ROLE OF CYTOKINES IN RESISTANCE TO AFRICAN TRYPANOSOMES

A Thesis

Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements for

The Degree of Doctor Philosophy

in the

Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

By

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March, 1998

DEDICATION

To Regina. Oliveth. Innocent and Donatus

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ABSTRACT

The early expression and role of cytokines in resistance to experimental *Trypanosoma congolense* infections in the highly susceptible BALB/c and relatively resistant C57BL/6 mice was studied.

Higher levels of IL-4, IL-10 and IFN-γ were detected in the plasma of infected BALB/c than in those of C57BL/6 mice. In contrast, plasma levels of TNF-α were higher in infected C57BL/6 than in BALB/c mice. IL-10 and IFN-γ mRNA transcripts accumulated earlier and in higher concentrations in the spleens of susceptible than in those of resistant mice. TNF-α mRNA levels in the spleens were similar, but the hepatic TNF-α mRNA levels were higher in resistant than in susceptible mice on day 9.

The patterns and kinetics of IL-4, IL-10 and IFN-γ spot-forming cells in the spleens were essentially similar but significantly higher numbers were detected in BALB/c than in C57BL/6 mice. In contrast, unstimulated and concanavalin A- (Con A) stimulated splenocytes from BALB/c mice secreted dramatically high amounts of IL-4, IL-10 and IFN-γ in cultures starting from day 4. Secretion of IL-4 by splenocytes from infected C57BL/6 mice was undetectable throughout the period tested. Secretion of IL-10 and IFN-γ became appreciable on day 6 but were down-regulated by day 8. Treatment of infected BALB/c mice with Berenil resulted in cure and caused a dramatic decline in the secretion of IL-10 and IFN-γ by BALB/c splenocytes.

Con A-induced proliferation of splenocytes from infected BALB/c mice was progressively suppressed. This suppression was effectively reversed by anti-IL-10 or anti-IFN-γ antibodies. Whereas *in vivo* administrations of anti-IL-10 antibodies to BALB/c mice early during infection only moderately prolonged their survival period, anti-IFN-γ antibodies substantially shifted the phenotype of these susceptible BALB/c mice to a resistant-like phenotype.

Most of the IL-4, IL-10 and IFN-γ found in the infected BALB/c mice were produced by adherent $Thy1.2^{-}CD4^{-}8^{-}$ splenocytes in synergy with adherent $Thy1.2^{-}$ cells. These adherent cells suppressed T and B cell responses.

Infected BALB/c mice mounted an earlier IgM response to various antigens of *T. congolense* than C57BL/6 mice. In contrast, C57BL/6 mice made a strong and sustained IgG2a and IgG3 response to these antigens. It is hypothesized that enhanced resistance to *T. congolense* infection in mice is mediated by a TH1 cell response.

ACKNOWLEDGMENTS

The successful completion of this thesis was as a result of contributions and steadfast assistance of many people.

I would like to thank my supervisor, Dr. Henry Tabel, who accepted me as a Ph.D. student and gave me the opportunity to work in his laboratory. He believed in me and gave me constant guidance, encouragement and constructive criticisms throughout this period. I also acknowledge and appreciate the constructive criticisms and invaluable suggestions of members of my advisory committee- Drs John Gordon, Debbie Haines, Peter Bretscher and Vikram Misra. Their contribution to the research project helped in producing a better and logical research work.

I would also like to thank Dr. Lydden Polley for his efforts in bringing me to the Department of Veterinary Microbiology. I thank Mr. Brian Chelack for all the FACS analyses. His patience and encouragement were sources of inspiration to me. I appreciate the invaluable assistance of Ying Zhang, our technician. Her "magic touch" made most of the techniques seem very easy. Dr. John Allen's critical review of some of the manuscripts is appreciated. I acknowledge the generous gift of monoclonal anti-IL-10 antibody-secreting hybridoma from Dr. Tim Mosmann, University of Alberta.

Special thanks go to my friend and colleague, Dr. Radhey Shyam Kaushik, for his support, encouragement and interpretations of some results. His suggestions most often result in exciting findings. I am grateful for the support of friends and graduate students in the Department of Veterinary Microbiology-Angie Schneider, Bin Bin Yue, Susan Kutz, Sanipa Suradhat, and Keith West. I am thankful to Ann Margot for all her care, support and contribution in typing this thesis.

I thank my parents, late Mr. and Mrs. Michael Ezeh, for instilling in me moral discipline and respect for societal values and norms as well as in building the foundation of solid education for me. My father, in particular, constantly reminded me of the values of good education in the present contemporary society. I am grateful to my cousins, Mr. Donatus Ezeh and Mr. Innocent Okeke, and my sisters, Benedeth, Elizabeth, and Regina, for their contributions to my education.

I am grateful to Sarah Liu, Evelyn Rukundo, Dele Ogunremi, Mr. & Mrs. Raphael Idem, Ike Oguocha, XhiaHong Li, Ray Lu and Ping Yang for their friendship over the years.

Last but not the least, I thank Pat Thompson and members of staff of the GMP and Animal Care Unit of the Western College of Veterinary Medicine for their help.

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ABBREVIATIONS

^{3}[H]TdR	Triturated thymidine
ABTS	2,2' azino-di 3-ethyl-benz-thiazoline sulphonate
ANOVA	Analysis of variance
APC	Antigen-presenting cell
BSA	Bovine serum albumin
CD	Cluster of Differentiation
cDNA	complementary DNA
Con A	Concanavalin A
CR	Complement receptor
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Ezyme linked immunosorbent assay
F1	First filial generation
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
IFN	Interferon
IL	Interleukin
IL-2R	Interleukin-2 receptor
IU	International unit
kb	kilobase
kD	Kilodalton
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MACS	Magnetic activated cell sorter
MHC	Major histocompatiblity complex
mRNA	messenger RNA
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl terazolium bromide
N^{G}MMA	N^{G}-momomethyl-L-arginine
NO	Nitric oxide
NOS	nitric oxide synthase
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-FBS	Phosphate buffered saline containing 10% fetal bovine serum
PBST	Phosphate buffered saline containing Tween 20
PE	Phycoerythrin
PFC	Plaque-forming-cell
PMA	Phorbol myristic acid
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SE	Standard error
SRBC	Sheep red blood cell
TC	Cytotoxic T cell
TC13	*Trypanosoma congolense* clone 13
TH	T helper
TNF	Tumor necrosis factor
TSG	Tris saline glucose
u.v	Ultraviolet
VAT	Variant antigen type
VSG	Variant surface glycoprotein

1.0 LITERATURE REVIEW

1.1 African Trypanosomes

1.1.1 Introduction

African trypanosomes are protozoal parasites that include the causative agents of important human and animal diseases. *Trypsanosoma congolense*, *T. brucei* and *T. vivax* are the major species that primarily cause disease in domestic livestock.

The occurrence of animal trypanosomiasis coincides with the distribution of their tsetse fly vectors. This includes large expanses of sub-Saharan Africa between latitudes 14°N and 29°S. This area represents a vast region covering about 10 million square kilometers and 37 countries (Hursey and Slingenbergh, 1995).

The economic impact of African trypanosomiasis is enormous. It results in an estimated three million cattle deaths annually and the estimated direct production loss in cattle alone is between US$6,000 million and US$12,000 million per year (Hursey and Slingenbergh, 1995). It is projected that if the disease is controlled, the region could support a further 120 million cattle translating to an additional 1.5 million tons of meat annually (Nantulya, 1986).

Several methods of control of trypanosomiasis are currently employed and include chemotherapy, breeding of resistant breeds of cattle, and measures directed against the insect vector. Other measures include bush clearing to eliminate the fly habitats, spraying with insecticides and trapping of the fly (Taylor, 1998). The efficacy of chemotherapy and chemoprophylaxis is hindered by the rapid evolution of drug-resistant trypanosomes and this has resulted in widespread outbreaks in cattle (Holmes and Scott, 1982, Leach and Roberts, 1981). Furthermore, the lack of development of new drugs and logistical

difficulties in maintaining treatment regimes make this method of control unpopular. Measures directed against the tsetse fly vector such as destruction of the fly habitats by bush clearing and extensive spraying of insecticides have been effective. However, these measures are no longer desirable for ecological concerns and their deleterious effects to the environment. Sterilization of male flies in an attempt to reduce the degree of productive mating and hence the incidence of the vector has been attempted. The logistical problems in assessing the efficacy of this measure has lead to the failure of this scheme. Tsetse flies can be locally controlled by the use of traps (Dransfield et al., 1990). Again, this method requires frequent maintenance and the costs might be enormous.

Vaccination has been considered to be the most desirable method of control. However, the ability of the African trypanosome to continually express antigenically distinct VSG genes (reviewed in Borst et al., 1997) reduces the likelihood of an effective VSG-based vaccine. Invariant parasite antigens are being sought as candidates for vaccine designs but only partial and minimal cross-protection has been reported so far (Mkunza et al., 1995). Most researchers therefore, have focused on understanding the parasite and host factors involved in either parasite or disease control. It is believed that understanding these mechanisms might be important in the design of appropriate immunotherapeutic interventions aimed at reducing the disease and pathology in infected livestock.

Infections with African trypanosomes are associated with profound alteration of the immune system of the host. Many of these alterations may be important in the induction of the disease process as well as in control of parasitemia. Modulation of the immune system by the parasite might be advantageous for the parasite in subverting the

host's immune responses and consequently its survival. Identification of mechanisms and molecules causing immunomodulations might make it possible to design appropriate strategies for intervention aimed at preventing certain immunopathologies and enhancing effective immune responses by the host against the parasite.

1.1.2 Classification

Trypanosomes are flagellated protozoal parasites that belong to the class Zoomastigophores, order Kinetoplastida, family Trypanosomatidae. Members of this order are elongate, slender and possess a single nucleus and a kinetoplast which is situated near the origin of a single flagellum. The pathogenic trypanosomes belong to the subgenus *Trypanosoma* and have been divided into two sections on the basis of their life cycle in the insect vector and mode of transmission (Hoare, 1972).

The section salivaria consists of groups that complete their developmental cycle within the salivary glands of the insect vector. Almost all of the species in this group are transmitted by tsetse flies (*Glossina* spp) with the exception of *T. equiperdum and T. evans*i that are transmitted venerally and mechanically respectively. Most members of this section are important pathogens either to man or to domestic animals. Because development takes place in the salivary glands, transmission occurs only by bite of the infected insect vector during a blood meal. The section salivaria consists of four subgroups namely *Duttonella* (*T. vivax*), *Nannomonas* (*T. congolense*), *Trypanozoon* (*T. brucei brucei*) and *Pycnomonas* (*T. suis*).

Members of the section stercoraria complete their developmental cycle in the hindgut of the insect vector. As a result, they are passed out in the vector's feces or may

be liberated if the vector is crushed. Infection occurs when the feces containing the infective metacyclic form of the parasite is rubbed into lesions or wounds produced by scratching the skin in response to irritation of the insect's bite. They may also actively penetrate punctures produced by the proboscis of the insect or the mucous membranes of the mouth if the animal licks up the insect feces. There are three subgroups in this section namely, *Megatrypanum* (*T. theileri*), *Herpestoma* (*T. lewisi*, *T. musculi*) and *Schizotrypanum* (*T. cruzi*). Most members of the section Stercoraria are relatively non-pathogenic, however, *T. cruzi* is of medical importance because it causes Chagas disease in man in South and Central America.

1.1.3 Morphology and characterization

African trypanosomes are characterized and distinguished on the basis of shape, size, position of the nucleus, location and size of the kinetoplast as well as host range and geographical distribution. Generally, they are elongate, spindle-shaped and possess a single flagellum originating from the basal body near the kinetoplast. The flagellum runs along the body from the posterior to the anterior end forming folds on the body pellicle known as the undulating membrane (Hoare, 1972).

African trypanosomes range from 8-30μm in length. *Trypanosoma congolense* is a small organism and varies in length between 8 and 24 μm with a mean of 12.2-17.6 μm (Soltys and Woo, 1977). It has a central nucleus and a marginal kinetoplast. The undulating membrane is usually not well marked and movement appears sluggish under the microscope. *T. congolense* is usually monomorphic (Soltys and Woo, 1977).

1.1.4 Life cycle

1.1.4.1 Development in insect vectors

Almost all the pathogenic salivarian trypanosomes of livestock are transmitted cyclically by tsetse flies (*Glossina spp*). *Trypanosoma congolense* is capable of developing in all the three groups of this genus consisting of more than 34 species and subspecies (Hoare, 1972). The parasites are ingested by the female tsetse fly during a regular blood meal from an infected mammalian host. In the hindgut of the fly, the ingested parasites lose their VSG and undergo a series of developmental and morphological changes (reviewed by Stephen, 1986) resulting in multiplication. They migrate anteriorly through the cell linings of the gut wall and via the proventricular to the esophagus of the insect (Newton et al., 1973). From here, they migrate to the hypopharynx and transform into the epimastigote form (Newton, et al., 1957). The epimastigotes migrate to the mouth pads of the proboscis and transform into the effective metacyclic trypanosomes. This transformation is associated with the reappearance of the VSG which was lost in the midgut (Vickerman, 1974). The entire cycle of development in the *Glossina* may take from 7 (Nantulya et al., 1978) to 53 (Hoare, 1972) days depending on the species of the insect vector and ambient temperature. Once infected, the tsetse fly harbors the parasite and is able to transmit it for a considerable length of time and in certain instances, for life (Molyneux and Ashford, 1983).

1.1.4.2 Development in mammalian hosts

Infection of the mammalian host occurs by the deposition of the metacyclic trypanosomes in the saliva of the insect vector within the dermal connective tissue of the

animal during a regular blood meal. Here, they multiply extensively as typical blood forms within a few days, producing a raised cutaneous swelling commonly referred to as chancre (Akol et al., 1982). These cutaneous trypanomastigotes eventually spread from the chancre via the blood and local lymph vessels and enter the blood stream. Certain blood stream forms of many species may invade the connective tissues of the animals, but this is not usually observed with *T. congolense* (Losos and Ikede, 1972). The length of the prepatent period is variable and depends on many factors including the number of infecting parasites, the route of inoculation and the genetic makeup of the host. Usually, parasitemia becomes apparent within 1-2 weeks after natural infection and may persist for months, occuring in waves, until the animal dies (Gray and Luckins, 1976).

During the period of growth in the blood stream, certain changes in metabolism result in morphological pleomorphism among various subspecies of *T. brucei* (Opperdoes et al., 1987). Morphological variants range from short, stumpy, usually non-dividing forms to long slender forms that are in a state of active cell division. Intermediate forms are also frequently observed and may represent a transition stage from long slender to stumpy form (Opperdoes et al., 1987). The slender forms predominate during the early logarithmic growth phase of infection (Lumsden, 1972) while the stumpy forms predominate during the phase of parasite remission (McLintock et al., 1990). It has been suggested that the transformation from slender dividing forms to the short stumpy non-dividing forms is necessary for cyclical development in the insect vector (Seed and Sechelski, 1989). Although Stephen (1986) reported morphological variants of *T. congolense*, the general agreement among workers is that *T. congolense* does not undergo physical transformation.

1.1.5 Course of the infection

The severity and clinical signs in animals following infection with pathogenic trypanosomes vary depending on the virulence of the infecting organisms and susceptibility of the host. Various strains within a species are capable of causing a wide range of clinical responses ranging from acute, chronic to asymptomatic carrier syndromes (Stephen, 1970; Maxie et al., 1979). Also, even under similar environmental conditions, pronounced differences in susceptibility of individual animals within a homogenous population are observed (Ikede and Losos, 1972).

In ruminants, acute infection with *T. congolense* is associated with intermittent fever, depression, anemia, subcutaneous edema of the mandible, and a prominent jugular pulse (Stephen, 1970; Losos, 1986). The appetite is depressed and there is rapid loss of weight. Death is most often related to severe anemia and circulatory collapse. Chronic syndromes may last for months and results in extreme emaciation and anemia. Profound lymphadenopathy commonly seen in infections with other species is not a common feature of *T. congolense* infection in cattle (Maxie et al., 1979; Wellde et al., 1974).

1.1.6 Pathology

There are no pathognomonic lesions in organs and tissues of animals that die of trypanosomiasis. Most lesions are either due to immunocomplexes or related to the circulatory disturbances resulting from anemia. Animals that died of acute syndrome have pale carcasses and variable atrophy of adipose and skeletal muscle tissues (Losos, 1986; Molyneux and Ashford, 1983). In chronic cases, there is extensive muscle wasting

and marked enlargement of the spleen with very prominent white pulp (Valli and Forsberg, 1979; Morrison and Murray, 1979). On histology, there are marked foci of necrosis in these organs and trypanosomes are found localized in the small blood vessels especially those of the heart and brain.

The mechanisms of pathogenesis of trypanosomiasis have not been clearly established, but both trypanosome-derived substances and immune complexes of trypanosomal antigens and their antibodies have been implicated. Several biologically active trypanosome metabolites with inflammatory and complement activating properties have been purified from trypanosomes and are thought to be released from disintegrating intravascular parasites (Tizard and Holmes, 1976; Tizard et al., 1978; Nielsen et al., 1979). Formation and deposition of immune complexes of trypanosome antigens and parasite-specific IgM or IgG and complement on the vascular and extravascular spaces of tissue could result in pathology (Mansfield, 1990). Such complexes have been detected in several tissues from infected animals and humans including brain, heart, skeletal muscle and kidney (Nagle et al., 1974; Facer et al., 1978; Lambert and Houba, 1974). It is believed that formation of immunocomplexes result from the repeated stimulation of the host immune system by the invariant antigens generated by the destruction of different variant populations during chronic infections (Mansfield, 1990).

1.1.7 The variant surface glycoprotein (VSG)

The plasma membrane of African trypanosomes is covered by a homogenous dense coat, the variant surface glycoprotein (VSG), composed of millions of glycoprotein molecules of a single molecular species. Purified VSG has a molecular weight of between

53-65 KD and different VSGs of different variant antigen types (VATs) of the same serodeme may differ in their isoelectric points, amino acid composition and carbohydrate percentage (Cross, 1978; 1990). The VSG is only expressed by the infective metacyclic form in the insect (Vickerman, 1974) and it has been proposed that the VSG protects the metacyclic forms from lysis by the host's serum factors (Ghitto et al., 1979).

The VSG molecules on a live trypanosome are packed as a dense array of rigid monomers or dimers thereby exposing similar N-terminal epitopes (Vickerman et al., 1993). As a result of this repetitive nature, the VSG molecules induces strong T-cell independent immune responses (Mansfield, 1990). Thus, not all of the epitopes on the VSG are exposed to the host immune system on the live trypanosome. Also, although most of the carbohydrate determinants within the C-terminus are invariant, they are covered by the N-terminal variable amino acids and are therefore apparently not accessible to antibodies in an intact VSG on a live trypanosome (Cross, 1979).

The VSG is anchored to the plasma membrane by the glycosyl-phosphatidylinositol [GPI] (Ferguson et al., 1985; 1988). Many proteins of eukaryotes are anchored to the plasma membrane by the GPI, including the decay-accelerating factor and Thy1 antigen (McConville and Ferguson, 1993). The lipophospho-glycan and the glycoinositol-phospholipids of *Leishmania* (Thomas et al., 1992; McConville et al., 1990) and the epimastigote proteins of *Trypanosoma cruzi* (Bertello et al., 1995, Ferguson, 1997) are also anchored by the GPI moiety.

The biological significance of the GPI anchor of the VSG is not known as yet. One function, however, appears to be related to the release and shedding of the VSG. Trypanosomes contain GPI-specific phospholipase C, which when activated, causes the

cleavage and release of the VSG from the plasma membrane (Fox et al., 1986; Hereld et al., 1988). In *Mycobacterium tuberculosis*, lipoarabinomannan (LAM), which is the major cell wall constituent, induces synthesis of monokines including IL-10 from monocytes (Barnes et al., 1992). The major effective moiety of LAM that induces this monokine secretion was shown to be phosphatidylinositol (Barnes et al., 1992). Similarly, the glycolipids of *M. tuberculosis* have been shown to induce cytokine secretion by a subpopulation of double negative γδ and αβ T cells (Porcelli, et al., 1992; Beckman et al, 1994). These antigens were shown to be presented by non-classical MHC class 1 (CD1) molecules and are processed in a manner quite different from antigens presented by class II or class I pathways (Porcelli, et al., 1992; Beckman et al., 1994; Bendelac, 1997). It is conceivable that similar presentation of GPI-derived trypanosomal antigens occur during immune responses in animals infected with African trypanosomes.

1.1.8 Antigenic variation

In the host, the VSG due to its highly immunogenic nature, induces a strong humoral response that results in rapid elimination of all but a tiny proportion of the parasites. These tiny variant individuals give rise to a new population expressing a new population of VSG. The new variants multiply and the immune system raises another set of antibodies that eventually succeed in eliminating the majority of these variants. This results in a continuous cycle of undulating parasitemia (reviewed by Donelson and Turner, 1985; Barry, 1997). Thus, the undulating wave of parasitemia observed in infected animals is clearly a result of interactions between the host immune factors and

the parasite. However, available evidence indicates that antibodies do not induce antigenic variation because antigenic variation has been observed *in vitro* (Doyle et al., 1980). But, it has been suggested that antibodies may select a variant that initiates a new population by its inability to quickly eliminate a particular new variant (Barry, 1997). It is estimated that coat switching occurs at a frequency of $10^{-6} - 10^{-7}$ per cell division in established rodent strains, but at a much higher rate immediately after passage through insect vectors (Barry and Turner, 1991).

The parasite's ability to change its VSG coat relies on the presence of many VSG genes. It is estimated that each trypanosome contains more than 1000 genes, each of which is complete (Barry, 1997; Borst, 1997). The expression of a new VSG gene requires the transcription of the new gene at the expression sites located at the teleomeric positions of the chromosomes (Pays, 1989; Borst, 1997). Only one teleomeric site is active at a time (Pays et al., 1989) and the new gene copy at this site is known as the expression-linked copy (Barry et al., 1997). Activation and inactivation of expression sites appear to be due to infrequent stochastic events (Borst et al., 1997), since there are no sequence differences between the promoter regions of the active and inactive expression sites (Zomedijk et al., 1990). Antigenic switching occurs either by translocation of a basic copy of a new gene into an active expression site or by differential control of expression sites (Van der Ploeg et al., 1992; Borst et al., 1997). In order to be translocated, the basic copy of the VSG gene has to be duplicated (Van der Ploeg et al., 1992). However, an already telomeric-linked VSG gene need not be duplicated but is converted into an active gene (Donelson and Turner, 1985). Other non-duplicational mechanisms of DNA rearrangements that lead to antigen switching include reciprocal

exchange of trypanosome telomere (Delange et al., 1983; Pays et al., 1985) and recombinational events (Donelson and Turner, 1985).

As previously stated, the precise molecular events that trigger the switch from one VSG gene to another are still not understood. Certainly, antigenic variation is advantageous to the parasite in the evasion of the host immune defenses. Because of the complexity and varied nature of these mechanisms, it is almost impossible to circumvent them and so highly unlikely that it will be feasible to develop an effective vaccine against blood stream trypanosomes.

1.1.9 Common antigens

These are the invariant antigens of trypanosomes that do not change from one variant type to another during the course of infection. They include enzymes, structural and nuclear proteins, trypanosomal membranes and some receptors such as those for low density lipoprotein and transferrin (Pays et al., 1994), high density lipoprotein (Hadjuk et al., 1994), TNF-α (Lucas et al., 1994) and IFN-γ (Olsson et al., 1993). In addition, certain enzymes such as phospholipase C (Fox et al., 1986) and peptidases (Knowles et al., 1989) are common to all species of trypanosomes. The conservation of these enzymes suggests that they may be important for parasite survival and therefore might play some role in pathogenesis and/or evasion of host defense mechanisms. The carbohydrate rich C-terminal portion of the VSG is also considered as a common antigen and has been shown to show a high degree of sequence homology across members of a species (Rice-Fitch et

al., 1981). The flagellar pocket of African trypanosomes is not covered by the VSG and therefore is invariant among members of the same species.

The invariant antigens are released after destruction of the parasite by anti-VSG antibodies or other immune factors. It has been suggested that these antigens may be involved in inducing pathology by forming immune complexes (Mansfield, 1990). However, in cattle infected with *T. congolense*, antibody responses to 33kd (Authie et al., 1993a) and 69kd (Authie et al., 1993b; Boulange and Authie, 1994) invariant cysteine proteases have been associated with trypanotolerance. The trypanotolerant N'dama cattle infected with *T. congolense* made a superior IgG and low IgM antibody response to these invariant proteins (Authie et al., 1992; 1993a; 1993b). In contrast, similarly infected trypanosusceptible Boran cattle mounted predominantly IgM but no IgG1 response to these proteases. Crosses between N'dama and Boran cattle, which exhibit an intermediate susceptibility, had intermediate levels of these antibodies. In these studies, evidence was presented that the 33kd cysteine protease was either being released from the parasite or was accessible on living trypanosomes (Authie et al., 1993b). In another study in cattle, immunization with antigens derived from the flagellar pocket of *T. brucei rhodesiense* resulted in significant protection against a challenge with. *T. congolense* and *T. vivax* (Mkunza et al., 1995). Although the mechanisms of protection in these animals were not studied, it is quite clear that they do not include immune responses to the VSG because the flagellar pockets are not covered by VSG.

Thus, it is now clearly evident that the long-held notion that the common antigens of trypanosomes do not have any relevance for protection (Mansfield, 1990) may not be totally correct. On the contrary, immune responses to these antigens appear to influence

the relative susceptibility or resistance during trypanosome infections in cattle. Detailed analysis of antibody responses to common antigens in mice is not yet known.

1.2 Immunomodulation

1.2.1 Polyclonal B-cell activation

Polyclonal activation of B-cells is a prominent feature of African trypanosome infections. There is a greatly increased concentration of serum IgM levels in infected animals (Houba et al., 1969; Clarkson, 1976). The elevated serum IgM levels result from the gross expansion of the B cells in the spleen and lymph nodes (Murray et al., 1974; Morrison et al., 1978; Morrison and Murray, 1979a). In humans, this elevated serum IgM level is used as an important diagnostic test for sleeping sickness in endemic areas of sub-Saharan Africa (Cunningham et al., 1967).

Studies on the specificity of the serum antibodies produced in response to trypanosome infections have shown that both trypanosome antigen-specific and non-specific antibodies are produced, including autoantibodies. Antibodies to a variety of unrelated antigens including sheep red blood cells, pneumococcal polysaccharides and haptens (Kobayakwa et al., 1979; Hudson et al., 1976) have been detected in mice infected with *T. brucei*. As a result of these findings, it was postulated that African trypanosomes possess B cell mitogens that can cause polyclonal activation of B cells.

Many workers have tried to purify a mitogen factor from trypanosomes. Esuruoso (1976) demonstrated that the membranes of trypanosomes were mitogenic for spleen cells of normal athymic and cyclophosphamide-treated mice *in vitro*. Similarly, whole or fractionated homogenates of trypanosomes have been reported to cause polyclonal

activation of lymphocytes (Mansfield et al., 1976; Greenwood et al., 1978). The purified VSG molecules have also been reported to be mitogenic for B cells (Diffey, 1983) and mice immunized with purified VSG have marked enlargement of B cell compartments in the spleen and an increase in serum IgM levels mostly due to the production of heterospecific antibodies (Diffley, 1983).

Polyclonal activation may also be a consequence of binding of the VSG molecule linked to a C3 fragment to a complement receptor on B cells. Liu et al. (1991; 1993) have shown that a soluble fragment of the VSG of *T. congolense* forms a covalent product with a breakdown component of C3 (VSG-C3b) in the presence of variant specific antisera. It is known that binding of an antigen by the B cell receptor and simultaneous cross-linking of the complement receptor 2 (CR2) on B cells has a synergistic effect on B cell activation (Klaus and Humphrey, 1986; Tedder et al., 1997).

The finding that the membranes of insect stages of trypanosomes (which do not possess VSG) do not cause polyclonal activation (Oka et al., 1988) suggests that bloodstream forms of African trypanosomes may induce polyclonal B cell activation as an evasion mechanism. Polyclonal B cell activation may prevent the maximum stimulation and proliferation of parasite-specific lymphocyte clones. In addition, because affinity maturation does not occur during polyclonal B cell activation activation (Roitt et al., 1997), selective proliferation and production of high affinity antibodies against the parasite is hence prevented.

1.2.2 Immunosuppression

One of the striking features of African trypanosomiasis in cattle, human and mice is profound suppression of the host immune system (reviewed by Roelants and Pinder, 1984; Askonas, 1985; Sileghem et al., 1994a; Taylor, 1998). Suppressed B and T cell responses to trypanosome and non-trypanosome antigens have been documented in most hosts, with the exception of trypanoresistant wildlife (Mulla and Rickman, 1988). Generalized immunosuppression was observed in patients suffering from trypanosomiasis and was proposed to be the major cause of increased susceptibility of trypanosome-bearing individuals to opportunistic infections (Greenwood et al., 1973). Similarly, infections of cattle with *T. vivax* and *T. congolense* cause suppression of antibody responses to a variety of vaccines (Rurangirwa et al., 1978; Ilemobade et al., 1982). Malu and Tabel (1986) reported suppressed antibody response to *Brucella abortus* in sheep infected with *T. congolense*. Similarly, in dogs, infections with *T. congolense* have been shown to suppress antibody response to *Brucella abortus* vaccine (Anene et al., 1986). Also, it has been reported that water buffaloes infected with *T. evansi* have an increased prevalence of brucellosis (Bajyana Songa et al., 1987).

The suppression of both B and T cell responses have been demonstrated and studied in detail in laboratory rodent models. Various B cell functions have been shown to be suppressed in mice infected with African trypanosomes, including *in vitro* release of antibody measured by hemolytic plaque-forming assays (Murray et al., 1974), mitogen-induced proliferation (Corsini et al., 1977; Jayawardena and Waksman, 1977), and production of antibodies (IgG and IgE) to *Nippostrongylus braziliensis* (Urquhart et al., 1973). Various T cell responses are also severely affected including allogeneic graft

rejection (Pearson et al., 1978), delayed type hypersensitivity (Mansfield and Wallace, 1984), mixed lymphocyte reaction (Pearson et al., 1978; 1978a; 1979; Roelants et al., 1979a) and mitogen-induced proliferation (Jayawarden and Waksman, 1977, Pearson et al., 1978; 1978a; 1979; Morrison et al., 1978; Kar et al., 1979; Sileghem et al., 1989; 1992b).

Various mechanisms have been held responsible for immunosuppression in experimental murine trypanosomiasis and these operative mechanisms appear to be influenced by a number of factors including host, parasite species and the lymphoid compartment studied.

1.2.2.1 Mechanisms of immunosuppression

Infections with African trypanosomes are associated with increases in total serum IgM levels and gross expansion of B cell populations (Murray et al., 1974). Most of the serum IgM antibodies have heterophilic and autoimmune specificities (Hudson et al., 1976) and this has led to the postulate that trypanosomes possess a mitogen (i.e.molecule) which causes non-specific activation of B cells (Urquhart et al., 1973). As a consequence, a progressive depletion or exhaustion of antigen-reactive B cells due to polyclonal activation would later result in suppression. Esuruosu (1976) first demonstrated the mitogenic capacity of *T. brucei* on B-cells *in vitro*. Mansfield et al. (1976) reported that extracts from *T. brucei* and *T. congolense* were stimulatory for normal rabbit spleen cells and peripheral blood lymphocytes. However, such extracts failed to stimulate spleen cells from normal mice, rats or guinea pigs (Mansfield et al., 1976). Both polyclonal activation

and immunosuppression were induced *in vivo* by the administration of trypanosome membrane fragments (Clayton et al., 1979; Sacks et al., 1982).

Immunization of mice with purified VSG induces polyclonal activation but does not affect B cell proliferation (Diffley, 1983) indicating that polyclonal activation might be distinct from suppression of lymphocyte proliferation. Moreover, although parasite-unrelated immune responses are depressed, variant specific immune responses appear to be spared (Mansfield, 1981; 1990) and recovery of B cell function is very rapid following trypanocidal chemotherapy (Clayton et al., 1980). These observations are inconsistent with the concept of clonal exhaustion resulting from polyclonal activation. It is likely that polyclonal activation and immunosuppression are not necessarily conflicting hypotheses but might represent coexisting mechanisms. Thus, other mechanisms have been proposed as the cause of immunosuppression during experimental and natural African trypanosomiasis.

1.2.2.2 Suppressor cells

There is considerable evidence that antigen nonspecific suppressor cells present in the lymphoid compartments of trypanosome-infected animals are involved in immunosuppression (Jayawardena and Waksman, 1977; Corsini et al., 1977; Pearson et al., 1978a; 1979; Roelants et al., 1979; Wellhausen and Mansfield, 1979; 1980; Askonas, 1985; Schleifer and Mansfield, 1993; Sileghem et al., 1994). The first evidence for the involvement of suppressor cells in trypanosome-induced immunosuppression was put forward by Jayawardena and Waksman (1977). Spleen cells from infected mice were shown to passively transfer unresponsiveness to spleen cells from uninfected mice.

Adoptive transfer of spleen cells from infected mice suppressed both mitogen-induced proliferation (Jayawardena and Waksman, 1977) and *in vitro* PFC responses to SRBC (Eardley and Jayawardena 1978; Wellhausen and Mansfield 1979). Other investigators reported that peritoneal macrophages from *T. brucei* infected mice were also capable of passively transferring suppression to normal spleen cells (Corsini et al., 1977; Clayton et al., 1979). The involvement of suppressor cells in *T. congolense*-elicited suppression of Con A- and allogeneic cell-induced proliferation of spleen cells from infected mice has also been reported (Pearson et al., 1978a; 1979).

There is controversy as to the phenotype of the suppressor cells in the lymphoid organs of mice infected with trypanosomes. A potent suppressor T cell removable by treatment with anti-Thy1 antibodies and complement has been postulated to act directly or indirectly by arming adherent macrophages to mediate suppression (Jayawardena and Waksman, 1977; Eardley and Jayawardena, 1977; Jayawardena et al., 1978). Other authors reported that peritoneal macrophages from *T. brucei* infected mice were potentially suppressive (Clayton et al., 1979; Groskinsky and Askonas, 1983; Askonas, 1985). Wellhausen and Mansfield (1980) showed that suppressor cells in the spleen of mice infected with *T. brucei-rhodesiense* were adherent to nylon wool and plastic and were insensitive to treatment with anti-Thy1 serum and complement. Because the suppressor cell populations were insensitive to radiation and mitomycin C treatment but were inhibited by exposure to silica particles, these workers concluded that the suppressor cells were macrophages (Wellhausen and Mansfield, 1979; 1980). However, these authors failed to restore *in vitro* PFC responsiveness of infected spleen cells to SRBC by

addition of indomethacin, an agent known to inhibit the activity of T-independent macrophage-mediated suppression, indicating that T cells might be indirectly involved.

Pearson et al (1979) found that the suppressor cells in the spleens of *T. congolense*-infected mice were a sub-population of mitomycin C-sensitive Thy1$^+$ cells that were not restricted by differences in the H-2 region. Because the suppressor cells were also present in the nylon wool-adherent cell populations, these workers concluded that macrophages were also involved in the induction of suppression (Pearson et al., 1978a; 1979). Suppression of the lymph node compartment in mice infected with *T. brucei* appears to be mediated entirely by macrophage-like cells (Sileghem et al., 1987; 1989; 1994). Depletion of Mac-1$^+$ fraction causes 100% reversal of suppression of proliferative responses to mitogens by lymph node cells whereas depletion of Thy1$^+$ cells has no effect. However, in *T. congolense* infections in mice, unresponsiveness or suppressive activities are absent in lymph node cells (Kar et al., 1979). These contradictory results and conclusions about the nature of the suppressor cells could be attributed to the differences in the *in vitro* systems, strains and species of the parasites as well as differences in mouse strains used by various groups. It appears that both suppressor T cells and macrophages are involved and mediate their suppressive activity via release of soluble factors (Roelants and Pinder, 1984; Sileghem, et al., 1994).

1.2.2.3 Role of cytokines

Suppression of lymph node T cells in mice infected with *T. brucei* results from suppression of IL-2 secretion and IL-2 receptor expression (Sileghem et al., 1987; 1989; 1992a). Suppression of IL-2 secretion was shown to be caused by prostaglandins secreted

by suppressor macrophages (Sileghem et al., 1989), while the suppression of IL-2R expression was associated with endogenously produced IFN-γ (Darji et al., 1993; 1996). In this system, interaction of macrophages with *T. brucei* rendered them stimulatory for $CD8^-$ T cells, leading to IFN-γ release by the latter cells (Darji et al., 1996). It was suggested that this cytokine then acts on the macrophages, leading to the release of a soluble factor associated with suppression of lymph node cell proliferative responses (Darji et al., 1996). The crucial role of IFN-γ in this system was substantiated *in vivo* by treatment of trypanosome-infected animals with anti-IFN-γ antibodies and subsequent abrogation of suppression in co-cultures of infected and uninfected splenocytes (Darji et al., 1996).

Apart from IFN-γ, TNF-α has also been implicated in suppression of lymph node cells in *T. brucei*-infected mice (Lucas et al., 1993; Darji et al., 1996). In this system, it was suggested that membrane-bound TNF-α produced by macrophages following uptake of opsonized trypanosomes acts as a co-stimulatory molecule in the sensitization of $CD8^-$ T cells for IFN-γ production (Darji et al., 1996). However, TNF-α has been shown to possess both static and lytic properties for *T. brucei* (Magez et al., 1993; 1997; Lucas et al., 1993; 1994).

The role of IFN-γ and TNF-α in immunosuppression during *T. congolense* infections has not been studied. Also the role of interleukin-10, which is a potent inhibitory cytokine (Moore et al., 1993), in African trypanosomiasis is unknown despite the fact that this cytokine has been shown to be involved in immunosuppression in other parasitic infections (Ding and Shevach, 1992; Denis and Ghadirian, 1993; Khan et al.

1995). In cattle infected with *T. congolense*, IL-10 mRNA has been shown to be upregulated in the spleen, lymph nodes and PBMC of the trypanosusceptible Boran cattle (Taylor et al., 1996), and a suggestion was made that IL-10 might be involved in immunosuppression in these animals (Taylor, 1998).

1.2.2.4 Role of nitric oxide (NO)

Production of high levels of NO have been observed in splenic and peritoneal macrophages of mice infected with *T. brucei* (Sternberg and McGuigan, 1992; Sternberg et al., 1994; Mabbot et al., 1995), and *T. brucei rhodesiense* (Schleifer and Mansfield, 1993). This high level of NO was shown to mediate suppression of splenic T cell responses which could be partially reversed by N^G-monomethyl-L-arginine (N^GMMA), a competitive inhibitor of arginine-dependent NO synthetase (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993). The production of immunosuppressive NO by splenic macrophages in mice infected with *T. brucei* is dependent on IFN-γ and TNF-α (Schleifer and Mansfield, 1993). However, using lymph node cells from mice infected with *T. brucei*, Darji et al. (1996) failed to observe suppressive activity attributable to NO. There is as yet no report of NO mediating suppression of T cell proliferative responses in mice infected with *T. congolense*. However, in cattle infected with *T. congolense* and *T. vivax*, production of NO by macrophages and monocytes is depressed and NO does not mediate the suppression of T cells observed in these animals (Taylor et al., 1996). It therefore appears that the role of NO in mediating suppression of T cell responses during trypanosomiasis may depend on the strain and species of parasites as well as the host.

1.3 Resistance to African trypanosomes

The degree of susceptibility or resistance of a host to most parasitic infections is generally a consequence of the host's immune response. However, many factors can act to modify the host's responses to the parasite. Some of these factors are related to the parasite while others are contributed by the host's non-immune defense mechanisms. The host's factors that determine resistance or susceptibility to African trypanosomes can be classified into non-specific and specific mechanisms.

1.3.1 Non-specific mechanisms

1.3.1.1 Inhibition of growth by human serum

Trypanosoma brucei brucei and several other species non-pathogenic to man (including *T. congolense*, *T. evansi* and *T. equiperdum*) are lysed when incubated in the presence of human serum (reviewed by Hajduk et al., 1994). In contrast, the human pathogenic strains (*T. b. rhodesiense* and *T. b. gambiense*) are resistant to lysis in human serum. Recently, the lytic factor in human serum has been shown to be a protein related to haptoglobin which occurs as an apoprotein in an association with a minor subclass of high density lipoprotein (Hager and Hajduk, 1997). In another report, a large complex protein termed trypanosome lytic factor 2 was shown to be responsible for the lytic effect of human serum on *T. brucei* (Raper et al., 1996). It is believed that the trypanosome lytic factor binds to an as yet unidentified receptor on trypanosomes, and is then internalized, leading to trypanosome lysis (Hager and Hajduk, 1997). Resistant strains of *T. brucei* bind but do not internalize this lytic factor (Hager and Hajduk, 1997).

The serum of cotton rats is lytic for *T. vivax* and as such these animals are refractory to infection with this parasite, even when challenged with very high numbers of trypanosomes (Hudson, 1972). This activity of cotton rat serum is specific only for *T. vivax* (Terry, 1957). The active molecule of cotton rat serum involved in lysis of *T. vivax* is a protein of molecular weight 550 kD and has been referred to as "natural antibody" (Hudson, 1972).

1.3.1.2 Role of NO in resistance

Peritoneal and splenic macrophages from mice infected with *T. brucei* (Sternberg and McGuigan, 1992; Sternberg et al., 1994) and *T. rhodesiense* (Schleifer and Mansfield, 1993) produce large amounts of nitric oxide. Nitric oxide produced by activated murine macrophages is cystostatic for *T. brucei* and *T. gambiense in vitro* (Vincendeau et al., 1992). Also, mice infected with NO-treated parasites had an increased prepatent period and longer survival period when compared with groups infected with untreated parasites (Vincendeau and Daulouede, 1991). Nitric oxide has been implicated in the microbistatic and microbicidal activities of macrophages against a variety of parasite targets, including intracellular protozoa, extracellular helminth larvae and extracellular pathogenic fungi (James and Hibbs, 1990). However, NO has been implicated in trypanosome-induced suppression of lymphoid cell proliferative responses (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993) and inhibition of NO production has been reported to cause reduction in parasitemia in murine *Trypanosoma brucei* infection (Sternberg et al., 1994).

1.3.1.3 Role of complement in resistance

Both the alternative and classical pathways of complement are activated during infection with African trypanosomes (Greenwood and Whittle, 1980). Although clearance of trypanosomes by phagocytic cells does occur in the absence of complement (Ngaira et al., 1983), the efficiency of parasite clearance and immune complex removal is greatly enhanced by complement (Stevens and Moulton, 1978; Takayanagi et al., 1987). Trypanosomes coated with variant-specific antibody have been shown to activate complement via the alternative pathway (Diggs et al., 1980). Mice depleted of C3 by treatment with cobra venom factor exhibit severe impairment in the hepatic uptake of opsonized *T. brucei brucei* (MacAskill et al., 1980). However, partial depletion of C3 in mice infected with *T. brucei brucei* (Shirazi et al., 1980) or *T. brucei rhodesiense* (Dempsey and Mansfield, 1983) had no effect on the control of parasitemia or phagocytosis.

Hypocomplementemia is a prominent feature of African trypanosomiasis in cattle (Tabel et al., 1982; Authie and Pobel, 1990), sheep (Malu and Tabel, 1986) and mice (Otesile et al., 1991). Reduction in total hemolytic complement activity and C3 are more marked in the trypanosusceptible Zebu than trypanotolerant Baoule cattle (Authie and Pobel, 1990). Otesile et al. (1991) analysed the levels of proteins of the alternative pathway of the complement system in mice strains that differ in resistance to infection with *T. congolense.* They observed that the preinfection levels of factor H were significantly higher in the resistant C57BL/6 mice than in the susceptible BALB/C mice. In addition, the amount of factor B in the plasma of infected mice during the later stages of infection was positively correlated with survival time and was higher in the

trypanotolerant C57BL/6 mice. Similarly, Ogunremi et al. (1993) demonstrated that the activity of the alternative pathway of complement activation as measured by deposition of C3b was significantly higher in the plasma of resistant C57BL/6 compared to susceptible BALB/C mice infected with *T. congolense*. Although these reports would suggest an important role for complement in the resistance to trypanosomes, there is evidence that complement-mediated lysis of trypanosomes demonstrable *in vitro* may not play a significant role in resistance to trypanosomiasis in mice since no significant differences were observed in either the hepatic uptake of parasites (MacAskill et al., 1980) or in the survival periods (Jones and Hancock, 1983) of C5-sufficient and C5-deficient mice.

1.3.2 Specific mechanisms

1.3.2.1 Role of antibodies in resistance

African trypanosomes are extracellular parasites and therefore humoral immune responses are mostly mounted by the host during infection. The VSG of the trypanosomes is highly immunogenic and dominant immune responses are mounted against it. Most B-cell responses are mounted against the exposed N-terminal epitopes of the VSG molecule. These responses are both T cell-independent and T cell-dependent (Reinitz and Mansfield, 1990).

The clearance of parasites from the blood of mice infected *with T. brucei rhodesiense* (Dempsey and Mansfield, 1983; Levine and Mansfield, 1984), *T. brucei brucei* (Black et al., 1986), *T. congolense* (MacAskill et al., 1983; Mitchell and Pearson, 1986) and *T. vivax* (DeGee et al., 1982; Mahan et al., 1986) is associated with the appearance of parasite-specific antibodies. Several experiments have demonstrated the

role of VSG-specific B-cell responses in protection. Passive transfer of VSG-specific antibody or primed B cells to immunocompromised animals results in transfer of variant-specific immunity (Seed and Gam, 1966; Campbell and Phillips, 1976; Otesile and Tabel, 1987). In contrast, passive transfer of T cells does not result in protection (Campbell and Phillips, 1976). Furthermore, mice rendered B cell-deficient by neonatal treatment with anti-μ chain specific antiserum are highly susceptible to *T. brucei rhodesiense* infections, whereas athymic nude mice can control their first wave of parasitemia (Campbell et al., 1977; 1978). However, in a study of *T. congolense* infections in BALB/c mice, Otesile and Tabel (1987) concluded that variant-specific antibody is necessary, but not sufficient, for control of infection.

The role of differences in antibody production in resistance to trypanosomes by different strains of mice has been extensively investigated. Morrison and Murray (1985) reported that the highly susceptible AJ mice produce little or no detectable antibody against the VSG during infection with *T. congolense* compared to the resistant C57BL mice. Because these mice had greatly increased numbers of plasma cells, they concluded that the lower antibody production in the AJ mice was due to a defect associated with antibody secretion. Similar results were reported by Black et al. (1986) and Newson et al. (1990) in mice infected with *T. brucei brucei.* Spleen cells from resistant mice infected with *T. brucei* exhibited superior antibody synthesis and secretion *in vitro* compared to those from susceptible mice. However, when mice were immunized with non-multiplying trypanosomes or purified VSG, susceptible and resistant mice produced almost similar levels of anti-VSG antibodies with almost identical kinetics (Morrison and Murray, 1985;

Pinder et al., 1986). Although Mitchell and Pearson (1983) reported superior qualitative and quantitative antibody response by the resistant mice following immunization with trypanosomal antigens, they also observed that the susceptible mice equally made a substantial antibody response to these antigens. In addition, when susceptible mice were treated with a trypanostatic (Newson et al., 1990) or trypanolytic (Black et al., 1986; Otesile and Tabel, 1987) drug, they mounted high antibody responses to the infecting variant organisms.

