DNA released following the 1st peak of parasitemia and upon IL-12 secretion stimulate PBMCs to secrete IFN- γ . With the presence of TLR9 on B cells a role for IL-12 here may also be considered. However with only a slight reduction in IgG2a responses during the chronic stages as reported in this paper as well as in the paper by Drennan et al., 2005, it is more likely that other factors play a more significant role in proliferation and differentiation of these cells. In contrast

to IL-12 deficient mice, TLR 9 deficient mice produce more IFN- γ during the chronic stages of *T. b. brucei* infection. Reduced IFN- γ production may explain the increased susceptibility in IL-12 deficient mice compared to TLR9 deficient mice which showed similar resistance as wild type mice. The function of IL-12 may thus be to link TLR9 signaling in macrophages during the chronic stage of *T. b. brucei* infection and the up-regulation of IFN- γ secretion in PBMCs necessary for effective parasitemia control and survival.

The parasitemia of wild type mice infected with *T. evansi* differed to that of *T. b. brucei*. *Trypanosoma evansi* infection in wild type mice showed severe inability to efficiently clear parasitemia waves, deteriorating after each successive wave, suggesting a progressive erosion of immune control. Interleukin-12 deficient mice infected with *T. evansi* were unable to reduce parasitemia compared to wild type mice. However, later during infection, no differences between IL-12 deficient and wild type mice were found. This suggests a role for IL-12p70 signaling for early parasitemia control. During the initial and late time points examined, plasma levels of IFN- γ were severely reduced but, in contrast to *T. b. brucei*, only moderately lower for TNF. *Trypanosoma evansi* infected IFN- γ R deficient mice were highly susceptible and were unable to clear the 1st parasitemic peak and rapidly died thereafter. This clearly demonstrates the importance of IFN- γ , although a direct comparison with IL-12 deficient mice is hampered by the difference in the genetic background of the deficient strains and mortality of wild type controls in *T. evansi* infection. Tumor necrosis factor was not severely reduced in IL-12 deficient mice and has been found not to be important in survival or parasitemia control [38]. In terms of anti-VSG B-cell responses, it has been recently shown that IgM is crucial for parasitemia control and survival [39]. However we found no obvious

differences between wild type and IL-12 deficient mouse strains. Therefore the mechanism of impaired parasitemia control in *T. evansi* infection is dependent on IL-12 with a possible role for IFN- γ as found in the *T. b. brucei* infection.

A comparison between the results from the C57Bl/6 IFN- γ deficient mice obtained by Baral et al., [39], and the results from the 129Sv/Ev IFN- γ R deficient mice presented in this paper show opposing results. This difference could be due to the emergence of a more virulent strain in our experiments as the *T. evansi* parasites used are non-clonal field stabilates and have been passaged for stock maintenance. In addition the SPF conditions at our facilities may exclude concurrent infections that may independently initiate an IL-12 driven Th1 response necessary for optimal resistance. These effects, although untested, adds impetus for further investigation into the immunology of *T. evansi*.

In conclusion the data presented in this paper showed that IL-12p70 but not the IL-12p80 homodimer or IL-23 is necessary for optimal resistance to *T. evansi* and *T. b. brucei* infection in terms of mortality and parasitemia control. The resistance attributed to IL-12 is mediated through IFN- γ , the production of which was significantly decreased in the absence of IL-12. Furthermore a role for IL-12 in parasitemia control during the chronic stage of *T. b. brucei* infection was shown where the presence of IL-12 is needed for optimal TNF and IFN- γ production reducing parasitemia burden.

Acknowledgements

We are grateful for the assistance from the staff of the University of Cape Town, including the mouse genotyping staff (Erica Smit, Wendy Green and Reagon Petersen), Animal Unit staff (Hiram Arendse and Jaap Visser), and Histology staff (Lizette Fick, Marilyn Tyler and Zoë Lotz). Also, Dr's Reto Guler and Antony J. Cutler are thanked for their critical evaluation of the manuscript.

University of Cape Town

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Table I. Interleukin-12 deficient mice infected with *T. b. brucei* and *T. evansi* show increased mortality compared to wild type mice.

Parasite species ^a	Strain	Mean survival (SD)	No. of mice/group ^b	<i>p</i> value compared to Wild Type mice ^c
<i>T. b. brucei</i>				
	Wild Type	40.6 ± 8.8	29	
	IL-12p35 ^{-/-} /p40 ^{-/-}	35.1 ± 6.4	15	<i>p</i> < 0.05
	IL-12p40 ^{-/-}	32.0 ± 3.7	15	<i>p</i> < 0.0001
	IL-12p35 ^{-/-}	33.3 ± 4.2	15	<i>p</i> < 0.001
<i>T. evansi</i>				
	Wild Type	47.0 ± 24.1	33	
	IL-12p35 ^{-/-} /p40 ^{-/-}	31.4 ± 14.3	35	<i>p</i> < 0.001
	IL-12p40 ^{-/-}	31.1 ± 13.1	21	<i>p</i> < 0.001
	IL-12p35 ^{-/-}	30.3 ± 20.6	34	<i>p</i> < 0.01

^a Mice were infected with 5x10³ trypanosomes *T. b. brucei* and 1x10³ trypanosomes *T. evansi* intraperitoneally and monitored daily for survival.

^b Three independent experiments were combined.

^c Tests for significance were performed using the Kaplan-Meier test.

Figure 1. Parasitemia profiles of *T. b. brucei* (**A**), and *T. evansi* (**B**) infected wild type (■), IL-12p35^{-/-}/p40^{-/-} (□), IL-12p40^{-/-} (△), and IL-12p35^{-/-} (◇) mice. Mice were

infected intraperitoneally with 5×10^3 *T. b. brucei* trypanosomes and 1×10^3 *T. evansi* trypanosomes with a lower detection limit of infected mice of 2.7×10^5 and 4.0×10^5 parasites/ml, respectively. Data points are the mean from 2-3 pooled experiments of 10-15 individual mice per group. Inferred data points appearing below the detection limit are denoted as; ϕ . The x-axis of (**B**) has been cropped from 100 days to 65 days to show detail. Statistical significance between infected wild type and IL-12 deficient mice at each time point was determined by one-way ANOVA (Dunnett post test) and are indicated by; *, $p < 0.05$, and **, $p < 0.01$.

