COMPLEMENT RECEPTOR 2 (CR2/CD21) IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

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ABSTRACT

African trypanosomes are protozoan blood parasites that infect both humans and livestock. BALB/c mice are highly susceptible to experimental infections by *Trypanosoma congolense* while C57BL/6 mice are relatively resistant, as measured by degree and pattern of parasitemia and survival time. Rapid death observed in highly susceptible BALB/c mice is due to a systemic inflammatory response syndrome (SIRS). A small subset of pathogenic, MHC class II-restricted $CD4^+$ T cells, activated during the course of *T. congolense* infections, mediates early mortality in infected highly susceptible BALB/c mice via excessive synthesis of the cytokine IFN-γ. Since these pathogenic T cells are matrix–adherent, they could be distinguished from conventional Th1 cells. There is a possibility that this subpopulation of T cells has unique surface markers.

The complement system is highly activated in African trypanosomiasis, leading to persistent hypocomplementemia. Amplification of the alternative pathway of complement is faster in BALB/c mice than in C57BL/6 mice and the degradation of complement component C3b to complement component C3d, during the amplification of the alternative pathway of complement, proceeds faster in BALB/c than in C57BL/6 mice (Ogunremi et al., 1993). *T. congolense*-infected BALB/c mice have more immune complexes containing trypanosomal variant surface glycoprotein (VSG) than C57BL/6 mice in their plasma (Pan & Tabel, unpublished). *T. congolense*-infected BALB/c mice might have more VSG-C3d immune complexes than infected C57BL/6 mice. The receptor for complement component C3d is the cell surface molecule CR2, also referred to as CD21. It is known that CR2 is widely expressed on B lymphocytes and follicular dendritic cells. There is also some evidence that CR2 is expressed on a subpopulation of activated T cells. Binding of VSG-C3d immune complexes to

the complement receptor CR2 might costimulate the $CR2^+$ T cells to produce IFN-γ. I hypothesize that IFN-γ-producing T cells in *T. congolense*-infected BALB/c mice are $CR2^+$ and that the $CR2^+$ T cells increase in numbers in experimental murine *T. congolense* infections.

Kinetic studies were carried out by staining spleen cells of *T. congolense*-infected BALB/c mice for the presence of CR2 on T cells ($CD3^+$ cells). Total numbers of spleen cells showed a 5-fold increase with progressive *T. congolense* infections. The total numbers of T cells in the spleen showed a 7-fold increase at day 8 post infection. The total numbers of $CR2^+$ T cells in the spleen showed a 3 to 7-fold increase with progressive infection. Parallel studies on B lymphocytes ($CD19^+$ cells) showed that absolute numbers of B cells in the spleen had a 5 to 6-fold increase with progressive infection. Absolute numbers of $CR2^+$ B cells in the spleen showed a 4-fold increase at day 7 post infection. The total numbers of $CR2^+$ cells in the spleen showed an increase while the mean numbers of CR2 molecules per cell showed a reduction with progressive infection.

These results show that $CR2^+$ T cells in the spleen increase in numbers with progressive *T. congolense* infections in BALB/c mice. I suggest that $CD4^+CR2^+$ T cells might play a role in the pathogenesis of *T. congolense* infections.

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ABBREVIATIONS

APC	antigen-presenting cell
BSA	bovine serum albumin
BES	bloodstream telomere-linked VSG gene expression site
Bp	base pairs
BCG	bacillus calmette-guérin
CD	cluster of Differentiation
CR	complement receptor
DAF	decay-accelerating factor
DNA	deoxyribonucleic acid
DEAE	diethylaminoethyl
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescene isothiocynate
FoxP3	forkhead box P3
GIP	glycosyl-inositol-phosphate
GPI	glycosylphosphatidylinositol
GPI-PLC	GPI-specific phospholipase C
HCV	hepatitis C virus
HTLV	human T-lymphotropic virus
HIV	human immunodeficiency virus
IFN	interferon