Studies on the nature of antibody class associated with resistance in infected or immunized mice are equivocal. Some investigators reported that IgM antibodies were better in mediating trypanosome agglutination (Seed, 1977, Pinder et al., 1985) and neutralization of infectivity (Takayanagi and Enriquez, 1973). Seed (1977) reported that IgG antibodies gave better passive protection against *T. brucei gambiense*, while Dempsey and Mansfield (1983) reported that both IgM and IgG were both protective against *T. brucei brucei*. Using a panel of monoclonal antibodies against the VSG of *T. brucei rhodesiense*, Hall and Esser (1984) reported that antibody-mediated immunity against this parasite is not associated with any particular subclass of antibody. Both IgM, IgG, IgG2a, IgG2b, IgG3 and IgA subclasses were shown to mediate comparable immune clearance of trypanosomes. Nude mice which lack T helper cells, when infected with *T. congolense*, developed uncontrolled parasitemia and survived for shorter period of time compared to thymus-intact litter mates (Morrison et al., 1978). These workers concluded that the enhanced susceptibility in these mice was due to lack of an IgG response against the parasite. In contrast, nude mice infected with either *T. brucei rhodesiense* (Campbell et al., 1978) or *T. brucei brucei* (Clayton et al., 1979) showed a reduced first peak

parasitemia and relatively longer survival than thymus intact mates. Thus, there is no consensus on the relative importance antibody class in the control of parasitemia in mice infected with trypanosomes.

The overall effect of any antibody class will depend on many factors including its concentration, affinity, avidity and ability to activate complement or bind to macrophages (Roitt et al., 1985). Various classes of immunoglobulins have different effector functions related to their Fc portion. Removal of antibody-coated trypanosomes is primarily mediated by phagocytosis on the part of liver macrophages (MacAskill et al., 1980; Dempsey and Mansfield, 1983). It is therefore conceivable that parasites coated with IgG would be more readily cleared than those coated with IgM, since macrophages have high affinity receptors for the Fc of IgG. However, Ngaria et al., (1983) has demonstrated adherence of trypanosomes to macrophages with subsequent phagocytosis in the presence of IgM antibodies, indicating that phagocytosis can be mediated by receptors on macrophages other than those for the Fc of IgG. The effectiveness of antibody may also be influenced by other factors including ability to mediate aggregation, neutralization, induce shedding of the VSG and/or activate complement to induce lysis. Takayanagi et al. (1993) observed that IgG3 caused aggregation of *T. gambiense* and their adherence to macrophages and suggested a role for this isotype in protection against *T. gambiense* infection. Similarly, an IgG3 monoclonal antibody against *T. congolense* caused rapid immobilization of this parasite *in vitro* as well as parasite neutralization *in vivo* (Wei et al., 1993). It is conceivable that such immobilization *in vivo* would result in prolonged adherence of antibody-coated parasites to macrophages and thereby facilitate phagocytosis and clearance.

Although resistant mice produce higher VSG-specific antibody levels than the susceptible mice during infection with trypanosomes, genetic analyses have shown that production of VSG-specific antibodies and overall resistance are distinct traits that segregate independently (DeGee and Mansfield, 1984; 1988; Seed and Sechelski, 1989; Ogunremi and Tabel, 1995). Using bone marrow chimeras, DeGee and Mansfield (1984) showed that the ability to make an IgM anti-VSG antibody response is not linked to resistance. Irradiated susceptible mice adoptively reconstituted with bone marrow cells from H-2-compartible resistant mice made very good IgM anti-VSG antibody responses but were still susceptible to infection. They concluded that in addition to antibodies, resistance requires other factors associated with radioresistant cells. Similarly, Otesile and Tabel (1987) have shown that although antibodies were necessary for control of variant-specific parasitemia in susceptible mice infected with *T. congolense*, they were insufficient to mediate cure from the infection. These reports demonstrate that the longer survival period in resistant mice is due to anti-VSG antibody responses and at least, in part, to as yet unidentified additional factors which do not segregate with VSG-specific antibody responses. It has been suggested that among other factors, the degree of immunosuppression among resistant and susceptible mice is important (Black, 1985; Pinder et al., 1986; Sileghem et al., 1994).

Several reports have demonstrated similar as in mice, antibodies may be important in protection against bovine trypanosomiasis. Bovine macrophages can phagocytose trypanosomes *in vitro* in the presence of variant-specific antibodies (Takayanagi et al., 1974; Ngaira et al., 1983). In addition, immunization of cattle with VSG or irradiated

trypanosomes results in variant-specific immunity (Morrison et al., 1982; Wells et al., 1982).

As in mice, the trypanotolerant cattle breeds appear to produce a superior variant-specific antibody compared to the trypanosusceptible breeds (Chandler, 1958; Dwinger et al., 1992). However, Pinder et al. (1984, 1988) and Murray et al. (1982) did not find differences in the antibody responses between the resistant and susceptibile breeds. In one study, although no difference was observed between the tolerant and susceptible cattle in variant-specific antibodies to the infecting trypanosomes, later during the infection, tolerant cattle had higher titers of neutralizing antibodies than susceptible cattle (Akol et al., 1986). The differences among results from these experiments may be due to the difficulty in obtaining animals of homogenous immune status free from mixed infections.

Analysis of antibody classes indicate that resistant cattle produce more IgG antibodies against the VSG (Williams et al., 1996; Taylor et al., 1996) and invariant antigens (Authie et al., 1992; 1993a; 1993b) than susceptible cattle. In contrast, susceptible cattle made higher IgM responses to these antigens than resistant ones (Taylor et al., 1996; Williams et al., 1996). Superior IgG responses to the invariant antigens have been associated with enhanced resistance observed in the trypanotolerant cattle breeds (Authie et al., 1993a; 1993b; Agur and Mehr, 1997). Thus, while clear demonstration of antibody-mediated protection in cattle has not been shown, there appears to be an association between higher antibody production and resistance.

1.3.2.2 Role of liver macrophages

Several studies in rodent models of trypanosomiasis indicate that the mononuclear phagocytic system plays an important role in the elimination of trypanosomes from blood of infected animals (Takayanagi et al., 1974; MacAskill et al., 1980; Dempsey and Mansfield 1980). Mice infected with trypanosomes have greatly expanded mononuclear phagocytic system with macrophages from the liver, spleen, lymph nodes and bone marrow displaying morphological features of activation and increased activity (Murray et al., 1974). Antibody-coated trypanosomes are readily phagocytosed by mononuclear phagocytic cells of rodents (Takayanagi et al., 1974; MacAskill et al., 1980).

Several studies have shown that Kupffer cells of the liver are the major cells involved in the clearance of antibody-coated trypanosomes (MacAskill et al., 1980; Dempsey and Mansfield 1983; Albright et al., 1990). Injection of *T. brucei brucei* into mice immune to the infecting variant results in rapid sequesteration of the parasites within the Kupffer cells (Dempsey and Mansfield, 1983). Following phagocytosis, trypanosomes are rapidly destroyed within the phagolysosomes of the Kupffer cells (Mansfield, 1990).

The exact mode of attachment and uptake of antibody-coated trypanosomes by macrophages is not known. However, it is generally agreed that attachment of trypanosomes with subsequent phagocytosis by macrophages requires the presence of variant-specific antibody (Mosser and Roberts, 1982). Uptake of trypanosomes by monocytes occurs in the presence of IgM or IgG antibodies (Ngaira et al., 1983) indicating that both Fc receptor-independent and -dependent mechanisms may be operative. Binding of IgM antibodies to the VSG may lead to perturbations of the surface

coat of the blood stream trypanosomes thereby exposing the plasma membranes. This exposes some carbohydrates which may serve as ligands for carbohydrate receptors on macrophages thereby facilitating phagocytosis. This theory is supported by the finding that the procyclic forms of trypanosomes (which do not have VSG on their membrane) readily adhere to macrophages (Mosser and Roberts, 1982) and are phagocytosed *in vitro* by monocytes in the absence of antibody (Abolarin et al., 1983). Binding of IgM antibodies to the surface of trypanosomes leads to complement activation via the alternative pathway (Diggs et al., 1980) resulting in the deposition of C3 cleavage products (Devine et al., 1986). Because macrophages have receptors for C3 cleavage products, trypanosomes coated with these molecules will be readily phagocytosed (Mansfield, 1990).

In cattle, indirect evidence suggests that phagocytosis may also be important in parasite clearance from the blood of infected animals. Trypanosomes coated with variant-specific antibodies were shown to adhere to the surface of monocytes (Ngaira et al., 1983). Furthermore, monocytes isolated from the peripheral blood of cattle infected with *T. brucei* or *T. congolense* were shown to require shorter periods of *in vitro* activation to bind and phagocytose opsonized parasites than those from uninfected cattle (Taylor, 1998). This was interpreted to mean that monocytes of trypanosome-infected cattle have increased FcγR function compared to those from uninfected cattle.

The overall contribution of parasite clearance trypanotolerance is equivocal. Kamanga-Sollo et al. (1991) reported that antibody-coated trypanosomes adhere better to mononuclear cells from N'Dama than to those from Boran cattle. Kissling et al. (1982)

observed that the blood of uninfected N'Dama cattle contains a greater number of neutrophils and has higher phagocytic activity than that of Zebu cattle. This phagocytic activity was further increased in *T. congolense* infected N'Dama, but not Boran, cattle (Kissling et al., 1982). However, Sileghem et al. (1991) did not observe differences in trypanosome phagocysis by monocytes and macrophages obtained from *T. congolense*-infected N'Dama and Boran cattle.

1.4 Genetics of resistance to African trypanosomes

Infections with African trypanosomes result in a spectrum of diseases in cattle and inbred mouse strains. Extensive studies in mice of different breeds found a wide array of susceptibility/resistance to different species and strains of African trypanosomes (Morrison et al., 1978; 1978a; 1979; Jennings et al., 1978; DeGee et al., 1982; Levine and Mansfield 1981; Pinder, 1984; Greenblatt et al., 1984; Ogunremi and Tabel, 1995). Some inbred strains are more resistant (as measured by lower parasitemia and longer survival period) than other strains. Studies on strains congenic for the major histocompatability complex indicate that resistance or susceptibility is not linked to these genes (Morrison et al., 1978; Pinder et al., 1985; Levine and Mansfield 1981). Studies on the inheritance pattern of resistance and susceptibility between F1 crosses of resistant and susceptible inbred parents were equivocal. Whereas Pinder (1984) and DeGee et al. (1988) found susceptibility to be a dominant trait, Greenblatt et al. (1984) and Morrison and Murray (1979) found resistance to be dominant. In contrast, Ogunremi and Tabel (1995) reported a wide range of susceptibility to *T. congolense* infection among the F1 offsprings of the resistant C57BL/6 and susceptible BALB/c mice. Studies with F2 and

F1 backcrosses demonstrated that resistance/susceptibility is under a polygenic control (Greenblatt et al., 1984; Blackwell 1988; Ogunremi and Tabel, 1995).

The mechanisms underlying the differences in resistance/susceptibility of inbred mouse strains to African trypanosomes are poorly understood. However, it has been shown that, at least in *T. congolense* infection, the relative resistance of C57BL/6 mice requires an intact immune system. Their relative resistance is ablated by subclinical irradiation (Morrison and Murray, 1985). Moreover, T cell-deficient C57BL/6 mice infected with *T. congolense* have higher parasitemias and shorter survival periods than their similarly infected T cell-sufficient littermates (Pinder et al., 1986). An association has been made between the degree of resistance and the kinetics of appearance and level of VSG-specific antibody to the infecting variant (Mansfield, 1990). Infected resistant mice mount early and higher anti-VSG antibody responses that are associated with clearance of the infecting variant while susceptible mice fail to make detectable VSG-specific antibody responses or control parasitemia (Morrison and Murray, 1979; Levine and Mansfield 1984; Mansfield, 1990). Significant differences in the quality of antibody responses reported among the resistant and susceptible breeds (Seed et al., 1977; Morrison et al., 1978; Mitchell and Pearson, 1983; 1986) could play a significant role in host susceptibility to trypanosomes.

Another factor reported to contribute to differential resistance to trypanosome infections in mice is the degree of immunosuppression. Morrison et al. (1978a) showed that the kinetics and degree of immunosuppression correlated with the degree of susceptibility to infection. Other investigators (Roelants and Pinder, 1984; Pinder et al., 1986) have reached similar conclusions. Recently it was shown that the nitric oxide

(Sternberg and McGuigan 1992; Schleifer and Mansfield, 1993; Mabott et al., 1995), and IFN-γ (Darji et al., 1993; 1996) produced in trypanosome-infected mice are immunosuppressive. IFN-γ is produced in higher amounts in susceptible than in resistant mice. Thus, differential induction and kinetics of secretion of this cytokine could influence the outcome of infection in different strains of mice.

In cattle, it has been observed that certain indigenous west African breeds can survive in areas of high tsetse fly infestation where trypanosomiasis is endemic (Murray et al., 1982). In contrast, exotic cattle breeds do not survive in these regions in the absence of regular strategic chemotherapy. The trypanotolerant breeds (e.g. N'Dama) possess superior ability to withstand disease as assessed by less weight loss, less anemia and longer survival (Murray et al., 1982). The ability to suppress the development of anemia is one of the major criteria for measuring trypanotolerance in cattle (Murray et al., 1982; Murray et al., 1991). Trypanotolerant breeds of cattle are less anemic than the trypanosensitive breeds when infected with different strains and species of African trypanosomes (Murray et al., 1991; Murray and Morrison, 1979; Authie and Pobel, 1990). The genetic or immunological basis for the control of anemia by the trypanotolerant breeds is not understood. However, higher levels of parasitemia and IgM found in susceptible cattle has been suggested to contribute to higher rates of erythrocyte destruction (Jenkins and Facer, 1985).

An immunological basis for trypanotolerance has been proposed by many investigators. Most of these studies have focused on the kinetics of induction and quality of antibody responses to the infecting organisms. In general, trypanotolerant cattle

produce an earlier and higher variant-specific antibody response than the trypanosusceptible cattle (Chandler, 1958, Dwinger et al., 1992; Akol et al., 1986). Trypanotolerant cattle also produced higher IgG responses to the buried epitopes of the VSG relative to the susceptible cattle (Williams et al., 1996; Taylor et al., 1996). It has been demonstrated that the tolerant cattle produce more IgG1 antibodies against the invariant antigens of *T. congolense* whereas the susceptible cattle made predominantly an IgM antibody response (Authie et al., 1992; 1993). These IgG1 antibodies to invariant antigens have been associated with resistance (Authie, 1994). Since clearance of parasites from the blood is mediated by variant-specific antibodies (Mansfield, 1990), it is conceivable that an earlier and higher antibody response by tolerant cattle confers some advantage in resistance.

The role of other immune factors in trypanotolerance has not been extensively investigated in cattle. Antigen-specific proliferation of PBMC of trypanosome-infected N'Dama cattle were higher than those of Boran cattle (Flynn et al., 1992), an observation that could be explained by more severe immunosuppression in trypanosusceptible cattle. Furthermore, lymph node cells of infected Boran cattle produce more IFN-γ than those of infected N'Dama cattle following mitogen stimulation (Sileghem and Flynn, 1992b; Lutje et al., 1996). Elevated levels of IL-10 mRNA transcripts have been reported in the PBMC, lymph nodes and spleens of trypanosome-infected Boran (Taylor et al., 1996) and it was suggested that the suppression of macrophage monokines observed in infected Boran cattle may be related to this high level of IL-10. Because IFN-γ (Darji et al., 1993; 1996), has been shown to contribute to immunosuppression and enhanced trypanosome

susceptibility in mice, it is conceivable that the higher levels of these cytokines observed in the susceptible cattle might be involved in their enhanced susceptibility to trypanosome infections.

1.5 T cells and T cell-derived cytokines

1.5.1 Murine helper T cell subsets

T helper lymphocytes can be divided into two distinct subsets of effector cells based on their functional characteristics and the profile of cytokines they produce (reviewed by Mosmann and Sad, 1997). TH1 subsets of $CD4^+$ T cells secrete IL-2, IFN-γ and TNF-β, and TH2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann et al., 1986; Cherwinski et al., 1987; Mosmann et al., 1990; Mosmann and Sad, 1997). A third $CD4^+$ T cell subset, called TH0 cells, have been defined on the basis of secretion of mixed cytokines - IL-2, IL-4, IL-5 and IFN-γ (Gajewski and Fitch, 1988; Firestein et al., 1989; Street et al., 1990). Although TH0 cells have been described in a variety of conditions, it has been suggested, based on results from *in situ* mRNA hybridization (Carding et al., 1989) and intracellular protein staining (Openshaw et al., 1995), that the overlapping cytokine profiles associated with this subset are due to the presence of mixed populations of $CD4^+$ T cell subsets. Recently, T helper cells producing high amounts of TGF-β have been termed TH3 (Chen et al., 1994) and additional phenotypes have been described among long-term clones (Mosmann and Sad, 1997). The heterogenicity and quantitative differences in cytokine expression may represent distinct phenotypes, developmental stages, or transient responses to different stimulatory conditions.

1.5.2 Helper T cell subsets in humans

Initial work with human $CD4^+$ T cells failed to show distinct TH subsets similar to those observed with the murine $CD4^+$ T cells (Paliard et al., 1988). However, T cell clones derived from individuals with certain pathological or physiological states produced distinct cytokines suggestive of polarized T helper subsets. The majority of allergen-specific T cell clones derived from atopic individuals were shown to produce IL-4 and IL-5 with little or no IFN-γ (Wierenga et al., 1990). In contrast, T cell clones specific for the purified protein derivative (PPD) of *Mycobacterium tuberculosis* derived from the same donors sensitized to this antigen, produced mainly IL-2 and IFN-γ but no IL-4 (Del Prete et al., 1991; Parronchi et al., 1991). Thus, similar to the murine CD4 TH cells, human TH1 subsets secrete IFN-γ and TNF-β while TH2 subsets secrete IL-4, IL-5, IL-10 and IL-13 (reviewed by Romagnani, 1997).

1.5.3 TC1 and TC2 $CD8^+$ T cells

Most activated $CD8^+$ T cells exhibit a TH1-like cytokine pattern. These cells have been termed TC1 cells (Mosmann and Sad, 1996; Romagnani, 1997). There is increasing evidence for the existence of TH2-like $CD8^+$ T cells in humans and mice (Salgame et al., 1991; Croft et al., 1994). The TC2 cells are thought to arise under some pathological conditions (Romagnani, 1997). Although $CD8^+$ TC2 cells secrete IL-4 and IL-5, they still retain their cytotoxic abilities (Croft et al., 1994; Sad et al., 1995) and do not appear to help B cells in a cognate helper assay (Sad et al., 1995). However, it is possible that non-

cognate, or bystander B cell help by $CD8^+$ TC2 cells may occur *in vivo*, perhaps due to their expression of CD40L (Cronin et al., 1995) or secretion of type 2 cytokines, which aid B cells previously activated by a $CD4^+$ T helper cell.

1.5.4 Polarization of TH1 and TH2 responses

The factors responsible for the polarization of immune responses with a predominant TH1 or TH2 profile have been extensively studied in mice and humans (Mosmann and Sad, 1996; Constant and Bottomly, 1997; Romagnani, 1997). Studies in transgenic mice have shown that TH1 and TH2 cells can develop from the same $CD4^+$ T cell precursor under the influence of environmental and genetic factors acting at the level of antigen presentation (Constant and Bottomly, 1997; Romagnani, 1997). Such environmental factors include the route of antigen entry, the physical form of antigens, the type of adjuvant and the dose of antigen (Constant and Bottomly, 1997).

The environmental and genetic factors exert their influence mainly by affecting the preponderance of a given cytokine in the microenvironment of the responding T helper cell(s). IFN-γ and IL-12 are thought to be the major cytokines that promote TH1 differentiation (Seder and Paul, 1994; Constant and Bottomly, 1997). The early presence of IL-4 is the most potent stimulus for TH2 differentiation (Swain et al, 1990; LeGross et al., 1990). IL-12 is produced by many cells, but mostly by macrophages in response to certain microbial products (Scott, 1993) and natural killer (NK) cells synthesize IFN-γ in response to IL-12 (Scott, 1993). These cells are believed to be the primary sources of IFN-γ and IL-12 that drive a naïve $CD4^+$ T cells to differentiate into a TH1 subset during a

primary immune response. The mechanisms responsible for the early IL-4 production involved in the differentiation of naïve $CD4^+$ T cells to TH2 cells are not fully understood. In mice, early IL-4 production may be contributed by a subpopulation of $CD3^+$ $CD4^+$ $NK1.1^+$ T cells that are restricted by CD1 molecules (Bendelac et al., 1997). These cells have a restricted TCR repertoire and produce large amounts of IL-4 within few hours of polyclonal stimulation (Zlotnik et al., 1992; Arase et al., 1993; Bendelac et al., 1997).

1.5.5 Cross regulation of TH1 and TH2 cells by cytokines

TH1 and TH2 responses observed in certain physiological states and following immunization with strong immunogens tend to be exclusive of one another in most cases. The cytokines produced by each of the two major $CD4^+$ subset have a regulatory effect on the proliferation or functional activity of the opposing subset. IFN-γ produced by TH1 cells inhibits the growth and proliferation of TH2 cells (Gajewski et al., 1989; Gajewski and Fitch, 1988). IL-12, which is a potent enhancer of IFN-γ production (Seder and Paul, 1994), indirectly diminishes priming for IL-4 production and hence inhibits the expansion of TH2 cells. Similarly, IL-4 preferentially stimulates the generation of TH2 cells and prevents priming for IFN-γ production (Seder and Paul, 1994). IL-4 has also been shown to inhibit IFN-γ release by NK cells (Peteman et al., 1989) and to inhibit TNF transcription by activated macrophages (Lehn et al., 1989). IL-10, formerly known as cytokine synthesis inhibitory factor (Mosmann, 1990), inhibits cytokine (IL-2 and IFN-γ) synthesis by TH1 cells (Mosmann, 1990). This inhibitory effect is mediated by acting via the antigen presenting cells (Fiorentino et al., 1989; Sher et al., 1991).

This cross-regulation of TH1 and TH2 cells by cytokines partially explains the strong and polarization of immune responses during many parasitic infections in mice and humans. In several cases, alteration of these patterns by cytokines or anti-cytokine reagents reverses host resistance or susceptibility to infection.

1.5.6 TH1 and TH2 CD4$^+$ T cells perform distinct functions

The production of distinct cytokines by TH1 and TH2 CD4$^+$ T cells correlates with distinct functions *in vivo*. TH1 cells are responsible for classical delayed-type hypersensitivity (DTH) responses and activation of macrophages for cell mediated immunity. Several TH1 cytokines activate cytotoxic and inflammatory functions (Mosmann and Coffman, 1989) and IFN-γ is commonly expressed at sites of DTH reactions (Yamamura et al., 1991; Tsicopoulos et al., 1992). IFN-γ also enhances the microbiocidal activity of macrophages (Murray et al., 1988; Stout and Bottomly, 1989). As a result, IFN-γ associated responses have been shown to be protective against several intracellular pathogens including *Leishmania major* (Sadick et al., 1987; Nathan et al., 1983; Murray et al., 1983), *Listeria monocytogenes* (Buchmeier and Schneiba, 1985), *Toxoplasma gondii* (Gazzinelli et al., 1991), *Trypanosoma cruzi* (Nickell et al., 1993) and *Cryptosporidium* (Unger et al., 1991). Some B cell help can be provided by TH1 cells and IFN-γ has been shown to mediate class switching in B cells from IgM to IgG2a isotype (Snapper and Paul, 1987, Boehm et al., 1997). However, at higher TH1 cell numbers, B cell responses may be suppressed (Coffman et al., 1988; Del Prete et al., 1991).

TH2 cells encourage antibody production by providing cognate help to B cells. In addition, TH2 cytokines stimulate B cell growth and differentiation into antibody-producing cells. IL-4 mediates class switching to IgG1 and IgE (Paul and Ohara, 1987; Banchereau et al., 1991) and mice with disruption of IL-4 genes do not have detectable levels of serum IgE despite having normal numbers of B and T cells (Kuhn et al., 1991). When these mice were infected with *Nippostrongylus braziliensis*, a parasite known to induce strong IgE responses, they were unable to make an IgE response indicating a strong dependence of IgE responses on IL-4 (Kuhn et al., 1991).

IL-5 plays an important role in the development of stimulated B cells into antibody-secreting cells (Hamaoka and Ono, 1986). It also mediates class switching to IgA (Stevens et al., 1988). Both IL-6 (Kishimoto, 1989) and IL-10 (Roussel et al., 1992) promote Ig secretion by activated B cells.

Because of the profound effects of TH2 cytokines on B cell growth, differentiation and antibody secretion, they are commonly found in association with strong antibody and allergic responses.

1.6 Regulation of immunity to parasites by T cell-derived cytokines

Most parasitic infections tend to be chronic in nature and the resultant persistent antigenic stimulation often leads to polarization of T cell subsets (Sher and Coffman, 1992). In addition, the biological diversity of parasites and differences in their *in vivo* residence within the host results in distinct forms of antigen presentation and T cell activation. Furthermore, there appears to be genetic differences in the immune responses and control of many parasitic infections by the host (Sher and Coffman, 1992). As a

result of these variables, different effector mechanisms are recruited in response to different parasites and both $CD4^+$ and $CD8^+$ T cells have been shown to play regulatory functions in both helminth and protozoal infections (Sher and Coffman, 1992).

1.6.1 Immunity to gastrointestinal nematodes

Studies with gastrointestinal nematodes have shown that immunity, characterized by worm expulsion and reduction in egg output, is dependent upon IL-4 secreted by $CD4^+$ T cells (Finkelman et al., 1997). Immunity to experimental *Trichurus muris* and *Heligmonsomoides polygyrus* is highly dependent on IL-4 and anti-IL-4 or anti IL-4R monoclonal antibodies block host immunity to these infections (Urban et al., 1991; Else et al., 1994). Administration of recombinant IL-4 alone or in complexes that prolong its half life have been shown to cure mice of established primary *T. muris* and *H. polygyrus* infections (Finkelman et al., 1993; Else et al., 1994; Urban et al., 1995). IL-4 has also been implicated in protective mechanisms against *Nippostrongylus brasilienses* and *Trichinella spiralis* (Urban et al., 1995, Madden et al., 1991). The protective role of IL-4 against *T. spiralis* is believed to be mediated by its enhancing effect on IgE antibody production which causes worm expulsion (Ahmad et al., 1991; Dessein et al., 1981).

Most gastrointestinal nematode infections stimulate eosinophils and increase IL-5 production (Finkelman et al., 1997). Eosinophils kill some parasite larvae *in vitro* (David et al., 1980) and ablation of eosinophils *in vivo* by anti-IL-5 antibody treatment has been shown to enhance the survival of certain nematode parasites (Sasaki et al., 1992; Korenga et al., 1994). However, administration of anti-IL-5 antibodies has no effect on the control

of *T. muris*, *H. polygyrus* or *N. brasiliensis* (Urban et al., 1991; Herndon and Kayes, 1992; Coffman et al., 1989).

In contrast, mouse strains that produce a strong TH1 (IFN-γ) response to primary *T. muris* infection develop chronic infections and neutralization of this cytokine results in protection (Else et al., 1994). Furthermore, treatment of mice infected with *N. brasiliensis* with recombinant IL-12 or IFN-γ enhances egg output and prolongs the course of infection (Finkelman et al., 1994).

Thus it appears that effective immunity against nematodes is associated with TH2 CD4 T cell responses and TH2-associated cytokines (IL-4 and IL-5)

1.6.2 Immunity to *Schistosoma mansoni*

There is strong evidence that established murine schistosomiasis is associated with strong TH2 responses and protection induced by vaccination with irradiated cercariae correlates with a more responsive TH1 cytokine production (Grzych et al., 1991; Pearce et al., 1991). This protection can be ablated partially by treatment of vaccinated animals with antibodies against IFN-γ (Sher et al., 1990). Depletion of IgG2a antibodies from serum of infected rats showing a strong resistance to reinfection, results in a marked diminution of the capacity of such serum to transfer resistance to the schistosomulum (Butterworth, 1990). Similarly, immunity can be transferred with an IgG2a monoclonal antibody against a schistosomulum membrane antigen (Butterworth, 1990). IFN-γ, a TH1 cytokine, favors the switching of B cells from IgM to IgG2a antibody isotype (Snapper and Paul, 1987; Boehm et al., 1997). However, TH1 cytokines (IL-2 and IFN-γ) have

been implicated in fibrosis and granuloma formation in schistosomiasis (Mather et al., 1990).

1.6.3 Immunity to protozoan parasites

1.6.3.1 *Leishmania major*

L. major is an intracellular parasite of macrophages. Cutaneous infections of inbred strains of mice with *L. major* results either in a localized lesion that resolves spontaneously or in a progressive disseminated visceral disease that is usually fatal (Locksley and Reiner, 1995). It is now known that resistance (healing) and susceptibility (non-healing) are conditional traits that represent the development of strong TH1 and TH2 responses, respectively. Analysis of cytokines produced by T cells from infected mice following *in vitro* restimulation with leishmania antigen show extreme polarization and dichotomy in IFN-γ and IL-4 production. Healer mice typified by C57BL/6 and CBA mice produce substantially higher amounts of IFN- γ and are able to mount a strong DTH response, but a poor antibody response (Liew et al., 1989; Sadick et al., 1986, Boom et al., 1990). In contrast, susceptible mice produce very high amounts of IL-4 and IL-5 and make strong antibody responses with very high IgE levels (Heinzel et al., 1989; Boom et al., 1990; Heinzel et al., 1991). A single injection of monoclonal anti-IFN-γ antibody converts resistant into susceptible mice (Belosevic et al., 1989) while anti-IL-4 antibodies can convert susceptible mice into resistant ones (Sadick et al., 1990). Administration of recombinant IL-12 promotes resistance by stimulating IFN-γ production and inhibiting IL-4 production (Locksley and Reimer, 1995).

The correlation of TH1 responses with resistance and TH2 responses with susceptibility results from the differential effects of the cytokines produced by these $CD4^+$ subsets on macrophages. IFN-γ activated macrophages acquire microbiocidal abilities against *L. major* via the release of NO (Liew et al., 1990). In contrast, IL-4 inhibits this activation (Nacy and Meltzer, 1991).

1.6.3.2 *Trypanosoma cruzi*

Resistance to *T. cruzi* infections in mice has been associated with TH1-like cytokine responses. In particular, IFN-γ produced by both $CD4^+$ T cells (Aranjo, 1989; Rottenberg et al., 1993) and $CD8^+$ T cells (Tarleton, 1990; Tarleton et al., 1992) have been shown to mediate resistance to *T. cruzi* infections in mice. *In vivo*, IFN-γ has been shown to be essential for clearance of this intracellular parasite in naturally resistant mouse strains (Minoprio et al., 1993) and treatment with anti-IFN-γ antibodies increases parasitemia levels and mortality in infected mice (Torrico et al., 1991; Petray et al., 1993). Administration of recombinant IFN-γ to susceptible mice diminishes parasitemia and prevents death during experimental infections (Reed, 1988). In contrast, TH2 cytokines (IL-4, IL-10) are associated with enhanced susceptibility to the infection (Silva et al., 1992; Reed et al., 1994). Spleen cells from mice rendered resistant by immunization with *T. cruzi* antigens secrete large amounts of IFN-γ and no detectable or very low amounts of IL-4, IL-5 and IL-10 (Hansen et al., 1996; Taibi et al., 1996).

1.6.3.3 *Toxoplasma gondii*

T. gondii is also an intracellular parasite. Similar to *T. cruzi* infections, both $CD4^+$ and $CD8^+$-derived IFN-γ mediate resistance *to Toxoplasma gondii* infections in mice (Gazzinelli et al., 1991). *In vivo* neutralization of IFN-γ ablates active immunity (Gazzinelli et al., 1991; Suzuki et al., 1988) and the resistance can be adoptively transferred by purified $CD8^+$ populations (Suzuki and Remington, 1990). TH2 cytokines do not contribute to resistance against *T. gondii* infections in mice (Sher and Coffman, 1992) and IL-10 has been shown to mediate immunosuppression in infected mice (Khan et al., 1995).

1.6.3.4 *Plasmodium spp*

Protective immunity in rodents vaccinated with the circumsporozoite protein of the malaria parasite is T cell-dependent and is mediated by both antibody and cellular responses (Sher and Coffman, 1992). Antibody coating of the sporozoite can cause lysis and thus prevent infection of the liver cells by these stages. However, very low concentrations of IgM anti-circumsporozoite antibody can enhance infection of liver cells *in vitro*. Cellular mechanisms are directed against infected liver cells and involves inhibition of intracellular parasite development by IFN-γ (Ferreira et al., 1988) or direct lysis of the target cells by $CD8^+$ cytotoxic T-cells (Weiss et al., 1988; Romero et al., 1989). Also, $CD4^+$ TH1-like cells have been shown to confer protection against murine malaria (Tsujin et al., 1990). Thus, similar to *T. gondii*, both $CD8^+$ and $CD4^+$ T cells

secreting IFN-γ appear to be important in resistance against the hepatic (cellular) stages of malaria parasite.

1.7 Cytokines in African trypanosomiasis

The roles of cytokines in the pathogenesis of African trypanosomiasis have not been extensively studied. One of the paradoxes of trypanosomiasis is that despite the apparent impairment of proliferative responses, lymphoid cells retain their ability to secrete large amounts of cytokines especially IFN-γ (Bakhiet et al., 1990; 1993; Darji et al., 1993; 1996; Olsson et al., 1993; Schleifer et al., 1993). The first direct evidence of stimulation of VSG-specific TH cells during trypanosome infection was obtained by Schleifer et al. (1993). $CD4^+$ TH cells specific for VSG were found predominantly in the peritoneum of infected resistant mice and were exclusively of the TH1 phenotype secreting IL-2 and IFN-γ but not IL-4 (Schleifer et al., 1993). In contrast, when mice were immunized with purified VSG, both TH1 and TH2 VSG-specific cells were induced in both the peritoneum and lymphoid organs. These VSG-specific TH cells were shown to be $Thy1^+$, $CD4^+$, $CD8^-$, $CD3^+$, $\alpha\beta$ TCR^+, $\gamma\delta$ TCR^-, and were restricted by MHC class II (Schleifer et al., 1993). Unfortunately, TH cell responses in infected susceptible mice were not studied.

In other studies, the production of IL-4 and IFN-γ were reported to be induced by polyclonal activation of $CD8^+$ T cells by a T lymphocyte triggering factor released by *T. brucei* (Bakhiet et al., 1993; 1996). In another study, the production of IFN-γ was similar in athymic mice compared to euthymic littermates indicating that cells other than thymus-

derived lymphocytes cells are involved in the secretion of this cytokine during *T. brucei* infections (Bakhiet et al., 1996a). Darji et al. (1996) showed that a macrophage hybridoma cell line pulsed with opsonized trypanosomes acquired the ability to induce $CD8^-$ cell stimulated with Con A cells to secrete large amounts of IFN-γ.

Thus, the cytokine-secreting cells have been identified as $CD4^+$ TH cells in *T. brucei rhodesiense* (Schliefer et al., 1993) and $CD8^+$ in *T. brucei brucei* infections (Bakhiet et al., 1993; Darji et al., 1996). There are no reports on the phenotype of cytokine-secreting cells in *T. congolense* infection in mice.

There appears to be complex (both direct and indirect) effects of host cytokines on trypanosome survival and pathogenesis. IFN-γ has been reported to act as a growth factor for *T. brucei* (Olsson et al., 1991). However, Kaushik et al. (1997) failed to demonstrate growth stimulatory effects of IFN-γ on *T. congolense*. Mice with disrupted IFN-γ genes have decreased parasitemia and increased survival time (Bakhiet et al., 1996) and this effect was attributed to reduction of parasite multiplication as a result of lack of an IFN-γ-induced stimulatory effect. However, because IFN-γ is known to mediate suppression of T cell responses in *T. brucei brucei* infections (Darji et al., 1993; 1996) it is likely that the enhanced resistance observed in IFN-γ gene knockout mice may be due to reversal of immunosuppression. This would result in a more effective immune response against the parasites. IFN-γ has also been shown to synergize with a crude soluble extract from *T. brucei brucei* to stimulate nitric oxide production from peritoneal macrophages *in vitro*, leading to suppression of T cell responses (Mabbott et al., 1995).

Soluble extracts of blood stream forms of various salivarian trypanosomes induce TNF-α production by macrophage cell lines *in vitro* (Magez et al., 1993). Experimental infections of mice with *T. brucei* were found to sensitize macrophages to increased production of monokines including TNF-α (Sileghem, 1988). The role of TNF-α in trypanosomiasis is equivocal. It has been associated variously with immunosuppression (Lucas et al., 1993; Darji et al., 1996) and enhanced resistance (Lucas et al., 1993; Magez et al., 1993; 1997). Membrane-bound TNF-α on a macrophage hybridoma cell line pulsed with trypanosomes was reported to act as a co-stimulatory molecule for induction of IFN-γ secretion by $CD8^+$ T cells, thereby possibly leading to immunosuppression (Darji et al., 1996). TNF-α has also been shown to be a negative regulator in trypanosome growth control. Treatment of *T. brucei*-infected mice with anti TNF-α antibodies caused a significant increase in parasitemia (Magez et al., 1993; 1997). Also, injection of soluble trypanosome components into mice before infection with *T. brucei* completely abolished parasite development and this effect was blocked by treatment with anti-TNF-α antibodies (Magez et al., 1993). Lucas et al. (1994) demonstrated that TNF-α is both trypanolytic and trypanostatic for *T. brucei brucei* and *T. brucei rhodesiense in vitro.* The trypanolytic effect of TNF-α occurs via a novel lectin-like affinity receptor on *T. brucei* that is functionally and spatially distinct from the mammalian TNF-α binding site (Lucas, 1994). There are no reports of TNF-α in resistance to *T. congolense* infections in mice.

Very few studies have focused on the role of TNF-α in trypanosome-associated pathological reactions. Rouzer and Cerami (1980) showed that excessive fat metabolism and the emaciation observed in *T. brucei brucei*-infected rabbits were mediated by

cachectin, a molecule later identified to be TNF-α (Beutler and Cerami, 1986). Sileghem et al. (1994b) demonstrated an association between TNF-α production and the severity of erythrophagocytosis and thus suggested a role for this cytokine in anemia. Infection with *T. vivax*, which causes a more severe anemia, was shown to induce higher TNF-α production by monocytes from both trypanotolerant and trypanosusceptible cattle (Sileghem et al., 1994b). The monocytes from less anemic trypanotolerant cattle produced significantly lower amounts of TNF-α than those from the anemia prone, trypanosusceptible breed. Hunter et al. (1991) showed that mice chronically infected with *T. brucei brucei* and treated with subcurative doses of trypanocidal drugs develop inflammatory lesions in the central nervous system. Higher TNF-α mRNA transcripts were detected in the brains of such mice but not in either uninfected treated or untreated infected animals (Hunter, 1991). They concluded that the TNF-α mRNA was locally induced by membranes of degenerating trypanosomes. Recently, treatment of *T. evansi*-infected rabbits and mice with trypanocidal drugs was shown to result in a massive increase in serum TNF-α levels and a concomitant decrease in parasite numbers (Lucas et al., 1993). Transfer of blood from such animals to naïve recipients resulted in pronounced lethality, inhibitable by pre-treating the recipients with neutralizing anti-TNF-α antibodies, indicating that TNF-α is involved in the observed lethality (Lucas et al., 1993).

In general, TNF-α appears to have a profound effect on the pathogenesis of African trypanosomiasis. The role of this cytokine may depend on a lot of factors,

including its interaction with other cytokines, the trypanosome species as well as the genetic status of the trypanosome-bearing host.

Relatively little is known about the role of other cytokines in African trypanosomiasis. Higher levels of TGF-β and lower levels of TNF-β mRNA have been detected in the spleens of resistant and susceptible mice respectively following infections with *T. brucei* (Bakhiet et al., 1996). The significance of these observations is not known. In cattle infected with *T. congolense*, higher levels of IL-10 mRNA transcripts were detected in the spleens, lymph nodes and PBMC of susceptible than in the resistant breeds (Taylor et al., 1996). It was suggested that higher levels of IL-10 might be associated with the higher levels of immunosuppression observed in susceptible breeds (Taylor, 1998). In *T. brucei brucei* infections, IL-10 and IL-12 mRNA were undetectable in the spleens of both resistant and susceptible mice (Bakhiet et al., 1996). There is no report on the production and role of these cytokines during *T. congolense* infection in mice.

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Introduction

Infection of different breeds of cattle and mice with various species of African trypanosomes results in a spectrum of disease. The indigenous West African N'Dama cattle are more resistant than Zebu cattle to both natural and experimental infections with *T. congolense* (Murray et al., 1982; 1991). Similarly, in a mouse model system, BALB/c mice are highly susceptible to experimental *T. congolense* infections whereas C57BL/6 mice are relatively resistant, as measured by the level of parasitemia, immunosuppression and survival period (Pinder et al., 1986; Otesile et al., 1991; Ogunremi and Tabel, 1995). Genetic analyses show that differences in resistance among different breeds of mice are not due to differences in the MHC genes (Morrison et al., 1978; Pinder et al., 1985; Levine and Mansfield, 1981). A recent report by Ogunremi and Tabel (1995) showed that the efficiency of clearance of the first wave of parasitemia among the F2 offspring of crosses between BALB/c and C57BL/6 mice infected with *T. congolense* correlated with long-term survival. This suggests that the pattern of early responses by infected animals could determine the overall outcome of the infection.

The mechanisms underlying the differences in susceptibility among inbred mouse strains to infections by African trypanosomes are still poorly understood. However, differences in the kinetics, quantity and quality of antibody responses to the infecting variant (Morrison and Murray, 1979; Levine and Mansfield 1984; Mitchell and Pearson, 1986; Mansfield 1990) as well as differences in the degree of immunosuppression (Morrison et al., 1978; Roelants et al., 1979; Pinder et al., 1986) appear to be contributing factors. Clearance of trypanosomes from the blood is the result of antibody-mediated

uptake predominantly by Kupffer cells in the liver, but also by macrophages in the spleen (MacAskill et al., 1980; Dempsey and Mansfield, 1983). Studies indicate that mice that are highly susceptible to trypanosomiasis produce little or no detectable antibody against the VSG during infection (Black et al., 1983; Morrison and Murray, 1985; Newson et al., 1990). However, De Gee and Mansfield (1984) reported a lack of correlation between IgM anti-VSG antibody response and overall host resistance. In cattle, an association has been made between resistance and the ability to produce IgG antibodies to invariant antigens of trypanosomes (Authie et al., 1992; Authie, 1994). Studies on the kinetics as well as quality of antibody responses not only to VSG but also to the whole antigens of *T. congolense* in mice are needed to shed more light on the mechanisms of differential resistance and the role of antibodies in this phenomenon.

Trypanosome-induced immunosuppression is considered one of the major mechanisms of the parasite to escape the host's immune defense mechanisms (Sileghem et al., 1994; Darji et al., 1996). The degree of immunosuppression of lymphoid cells correlates with the degree of susceptibility in mice during infections (Morrison et al., 1978a; Roelants and Pinder 1984; Pinder et al., 1986). Reduced responsiveness of the lymphoid cells of infected animals to parasite-related and unrelated antigens has been attributed variously to suppressor macrophages (Akonas, 1985; Flynn et al., 1991; Schleifer and Mansfield, 1993), suppressor T cells (Jayarwadena and Waksman 1977; Jayarwadena et al., 1978) or a combination of these cell types (Corsini et al., 1977; Pearson et al., 1978a; 1979; Roelants and Pinder 1984). Recently, IFN-γ (Darji et al., 1993; 1996) and NO (Schleifer and Mansfield 1993; Sternberg et al., 1996) have been

shown to mediate suppression and enhanced susceptibility to *T. brucei* infections. NO-mediated suppression of lymph node cells does not occur in cattle infected with *T. congolense* (Taylor et al., 1996). This is not surprising in view of the differences between *T. brucei* and *T. congolense*. *Trypanosoma congolense*, unlike *T. brucei*, does not invade the interstitial tissues. The role of cytokines in *T. congolense*-induced immunosuppression is not yet known.

The patterns of cytokine response during many parasite infections determine or are at least strongly related to the relative susceptibility of the host (Sher and Coffman 1992; Finkelman et al., 1997). Parasitic infections often result in extreme polarization of T cell subsets with distinct and non-overlapping cytokine patterns, and consequently distinct effector functions. Studies with different parasites have shown that TH2 cytokine responses (because of their stimulatory effect on B cell growth and differentiation) are associated with the control of extracellular parasites (Sher and Coffman, 1992; Finkelman et al., 1997). However, because of the pleiotropic, redundant, synergistic and immunomodulatory properties of cytokines, it is often very difficult to precisely establish a role for a particular cytokine in any disease process. Furthermore, the findings that cells other than the conventional $CD4^+$ helper T cells do secrete large amounts of cytokines hitherto attributed to $CD4^+$ T helper cells, suggests that the role of a particular cytokine would depend on its source and the cellular interactions among the producing and non producing cells.

Studies in rodents have shown that certain cytokines influence the outcome of infection with *T. brucei*. IFN-γ reportedly produced by polyclonal activation of $CD8^+$ T

cells has been reported to act as a growth factor for *T. brucei* and IFN-γ knockout mice exhibited enhanced resistance to *T. brucei* infections (Olson, 1991; Bakhiet et al., 1993; 1996). In another system, IFN-γ was reported to cause immunosuppression in lymph node cells of *T. brucei* infected mice (Darji et al., 1993, 1996). There is as yet no extensive study on the patterns of secretion and role of cytokines in *T. congolense* infections in rodents or cattle. However, our preliminary evidence indicates that IL-4, IL-10 and TNF-α are present in the plasma of *T. congolense* infected mice (Tabel et al., 1994) and that these cytokines do not have a direct growth stimulatory effect on the parasite (Kaushik et al., 1997). In view of the fact that the speed of control of the first wave of parasitemia during *T. congolense* infections in mice is correlated with long-term survival (Ogunremi and Tabel, 1995), it is desirable to investigate the pattern and role of early cytokine responses to *T congolense* infections in mice.

2.2 Hypothesis

The early cytokine response to *T. congolense* infections in mice plays a major role in resistance.

2.3 Objectives

1. To determine the kinetics of mRNA expression and patterns of secretion of IL-4, IL-10, TNF-α and IFN-γ by splenocytes from *T. congolense*-infected highly susceptible BALB/c and relatively resistant C57BL/6 mice.

2. To characterize the phenotype of cells secreting IL-4, IL-10 and IFN-γ in the spleens of *T. congolense*-infected BALB/c mice.

3. To determine the role of IL-4, IL-10 and IFN-γ in the pathogenesis of *T. congolense* infection in mice.

4. To determine whether differences occur in the kinetics, quantity and quality of antibody responses to the purified VSG, cryptic epitopes of the VSG and whole antigens of *T. congolense* among infected BALB/c and C57BL/6 mice.