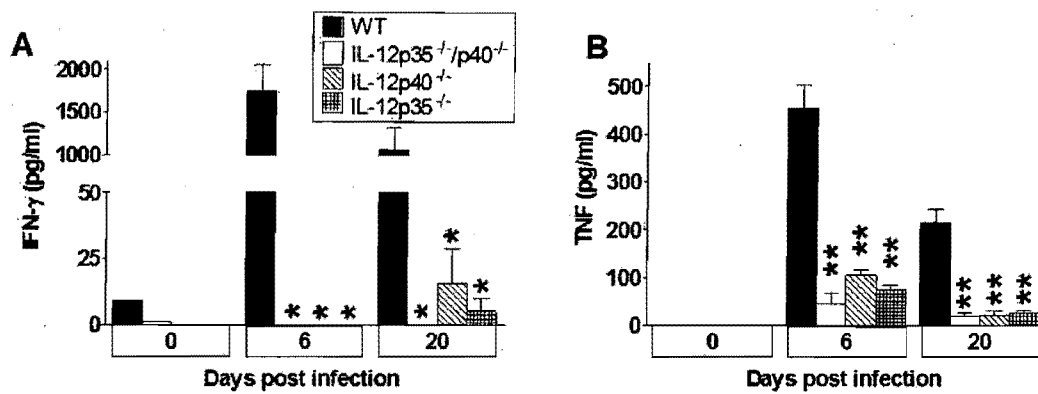
Figure 2. Interferon- γ and TNF plasma levels were measured by ELISA in wild type (Filled bars), IL-12p35^{-/-}/p40^{-/-} (Open bars), IL-12p40^{-/-} (Striped bars) and IL-12p35^{-/-} (Hatched bars) mice infected with *T. b. brucei* (**A** and **B**), and *T. evansi* (**C** and **D**). Results are representative of two independent experiments; however TNF levels in IL-12p40 deficient mice were not consistently significantly reduced. Data expressed as means ($\pm$ SEM) from 2-3 individual mice. Statistical significance determined by unpaired Student's t-test are indicated by; *, $p < 0.05$ or **, $p < 0.01$.

Figure 3. Immunoglobulin G2a and IgM plasma antibody titres from *T. brucei* (Day 6 (**A**) and day 20 (**B**)) and *T. evansi* (Day 8 (**C**) and day 28 (**D**)) infected C57BL/6 (wild type) (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-} p40^{-/-} (□) mice. Pre-immune plasma was used as a non-infected control (●). Data points represent mean and SEM from plasma of 2-3 individual mice.

Figure 4. Survival and parasitemia profiles of wild type (■) and IFN- γ R deficient (□) mice infected with *T. b. brucei* (**A**, **B**) and *T. evansi* (**C**, **D**). Mice were infected

intraperitoneally with 5×10^3 *T. b. brucei* trypanosomes (n=11 wild type and 12 deficient mice) and 1×10^3 *T. evansi* trypanosomes (n=14 wild type and 17 deficient mice) per mouse. Parasitemia detection limit for *T. b. brucei* is 4.4×10^5 and 1.0×10^6 parasites/ml for *T. evansi*. Mortality and parasitemia data were pooled from two independent experiments with 4-5 mice per group used for parasitemia measurement for either experiment. Inferred data points appearing below the detection limit are denoted as; ϕ . The Kaplan-Meier test for mortality and for parasitemia the unpaired Student's t-test was used; *, $p < 0.05$.

T. b. brucei



T. evansi

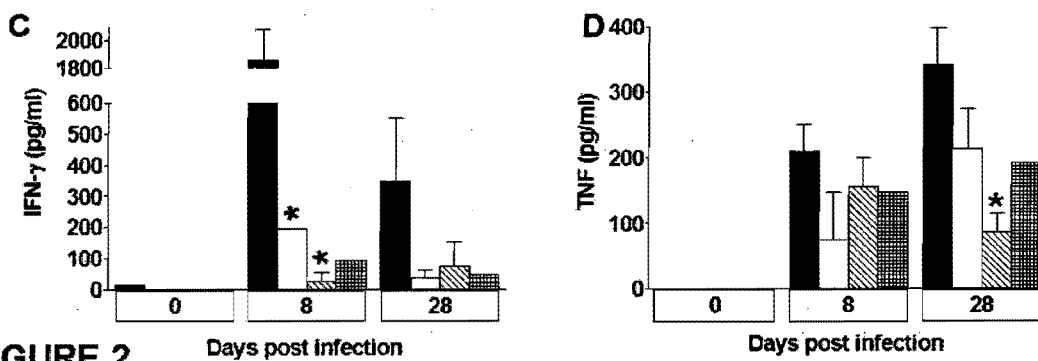
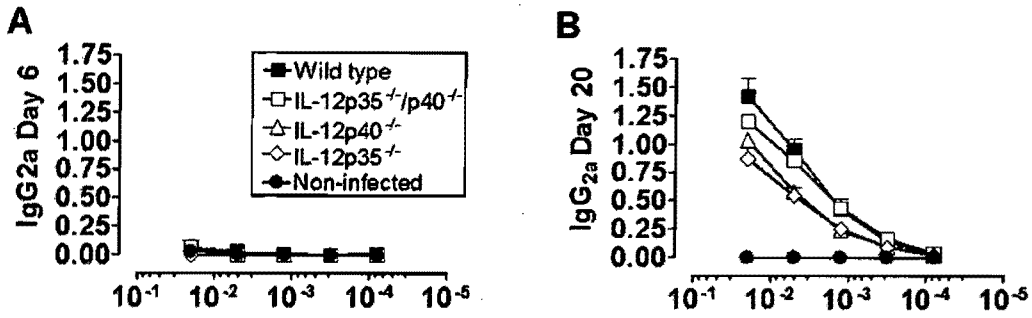


FIGURE 2.

T. b. brucei



T. evansi

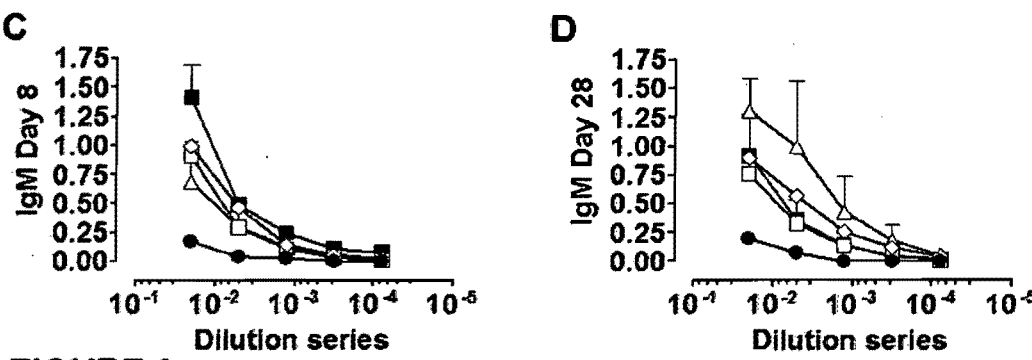


FIGURE 3.