Ig	immunoglobulin
IL	interleukin
iNOS	inducible Nitric oxide synthase
kD	kilodalton
LAL	Limulus Amebocyte Lysate
LPS	lipopolysaccharide
MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
Mφ	macrophages
mRNA	messenger ribonucleic acid
mVSG	membrane form of VSG
MW	molecular weight
NF-κB	nuclear factor-kappa B
NK	natural killer cells
NKT	natural killer T cells
NO	nitric oxide
OD	optical density
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing Tween 20
PE	phycoerythrin
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SIRS	systemic inflammatory response syndrome

sVSG	soluble VSG
TB	tuberculosis
TC13	*Trypanosoma congolense* clone 13
TGF	transforming growth factor
T_H	T helper
TCR	T cell receptor
TNF	tumor necrosis factor
Tregs	regulatory T cells
TSG	Tris saline glucose
VAT	variant antigen type
VSG	variant surface glycoprotein
WT	wild type

1.0 LITERATURE REVIEW

1.1. African Trypanosomes

1.1.1. Introduction

African trypanosomes are single-cell, extracellular blood parasites that cause disease and death in humans and livestock. *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cause sleeping sickness in humans. *Trypanosoma congolense*, *Trypanosoma brucei brucei* and *Trypanosoma vivax* are pathogens for livestock. Of these species *T. congolense* is the most virulent pathogen in livestock, causing the greatest losses in livestock (Mulligan, 1970a; Mulligan, 1970b).

African trypanosomes are mainly transmitted to mammalian hosts by tsetse flies. The occurrence of animal trypanosomiasis coincides with the distribution of tsetse fly vectors which includes the regions between latitudes 14°N and 29°S. About 10 million square kilometers and 37 countries are covered in this tsetse fly "belt", a geographical area equivalent to the combined size of the United States, India and Western Europe (Hursey, 1995).

African trypanosomiasis causes a great economic loss in the livestock industry with an estimated three million cattle death annually. Estimated direct production losses in cattle are between US$ 6,000 million and US$ 12,000 million per year (Hursey, 1995). It is estimated that if the disease is controlled, the region could support a further 120 million cattle resulting in an increase in annual meat production by 1.5 million tons (Nantulya, 1986).

Currently employed control methods of trypanosomiasis are chemotherapy, breeding resistant breeds of cattle and control of tsetse fly vectors. Other control methods include bush clearing to destroy the tsetse fly habitats, spraying insecticides and trapping of tsetse flies (Taylor, 1998). But the control measures like bush clearing and the use of insecticides are no longer desirable due to their deleterious effects on the environment. Even though the first

trypanocidal drug was developed in 1905, the number of drugs available for the treatment of African trypanosomiasis is very limited (Seed, 2001). Rapid evolution of drug-resistant trypanosomes reduces the efficacy of chemotherapy and chemoprophylaxis causing widespread outbreaks in cattle (Leach and Roberts, 1981; Holmes, 1982).

Vaccination is considered to be one of the best methods of controlling infections. Presently, it is widely believed that the ability of African trypanosomes to continually express antigenically distinct VSG genes (Borst et al., 1997) reduces the likelihood of the development of an effective VSG-based vaccine. Therefore, invariant parasite antigens are being considered as vaccine candidates but only partial and minimal cross-protection has been reported so far (Mkunza et al., 1995). Mice immunized with the recombinant beta-tubulin from *T. evansi*, were protected from lethal challenge with *T. evansi* STIB 806, *T. equiperdum* STIB 818 and *T. brucei brucei* STIB 940, showing 83.3%, 70% and 76.7% protection, respectively (Li et al., 2007). A better understanding of the parasite and host factors involved in pathogenesis of the disease might be helpful in the design of more effective vaccines against trypanosomiasis. Therefore, most research is focused on understanding the pathogenesis of the disease, especially the induction of immunosuppression by the infection.

1.1.2 Classification

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

The section salivaria consists of the trypanosomes that complete their developmental cycle in the salivary glands of their insect vector. Transmission of the parasite to the mammalian host occurs by bite of the insect vector during a blood meal. The section salivaria consists of four subgroups; Duttonella (*T. vivax*), Nannomonas (*T. congolense*), Trypanozoon (*T. brucei brucei*) and Pycnomonas (*T. suis*). Most members of the group salivaria are transmitted by tsetse flies and they are important pathogens either to man or to domestic animals.