3.0 IMMUNOREGULATION IN EXPERIMENTAL MURINE *TRYPANOSOMA CONGOLENSE* INFECTION: ANTI-IL-10 ANTIBODIES REVERSE TRYPANOSOME-MEDIATED SUPPRESSION OF LYMPHOCYTE PROLIFERATION *IN VITRO* AND MODERATELY BUT SIGNIFICANTLY PROLONG THE LIFESPAN OF GENETICALLY SUSCEPTIBLE BALB/C MICE.

3.1 Abstract

We infected highly susceptible BALB/c and relatively resistant C57BL/6 mice with cloned *Trypanosoma congolense* and followed the effects of these infections on the circulating parasite numbers, mouse mortality and cytokine expression. C57BL/6 mice controlled their parasitemia and survived for up to 163 $\pm$ 12 days, while BALB/c mice could not control their parasitemia and succumbed to the infection within 8.4 $\pm$ 0.5 days. Susceptible BALB/c mice had dramatically higher plasma levels of IL-10 than the resistant C57BL/6 mice from day 7 forward. This was preceded by an earlier and higher level induction of splenic IL-10 messenger RNA (mRNA) expression in the infected BALB/c mice. There was a strong negative correlation ($r = -0.75$; $p < 0.03$) between the splenocyte proliferative responses to concanavalin A (Con A) and their production of IL-10 in infected BALB/c mice. Co-treatment of the Con A-stimulated spleen cell cultures with monoclonal anti-IL-10 antibodies, but not isotype-matched control antibodies, could completely reverse this suppression of the splenocyte proliferative response. Finally, in 3 experiments, anti-IL-10 antibody treatment *in vivo* reduced the peak circulating parasitemia of infected BALB/c mice by 43% and increased their median survival periods

by 38% relative to isotype-matched control antibody-treated mice. These results demonstrate that IL-10 has a significant inhibitory effect on the immune system of trypanosome-infected BALB/c mice as measured by lymphocyte proliferation *in vitro*, and control of parasitemia and mortality *in vivo*, but they also show that neutralization of this cytokine does not, by itself, reverse the ultimate outcome of the disease.

3.2 Introduction

Infections with African trypanosomes are associated with profound immunosuppression in both man and animals (reviewed in Darji et al., 1992, Sileghem et al., 1994). A remarkable characteristic of this suppression is a down-regulation of both cell-mediated and humoral responses, leading to an increased susceptibility to secondary infections and poor responsiveness of trypanosome-bearing individuals to unrelated vaccinations (Greenwood et al., 1973). Lymphocytes from infected animals show suppressed responsiveness to T-cell mitogens, probably as a result of their decreased IL-2 secretion and IL-2 receptor (IL-2R) expression (Sileghem and Flynn, 1992a; Sileghem et al., 1986; Darji et al., 1993). Suppression of IL-2R expression in mice infected with *T. brucei* is associated with a non-prostaglandin product released by IFN-γ–activated macrophages, while the IL-2 secretion is apparently downregulated via prostaglandin-dependent effects (Askonas, 1985; Darji et al., 1992; 1993; Sileghem et al., 1994a).

IL-10 is produced relatively late following activation of T-cells or monocytes/macrophages (reviewed in Moore et al., 1993). Its effects have been reported to include an inhibition of $CD4^+$ and $CD8^+$ T-cell proliferation and cytokine synthesis

following stimulation with T-cell mitogens, superantigens or alloantigens (Ding and Shevach, 1992; Moore et al., 1993). This effect is mediated in part by an inhibition of antigen presenting cell-dependent stimulation of IL-2 production and a marked decrease in the expression of the p55 chain of the IL-2R (Ding et al., 1992). The production of high amounts of IL-10 by splenocytes from *Trypanosoma cruzi*-infected mice have been shown to mediate susceptibility to the disease (Silva et al., 1992, Reed et al., 1994) and *in vivo* neutralization of IL-10 enhanced the resistance of the highly susceptible mice (Reed et al., 1994). Because of the similarities of the effects of IL-10 and trypanosome infection on T-cell proliferation and IL-2 and IL-2R expression, we sought to determine whether this cytokine has a role in the immunosuppression observed in experimental African trypanosomiasis as well as in enhanced susceptibility to the disease. In agreement with our previous preliminary report (Tabel et al., 1994), we show here that, following infection with *T. congolense*, the plasma of the highly susceptible BALB/c mice contains significantly higher levels of IL-10 than that of relatively resistant C57BL/6 mice. Also, the splenocytes of infected BALB/c mice constitutively contained increasingly higher steady levels of IL-10 mRNA and produced more IL-10 than those of the resistant mice. In contrast, the proliferative responses to Con A of the spleen cells from the susceptible mice were progressively suppressed during *T. congolense* infections, and exhibited a reciprocal relationship with IL-10 production. Most importantly, neutralizing anti-IL-10 antibodies restored the *in vitro* proliferative responses of spleen cells from infected BALB/c mice and, *in vivo*, these antibody treatments significantly reduced the parasitemia and increased the survival period of the highly susceptible mice.

3.3 Materials and Methods

3.3.1 Mice

Female BALB/cAnNCrlBR (BALB/c) and outbred CD1 mice were obtained from the Animal Resource Center of the University of Saskatchewan, and female C57BL/6NCrlBR (C57BL/6) mice were purchased from Charles River Laboratories (St. Constant, Quebec). BALB/c and C57BL/6 mice were between 8 to 10 weeks of age and CD1 mice were 5 weeks old. All mice were maintained according to the recommendations of the Canadian Council of Animal Care.

3.3.2 Parasite

T. congolense, variant antigenic type (VAT) TC13 (Tabel, 1982) was used in these experiments. Parasites were passaged in immunosuppressed CD1 mice as described previously (Tabel, 1982). The parasites for infection of the BALB/c and C57BL/6 mice were isolated from the blood of the CD1 mice by DEAE-cellulose chromatography (Lanham & Godfrey 1970).

3.3.3 Experimental design

Groups of four to five BALB/c and C57BL/6 mice were infected intraperitoneally (i.p.) with 10^3 organisms of *T. congolense* TC13 and killed with CO_2 on days 1-10 post-infection. In some experiments, infected mice were treated with berenil (14 mg/kg of diminazene aceturate; Sigma Chemical Co., St. Louis, Missouri) on day 6 and 7 post-infection to cure their infections. On each of the indicated days blood was withdrawn

from the vena cava into syringes containing heparin (final concentration ~20 IU/ml), and the plasma collected after centrifugation of the blood at 1,000 x g for 30 min. The plasma was then centrifuged at 13,000 x g for 15 min and the supernatant was stored at -35°C until used. Each experiment and cytokine determination was performed at least twice.

3.3.4 Estimation of parasitemia and survival period

To estimate the circulating parasite numbers, a drop of blood from the tail vein of each infected mouse was examined at 400x magnificaton by phase contrast microscopy. Parasitemia was most often estimated by counting the number of parasites present in at least 10 fields, but unusually heavy parasite loads were quantified according to Herbert and Lumsden (1976). The survival period was defined as the number of days post-infection that the infected mice remained alive. Moribund mice were euthanized with CO_2.

3.3.5 Splenocyte cultures for measurement of IL-10 synthesis

Single cell suspensions of spleen cells from normal and infected mice were adjusted to $2x10^6$ cells/ml complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 100 IU/ml penicillin/streptomycin) and cultured in 24 well plates (Corning, New York) at 1.5 ml/well in a humidified 37°C, 5% CO_2 atmosphere. Some cells were stimulated with 5 µg/ml concanavalin A (Con A; Calbiochem, Behring Diagnostics, La Jolla, CA). All

supernatants were collected after 48 hours, centrifuged at 1,500 x g for 10 min and stored at -80°C until used.

3.3.6 Splenocyte proliferation assay

Splenocyte proliferation was quantified by the MTT assay as described by Mosmann (1983). Briefly, cells from infected mice were cultured for 72 h in 200 µl of complete medium at a density of 10^5 cells/well in 96-well flat bottom plates (Falcon®; Becton Dickinson, Lincoln Park, NJ), using quadruplicate cultures of pooled cells from groups of 4 mice. Cells were either left untreated or were treated with Con A (5 µg/ml); some wells were treated with either 10 µg/ml of rat anti-mouse IL-10 (SXC-2), anti-rat IL-4 (11B11, American Type Culture Collection [ATCC], Rockville, MD) or control rat IgM hybridoma (HECA 452, ATCC #HB11485) antibodies. After 72 hours, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co) was added to a final concentration of ~0.48 µg/ml for an additional 3 hr, then lysed the cells with acidified isopropanol and quantified the mitochondrial formazan precipitates with an ELISA plate reader, by measuring the absorbance at 595 nm wavelength. Results are presented as percent proliferation relative to Con-A-stimulated splenocytes from normal non-infected mice.

3.3.7 Recombinant murine IL-10, anti-IL-10 antibodies and IL-10 ELISA

Recombinant murine IL-10 was purchased from BACHEM (Feinchemikalien Ag, Bubendorf, Switzerland), an ELISA kit for measurement of plasma IL-10 (Endogen Inc.,

Boston, MA), and paired antibodies for sandwich ELISA assays of splenocyte IL-10 production (PharMingen, San Diego CA). Each was used according to the manufacturer's suggested protocol.

3.3.8 Northern blot analysis

Total cellular RNA was extracted from the spleens of normal and *T. congolense*-infected BALB/c and C57BL/6 mice from days 1-9 post-infection using the guanidinium isothiocyanate/cesium chloride method (Chirgwin et al., 1979). For each sample, 20 μg of RNA was electrophoresed in a 1.2% denaturing agarose gel, transferred to a nylon membrane (Zetabind™, CUNO Lab., Meridien, CN.) by capillary action and cross-linked by u.v. irradiation (Stratalinker™, Stratagene, La Jolla, CA). The membranes were blocked with 0.1X SSC/0.5% SDS for 1 hr at 65°C and pre-hybridized overnight at 42°C in a solution containing 50% de-ionized formamide, 0.1 M HEPES, 0.5 M NaCl, 5 mM EDTA (pH 8.0), 5X Denhardt's solution, 1% SDS, 0.5% sodium pyrophosphate and 12.5 μg/ml salmon sperm DNA. ^{32}P-labeled cDNA probes were prepared by the random hexamer-labeling technique (Oligolabeling kit, Pharmacia, Uppsala, Sweden) according to the manufacturer's suggested protocol. The mouse IL-10 cDNA consisted of a 550 bp Xba I/Not I fragment (kindly provided by Dr. John Elliot, University of Alberta) while the human γ-actin cDNA comprised a 910 bp EcoRI/Hind III fragment (provided by Drs. B. Murphy and V. Misra, University of Saskatchewan). The membranes were hybridized overnight at 42°C in pre-hybridization buffer containing 1 x 10^6 cpm/ml of labeled probes and then washed two times with 2X SSC/0.1% SDS for 5 min at room temperature and

another two times for 30 min with 0.2X SSC/0.1% SDS at 42°C. Autoradiography was performed at -80°C for 1-10 days. Densitometry of the autoradiograph signals was done using the Image 1 program from Universal Imaging Corporation (Westchester, PA) and the results presented graphically as the ratios of the IL-10 to γ-actin signal density for each sample (in order to accommodate any lane-to-lane variation in RNA loading).

3.3.9 Anti-IL-10 antibodies for *in vivo* experiments

SXC 2, a rat IgM anti-mouse IL-10 antibody-secreting hybridoma (Mosmann, et al., 1990) was a kind gift of Dr. Tim Mosmann (University of Alberta) while isotype-matched antibodies (HECA 452, ATCC) directed against a human cutaneous antigen were used for control treatments. Hybridomas were grown either in serum- and protein-free medium (Gibco BRL, Life Technologies, Mississauga, ON) or in complete medium. The hybridoma supernatants, as well as control culture medium (i.e., complete medium), were precipitated with 33% w/v crystalline ammonium sulfate and dialyzed extensively against phosphate-buffered saline (PBS; pH 7.0). Protein levels were quantified using the Bradford method (BIORAD Protein Assay; BIO-RAD Laboratories, Richmond, CA). In some preparations, IgM antibodies were further purified on Sephacryl G200 (Pharmacia, Uppsala, Sweden) gel filtration columns. Fractions containing the first protein peak eluted from the column were collected, pooled and concentrated with Amicon Centriprep 100 concentrators (Amicon, W.R. Grace and Co., Beverly, MA.). The levels of IgM antibodies in the concentrates was determined by single radial immunodiffusion using

goat anti-rat IgM antibodies (Cappel, Organon Teknika, Inc., Scarborough, Ontario) and purified rat IgM (Cappel) standards.

3.3.10 Injection of mice with monoclonal antibodies or rIL-10

Groups of six BALB/c mice were injected intraperitoneally (i.p.) on days 0, 2, 5 and 7 post-infection with 200 μg of purified anti-IL-10 antibodies in 200 μl of PBS. Control mice received an equivalent amount of either similarly-treated isotype-matched control antibody or PBS alone. In another experiment, groups of six C57BL/6 mice received 600 ng of recombinant murine IL-10 per mouse i.p. on days 0, 2, 5 and 7 post infection. Treated and control mice were monitored daily for parasitemia and mortality.

3.3.11 Statistical analysis

Data are represented as means ± standard error (SE). Significance of differences was determined either by Student's t-test or Kruskal-Wallis nonparametric one-way Analysis of Variance (ANOVA) using the StatView™.SE 1988 Software (Abacus Concepts Inc, Berkeley, CA.).

3.4 Results

3.4.1 IL-10 is differentially regulated in genetically susceptible BALB/c and resistant C57BL/6 mice infected with *T. congolense*

In agreement with what has been reported previously (Ogunremi and Tabel, 1995), BALB/c mice infected with 10^3 organisms of *T. congolense* were unable to control the

parasitemia and succumbed to it with mean survival times of 8.4 ± 0.5 days (Figure 3.1A). In similarly infected but relatively resistant C57BL/6 mice, the first wave of parasitemia reached its peak at day 7, declined at day 8, and was almost undetectable by day 9 post-infection (Figure 3.1A). As noted previously, the median survival time of *T. congolense* (TC13)-infected C57BL/6 mice was 163.0 ± 12 days (Ogunremi and Tabel, 1995).

IL-10 was detected at high levels in the plasma of the susceptible BALB/c mice, but only at very low levels in the resistant C57BL/6 mice. IL-10 first became detectable in the susceptible mice on day 6 post-infection, and increased steadily in concentration to a peak on day 10 (Figure 3.1B). There was a strong positive correlation ($r = 0.87$; $p \leq 0.003$) between the measured parasitemia and plasma IL-10 level. Overall, the plasma levels of IL-10 were significantly higher in BALB/c mice than in the C57BL/6 mice ($p \leq 0.05$).

The expression of IL-10 mRNA in the spleens of infected BALB/c and C57BL/6 mice was assessed by Northern Analysis and the data expressed graphically as the ratio for each sample of the IL-10 mRNA signal density to that of γ-actin. As shown in Figure 3.1C, BALB/c mice had a higher level induction of IL-10 mRNA than did C57BL/6 mice. The induction of IL-10 mRNA first became apparent on day 3 post-infection, increased markedly on day 4 and remained elevated thereafter in BALB/c mice. In contrast, in C57BL/6 mice, the expression of IL-10 mRNA remained relatively low throughout the infection period.

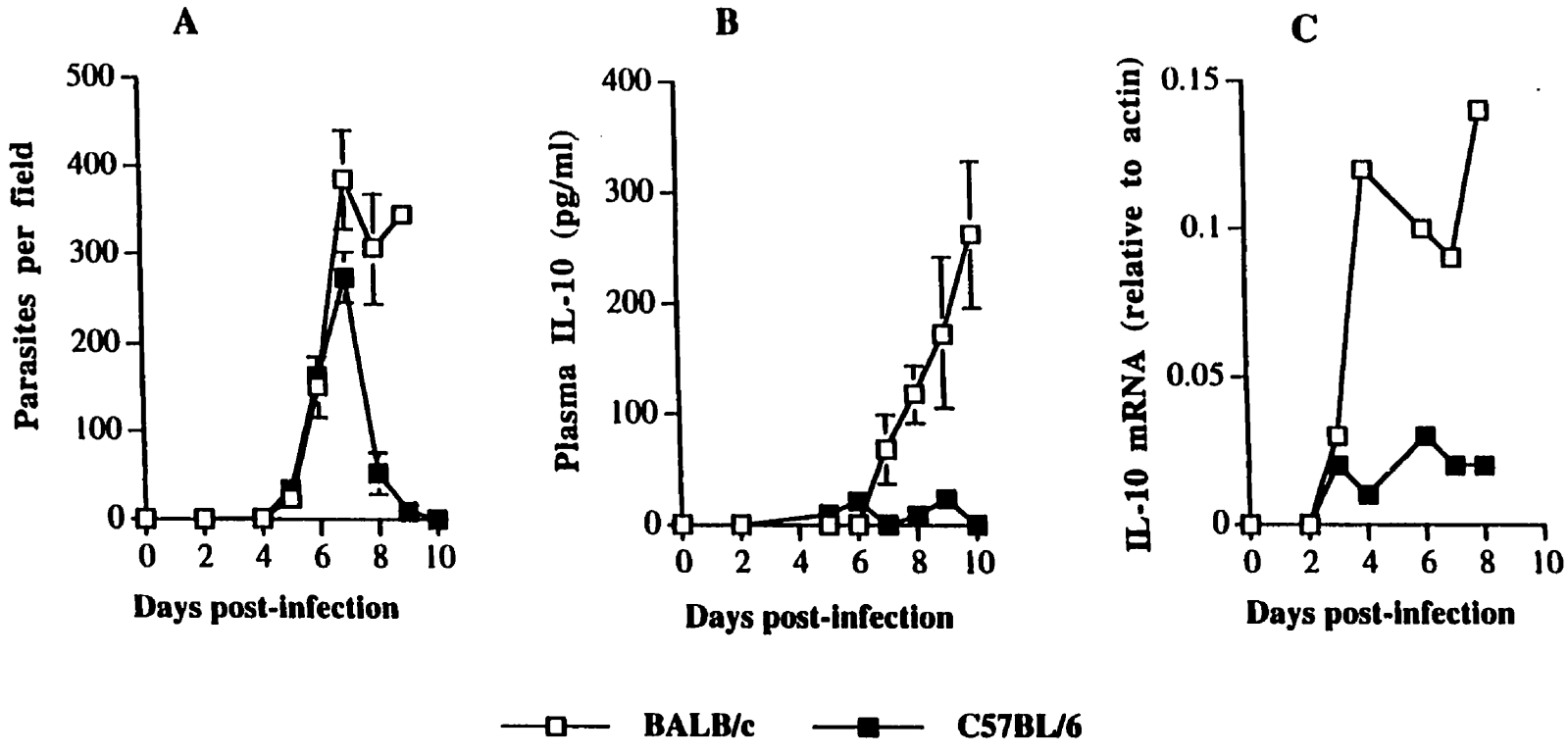

Figure 3.1 Parasitemia, plasma levels of IL-10 and expression of IL-10 mRNA in the spleen of BALB/c (open squares) and C57BL/6 (closed squares) mice infected with *T. congolense*. Parasitemia (A) was estimated by examining a drop of blood from the tail vein on a microscopic slide at 400x power by phase contrast microscopy. (B) Plasma levels of IL-10 in infected BALB/c and C57BL/6 mice. Groups of 4 infected mice were killed daily by CO_2 anesthesia and blood was collected for plasma (Materials and Methods). IL-10 levels were measured by sandwich ELISA. Results indicate mean ± SE of the mean. (C) Induction of IL-10 mRNA in the spleen tissues of infected BALB/c and C57BL/6 mice. The levels of IL-10 and actin mRNA were assessed by Northern analysis and scanning densitometric analysis of the blots. In order to correct for variability in the amounts of RNA loading, the results are presented as a histogram after normalization of the IL-10 signals to the respective actin signals. Figure legends for (B) and (C) are as in (A). Results of a representative experiment repeated two times.

3.4.2 Splenocytes from infected BALB/c mice produce high levels of IL-10 relative to resistant C57BL/6 mice

Spleen cells from infected susceptible BALB/c and resistant C57BL/6 mice were cultured *in vitro* for 48 hr either alone or in the presence of Con A and the levels of IL-10 in the supernatants were determined by sandwich ELISA (Figure 3.2). On each day tested, both spontaneous and Con A-induced secretion of IL-10 were significantly higher in BALB/c cultures than in those from their C57BL/6 counterparts. By day 4 post-infection, IL-10 production by the splenocytes from BALB/c mice was high under both conditions and remained so throughout the remainder of the infection. In contrast, the splenocytes from the resistant C57BL/6 mice only produced substantial amounts of IL-10 on days 6 and 7, and by day 8 their production of this cytokine had waned. There was a strong correlation ($r = 0.84$, $p < 0.02$) between the measured blood parasite numbers and the spontaneous secretion of IL-10 in cultures by splenocytes of infected BALB/c mice. In general, Con A-stimulation resulted in 7- to 50-fold increases in the levels of IL-10 secretion by spleen cells of both susceptible and resistant mice (Figure 3.2), indicating that T-cells are involved in the secretion and/or regulation of this cytokine during experimental *T. congolense* infections in mice.

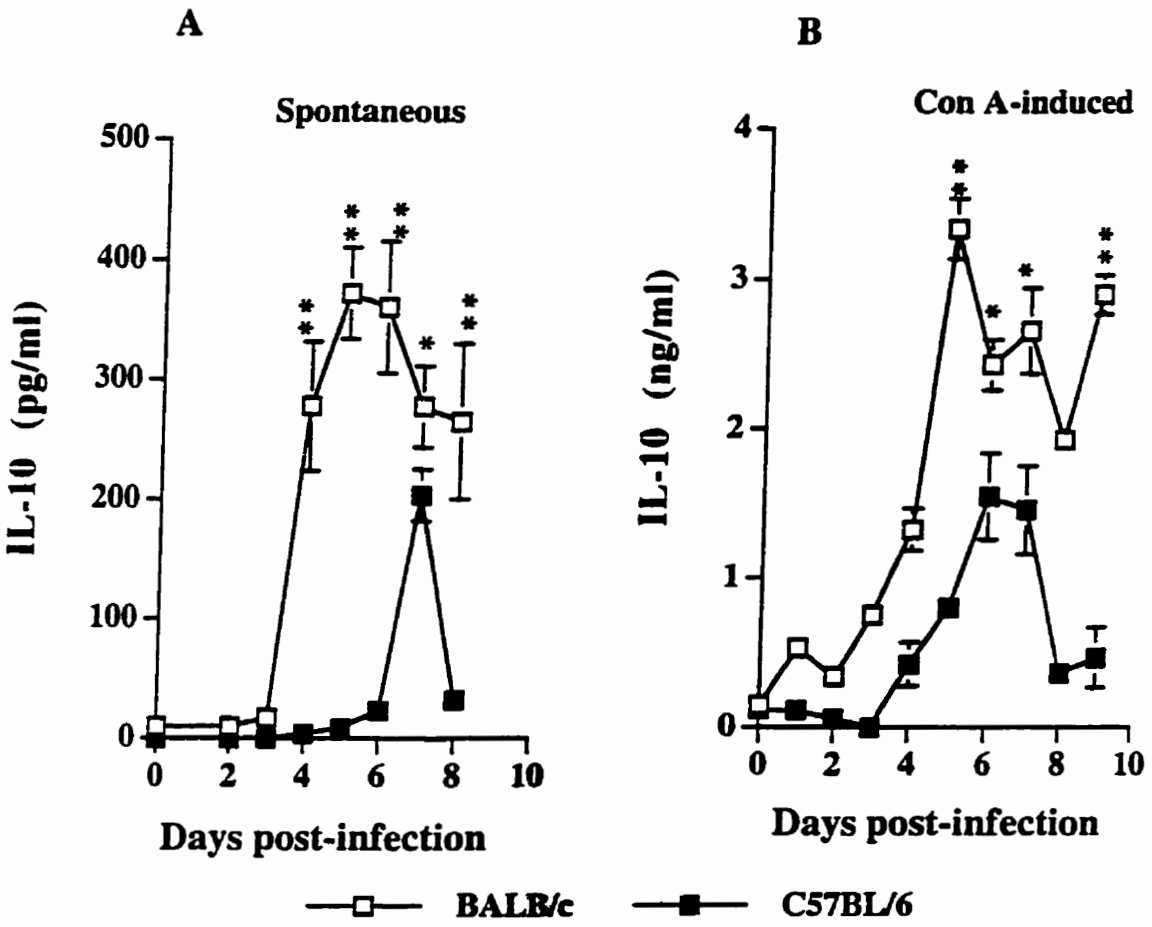

Figure 3.2 Spontaneous and Con A-induced secretion of IL-10 by splenocytes from infected BALB/c (open squares) and C57BL/6 (closed squares) mice. Normal (Day 0) and infected (Days 1-9) spleen cells were cultured in 200 µl of medium in 96-well tissue culture clusters at 5 x 10^5 cells per well for 48 hr in the absence or presence of 5 µg/ml Con A, and supernatants were collected and assayed for IL-10 by ELISA Results are from a representative experiment which was repeated 3 times. * $p < 0.05$; ** $p < 0.001$.

3.4.3 IL-10 mediates suppression of splenocyte proliferative responses that occurs in infected BALB/c mice

It is well established in the literature that trypanosome infections lead to a very significant suppression of lymphoid reactivity to mitogens and antigens (Darji et al., 1993; 1996; Sileghem et al., 1986; 1987; 1989). While we found that within one day of infection the splenocytes of the infected mice exhibited an augmented proliferative response to Con A (133 $\pm$ 7 % of the value for the control, uninfected mice), by day 4 post-infection these responses had returned to baseline, and by day 8 post-infection the Con A responses of these cells were suppressed (i.e., only 60 $\pm$ 3 % of the uninfected control mouse responses; Figure 3.3A). This suppressive effect was reciprocally related to the splenocyte IL-10 production kinetics noted above ($r = -0.75$; $p < 0.03$).

Since it has been shown that IL-10 is involved in the down-regulation of T-cell responses in many parasitic infections (Denis and Ghadirian 1993; Khan et al., 1995), we wished to know if the high plasma and splenic IL-10 levels in infected BALB/c mice could mediate the observed suppression of the T-cell responses to Con A. We cultured splenocytes from infected and control BALB/c mice, but then added either anti-IL-10 or control antibodies to the cultures and assessed the proliferation of the cells (Figure 3.3b). Anti-IL-10 antibodies had no significant effect on the responses of cells from uninfected mice to Con A stimulation, but fully reversed (i.e. by 98.9 $\pm$ 2%; $p < 0.001$) the suppression of proliferation of splenocytes from *T. congolense* -infected BALB/c mice. Control IgM antibodies had no such effect in this system (~1.0% reversal; $p > 0.05$).

Figure 3.3 Reversal of *T. congolense*-elicited suppression of proliferation of splenocytes from infected mice. (A) Con A-induced proliferation of spleen cells of BALB/c mice is progressively suppressed during experimental *T. congolense* infection and is negatively correlated ($r = -0.74$, $p < 0.03$) with the spontaneous secretion of IL-10 in the culture supernatants. (B) Addition of neutralizing anti-IL-10 antibodies into the cultures of day 7 or 8 infected spleen cells completely reversed the suppression of the Con A-induced proliferative responses. Anti-IL-4 or control rat IgM antibodies had no such effect. Results are representative of 3 experiments. * $p < 0.05$ compared to normal splenocytes; ** $p < 0.05$ compared to isotype-matched control antibody-treated splenocytes.

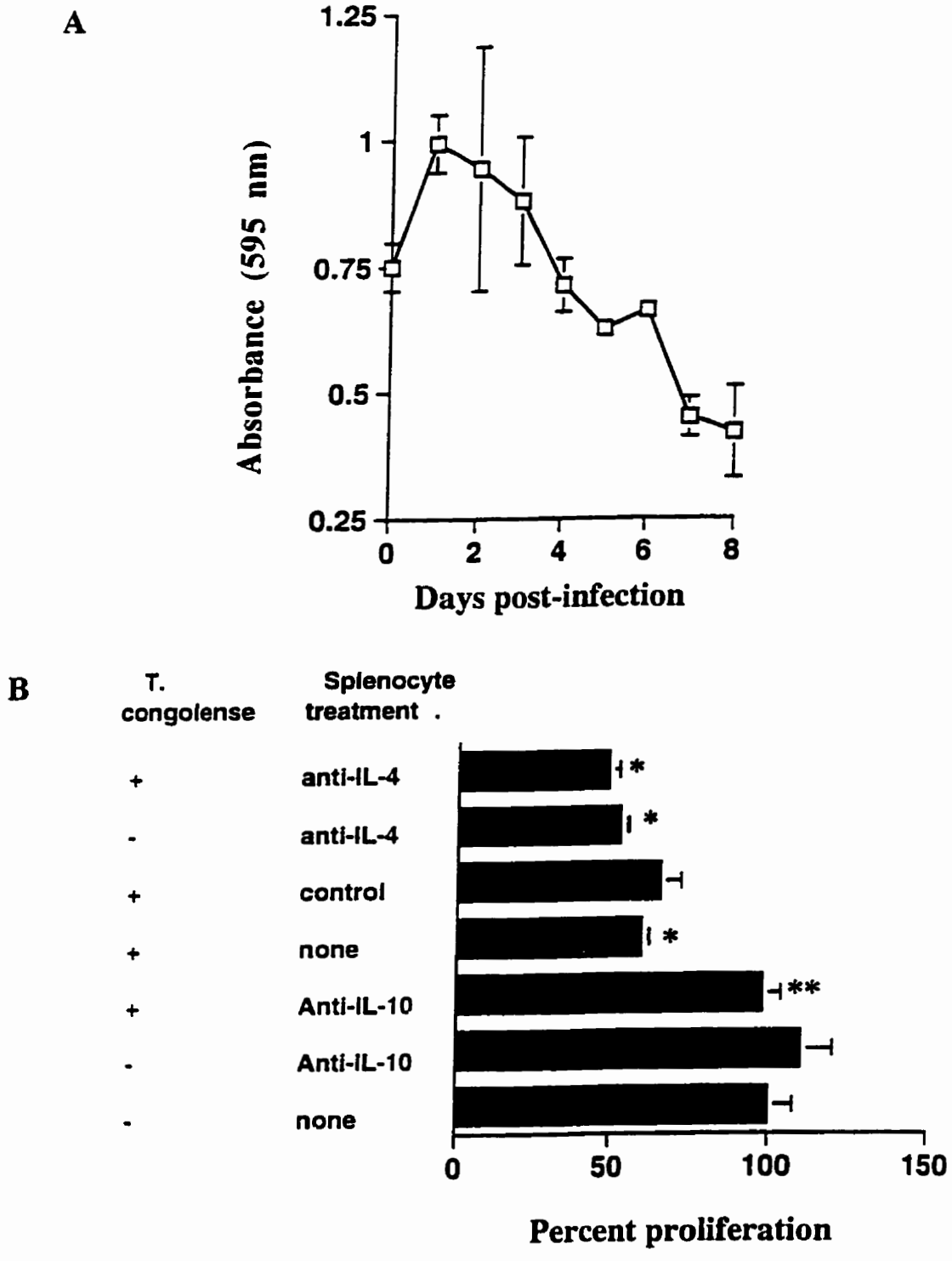
A
1.25
1
0.75
0.5
0.25
Absorbance (595 nm)
0
2
4
6
8
Days post-infection
B
T. congolense
Splenocyte treatment
+
anti-IL-4
-
anti-IL-4
+
control
+
none
+
Anti-IL-10
-
Anti-IL-10
-
none
*
*
*
**
0
50
100
150
Percent proliferation

Figure 3.3

These results demonstrate that IL-10 is important in the immunosuppression observed among the splenocytes of *T. congolense*- infected BALB/c mice.

Because we have found previously that splenocytes from these mice also secrete high levels of IL-4 both spontaneously and in response to Con A challenge (see chapter 4), we also tested the effects on these responses of neutralizing the endogenous IL-4 in this system. Unlike the anti-IL-10 antibodies, anti-IL-4 antibodies did not have any significant effect on the proliferative response of splenocytes from infected mice, but they did significantly decrease (by 50 $\pm$ 4%; $p < 0.01$) the proliferative response of Con A-challenged splenocytes from control mice .

3.4.4 The effects of Berenil chemotherapy on the circulating IL-10 levels in *T. congolense*-infected BALB/c mice

As noted above, day 9 corresponds to the time at which C57BL/6 mice clear their first wave of parasitemia and BALB/c mice succumb to the disease. Chemotherapeutic treatment of BALB/c mice with Berenil on days 6 and 7 post-infection lead to an apparent clearance of parasites from the blood on day 8. Berenil also reduced the plasma levels of IL-10 back to normal (i.e. undetectable) levels and significantly decreased the spontaneous and Con A-induced secretion of IL-10 by splenocytes from animals infected for 8 or 9 days. On days 8 and 9, the levels of IL-10 in the 48 hr unstimulated splenocyte cultures of infected, untreated versus infected berenil-treated mice were 241.0 $\pm$ 17.5 vs. 72.3 $\pm$ 8.9 pg/ml (day 8; $p < 0.01$) and 338.0 $\pm$ 12.4 vs. 51.3 $\pm$ 11.2 pg/ml (day 9; $p < 0.01$). Similarly, the levels of IL-10 production in the Con A-stimulated splenocyte cultures were 1928 $\pm$ 101 versus 1096 $\pm$ 32 pg/ml (day 8; $p < 0.05$) and 2900 $\pm$ 132 versus

670 ± 96 pg/ml (day 9; $p < 0.01$). Berenil treatment of infected BALB/c mice also was associated with downregulation in the IL-10 mRNA levels on days 8 and 9 post-infection (data not shown), and a rapid restoration to near normal levels of the proliferative responses to Con A challenge of splenocytes from infected BALB/c mice (Appendix, Table 1). These results are consistent with the findings of Rhind et al. (1997), who showed that treatment of *T. brucei rhodesiense*-infected patients with melarsoprol was associated with a rapid decline in the hitherto high plasma levels of IL-10.

3.4.5 *In vivo* administration of anti-IL-10 reduces parasitemia and prolongs the median survival period of susceptible BALB/c mice

We next wished to investigate the potential role of IL-10 as a disease-enhancing agent in susceptible BALB/c mice infected with *T. congolense*. The reasons for this were three-fold: first, the plasma levels and splenocyte production of IL-10 in infected BALB/c mice were significantly higher than those of the C57BL/6 mice; second, our *in vitro* evidence strongly implicated IL-10 in *T. congolense*-mediated immunosuppression in BALB/c mice; and third, Berenil-cure of the BALB/c mice was associated with a dampening of IL-10 production and a reversal of splenocyte suppression in these mice.

We therefore injected purified monoclonal rat anti-mouse IL-10 antibodies or isotype-matched irrelevant control IgM antibodies (200 μg/injection) into groups of BALB/c mice on days 0, 2, 5, 7 post-infection with *T. congolense* clone TC13. We then monitored the circulating parasite levels of the mice daily. Anti-IL-10 administration caused a significant decrease in the parasitemia from day 6 post-infection compared to the

controls (Figure 3.4A). It also caused a significant decrease in the measured peak parasitemia of the treated group compared to the controls (43% decrease, $p < 0.01$; Figure 3.4A) and significantly increased the median survival period from 8 to 11 days (38% increase, $p < 0.01$; Figure 3.4B).

3.4.6 Treatment of genetically resistant C57BL/6 mice with IL-10 does not render them susceptible to trypanosome challenge

The severity of disease and immunosuppression in the highly susceptible BALB/c mice infected with *T. congolense* appeared to be associated with high levels of IL-10 in the plasma and spleen. The question arose then whether administration of IL-10 to a resistant mouse strain would enhance their susceptibility to the disease. Groups of 6 C57BL/6 mice were injected i.p. with 600 ng of recombinant murine IL-10 on days 0, 2, 4 and 6 post-infection. Control mice were injected with saline on the same time schedule. There were no significant differences in the levels of circulating parasites among the groups of mice treated with either recombinant IL-10 or saline, as determined during the middle of the first wave of parasitemia. Similarly, both groups appeared equally healthy at this time, suggesting that IL-10 treatments had no significant impact on their resistance to this disease.

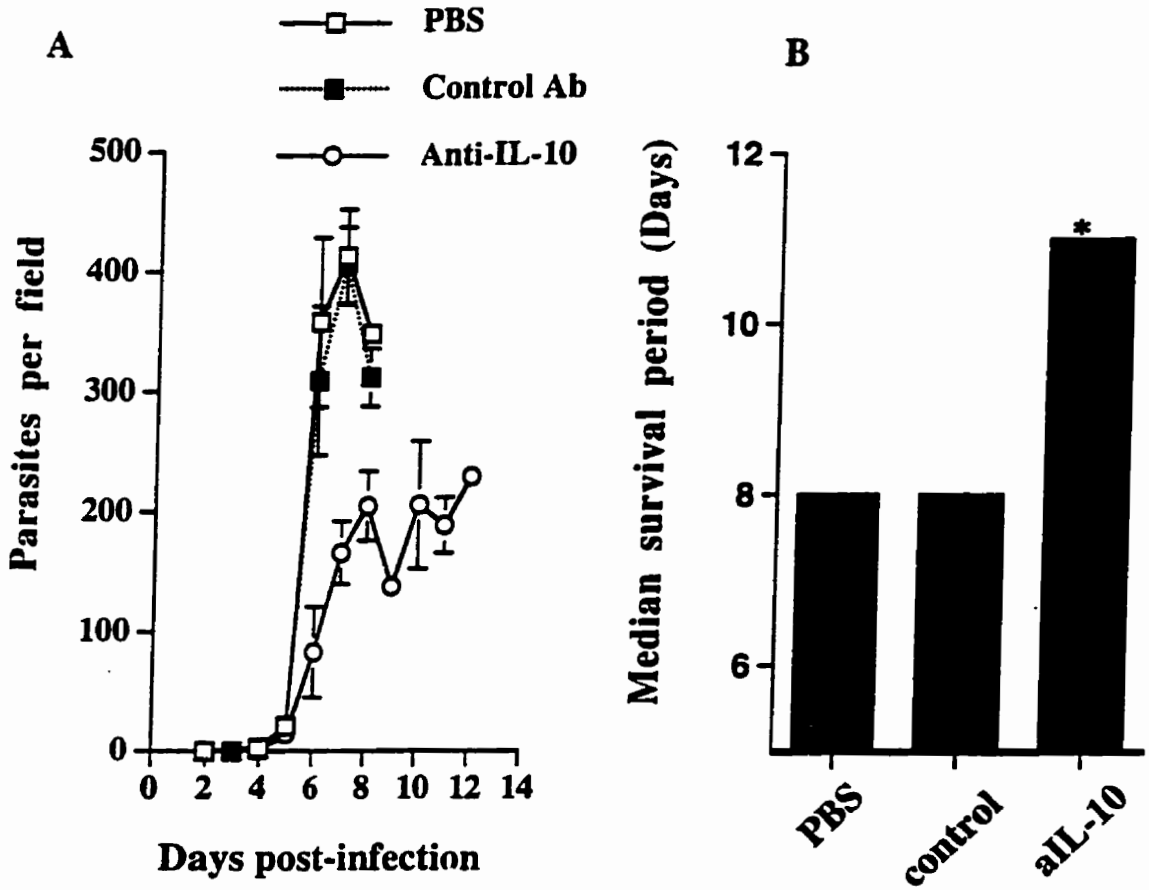

Figure 3.4 Parasitemia and survival period of *T. congolense*-infected BALB/c mice treated with anti-IL-10 antibody. In vivo administration of anti-IL-10 antibodies during *T. congolense* infections significantly reduces parasitemia (A) and increases the median survival period (B) of the highly susceptible BALB/c mice. Groups of 6 mice were injected i.p. with 200 μg of anti-IL-10, control antibody or PBS on days 0, 2, 5 and 7 post-infection. Mice were monitored daily for parasitemia and survival. * $p < 0.001$ compared to isotype-matched control antibody-treated group.

3.5 Discussion

Our data suggest that endogenously-produced IL-10 has a moderate disease-enhancing role in experimental trypanosome infections in genetically-susceptible BALB/c mice. In the present, IL-10 was associated with *T. congolense*-mediated suppression of splenic T-cell proliferative responses in infected mice and in the control of parasitemia and survival of this highly susceptible mouse strain. We have shown that the plasma levels and splenocyte production of IL-10 in infected BALB/c mice were significantly higher than in resistant C57BL/6 mice. This IL-10 production correlated with the rise in parasitemia ($r = 0.93$) and was negatively correlated with spleen cells proliferation in response to Con A ($r = -0.75$). Furthermore, Berenil treatment of infected BALB/c mice rapidly cleared the parasitemia and led concomitantly to a reduction in plasma IL-10 levels and, finally, *in vivo* administration of anti-IL-10 antibodies significantly reduced the parasitemia and increased the median survival period of the highly susceptible BALB/c mice.

The down-regulation of T-cell proliferation during African trypanosomiasis has been attributed to at least two mechanisms, with activated macrophages that have interacted with trypanosomes or trypanosomal membranes being central to this effect (Askonas 1985; Darji et al., 1992; Sacks et al., 1982; Sileghem et al., 1994a). Both prostaglandin-dependent and -independent suppressive mechanisms may be operative here (Darji et al., 1992; Kierszenbaum et al., 1991; Sileghem et al., 1986; 1994a), but IL-10 is an obvious candidate effector molecule in this suppressor function. It is produced by activated macrophages (Ding and Shevach 1992; de Waal Malefyt et al., 1991) and, indeed, we have evidence that following phagocytosis of opsonized trypanosomes, bone

marrow-derived macrophage cell lines from BALB/c mice secrete copious amounts of IL-10 relative to similar cells from C57BL/6 mice (R. S. Kaushik, et al., 1998; manuscript in preparation). IL-10 is also produced by a sub-population of $CD5^+$ B-cells (O'Garra et al., 1992) which has been shown to expand greatly during experimental *T. congolense* infections in cattle (Naessens and Williams, 1992). However, the finding that T-cell mitogen (i.e. Con A) stimulation was associated with up to 50-fold increases in IL-10 secretion by splenocytes from infected mice indicates that T-cells are at least potentially involved in the secretion of this cytokine during experimental *T. congolense* infections in mice. It may be noteworthy though that lipoarabinomannan (LAM), a cell wall component of *Mycobacterium tuberculosis* which induces IL-10 production by monocytes (Barnes et al., 1992), shares some biochemical similarities with the phosphatidylinositol moiety of the variant surface glycoprotein of the African trypanosomes (Barnes et al., 1992, Ferguson and Williams, 1988).

Treatment of infected BALB/c mice with berenil on days 6 and 7 lead to clearance of parasites from the blood and a rapid decline in the IL-10 mRNA expression and IL-10 secretion by splenocytes from infected mice. This suggests that the presence of trypanosomes in the blood is associated with IL-10 production. The finding that the differences in IL-10 production by splenocytes from infected BALB/c and C57BL/6 mice were apparent as early as day 4 post-infection (Figure 3.2A) despite that fact that parasitemia was essentially similar in both strains of mice up to day 6 post-infection (Figure 3.1A), indicates that the differences in IL-10 secretion were not due to higher parasite loads in the susceptible BALB/c mice. Rather, it suggests that the production of higher amounts of IL-10 by the susceptible BALB/c mice was as a result of intrinsic

physiological differences in responses of BALB/c mice to *T. congolense* infection relative to the resistant C57BL/6 mice.

The role of IL-10 in African trypanosomiasis is not as yet clearly defined. Elevated levels of IL-10 mRNA transcripts have been reported in the blood, lymph node and splenic leucocytes of *T. congolense*-infected trypanosusceptible Boran cattle (Taylor et al., 1996) and it has been suggested that this cytokine may be responsible for the inhibition of monokine secretion by macrophages from infected cattle (Taylor, 1998). Similarly, high levels of IL-10 have been detected in the plasma of human patients suffering from chronic *T. brucei rhodesiense* infections. Treatment of infected patients with melarsoprol caused a rapid decline in the plasma levels of this cytokine in these patients (Rhind et al., 1997). Our results demonstrate the involvement of IL-10 in *T. congolense*-elicited suppression of splenocyte proliferative response to Con A and in enhanced susceptibility of BALB/c mice to the disease.

We have observed that trypanosome-infected BALB/c mice also have significantly higher levels of IFN-γ in their plasma (Tabel et al., 1994) and spleen culture supernatants (see chapter 4) than the relatively resistant C57BL/6 mice. IFN-γ is a prototypic macrophage-activating factor and could thus indirectly induce the increases in IL-10 production observed in this report. Darji et al., (1996) have shown, for example, that $CD8^+$ T-cells that are exposed to *Trypanosoma brucei brucei*-pulsed macrophages secrete copious amounts of IFN-γ. This IFN-γ in turn indirectly effects a suppression of T-cell proliferative responses, reportedly via a soluble product released from IFN-γ-activated macrophages (Darji *et al.* 1992, 1996). We are presently studying the role(s) of this IFN-γ

expression and its interplay with IL-10 in the enhanced susceptibility to disease and immunosuppression seen in BALB/c mice.

Based on our *in vitro* results in which anti-IL-10 antibody treatments completely reversed the suppression of Con-A-induced splenocyte proliferation, we had anticipated longer survival periods for the anti-IL-10-treated *T. congolense*-infected BALB/c mice than were observed. It could be and is likely that, although IL-10 may be important to the pathogenesis of this disease, other immunomodulatory molecules are also critically involved. It has been shown, for example, that in *T. brucei brucei*-infected rodents, endogenous IFN-γ enhances susceptibility to the disease, such that IFN-γ gene knock-out mice survive longer than their IFN-γ sufficient counterparts (Bakhiet *et al.* 1996). It is almost undoubtedly true that the immunoregulation of host resistance to such a complex pathogen as *T. congolense* is very complicated, more so perhaps because of the pleiotropic and redundant biologic effects of the many cytokines involved in this disease. An alternate, though perhaps more trivial explanation for the unexpectedly low impact of the anti-IL-10 antibody treatments could potentially be related to the IgM isotype of the antibodies. While IgG antibodies would penetrate relatively easily through the tissues, the much larger IgM antibodies may not do so. Furthermore, IgM-IL-10 complexes may not be as efficiently removed from the circulation as IgG-cytokine complexes, and it has been shown that complexing of cytokines to antibodies *in vivo* can actually protect the cytokine from catabolic effects (Martens et al., 1993).

Attempts to enhance the susceptibility (increased parasitemia and shorter survival periods) of the relatively resistant C57BL/6 mice by administration of recombinant IL-10

were not successful. This could be due to insufficient dose to attain physiologically-relevant levels of circulating cytokine. This would be particularly important given that cytokines in general have very short half lives *in vivo*. In other instances wherein enhanced resistance has been obtained in susceptible mice by *in vivo* administration of anti-cytokine antibodies, similar attempts to make resistant strain mice more susceptible by the administration of recombinant cytokines have also failed (Sadick et al., 1991). Thus, our inability to render the relatively resistant C57BL/6 mice more susceptible to *T. congolense* infection by treatment with IL-10 does necessarily not contradict or conflict with our data on the susceptible mice.