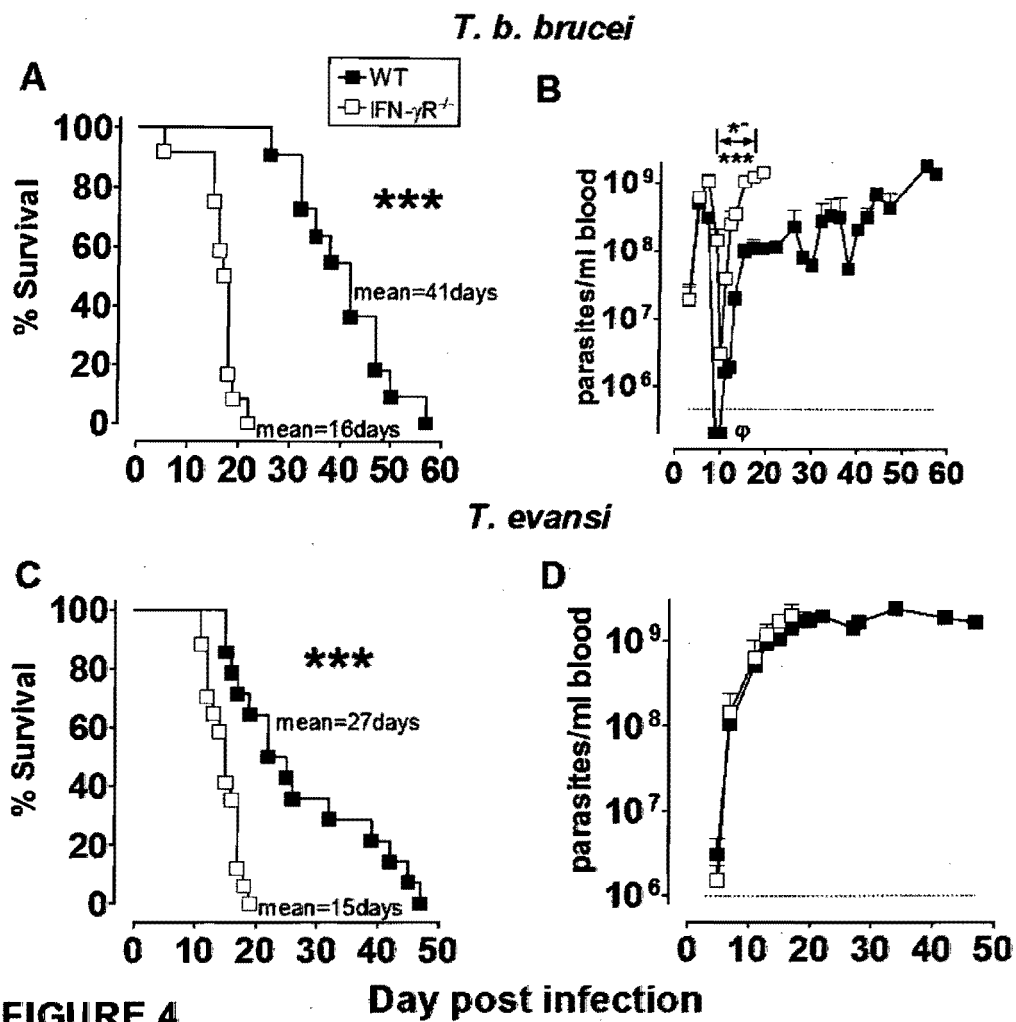


FIGURE 4.

PUBLICATIONS

Interleukin-12p70 Deficiency Increases Survival and Diminishes Pathology in *Trypanosoma congolense* Infection.

Mark Barkhuizen¹, Stefan Magez^{1,2,3}, Bernhard Ryffel⁴ and Frank Brombacher^{1*}

¹The Division of Immunology, Institute of Infectious Diseases and Molecular Medicine,
Faculty of Health Sciences, University of Cape Town (UCT), Cape Town, South Africa.

²Department of Molecular and Cellular Recognition, VIB, Brussels, Belgium.

³Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussels (VUB),
Brussels, Belgium.

⁴Université d'Orléans, Centre national de la Recherche Scientifique (CNRS), Molecular
Immunology and Embryology, 45071, Orléans, France.

Word count: Abstract: 144, Article text: 3195

Number of text pages: 23; Figures: 5.

Running title: Role of IL-12 in trypanosomiasis.

FOOTNOTES:

Conflict of Interest: The authors have no commercial or other association that may cause a conflict of interest.

Grant numbers and sources of support: This work was supported by the Flanders-South African Research Cooperation Program as well as the MRC, NRF and NHLS, South Africa. S.M. was a Research fellow for the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

* **Address for correspondence and reprint requests:** Prof. Frank Brombacher, University of Cape Town, IIDMM, Wernher Beit South, S27.1, Anzio road, Observatory, 7925, Cape Town, South Africa; Phone: +27-21-406-6616, Fax: +27-21-406-6029, e-mail: fbrombac@mweb.co.za

ABSTRACT

To determine the immunological role of IL-12 family members in *T. congolense* infection, IL-12p35^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} mice were used. While the two latter strains lack all IL-12 homologues, IL-12p35^{-/-} mice still produce IL-12p80 homodimers and IL-23. Compared to wild type mice, all infected IL-12 deficient mouse strains showed prolonged survival, while parasitemia levels were unaltered. Interferon- γ production in IL-12 deficient mice was strikingly reduced during the acute and chronic infection stages, coinciding with significantly reduced chronic stage hepatocellular damage, demonstrated by histological analysis and plasma AST measurements. In contrast, IL-10 production was not affected by the absence of IL-12. Together, these results show that during *T. congolense* infection, the absence of IL-12, but not the IL-12p80 homodimer or IL-23, leads to a reduction of IFN- γ production, which reduces hepatic pathology and improves host survival in conjunction with IL-10 without negatively affecting parasitemia control.

Keywords: Trypanosomiasis, Immunology, Gene-deficient mice, Cytokines.

INTRODUCTION

Trypanosomes are unicellular eukaryotic parasites that infect humans as well as a wide range of other mammals. While human 'sleeping sickness' is caused by the *T. brucei* subspecies *T. b. rhodesiense* and *T. b. gambiense*, the disease in cattle 'Nagana' is mainly caused by *T. congolense*. In contrast to *T. b. brucei* and *T. evansi* [1], *T. congolense* is strictly an intravascular blood parasite [2]. Several parasite defense mechanisms exist of which the most significant is antigenic variation, enabling trypanosomes to continually evade the hosts' immune response [3]. In *T. congolense* infection, both an antibody and a cellular mediated immune response are necessary for optimal host protection. Indeed, phagocytosis of opsonized trypanosomes by macrophages and Kupffer cells of the liver, mediated by both IgM and IgG antibodies, is considered as the main parasite clearing mechanism [4]. Subsequently, the release of inflammatory cytokines occurs in order to drive a rapid host-anti-parasite response.

Using a compendium of various gene deficient mice we illustrated the requirement for a Th1 response for effective control of the acute phase of *T. congolense* infection [5]. Here, interferon- γ (IFN- γ) signaling was shown to be necessary for the upregulation of inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF) production, both required for prolonged survival and parasitemia control [5] [6] [7]. On the other hand, interleukin-10 (IL-10) has been shown to be necessary to limit the pro-inflammatory IFN- γ response [8] which was implicated in trypanosomiasis-associated liver pathology [9].

The establishment of a Th1 immune response during *T. congolense* infection focuses attention onto the role of interleukin-12 (IL-12) [10] [11]. The IL-12 molecule is a heterodimeric protein comprised of two subunits of 35 and 40 kDa [12]. Interleukin-23, which shares the p40 subunit, is able to promote a T cell subset that is distinct to that of IL-12 [13]. Homodimers (IL-12p80) also form from the p40 subunits, and may act

antagonistically to the functioning of IL-12 and IL-23 [14] [15], conversely agonist activity is also possible by its ability to induce cell-mediated Th1 immune responses [16] [15].

In the present study the role of IL-12 and IL-23 together with IL-12p80 were examined using three IL-12 gene deficient mouse strains namely; IL-12p35, IL-12p40 and IL-12p35/p40 in *T. congolense* infection. Interleukin-12 but not IL-12p80 and/or IL-23 were necessary for optimal IFN- γ and iNOS production. In contrast to previously reported results obtained with *T. b. brucei* and *T. evansi* infected IL-12 deficient mice [17], these mice showed increased survival when infected with *T. congolense*, albeit parasitemia control itself was comparable to wild type controls. From the data presented we conclude that IL-12, most likely through the action of IFN- γ production, contributes to the formation of chronic stage pathological lesions in liver of *T. congolense* infected mice, hence negatively affecting the hosts' survival time.