The section stercoraria consists of groups that complete their developmental cycle in the hindgut of the insect vector. Transmission of these species occurs when the feces containing the infectious metacyclic form of the parasite is rubbed into the lesions produced at the site of the insect bite. Also, they may actively penetrate punctures made by the proboscis of the insect or the mucous membrane of the mouth if the animal licks the feces of the vector. There are three sub groups in the section stercoraria namely, Megatrypanum (*T. theileri*), Herpestoma (*T. lewisi* and *T. musculi*) and Schizotrypanum (*T. cruzi*) (Hoarse, 1972). *T. cruzi* causes Chagas disease in man in South and Central America while most of the other members of section stercoraria are considered relatively non-pathogenic.

1.1.3 Morphology and characterization

African trypanosomes are characterized on the basis of their size, shape, position of the nucleus, size and location of the kinetoplast, host range and geographical distribution. Generally they are elongated, spindle-shape organisms with a single flagellum. The flagellum originates from the basal body near the kinetoplast and runs the length of the trypanosome. The pellicle, the layer bordering the cytoplasm, while maintaining a definite shape, is flexible enough to permit a certain degree of body movement. The pellicle and the cytoplasm are

pinched up into a thin sheet of tissue along the length of the body forming the undulating Membrane (Hoarse, 1972).

African trypanosomes are 8-30 μm in length. *T. congolense* is a small monomorphic organism with variable length of 8-24 μm (Soltys, 1987). It has a central nucleus and a medium sized marginal kinetoplast. The kinetoplast has important functions in reproduction and metabolism (Hoarse, 1964). The trypanosome has sluggish movements and has an undulating membrane (Hoarse, 1972; Soltys, 1987).

1.1.4 Life cycle

The life cycle of African trypanosomes consists of two phases. One is the development in their insect vectors and the other phase is the development in mammalian hosts.

1.1.4.1. Development in insect vectors

Transmission of African trypanosomes is mediated by tsetse flies (*Glossina* species). The genus *Glossina* consists of three groups called *fusca* group (forest group), *palpalis* group (riverine group), and *morsitans* group (savannah group). These three groups have more than 34 species and subspecies and *T. congolense* is capable of developing in all of these species and subspecies (Hoarse, 1972). The female tsetse fly acquires a trypanosomal infection during a regular blood meal from an infected mammalian host. The ingested parasites lose their VSG and undergo a series of developmental and morphological changes resulting in multiplication in the hindgut of the fly (Stephen, 1986). They migrate interiorly through the cell linings of the gut wall and to the esophagus of the insect (Newton et al., 1973). Then they migrate to the hypopharynx and transform into the epimastigote form (Newton et al., 1973). The epimastigotes migrate into the mouth pads of the proboscis and transform into metacyclic trypanosomes. This transformation is associated with the regaining of the VSG which the trypanosomes lost in the midgut (Vickerman, 1974). The entire developmental cycle in the

insect vector may take from 7 (Nantulya et al., 1978) to 53 (Hoarse, 1972) days depending on the species of the insect vector and the ambient temperature. The infected tsetse fly harbors the parasite and is able to transmit the parasite for a considerable length of time and sometimes, for life (Molyneux, 1983).

1.1.4.2. Development in mammalian hosts

Infection of the mammalian host occurs when the insect vector deposits metacyclic trypanosomes within the dermal connective tissue of the animal during a blood meal. At the site of infection the trypanosomes multiply extensively as typical blood forms within a few days, resulting in a raised cutaneous swelling called a chancre (Akol and Murray, 1982). These trypanomastigotes spread from the chancre via the blood and local lymph vessels and enter to the blood stream. Blood stream forms of many *T. brucei* invade the connective tissues of the mammals, but this is not usually observed with *T. congolense* (Losos and Ikede, 1970).

Certain changes in the metabolism during the period of growth in the blood stream results in morphological pleomorphism among various subspecies of *T. brucei* (Opperdoes, 1987). Morphological variants ranging from long slender forms to short, stumpy, usually non-dividing forms could be found during the process of active cell division. Frequently observed intermediate forms may represent a transition stage from long slender form to stumpy form (Opperdoes, 1987). Predominate slender forms could be seen during the early logarithmic 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 of slender forms to the stumpy forms is necessary for the cyclical development in the insect vector (Seed and Sechelski, 1989). Even though there are reports on morphological variants of *T. congolense* (Stephen, 1986), the general agreement among researchers is that *T. congolense* does not undergo physical transformation.