The importance of cytokines in altering host resistance to protozoal infections is well documented. IL-4, IL-10 and TGF-β have all been shown to be associated with enhanced susceptibility to *T. cruzi* and *Leishmania major* infections in mice and administration of antibodies to these cytokines *in vivo* has been associated with enhanced resistance to these diseases (Reeds et al., 1994; Sadick et al., 1991). IFN-γ has been claimed to support growth of *T. brucei brucei* and mice treated with antibodies to IFN-γ showed reduced parasitemia and longer survival period (Olsson et al., 1991). Our data implicate IL-10 as a disease-enhancing cytokine during *T. congolense* infection in mice. At present time we are examining further the roles and interactions of other cytokines with IL-10 in the pathology of this disease.

4.0 DIFFERENTIAL INDUCTION, SECRETION AND ROLE OF CYTOKINES DURING EXPERIMENTAL *TRYPANOSOMA CONGOLENSE* INFECTIONS: ADMINISTRATION OF ANTI-IFN-γ ANTIBODIES ALTERS TRYPANOSOME-SUSCEPTIBLE MICE TO A RESISTANT-LIKE PHENOTYPE

4.1 Abstract

The immunologic mechanisms regulating resistance or susceptibility to the blood parasite *Trypanosoma congolense* have been enigmatic. Here, we address the roles of several cytokines (IL-4, IFN-γ and TNF-α) known to regulate either the immune response of the host or the pathogenesis of its infections. We assessed the production of these cytokines *in vivo* and *in vitro* using genetically susceptible (BALB/c) or resistant (C57BL/6) strains of mice infected with cloned *T. congolense*. We found that the plasma of infected BALB/c mice contained dramatically higher levels of IL-4 and IFN-γ than the plasma of infected C57BL/6 mice. Conversely, the plasma TNF-α levels were significantly elevated in the resistant mice relative to the susceptible ones. Differential kinetics for IFN-γ mRNA accumulation were observed in the spleens of susceptible and resistant mice, with the former having earlier and more sustained high steady state mRNA levels than the latter. Both unstimulated and concanavalin A (Con A)-induced secretions of IL-4 and IFN-γ by splenocytes from infected BALB/c mice were significantly higher than those from their C57BL/6 counterparts. Con A-induced proliferation of splenocytes from infected BALB/c mice was progressively suppressed. Nitric oxide was not involved in this suppression but

the suppression was positively correlated with the observed IFN-γ secretion. Addition of neutralizing monoclonal antibodies to IFN-γ to cultures of Con A-stimulated spleen cells from infected BALB/c mice effectively reversed this suppression. Furthermore, the administration of anti-IFN-γ antibodies to BALB/c mice early during infection dramatically shifted the phenotype of these susceptible mice to a more resistant-like phenotype, as expressed by a low and undulating pattern of parasitemia and a > 300% increase in survival period. These results strongly suggest that the enhanced induction and secretion of IFN-γ during *T. congolense* infections in mice contributes to the relative susceptibility of these animals to the disease.

4.2 Introduction

African trypanosomes are extra-cellular hemoprotozoan parasites that cause disease and death in man and animals in the sub-Saharan Africa. A remarkable feature of salivarian trypanosomes is their ability to continually change their surface protein coat, and thereby effectively evade the host's immune response (Gray and Luckins, 1976). Infections are associated with profound aberrations in the host's immune system, including a generalized immunosuppression that affects both the humoral and cellular immune compartments (Askonas, 1985; Darji et al., 1992; Sileghem et al., 1994a). This suppression encompasses responses to parasite-related and unrelated antigens and mitogens and is responsible for the increased susceptibility of infected animals to opportunistic infections as well as for the poor responsiveness of parasite-bearing animals to vaccinations against unrelated pathogens (Greenwood et al., 1973).

The indigenous West African N'dama breed of cattle are more resistant than the Zebu breed to both natural and experimental infections with *T. congolense* (Mansfield, 1990; Murray et al., 1982). Similarly, in a mouse model system, BALB/c mice are highly susceptible to experimental *T. congolense* infections whereas C57BL/6 mice are relatively resistant, as measured by the levels of parasitemia and immunosuppression and survival periods (Pinder et al., 1986; Otesile et al., 1991; Ogunremi and Tabel, 1995). Genetic analyses have shown that differences in resistance to related *T. brucei* infections in mice are not due to differences in the MHC genes (Levine and Mansfield, 1984; Mansfield, 1990) and that the survival period of BALB/c mice infected with *T. congolense* is significantly correlated with the speed of control of the first wave of parasitemia (Ogunremi and Tabel, 1995). The mechanisms underlying the differences in resistance among different breeds of cattle or among inbred mouse strains are poorly understood.

The patterns of cytokine response during some parasite infections determine or are at least strongly correlated to the relative susceptibility of the host (Sher and Coffman, 1992), although the role(s) of individual cytokines in resistance to trypanosomal infections remains equivocal. IFN-γ is expressed just preceding the peak of parasitemia in mice infected with *T. brucei* (Bancroft et al., 1981). It has been associated with immunosuppression, mediated in part by stimulation of nitric oxide (NO) production by macrophages (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993), although Darji et al., (1996) did not observe any NO-mediated immunosuppression caused by IFN-γ. IFN-γ has also been associated with enhanced host susceptibility to *T. brucei* infections and this effect is related to its direct stimulatory effect on the growth of *T. brucei* (Olsson

et al., 1991). On the other hand, IFN-γ also has been reported to mediate parasite control and host protection (DeGee et al., 1985). TNF-α appears to play a protective role during the initial phase of experimental infection with *T. brucei brucei* in mice (Magez et al., 1993). It has been shown to have a direct lytic effect on *T. brucei* (Lucas et al., 1994; Magez et., 1997). More recently, the expression of IL-4 mRNA was shown to be higher in the relatively resistance C57BL/6 than in susceptible C3H mice infected with *T. brucei brucei* and it was suggested that the differential expression of this cytokine might be responsible for the observed differences in resistance between these inbred mouse strains (Bakhiet et al., 1996). To further document these inter-strain differences, we examined the expression of IL-4, IFN-γ and TNF-α in mice infected with *T. congolense* and the roles of the former two cytokines in susceptibility to this parasite. We show here that following infection with *T. congolense*, BALB/c and C57BL/6 mice differentially express each of these cytokines, such that the plasma of the highly susceptible BALB/c mice contained significantly more IL-4 and IFN-γ and less TNF-α than that of the relatively resistant C57BL/6 mice. The splenocytes from infected BALB/c mice contained more IFN-γ mRNA and produced much more IL-4 and IFN-γ protein than those of the resistant C57BL/6 mice. The proliferative responses to Con A of splenocytes from infected BALB/c mice were progressively suppressed during infection and thereby showed an inverse relationship with IFN-γ production. The addition of neutralizing anti-IFN-γ antibodies into such cultures restored the proliferative responses. Furthermore *in vivo*, anti-IFN-γ antibody treatments, but not anti-IL-4 treatments, of infected BALB/c mice

significantly reduced the parasitemia of these highly susceptible mice and increased their life-span relative to untreated or control antibody-treated mice by more than 300%.

4.3 Materials and Methods

4.3.1 Mice

Female BALB/cAnNCrlBR (BALB/c) and outbred CD1 mice were obtained from the Animal Resource Center of the University of Saskatchewan. Female C57BL/6NCrlBR (C57BL/6) mice were purchased from Charles River Laboratories, St. Constant, Quebec. The BALB/c and C57BL/6 mice were 8-10 weeks of age and the CD1 mice were 5-6 weeks old. All mice were maintained according to the recommendations of the Guide for the Care and Use of Experimental Animals of the Canadian Council of Animal Care.

4.3.2 Parasites

The origin of the *T. congolense*, variant antigenic type (VAT) TC13 used in the present study has been previously described (Tabel, 1982). Cloned trypanosome populations were stored as frozen stabilates in liquid nitrogen. Parasites were passaged in CD1 mice as previously described (Tabel, 1982). The parasites for infection of BALB/c and C57BL/6 mice were isolated from the blood of CD1 mice three days after passage by DEAE anion exchange chromatography (Lanham and Godfrey, 1970).

4.3.3 Experimental design

Groups of four to six BALB/c or C57BL/6 mice were infected intraperitoneally (i.p.) with 10^3 organisms of *T. congolense* VAT TC13 and killed with CO_2 on days 1-10 post

infection. In some experiments, infected mice were treated with berenil (14 mg/kg of diminazene aceturate; Sigma Chemical Co., St. Louis, Missouri) on day 6 and 7 post-infection to cure their infections. On each day indicated, blood was withdrawn from the caudal vena cava into syringes containing heparin (final concentration ~20 IU/ml), and the plasma was collected after centrifuging the blood at 1,000 x g for 30 min. The plasma was centrifuged at 13,000 x g for 15 min and the supernatant plasma was stored at -35°C until used. Each experiment (and cytokine determination) was performed at least twice.

4.3.4 Estimation of parasitemia and survival period

To estimate the circulating parasite numbers, a drop of blood from the tail vein of each infected mouse was examined at 400x magnification by phase contrast microscopy. Parasitemia was most often estimated by counting the number of parasites present in at least 10 fields, but unusually heavy parasite loads were quantified according to Herbert and Lumsden (16). The survival period was defined as the number of days post-infection that the infected mice remained alive. Moribund mice were euthanized with CO_2. BALB/c mice infected with 10^3 organisms of *T. congolense* had a mean survival time of 8 $\pm$ 0.5 days and invariably succumbed to the first wave of parasitemia (see Figure 3.1A). In contrast, infected C57BL/6 mice controlled their first (Figure 3.1A) and many subsequent waves of parasitemia. Their survival period was not determined in this study but previously has been found to be 163 $\pm$ 12 days (Ogunremi and Tabel, 1995).

4.3.5 Splenocyte cultures for measurement of cytokine synthesis

Single cell suspensions of spleen cells from normal and infected mice were adjusted to 5 x 10^6 cells/ml of complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 100 IU/ml penicillin/streptomycin) and cultured in 96-well plates (Falcon, VWR Edmonton, AB) at 0.2 ml/well in a humidified 37°C, 5% CO_2 atmosphere. Some wells were stimulated with 5 μg/ml concanavalin A (Con A; Calbiochem, Behring Diagnostics, La Jolla, CA). All culture fluids were collected after 48 hours, centrifuged at 1,500 x g for 10 min and the supernatant fluids stored at -35°C until used.

4.3.6 Splenocyte proliferation assay

Splenocyte proliferation was quantified by the MTT assay as described by Mosmann (1983). Briefly, cells from infected mice were cultured for 72 h in 200 μl of complete medium at a density of 10^5 cells/well in 96-well flat bottom plates (Falcon®; Becton Dickinson, Lincoln Park, NJ), using quadruplicate cultures of pooled cells from groups of 4 mice. Cells were cultured either without or with Con A (5 μg/ml). Some wells were treated with either 5 μg/ml of purified monoclonal rat anti-mouse IFN-γ antibodies (XMG 1.2, American Type Tissue Collection [ATCC], Rockville, MD), isotype-matched rat IgG1 antibody (Cappel, Organon Teknika Inc. Scarborough, ON) or 500 μM (final concentration) of nitric oxide synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (N^GMMA, Sigma).. After 72 hours, we added MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma) to a final concentration of 0.48 μg/ml for an

additional 3 hr. Then the cells were lysed with acidified isopropanol and the dissolved mitochondrial formazan precipitates were quantified with an ELISA plate reader by measuring the absorbance at 595 nm wavelength. Data are presented as percent proliferation relative to splenocytes from uninfected mice stimulated with Con A only.

4.3.7 Recombinant cytokines and cytokine assays

Recombinant murine IL-4 and ELISA kits for the determination of plasma IL-4 were purchased from ENDOGEN Inc., (Boston, MA). ELISA kits used for determination of plasma levels of IFN-γ and TNF-α were purchased from Genzyme Diagnostics (Cambridge, MA). Recombinant murine IL-4, IL-10, IFN-γ and TNF-α and the paired antibodies used in our sandwich ELISAs for the determination of IL-4, IL-10, IFN-γ and TNF-α in spleen culture supernatants were produced by PharMingen Inc. (San Diego, CA). ELISA assays were done according to the manufacturer's suggested protocols. The functional activity of IFN-γ in the spleen culture supernatants was quantified by the viral cytopathic effect (CPE) reduction assay, using L929 cells and endomyocarditis (EMC) virus as described by Familletti et al., (1981). A functional assay was used to detect the plasma TNF-α bioactivities as previously described (Green et al., 1984).

4.3.8 Quantification of nitrite

Nitric oxide rapidly reacts with oxygen to form nitrate and nitrite, which are relatively stable *in vitro* and are therefore useful for NO quantification (Moncada et al., 1991). The concentration of nitrite in supernatant fluids from splenocyte cultures was determined by a

standard Griess reaction (Green et al., 1982). The detection limit of the test was 1 μM nitrite.

4.3.9 Northern blot analysis

Total cellular RNA was extracted from the spleens of normal and *T. congolense*-infected BALB/c and C57BL/6 mice from days 1-9 post-infection using the guanidinium isothiocyanate/cesium chloride method (Chirgwin et al., 1979). For each sample, 20 μg of RNA was electrophoresed in a 1.2% denaturing agarose gel, transferred to a nylon membrane (Zetabind™, CUNO Lab., Meridien, CN.) by capillary action and cross-linked by u.v. irradiation (Stratalinker™, Stratagene, La Jolla, CA). The membranes were blocked with 0.1X SSC/0.5% SDS for 1 hr at 65°C and pre-hybridized overnight at 42°C in a solution containing 50% de-ionized formamide, 0.1 M HEPES, 0.5 M NaCl, 5 mM EDTA (pH 8.0), 5X Denhardt's solution, 1% SDS, 0.5% sodium pyrophosphate and 12.5 μg/ml salmon sperm DNA. ^{32}P-labeled cDNA probes were prepared by the random hexamer-labeling technique (Oligolabeling kit, Pharmacia, Uppsala, Sweden) according to the manufacturer's suggested protocol. The mouse IFN-γ and TNF-α cDNAs consisted of 550 bp Pst I fragment and 990 bp EcoRI fragments respectively (kindly provided by Dr. John Elliot, University of Alberta and Dr. Rik Derynck, Genentech Inc. CA, respectively) while the human γ-actin cDNA comprised a 910 bp EcoRI/Hind III fragment (provided by Drs. B. Murphy and V. Misra, University of Saskatchewan). The membranes were hybridized overnight at 42°C in pre-hybridization buffer containing 1.5 x 10^6 cpm/ml of labeled probes and then washed two times with 2X SSC/0.1% SDS for 5 min at room

temperature and another two times for 30 min each with 0.2X SSC/0.1% SDS at 42°C. Autoradiography was performed at -80°C for 1-10 days. Densitometric analysis of the autoradiograph signals was carried out using the Image 1 program from Universal Imaging Corporation (Westchester, PA).

4.3.10 Anti-IFN-γ and anti-IL-4 antibodies for in vivo experiments

Anti-IFN-γ (XMG 1.2, IgG1 isotype, ATCC) and rat anti-mouse IL-4, (11B11, IgG1 isotype, ATCC) hybridomas were grown either in serum- and protein-free medium (GIBCO BRL, Life Technologies, Mississauga, ON) or complete medium. The hybridoma supernatants, as well as control culture medium (i.e., complete medium), were precipitated with 45% v/v saturated ammonium sulfate solution and dialyzed extensively against phosphate-buffered saline (PBS; pH 7.0). Antibodies were further concentrated with Amicon Centriprep 100 concentrators (Amicon, W.R. Grace and Co., Beverly, MA.), filtered through 0.2 m syringe filters (Amicon), and protein levels were quantified using the Bradford method (BIORAD Protein Assay; BIORAD Laboratories, Richmond, CA). The levels of IgG1 antibodies in the concentrates were determined by single radial immunodiffusion using goat anti-rat IgG antibodies (Cappel) and purified rat IgG1 (Cappel) standards.

4.3.11 Treatment of mice with monoclonal antibodies

For estimation of parasitemia and survival periods, groups of six BALB/c mice were injected i.p. on days 0, 2, 4 and 6 post-infection with 200 μg of purified anti-IL-4 or anti-

IFN-γ antibodies in 200-400 μl of PBS. Control mice received PBS alone. Treated and control mice were monitored daily for parasitemia and mortality. To determine the effect of in vivo injections of anti-IFN-γ antibodies on cytokine and nitric oxide production, groups of four BALB/c mice were injected i.p. with either PBS alone or 200 μg of purified anti-IFN-γ antibodies in PBS on days 0, 2 and 4 post-infection. Treated and control mice were killed on day 7 post-infection. Collection of plasma and spleens for splenocyte cultures were carried out as previously described. The levels of IL-10 in the plasma and IL-10 and IFN-γ in culture supernatants were determined by sandwich ELISA. The levels of nitrite in the culture supernatant fluids were determined by a standard Griess reaction (13).

4.3.12 Statistical analysis

Data are represented as means ± standard error (SE). Significance of differences was determined either by Student's t-test or Kruskal-Wallis nonparametric one-way Analysis of Variance (ANOVA) using the StatView™.SE 1988 Software (Abacus Concepts Inc., Berkeley, CA.).

4.4 Results

4.4.1 The levels of IL-4, IFN-γ and TNF-α in the plasma of *T. congolense*-infected BALB/c and C57BL/6 mice differ significantly

The levels of IL-4, IFN-γ and TNF-α in the plasma of BALB/c and C57BL/6 mice infected with *T. congolense* were measured by sandwich ELISA. IL-4 was detected in the plasma of BALB/c mice starting at day 5 post-infection and the levels rapidly increased to a peak at day 6 (138 pg/ml), and then gradually declined to insignificant levels by day 9 post-infection (Figure 4.1A). In contrast, the IL-4 levels in the plasma of C57BL/6 mice remained very low or were undetectable throughout the infection period. IFN-γ was first detected on day 5, also peaked on day 6, but then remained high until death in BALB/c mice (Figure 4.1B). The levels of IFN-γ in the BALB/c mice were consistently significantly higher ($p < 0.05$) than in those of the C57BL/6 mice. Treatment of infected BALB/c mice with Berenil on days 6 and 7 post-infection was associated with a dramatic decline in the plasma IFN-γ levels on days 8 and 9 post-infection (504 ± 31 pg/ml for untreated mice vs. 72 ± 9 pg/ml for treated animals on day 8, $p < 0.01$; and similarly 741 ± 10 pg/ml vs. 17 ± 9 pg/ml, $p < 0.001$ on day 9 respectively). TNF-α levels in plasma samples of normal BALB/c mice (as determined by a commercial ELISA kit), were surprisingly high (80 pg/ml, Figure 4.1C), although TNF-α was not detectable in these samples using a functional assay sensitive to 10 pg/ml of TNF-α (data not shown).

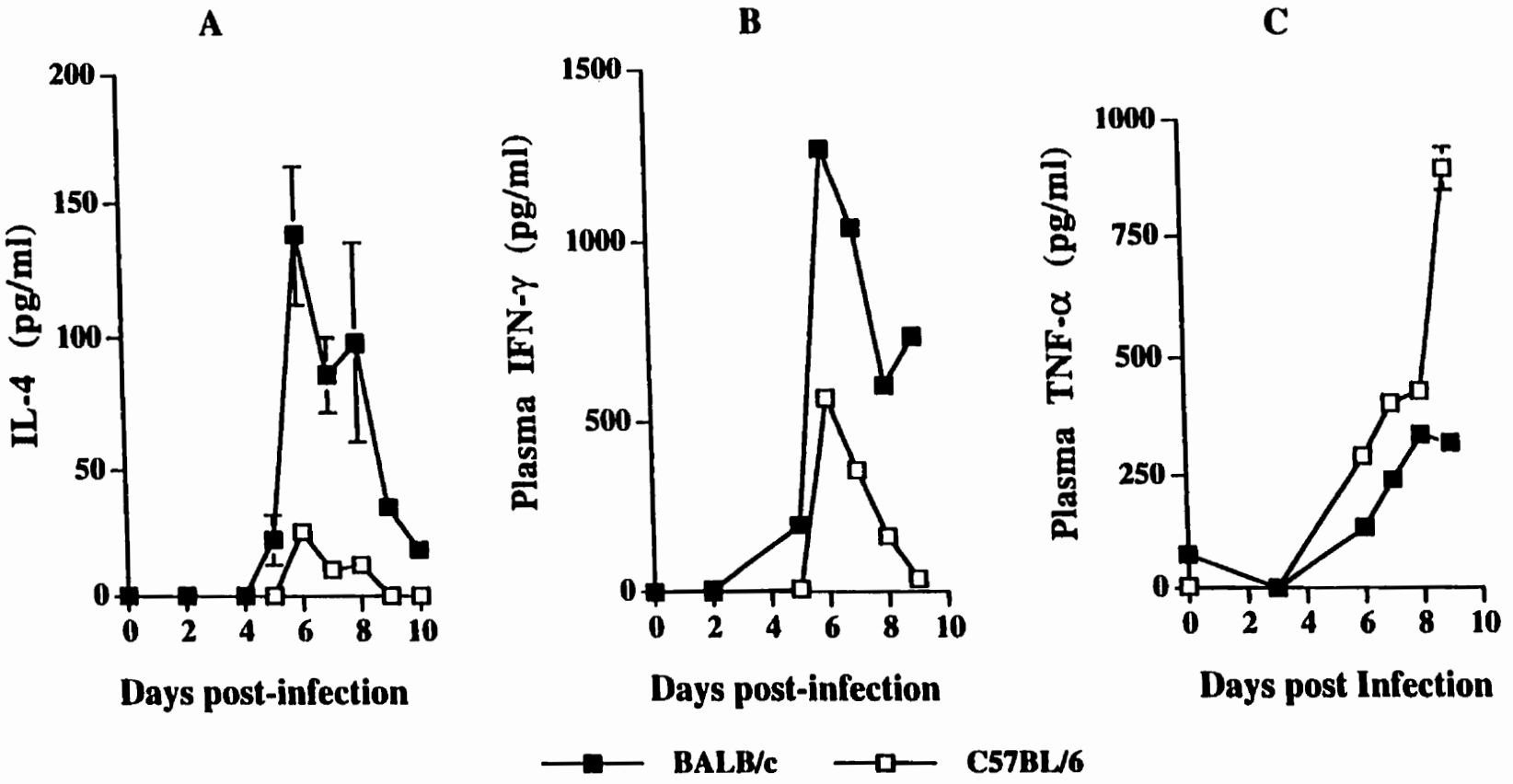

Figure 4.1 Plasma levels of IL-4, IFN-γ and TNF-α in BALB/c (closed squares) and C57BL/6 (open squares) mice infected with *T. congolense*. Plasma samples were collected from four mice on each day post-infection. IL-4 (A), IFN-γ (B) and TNF-α (C) levels were determined by sandwich ELISA. Data are presented as mean ± SE. The results presented are from one of two similar experiments.

Neither the ELISA nor functional assays could detect TNF-α in the plasma of normal C57BL/6 mice (Figure 4.1C). The levels of TNF-α in the infected animals of both strains of mice increased with the progression of the disease, although they were significantly higher ($p < 0.05$) in C57BL/6 mice than in BALB/c mice on days 6 to 9 post-infection (Figure 4.1C). By day 9 post-infection, C57BL/6 mice had about 2.5 times more TNF-α in their plasma than the BALB/c mice. This period corresponds to the time of control of first the wave of parasitemia in C57BL/6 and the time of death in BALB/c mice. The TNF-α proteins detected in the plasma of infected C57BL/6 mice by ELISA were tested by functional assay and found to be biologically active.

4.4.2 Splenic IFN-γ and TNF-α and hepatic TNF-α mRNA expressions are differentially regulated in susceptible BALB/c and resistant C57BL/6 mice infected with *T. congolense*

The steady state levels of IFN-γ and TNF-α mRNA in the spleens and TNF-α mRNA in the livers of infected BALB/c and C57BL/6 mice was assessed by northern analysis. A significant difference was observed in the kinetics of expression of IFN-γ mRNA by the splenocytes from infected BALB/c and C57BL/6 mice. IFN-γ mRNA accumulated to high levels in the spleens of infected BALB/c mice by day 3 post-infection and remained high thereafter. In the C57BL/6 mice, IFN-γ mRNA was not detected at very high levels until day 6 but then was quickly down-regulated thereafter so that by day 8 the steady state levels were about 25% of those at day 6 (Figure 4.2A). Treatment of infected

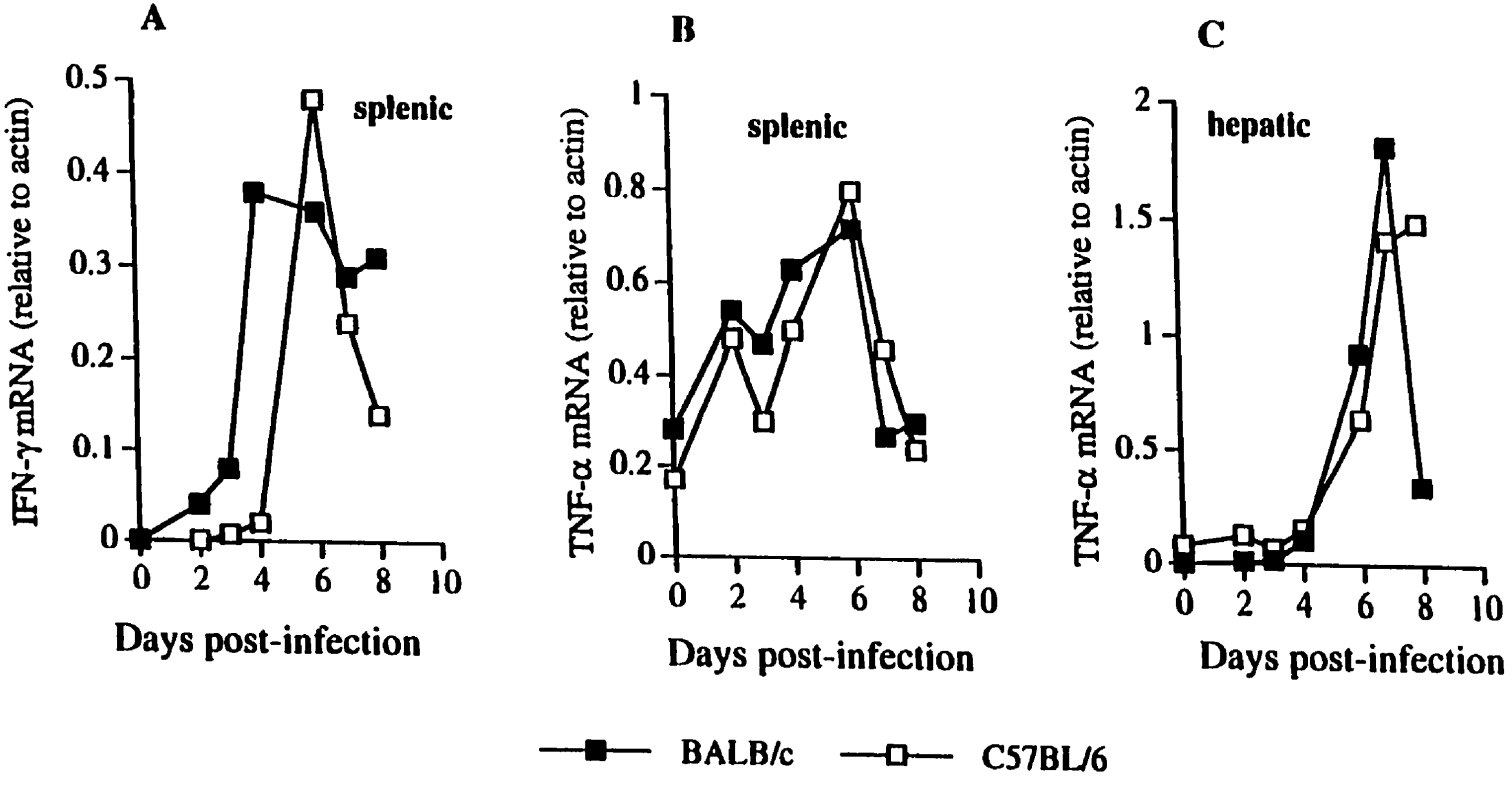

Figure 4.2 Splenic IFN-γ (A) and TNF-α (B) and hepatic TNF-α (C) mRNA expressions (C) in BALB/c and C57BL/6 mice infected with *T. congolense*. Northern blots of total cellular RNA from the spleens or livers of mice infected from 0-8 days were probed with ^{32}P-labeled IFN-γ, TNF-α and γ-actin cDNAs. The results are presented graphically as the ratios of the IFN-γ and TNF-α to γ-actin signals to accommodate any lane-to-lane variations in RNA loading. The results presented are from one of two similar experiments.

BALB/c mice with Berenil on days 6 and 7 post-infection markedly reduced the subsequent expression of IFN-γ mRNA in the spleen, such that on days 8 and 9 IFN-γ mRNA was undetectable by northern analysis (data not shown). The kinetics and patterns of expression of TNF-α mRNA in the spleens of infected mice were similar in the two strains (Figure 4.2B). However, TNF-α mRNA expression in the livers of BALB/c mice gradually rose to a peak on day 7 and markedly declined on day 8 post-infection. This drop in TNF-α mRNA levels corresponded with the peak of parasitemia and the onset of morbidity in these animals. In contrast, in infected C57BL/6 mice and akin to the plasma levels (Figure 4.1C), TNF-α mRNA levels steadily increased with the progression of the infection and were highest on day 9 post-infection by which time the first wave of parasitemia was almost controlled (Figure 4.2C).

4.4.3 Splenocytes from infected BALB/c mice produce higher levels of IL-4 and IFN-γ than spleen cells from infected C57BL/6 mice

Splenocytes from infected, highly susceptible BALB/c and relatively resistant C57BL/6 mice were cultured *in vitro* for 48 hr either alone or in the presence of Con A and the levels of IL-4 and IFN-γ in the supernatants determined using a sandwich ELISA. On each day tested, both unstimulated (Figure 4.3) and Con A-induced secretion of these cytokines (Appendix figures 1 & 2) were significantly higher in cultures of BALB/c cells than in those from C57BL/6 mice. The production of IL-4 by unstimulated BALB/c splenocytes first became apparent on day 4, peaked on day 6 and decreased by day 8 post-

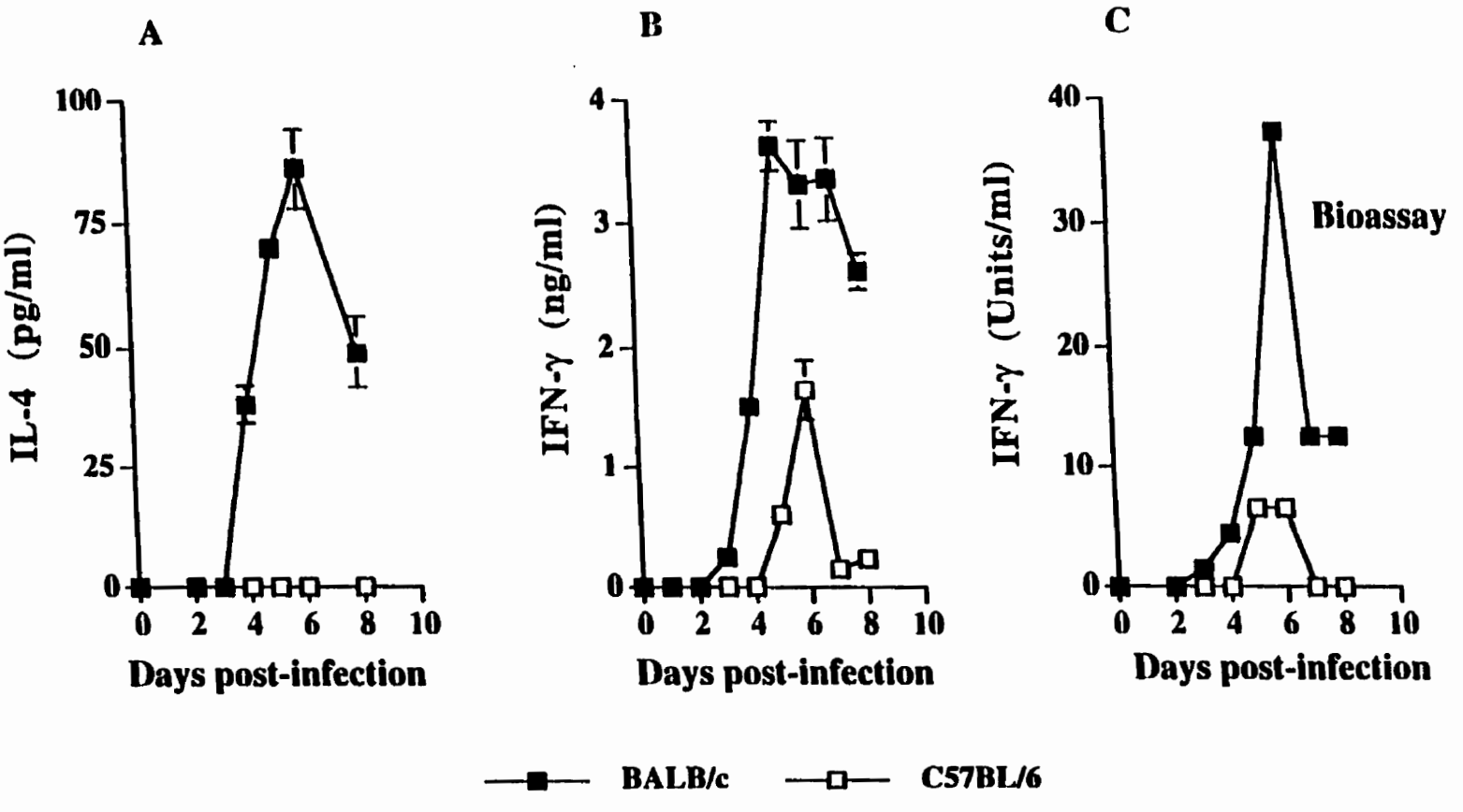

Figure 4.3 Secretion of IL-4 (A) and IFN-γ (B) as measured by ELISA or functional assay (IFN-γ only; frame C) in cultures of unstimulated splenocytes from BALB/c and C57BL/6 mice infected with *T. congolense*. Splenocytes from mice infected for varying periods of time were cultured for 48 hr and the supernatant fluids were assayed for IL-4 and IFN-γ as described in materials and methods. Data are presented as mean ± SE. The results presented are from one of three similar experiments.

infection (Figure 4.3A), but unstimulated C57BL/6 splenocytes produced no detectable IL-4 throughout the infection period tested. There was a gradual decline in Con A-induced production of IL-4 following infection in BALB/c mice, but by day 7 post-infection, the trend was reversed and Con A-driven IL-4 production increased markedly and remained high until the death of the mice (Appendix figure 1). In contrast, Con A-driven production of IL-4 by splenocytes from C57BL/6 mice remained either undetectable or were significantly lower ($p < 0.001$) than those from BALB/c mice (Appendix figure 1). The production of IFN-γ was significantly higher in BALB/c cultures than in the C57BL/6 cultures. By day 4 post-infection, IFN-γ production by unstimulated splenocytes from BALB/c mice was high and remained so throughout the remainder of the infection (Figure 4.3B). In contrast, the splenocytes from resistant C57BL/6 mice only produced significant amounts of IFN-γ on days 5 and 6 and by days 7 and 8 post-infection, their production of this cytokine had largely waned (Figure 4.3B). There was a strong correlation between the rise in parasitemia and the spontaneous secretion of IFN-γ in culture by splenocytes of infected BALB/c mice ($r = 0.89$, $p < 0.01$). Because of the potentially contradictory co-production of high levels of IFN-γ as well as IL-4 and IL-10 (see chapter 3) in the BALB/c mice, we wished to confirm the IFN-γ ELISA results using a functional assay for this cytokine. We observed similar patterns of splenocyte IFN-γ production using this assay and thereby also confirmed that the immunologically detectable IFN-γ in both BALB/c and C57BL/6 mice was functionally active (Figure 4.3C). There was no detectable TNF-α production by ELISA or bioassay in culture fluids of either the

unstimulated or Con A-stimulated splenocytes from infected BALB/c or C57BL/6 mice after 48 hr in culture.

4.4.4 IFN-γ mediates the suppression of mitogen-driven splenocyte proliferation in *T. congolense*-infected BALB/c mice

A prominent feature of African trypanosomiasis is suppression of lymphocyte reactivity to mitogens and antigens (Askonas, 1985; Darji et al., 1992; Sileghem et al., 1994b) and the degree of immunosuppression correlates negatively with the resistance exhibited by different strains of mice (Pinder et al., 1986). Because it has been shown that IFN-γ is involved in the down-regulation of T-cell responses in *T. brucei brucei* (Darji et al., 1993; 1996) and other parasitic infections (Leite de Moraes et al., 1994; Candolfi et al., 1995) in mice, we wished to determine whether the increased production of IFN-γ in *T. congolense*-infected BALB/c mice was involved in the suppression of their T-cell responses to Con A (see below).

Spleen cells from infected BALB/c mice showed a progressive suppression of their proliferative responses to Con A stimulation, most markedly on days 7 to 9 post-infection (82 $\pm$ 10% suppression relative to uninfected controls on day 8). To assess the involvement of IFN-γ in this suppression, we set up splenocyte cultures from day 8-infected and control BALB/c mice, adding either anti-IFN-γ or equivalent levels of isotype-matched control antibodies to the cultures and again assessed the proliferative response of the cells to Con A challenge. As shown in figure 4.4, anti-IFN-γ antibody had no significant effect on the Con A response of splenocytes from normal mice, but

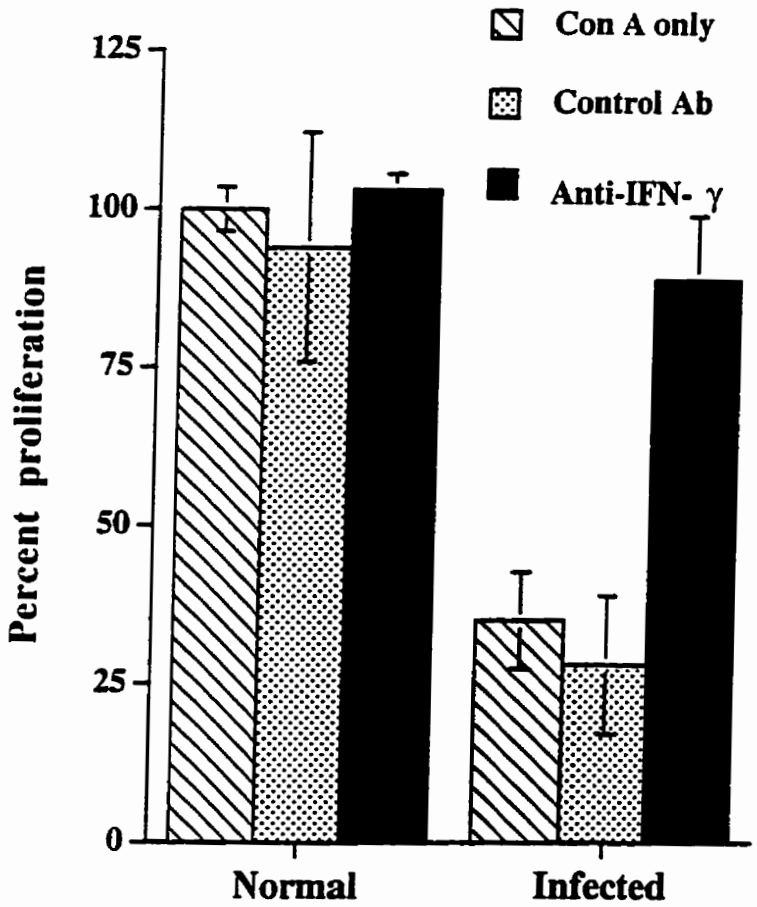

Figure 4.4 Reversal of *T. congolense*-elicited suppression of splenocytes from BALB/c mice infected with *T. congolense* by neutralizing anti-IFN-γ antibodies. Splenocytes from uninfected or day 7 infected mice were stimulated with Con A alone (hatched bars), Con A plus isotype-matched control antibody (dotted bars) or Con A plus anti-IFN-γ antibodies (solid bars). The proliferation of the unstimulated splenocytes from uninfected or infected mice in medium alone were less than 8%. Data are presented as mean ± % coefficient of variation. The results are from one of three similar experiments.

abrogated the suppressive effects of *T. congolense* infection on the proliferation of splenocytes from infected mice. In contrast, isotype-matched control antibody had no detectable effects on the Con A response of splenocytes from uninfected or infected mice.

Suppression of splenocyte proliferative responses to mitogens in mice infected with either *T. brucei brucei* (Sternberg and McGuigan, 1992) or *T. brucei rhodesiense* (Schleifer and Mansfield, 1993) have been reported to be partially mediated by NO produced by IFN-γ-activated macrophages. However, Taylor et al. (1996) reported that NO-mediated immunosuppression does not operate in cattle infected with *T. congolense*. In order to test whether NO in some way affects the suppression of splenocyte proliferative response observed in *T. congolense*-infected mice, we repeated our proliferation assays, but included a specific NOS inhibitor (N^GMMA) in the spleen cultures to block the production of NO. As shown in table 4.1, anti-IFN-γ antibodies again effectively reversed the suppression of Con A-driven proliferation of splenocytes from *T. congolense*-infected mice, but the addition of 500 μM N^GMMA to the cultures, which inhibited NO production by 93 ± 9.5%, had no significant effect on the proliferative responses of these splenocytes.

These results clearly show that IFN-γ is involved in the suppression of splenocyte proliferative response associated with *T. congolense* infections and suggest that this cytokine could be important in the disease-promoting immunosuppression observed in infected, genetically susceptible mice. They also demonstrate that, as in cattle (Taylor et al., 1996), NO does not mediate the suppression of splenocyte proliferative responses observed in *T. congolense*-infected mice.

Table 4.1. Nitric oxide-independent suppression of Con A-induced proliferation of splenocytes from *T. congolense*-infected BALB/c mice.

Culture condition[a]	Percent proliferation ± SE
(U) Splenocytes + Con A	100 ± 6
(I) Splenocytes + Con A	25 ± 9[b]
(I) Splenocytes + Con A + control antibody	24 ± 8[c]
(I) Splenocytes + Con A + anti-IFN-γ	93 ± 7.5
(I) Splenocytes + Con A + N^GMMA	28 ± 11[c]

[a]Splenocytes from uninfected (U) or *T. congolense*-infected (I) BALB/c mice were cultured at 10^5 cells/well in a 96-well tissue culture plates in the presence or absence of 5 μg/ml anti-IFN-γ, rat IgG1 (isotype-matched control antibody) or 500 μM of NOS inhibitor, (N^GMMA). The cultures were stimulated with 5 μg/ml Con A for 72 hr and proliferation of splenocytes was measured by the MTT dye incorporation assay.
[b]$p < 0.01$ vs. splenocytes from uninfected mice.
[c]$p < 0.01$ vs. anti-IFN-γ antibody-treated cultures.

4.4.5 In vivo anti-IFN-γ administration early during infection reduces parasitemia and dramatically prolongs the survival period of genetically susceptible BALB/c mice

We next wished to determine whether neutralization of endogenous IFN-γ in genetically susceptible BALB/c mice infected with *T. congolense* would influence the outcome of the disease. The reasons for this arose from: i) our earlier observation that the plasma and splenocyte culture supernatants from infected BALB/c mice consistently contained significantly more IFN-γ than those from their C57BL/6 counterpart mice; ii) the strong correlation of IFN-γ expression with the rise in parasitemia in susceptible mice; iii) the anti-IFN-γ-mediated reversal of suppression of the proliferative responses of splenocytes from infected BALB/c mice to Con A challenge; and iv) the observation that treatment of infected mice with the trypanocidal drug Berenil effectively abolished IFN-γ expression in infected BALB/c mice.

Groups of BALB/c mice were injected intraperitoneally with either purified monoclonal anti-IFN-γ antibodies, anti-IL-4 antibodies (200 μg in 200-400 μl of PBS per injection) or PBS on days 0, 2, 4 and 6 post-infection with *T. congolense*. The circulating parasitemia and survival of the mice were monitored daily. All of the PBS and anti-IL-4-treated mice succumbed to their infections within 7-9 days, with each becoming moribund at about the time their circulating parasite loads peaked (Figure 4.5 A &B). However, anti-IFN-γ-treated BALB/c mice effectively adopted a trypanosome-resistant phenotype.

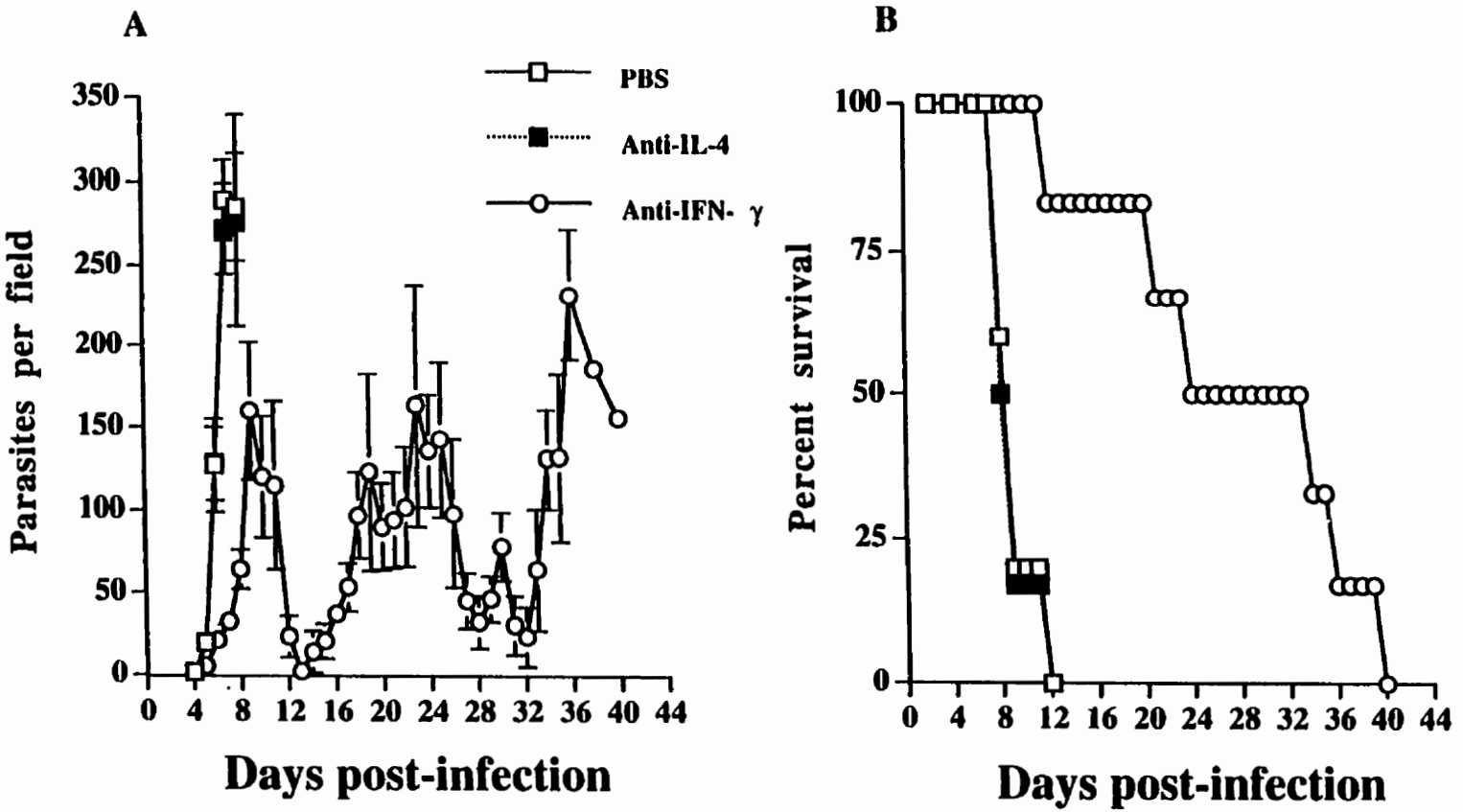

Figure 4.5 Parasitemia (A) and survival period (B) of *T. congolense*-infected BALB/c mice treated with anti-IFN-γ antibodies. Groups of six BALB/c mice were injected i.p. with either PBS (open squares), anti-IL-4 antibodies (closed squares) or anti-IFN-γ antibodies (open circles) as described in materials and methods. Parasitemia was monitored daily by direct assessment of peripheral blood. The results are from one of two similar experiments.