MATERIALS AND METHODS

Mice

Interleukin-12p35^{-/-} [18] and IL-12p40^{-/-} [19] on a 129Sv/Ev background, backcrossed five times to C57BL/6 mice, were maintained under specific pathogen-free conditions. For the generation of homozygous double-deficient mice, IL-12p35^{-/-} and IL-12p40^{-/-} mice were intercrossed and genotyped by PCR [18] [19]. Experiments were performed in accordance with the guidelines of the Animal Research Ethics Board of the University of Cape Town (Cape Town, South Africa) (Animal ethics approval number: 005/041).

Parasites

The clonal Tc13 antigen type of *T. congolense* was kindly provided by Dr. H Tabel from the University of Saskatchewan, Canada [20]. Trypanosome stabilates were maintained at -80°C and thawed for infection.

Infection & parasitemia

Each mouse was infected intraperitoneally with 1000 parasites from stabilate (100µL per mouse). Blood for parasite enumeration was obtained, diluted 1/200 in RPMI (10% FCS) (Highveld Biological, JHB, South Africa) and counted under a light microscope (Nikon, Randburg, South Africa).

Cytokine ELISAs

Cytokines in plasma was measured by ELISA as described previously [21] with modifications. Blood plasma and cytokine standards were serially diluted three- and twofold, respectively. Cytokine standards, coating and biotinylated antibodies; TNF, IFN-γ (BD PharMingen, San Diego, CA) and IL-10 (R&D Systems, Abingdon, UK) were used. Streptavidin labeled horseradish peroxidase (BD PharMingen) and 3,3',5,5-tetramethylbenzidine (KPL, Maryland, USA) were used for detection and development, respectively. Detection limit for IFN-γ and TNF was 16pg/mL, and IL-10 was 63pg/mL. Hepatocellular damage was determined by the aspartate transaminase assay (AST) (Sigma, Aston Manor, SA).

Anti-VSG plasma titres

Plasma IgG2a and IgG3 antibody responses specific for trypanosome-derived antigens were determined as previously described [22].

Histopathology

Organs were fixed in 4% buffered formalin and paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E) and assessed by light microscopy (Nikon, Randburg, South Africa). Images were captured using the DXM1200 camera and ACT software (Nikon).

Immunohistochemistry

Sections of frozen tissue were attached to coated slides then air-dried and fixed. Slides were treated with primary antibody against iNOS (gift from Prof. B. Ryffel) after which secondary antibody coupled with FITC (BMA Biomedicals, Switzerland) was added. Sections were mounted in Fluoromount (DAKO, Carpinteria, CA) and assessed by immunofluorescence microscopy (Nikon).

Graph and statistical analysis

Data are expressed as the means of individual determinations and SEM. Statistical analysis was performed using the unpaired Student's t-test for ELISA's and for mortality Kaplan-Meier with log rank test was used. Mortality data showed similar trends between experiments and was thus pooled. Parasitemia between wild type and IL-12 deficient mouse strains was analyzed using one-way ANOVA (Dunnett post-test) at each time point measured. Parasitemia data showed similar trends between experiments and was thus pooled.

RESULTS

Prolonged survival in Interleukin-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice infected with T. congolense.

In previous trypanosomiasis studies, survival of IL-12p35^{-/-}/p40^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-} mice was found to be decreased compared to wild type mice when infections were performed with *T. b. brucei* and *T. evansi* parasites [17]. Unexpectedly, mice deficient in either or both the p35/p40 subunits showed strikingly increased survival time compared to wild-type controls ($p < 0.0001$) (Figure 1A). Whereas the pooled mean survival time of wild-type mice was 97 days; IL-12p40, IL-12p35/p40 and IL-12p35 deficient mice showed a mean survival time of 163, 155 and 150 days respectively. The slight mean survival time difference between the three IL-12 deficient mouse strains was not significant, as determined from two individual pooled experiments consisting of 27 to 37 mice per strain.

Parasitemia control is independent of IL-12 in T. congolense infection.

Despite the significant role of IL-12 on host survival, no significant differences were found between the different strains of IL-12 deficient and wild type mice during 1st peak parasitemia control, clearance and subsequent waves of parasitemia (Figure 1B). In addition, no significant differences in anemia were found between IL-12 deficient and wild type mice (data not shown).

Interleukin-12 is necessary for optimal production of IFN- γ in T. congolense infection at the 1st parasitemic peak.

Both IFN- γ and TNF are known to be necessary for the control of parasitemia and survival in *T. congolense* infection [5] [7] [6], thus their plasma cytokine levels were determined during the 1st parasitemia peak at day 7 and at day 28 post infection (Figure

2). Infection induced strong systemic IFN- γ and TNF production in wild type mice at day 7 post infection (Figure 2A and B). However, in all IL-12 deficient mouse strains, IFN- γ ($p < 0.05$) was significantly impaired at day 7 post infection. Levels of TNF were variable between the IL-12 deficient strains with moderately reduced responses compared to wild type controls at both days 7 and 28 post infection. At day 28 post infection, IFN- γ was similarly present in both wild type and IL-12 deficient mouse strains, but at low levels. Plasma levels of IL-10 were similar in both wild type and IL-12 deficient mice at day 28 post infection (Figure 2C).

Similar IgG2a and IgG3 antibody titres were found between wild type and IL-12 deficient mice.

Results from B cell and IgM deficient mice as well as splenectomized mice infected with *T. congolense* have suggested that IgG2a and IgG3 anti-VSG antibody responses play a role in parasitemia control and survival [5]. Day 28 post infection is shown, as maximal titres were attained at this time point in both wild type and IL-12 deficient mice. Similar levels of IgG2a and IgG3 were found between wild type and IL-12 deficient mice (Figure 3A and B).

Reduced early iNOS production in liver during the acute stages of T. congolense infection in IL-12 deficient mice compared to wild type mice.

As both IFN- γ and TNF induce NO synthesis, important for resistance to *T. congolense* infection [5], for the detection of NO we analyzed iNOS production in spleen and liver. Inducible nitric oxide production was severely reduced in liver sections of IL-12 deficient mice regardless of subunit deletion compared to wild type mice at day 7 post infection (Figure 4A and B) but similar production was found in spleen sections (Figure 4C and

D). Furthermore, iNOS production examined in liver sections at day 105 post infection was highest in wild type mice with IL-12 deficient mice showing slightly reduced production (data not shown).

Interleukin-12 deficient mice display decreased IFN- γ and AST levels as well as liver pathology compared to wild type mice at day 105 post infection.

In order to get a better insight into the chronic stage; liver pathology of *T. congolense* infections, plasma cytokine levels as well as AST levels were measured in all mice on day 105 of infection. Figure 5 shows that IFN- γ levels were significantly reduced in all IL-12 deficient mice compared to wild type mice (Figure 5A). Although in the repeat experiment significance was not attained, the trend was altogether similar with mean plasma levels of 78.5 pg/mL and below detection limit (<16 pg/mL) for wild type and IL-12 deficient mice, respectively. Levels of IL-10 and TNF were similar in both wild type and IL-12 deficient mice, with high variation detected (Figure 5B and C). Circulating levels of AST were significantly lower in all IL-12 deficient mouse strains compared to wild type mice at day 105 post infection (Figure 5D).