1.1.5. Course of the infection

Clinical signs and the severity of the disease following infection vary depending on the virulence of the trypanosome and susceptibility of the host. The length of the prepatent period depends on many factors such as the number of infecting parasites, the route of inoculation and the genetic makeup of the host. Parasitemia becomes apparent within 1-2 weeks following natural infections and may persist for months, occurring in waves, until the host dies (Gray, 1976). Various strains of trypanosomes can cause a wide range of clinical responses ranging from acute, chronic to asymptomatic carrier syndromes (Stephen, 1970; Maxie et al., 1979). Even under similar environmental conditions, marked differences in susceptibility of individual animals within a homogenous population could be observed (Ikede and Losos, 1972).

Acute infection of *T. congolense* in ruminants is associated with intermittent fever, depression, anemia, subcutaneous edema of the mandible and prominent jugular pulse (Stephen, 1970; Losos, 1986). The appetite is decreased and there is a rapid weight loss. Often, death is related to severe anemia and circulatory collapse. Chronic syndromes often results in extreme emaciation and anemia. Lymphadenopathy is commonly seen in infections with other species of trypanosomes but not common in *T. congolense* infections in cattle (Maxie et al., 1979). The more rapid fall in myeloid:erythroid ratio in *T. congolense*-infected goats reflects the fact that *T. congolense* causes more severe pathological effects in goats than *T. brucei* (Biryomumaisho and Katunguka-Rwakishaya, 2007).

1.1.6. Pathology

There are no pathognomonic gross or histopathological lesions found in the animals that die of trypanosomiasis. Most of the observed lesions are due to the circulatory disturbances caused by anemia. Mice infected with *T. congolense* developed a severe anemia one week after infection which was accompanied by a marked increase in the plasma levels of acute phase proteins such as serum amyloid P component and haptoglobin (Ngure et al., 2008). Animals that die of acute infections have variable atrophy of muscle and adipose tissues and pale carcasses. Often the lymph nodes, spleen and the liver are enlarged. Also, there may be edema of the lungs, ascitis, hydrothorax and hydropericardium (Stephen, 1970; Molyneux, 1983; Losos, 1986). The skeletal muscles of the carcass may be wasted and pale in chronic cases. Marked splenomegaly with foci of necrosis and prominent white pulp is also observed in chronic cases (Morrison and Murray, 1979; Valli et al., 1979). Inflammatory foci are found in spleen, liver, lung, heart, skeletal muscles, brain, skin and kidney.

Even though the mechanisms of pathogenesis of trypanosomiasis is not clearly understood, trypanosome-derived substances and immune complexes comprising trypanosome antigens and parasite-specific IgM and/or IgG antibodies plus complement were shown to be involved in the pathogenesis (Mansfield, 1990). Immune complexes have been detected in several tissues of infected animals and humans including heart, brain, kidney and skeletal muscles (Lambert, 1974; Nagle et al., 1974). It has been suggested that trypanosomes generate toxic catabolites and biologically active metabolites with complement activating and inflammatory properties, such as cytokines, vascular amines and plasma proteases which can contribute to the pathogenesis of the disease (Tizard and Holmes, 1976; Tizard et al., 1978; Tabel, 1982; Liu et al., 1993). Highly susceptible BALB/c mice infected with *T. congolense* die of a systemic inflammatory response syndrome (SIRS) that is mediated by IFN-γ (Shi et

al., 2003; Shi et al., 2005). This SIRS was associated with elevated plasma levels of IL-6, IL-12p40, IL-10, and IFN-γ. Focal liver lesions of apoptotic parenchymal cells, 5-fold enlargement of Kupffer cells, apoptosis of 10% of Kupffer cells, enlarged capillary bed, hypotension, decreased body temperature, piloerection, hypomotility and death were also associated with SIRS (Shi et al., 2006a).