Although anti-IFN-γ-treatments were stopped on day 6 post-infection, more than half of the animals successfully controlled their disease through 3-4 successive waves of parasitemia over the next 25 days, almost eliminating trypanosomes from their blood twice (days 13 and 32; Figure 4.5A). Overall, the anti-IFN-γ-treated animals had substantially fewer circulating parasites (30% decrease; $p < 0.01$), and a dramatically prolonged mean survival period (> 325% prolonged, $p < 0.003$; Figure 4.5B) relative to the PBS or anti-IL-4-treated groups.

4.4.6 *In vivo* neutralization of IFN-γ dramatically reduces plasma IL-10 and spontaneous and Con A-induced secretion of IL-10 by splenocytes from *T. congolense*-infected BALB/c mice

We studied the effects of our in vivo anti-IFN-γ immunotherapy on IL-10 and IFN-γ production in *T. congolense*-infected BALB/c mice for two reasons. First, we have previously shown that anti-IL-10 antibodies completely reverse trypanosomiasis-associated suppression of splenocyte proliferative responses to Con A and also reduce the circulating parasitemia and increase the survival periods of infected BALB/c mice (see chapter 3). Second, although the biology of *T. brucei* infections in mice is substantially different than that of *T. congolense*, and anti-IFN-γ antibody treatments in *T. brucei*-infected mice were not as effective as those observed herein, it was shown in the *T. brucei* system that anti-IFN-γ antibodies inhibited the release of an unidentified soluble factor that is associated with suppression of lymph node cell proliferation from parasite-pulsed macrophages (Darji et al., 1996). We recently have obtained evidence that bone marrow-

derived macrophages from genetically susceptible but not resistant mice, when pulsed simultaneously with IFN-γ and *T. congolense* lysates, increase their secretion of IL-10 by 2-4 fold (Kaushik et al., manuscript in preparation).

Groups of mice were injected i.p. with either PBS or 200 μg of anti-IFN-γ antibodies on days 0, 2 and 4 post-infection. On day 7 the mice were killed and we determined the amounts of IFN-γ and IL-10 produced by the splenocytes *in vitro*. We also measured the levels of IL-10 in the plasma of the infected mice. As shown in Figure 6, anti-IFN-γ antibody treatments reduced the secretion of IFN-γ (Figure 4.6A) and IL-10 (Figure 4.6B) by unstimulated splenocytes and the Con A-induced secretion of IL-10 (Figure 4.6C) by splenocytes from infected mice, as well as the plasma levels of IL-10 (Figure 4.6D).

Because it has been reported that the production of NO by splenic macrophages in *T. brucei rhodesiense*-infected mice was mediated by IFN-γ (Schleifer and Mansfield, 1993) and inhibition of NO synthesis abolished immunosuppression and enhanced the resistance of *T. brucei brucei*-infected mice (Mabbott et al., 1995), we determined whether the beneficial effects of anti-IFN-γ antibodies observed in this study was mediated, in part, by inhibition of NO synthesis. Anti-IFN-γ antibody-treatment of *T. congolense*-infected BALB/c mice surprisingly enhanced the production of NO by splenocytes from these mice on day 7 after treatment relative to untreated controls (41.3 ± 1.3 μM vs. 27.4 ± 0.8 μM, $p < 0.002$; for anti-IFN-γ- and PBS-treated mice respectively).

Figure 4.6 Effects of in vivo administration of anti-IFN-g antibodies on plasma IL-10 and secretion of IL-10 by splenocytes from *T. congolense*-infected BALB/c mice. Groups of 4 infected BALB/c mice were injected with either PBS alone or 200 μg of anti-IFN-γ antibodies as described in materials and methods. On day 7 post-infection, the mice were killed to obtain plasma samples and to establish splenocyte cultures. The results depict unstimulated secretion of IFN-γ (A) and IL-10 (B) and Con A-induced IL-10 secretion (C) by splenocytes, as well as plasma levels of IL-10 (D) in anti-IFN-γ-treated mice. Data are presented as mean ± SE.

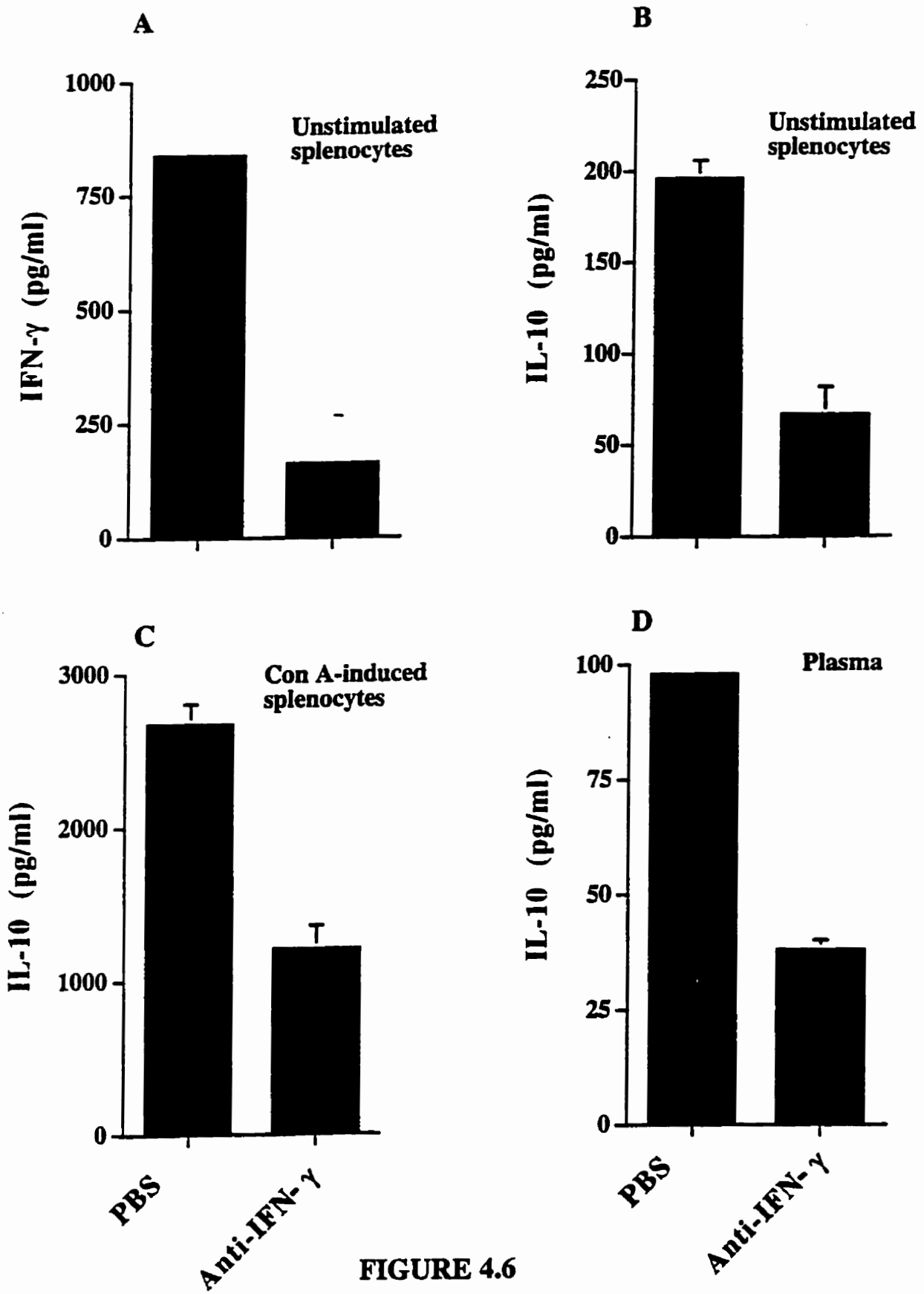
A
1000
750
500
250
0
IFN-γ (pg/ml)
Unstimulated splenocytes
B
250
200
150
100
50
0
IL-10 (pg/ml)
Unstimulated splenocytes
C
3000
2000
1000
0
IL-10 (pg/ml)
Con A-induced splenocytes
PBS
Anti-IFN-γ
D
100
75
50
25
0
IL-10 (pg/ml)
Plasma
PBS
Anti-IFN-γ

FIGURE 4.6

These results unequivocally link IFN-γ activity in infected BALB/c mice with the expression of IL-10. The results, at least in part, imply that the protective effect of anti-IFN-γ antibodies could be associated with the reduction of IL-10 secretion. This observation is consistent with our previous report wherein we demonstrated that IL-10 is a disease-enhancing cytokine in the context of experimental *T. congolense* infections in BALB/c mice (see chapter 3).

4.5 Discussion

The present study clearly shows that endogenously produced IFN-γ plays a disease-enhancing role in experimental *T. congolense* infections in highly susceptible BALB/c mice. The numbers of parasites in the blood were reduced and the survival periods were increased by more than 300% following in vivo injections of neutralizing anti-IFN-γ antibodies into such animals, and anti-IFN-γ antibodies reversed the splenocyte suppression observed within the infected mice *in vitro*. Furthermore, Berenil cure of these animals rapidly cleared the parasitemia and led to a dramatic reduction in the plasma levels and secretion of IFN-γ by splenocytes from infected mice.

Suppression of antigen- and mitogen-driven T cell proliferation in the spleen and lymph nodes is a prominent feature of African trypanosomiasis (Askonas, 1985; Darji et al., 1992; Sileghem et al., 1994a,). This has been suggested as one of the mechanisms responsible for the observed increase in the susceptibility of trypanosome-bearing hosts to opportunistic infections (Greenwood et al., 1973). In mice experimentally infected with *T. brucei brucei*, albeit a significantly different

parasite than *T. congolense*, the suppression of lymph node T cell proliferative responses is associated with endogenously secreted IFN-γ which mediated the observed decrease of IL-2 receptor (IL-2R) expression on $CD4^+$ T cells. In that system, anti-IFN-γ antibodies abolish this *in vitro* lymphoid suppression via the restoration of IL-2R expression (Darji et al., 1993; 1996). However, IFN-γ by itself was not enough to mediate this suppression. An unknown soluble factor(s) released by *T. brucei*-challenged macrophages also was required to fully implement this suppressive effect (Darji et al., 1996). Nitric oxide released by IFN-γ-activated macrophages has been shown to mediate immunosuppression in mice infected with *T. brucei brucei* (Sterberg and McGuigan, 1992; Mabbott et al., 1995) and *T. brucei rhodesiense* (Schleifer and Mansfield, 1993). However, Taylor et al. (1996) showed that NO does not contribute to immunosuppression in *T. congolense* infections in cattle and suggested that the expression of high levels of IL-10 mRNA transcripts in the spleens and lymph nodes in these animals may contribute to the observed immunosuppression in infected cattle. We have previously shown that endogenously produced IL-10 is associated with the suppression of splenocyte proliferative responses to Con A challenge in *T. congolense*-infected mice, with the highly suppressed splenocytes of susceptible BALB/c mice secreting significantly higher levels of this cytokine than the spleen cells of the resistant C57BL/6 mice (chapter 3). Also, anti-IL-10 antibodies completely reversed this *in vitro* suppression of proliferation the BALB/c splenocytes (chapter 3). We have evidence that IFN-γ treatment of bone marrow-derived macrophages from highly susceptible BALB/c (but

not relatively resistant C57BL/6 mice) combined with exposure to *T. congolense* lysates, causes 2-4-fold increases in their production of IL-10 in response to *in vitro* challenge with *T. congolense* (Kaushik et al., manuscript in preparation). In line with this, we observed herein that in vivo injections of anti-IFN-γ antibodies significantly reduced the plasma levels of IL-10 and the secretion of IL-10 by splenocytes from *T. congolense*-infected BALB/c mice. IL-10 down-regulates the expression on antigen-presenting cells, of the costimulatory molecule B7, which is necessary for T cell activation (Moore et al., 1993; Willems et al., 1994). Thus, it is quite likely that the immunosuppressive effect of IFN-γ observed in mice infected with *T. congolense*, could be mediated, at least in part, via IL-10 secreted by macrophages following their interactions with trypanosomes (or their products).

Some aspects of immunosuppression in mice infected with *T. brucei brucei* (Sternberg and McGuigan, 1992) and *T. brucei rhodesiense* (Schleifer and Mansfield, 1993) have been reported to be mediated by NO produced by IFN-γ-activated macrophages. However, Taylor et al (1996) did not observe any NO-mediated immunosuppression in cattle infected with *T. congolense*. Our data clearly indicate that, as in cattle (Taylor et al., 1996), NO does not mediate immunosuppression in BALB/c mice infected with *T. congolense*. This is consistent with our observation that splenocytes from *T. congolense*-infected BALB/c and C57BL/6 mice produce almost similar amounts of NO despite the apparently higher production of IFN-γ and greater immunosuppression in BALB/c than in C57BL/6 splenocytes (Appendix figure 3). Moreover, treatment of infected BALB/c mice with anti-IFN-γ antibodies actually

increased the production of NO by splenocytes from these mice relative to untreated control mice. While on the surface it may be surprising that such marked differences exist between *T. congolense* and *T. brucei*, especially in view of their close structural and molecular similarities, we suspect that, among other things, the inherently invasive nature of *T. brucei* as well as differences in some enzymes and in the strains of mice used could be important distinguishing factors between the two murine models of trypanosomiasis.

In experimental *T. brucei* infections in mice, IFN-γ has been claimed to have a direct growth stimulatory effect on the parasites (Olsson et al., 1991) and as such, the reductions in parasitemia and increases in survival periods following anti-IFN-γ antibody administration were attributed to an inhibition of this stimulatory effect by the antibodies (Bakhiet et al., 1990). We have been unable to demonstrate such a growth-stimulatory effect of IFN-γ on *T. congolense* (Kaushik et al., 1997). The reduction in parasitemia and the enhanced survival of anti-IFN-γ antibody-treated mice reported here would seem more likely to be due to an indirect effect, at least in part, resulting from the reversal of immunosuppression, with a subsequent induction of effective immune response against the parasites. In agreement with this hypothesis, we have recently characterized a population of Thy1.2^{+}CD4^{-}8^{-} cells in the spleens of *T. congolense*-infected BALB/c mice that produce very high amounts of IL-10 and IFN-γ. These antigen-specific cells co-purify with plastic-, nylon wool- and Sephadex G-10-adherent cells and nonspecifically suppress T and B cell responses to mitogens and sheep red blood cess respectively (see chapter 5).

We detected much more TNF-α in the plasma of the relatively resistant C57BL/6 mice than in the highly susceptible BALB/c mice and this difference increased markedly as the infections progressed. The role of TNF-α in the pathogenesis of African trypanosomiasis is not yet clearly known. TNF-α has been associated with a suppression of lymph node cell proliferative responses (Darji et al., 1992), but it has also been shown to be both trypanostatic and trypanolytic for *T. brucei brucei* and *T. brucei rhodesiense in vivo* and *in vitro* (Lucas et al., 1994; Magez et al., 1997). The trypanolytic effect of TNF-α on *T. brucei* occurs via a novel lectin-like affinity receptor on the trypanosomes that is functionally and spatially distinct from the previously characterized mammalian TNF-α receptor binding sites (Lucas et al., 1994). It is undetermined whether *T. congolense* has such binding sites for TNF-α, but the finding that the biologically active form of this cytokine was present at much higher levels in the plasma of the more resistant mice at the time of control of the circulating parasitemia is consistent with its being associated with more effective parasite control and enhanced resistance.

TNF-α mRNA accumulated to higher steady state levels in the livers than in the splenic tissues of all mice and, in addition, the hepatic TNF-α mRNA levels were higher on day 9 post-infection in the resistant C57BL/6 than in the susceptible BALB/c mice. This suggests that much of the TNF-α detected in the circulation could have been of hepatic origin, most probably Kupffer cell-derived. Elimination of trypanosomes from the blood of infected mice is reportedly mediated primarily by Kupffer cells, the primary macrophage population of the liver (MacAskill et al., 1980). Macrophages are a major source of TNF-α (Beutler and Cerami, 1989) and soluble extracts from *T. brucei* have

been reported to induce production of this cytokine by macrophages *in vitro* (Magez et al., 1993), as have the glycophosphatidylinositol (GPI) moieties derived from the variant surface glycoprotein of *T. brucei* (Tachado and Schofield, 1994). IFN-γ-primed peritoneal and bone marrow-derived macrophages from C57BL6 mice, upon interaction with *T. congolense* lysate, secrete significantly higher amounts of TNF-α than similarly treated BALB/c mice macrophages (Kaushik et al., manuscript in preparation).

The simultaneous presence of high levels of IL-4, IL-10 and IFN-γ in the plasma (Tabel et al., 1994) and splenocyte culture supernatants (see chapter 3) from infected BALB/c mice is intriguing, considering the ostensibly dichotomous cross-regulatory effects of these cytokines on TH1 and TH2 cells. The significantly higher levels of IL-4 detected in the plasma and culture supernatants of splenocytes from infected BALB/c mice contrasts sharply with the lack of or very low levels of IL-4 detected in the C57BL/6 mice. According to the contemporary immunologic theory, IL-4 is a major factor in driving the differentiation of uncommitted T-helper cells to the TH2 phenotype (Seder and Paul, 1994), the phenotype presently considered to favor more effective immunologic control of extracellular parasites (Sher and Coffman, 1992). This hypothesis might not necessarily apply to the control of African trypanosomes. We did find that treatment of resistant and susceptible mice with monoclonal antibodies to IL-4 during *T. congolense* infections did not alter their disease. However, it would be prudent to note that alternate explanations for this result could also be feasible, including, for example, inadequate neutralization of the cytokine by the antibodies.

It has been suggested that a balance between IFN-γ and TNF-α secretion in mice infected with *T. brucei* determines the course of parasitemia and the outcome of the disease (Darji, et al., 1992). If so, it is feasible that such a balance could also affect the disease outcome during *T. congolense* infections in mice. Although we presently have no direct evidence for the beneficial effects of TNF-α in *T. congolense*-infected mice, the finding that only this cytokine was elevated in the plasma of the resistant mice suggests an association between high levels of this cytokine and resistance. As mentioned, we have recently found that bone marrow-derived macrophages from resistant mice produce higher amounts of TNF-α upon interaction with *T. congolense* lysate than those from susceptible mice. High levels of IFN-γ could stimulate BALB/c mcrophages, following interaction with trypanosomes or their products, to release copious amounts of IL-10. IL-10, in turn, would contribute to a down-regulation of immune responses (see chapter 3) and TNF-α secretion by macrophages (Moore et al., 1993) leading to increased susceptibility. Conversely, high levels of TNF-α could restrict the parasite growth by its static and lytic effects on the trypanosomes (Lucas et al., 1993; Magez et al., 1993; 1997) thereby limiting the parasite load and allowing the host to mount an effective immune response against the multiplying parasites.

5.0 EXPERIMENTAL MURINE *TRYPANOSOMA CONGOLENSE* INFECTIONS: ROLE OF SPLENIC ADHERENT THY1$^+$CD4$^-$8$^-$ CELLS IN THE PRODUCTION OF IL-4, IL-10 AND IFN-γ AND IN TRYPANOSOME-INDUCED IMMUNOSUPPRESSION.

5.1 Abstract

Trypanosome-induced suppression of T and B cell responses to parasite-related and -unrelated antigens is considered a major mechanism of evasion of the host's immune defenses by the parasite. Reduced T and B cell responses have been attributed to suppressor T cells, suppressor macrophages, or both. We have previously shown that endogenously produced IL-10 and IFN-γ mediate the suppression of T cell responses in *Trypanosoma congolense*-infected mice. Here, we show for the first time that, Thy1.2$^-$CD4$^-$8$^-$ cells that co-purify with plastic-, nylon wool- or Sephadex G-10-adherent cell populations, in synergy with adherent Thy1.2$^-$ cells, are the major producers of IL-4, IL-10 and IFN-γ in *T. congolense*-infected mice. We further demonstrate the involvement of these cells in the suppression of T cell proliferative responses to mitogen and in B cell responses to an unrelated antigen.

5.2 Introduction

African trypanosomes are protozoan parasites that cause disease and death in humans and animals. *Trypanosoma congolense* infections result in severe acute or chronic debilitating disease in cattle and other domestic animals. *Trypanosoma congolense* is an extracellular parasite living in the blood stream of the host and as such, to survive, it must have developed very sophisticated mechanisms to evade the host's immune defenses. Trypanosome-induced immunosuppression is considered one of the major mechanisms of evasion from the host's immune defenses by the parasite (reviewed by Roelants and Pinder, 1984; Askonas, 1985; Darji et al., 1992; Sileghem et al., 1994). Immunosuppression has been described in both natural and experimental infections of domestic mammals as well as laboratory rodents and is suggested to be responsible for poor responsiveness of trypanosome-bearing hosts to vaccinations (Greenwood et al., 1973). Lymphocytes from infected hosts give a lower response to T cell mitogens or allogeneic cells *in vitro* (Sileghem et al., 1994). Both *in vivo* (Murray et al., 1977) and *in vitro* (Eardley and Jayawardena, 1977; Jayawardena and Waksman, 1977) T cell-dependent and T cell-independent B cell responses are suppressed. Similarly, suppression of antigen-specific T cell proliferative responses to unrelated antigens has been reported in infected animals (Darji et al., 1996). There is as yet no consensus about the mechanisms underlying trypanosome-induced suppression of lymphoid cells. Reduced T and B cell responsiveness has been attributed to suppressor T-cells (Eardley and Jayawardena, 1977; Jayawardena et al., 1978), suppressor macrophages (Wellhausen and Mansfield, 1979; 1980; Askonas, 1985; Sileghem et al., 1986; Flynn et al., 1991, Schliefer and Mansfield,

1993), or a combination of these cell types (Corsini et al., 1977; Roelants and Pinder, 1984).

In response to trypanosomal antigens generated during infection, the host's immune system produces an array of molecules including cytokines. In *T. brucei* infections, endogenous IFN-γ produced by $CD8^+$ T cells has been associated with the downregulation of lymph node cell proliferative responses to mitogens (Darji et al., 1996) and enhanced susceptibility to the disease (Bakhiet et al., 1990; Olsson et al., 1991). Depletion of $CD8^+$ cells has been shown to suppress both the growth of *T. brucei* and IFN-γ secretion in infected rodents (Bakhiet et al., 1990). In this system, it was reported that *T. brucei* releases a molecule that binds to the CD8 molecule on T cells and polyclonally activates the $CD8^+$ T cells to secrete copious amounts of IFN-γ, which, in turn, has been reported to stimulate parasite growth (Olsson et al., 1991).

BALB/c mice are highly susceptible to experimental *T. congolense* infections while C57BL/6 mice are relatively resistant, as measured by the degree and pattern of parasitemia and survival period (Otesile and Tabel, 1987; Ogunremi and Tabel, 1995). Genetic analysis indicates that the efficiency of control of the first wave of parasitemia in mice infected with *T. congolense* correlates with the survival period (Ogunremi and Tabel, 1995). The patterns of cytokine responses during some parasite infections determine or are at least strongly correlated to susceptibility of the host (Sher and Coffman, 1992). We have recently shown that during experimental *T. congolense* infections, the plasma of the highly susceptible BALB/c mice as well as the supernatant fluids of their spleen cell cultures contain significantly higher levels of IL-4, IL-10 and

IFN-γ than those from resistant C57BL/6 mice (see chapters 3 and 4). In addition, we found that IL-10 and IFN-γ contributed to the suppression of splenocyte proliferative responses to concanavalin A (Con A) in these mice (see chapters 3 and 4). Furthermore, *in vivo* neutralization of these cytokines significantly enhanced the resistance of the highly susceptible BALB/c mice to *T. congolense* infections, as measured by reductions in parasitemia and increased mean survival periods. However, in contrast to the report on *T. brucei* (Olsson et al; 1991), we have been unable to demonstrate any growth stimulatory effect of either IFN-γ or IL-10 on *T. congolense* (Kaushik et al., 1997), indicating that the beneficial effects of *in vivo* neutralization of these cytokines were indirect, at least in part due to a reversal of immunosuppression. Here, we show that in contrast to *T. brucei* infections, most of the IL-4, IL-10 and IFN-γ production in *T. congolense*-infected BALB/c mice is mediated by small numbers of $Thy1.2^+CD4^+8^-$ and $Thy1.2^+CD4^-8^-$ cells that co-purify with plastic-, nylon wool- or Sephadex G-10-adherent cells. We also show that the secretion of these cytokines requires cooperation between $Thy1.2^+$ and $Thy1.2^-$ cells in the spleens of infected mice rather than by polyclonal activation involving the direct binding of *T. congolense*-derived molecules to T cells as was reported for *T. brucei* (Olsson et al., 1991). We further demonstrate that these cells are involved in the *in vitro* suppression of B cell responses to a T cell-dependent antigen as well as in the suppression of T cell proliferative responses to Con A.

5.3 Material and Methods

5.3.1 Mice

Female BALB/cAnNCrlBR (BALB/c) and outbred CD1 mice were obtained from the Animal Resource Centre of the University of Saskatchewan. BALB/c mice were between 8 to 10 weeks and CD1 mice were 5-6 weeks old. All mice were maintained according to the recommendations of the *Guide to the Care and Use of Experimental Animals* of the Canadian Council for Animal Care.

5.3.2 Parasites

The origin of the *T. congolense*, variant antigenic type (VAT) TC13 used in the present study has been previously described (Otesile and Tabel, 1987). Cloned trypanosome populations were stored as frozen stabilates in liquid nitrogen. Parasites were passaged in CD1 mice as previously described (Tabel, 1982). The parasites for infection of BALB/c mice were isolated from the blood of CD1 mice three days after passage by DEAE anion exchange chromatography (Lanham and Godfrey, 1970).

5.3.3 Experimental design

BALB/c mice were infected intraperitoneally (i.p.) with 10^3 organisms of *T. congolense* VAT TC13 in 0.1 ml of Tris-saline-glucose supplemented with 10% heat inactivated fetal bovine serum (FBS). Infected mice had a prepatent period of 4 days and developed a fulminating and uncontrollable parasitemia that peaked on day 7-9 post-infection. Their mean survival period was 8 ± 0.5 days. On day 7 post-infection, groups of 6-8 infected and control uninfected mice were killed with CO_2 and their spleens were made into single

cell suspensions by teasing in complete medium. This medium consisted of RPMI-1640 (Sigma, Oakville, ON) supplemented with 10% FBS, 2 mM L-glutamine, 50 mM 2-mercarptoethanol and 100 I.U. each of penicillin and streptomycin (Gibco, Life Technologies, Grand Island, NY).

5.3.4 Depletion of splenocyte subsets

One ml aliquots of a splenocyte suspension containing 10^7 cells/ml in complete medium were added to polypropylene tubes containing 50 µl of purified monoclonal rat antibody to mouse CD4 (GK 1.5, American Type Tissue Collection, [ATCC], Rockville, MD), CD8 (TIB 211, ATCC) or Thy1.2 (TIB 99, ATCC) and incubated on ice for 45 min. After 2 washings in RPMI-1640 medium supplemented with 5% FBS (RPMI-5), the cells were resuspended in 1 ml RPMI-5 containing 100 µl of LOW-TOX-M rabbit complement (Cedarlane, Hornby, ON) and incubated at 37°C for 45 min. The cells were washed 2 times, resuspended in complete medium, counted and aliquots were stained either with FITC-anti-mouse CD4, PE-anti-mouse CD8 or PE-anti-mouse Thy1.2 (PharMingen, San Diego, CA) antibodies for FACS analysis. In all cases, $CD4^+$, $CD8^+$ and $Thy1.2^+$ cells depletions were $\geq$ 99%. The percentages of non-target cells ($CD8^+$ in case of $CD4^+$ depletions and vice versa) were not significantly affected by these treatments. Whereas depletion of $Thy1.2^+$ cells resulted in the complete loss of detectable $CD8^+$ cells, between 1.2-1.8% of $CD4^+$ cells were stained with anti-CD4 antibodies.

5.3.5 Separation of cells by adherence or non-adherence to plastic dishes

Splenocytes from infected mice were separated into plastic-adherent and non-adherent populations as described by Khan et al., (1995), with minor modifications. Briefly, 15 ml of cells (10^7/ml) in complete medium were seeded into 20 x 100 mm tissue culture-treated plastic petri dishes (Falcon #3001, VWR, Edmonton, AB) and incubated at 37°C in a humidified atmosphere containing 5% $C0_2$. After 3 hr, the non-adherent populations were collected and the petri dishes were washed 3 times with medium. The adherent cells were dislodged with ice-cold versene (0.02% EDTA in phosphate buffered saline [PBS], pH 7.2) and gentle scraping. Once separated, the cells were washed 2 times with complete medium and the purity was determined by staining a cytospin preparation for non-specific esterase (Ennist and Jones, 1983) and Giemsa stain. The adherent cells were greater than 85% macrophages as assessed by their microscopic morphology. The percentage of contaminating T cells as assessed by FACS analysis was less than 8% on all occasions.

5.3.6 Enrichment of T cells by non-adherence to nylon and Sephadex G-10

Nylon wool and Sephadex G-10 columns were prepared according to the methods described by Hathcock (1994a,b). Briefly, 8 ml of packed sterile nylon wool in 10 ml syringes and 30 ml of packed sterile Sephadex G-10 (Pharmacia, Uppsala, Sweden) in 35 ml syringes were equilibrated by running 50 ml complete medium through each column followed by incubation for 45 min at 37°C. Two milliliters and 5 ml of a pre-warmed (37°C) single-spleen cell suspension in complete medium (5 x 10^7 cells/ml) were applied to nylon wool or Sephadex G-10 columns, respectively and the columns were incubated for 30 min at 37°C. Non-adherent (i.e. effluent) cells were obtained by washing the

columns with 15 ml (nylon wool) or 35 ml (Sephadex G-10) of complete medium. The non-adherent cells were ≥ 80 and 65% (nylon wool and Sephadex G-10 respectively) Thy1.2$^+$ as assessed by FACS. To obtain the adherent cells, the nylon wool or the Sephadex G-10 slurry was dislodged from each column, flooded with warm medium, stirred for 2 min with a pipette and then incubated for 10 min at room temperature. The dislodged cells were washed 2 times; their viability, as assessed by trypan blue dye exclusion, was greater than 95%. The adherent cells from all columns contained less than 8% Thy1.2$^+$ cells as assessed by FACS.

5.3.7 Positive enrichment of CD4$^+$ and Thy1.2$^+$ cells

Enrichment of CD4$^+$ and Thy1.2$^+$ cells was done by positive selection using rat anti-mouse CD4 or rat anti-mouse Thy1.2 antibodies coupled to paramagnetic beads respectively (Miltenyi Biotec, Auburn, AL) according to the manufacturer's suggested protocols. Briefly, 1 ml aliquots of a single spleen cell suspension (4 x 10^7/ml and 3.0 x 10^7/ml for CD4$^+$ and Thy1.2$^+$ enrichments respectively) were washed 2 times with buffer (PBS supplemented with 5% FBS and 2 mM EDTA, pH 7.2) and resuspended in 0.5 ml of buffer. Then, 80 µl rat anti-mouse Thy1.2 antibodies attached to paramagnetic beads (Miltenyi) were added to each tube, which was incubated for 20 min at 4°C. After washing, the cells were resuspended in 0.5 ml of buffer and applied onto a MiniMACS Type MS+ column (Miltenyi) attached to a magnet. Non-adherent (i.e. negative) cells were collected by washing the column while still in contact with the magnet with 2.5 ml of buffer. To obtain the eluate (i.e. marker-positive) cells, the column was separated from

the magnet and bound cells were eluted by flushing 2 ml of buffer through the column with a plunger. Both effluent and eluate cells were washed 2 times with complete medium; their viability, as assessed by trypan blue dye exclusion, was always greater than 99%. Positively enriched cells were greater than 97% positive for their respective markers, as assessed by FACS analyses. The negative (effluent) populations contain between 5-8% Thy1.2$^+$ cells.

5.3.8 FACS analysis

One hundred microliter aliquots of 10^7 cells/ml in complete medium were incubated for 15 min on ice with 10 µl of rat anti-mouse CD16/CD32 (Fcγ II/III Receptor, Fc Block™, PharMingen) monoclonal antibody. Then 10 µl of appropriate antibodies diluted 1:5 in FACS wash solution (2% BSA in PBS, pH 7.2) was added and incubated for 30 min at 4°C. The cells were washed 3 times with FACS wash, resuspended in 100 µl of FACS wash and fixed by adding 100 µl of 2% paraformaldehyde. Fixed cells were analyzed using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA).

5.3.9 Assay for cooperation of cells and antigen specificity of cytokine secretion

Splenocytes from both normal and *T. congolense*-infected BALB/c mice were separated into Thy1.2$^+$ (eluate) and Thy1.2$^-$ (effluent) populations by positive selection using anti-Thy1 antibody-coated paramagnetic beads (above). The effluent cells, containing between 5-8% of Thy1.2$^+$ cells, were further depleted of the residual contaminating Thy1.2$^+$ cells by treatment with anti-Thy1.2 antibodies and complement. This treatment removed all

detectable Thy1.2$^+$ cells from the effluent populations as assessed by FACS analysis. Positively-enriched Thy1.2$^+$ cells were cultured either alone or in combination with varying concentrations (2.5-20%) of Thy1.2$^-$ cells.

In another series of experiments, cultures of enriched Thy1.2$^+$ cells from infected or uninfected mice were supplemented with whole spleen cells from uninfected mice completely depleted of Thy1.2$^+$ cells by treatment with anti-Thy1.2 antibodies and complement (APC). The cultures were subsequently pulsed with 10^6 trypanosome-equivalent of lysates obtained by subjecting parasites isolated by DEAE-cellulose chromatography (Lanham and Godfrey, 1977) from the blood of immunosuppressed CD1 mice through 3 cycles of freezing and thawing at -80°C.

5.3.10 Splenocyte cultures

Splenocytes were cultured at a concentration of 2.5 x 10^6 cells/ml in 200 µl volumes in 96-well tissue culture plates in a humidified incubator at 37°C, 5% CO_2 atmosphere. All cultures were harvested after 48 hr, centrifuged at 1,500 x g for 15 min and the supernatant fluids were stored at -35°C until used.

5.3.11 Cytokine assays

The levels of IL-4, IL-10, and IFN-γ in the splenocyte culture supernatants were determined by routine sandwich ELISA, according to the manufacturer's suggested protocols. Recombinant murine IL-4, IL-10 and IFN-γ used to generate standard curves and paired antibodies against murine IL-4, IL-10, and IFN-γ for sandwich ELISA assays

were produced by PharMingen Inc. (San Diego, CA) and purchased from Cedarlane Inc. (Hornby, ON). The limits of detection of the ELISAs were 15 pg/ml for IL-4 and 31 pg/ml for IL-10 and IFN-γ. All experiments were repeated at least two times.

5.3.12 Splenocyte proliferation assay

Splenocyte proliferation was quantified by the [^{3}H]TdR uptake assay. Briefly, cells from uninfected and 7-day infected mice were cultured in 200 µl complete medium at a density of 3 x 10^5 cells/well in 96-well flat bottom plates (Falcon®, VWR, Edmonton, AB). Quadruplicate cultures of pooled cells from groups of 4 mice were performed. To assess the suppressive effects of Sephadex G-10-adherent cells, co-cultures of 2 x 10^5 spleen cells from uninfected mice and 10^5 undepleted or Thy1.2$^+$ depleted adherent cells from infected mice were performed. Cells were cultured either with or without Con A (5 µg/ml). After 48 hr, the cultures were pulsed overnight with 0.5 µCi [^{3}H]TdR (1 mCi/ml; Amersham, Chicago, IL) and cells were harvested with an automated cell harvester (PHD cell harvester; Cambridge Technology Inc., Watertown, MA). Incorporation of [^{3}H]TdR was determined with a scintillation counter.

5.3.13 Assay for hemolytic plaque forming cells (PFC)

B cell responses to sheep red blood cells (SRBC) were assayed by PFC using the slide modification of Jerne technique (Dresser, 1978). Groups of mice (4 mice per group) were primed intravenously with 0.1% SRBC in PBS and boosted two days later with 10% SRBC. Seven days after primary immunization, the mice were killed, the spleens

collected and suspensions of single-spleen cells prepared. The cells (10^6/well) were cultured in 200 µl volumes in 96-well tissue culture plates in the presence or absence of SRBC (2.5 x 10^5/well). Some cultures received additional 2.5 x 10^5 undepleted or Thy1.2$^+$-depleted Sephadex G-10-adherent cells from uninfected or infected BALB/c mice. After four days of *in vitro* culture, the cells were harvested, washed and used for PFC assay against SRBC.

5.3.14 Statistical analysis

Data are presented as mean ± standard error (SE). Significance of differences was determined by Student's t-test using the StatView™ SE Software (Abacus Concepts Inc, Berkeley, CA).

5.4 Results

5.4.1 Depletion of Thy1.2$^+$ or CD4$^+$, but not CD8$^+$, cells abolishes spontaneous secretion of IL-4, IL-10 and IFN-γ by splenocytes from *T. congolense*-infected BALB/c mice

In agreement with our previous results (chapters 3 and 4), unstimulated splenocytes from BALB/c mice infected with *T. congolense* for 7 days, but not spleen cells from uninfected BALB/c mice, when cultured for 48 hr, secreted considerable amounts of IL-4, IL-10 and IFN-γ (Figure 5.1A-C). To determine which cell types were associated with the secretion of these cytokines by the splenocytes of infected mice, we carried out complement-mediated lysis of CD4$^+$, CD8$^+$ or Thy1.2$^+$ cells using monoclonal antibodies directed against these cell surface molecules. In each case, the treatment resulted in greater than 99% depletion of the appropriate cell type as assessed by FACS. Depletion of CD4$^+$ cells completely abolished the spontaneous secretion of IL-4 and IFN-γ and reduced (by $\geq$ 80%) the secretion of IL-10 by splenocytes from infected mice (Figure 5.1A-C). Depletion of CD8$^+$ cells had no significant effect on IL-10 and IFN-γ production but caused a minimal and significant increase ($\geq$ 25%; $p < 0.05$) in IL-4 production. Depletion of CD4$^+$ and CD8$^+$ cells together or depletion of Thy1.2$^+$ cells also effectively abolished the production of these cytokines (Figure 5.1A-C). These results clearly indicate that

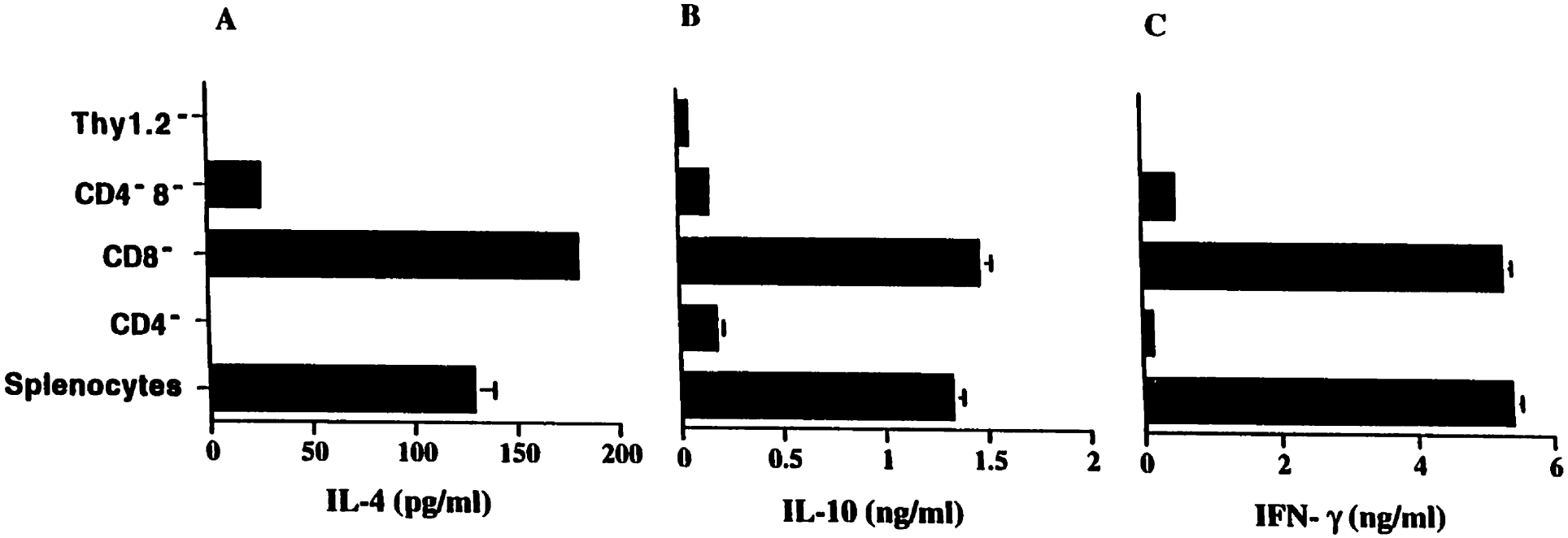

Figure 5.1 Production of cytokines by splenocytes from *T. congolense*-infected BALB/c mice. Whole spleen cell cultures (splenocytes) from infected mice spontaneously secrete IL-4, (A), IL-10 (B) and IFN-γ (C). Depletion of $Thy1.2^+$ or $CD4^+$ cells abolished the spontaneous secretion of these cytokines while depletion of $CD8^+$ cells had either minimal or no effect. Single-cell suspensions of spleens from BALB/c mice infected with *T. congolense* for 7 days were treated with monoclonal antibodies to CD4, CD8, CD4 and CD8 or Thy1.2 and complement. In all cases, depletion of the target cells was ≥ 99% as assessed by FACS analyses. Treated or untreated cells were cultured without mitogens at a density of 5×10^5/well in 96-well tissue culture plates. After 48 hr, the supernatant fluids were collected and assayed for IL-4, IL-10 and IFN-γ by ELISA. Parallel cultures of splenocytes from uninfected mice do not produce detectable amounts of IL-4, IL-10 or IFN-γ. The set of results presented is one of three similar experiments.

Thy1.2$^+$CD4$^+$8$^-$ cells are involved in the regulation and/or secretion of IL-4, IL-10 and IFN-γ by splenocytes from *T. congolense*-infected BALB/c mice. These results are in contrast with previous reports that showed CD8$^+$ T-cells to be the main producers of IFN-γ in experimental *T. brucei* infections in mice (Olsson et al., 1991; Darji et al, 1996).

5.4.2 Nylon wool- or Sephadex G-10-enriched T cells from the spleens of *T. congolense*-infected BALB/c mice do not secrete IL-4, IL-10 and IFN-γ

The above results suggested the possibility that conventional T-cells of the helper phenotype were the main sources of IL-4, IL-10 and IFN-γ during *T. congolense* infections in BALB/c mice. If this were the case, T cell-enriched splenocyte populations would be expected to produce much more of these cytokines than non-T cell-enriched splenocytes. We carried out T-cell enrichment by passing splenocytes from infected mice either through nylon wool or Sephadex G-10 columns (Hathcock, 1994a,b). FACS analyses of the effluent (non-adherent) cells showed the expected increases in the percentages of Thy1.2$^+$, CD4$^+$ and CD8$^+$ cells with fewer than 20% contaminating non-T cells. These cells also proliferated strongly (3-5-fold more than unfractionated controls) and secreted large amounts of IL-10 and IFN-γ in culture in response to stimulation with concanavalin A (Con A; data not shown). The enriched T cells were cultured for 48 hr and the culture supernatants assayed for IL-4, IL-10 and IFN-γ. Surprisingly, the T cell-enriched spleen cell populations from infected mice almost entirely lacked the ability to secrete these cytokines when cultured without mitogen (Figure 5.2A-C), suggesting that

the responsible cytokine-secreting cells were effectively removed by passage through these columns.