Chronic stage liver inflammation has previously been shown to be a hallmark of *T. congolense* infections. However, during the chronic stage of infection, liver sections from IL-12 deficient mice displayed smaller and fewer focal mononuclear cellular infiltrates compared to wild type mice (Figure 5E and F). Both IL-12 deficient and wild type mice had similar cellular compositions of focal mononuclear cell infiltrates consisting of activated macrophages with scattered lymphocytes and neutrophils. Subcapsular hemorrhagic infarctions present in the liver were also found in certain wild type mice but not in IL-12 deficient mice. Enlarged sinusoidal spaces were found in IL-12 deficient

mice as well as in wild type mice; however mononuclear cell infiltration in these areas was distinctly reduced in IL-12 deficient mice compared to wild type mice. All IL-12 deficient mice displayed a similar reduction of pathological lesions compared to wild type mice regardless of subunit deletion as well as normal liver structure compared to wild type mice.

DISCUSSION

The finding of increased survival with similar parasitemia burden in IL-12p35^{-/-}, IL-12p40^{-/-} and IL-12p35^{-/-}/p40^{-/-} mice in comparison to wild type mice shows evidence that IL-12p70 but not IL-23 or the IL-12p80 homodimer is detrimental during experimental murine *T. congolense* infection. We may eliminate a role for the IL-12p80 homodimer and IL-23 in *T. congolense* infection as no phenotypic differences were found between IL-12p35^{-/-} deficient mice, which produce both IL-12p80 homodimer and IL-23, and IL-12p35^{-/-}/p40^{-/-} mice which lack the IL-12p40 related products [10].

Interleukin-12 is important for a rapid activation of IFN- γ -producing cells in order to promote protective Type 1 responses, including T helper 1 differentiation and classical macrophage activation. The latter induces both IFN- γ and TNF production necessary for optimal iNOS synthesis [10]. Although global deletion of either TNF [6] [23] or IFN- γ [5] resulted in early mortality and uncontrolled parasitemia in *T. congolense* infections, suboptimal production of IFN- γ due to IL-12-independent pathways [24] during early and late time points had no deleterious effects on parasitemia control or survival in *T. congolense* infected IL-12 deficient mice. Thus, IL-12 dependent IFN- γ production is dispensable during *T. congolense* infection.

Nitric oxide catalyzed by iNOS and essential for parasitemia control and survival in *T. congolense* infection, is a highly effective killing effector radical for both intra- and extracellular pathogens [5]. Interleukin-12 deficient mouse strains infected with *T. congolense* produced residual iNOS, localized in the spleen, probably due to the presence of IL-12-independent IFN- γ . Remaining iNOS production seemed to be sufficient for the control of parasitemia and survival.

The detrimental role of IL-12 was seen to be mainly manifesting in liver pathology in accordance with studies associating liver pathology with mortality in *T. congolense* infection [8] [9]. The presence of immunosuppressive IL-10 in infected IL-12 deficient mice in combination with reduced IFN- γ production may explain the reduced liver histopathology and increased survival as apposed to wild type mice [9] [8]. Together, these results indicate that a qualitative rather than quantitative cytokine response to *T. congolense* infection would translate into effective parasite control and survival of the host.

Similar findings have been reported implicating IL-12 in promoting hepatic immunopathology in *Leishmania donovani* [25], *Plasmodium berghei* [26] and in *Schistosoma mansoni* infection models [27]. Together, these results suggest that IL-12 can play a role in pathogenesis during infection by its induction of pro-inflammatory immune responses, as demonstrated in *T. congolense* infection.

The results found in the present study are in striking contrast to the known beneficial role of IL-12 observed in *T. b. brucei* infection, where IL-12 is found to be essential for

parasite control and survival [17]. A reason for these differences may be that, as *T. congolense* and *T. b. brucei* belong to different subgenus's, the host may rely on different immune effector molecules for resistance. Downstream of the IFN- γ signaling pathway, NO is induced which is essential for resistance to *T. congolense* [5] and detected in similarly infected IL-12 deficient mice. In contrast to *T. congolense* infection the downstream mechanisms of IFN- γ in *T. b. brucei* [28] remains to be elucidated and, as our previous results demonstrated, the mechanism is also dependent on IL-12 signaling [17].

Furthermore, the induction of TNF in *T. b. brucei* infection is largely dependant on IL-12 signaling [17], however in *T. congolense*, production is only slightly impaired. As TNF is necessary for parasitemia control in both *T. b. brucei* and *T. congolense* infections, this may also explain the differences in terms of parasitemia control in IL-12 deficient mice infected with either parasite.

The findings from the IL-12 deficient mice parallel those of other studies describing characteristics linked to trypanotolerance in *T. congolense* infection. Recently, regulatory T cells have been shown to be necessary to limit the sustained type I immune response resulting in pathology in *T. congolense* infected mice [8]. In addition, the emergence of an alternatively activated macrophage profile has been documented during the chronic stages of disease exhibiting properties such as the down-regulation of pro-inflammatory responses [29]. The findings here, illustrating decreased pro-inflammatory responses together with decreased immunopathology in IL-12 deficient mice, parallel the above-mentioned studies and indicate that immune responses in wild type mice are ultimately detrimental to the host.

Residual IFN- γ responses seem also to be sufficient for optimal type 1 responses, as IL-12 deficient mice produced similar levels of VSG-specific IgG2a and IgG3 antibodies during *T. congolense* infection as wild type mice, a result also found in *T. b. brucei* and *T. evansi* infection [17]. Contrary to previous hypothesis, these results suggest that IL-12 together with high IFN- γ responses are not necessary to drive this B-cell switch in *T. congolense* infections [30] [31].

In summary the data presented here illustrates that the immune response against *T. congolense* infection in wild type animals is to an extent an over-shooting inflammatory response, mediated by IL-12p70 but not IL-12p80 homodimer or IL-23, resulting in pathological manifestations leading to mortality. The result of increased survival in *T. congolense* infected IL-12 deficient mouse strains compared to wild type mice is in contrast to the findings of other type 1 dependant diseases where decreased resistance is characteristically found in the IL-12 deficient mouse strains [10]. Our understanding of resistance to *T. congolense* is therefore incomplete and the results presented here suggests a finely tuned immune response that is not pathogenic for the host but at the same time anti-parasitic, is the key for effective trypanotolerance. Furthermore this study also highlights major immunological differences between *T. congolense* and *T. b. brucei* as well as *T. evansi* that should be taken into consideration in future studies.

Acknowledgements

We are grateful for the assistance from the staff of the University of Cape Town, including the mouse genotyping staff (Erica Smit, Wendy Green and Reagon Petersen), Animal Unit staff (Hiram Arendse and Jaap Visser), and Histology staff (Lizette Fick, Marilyn Tyler and Zoë Lotz). Also, Dr Antony J. Cutler is thanked for his critical evaluation of the manuscript.