1.1.7. Trypanosomal common antigens

The trypanosomal antigens can be divided into two groups namely invariant or common antigens and variant antigens, based on their immunological specificity. Invariant antigens of trypanosomes do not change from one variant type to another type during the course of infection. These include enzymes, trypanosomal membranes, structural and nuclear proteins and some receptors such as those for transferrin and low density lipoproteins (Pays et al., 1994), high density lipoproteins, receptor for TNF-α (Lucas et al., 1994) and receptor for IFN-γ (Olsson et al., 1993). Some 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 the survival of parasites and also they might play a role in pathogenesis and/or evasion of host defense mechanisms. The carbohydrate determinants in the C-terminal portion amino acid sequence of the variant surface antigen (VSG) is also considered as a common antigen and show a high degree of homology among the members of a species (Rice-Ficht, 1981; Rice-Ficht et al., 1982). Flagellar pockets of African trypanosomes are not covered by the VSG and are invariant among the members of a species. In cattle, immunization with antigens derived from flagellar pockets of *T. brucei rhodesiense* resulted in significant protection against challenges with *T. congolense* and *T. vivax* (Mkunza et al., 1995). Since the flagellar pockets are not

covered by VSG, it is clear that the immune response to VSG is not included in this protection.

It has been suggested that the invariant antigens released by destruction of the parasite by anti-VSG antibodies and other immune factors, may be involved in inducing pathology by forming immune complexes (Mansfield, 1990). However, in *T. congolense*-infected cattle high serum antibody levels to 33kd (Authie et al., 1993) and 69kd (Boulange and Authie, 1994) invariant cystein proteases have been associated with trypanotolerance. *T. congolense*-infected trypanosusceptible Boran cattle mounted predominant IgM response to invariant proteins while similarly infected trypanotolerent N'dama cattle made a superior IgG and low IgM response (Authie et al., 1993). Crosses between Boran and N' dama cattle which are of intermediate susceptibility had intermediate levels of these antibodies (Mkunza et al., 1995).

1.1.8. Variant surface glycoprotein (VSG)

The plasma membrane of trypanosomes is covered by a homogenous dense coat called variant surface glycoprotein, consists of millions of glycoprotein molecules of a single molecular species.

1.1.8.1. Organization of the VSG gene

The genome of African trypanosomes contains about 1000 different VSG genes (Van der Ploeg et al., 1982). Only one VSG gene is expressed at a time in a given bloodstream parasite under normal conditions (Munoz-Jordan et al., 1996). The unexpressed VSG genes are scattered among the different chromosomes (Cross et al., 1998). VSG genes need to be located in a specialized telomeric environment, which is known as a bloodstream telomere-linked VSG gene expression site (BES) in order to be transcribed. Bloodstream VSG genes have been found to be transcribed from telomere-linked expression sites and contain a VSG

promoter, a variable number of 70-76 bp repeats, and the VSG gene is followed by subtelomeric and telomeric DNA repeats (Kang et al., 2002). At any time, only one BES is active and only one VSG gene is transcribed. The molecular events in a given trypanosome that induce transcription at one BES and silence expression at the other BESs are not understood (El-Sayed et al., 2000).

1.1.8.2 Structure of VSG

The molecular weight of VSG varies between 53-65 KD and VSGs of different variant antigen types of the same serodeme may differ in their amino acid composition, isoelectric points and carbohydrate percentage (Cross, 1978; Cross, 1990). Tryptic digestion of the C terminal portion of the VSG molecule can reduce the MW from 53 to 48 kDa. In the insect, VSG is only expressed in infective metacyclic form (Vickerman, 1974) and it has been proposed that the VSG prevents the lysis of metacyclic forms by the host's serum factors (Ghiotto et al., 1979). VSGs, cover the entire parasite surface, and are arranged in a tightly packed monolayer of homodimers. They form a 12-15 nm thick coat which functions as a barrier to lytic serum components but allows nutrients such as glucose to reach transporters in the membrane of the flagellar pocket (Borst and Fairlamb, 1998). Each parasite has10^7 densely packed identical VSG molecules on their surface (Cross, 1990; Gerold et al., 1996). VSG constitute about 10% of the total protein of trypanosomes and is synthesized at a high rate (Kang et al., 2002). VSG is very immunogenic and therefore the target of the very potent immune response (Vanhamme et al., 2001).