5.4.3 IL-4-, IL-10- and IFN-γ-producing cells co-purify with cell populations adherent to plastic, nylon wool or Sephadex G-10

Because the nylon wool- or Sephadex G-10-enriched T cells from infected mice failed to secrete IL-4, IL-10 or IFN–γ when cultured without mitogen stimulation, we hypothesized that the cytokine-producing cells have co-purified with the column-adherent cell populations. To test this, we allowed splenocytes from BALB/c mice infected for 7 days to adhere either to plastic petri dishes, nylon wool or Sephadex G-10, and then cultured both the non-adherent and adherent cells for 48 hr and assayed their supernatants for IL-4, IL-10 and IFN–γ. As noted above, the production of IL-4, IL-10 and IFN-γ by cells non-adherent to nylon wool, Sephadex G-10 or plastic was again dramatically reduced compared with that by untreated whole splenocyte suspensions from infected mice (Figure 5.3A-C). In contrast, each of these cytokines was produced at high levels by the adherent cells (Figure 5.3). Indeed, the plastic-adherent spleen cells produced 3-4 fold more IL-4 and 1.5 fold more IL-10 and IFN-γ than did the unfractionated spleen cells (Figure 5.3A-C); and nylon wool- or Sephadex G-10-adherent cells produced more IL-10 and as much IFN-γ as did the unfractionated cells (Figure 3). Neither plastic-, nylon wool- nor Sephadex G-10-adherent cells from normal uninfected mice produced detectable levels of

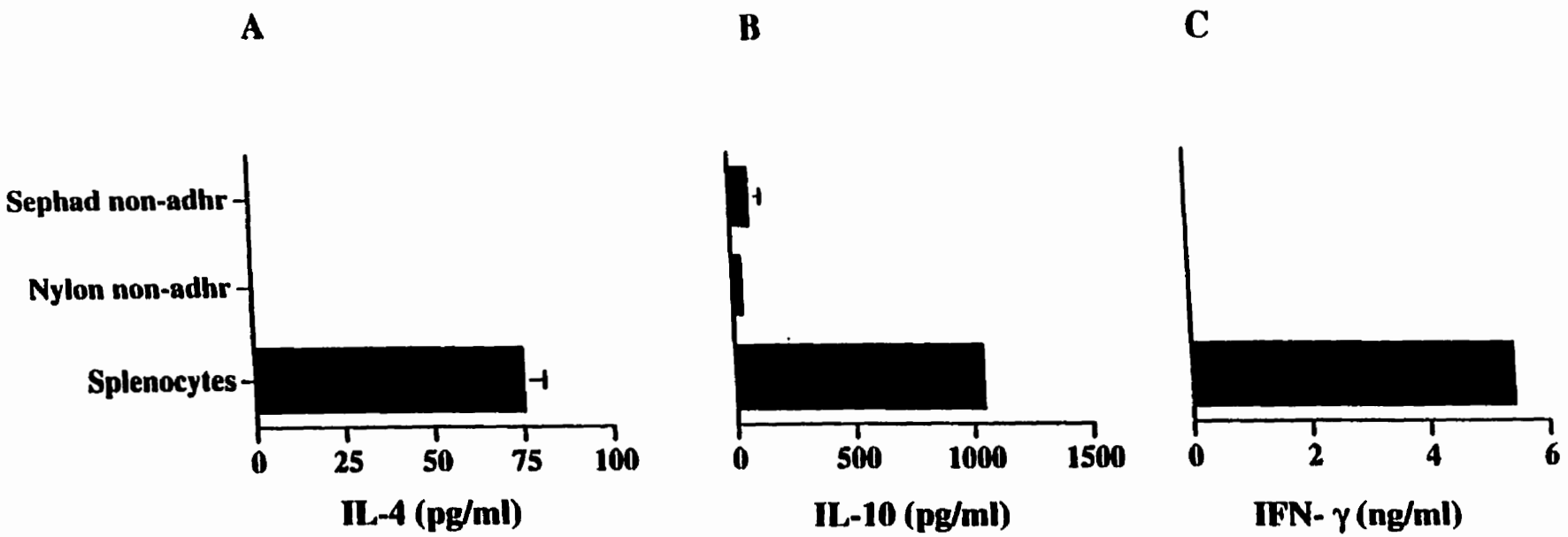

Figure 5.2 Nylon wool- or Sephadex G-10-enriched T cells from the spleens of *T. congolense*-infected mice do not produce IL-4 (A), IL-10 (B) or IFN-γ (C) spontaneously *in vitro*. Single-cell suspension of spleens from BALB/c mice infected with *T. congolense* for 7 days were passed through nylon wool or Sephadex G-10 columns and the effluent (non-adherent) cells were collected. Whole spleen cell suspensions (splenocytes) of infected mice or column-fractionated spleen cell suspensions, i.e. nylon wool effluent (nylon non-adhr) and Sephadex G-10 effluent (sephad non-adhr) were cultured at 5 x 10^5/well in a 96-well tissue culture plates without mitogens. After 48 hr, the supernatant fluids were collected and assayed for IL-4, IL-10 and IFN-γ by ELISA. The set of results presented is one of three similar experiments.

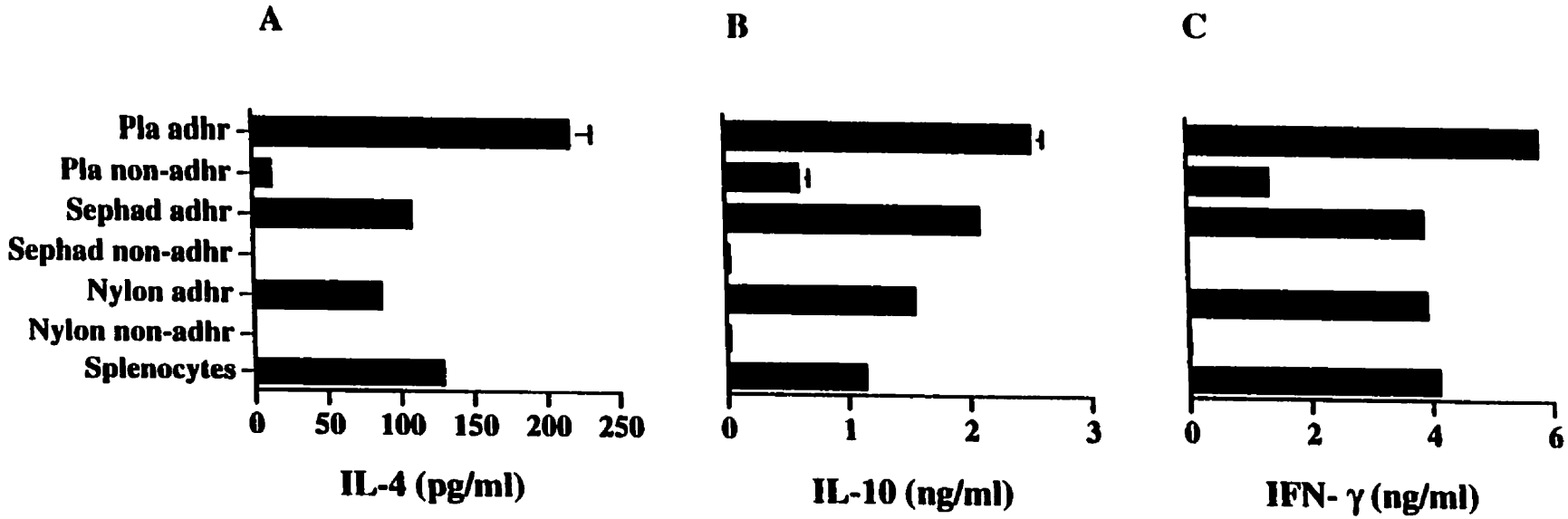

Figure 5.3 IL-4 (A), IL-10 (B) and IFN-γ (C) producing cells co-purify with plastic-, nylon wool- and Sephadex G-10-adherent cell populations. Splenocytes from BALB/c mice infected with *T. congolense* for 7 days were separated into adherent and non-adherent populations by incubating on a plastic petri dish for 3 hr or passage over nylon wool or Sephadex G-10 columns (materials and methods). Plastic-adherent (Pla-adhr) and -non-adherent (Pla non-adhr), as well as nylon wool- or Sephadex G-10-adherent (nylon adhr or sephad adhr respectively) and -non-adherent (nylon non-adhr or sephad non-adhr respectively) cells were cultured at a density 5 x 10^5/well without mitogens. Cultures of unfractionated spleen cell suspensions (splenocytes) from infected BALB/c mice were also included. After 48 hr, the supernatant fluids were collected and assayed for IL-4, IL-10 and IFN-γ by ELISA. Plastic-, nylon wool- or Sephadex G-10-adherent or -non-adherent cells from uninfected mice do not secrete any detectable amounts of IL-4, IL-10 or IFN-γ in cultures (data not shown). The set of results presented is one of three similar experiments.

IL-4 (< 15 pg/ml), IL-10 or IFN-γ (< 31 pg/ml) in culture, indicating that the secretion of these cytokines was dependent on the *T. congolense* infection.

5.4.4 Thy1.2$^+$CD4$^+$8$^-$ and Thy1.2$^+$CD4$^-$CD8$^-$ adherent cells produce and/or regulate the production of IL-4, IL-10 and IFN-γ by splenocytes of *T. congolense*-infected BALB/c mice

Since depletion of CD4$^+$ and Thy1.2$^+$ cells from whole splenocytes of *T. congolense*-infected mice resulted in abolition or marked reduction in IL-4, IL-10 and IFN-γ production, it was surprising that cells that co-purified with plastic- or matrix-adherent cell populations were the major producers of these cytokines. Adherent cells of normal mice reportedly comprise mostly macrophages and few other accessory cells (Hatchcock 1994b), none of which secrete IL-4 or IFN-γ. We then analyzed the adherent cells from infected mice by FACS to assess their expression of each T cell marker, and found that they contained 5-8% Thy1.2$^+$ cells. We therefore repeated the plastic-adherent cell purification, but subsequently depleted the purified adherent cells of residual CD4$^+$, CD8$^+$ or Thy1.2$^+$ cells by complement-mediated lysis and then assessed the abilities of the remaining adherent cells to produce IL-4, IL-10 and IFN-γ (Figure 5.4A-C). The depletion procedures were ≥ 99% effective in removing the target cell populations, as determined by FACS analyses. Depletion of CD4$^+$ cells substantially but incompletely reduced IL-4, IL-10 and IFN-γ production by the adherent splenocytes. Depletion of CD8$^+$ cells had no

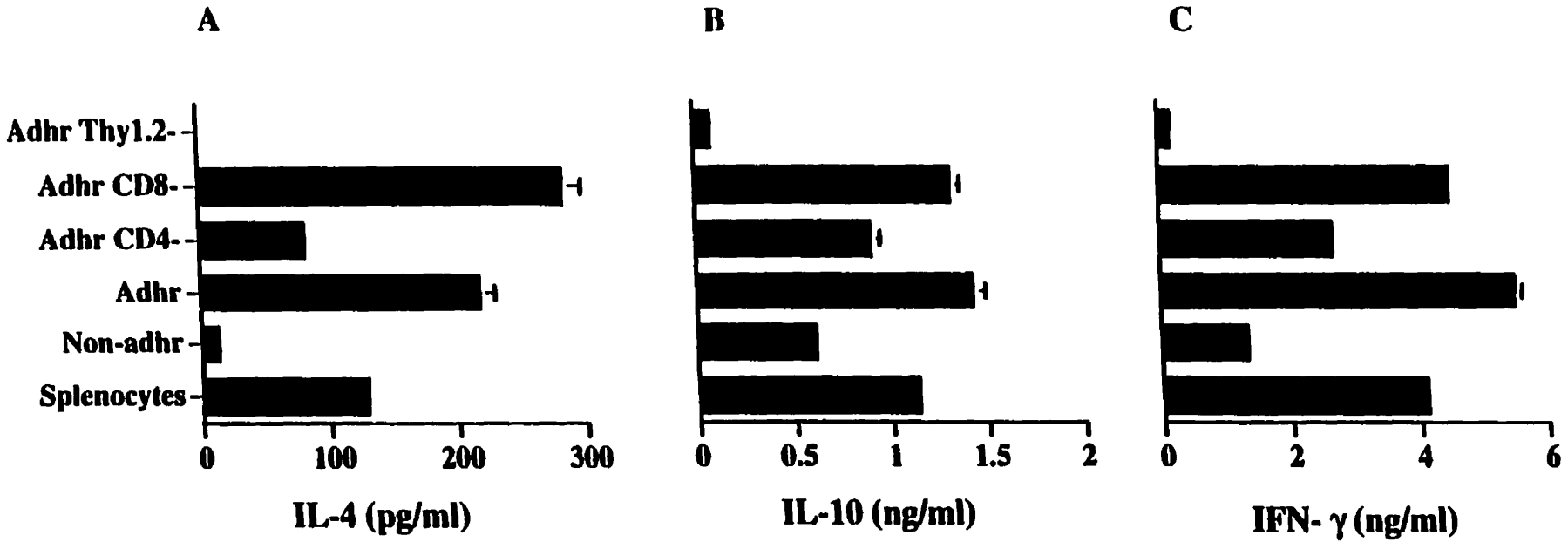

Figure 5.4 Adherent Thy1.2$^+$CD4$^+$8$^-$ and Thy1.2$^+$CD4$^-$8$^-$ secrete IL-4 (A), IL-10 (B) and IFN-γ (C). Splenocytes from BALB/c mice infected for 7 days were separated into plastic-adherent (Adhr) and non-adherent (Non-adhr) populations as described in materials and methods. The adherent populations were depleted of CD4$^+$, CD8$^+$ or Thy1.2$^+$ cells by treatment with anti-CD4, anti-CD8 or anti-Thy1.2 antibodies and complement respectively. This treatment resulted in ≥ 99% depletion of target cells. The resulting cell populations as well as whole spleen-cell suspensions (splenocytes) from infected mice were then cultured without mitogens at a density of 5 x 10^5/well. After 48 hr, their supernatant fluids were collected and assayed for IL-4, IL-10 and IFN-γ by ELISA. Depletion of CD4$^+$ cells caused a reduction in the level of secretion of these cytokines while depletion of CD8$^+$ cells had either no or minimal effect. Depletion of Thy1.2$^+$ cells lead to a total abolition in secretion of these cytokines. Similar results were obtained with either nylon wool- or Sephadex G-10-adherent cells. The set of results presented is one of two similar experiments.

effect on IL-10 production, but increased significantly the level of IL-4 secretion ($p < 0.01$) and minimally decreased the IFN-γ secretion ($p < 0.05$) by the plastic-adherent cells. In contrast, depletion of Thy1.2$^+$ cells completely abolished the secretion of each of these cytokines by the adherent cells.

To find out whether the cytokine-secreting Thy1.2$^+$ cells that co-purified with the adherent cell populations could be replaced by conventionally purified T cells (which were $\geq$ 80% Thy1.2$^+$ as assessed by FACS), we depleted our plastic-adherent cells from infected mice of Thy1.2$^+$ cells but then added back 10% of T cells (i.e. nylon non-adherent cells) and again assessed the abilities of the T cell-reconstituted Thy1.2-depleted plastic-adherent spleen cells to secrete IL-4, IL-10 and IFN-γ. This T cell reconstitution restored only about 3% of the IFN-γ synthesis (320 $\pm$ 25 pg/ml versus 10,500 $\pm$ 490 pg/ml, $p < 0.001$; for Thy1.2-depleted T cell reconstituted adherent and undepleted adherent cells respectively) and had no restorative effects on IL-4 and IL-10. Thus, the Thy1.2$^+$ cytokine-secreting cells among the adherent cell populations of the infected mice appear to be of a unique phenotype.

Since Thy1.2$^+$ cell depletion completely ablated cytokine production, but CD4$^+$ depletion only moderately affected IL-4, IL-10 and IFN-γ secretion, the data suggest that there exists among the adherent cells a small but important sub-population of Thy1.2$^+$, CD4$^-$, CD8$^-$ cytokine-secreting cells. Our results with the CD8$^+$ cell depletion experiments also suggest that CD8$^+$ cells among the adherent populations might negatively affect IL-4 production by these cells.

5.4.5 IL-4, IL-10 and IFN-γ production requires synergy of Thy1.2$^+$ and Thy1.2$^-$ cells

An apparent paradox associated with African trypanosomiasis is the observation that despite the impairment of their proliferative capacity, splenocytes and lymph node cells from infected animals secrete large amounts of cytokines, particularly IFN-γ (Sileghem et al., 1989; Olsson et al., 1991; Schleifer et al., 1993; Darji et al., 1996; chapters 3 and 4). This has led to the suggestion that the cytokine-producing cells are induced or activated in an antigen non-specific manner, perhaps by a mechanism such as polyclonal T cell activation mediated by a direct interaction of trypanosome-derived molecules with the T cells (Olsson et al., 1993). To address this issue, we carried out positive selection of CD4$^+$ and Thy1.2$^+$ cells from splenocytes of *T. congolense*-infected BALB/c mice by using columns of magnetic beads coated with specific antibodies (MACS technology; see below). Positively enriched cells were ≥ 97% pure for the CD4$^+$ or Thy1.2$^+$ markers respectively as assessed by FACS analyses. As shown in Figure 5.5, neither the MACS Thy1.2-positive nor -negative populations secreted substantial amounts of IL-4, IL-10 or IFN-γ relative to the unfractionated spleen cells suggesting that the production of these cytokines by the Thy1.2$^+$ cells might require cooperation with Thy1.2$^-$ cells.

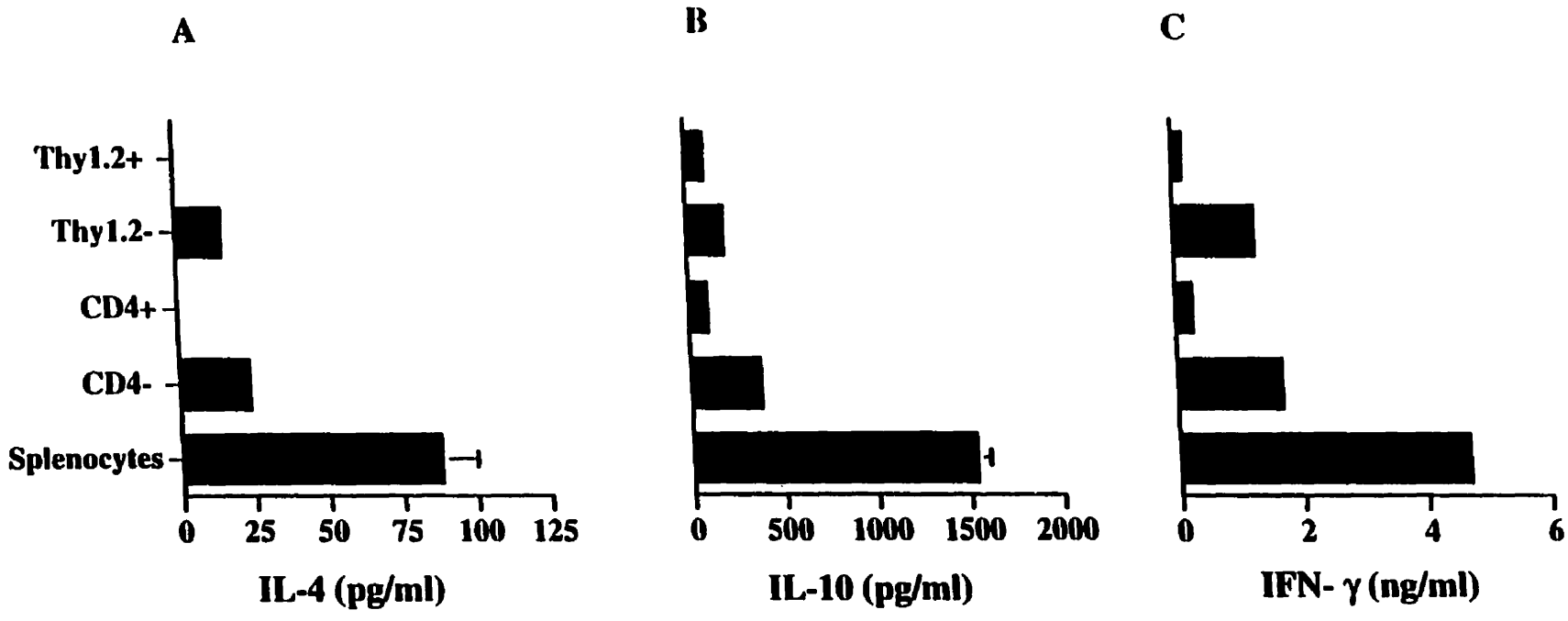

Figure 5.5. Enriched Thy1.2$^+$ and CD4$^+$ splenocytes of *T. congolense*-infected mice lacked the ability to secrete IL-4 (A), IL-10 (B) and IFN-γ (C). Single-cell suspensions were prepared from the spleens of BALB/c mice infected with *T. congolense* for 7 days. Positive selection of Thy1.2 and CD4 cells was carried out with paramagnetic beads coated with antibodies to Thy1.2 and CD4 respectively. The enriched cells were ≥ 97% pure for their respective target cells as assessed by FACS analyses. Positive (Thy1.2$^+$) and negative (Thy1.2$^-$) cells as well as whole spleen cell suspensions (splenocytes) from infected mice were cultured without mitogens at a density of 5 x 10^5/well in a 96-well tissue culture plate. After 48 hr, the supernatant fluids were collected and assayed for IL-4, IL-10 and IFN-γ by ELISA. The set of results presented is one of three similar experiments.

In another set of experiments, Thy1.2$^+$ cells were cultured either alone or in the presence of varying concentrations of Thy1.2-negative cells which had been completely depleted of all residual Thy1.2$^+$ cells by treatment with anti-Thy1.2 antibodies and complement. Supplementation of positively-selected Thy1.2$^+$ cells from infected mice with Thy1.2$^-$ cells derived from the spleens of infected (Figure 5.6), but not uninfected (data not shown), mice resulted in a dose-dependent restoration of cytokine production by the T cells. Thy1.2$^-$ cells from the infected mice did not induce positively-selected Thy1.2$^+$ spleen cells from uninfected mice to secrete detectable levels of IFN-γ and induced only low/background production of IL-10 (Table 5.1). These results demonstrate that the secretion of IL-10 and IFN-γ by splenocytes from *T. congolense*-infected mice operates via the cooperation of Thy1.2$^+$ and Thy1.2$^-$ cells derived from the spleens of *T. congolense*-infected mice.

To test whether the production of IL-10 and IFN-γ by enriched Thy1.2$^+$ cells from the spleens of infected mice might occur by an antigen-specific mechanism, we supplemented cultures of Thy1.2$^+$ cells with whole spleen cells from uninfected mice which had been completely depleted of Thy1.2$^+$ cells by treatment with anti-Thy1.2 antibodies and complement (APC). These cultures were then pulsed with 10^6 parasite-equivalent of *T. congolense* lysate. As shown in table 5.2, supplementation with APC restores the abilities of Thy1.2$^+$ cells from infected, but not uninfected, mice to secrete IL-10 and IFN-γ in response to *T. congolense* lysates. Direct pulsing of Thy1.2$^+$ cells from

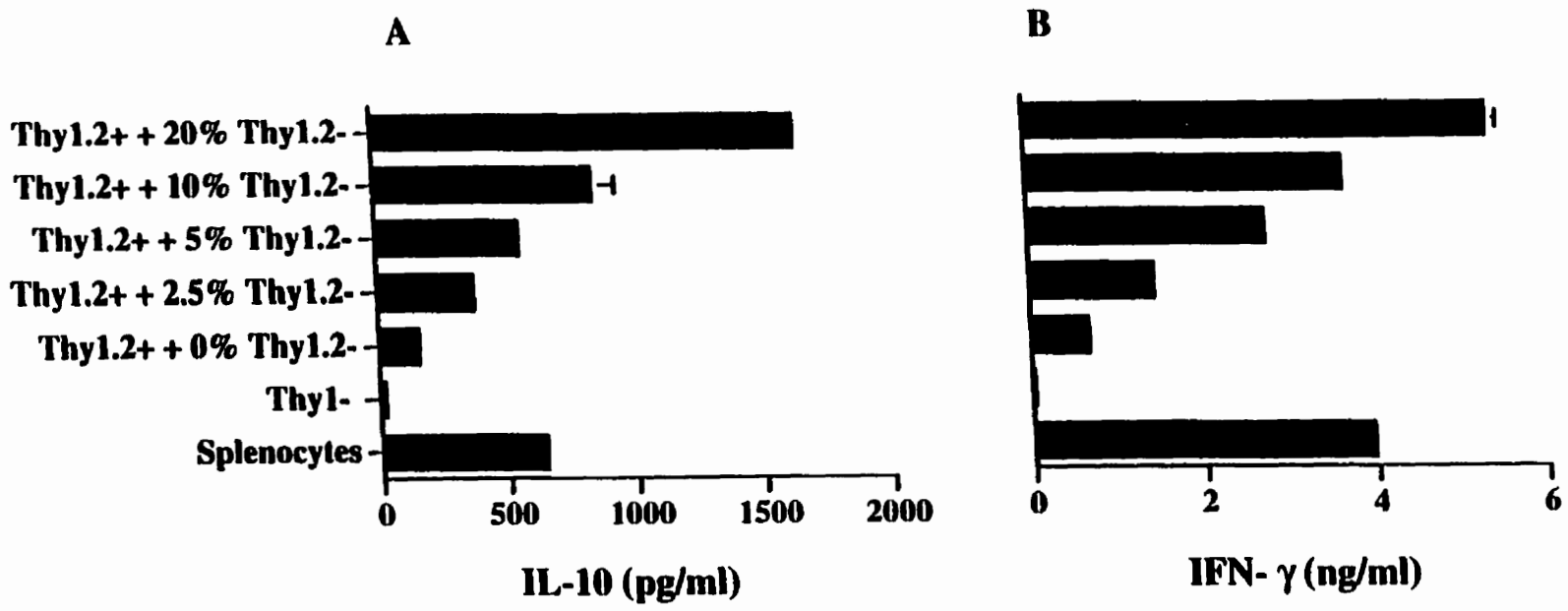

Figure 5.6 Secretion of IL-10 and IFN-γ by enriched Thy1.2$^+$ cells from the spleens of infected mice is drastically reduced. Thy1.2$^-$ cells restored IL-10 and IFN-γ secretion in a dose-dependent manner. Thy1.2$^+$ enrichment was carried out with paramagnetic beads coated with anti-Thy1.2 antibodies (materials and methods). The effluent cell populations (Thy1.2$^-$) were treated with anti-Thy1.2 antibodies and complement to completely deplete them of any residual Thy1.2$^+$ cells. Enriched Thy1.2$^+$ cells were cultured at a density of 5 x 10^5/well without mitogens either alone or with varying concentrations of Thy1.2$^-$ cells. Cultures were also set up with 5 x 10^5 cells/well of whole spleen cell suspensions (splenocytes) from infected BALB/c mice as well as effluent cells (Thy1.2$^-$) cells alone. After 48 hr, the supernatant fluids were collected and assayed for IL-10 and IFN-γ by ELISA. The set of results presented is one of two similar experiments.

TABLE 5.1. Optimal secretion of IL-10 and IFN-γ requires cooperation of positively enriched Thy1$^+$ cells and Thy1.2$^-$ cells derived from the spleens of *T. congolense*-infected, but not uninfected, mice[a].

Culture condition	Concentration of cytokine in culture fluid (pg/ml)	
	IL-10	IFN-γ
Infected[b], splenocytes[c]	1805 ± 42	5134 ± 64
Infected, Thy1.2$^+$ cells	412 ± 6[e]	500 ± 24[e]
Infected, Thy1.2$^-$ cells	39	50 ± 10
Infected, Thy1.2$^+$ cells + Infected, Thy1.2$^-$ cells	2342 ± 25	4708 ± 46
Infected, Thy1.2$^+$ cells + Normal, Thy1.2$^-$ cells	130 ± 18	43 ± 12
Normal[d], splenocytes	65 ± 12	ND
Normal, Thy1.2$^-$ cells	38 ± 3	ND
Normal, Thy1.2$^-$ cells + Infected, Thy1.2$^-$ cells	103 ± 56[f]	ND

[a] Single-cell suspension of spleens from uninfected or infected mice were separated into Thy1.2$^+$ and Thy1.2$^-$ populations using anti-Thy1.2 antibody-coated paramagnetic beads. The Thy1.2$^-$ populations were further treated with anti-Thy1.2 antibody and complement to completely deplete them of any residual Thy1.2$^+$ cells. The enriched Thy1.2$^+$ cells were cultured at 5 x 10^5 cells/well either alone or with 5 x 10^4 Thy1.2$^-$ cells in 96-well tissue culture plates. After 48 hr, the supernatant fluids were collected and assayed for IL-10 and IFN-γ by ELISA. Values presented represent the mean ± S.E of four wells. The results shown are representative of two similar experiments.

[b]cells derived from infected mice

[c]whole spleen cell cultures

[d]cells derived from uninfected mice

[e] $p < 0.001$ versus whole spleen cells from infected mice

[f] $p < 0.001$ versus Infected, Thy1.2$^+$ + 5 x 10^4 Infected, Thy1.2- cells

ND = Not detected (i.e. below the sensitivity of the ELISA)

TABLE 5.2. Secretion of IL-10 and IFN-γ by Thy1$^-$ cells in the spleens of *T. congolense*-infected BALB/c mice is trypanosome-specific and requires splenic Thy1.2$^-$ cells as antigen-presenting cells.

Culture condition[a]	Concentration of cytokine in culture fluid (pg/ml)	
	IL-10	IFN-γ
Infected[b], splenocytes[c]	726 ± 12	2256 ± 14
Infected, Thy1.2$^-$ cells	ND	80 ± 15
Infected, Thy1.2$^+$ cells + Tryps[d]	ND	ND
Infected, Thy1.2$^+$ cells + APC[e] + Tryps	1020 ± 41	1744 ± 36
Normal[f], splenocytes	ND	ND
Normal, Thy1.2$^-$ cells	ND	ND
Normal, Thy1.2$^+$ cells + APC + Tryps	ND	ND
APC + Tryps	ND	ND

[a] Single-cell suspensions of spleens from uninfected or infected mice were enriched for Thy1.2$^+$ cells by anti-Thy1.2 antibody-coated paramagnetic beads. Enriched Thy1.2$^+$ cells (5 x 10^5 cells/well) were supplemented with 10^5 uninfected spleen cells (APC) completely depleted of Thy1.2$^+$ cells by treatment with anti-Thy1.2 antibodies and complement and cultured in a 96-well tissue culture plates in the presence or absence of *T. congolense* lysate. After 48 hr, the supernatant fluids were collected and assayed for IL-10 and IFN-γ by ELISA. Values presented represent the mean ± S.E of four wells.

[b] Cells derived from infected mice

[c] Whole spleen cell cultures

[d] *Trypanosoma congolense* lysate

[e] Whole spleen cells from uninfected mice treated with anti-Thy1.2 antibodies + complement

[f] cells derived from uninfected mice

ND = Not detected (values below the sensitivity of the ELISA)

either uninfected or *T. congolense*-infected mice with *T. congolense* lysates did not induce either IL-10 or IFN-γ secretion. These results suggest that the production of IL-10 and IFN-γ by splenocytes from *T. congolense*-infected BALB/c mice might occur by an antigen-specific mechanism rather than by direct binding of *T. congolense*-derived molecules to T cells as was reported for *T. brucei* (Olsson et al., 1993).

5.4.6 Adherent Thy1.2$^+$ spleen cells from *T. congolense*-infected mice suppress T and B cell responses to mitogen and parasite-unrelated antigen

One of the prominent features of trypanosomiasis is a suppression of T and B cell responses to parasite-related and -unrelated antigens (Roelants and Pinder, 1984; Askonas, 1985; Sileghem et al., 1994). Suppressor T cells (Eardley and Jayawardena, 1977; Jayawardena et al., 1978), or macrophages (Askonas, 1985; Sileghem et al., 1986; Schliefer and Mansfield, 1993), or a combination of these cell types (Corsini et al., 1977; Roelants and Pinder, 1984) have variously been implicated in this effect. Because IL-10 (chapter 3) and IFN-γ (Darji et al., 1996; see chapter 4) have been shown to be involved in suppression of T cell responses in trypanosome-infected animals, we wished to know if the matrix-adherent Thy1.2$^+$ cells which secrete large amounts of IL-10 and IFN-γ (above) might effect a suppression of the T and B cell responses. To test the potential effects on T cell responses, we co-cultured spleen cells from uninfected mice with Sephadex-G-10-adherent cells purified from the spleens of either uninfected or *T. congolense*-infected BALB/c mice and challenged the cells with Con A. In some co-cultures, the Sephadex G-10-adherent cells were further Thy1.2-depleted as above before being used in the cultures.

Co-culture of spleen cells from uninfected mice with Sephadex-adherent cells from infected, but not uninfected, mice resulted in a significant (48 ± 5%) suppression of the proliferative response to Con A. Depletion of Thy1.2$^+$ cells from the adherent cells reversed this suppression (Figure 5.7A).

In another set of experiments, spleen cells from BALB/c mice immunized 7 days previously with sheep red blood cells (SRBC) were stimulated *in vitro* with SRBC in the absence or presence of Sephadex G-10-adherent cells from either uninfected or *T. congolense*-infected mice. Four days later, the B cell responses to SRBC were measured using a hemolytic plaque-forming cell (PFC) assay. As shown in Figure 5.7B, adherent cells from the spleens of infected, but not those from uninfected, mice significantly suppressed (80 ± 3%) the B cell response to SRBC. Again, depletion of Thy1.2$^+$ cells from the adherent cells of infected mice effectively reversed this suppression.

Taken together, these results suggest that adherent Thy1.2$^+$ cells from the spleens of *T. congolense*-infected mice are involved in the suppression of T and B cell responses observed in these animals. They demonstrate also that this suppression is not effected in an antigen-specific fashion.

Figure 5.7 Adherent Thy1.2$^+$ cells suppress T and B cell responses. Sephadex-G-10-adherent cell populations were obtained from single-cell suspensions of spleens from uninfected (norm.) or BALB/c mice infected for 7 days with *T. congolense* (infect.). Depletion of residual Thy1.2$^+$ cells was carried out by treatment with antibody and complement of adherent cells derived from uninfected (Thy1.2$^-$ norm.) or infected (Thy1.2$^-$ infect.) mice. (A) Whole spleen-cell suspensions from uninfected or infected mice were cultured either alone (3 x 10^5 cells/well) or with undepleted or Thy1.2-depleted Sephadex-G-10-adherent cells (10^5/well) from uninfected or infected mice in the presence of 5 μg/ ml Con A. After 48 hr, the cultures were pulsed with 0.5 μCi [^{3}H]TdR overnight and the uptake of [^{3}H]TdR was determined. (B) BALB/c mice were primed with 0. 1% SBRC and boosted with 10% SBRC 3 days later. Seven days after primary immunization, the mice were euthanized and single-cell suspensions were made from their spleens. Splenocytes (10^6/well) were cultured *in vitro* in the presence of 2.5 x 10^5 SRBC and 2.5 x 10^5/well undepleted or Thy1.2-depleted Sephadex-G-10-adherent cells from normal or infected mice. After 4 days of culture, the number of anti-SRBC plaque-forming cells (PFC) was determined.

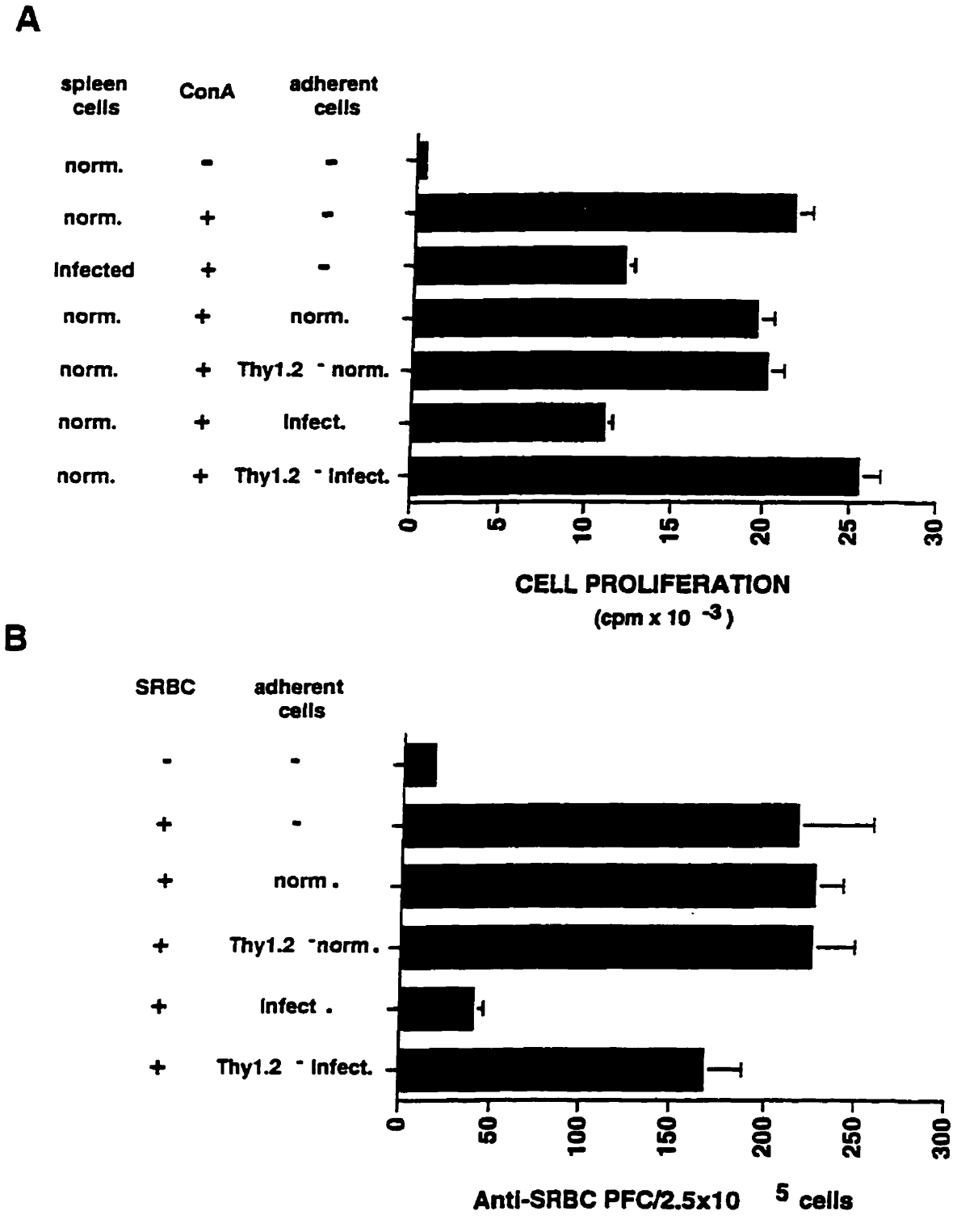
A
spleen cells
ConA
adherent cells
norm. - -
norm. + -
Infected + -
norm. + norm.
norm. + Thy1.2 - norm.
norm. + Infect.
norm. + Thy1.2 - Infect.
0 5 10 15 20 25 30
CELL PROLIFERATION
(cpm x 10 -3)
B
SRBC
adherent cells
- -
+ -
+ norm.
+ Thy1.2 -norm.
+ Infect.
+ Thy1.2 - Infect.
0 50 100 150 200 250 300
Anti-SRBC PFC/2.5x10 5 cells

Figure 5.7

5.5 Discussion

This work identified a unique set of T cells that is involved in the regulation and/or secretion of IL-4, IL-10 and IFN-γ in the spleens of BALB/c mice infected with *T. congolense*. According to the data presented here, both Thy1.2$^+$CD4$^-$8$^-$ and Thy1.2$^-$CD4$^-$ 8$^-$ cells are involved in this process. In *T. brucei*-infected rodents, CD8$^+$ but not CD4$^+$ cells have been shown to be the major producers of IFN-γ (Bakhiet et al., 1990; 1993). In that system, direct binding of a trypanosomal component to the CD8 molecule on CD8$^+$ T cells was reported to trigger the production of large amounts of IFN-γ (Olsson et al., 1991; Bakhiet et al., 1990). IFN-γ was further reported to have a direct growth-stimulatory effect on the parasite (Bakhiet et al., 1990; 1996), such that depletion of these cells *in vivo* during infection was associated with a reduction in parasitemia and enhanced resistance to the disease (Bakhiet et al., 1991). In addition, CD8-, but not CD4-knockout mice exhibited an enhanced resistance to *T. brucei* infections (Rottenberg et al., 1993) and this effect was reportedly mediated via a suppression of IFN-γ secretion. In *T. congolense*-infected mice, we found higher levels of IL-10 and IFN-γ mRNA induction and cytokine expression by spleen cells of infected highly susceptible BALB/c than by those of relatively resistant C57BL/6 mice (see chapters 3 and 4). However, we were unable to show any growth stimulatory effect of IFN-γ on *T. congolense* (Kaushik et al., 1997). Our data rather indicate that the production of higher levels of IL-10 and IFN-γ by the susceptible mice contributes to their enhanced susceptibility to the disease, in part through the immunosuppressive effects of these cytokines (see chapters 3 and 4). Our current data

also differ from that obtained in the *T. brucei*-infected mice, in that they show that Thy1.2$^+$CD4$^+$8$^-$ and Thy1.2$^+$CD4$^-$8$^-$ cells are the major producers and/or regulators of IL-4, IL-10 and IFN-γ in BALB/c mice infected with *T. congolense*. In fact, our evidence suggests that CD8$^+$ cells could be involved in a down-regulation of these cytokines, since depletion of CD8$^+$ cells often led to a relative increase in the secretion of some of these cytokines (Figures 5.1 & 5.4). This finding is in agreement with recent observations that depletion of CD8$^+$ cells in *T. congolense*-infected susceptible Zebu cattle has no effect in the outcome of the disease (Sileghem and Naessens, 1995), despite the fact that spleen and lymph node cells from infected animals secrete high levels of IFN-γ in vitro (Flynn et al., 1992). While on the surface it may be surprising that such marked differences exist between *T. brucei* and *T. congolense*, especially in view of their close structural and molecular similarities, we suspect that, among other things, the inherently invasive nature of *T. brucei* as well as the differences in the strains of mice used could be important distinguishing factors between the two model systems.

We provided clues that secretion of cytokines (IL-4, IL-10 and IFN–γ) by the Thy1.2$^+$ cells from the spleens of *T. congolense*-infected BALB/c mice might occur by an antigen-specific manner rather than by mechanisms of polyclonal activation involving direct binding of *T. congolense*-derived molecules to T cells as was reported for *T. brucei* infections (Bakhiet et al., 1990; Olsson et al., 1993). Several lines of evidence support this. Firstly, cytokine secretion was restricted to a small percentage of T cells present in the plastic-, nylon wool- or Sephadex G-10-adherent cell populations. Furthermore, positively selected Thy1.2$^+$ cells could not produce IL-4, IL-10 or IFN-γ unless these

purified T cells were supplemented with purified Thy1.2$^-$ cells from infected mice. Supplementation with only Thy1.2$^-$ spleen cells (APC) from uninfected mice did not restore cytokine secretion by Thy1.2$^+$ cells from infected mice. However, in the presence of *T. congolense* lysate, these APC were able to restore cytokine secretion by Thy1.2$^+$ splenocytes from infected, but not uninfected, mice. This cytokine secretion response could not be restored by pulsing purified Thy1.2$^+$ T cells with optimal concentrations of *T. congolense* antigens alone. The purified Thy1.2$^-$ cells from infected mice were unable to induce cytokine secretion by positively-enriched Thy1.2$^+$ cells from uninfected mice. This is probably due to the fact that the few antigen-specific Thy1.2$^+$ cells which would be expected to exist in the spleens of uninfected mice would require much more than 48 hr of primary *in vitro* culture for expansion and cytokine secretion, whereas those from mice infected for 7 days have been primed and expanded *in vivo*.

We established a potential role for such adherent Thy1.2$^+$ cells in the pathogenesis of African trypanososmiasis by demonstrating their involvement in the suppression of T and B cell responses to mitogen and SRBC respectively. Co-culture of the adherent cells from infected mice with whole spleen cells from uninfected mice resulted in a suppression of the normal proliferative responses to Con A, and this effect was abolished by depletion of Thy1.2$^+$ cells from the adherent cell populations (Figure 5.7A). Furthermore, the dramatic suppression of B cell response to SRBC *in vitro* by the adherent cell populations was also ablated by depletion of these Thy1.2$^+$ cells (Figure 5.7B). Adoptive transfer experiments have shown that both T cells (Eardley and Jayawardena, 1977; Jayawardena et al., 1978) and adherent macrophage-like cells (Askonas, 1985; Sileghem et al., 1986; Flynn et al., 1991; Schleifer and Mansfield, 1993) are involved in the suppression of

lymphoid cell responses in trypanosome-infected mice. In a review of the mechanisms of trypanosome-induced immunosuppression, Roelants and Pinder (1984) suggested that both cell types are important. Our present data demonstrate that adherent T cells or T cells that co-purify with adherent cell populations are involved in the pathogenesis of immunosuppression observed in *T. congolense*-infected mice. We have previously shown that IL-10 and IFN-γ produced during *T. congolense* infections mediate suppression of lymphoid cell proliferative responses (see chapters 3 and 4). Our finding that the secretion of IL-10 and IFN-γ by spleen cells from infected mice requires cooperation between adherent-Thy1.2$^+$ and -Thy1.2$^-$ cells further supports the reports of Corsini et al. (1978) and suggestions of Roelants and Pinder (1984) that both T cells and macrophages are important in trypanosome-induced immunosuppression. Suppressor T cells could alter (via cell to cell contact or cytokines) macrophage functions and its cytokine patterns thereby rendering them suppressive. In line with this, we have obtained recent evidence that simultaneous exposure of bone marrow-derived macrophages from trypanosome-susceptible but not -resistant mice to IFN-γ and trypanosomal lysate increases 2-4 fold their IL-10 secretion (Kaushik et al., manuscript in preparation). IL-10 is a potent immunosuppressive cytokine (Moore et al., 1990) and its increases contribute to trypanosome-induced immunosuppression (see chapter 3).

Several lines of evidence suggest that the cell(s) producing IL-4, IL-10 and IFN-γ in the spleens of *T. congolense*-infected BALB/c mice are not conventional helper T-cells. First, nylon wool- and Sephadex G-10-non-adherent cells, which are enriched for conventional T-cells, did not secrete these cytokines (Figure 5.2) and the production of

these cytokines by cells non-adherent to plastic was dramatically reduced. Second, plastic-adherent cell populations produced 2-5 fold more of each cytokine than did the non-adherent cells, despite the fact that they contained only about 5-8% of $CD4^+$ and/or $CD8^+$ cells. Furthermore, nylon wool- and Sephadex G-10-adherent cells containing fewer than 5% of $CD4^+$ and/or $CD8^+$ cells produced as much or even more of each cytokine than did unfractionated splenocytes from infected mice. Moreover, depletion of $CD4^+$ cells from the adherent cell populations did not completely abolish the secretion of these cytokines, and $CD8^+$ cell depletion had very little negative effect; only the depletion of $Thy1.2^+$ cells from the adherent cells could completely abolish cytokine secretion.

It has not yet been tested whether IL-4-, IL-10- and IFN-γ-secreting cells were $NK1^+$ T-cells. $NK1^+$ T cells, which can either be $Thy1.2^+CD4^+8^-$ (60%) or $Thy1.2^+CD4^-8^-$ (40%), are known to secrete large amounts of IL-4, IL-10 and IFN-γ upon appropriate stimulation, and comprise about 0.5 - 1% of splenocytes from normal mice (reviewed by Bendelac, 1995; Vicari and Zlotnik, 1996; Bendelac et al., 1997). Their activation is restricted by the CD1 molecule and they recognize both hydrophobic peptides and lipid antigens presented in association with CD1 (Porcelli et al., 1992; Bendelac et al., 1997). Because they have a conventional αβ TCR (with the associated CD3 molecules), they are activated by anti-CD3 or anti-αβ TCR antibodies to release large amounts of cytokines, including IL-4, IL-10 and IFN-γ (Zlotnik et al., 1992; Arase et al., 1993; Vicari and Zlotnik, 1996). This might explain why splenocytes from *T. congolense*-infected BALB/c mice secrete large amounts of IL-4, IL-10 and IFN-γ following Con A stimulation despite the fact that proliferative responses are usually suppressed (chapter 3). Double negative

NK1.1 T cells have been reported to act as suppressor cells in hemopoesis (Yankelevich et al., 1989; Kikly and Dennert, 1992) and during tumor development (Tamada et al., 1997). Also, these cells have been shown to have regulatory properties by suppressing the induction of autoreactive lymphocytes (Takeda and Dennert, 1993; Sumida et al., 1995; Meiza et al., 1996).

The mechanisms of induction and activation of adherent Thy1.2$^+$CD4$^-$8$^-$ and Thy1.2$^-$CD4$^-$8$^-$ cells during African trypanosomiasis is as yet unknown. However, it is conceivable that they may be induced following recognition of some trypanosomal antigen, e.g. the lipid portion of the variant surface glycoprotein. Whatever the mechanisms are, our results indicate that the stimulation of adherent Thy1.2$^-$CD4$^-$8$^-$ and Thy1.2$^-$CD4$^-$8$^-$ cells to produce IL-4, IL-10 and IFN-γ requires cooperation between these cells and Thy1.2$^-$ cells derived from the spleens of *T. congolense*-infected mice and that these cells are involved in immunosuppression observed in infected animals.