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FIGURE 1. Interleukin-12 deficiency results in increased resistance to infection with *T. congolense*. Mortality (A) and parasitemia (B) profiles of *T. congolense* infected wild type (■), IL-12p35^{-/-}/p40^{-/-} (□), IL-12p40^{-/-} (△), and IL-12p35^{-/-} (◇) mice are shown from two independent pooled experiments. Infectious dose was 1x10³ trypanosomes per mouse and were monitored for; survival and parasite burden from peripheral blood obtained from tail snips (10 individual mice per strain) with a lower detection limit of: 4.0x10⁵ parasites/ml. Statistical tests performed by the Kaplan-Meier with log rank test for mortality and presented as; ***, *p*<0.001. A portion of the entire parasitemia profile is shown; days 2 to 17 and 55 to 106. Statistical tests between parasitemia levels of wild type and IL-12 deficient mice were determined at individual time points by one way ANOVA with Dunnett as post test (±SEM).

FIGURE 2. Interleukin-12 is necessary for an optimal interferon-gamma (IFN-γ) response to infection with *T. congolense* during the 1st parasitemic peak. Interferon-γ (A), tumor necrosis factor (TNF) (B) and interleukin-10 (IL-10) (C) plasma levels were measured by ELISA in wild type (Filled bars), IL-12p35^{-/-}/p40^{-/-} (Open bars), IL-12p40^{-/-} (Striped bars) and IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense*. Time points analyzed were; day 7 and day 28 post infection. Data are from two independent experiments. Data expressed as means (±SEM) from 2-3 individual mice. Detection limit for both IFN-γ and TNF assays was 16pg/mL, and IL-10; 63pg/mL. Statistical tests between infected wild type and IL-12 deficient mice was determined by unpaired Student's t-test and are indicated by; *, *p* < 0.05. nd: not detected.

FIGURE 3. Immunoglobulin G2a (A) and IgG3 (B) plasma antibody titres from *T. congolense* infected C57BL/6 wild type (■), C57BL/6 IL-12p35^{-/-} (◇), C57BL/6 IL-

12p40^{-/-} (△) and C57BL/6 IL-12p35^{-/-}/p40^{-/-} (□) mice at day 28 post infection. Pre-immune plasma was used as non-infected controls (●). Data points represent mean and SEM from plasma of 2-3 individual mice.

FIGURE 4. Interleukin-12 is necessary for inducible nitric oxide synthase (iNOS) production in liver but not spleen during the acute infection. Frozen tissue sections of liver (A & B) and spleen (C & D) from infected wild type (A & C) and IL-12p35^{-/-}/p40^{-/-} (B & D) mice at day 7 post infection were stained with monoclonal antibodies against iNOS followed by FITC conjugated secondary antibody. Images are representative of 5-6 individual mice. Interleukin-12p35^{-/-}/p40^{-/-} mice are shown as representative of all three IL-12 deficient mouse groups as they were similar. White pulp (WP) and red pulp (RP) are shown (Magnification for immunofluorescent images: 200×).

FIGURE 5. Interleukin-12 deficiency results in decreased liver pathology in conjunction with a decreased pro-inflammatory response compared to wild type mice at day 105 post infection. Interferon-γ (IFN-γ) (A), tumor necrosis factor (TNF) (B) and interleukin-10 (IL-10) (C) plasma levels were measured by ELISA and aspartate transaminase assay (AST) levels (D) in wild type (Filled bars), IL-12p35^{-/-}/p40^{-/-} (Open bars), IL-12p40^{-/-} (Striped bars) and IL-12p35^{-/-} (Hatched bars) mice infected with *T. congolense* at day 105 post infection. Data are representative from two independent experiments. Data expressed as means (±SEM) from 2-3 individual mice. Detection limit for both IFN-γ and TNF assays was 16pg/mL, IL-10; 63pg/mL and for AST; 6 U/L. Statistical tests between infected wild type and IL-12 deficient mice was determined by unpaired Student's t-test and indicated by; *, $p < 0.05$; **, $p < 0.01$. nd: not detected. Representative images of liver sections stained with hematoxylin and eosin (H&E) are shown of infected wild type

(E) and IL-12p35^{-/-}/p40^{-/-} (F) mice at day 105 post infection. Arrowheads indicate regions of inflammatory cell foci and the line denotes a region of an infarct at the bottom left. Images are representative of 5-6 individual mice. Interleukin-12p35^{-/-}/p40^{-/-} mice are shown as representative of all three IL-12 deficient mouse groups as they were similar (Magnification: 200×).

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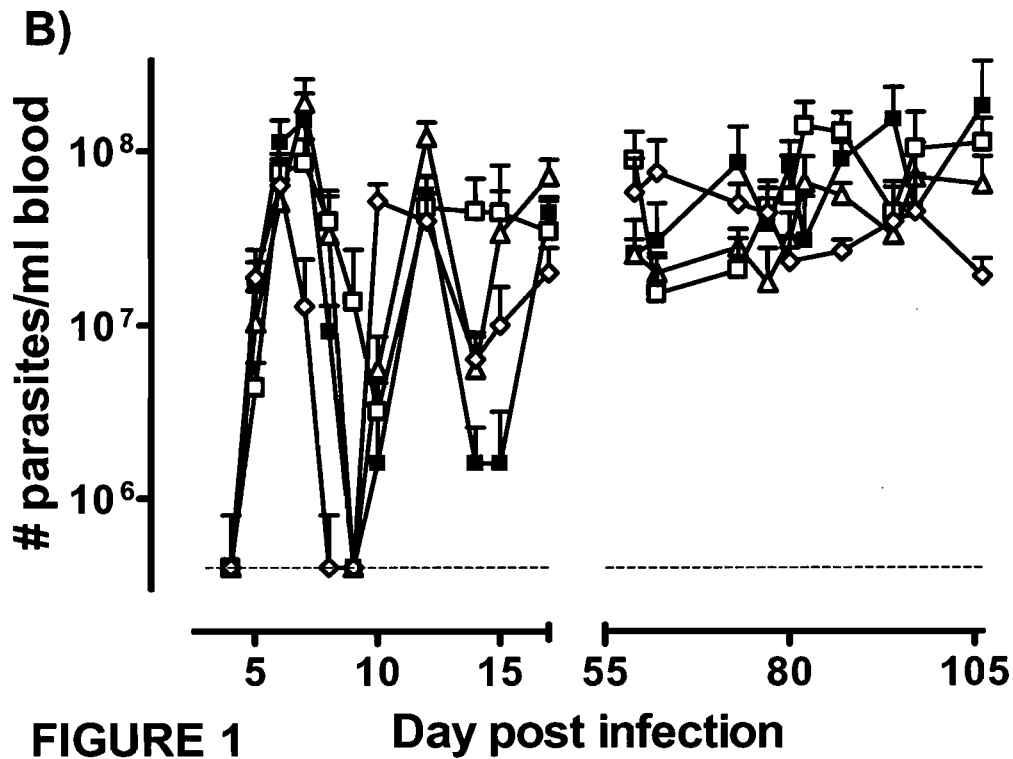
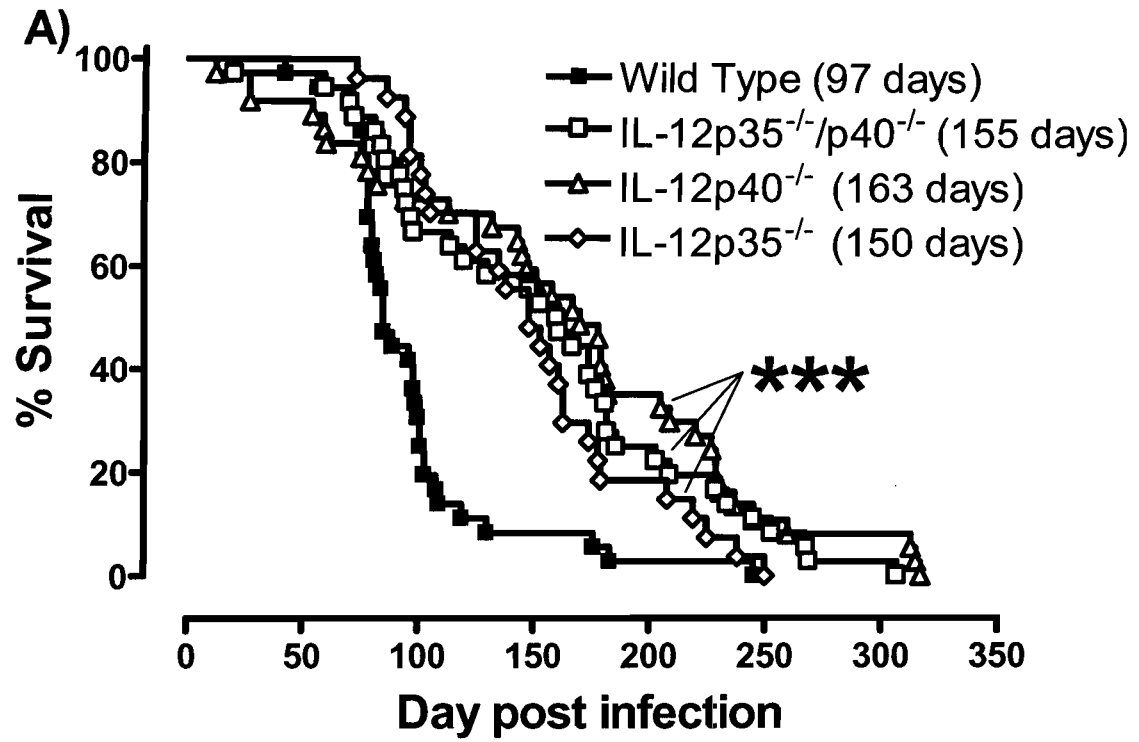