Each VSG monomer contains an N-terminal signal sequence and a hydrophobic C-terminal domain and is covalently attached to a glycosylphosphatidylinositol (GPI) membrane anchor (Kang et al., 2002). Many proteins of eukaryotes are anchored to the plasma membrane by the GPI, including Thy 1 antigen and decay-accelerating factor (McConville and Ferguson,

1993). N-terminal sequence of VSG is highly variable. The three-dimensional structures of the N-terminal two-thirds of two VSGs have been determined by X-ray crystallography and found to be very similar rod-like shapes despite having quite different amino acid sequences (Blum, 1993). The VSG molecules can be cleaved by trypanosomal GPI-specific phospholipase C. This GPI-PLC cleaves the GPI anchor, leaving the dimyristoylglycerol compound of the GPI anchored in the membrane, and releases the glycosyl-inositol-phosphate (GIP)-VSG part which is also called soluble VSG (Magez et al., 1998).

1.1.8.3. Release of soluble VSG

VSG could be detected in the plasma of mice and rats infected with *T. brucei brucei* (Diffley et al., 1980) indicating that trypanosomes release their VSG during the course of infection. Even though the VSG coats are stably associated with the plasma membrane of the trypanosomes, it can be isolated as membrane form of VSG (mVSG) (Bulow and Overath, 1986) or as the water soluble form (sVSG) after lysis of the cells. mVSG is converted into sVSG during the lysis of cells by the action of an endogenous enzyme, GPI-specific phospholipase C (GPI-PLC) (Ferguson, 1999). GPI-PLC is involved in degradation, shedding and recycling of VSG (Carrington et al., 1991; Rolin et al., 1996).

Trypanosomal lysates contain vast amounts of sVSG produced from mVSG by the action of GPI-PLC, which gains access to mVSG by hypotonic lysis of the cells (Fox et al., 1986). However, the role of GPI-PLC in hydrolysis of GPI is still not clear because cytosolic GPI-PLC is localized away from cell surface VSG. GPI-PLC null mutants are fully viable, and GPI-PLC null mutants undergo antigenic variation (Cardoso De Almeida et al., 1999). It was shown that shedding of VSG is mediated by hydrolysis of GPI. Surface biotinylation assays suggest that GPI-PLC does gain access to extracellular VSG. The above results indicate the importance of GPI-PLC in release of VSG (Gruszynski et al., 2003).

The cell surface VSG pool is turned over within 12 minutes. Recycling of VSG happens through endocytosis in large clathrin-coated vesicles that bud from the flagellar pocket membrane, and then are delivered to endosomes or lysosomes. The VSG returns to the cell surface at the flagellar pocket by exocytosis (Engstler et al., 2004).

Antibodies against *T. congolense* can induce shedding of soluble VSG and the formation of a soluble covalent complex of VSG and bovine complement component C3b, in the presence of fresh bovine serum (Liu et al., 1993).

1.1.8.4 Effect of VSG on the immune system of the host

10% of the total protein content of trypanosomes consists of GPI-linked VSG. Since the trypanosomes are extra cellular parasites and they release vast amounts of sVSG into the circulation, the immune system of the infected mammalian host is continuously exposed to the sVSG (Magez et al., 2002). There is evidence that blood stream forms of *T. congolense* evade complement lysis by shedding their immune complexes (Frevert and Reinwald, 1990) and endocytosis of immune complexes (Engstler et al., 2007). VSG of *T. brucei* cause consumption of complement proteins, which may occur via the massive amounts of immune complexes generated during antibody-mediated clearance of each wave of parasitaemia (Musoke and Barbet, 1977). Immunostimulatory and regulatory activity of protozoan derived GPI anchors has been documented (Ropert and Gazzinelli, 2000). It has been suggested that soluble VSG which carries the carbohydrate core (GIP-VSG), once released from the parasite surface, is affecting the functions of macrophages, including induction of cytokine synthesis (Magez et al., 2002). It has been shown that NO is trypanostatic for *T. congolense*, *T. musculi*, *T. gambiense* and *T. brucei* in vitro (Vincendeau et al., 1992; Kaushik et al., 1999a). Soluble VSG could inhibit IFN-γ induced nitric oxide production by macrophages (Coller et al., 2003).