6.0 CYTOKINE AND ANTIBODY RESPONSES TO EXPERIMENTAL *TRYPANOSOMA CONGOLENSE* INFECTIONS IN TWO INBRED STRAINS OF MICE THAT DIFFER IN RESISTANCE

6.1 Abstract

We studied IL-4, IL-10 and IFN-γ secretion by splenocytes and the plasma levels of different antibody isotypes against various antigens of *Trypanosoma congolense* in highly susceptible BALB/c and relatively resistant C57BL/6 mice during the early course of infection with *T. congolense*. The pattern and kinetics of cytokine spot-forming cells in the spleens were essentially similar in the two mouse strains although higher numbers were detected in the spleens of BALB/c than C57BL/6 mice on some days post-infection. However, the amount IL-4, IL-10 and IFN-γ secreted into the culture fluids was dramatically different. From day 4 forward, splenocytes from BALB/c mice secreted very high levels of these cytokines. In contrast, unstimulated splenocytes from infected C57BL/6 mice did not secrete detectable levels of IL-4 throughout the period tested. The secretion of IL-10 and IFN-γ by C57BL/6 splenocytes only became appreciable on day 6 and was down-regulated by day 8, when the first wave of parasitemia is being controlled. Infected BALB/c mice mounted an earlier IgM antibody response to VSG, formalin-fixed *T. congolense* and whole *T. congolense* lysates than did infected C57BL/6 mice. However, infected BALB/c mice failed to make any detectable IgG3 and IgG2a antibody responses to these antigens whereas infected C57BL/6 mice made strong IgG3 and IgG2a

response. We hypothesize that enhanced resistance against *T. congolense* infections in mice may be mediated by a TH1 cell response.

6.2 Introduction

Trypanosomes are extra-cellular hemoprotozoan parasites that cause disease and death in man and animals in the sub-Saharan Africa. *Trypanosoma congolense* infections result in acute or chronic debilitating disease in cattle and other domestic animals. Cattle and inbred strains of mice differ in their susceptibility to infections with different species and strains of African trypanosomes. The indigenous west African taurine breed of cattle are more resistant than the Zebu breed, as measured by the level of parasitemia, anemia and survival period after both natural and experimental infections with various species of African trypanosomes (reviewed by Murray et al., 1982). Differences in the ability to control parasitemia and the duration of survival has also been observed in inbred strains of mice infected with African trypanosomes (Morrison et al., 1978; Levine and Mansfield, 1984; Greenblatt et sl., 1985; Pinder et al., 1986; Ogunremi and Tabel, 1995). Generally, the highly susceptible mice are unable to control the first wave of parasitemia after infection whereas the relatively resistant mice are able to control many waves of parasitemia before dying. Genetic analyses showed that the differences in ability to control parasitemia among various strains of mice is not linked to the MHC genes (Levine and Mansfield, 1981; 1984) and may be under a polygenic control (Pinder, 1984; DeGee et al., 1988; Ogunremi and Tabel, 1995). Further studies utilizing radiation chimeras indicated that resistance is influenced by both radio-resistant and -sensitive cells residing in the bone marrow or the spleen (DeGee and Mansfield, 1984; Greenblatt et al., 1985).

Comparative serological studies of trypanosome-infected susceptible and resistant strains of mice show that the efficiency of clearance of first wave of parasitemia is correlated with the serum levels of antibodies specific for the variant specific variant surface glycoprotein (VSG). Resistant strains of mice have earlier and higher serum anti-VSG antibody response than susceptible strains (Whitelaw et al., 1983; Mitchell and Pearson, 1986; Black et al., 1983; 1986; Otesile and Tabel, 1987). Because it has been shown that nude mice infected with *T. brucei rhodesiense* were capable of controlling their first wave of parasitemia (Campbell et al., 1978), one could argue that an IgM anti-VSG antibody response is sufficient to control variant specific parasitemia (Mansfield, 1990). However, DeGee and Mansfield (1984) showed that the levels of IgM anti-VSG antibody was not correlated with protection in mice infected with *T. brucei rhodesiense*. Similarly, Otesile and Tabel (1987) have shown that anti-VSG antibodies are necessary, but not sufficient for the control of *T. congolense* infections in BALB/c mice.

The patterns of cytokine responses during some parasite infections determine or are at least strongly correlated to the susceptibility of the host (reviewed by Sher and Coffman, 1992). IL-4 produced by TH2 cells has been shown to promote B cell growth and differentiation into antibody-secreting cells as well as switch from IgM to IgG1 antibody isotype (Finkelman and Holmes, 1990). IFN-γ produced by TH1 cells on the other hand promotes cell-mediated immunity and production of IgG2a and IgG3 antibody isotypes (Finkelman and Holmes, 1990; Boehm et al., 1997). The relationships between the cytokine and antibody responses in the resistant and susceptible mice infected with African trypanosomes have not been studied in detail. During infections with *T. brucei*

rhodesiense, TH1 cells secreting IL-2 and IFN-γ were detected predominantly in the peritoneal cavity and rarely in the spleen of infected resistant mice (Schleifer et al., 1993). In another study using in situ hybridization, no differences were observed in the levels of IFN-γ and IL-10, IL-12 and TNF-α mRNA transcripts between the splenocytes of *T. brucei brucei*-infected susceptible (C3H.Q) and resistant (B10.Q) mice (Bakhiet et al., 1996). The expression of IL-4 mRNA transcripts were, however, higher in the resistant mice, but this cytokine did not seem to be positively correlated with resistance to infection, as deletion of the IL-4 gene in these mice was associated with enhanced resistance to the infection (Bakhiet et al., 1996).

Trypanosoma congolense organisms are intravascular but extra-cellular parasites. Their elimination from the blood of infected animals depends largely on the antibody responses to various antigens of the parasite. The regulatory mechanisms that influence the differences in the quantity and quality of antibody response to the VSG and other antigens of African trypanosomes are still not known, but are undoubtedly influenced by T cells (Reinitz and Mansfield, 1990; Mansfield, 1990). Various cytokines secreted by T cells may, in addition to having a direct effect also indirectly influence B cell responses by causing immunosuppression. We have previously shown that endogenously produced IL-10 and IFN-γ mediate the suppression of mitogen-driven proliferative responses of splenocytes from BALB/c mice infected with *T. congolense* (see chapters 3 and 4). Neutralization of these cytokines in vivo enhanced the resistance of the highly susceptible BALB/c mice to *T. congolense* infection. Here, we show that differences exist in the number of IL-4-, IL-10- and IFN-γ-secreting cells in the spleens, as well as in the levels of

these cytokines secreted in cultures by splenocytes from *T. congolense*-infected BALB/c and C57BL/6 mice. We also show marked differences between BALB/c and C57BL/6 mice regarding the Ig antibody responses to various antigens of *T. congolense*.

6.3 Materials and Methods

6.3.1 Mice

Female BALB/cAnNCrlBR (BALB/c) and outbred CD1 mice were obtained from the Animal Resource Center of the University of Saskatchewan, and female C57BL/6NCrlBR (C57BL/6) mice were purchased from Charles River Laboratories (St. Constant, Quebec). BALB/c and C57BL/6 mice were between 8 to 10 weeks of age and CD1 mice were 5 weeks old. All mice were maintained according to the recommendations of the *Canadian Council of Animal Care*.

6.3.2 Parasites

Trypanosoma congolense, variant antigenic type (VAT) TC13 (Otesile and Tabel, 1987) was used in these experiments. Parasites were passaged in immunosuppressed CD1 mice as described previously (Tabel, 1982). The parasites for infection of the BALB/c and C57BL/6 mice were isolated from the blood of the CD1 mice by DEAE-cellulose chromatography (Lanham and Godfrey, 1970).

6.3.3 Experimental design

Groups of four to five BALB/c and C57BL/6 mice were infected intraperitoneally (i.p.) with 10^3 organisms of *T. congolense* TC13 and killed with CO_2 on days 1 to 8 post-infection. On each day indicated, blood was withdrawn from the caudal vena cava into syringes containing heparin (final concentration ~20 IU/ml), and the plasma collected after centrifuging the blood at 1,000 x g for 30 min. Subsequently, the plasma was centrifuged at 13,000 x g for 15 min and the supernatant plasma was stored at -35°C until used.

6.3.4 Estimation of parasitemia and survival period

To estimate the circulating parasite numbers, a drop of blood from the tail vein of each infected mouse was examined at 400x power by phase contrast microscopy. Parasitemia was estimated by counting the number of parasites present in at least 10 fields. The survival period was defined as the number of days post-infection that the infected mice remained alive. Moribund mice were euthanized with CO_2.

6.3.5 Single-spleen cell suspensions

The spleens from euthanized mice (above) were made into single-cell suspensions by teasing in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum [FBS], 50 mM 2-mercaptoethanol, 2 mM L-glutamine and 100 Units each of penicillin/streptomycin). The single-spleen cell suspensions were then used for cultures or to detect single cells secreting cytokines (see below).

6.3.6 Single-cell assay for cytokine secretion

The ELISPOT method described by Czerkinsky et al. (5) was used to detect single cytokine (IL-4, IL-10 and IFN-γ)-producing cells. Briefly, 96-well microtiter plates in which the bottom was made of nitrocellulose (Polyfiltronics Inc., Rockland, MA) were coated overnight with 50 µl of 2 µg/ml monoclonal rat anti-mouse IL-4, IL-10 or IFN-γ (PharMingen, San Diego, CA). The plates were washed two times with RPMI 1640 medium (Sigma, Oakville, ON) and blocked for 2-4 hr at room temperature with 200 µl complete medium. Single cell suspensions of spleens from infected mice were applied in 200 µl aliquots at a density of 5 x 10^5 cells/well and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. The next morning, the plates were given a brief agitation in an ELISA plate shaker and the cells were removed by flicking the plates. Following repeated washings in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T), 100 µl of 1 µg/ml biotinylated rat anti-mouse IL-4, IL-10 or IFN-γ (Pharmingen) diluted in PBS/T was applied to each well and incubated for 2 hr at 37°C. After washing, 100 µl of 1:700 dilution of alkaline phosphatase streptavidin (Vector Lab. Burlingame, CA) was added and the plates were incubated for 30 min at 37°C. The spots were developed by adding 100 µl 5-bromo-4-chloro-indolylphosphate p-toluidine and nitroblue tetrazolium chloride solution (Gibco). The spots corresponding to single cells that had secreted cytokine were counted by the use of a dissecting microscope equipped with a grid.

6.3.7 Splenocyte Cultures

Splenocytes were cultured at a concentration of 2.5 x 10^6 cells/ml in 200 µl volumes in 96-well tissue culture plates in a humidified incubator at 37°C, 5% CO_2 atmosphere. All cultures were harvested after 48 hr, centrifuged at 1,500 x g for 15 min and the supernatant fluids were stored at -35°C until used.

6.3.8 Cytokine assays

The levels of IL-4, IL-10, and IFN-γ in the splenocyte culture supernatants were determined by routine sandwich ELISA, according to the manufacturer's suggested protocols. Recombinant murine IL-4, IL-10 and IFN-γ used to generate standard curves and paired antibodies against murine IL-4, IL-10, and IFN-γ for sandwich ELISA assays were made by PharMingen and purchased from Cedarlane (Hornby, ON). The sensitivities of the ELISA assays were 15 pg/ml for IL-4 and 31 pg/ml for IL-10 and IFN-γ.

6.3.9 ELISA assays for IgG antibodies

6.3.9.1 Purified VSG ELISA

VSG was purified from *T. congolense* VAT TC13 by a combination of ion exchange and gel filtration chromatography as previously described by Liu et al., (1993). The purified VSG migrated as a single molecular species with an apparent molecular weight of 54 kDa under non-reducing conditions. The identity was confirmed by Western blot using polyclonal rabbit anti-TC13 serum. ELISA plates were coated with 12.5 µg/ml (50 µl/well) and incubated at 4°C overnight. The plates were washed twice in PBS/T and non-

specific binding sites were blocked for 2hr at room temperature with 200 µl PBS containing 10% heat-inactivated fetal calf serum (PBS-FBS). Serum samples, diluted 1:50 in PBS-FBS were added to each well and incubated for 2 hr at 37°C. After washing (4 times), 100 µl of previously determined dilutions of rabbit anti-mouse isotype specific antibodies (Cappel, Organon Teknika Corporation, Durham, NC) in PBS-FBS were added to each well and incubated for 1hr at 37°C. The plates were washed 6 times and 100 µl of 1:10,000 dilution of biotinylated mouse monoclonal anti-rabbit antibodies (Sigma Immunochemicals, Oakville, ON) was added to each well and incubated for 45 min at 37°C. After 8 washings, 100 µl of 1:5000 dilution of streptavidin-horse radish peroxidase (Vector Laboratories Inc., Burlingame, CA) was added to each well followed by another incubation for 30 min at 37°C. The plates were washed 10 times and color development was achieved by adding 100 µl of 2,2' azino-di 3-ethyl-benz-thiazoline sulphonate (ABTS, Kirkegaard & Perry Lab, Gaithersburg, MD) and incubating for 15-30 min at room temperature. Optical densities were read in a microtitre plate reader at a wavelength of 405 nm.

6.3.9.2 Fixed trypanosome ELISA

Freshly isolated trypanosomes were diluted in phosphate buffered saline (PBS) to a final concentration of 2.5 x 10^6/ml and 50 µl was added to each well of an ELISA plate (Immunlon 2, Dynatech, VWR Edmonton, AB). The plates were centrifuged at 500 x g for 5 min and then 50 µl of 2% paraformaldehyde in PBS was added to each well and incubated overnight at 4°C. The plates were washed two times with PBS/T and blocked

with 200 µl of PBS-FBS for 2 hr at room temperature. Thereafter, the ELISA procedure described above was then followed.

6.3.9.3 Whole trypanosome lysate ELISA

Whole trypanosome lysate was prepared by 3 cycles of freezing and thawing of freshly isolated trypanosomes at -80°C in the presence of 5 mM N-tosyl-L-lysine chloromethylketone (TLCK; Sigma) and the total protein content was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). ELISA plates were coated overnight at 4°C with 50 µl of the lysate containing 25 µg/ml total protein. The plates were washed 2 times with PBS-T and non-specific binding sites were blocked with PBS-FBS for 2 hr at room temperature. Thereafter, the ELISA procedure described above was followed.

6.3.10 Quantitative IgM ELISA

Essentially, the same protocol as that employed for the IgG ELISA was used with minor changes. After blocking, the plates were washed 4 times with PBST and 100 µl of 1:300 dilution of goat anti-mouse IgM antibodies (Cappel) was added to each well and incubated for 2 hr at 37°C. The plates were washed 6 times, and then 100 µl of 1:2,500 dilution of peroxidase-conjugated rabbit anti-goat IgG (Cappel) to each well. Subsequently, the plates were incubated for 1 hr at 37°C. After washing, the plates were developed by adding 100 µl of ABTS substrate to each well.

6.3.11 Statistical analysis

Data are represented as means ± standard error (SE). Significance of differences was determined by Student's t-test using the StatView™.SE 1988 Software (Abacus Concepts Inc. Berkley, CA).

6.4 Results

6.4.1 Survival time and parasitemia

BALB/c and C57BL/6 mice infected with 10^3 organisms of *T. congolense* had a pre-patent period of 4 days. There was no significant difference in parasitemia on days 5 and 6 in between the two mouse strains (see Figure 3.1A). From day 7 on, BALB/c mice showed significantly higher parasitemia than C57BL/6 mice and succumb to the infection with a mean survival period of 8.4 ± 0.5 days. In contrast, in infected C57BL/6 mice, the first wave of parasitemia peaked at day 7 and was almost controlled day 9 post infection (Figure see 3.1A). Their mean survival time was not tested in these series of experiments but has previously been shown to be 163.0 ± 12 days (Ogunremi and Tabel, 1995).

6.4.2 Frequency of IL-4-, IL-10- and IFN-γ-secreting cells in the spleens of *T. congolense*-infected BALB/c and C57BL/6 mice

As a first step, we assessed the number of cytokine-secreting cells in the spleens from the highly susceptible BALB/c and relatively resistant C57BL/6 mice infected with *T. congolense*. Both mouse strains showed a similar pattern in the kinetics of splenocytes secreting IL-4, IL-10 or IFN-γ (Figure 6.1). Cytokine-secreting cells became detectable by

day 4 in the two mouse stains and gradually increased in numbers as the infection progressed. The frequency of IL-4-secreting cells in the spleens from both strains of mice peaked on day 8, with their numbers being significantly ($p < 0.05$) higher in BALB/c than C57BL/6 mice on days 4, 5 and 8 (Figure 6.1A). IL-10 secreting cells became apparent on day 5 post-infection, peaked on day 7 and were slightly reduced by day 8 in the two mouse strains Generally, the numbers of IL-10-secreting cells were higher in the spleens from infected BALB/c than in those from C57BL/6 mice (Figure 6.1B). IFN-γ-secreting cells were detected by day 4 in the spleens of the two stains of mice. The number of these cells increased steadily, peaked on day 6 in both mouse strains and remained high up to day 8 in BALB/c mice (Figure 6.1C). In contrast, the frequency of IFN-γ-secreting cells in the spleen of infected C57BL/6 mice gradually declined after day 6. Overall, the frequency of IFN-γ-secreting cells was significantly ($p < 0.05$) higher in spleens from infected BALB/c than in those from C57BL/6 mice from day 4 on.

6.4.3 Levels of cytokine protein in cultures of splenocytes from infected BALB/c and C57BL/6 mice

While the pattern and kinetics of appearance of IL-4-, IL-10- and IFN-γ-secreting cells were essentially similar in infected BALB/c and C57BL/6 mice, the actual amount of protein secretion in cultures by these cells were paradoxically different. The secretion of IL-4, IL-10 and IFN-γ in cultures by splenocytes from infected BALB/c mice became

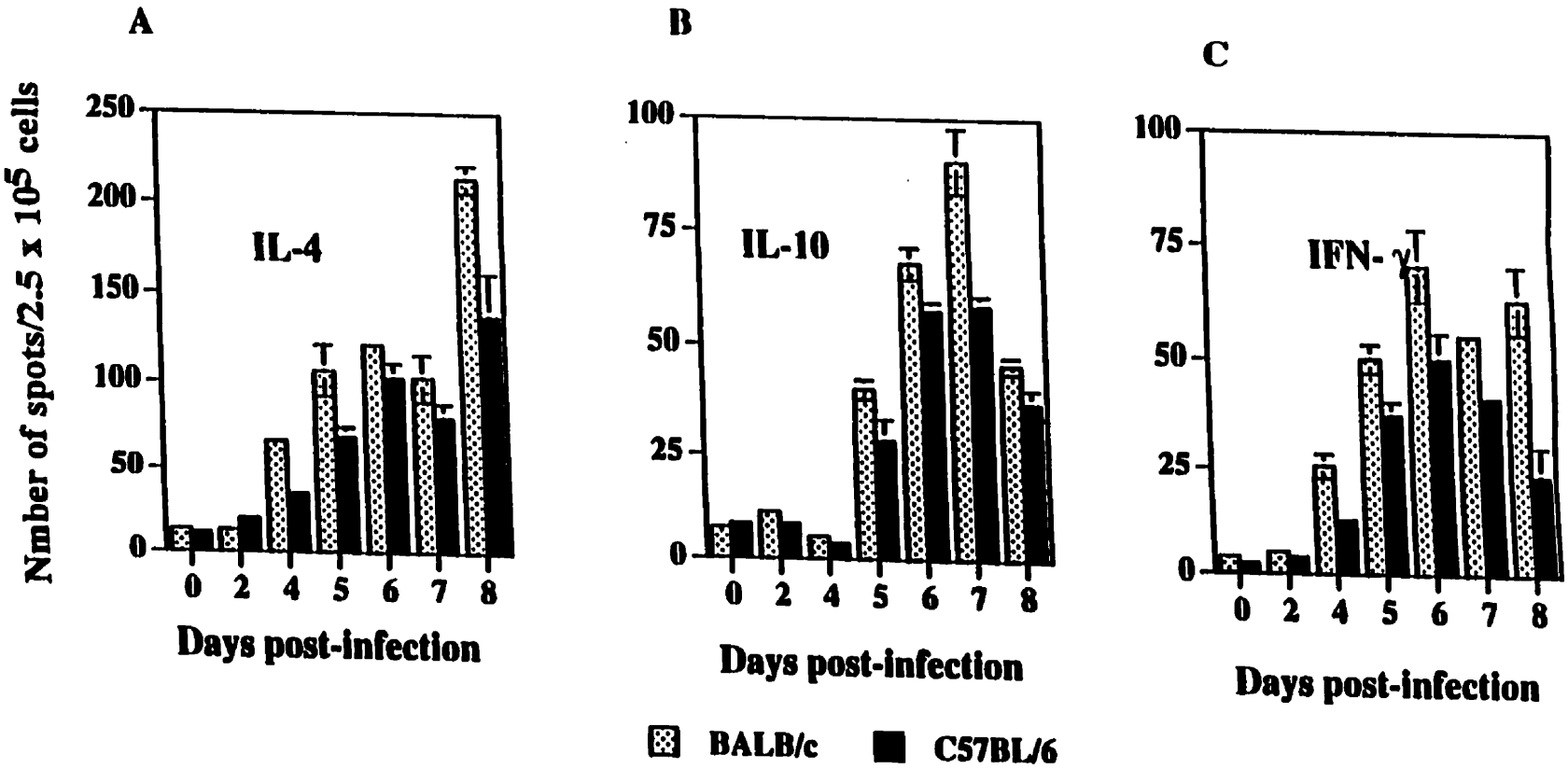

Figure 6.1 IL-4- (A), IL-10- (B) and IFN-γ– (C) spot-forming cells in the spleens of *T. congolense*-infected BALB/c (dotted bars) and C57BL/6 (solid bars) mice. Single-spleen cell suspensions were prepared on the days indicated from the spleens of *T. congolense*-infected BALB/c and C57BL/6 mice (four mice per group) and cultured for 24 hr in 96-well ELISPOT plates coated with antibodies against the indicated cytokine. After washing, the spots were developed and counted with the aid of a dissecting microscope (see materials and methods). Note the similar kinetics of appearance of IL-4-, IL-10- and IFN-γ-spot-forming cells in the spleens of infected BALB/c and C57BL/6 mice. Data are presented as mean ± SE. The set of results shown is a representative of two similar experiments.

apparent by day 4, peaked on day 6 and remained high by day 8 post-infection (see figures 3.2A and 4.3A & B). In contrast, secretion of IL-4 by splenocytes from infected C57BL/6 mice remained undetectable throughout the period of infection (see figure 4.3A) while secretion of substantial amounts of IL-10 and IFN-γ became only apparent by day 6 and were quickly down-regulated by day 8 (see figures 3.2A and 4.3B). In general, the secretion of IL-10 and IFN-γ in cultures by splenocytes from infected BALB/c mice were significantly ($p < 0.001$) higher than those from C57BL/6 mice.

Two important observations were made regarding the number of cytokine-secreting cells as measured by ELISPOT assay and the level of cytokines secreted into culture fluids as measured by ELISA. Whereas the differences between the number of cytokine-secreting cells detected in the two mouse strains were marginal (Figure 6.1), very marked differences were observed in the levels of protein detected in culture fluids (Figure 3.2A and 4.3 A & B), with BALB/c splenocytes apparently secreting significantly ($p < 0.001$) higher amounts of cytokines than their C57BL/6 counterparts. Also, although the frequency of IL-4-secreting cells were much higher than that of IL-10 or IFN-γ (approximate ratio 2:1 for IL-4- vs. IFN-γ-secreting cells), the IL-10 and IFN-γ-secreting cells produce much more cytokine on a cell-per-cell basis than did IFN-γ-producing cells (ratio 1:85 for IL-4 vs. IFN-γ on day 6 post-infection). These results clearly show that there was no correlation between the frequency of cytokine-secreting cells and the actual amount of cytokine secretion by the cells.

6.4.5 Antibodies to purified VSG

The levels of IgM, IgG1, IgG2a and IgG3 antibodies against purified VSG of *T. congolense* clone TC13 in the plasma of infected BALB/c and C57BL/6 mice were measured by ELISA. As shown in figure 6.3A, IgM anti-VSG antibodies appeared earlier in BALB/c than in C57BL/6 mice. IgM anti-VSG antibodies were measurable by day 4, peaked on days 5 and 6 and slightly declined on days 7 and 8 post-infection (Figure 6.2A). In contrast, IgM antibodies in the plasma of infected C57BL/6 mice remained undetectable until day 7 and increased to high levels on day 8. Similarly, infected BALB/c mice contained significantly higher levels of IgG1 anti-VSG antibodies in their plasma than did infected C57BL/6 mice (Figure 6.2B). In contrast, the levels of IgG2a and IgG3 anti-VSG antibodies were significantly higher ($p < 0.001$) in the plasma of infected C57BL/6 than in those of BALB/c mice from days 6 post-infection (Figures 6.2C & D). In fact, BALB/c mice did not make any detectable IgG3 antibody response to the purified VSG throughout the infection period. These results demonstrate that the highly susceptible BALB/c mice could mount an early and higher IgM anti-VSG antibody response but were incapable of mounting either IgG2a or IgG3 antibody responses to the VSG.

Figure 6.2 Antibodies to purified VSG of *T. congolense*. IgM (A), IgG1 (B), IgG2a (C) and IgG3 (D) antibody responses against the purified VSG in BALB/c (open squares) and C57BL/6 (closed squares) mice infected with *T. congolense*. Groups of 4-5 infected mice were killed on each day indicated with CO_2 and blood was collected into heparinized syringes for plasma (materials and methods). ELISA plates were coated overnight with 12.5 μg/ml (50 μg/well) of purified VSG in PBS and plasma samples were used at 1:50 dilution. Note the earlier IgM and higher IgG1 antibody responses mounted by the BALB/c than the C57BL/6 mice. Data are presented as mean ± SE. The set of results shown is a representative of two similar experiments.

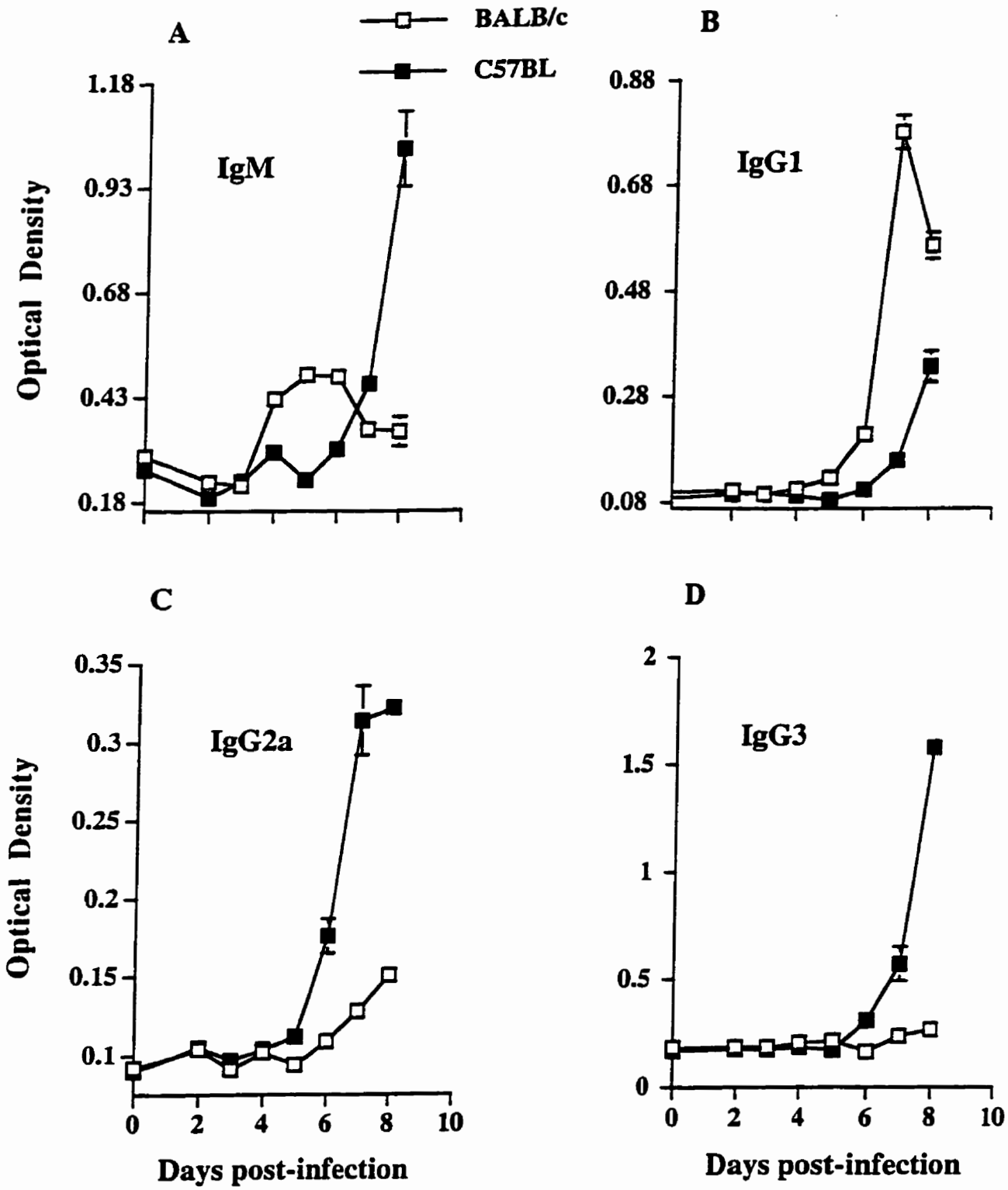
A
BALB/c
C57BL
IgM
Optical Density
1.18
0.93
0.68
0.43
0.18
B
IgG1
0.88
0.68
0.48
0.28
0.08
C
IgG2a
Optical Density
0.35
0.3
0.25
0.2
0.15
0.1
0
2
4
6
8
10
Days post-infection
D
IgG3
2
1.5
1
0.5
0
0
2
4
6
8
10
Days post-infection

Figure 6.2

6.4.5 Antibodies to the cryptic VSG epitopes

Trypanosoma congolense clone TC13 on the ELISA plates was fixed using the cross-linking agent, paraformaldehyde. Fixation disrupts the surface coats of trypanosomes leading to the exposure of cryptic epitopes on the VSG (Reinitz and Mansfield, 1990). Infected BALB/c and C57BL/6 mice mounted essentially equivalent IgM responses to the cryptic epitopes of the VSG (Figure 6.3A), although these antibodies were detected by day 4 in the BALB/c mice and were significantly elevated ($p < 0.05$) relative to the C57BL/6 mice until day 6. Thereafter, the levels of IgM in the plasma of the two strains rose steadily, but with C57BL/6 mice significantly ($p < 0.05$) producing more than BALB/c mice. Infected BALB/c mice did not make any appreciable IgG (IgG1, IgG2a and IgG3) antibody response to the cryptic epitopes of the VSG. In contrast, a strong IgG response was mounted by infected C57BL/6 mice starting on day 5 (IgG3) or 6 (IgG1 and IgG2a) and peaking on day 7 (Figure 6.3B-D). The high IgG antibody levels in the plasma of infected C57BL/6 mice persisted up to day 9 (not shown) by which time the first wave of parasitemia were apparently controlled by these mice.

Figure 6.3 Antibodies to formalin-fixed *T. congolense.* Levels of IgM (A), IgG1 (B), IgG2a (C) and IgG3 (D) antibodies against formalin-fixed trypanosomes in the plasma of infected BALB/c and C57BL/6 mice. ELISA plates were coated with 2.5 x 10^5 fixed trypanosomes per well and the plasma samples were used at 1:50 dilution. Data are presented as mean ± SE. The set of results shown is a representative of two similar experiments. Legends are as for figure 6.4.

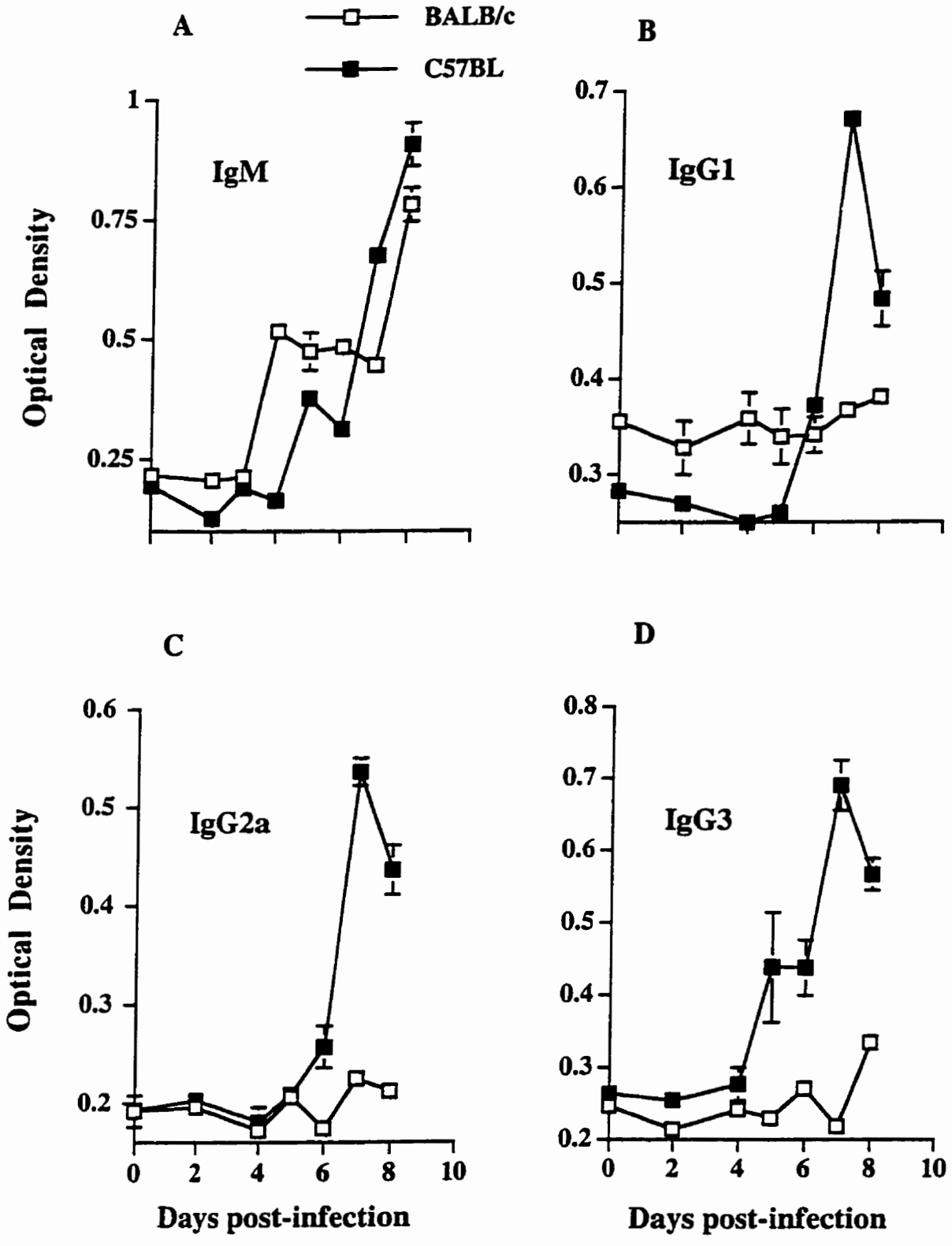
A
BALB/c
C57BL
IgM
Optical Density
1
0.75
0.5
0.25
B
IgG1
0.7
0.6
0.5
0.4
0.3
C
IgG2a
Optical Density
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
Days post-infection
D
IgG3
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
Days post-infection

Figure 6.3

6.4.6 Antibodies to whole *T. congolense* lysate

Although it has been suggested that anti-VSG antibodies are necessary for the control of variant specific parasitemia, recent reports indicate that antibodies to the invariant antigens of trypanosomes are also associated with resistance to trypanosome infections in cattle (Boulange and Authie1993; Authie et al., 1992; 1993a; Authie et al., 1993; Authie, 1994). To measure the antibody response to the whole *T. congolense* TC13 antigens, freeze-thawed trypanosomes were used to coat ELISA plates.

Plasma from infected BALB/c and C57BL/6 mice collected at different days after infection contained high levels of IgM antibodies to the whole parasite lysate. The kinetics and patterns of appearance of IgM antibodies were similar in the two strains of mice throughout the infection period (Figure 6.4A). In contrast, the levels of IgG antibodies in the plasma of the two mice strains were remarkably different. While IgG antibodies against the whole parasite lysate remained at the pre-infection levels in the plasma of infected BALB/c mice, a steady rise was observed in those of C57BL/6 mice starting from day 6 post-infection (Figure 6.4B-D). A strong IgG2a and IgG3 antibody response occurred in C57BL/6 mice starting on day 6 and increasing steadily through days 7-8. IgG1 antibody response started on day 7 and remained high by day 8 in C57BL/6 mice.

Figure 6.4 Antibodies to whole *T. congolense* lysate. IgM (A), IgG1 (B), IgG2a (C) and IgG3 (D) antibody responses to the whole *T. congolense* lysate in infected BALB/c and C57BL/6 mice. ELISA plates were coated with 1.25 μg/well of whole *T. congolense* clone TC13 lysate and plasma samples were used at 1:50 dilution. The set of results presented is a representative of two similar experiments. Legends are as for figure 6.4.

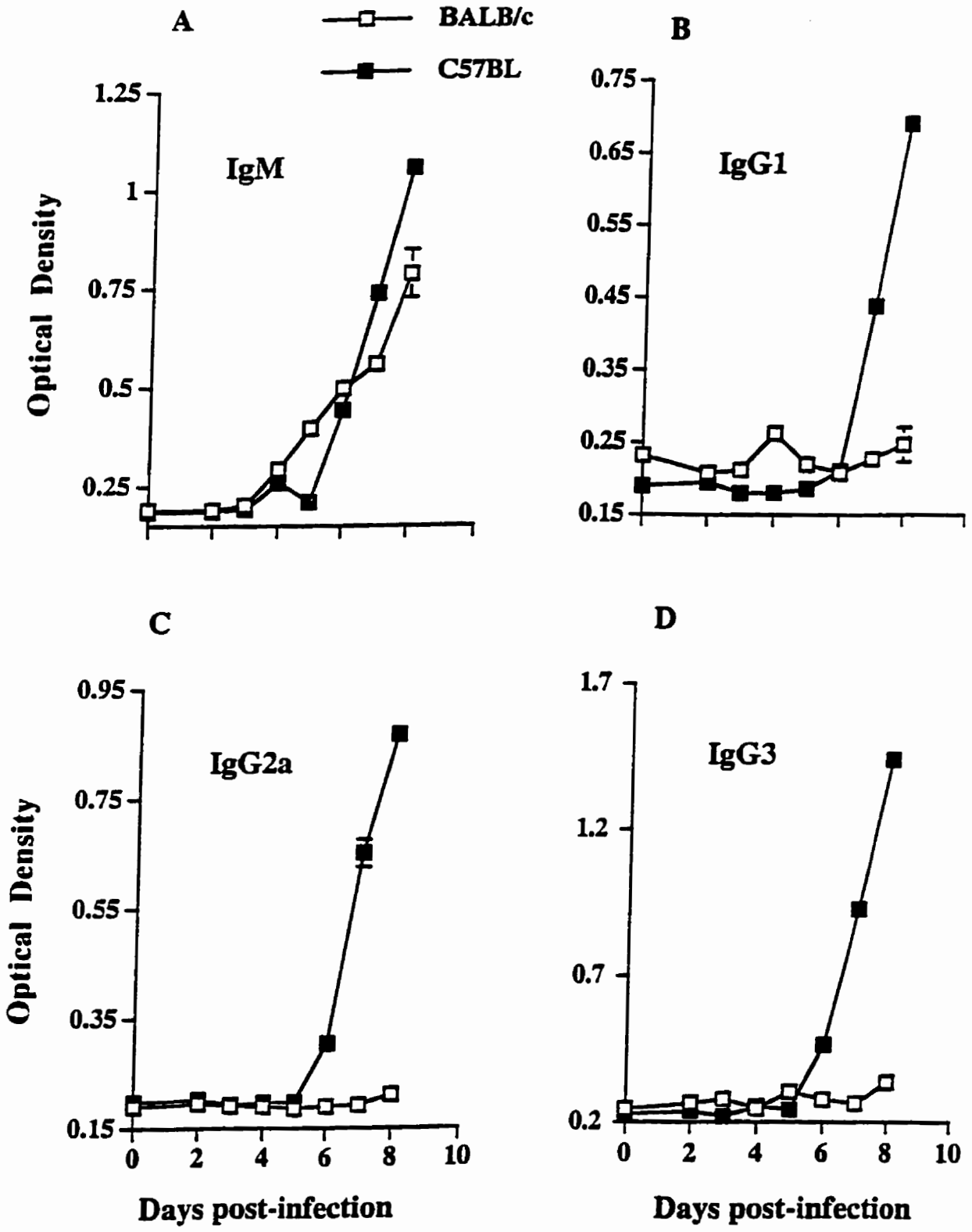
A
BALB/c
C57BL
IgM
Optical Density
1.25
1
0.75
0.5
0.25
B
IgG1
0.75
0.65
0.55
0.45
0.35
0.25
0.15
C
IgG2a
Optical Density
0.95
0.75
0.55
0.35
0.15
0
2
4
6
8
10
Days post-infection
D
IgG3
1.7
1.2
0.7
0.2
0
2
4
6
8
10
Days post-infection

Figure 6.4

6.5 Discussion

The present study shows that differences exist in the secretion of IL-4, IL-10 and IFN-γ in mouse strains of varying susceptibility to *T. congolense* infections. The susceptible BALB/c mice had significantly higher numbers of cells secreting these cytokines than the resistant C57BL/6 mice. Although the kinetics and pattern of appearance of IL-4, IL-10 IFN-γ-secreting cells were essentially similar, the frequency of these cells were moderately but significantly ($p < 0.05$) higher in BALB/c than in C57BL/6 mice on some days after infection. However, a striking difference was observed in the amounts of cytokine secreted in culture by splenocytes from these mice. For instance, although IL-4-secreting cells were detectable by ELISPOT among the splenocytes of infected C57BL/6 mice, these populations did not secrete detectable amounts (< 15 pg/ml) of IL-4 in culture, when assayed by ELISA (compare figures 4.3A and 6.1A). Similarly, although greater numbers of IL-4- than IFN-γ-secreting splenocytes (approximate ratio, 2:1) were detected on day 6 in the BALB/c mice, the ratio of IL-4 to IFN-γ protein secreted in culture was dramatically different (IL-4:IFN-γ = 1:85). Furthermore, dramatic differences were observed in the secretion of IL-10 and IFN-γ protein in the splenocyte cultures from the two mouse strains. The secretion of these cytokines occurred earlier in BALB/c mice and remained high throughout the infection. These observations suggest that the use of the number of cytokine-secreting cells as measured by ELISPOT to determine the type of helper T cell response in an immune response may not be very accurate, as there appears to be marked apparent differences between commitment to secretion and the actual amount secreted by the committed cell (Figures 4.2 & 6.1).

The splenocytes from the susceptible mice produced greater amounts of IL-4, IL-10 and IFN-γ in culture than did those from the relatively resistant mice. IL-10 has a profound down-regulatory effect on macrophages (Fiorentino et al., 1991; Moore et al., 1993), including inhibition of cytokine synthesis by these cells (Fiorentino et al., 1991). IL-10 also inhibits antigen-specific proliferation of T-cells (Moore et al., 1993) and this effect appears to be, in part, due to inhibitory effects on antigen presenting cells. We have shown previously that IL-10 and IFN-γ contribute to suppression of mitogen-induced proliferative responses of splenocytes from *T. congolense*-infected BALB/c mice and also to an enhanced susceptibility to the disease (see chapters 3 and 4). Recently, we have obtained evidence that when pulsed wih *T. congolense* lysates, IFN-γ-primed bone marrow-derived macrophages from BALB/c, but not those from C57BL/6 mice, up-regulate their IL-10 secretion by 2-4 fold (Kaushik et al., manuscript in preparation). Thus, the higher levels of IFN-γ secretion by splenocytes from infected BALB/c mice could potentially enhance IL-10, secretion leading to a more pronounced immunosuppression and an enhanced susceptibility in these animals. IL-10 has also been shown to inhibit IFN-γ-mediated nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) release by macrophages (Fiorentino et al., 1991; Gazzinelli et al., 1992). TNF-α has both static (Lucas et al., 1994) and lytic (Magez et al., 1997) effects on trypanosomes *in vitro* and inhibits trypanosome growth *in vivo* (Magez et al., 1993), while NO has a static effect on *T. brucei in vitro* (Vincendeau et al., 1992). Thus, high levels of IL-10 secretion by splenocytes from infected BALB/c mice could down-regulate the production of TNF-α

as well as NO by macrophages, thereby preventing their inhibitory effects on parasite multiplication and survival.

Both mouse strains were capable of mounting an IgM response against the purified VSG, cryptic (fixed) VSG, and whole trypanosome lysates. In fact, BALB/c mice mounted an earlier IgM antibody response to these antigens than did the C57BL/6 mice. Our data suggest that IgM antibody responses against various antigens of *T. congolense* may not be associated with the control of parasitemia, since the BALB/c mice made an earlier and higher specific IgM antibody response than the C57BL/6 mice. It is worth emphasizing that both mouse strains had almost identical parasite loads up to day 6 post-infection and during this period, higher levels of parasite-specific IgM antibodies were present in the plasma of infected BALB/c than in those of resistant C57BL/6 mice. These results are consistent with the reports of DeGee and Mansfield, (1984) that showed that the plasma levels of IgM antibodies to *T. brucei rhodesiense* in infected mice did not correlate with resistance to the infection.

The plasma levels of IgG antibodies were dramatically different in the two mouse strains. Only IgG1 antibodies to purified VSG were higher in the plasma of BALB/c than in that of C57BL/6 mice. In contrast, infected BALB/c mice were unable to make any substantial amounts of IgG2a and IgG3 antibody responses to the purified VSG and also failed to make any IgG (IgG1, IgG2a and IgG3) response to the cryptic epitopes of the VSG (fixed trypanosomes) or whole trypanosome lysate. The fact that these differences were apparent only at the level of IgG antibody isotypes suggests that this was not probably due to the antibody being absorbed out by an excess antigen load related to a higher parasitemia in the BALB/c mice, as has been suggested by Newson et al. (1990).

Although results of *in vitro* and *in vivo* studies on the relative potentials of IgM and IgG anti-trypanosome antibodies to confer protection have been equivocal, most evidence suggests that IgG may be more efficient than IgM in controlling *T. congolense* infections in mice (Morrison et al., 1978) and cattle (Williams et al., 1996). In cattle, higher frequencies of cells secreting IgM specific for the linear epitopes of the VSG as well as invariant antigens of *T. congolense* were found in the infected trypanosusceptible Boran (Taylor et al., 1996). In contrast, infected trypanotolerant N'Dama cattle have higher frequencies of spleen cells secreting VSG-specific IgG antibodies than do Boran cattle. Also, serum levels of VSG- (Williams et al., 1996) and *T. congolense* invariant antigen-specific IgG antibodies (Authie et al., 1992; 1993a,b) were higher in N'Dama than Boran cattle infected with *T. congolense*. These superior IgG antibody responses to the cryptic epitopes of the VSG (Williams et al., 1996) and invariant antigens of *T. congolense* (Authie et al., 1992; 1993a,b) have been suggested to be of importance in trypanotolerance (Authie 1994; Agur and Meher, 1997; Taylor, 1998).