FIGURE 1

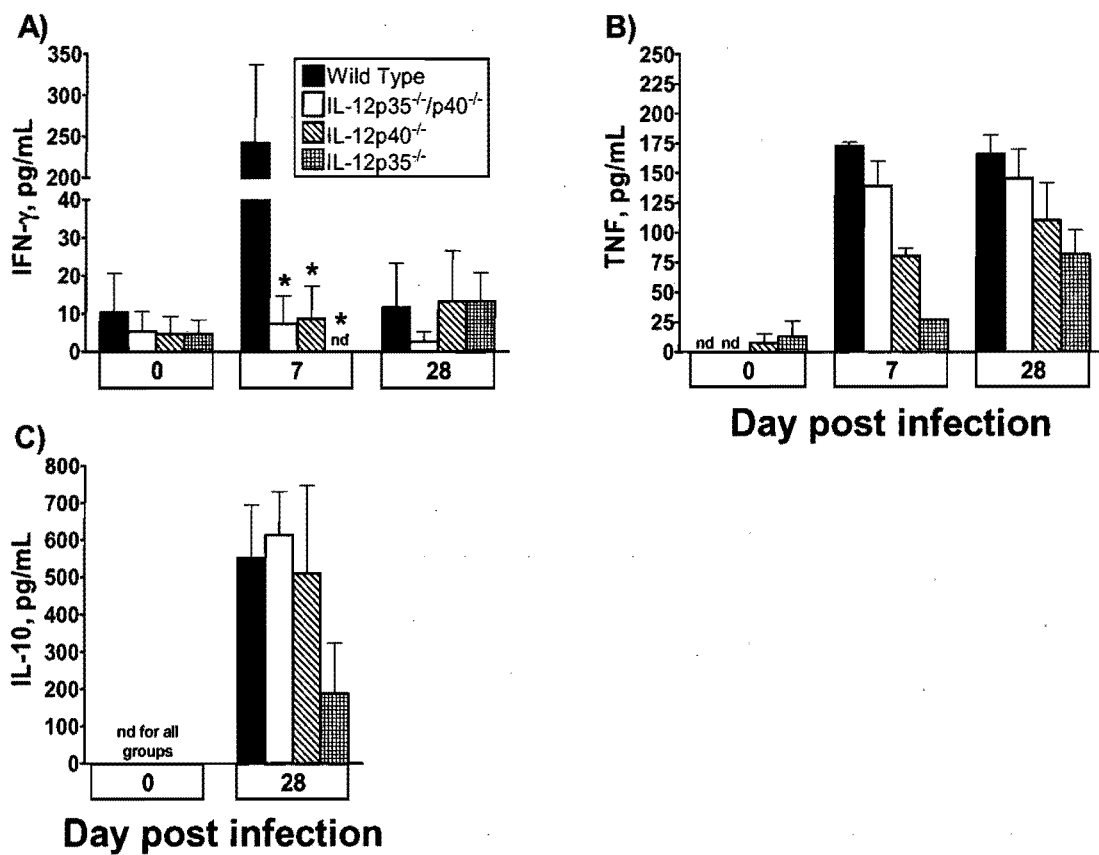


FIGURE 2

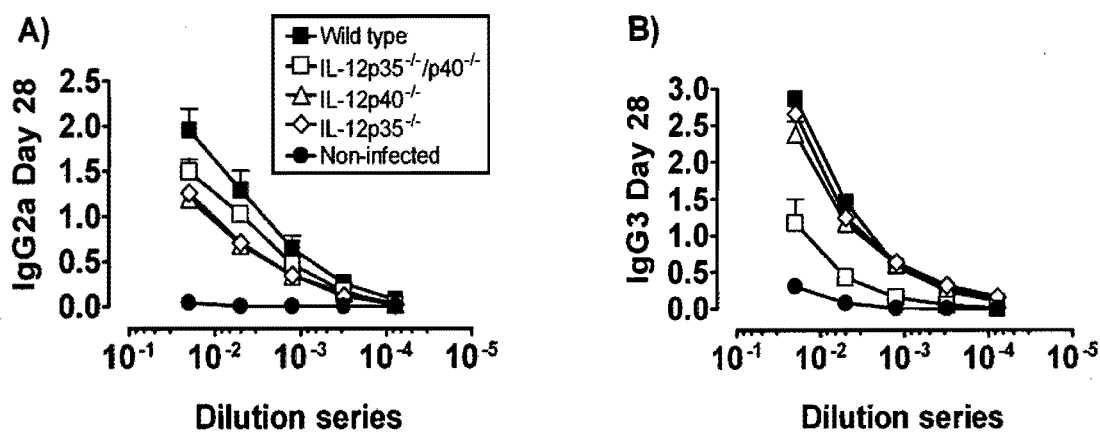