We found no correlation between cytokine production by splenocytes from infected mice and the isotype of antibodies against the various antigens of the *T. congolense*. IL-4 produced by TH2 cells has been shown to favor the switch from IgM to IgG1 and IgE antibodies whereas IFN-γ produced by TH1 cells favors IgG2a and IgG3 isotype switching (Finkelman and Holmes, 1990; Boehm et al., 1997). The fact that BALB/c mice were unable to make IgG2a and IgG3 antibodies despite the secretion of high levels of IFN-γ by their splenocytes suggests that the measured IFN-γ may not be produced by conventional T helper cells and this might not have been available within the

immediate vicinity of cooperating TH-B cells. We have obtained evidence that most of the IL-10 and IFN-γ detected in *T. congolense*-infected BALB/c mice is produced by small numbers of $Thy1.2^{+}CD4^{-}8^{-}$ splenocytes that co-purify with plastic-, nylon wool- or Sephadex G-10-adherent cell populations (see chapter 5). These unique cells are stimulated in an antigen-specific manner, rather by polyclonal activation, and are involved in *T. congolense*-elicited suppression of T and B cell responses (see chapter 5). The lack of correlation between the high levels of IFN-γ secretion and IgG2a response to the VSG has also been reported in mice infected with *T. brucei rhodesiense* (Mansfield, 1994). The secretion of greater amounts of IL-4 by splenocytes from infected susceptible BALB/c than that from resistant C57BL/6 mice suggests that this cytokine may be associated with enhanced susceptibility to *T. congolense* infections in mice. This also seems to be the case with *T. brucei* infections since deletion of the IL-4 gene enhanced the survival times of mice infected with *T. brucei brucei* (Bakhiet et al., 1996).

It is intriguing that the IgG antibody levels against the various antigens of *T. congolense* were higher in the plasma of C57BL/6 mice, despite the lower levels of splenocyte IFN-γ and IL-4 secretion by these mice relative to the BALB/c mice. It could be that most of the IFN-γ, which is known to favor IgG2a and IgG3 isotype switching by B cells (Finkelman and Holmes 1990; Boehm et al., 1997), was secreted by TH1 cells in C57BL/6 mice. An alternative explanation for this might be related to differences in monokine secretion by macrophages from the two mouse strains following their interaction with trypanosomes. In this regard, we have evidence that, when pulsed with *T. congolense* lysates, IFN-γ-primed bone marrow-derived macrophages from BALB/c mice

secrete significantly higher amounts of IL-10 than those from C57BL/6 mice. In contrast, C57BL/6 macrophages secrete significantly more IL-12 p40 than do BALB/c macrophages (Kaushik et al., manuscript in preparation). We suspect that this differential production of IL-10 and IL-12 by macrophages from the two strains could have diverse effects on the B and T cells of the infected mice, including growth and differentiation effects, and isotype switching (B cells) or suppression. IL-10, a potent TH1 down-regulatory cytokine (Moore et al., 1993), suppresses splenocyte proliferative responses to Con A and enhances susceptibility to *T. congolense* infections in BALB/c mice (see chapter 3). Thus, the production of high levels of IL-10 by BALB/c mice could potentially down-regulate TH1 cell-dependent B cell responses, and thereby lead to such lower IgG2a and IgG3 antibody responses as were observed in this study. In contrast, the high level production of IL-12 by macrophages from the resistant C57BL/6 mice would favor TH1 cell development (Trinchieri, 1995) and thus IgG2a and IgG3 isotype switching (Boehm et al., 1997), as also was observed in this study.

In conclusion, we hypothesize that enhanced resistance (effective control of parasitemia and increased survival period) to *T. congolense* infections in mice is mediated by a TH1 cell response.

7.0 GENERAL DISCUSSION

7.1 Cytokine patterns during *T. congolense* infections

The present study shows that, during *T. congolense* infections, susceptible BALB/c mice have higher levels of IL-4, IL-10 and IFN-γ in their plasma than do resistant C57BL/6 mice. In contrast, higher levels of TNF-α were detected in the plasma of infected resistant mice than in the susceptible mice. Consistent with the levels of IL-10 and IFN-γ in the plasma, the steady state levels of mRNA for these cytokines in the splenic tissues increased earlier following infection and were higher in infected BALB/c mice than in C57BL/6 mice. As expected based on the mRNA results, both the spontaneous and Con A-induced production of IL-4, IL-10 and IFN-γ by splenocytes of infected BALB/c mice also were higher than with those of infected C57Bl/6 mice. Although the patterns and kinetics of appearance of IL-4, IL-10 and IFN-γ-spot-forming cells were similar, greater numbers of cytokine-secreting cells were detected in the spleens of BALB/c mice than in C57BL/6 mice on some days.

The marked difference between the numbers of cytokine-secreting cells as detected by the ELISPOT assay and the amount of cytokine secreted into the culture fluids as measured by ELISA was unexpected (see Figure 4.3 and 6.1). For instance, the numbers of IL-4-secreting cells were higher than IFN-γ-secreting cells whereas IFN-γ-secreting cells were by far the most producers on per cell basis in cultures. Moreover, although there were marginal but significant differences in numbers of IL-4-, IL-10- and

IFN-γ-secreting cells in the spleens of infected BALB/c and C57BL/6 mice, dramatic differences were observed in the production of these cytokines in cultures with BALB/c splenocytes producing significantly higher amounts than C57BL/6 splenocytes. These observations indicate that there is a marked difference between commitment to production of a cytokine and the quantity of the cytokine secreted in culture fluids by the committed cell. Whereas ELISPOT assay could detect cells producing as few as 100 molecules of a specific cytokine (Klinman and Nutman, 1994), the minimun sensitivity of the ELISA assay is in the order of picograms. Thus, this difference in the sensitivities of the two assays might explain the differences in the results obtained by ELISPOT and ELISA assays observed in this study.

Detailed studies on the differential expression of cytokines in mice exhibiting varying degrees of resistance to African trypanosomes are scanty. Schleifer and Mansfield (1993a) demonstrated that $CD4^+$ T cell clones derived from the peritoneal cavities of resistant mouse strains infected with *T. brucei rhodesiense* were of the TH1 phenotype (i.e. secreted high levels of IL-2 and IFN-γ). Unfortunately, cytokine responses by infected susceptible mouse strains were not analyzed in this study. In contrast, Bakhiet et al. (1996) reported that IFN-γ secretion by splenocytes from *T. brucei brucei*-infected mice was similar in the resistant and susceptible strains, while only the resistant mice had cells secreting IL-4. These workers concluded that resistance to this parasite was related to the ability to produce IL-4. However, the deletion of IL-4 genes in the resistant mice actually enhanced their resistance to *T. brucei brucei* infection suggesting that enhanced resistance to this parasite does not depend on the production IL-4.

The concomitant presence of high levels of IL-4, IL-10 and IFN-γ in the plasma and splenocyte cultures of infected BALB/c mice stimulated our curiosity, primarily because of the known reciprocal cross-regulation by these cytokines of TH1-TH2 responses. Consequently, questions were asked regarding the cellular source(s) and role(s) of these cytokines in the pathogenesis and enhanced susceptibility of BALB/c mice to *T. congolense* infections.

7.2 IL-10 and IFN-γ mediate *T. congolense*-induced immunosuppression

The role of cytokines in the pathogenesis and enhanced susceptibility of BALB/c mice to *T. congolense* infections was a primary research aim of this thesis work. Generalized immunosuppression affecting both humoral and cellular immune compartments is a common feature associated with African trypanosomes infections (Askonas, 1985; Darji et al., 1992; Sileghem et al., 1994a). It has been suggested that this is one of the factors responsible for the increased susceptibility of trypanosome-bearing individuals to opportunistic infections (Greenwood et al., 1973; Maxie et al., 1979). The present study clearly showed an involvement of endogenously produced IL-10 and IFN-γ in the *T. congolense*-mediated suppression of splenic T cell proliferative responses to a mitogen.

The downregulation of T cell proliferation during *T. brucei* infections has been attributed to at least two unlinked mechanisms, involving decreases in IL-2 secretion on one hand and IL-2R expression on the other (Sileghem et al., 1994a). The suppression of

IL-2 secretion is reportedly mediated by prostaglandins (Sileghem et al., 1986; 1994a) while the suppression of IL-2R expression is mediated by IFN-γ (Darji et al., 1996), although IFN-γ expression by itself was not sufficient to mediate this suppression. An unknown soluble factor released by *T. brucei*-pulsed macrophages was required to mediate this IL-2-related suppression (Darji et al., 1996). Our studies may well provide some insights into the nature of this unknown soluble factor. Exposure to *T. congolense* lysates, of IFN-γ-primed bone marrow-derived macrophages from highly susceptible BALB/c, but not relatively resistant C57BL/6 mice, causes 2-4 fold increases in their IL-10 production (Kaushik et al; manuscript in preparation). Thus, it is feasible that the IFN-γ-mediated suppression of proliferative responses of lymphoid cells observed in the present study (Figure 4.4) and other studies (Darji et al., 1993; 1996) may be indirect, at least in part, mediated via the up-regulation of IL-10 secretion by macrophages. In line with this is the observation that administration of neutralizing anti-IFN-γ antibodies to *T. congolense*-infected BALB/c mice dramatically reduced their plasma levels of IL-10 as well as the spontaneous and Con A-induced secretion of IL-10 by their splenocytes (Figure 4.6A,B).

Some aspects of immunosuppression in *T. brucei* infections in mice are reported to be mediated by NO produced by IFN-γ-activated macrophages (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993; Sternberg et al., 1994; Mabott et al., 1996). However, Darji et al. (1996) failed to observe any NO-mediated immunosuppression of lymph node cells from mice infected with *T. brucei*, and furthermore, in cattle infected with *T. congolense* and *T. vivax*, NO production is depressed and NO does not mediate the

T cell suppression observed in these animals (Taylor et al., 1996). In addition, both peritoneal and bone marrow-derived macrophages from the relatively resistant C57BL/6 mice, upon interaction with *T. congolense* lysates in the presence of IFN-γ, produce more NO than similarly treated cells from susceptible BALB/c mice (Kaushik et al., manuscript in preparation). Moreover, there is no difference in the production of NO by splenocytes from *T. congolense*-infected BALB/c and C57BL/6 mice (Appendix figure 3), yet the proliferative responses of splenocytes from BALB/c were more suppressed than those from C57BL/6 mice. Furthermore, the addition of the inducible nitric oxide synthase inhibitor into cultures of Con A-stimulated splenocytes from day 7 infected BALB/c mice does not reverse the suppression of proliferative responses observed in these splenocytes. Also, splenocytes from anti-IFN-γ-treated BALB/c mice secrete higher amounts of NO than their PBS-treated controls (see section 4.4.6). These observations strongly indicate that, like in cattle (Taylor et al., 1996), NO-mediated suppression does not operate during *T. congolense* infections in mice. Thus, the restoring effect of anti-IFN-γ treatment on proliferation of splenocytes from *T. congolense*-infected mice observed in the present study was not mediated by reduction in NO synthesis.

7.3 Cellular sources of IL-4, IL-10 and IFN-γ in the spleens of infected BALb/c mice

This study identified a unique set of T cells that is involved in the regulation and/or secretion of IL-4, IL-10 and IFN-γ in the spleens of *T. congolense*-infected mice. These Thy1.2$^+$CD4$^-$8$^-$ cells co-purify with plastic-, nylon wool- or Sephadex G-10-

adherent cell populations. The production of IL-4, IL-10 and IFN-γ by the Thy1.2$^+$ cells requires cooperation between these cells and Thy1.2$^-$ cells present in the spleens of *T. congolense*-infected, but not uninfected, mice and occurs by an antigen-specific mechanism. In *T. brucei*-infected rodents, CD8$^+$ T cells have been reported to be the major producers of IL-4 and IFN-γ (Bakhiet et al., 1990; 1993; 1996). In that system, direct binding of a trypanosome-derived lymphocyte triggering factor to the CD8 molecule on CD8$^+$ T cells was reported to trigger these cells to produce large amounts of IFN-γ (Bakhiet et al., 1990; Olsson et al., 1991). The results reported here differ from those of the *T. brucei* system in that they clearly show that Thy1.2$^+$CD4$^+$8$^-$ and Thy1.2$^+$CD4$^-$8$^-$ cells are the major producers of IL-4, IL-10 and IFN-γ in the spleens of BALB/c mice infected with *T. congolense*.

During *T. congolense* infections in mice, progressive increases in the numbers of Ig$^-$Thy1$^-$ cells in the spleens of infected mice have been reported (Roelants et al., 1978; 1979; Kar et al., 1979; Roelants and Pinder, 1984). The characterization of these cells was based on microscopic examination of cells treated with fluorescein-labeled anti-Thy1 antibodies, a method which might not detect low level expression of Thy1.2 antigen. It is conceivable that these Ig$^-$Thy1$^-$ cells (so-called "null cells") were in reality Thy1$^+$ and equivalent to our Thy1.2$^+$CD4$^-$8$^-$ cells. This is likely to be so since this same research group reported in another study the existence of immunosuppressive Thy1^{+weak} cells in the spleens of *T. congolense*-infected mice (Pearson et al., 1979a). It would be worthwhile to investigate whether the Thy1.2$^+$CD4$^-$8$^-$ cells from our model express lower levels of

Thy1.2 antigens than do Thy1.2$^+$CD4$^+$ and Thy1.2$^+$CD8$^+$ cells and whether their numbers increase progresively in the spleen of *T. congolense*-infected mice.

A potential role these adherent Thy1.2$^+$CD4$^-$8$^-$ cells in the pathogenesis of *T. congolense* infection was established by demonstrating their involvement in suppression of T and B cell responses to mitogen and SRBC respectively. Previous reports from independent laboratories have shown that both T cells (Eardley and Jayawardena, 1977; Jayawardena et al., 1978, Pearson et al., 1979) and adherent macrophage-like cells (Askonas, 1985; Sileghem et al., 1986; Flynn et al., 1991; Schleifer and Mansfield, 1993) are involved in the suppression of lymphoid responses in trypanosome-infected mice. In a thorough review of the mechanisms of trypanosome-induced immunosuppression, Roelants and Pinder (1984) suggested that both cell types are important. The finding in the present study that the production of IL-10 and IFN-γ (both of which can be immunosuppressive) by spleen cells from infected mice requires cooperation between adherent Thy1.2$^+$ and Thy1.2$^-$ cells further supports the reports of Corsini et al. (1978) and Pearson et al (1978a; 1979) and the suggestion of Roelants and Pinder (1984) that both T cells and macrophages are important in trypanosome-induced immunosuppression. Suppressor T cells could alter (via cell to cell contact or cytokines) macrophage functions and cytokine patterns, thereby rendering them suppressive. On the other hand, macrophages could become suppressive after interaction with trypanosomes or their products and such suppressive activity may be transferred to T cells during antigen presentation.

What marker(s) distinguish these Thy1.2$^-$CD4$^-$8$^-$ adherent-like cells from conventional T cells and what is the nature of the trypanosomal antigen that they recognize? These questions were not addressed in this work but it is conceivable that these were NK1$^+$ T cells. NK1$^+$ T cells, which can either be Thy1.2$^-$CD4$^+$8$^-$ (60%) or Thy1.2$^-$CD4$^-$8$^-$ (40%), can secrete large amounts of IL-4, IL-10 and IFN-γ upon appropriate stimulation, and comprise about 0.5 - 1% of splenocytes from normal mice (Bendelac, 1995; Vicari and Zlotnik, 1996; Bendelac et al., 1997). Their activation is restricted by the CD1 molecule and they recognize both hydrophobic peptides and lipid antigens presented in association with CD1 (Porcelli et al., 1992; Bendelac et al., 1997). Double negative NK1.1$^+$ T cells that recognize a lipid antigen derived from the lipoarabinomanan of *M. tuberculosis* have been reported (Porcelli et al., 1992). The variant surface glycoprotein of African trypanosomes is inserted into the plasma membrane via the glycosyl phosphatidylinositol (GPI) similar to the lipoarabinomanan of *M. tuberculosis* (Ferguson et al., 1985; 1988; Ferguson, 1997). It is conceivable that similar antigen processing and presentation of lipid antigens derived from the GPI anchor of the VSG to Thy1.2$^-$CD4$^-$8$^-$ cells could well occur during infections with *T. congolense*.

What advantages could the induction of Thy1.2$^-$CD4$^-$8$^-$ cells confer to the trypanosomes in the infected host? The answers to this question are all purely speculative. Clearly, the copious secretion of large amounts of IL-10 and IFN-γ by these cells mediates immunosuppression within the host (discussed in section 7.2). The induction of immunosuppression might prevent effective immune responses by the host and thus delay the clearance of the parasite from the circulation, thereby allowing additional time for new

variants to arise. It has been reported that IFN-γ has a growth-stimulatory effect for *T. brucei* (Olsson et al., 1991), although we have tested this and been unable to demonstrate such an effect with *T. congolense* (Kaushik et al., 1997). However, it is not unlikely that under certain conditions, such an effect could occur. In such conditions, the activation of $Thy1.2^{+}CD4^{-}8^{-}$ cells would be advantageous for the parasite's survival.

7.4 The role of IL-10 and IFN-γ in susceptibility to *T. congolense* infections

A role for IL-10 and IFN-γ in the enhanced susceptibility of BALB/c mice to *T. congolense* infections was clearly demonstrated. Whereas in vivo administration of anti-IL-10 antibodies to BALB/c mice early during infection moderately but significantly prolonged their survival period, the administration of anti-IFN-γ antibodies dramatically shifted the phenotype of most of these highly susceptible mice to a resistant-like phenotype. Overall, the anti-IFN-γ-treated mice expressed low and undulating parasitemias and had a greater than 300% increase in the mean survival period. The anti-IFN-γ treatment was associated with a dramatic decline in the plasma levels of IL-10 as well as a reduction in both the spontaneous and Con A-induced secretion of IL-10 by splenocytes from the infected mice.

Based on the *in vitro* results in which anti-IL-10 antibody treatment completely reversed the suppression of Con A-induced splenocyte proliferation, a much longer survival periods for the anti-IL-10-treated *T. congolense*-infected mice was anticipated than were observed. An explanation for this low impact of anti-IL-10 antibody *in vivo* could potentially be related to the IgM isotype of the antibodies. While IgG antibodies

penetrate relatively easily through the capillary wall and into the tissues, the much larger IgM antibodies do not and thus are restricted much more to the intravascular compartment (Nakamura et al., 1968, Spiegelberg, 1974). Consequently, these antibodies would not be available at optimal concentrations within the interstitial tissues, where cytokines would predominantly mediate their local effects. It is proposed that an IgG anti-IL-10 antibody would present better effects *in vivo* than those obtained in the present study. An alternative approach to further investigate the role of IL-10 would be the use of IL-10 gene-knock out mice.

It is conceivable that the beneficial effects of *in vivo* neutralization of IL-10 and IFN-γ relate more to the reversal of immunosuppression associated with the infections which would then allow the infected animals to mount efficient and effective immune responses against the parasites. IL-10, formerly known as cytokine synthesis inhibitory factor (Mosmann et al., 1990) indirectly inhibits, via its effects on the antigen presenting cells, proliferation and cytokine production by TH1 cells (Mosmann and Coffman, 1989; Moore et al., 1993). TH1 cells and their cytokines promote B cell growth and production of IgG2a and IgG3 antibodies (Boehm et al., 1997) which would mediate a different kind of adherence of trypanosomes to macrophages via the Fcγ receptors (see section 7.5). Thus, the inability observed in this study of infected BALB/c mice to make antibodies of these isotypes may be associated with the production of high amounts of IL-10 by splenocytes from these mice.

On the other hand, neutralization of IL-10 and IFN-γ might enhance the production of TNF-α and NO by macrophages (Kupffer cells). Both NO (Vincendeau et

al., 1992) and TNF-α (Magez et al., 1993; Lucas et al., 1994) are trypanostatic for African trypanosomes. IL-10 inhibits IFN-γ-mediated NO and TNF-α production by macrophages (Fiorentino, et al., 1991; Gazinelli, et al., 1992; Moore et al., 1993). Thus, the high level production of IL-10 by infected BALB/c mice relative to that observed in C57BL/6 mice in this study may be responsible for the lower levels of TNF-α found in the plasma of the BALB/c mice. Consistent with this, we observed that the administration of anti-IFN-γ antibodies to *T. congolense*-infected BALB/c mice resulted in a dramatic decrease in IL-10 and a subsequent increase in NO production by splenocytes from these mice. Also, IFN-γ-primed bone marrow-derived macrophages from BALB/c mice pulsed with *T. congolense* lysate produce more IL-10 but less TNF-α than similar cells from C57BL/6 mice (Kaushik et al., manuscript in preparation). Thus, an effective suppression of NO and TNF-α production by the high levels of IL-10 in the BALB/c mice could prevent the trypanostatic and trypanolytic effects of NO and TNF-α, thereby allowing the parasites to multiply rapidly. In contrast, the production of low levels of IL-10 within infected C57BL/6 mice would facilitate the production of high levels of NO and TNF-α which would be beneficial for parasite control.

How do the anti-IFN-γ antibodies abolish IL-10 synthesis by splenocytes of *T. congolense*-infected mice? In the LPS model of macrophage activation, IFN-γ inhibits IL-10 synthesis (Moore et al., 1993) via inhibition of IL-10 gene transcription (Shakhov et al., 1996); but, in macrophages pulsed with *T. congolense* lysate, IFN-γ actually up-regulates IL-10 production. Specifically, priming with IFN-γ prior to interaction with *T. congolense* lysates leads to 2-4-fold increases in IL-10 production by bone marrow-

derived macrophages of BALB/c but not C57BL/6 mice (Kaushik et al., manuscript in preparation). Thus, during infection, *T. congolense* alters the behavior of macrophages from BALB/c mice such that their responses to IFN-γ are skewed towards IL-10 production. It is possible that this differential response of macrophages from BALB/c and C57BL/6 mice to *T. congolense* might be one of the genetic factors controlling the differential resistance of the two mouse strains to infections with *T. congolense*.

7.5 Antibody response to *T. congolense* in BALB/c and C57BL/6 mice

Infected BALB/c mice mounted earlier IgM antibody responses to the purified VSG and fixed VSG as well as to the whole *T. congolense* lysate than did the C57BL/6 mice. In contrast, BALB/c mice failed to mount any detectable IgG2a and IgG3 antibody responses to these antigens whereas infected C57BL/6 mice made vigorous and sustained IgG responses that peaked at the time of control of parasitemia.

Detailed analyses of the roles of anti-VSG antibodies in resistance to *T. brucei rhodesiense* infections in mice showed that there is no correlation between the production of specific IgM antibodies and resistance (DeGee and Mansfield, 1984; 1988). The present work further supports these reports, since highly susceptible BALB/c mice mounted earlier and initially higher IgM responses to the various antigens of *T. congolense* than did the resistant C57BL/6 mice. On the contrary, the data suggest that IgG2a and IgG3 antibodies to the cryptic epitopes of the VSG and whole trypanosome lysate may be associated with control of parasitemia and enhanced resistance to *T. congolense* infections in mice, since significantly higher levels of these antibodies were

made by the resistant C57BL/6 than the susceptible BALB/c mice. In cattle, superior IgG antibody responses against the invariant antigens of *T. congolense* have been associated with trypanotolerance (Authie et al., 1993a,b; Authie 1994; Agur and Meher, 1997).

How could IgG anti-trypanosome antibodies mediate better protection than IgM antibodies? Clearly, the mechanism can not be through enhanced phagocytosis, since both IgM and IgG antibodies mediate comparable uptake of trypanosomes by macrophages (Ngaira et el., 1983; Kaushik et al., unpublished data). It is speculated that the difference lies in the differential capacities of these antibodies to mediate adherence of trypanosomes to macrophages during the early phases of the antibody response. Such adherence would facilitate the exposure of the trypanosomes to the direct static and lytic effects of NO and TNF-α being produced by the macrophage (see section 7.4 above). Binding of IgM antibodies to the VSG induces shedding of the VSG from the parasite (H. Tabel, personal communication) thereby exposing the carbohydrate residues on the VSG as well as the underlying plasma membranes, leading to rapid phagocytosis via the carbohydrate receptors on the macrophages (Mansfield, 1990). In contrast, binding of the few available molecules of IgG antibodies to the trypanosomes during the initial phases of the antibody response could conceivably result in a prolonged Fcγ receptor-mediated adherence but only minimal phagocytosis by macrophages.

There was no correlation between the cytokines produced by the splenocytes of infected mice and the isotypes of antibodies against the various antigens of the parasite. The fact that BALB/c mice were unable to make IgG2a and IgG3 antibodies despite the secretion of high amounts of IFN-γ by their splenocytes suggests that the measured IFN-γ

was not produced by conventional T helper cells. Indeed, it was shown in this study that most of IL-10 and IFN-γ produced by splenocytes from infected BALB/c mice arises from small numbers of Thy1.2^{-}CD4^{-}8^{-} cells that are plastic-, nylon wool- or Sephadex G-10-adherent. On the other hand, the fact that strong IgG2a and IgG3 antibodies were made by the C57BL/6 mice suggests that some of the IFN-γ measured in those mice might have been produced by TH1 cells. Such a lack of correlation between the high levels of IFN-γ secretion and an IgG2a isotype response to the VSG has also been reported in mice infected with *T. brucei rhodesiense* (Mansfield, 1994).

Differences in IL-10 and IL-12 production by macrophages from BALB/c and C57BL/6 mice following their interaction with trypanosomes may be responsible for the differential antibody isotype production in these mice. As noted previously, IFN-γ-primed bone marrow-derived macrophages from BALB/c mice pulsed with *T. congolense* lysate secrete significantly more IL-10 than do those from C57BL/6 mice. In contrast, the secretion of IL-12 p40 by these macrophages was significantly greater in the C57BL/6 than in the BALB/c mice. IL-10, a potent down-regulatory cytokine (Moore et al., 1993), mediates a suppression of splenocyte proliferative responses to Con A and an enhanced susceptibility to *T. congolense* infections in BALB/c mice (see section 3). IL-10 also inhibits cytokine synthesis by TH1 cells (Mosmann et al., 1990). Thus, the production of high levels of IL-10 by infected BALB/c mice might be expected to down-regulate TH1-dependent B cell responses, leading to lower IgG2a and IgG3 antibody responses as observed in this study. On the contrary, the high level production of IL-12 by macrophages from the resistant mice would favor TH1 cell development (Trinchieri,

1995) and thereby IgG2a and IgG3 isotype production (Boehm et al., 1997). These antibodies might, in turn, mediate an adherence of trypanosomes to macrophages that enhances the cytostatic effects on trypanosomes of NO and TNF-α produced by the macrophages. It is hypothesized that control of parasitemia and increased survival in mice infected with *T. congolense* is mediated by a TH1 cell response.

7.6 Future directions

The studies presented in this thesis have brought up many questions that, if answered, would shed more light on the mechanisms mediating the pathogenesis and enhanced resistance to *T. congolense* infections. These questions include the following:

1) By what mechanism(s) does in vivo neutralization of IL-10 and IFN-γ enhance the resistance of the highly susceptible BALB/c mice to *T. congolense* infections? Clearly, it was shown in this report that IL-10 and IFN-γ are involved in trypanosome-induced suppression of splenocyte proliferative responses. It was therefore suggested that neutralization of these cytokines abrogated trypanosome-induced immunosuppression, thereby enabling these mice to make an effective immune response. This suggestion is based on the premise that the degree of immunosuppression in different strains of mice infected with African trypanosomes is correlated with the degree of susceptibility (Morrison et al., 1978; Roelants and Pinder, 1984; Pinder et al., 1986). However, an alternative hypothesis could be formulated based on the inhibitory effects of IL-10 on NO and TNF-α production by macrophages (Fiorentino et al., 1991, Gazinelli et al., 1992). NO is cytostatic for *T. b. rhodesiense* in vitro (Vincendeau et al., 1992) and TFN-α has

both static and lytic effects on *T. brucei in vitro* and *in vivo* (Magez et al., 1993; 1997; Lucas et al., 1994). It is conceivable that the high levels of IL-10 in the susceptible BALB/c mice potently inhibit the production of these molecules and consequently their static and lytic effects, thereby allowing the parasites to further multiply unchecked. Further studies are needed to dissect the mechanisms by which anti-IL-10 and anti-IFN-γ antibody treatments in BALB/c mice are beneficial.

2) It was clearly shown that splenic adherent Thy1.2$^+$CD4$^-$8$^-$ cells are the major T cells involved in the production of IL-4, IL-10 and IFN-γ in BALB/c mice infected with *T. congolense* clone TC13 and that these cells could mediate suppression of T and B cell responses to mitogens and unrelated antigens. By what mechanism(s) are these cells activated? Are they activated by trypanosomal antigen(s)? If so, what is the nature of the antigen(s)? How is this trypanosomal antigen(s) processed and presented? What is the antigen-presenting cell(s)? What is the restricting element(s)? These are very interesting and mind-searching questions that require answers in order to fully understand and appreciate the pathogenesis of African trypanosomiasis.

3) It was not tested whether IL-4-, IL-10- and IFN-γ-secreting adherent Thy1.2$^+$CD4$^-$8$^-$ cells were NK1$^+$ T-cells. NK1$^+$ T cells, which can either be Thy1.2$^+$CD4$^+$8$^-$ (60%) or Thy1.2$^+$CD4$^-$8$^-$ (40%), are known to secrete large amounts of IL-4, IL-10 and IFN-γ upon appropriate stimulation, and comprise about 0.5 - 1% of splenocytes from normal mice (Vicari and Zlotnik, 1996; Bendelac et al., 1997). Their activation is restricted by the

CD1 molecule and they recognize both hydrophobic peptides and lipid antigens presented in association with CD1 molecules (Porcelli et al., 1992; Bendelac et al., 1997). Double negative NK1.1 T cells have been reported to act as suppressor cells in hemopoesis (Kikly and Dennert, 1992), tumor development (Tamada et al., 1997) and autoimmunity (Sumida et al., 1995; Meiza et al., 1996). It will be highly desirable to know whether the Thy1.2$^-$CD4$^-$8$^-$ cells of this investigation are indeed NK1.1 T cells.

4) Most of the studies reported in this thesis were done in the highly susceptible BALB/c mice. This choice was guided, in part, by the findings that the patterns of early responses may influence the overall outcome of the disease (Ogunremi and Tabel, 1995). It will be desirable to study the involvement of the adherent Thy1.2$^+$CD4$^-$8$^-$ cells in the secretion of IL-10 and IFN-γ in infected relatively resistant C57BL/6 mice. *Trypanosoma congolense*-infected C57BL/6 mice produce very low amounts of IL-10 and IFN-γ, yet they are immunosuppressed (albeit to a relatively lesser degree than BALB/c mice). It will be desirable to investigate the roles of IL-10 and IFN-γ in this suppression in order to determine whether other mechanisms, such as NO-mediated suppression, operate in these mice.

5) It is hypothesized that effective control of parasitemia and increased survival periods in *T. congolense*-infected mice are correlated with TH1 cell responses to variant and invariant antigens. The present study suggested that IgM anti-VSG antibodies may not be associated with resistance, as was previously reported (DeGee and Mansfield, 1984).

Rather, IgG2a and IgG3 antibodies against the cryptic epitopes of the VSG and whole parasite lysate were higher in the resistant C57BL/6 mice and the levels of these antibodies peaked during the period of control of first wave of parasitemia. It would be desirable to measure the cytokine and antibody isotype responses to the variant and invariant parasite antigens in the few C57BL/6 mice that self cure. It is predicted that IgG2a and IgG3 antibodies against the invariant antigens will be high in the plasma of these mice.

8.0 CONCLUSIONS

The main objectives of this study were to study the patterns of cytokine secretion and the role of cytokines in resistance to *T. congolense* employing highly susceptible BALB/c and relatively resistant C57BL/6 mice. The kinetics and patterns of appearance of IL-4-, IL-10- and IFN-γ-secreting cells in the spleens of infected mice were essentially similar. However, a dramatic difference was observed in the secretion of cytokines in cultures. The infected BALB/c mice produced higher levels of these cytokines than infected C57BL/6 mice. The production of high levels of IL-4, IL-10 and IFN-γ was restricted mainly to a $Thy1.2^{+}CD4^{-}8^{-}$ splenocyte population that are plastic-, nylon wool- or Sephadex G-10-adherent and which require some interaction with $Thy1.2^{-}$ cells to produce these cytokines. These cells effectively suppressed Con A-induced proliferation of splenocytes from uninfected mice as well as B cell responses to SRBC.

Both endogenously produced IL-10 and IFN-γ mediate suppression of splenocyte proliferative responses to Con A and enhanced susceptibility to the infection. Neutralization of these cytokines *in vivo* enhanced the resistance of the highly susceptible BALB/c mice to *T. congolense* infection. The beneficial effect of anti-IFN-γ was associated with the reduction of IL-10 production.

There was no correlation between the production of high levels of cytokines and production of antibodies against various antigens of *T. congolense*. Infected BALB/c mice made earlier IgM antibody responses but were unable to produce IgG2a and IgG3

antibodies to these antigens. In contrast, the production of IgG2a and IgG3 antibodies was correlated with the control of parasitemia in C57BL/6. It is hypothesized that enhanced resistance (effective control of parasitemia and increased survival period) to *T. congolense* infection in mice is correlated with a TH1 cell response.

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10.0 APPENDIX

10.1 Restoration of Con A-induced proliferative response of splenocytes from *T. congolense*-infected BALB/c mice following treatment with Berenil.

Table 1. Berenil treatment[a] reverses suppression of Con A-induced proliferation of splenocytes from infected mice[b]

DAYS POST-INFECTION	ABSORBANCE (595 nm) ± SD	
	Untreated	Berenil-treated
0	0.751 ± 0.048	ND
6	0.664 ± 0.024	ND
7	0.450 ± 0.039	0.458 ± 0.073
8	0.420 ± 0.090	0.541 ± 0.072
9	0.250 ± 0.029	0.684 ± 0.022

[a] Infected mice were treated with Berenil (14 mg/kg) on days 6 and 7 post-infection.
[b] Cultures of splenocytes (10^5 cells/well) from uninfected or *T. congolense*-infected BALB/c mice were stimulated with 5 μg/ml Con A. After 72 hr, proliferative response was measured by the MTT dye incorporation assay. Data are presented as mean ± standard deviation. ND = Not done.

10.2 Con A-induced secretion of IL-4 by splenocytes from *T. congolense*-infected BALB/c and C57BL/6 mice.

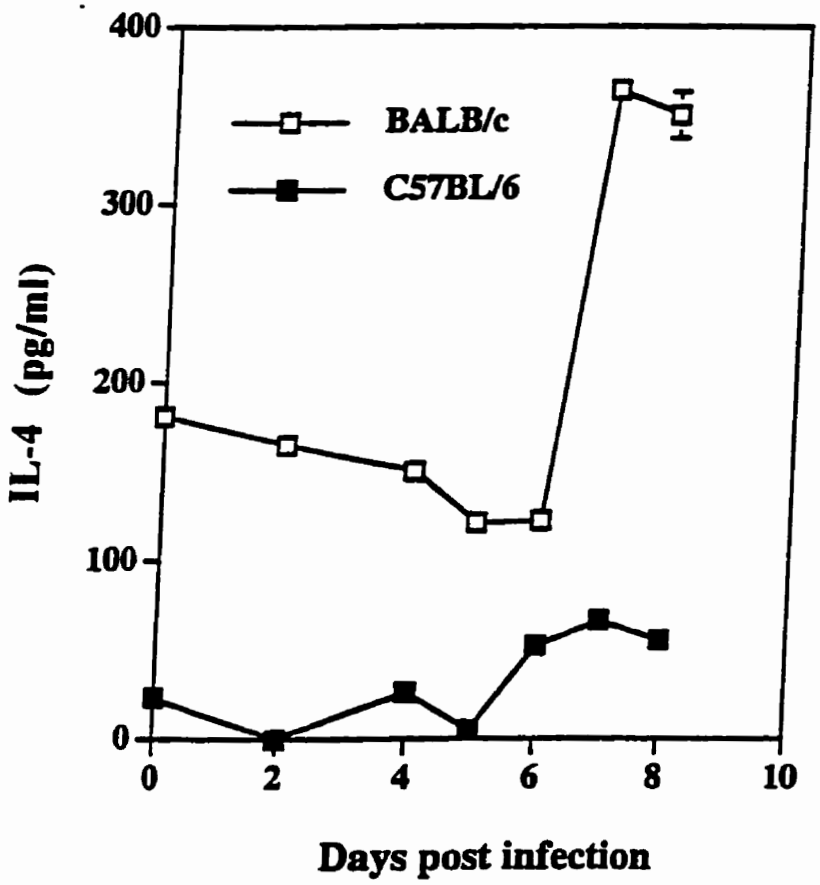


Figure 1. Con A-induced secretion of IL-4 by splenoctyes from *T. congolense*-infected BALB/c and C57BL/6 mice.

10.3 Con A-induced secretion of IFN-γ by splenocytes from *T. congolense*-infected BALB/c and C57BL/6 mice.

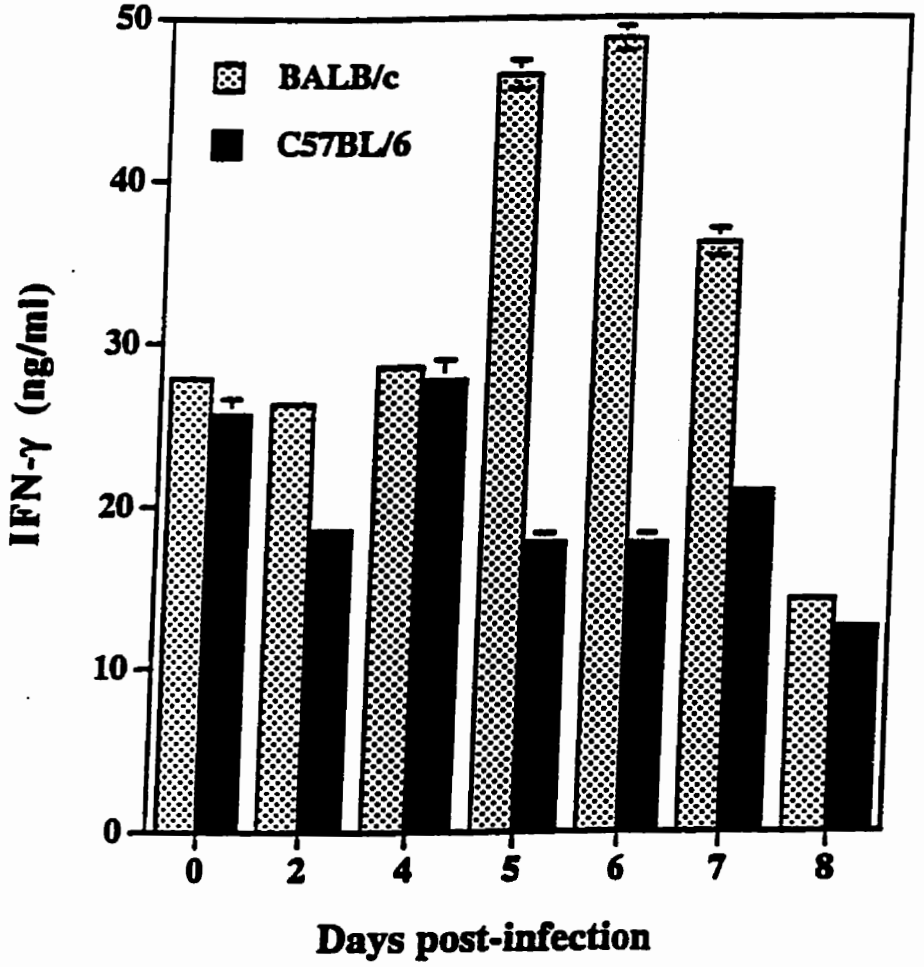


Figure 2. Levels of IFN-γ in culture fluids of Con A stimulated splenocytes from *T. congolense*-infected BALB/c and C57BL/6.

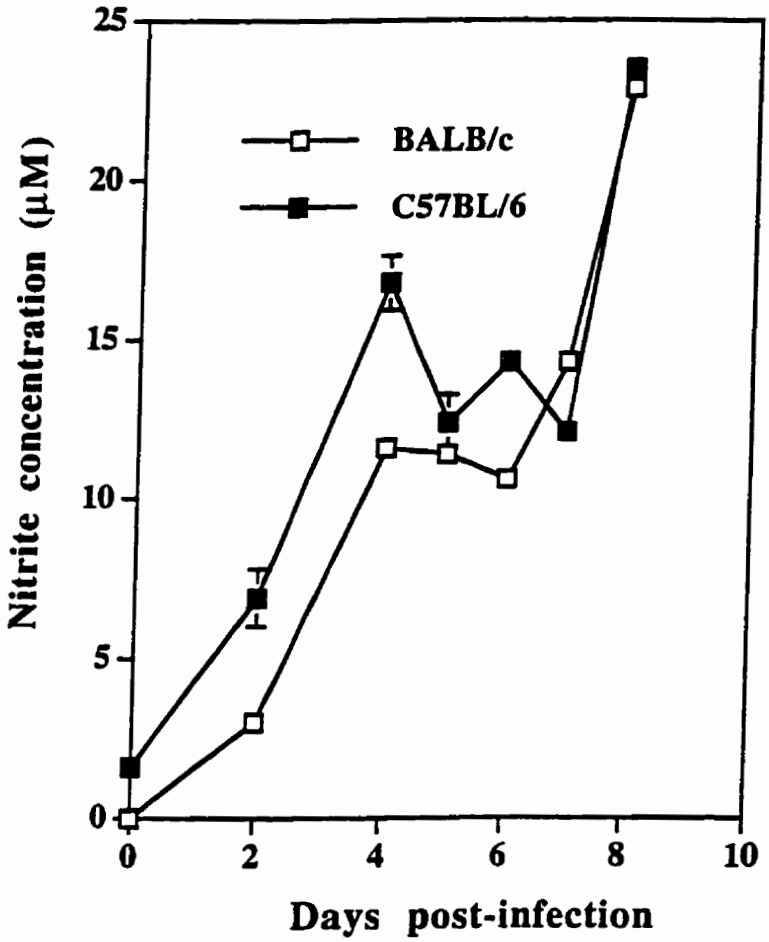
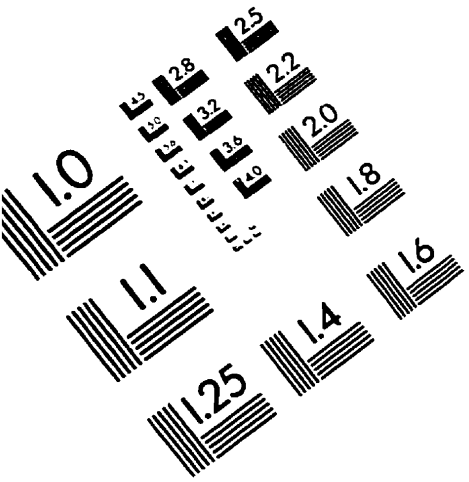
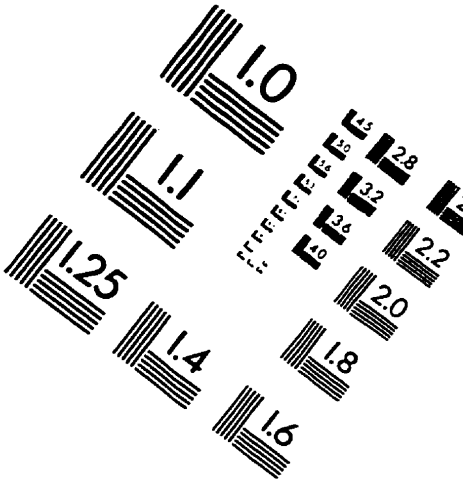
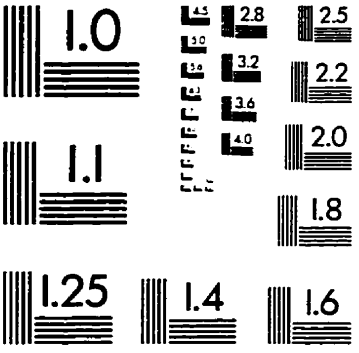


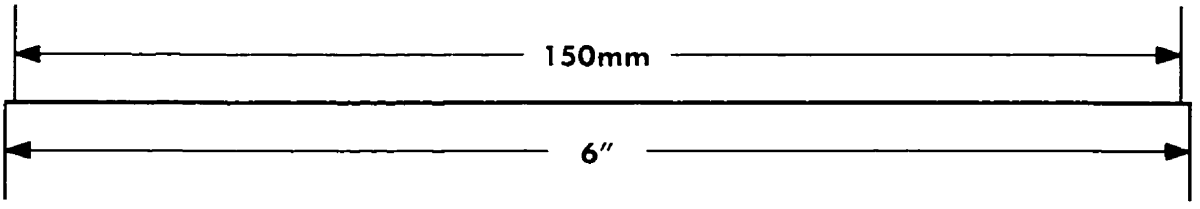
Figure 3. Production of nitric oxide by splenocytes from *T. congolense*-infected mice. Splenocytes from infected BALB/c and C57BL/6 mice were cultured in the presence of 5 μg/ml Con A. After 48 hr, the supernatant fluids were collected and the level of nitrite in the fluids was determined by the Griess reaction.

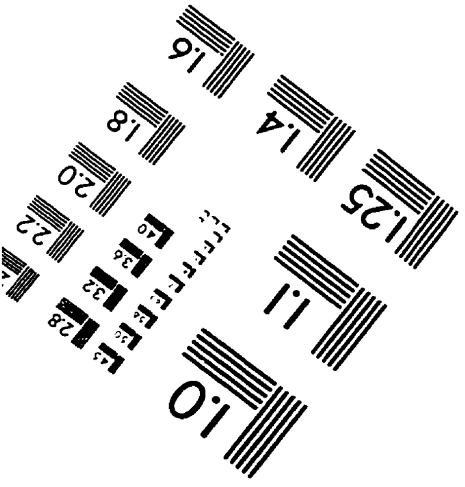
IMAGE EVALUATION
TEST TARGET (QA-3)

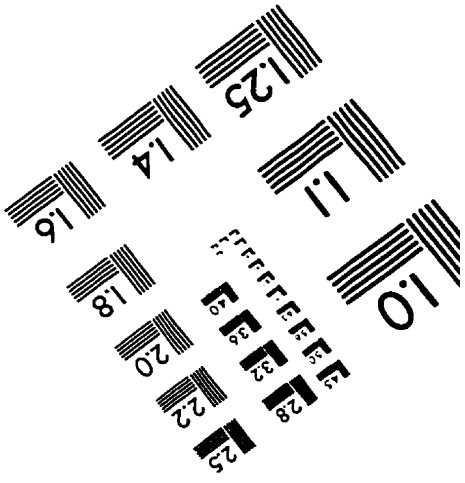












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