FIGURE 3

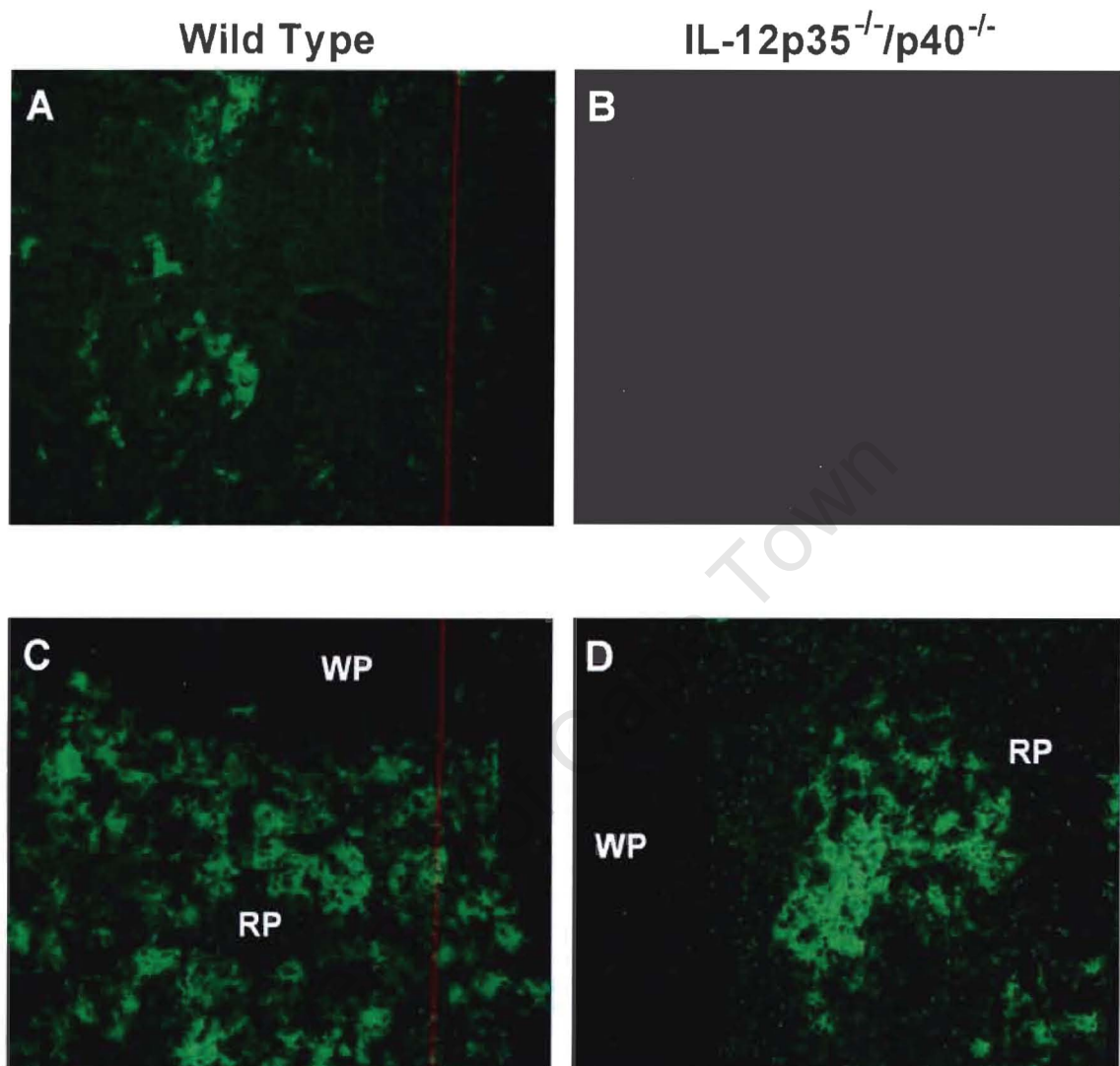


FIGURE 4

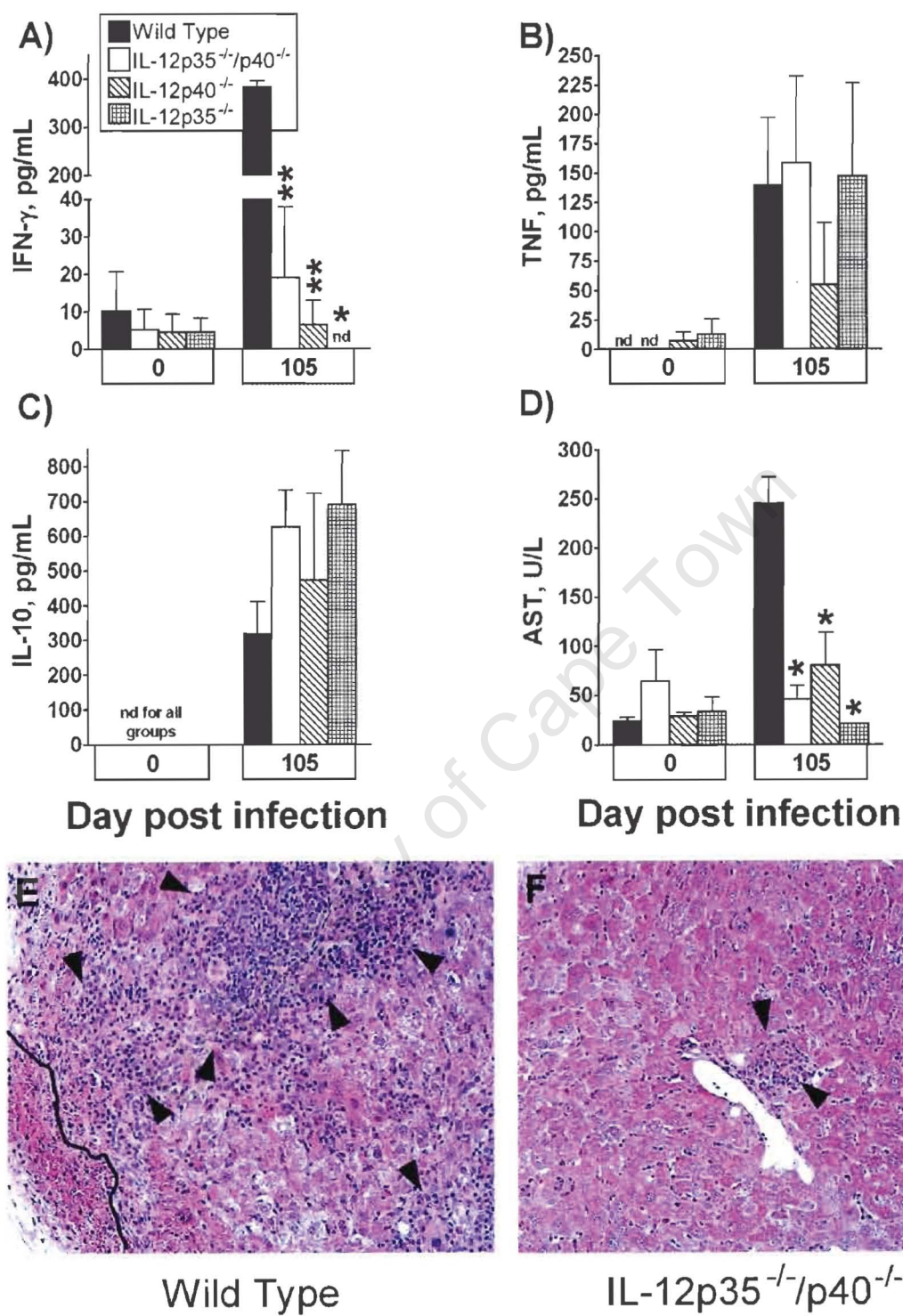


FIGURE 5