All the above findings suggest that sVSG released from the parasites has negative effects on control of the infection.

1.1.9. Antigenic variation

Highly immunogenic nature of VSG leads to a strong humoral immune response resulting in rapid elimination of all but a very small proportion of trypanosomes. These remaining individuals form a new population expressing a new variant of VSG. When the new population of parasites multiplies, the immune system raises another set of antibodies to eliminate these parasites and eventually eliminates the majority of these parasites. This process results in successive waves of parasitemia (Donelson and Turner, 1985). The undulating wave of parasitemia in infected animals is a result of interactions between the parasite and the host's immune factors. It has been shown that antibodies are not necessary to induce antigenic variation because antigenic variation has been observed *in vitro* (Doyle et al., 1980). In established rodent strains, it is estimated that coat switching occurs at a frequency of 10^{-6}-10^{-7} per cell division and this rate is much higher immediately after passage through insect vectors (Barry and Turner, 1991).

Even though the precise molecular events lead to the antigenic variation are still not understood, it is clear that antigenic variation is advantageous to the parasite in its evasion of the host immune defenses because the host is always a step behind the switching trypanosomes.

1.2. Immune responses of hosts against African trypanosomes

African trypanosomes are exposed to the host immune system from the time of infection. Since a single trypanosome is a package of about 10 million copies of a single VSG and thousands of invariant antigens (Vickerman, 1985), the immune system of the host is continuously assaulted by excessive amounts of invariant and variant antigens.

1.2.1. Innate resistance

Antigen-nonspecific defense mechanisms that are designed to recognize highly conserved structures present in many different microorganisms and called pathogen-associated molecular patterns, are considered to be a form of innate immunity (Aderem and Ulevitch, 2000). Trypanotolerance or the resistance to African tryapanosomiasis is characterized by lowered parasitemia and less marked anemia (Dargie et al., 1979). A/J and BALB/c mouse strains are susceptible for *T. congolense* infections while C57BL/6 mouse strain is relatively resistant. The speed of control of the first wave of parasitemia positively correlates with the survival period of infected mice (Ogunremi and Tabel, 1995). In response to *T.congolense* infections, macrophages of susceptible BALB/c mice produce less amounts of nitric oxide than the macrophages of relatively resistant C57BL/6 mice (Kaushik et al., 1999a). The differences in susceptibility and resistance in BALB/c and C57BL/6 mice is controlled by five quantitative trait loci namely Tir1, Tir2, Tir3a, Tir3b and Tir3c, located on mouse chromosomes 17, 5 and 1 (Iraqi et al., 2000).

1.2.1.1. Role of complement system

1.2.1.1.1. Complement system

Complement is a major clearance and defense system in the blood which can be activated via immunoglobulins once a foreign particle or an organism has been recognized by the antibody (Law, 1988). The alternative complement pathway can be amplified directly via a foreign particle or an organism while the classical complement pathway gets activated via antigen-antibody complex as summarized in Figure 1.1. Complement is an important component in host immune system which functions together with the adaptive immune responses to provide effective defense mechanisms such as killing microorganisms by lysis,

initiating inflammation and facilitating the clearance of microorganisms and immune complexes by facilitating phagocytosis (Law, 1988) .

The complement system is composed of more than 30 plasma and membrane-associated proteins as well as receptors for complement components (Table 1.1). Complement component C3 (M.W. = 185kD) is the central component of complement system and it is the most prevalent complement component in the serum being present at a concentration about 1.3 mg/ml. C3 plays a central role in the complement system by merging the classical and alternative pathways at the C3 activation step (Law, 1988). Both classical and alternative pathways can activate the same terminal complement pathway (C5-C9) which leads to the formation of the membrane attack complex and cause cytolysis (Rother, 1988).

The crucial intermediate step of the complement activation is the formation of C3/C5 convertase. Activated C3, referred as C3b opsonizes the target by covalently binding to it and facilitates the phagocytosis of the target. When C5b is generated, it induces the assembly of C5b-C9 membrane attack complex. Biologically active cleavage products such as C3a, C4a and C5a cause inflammation and anaphylactic activities.

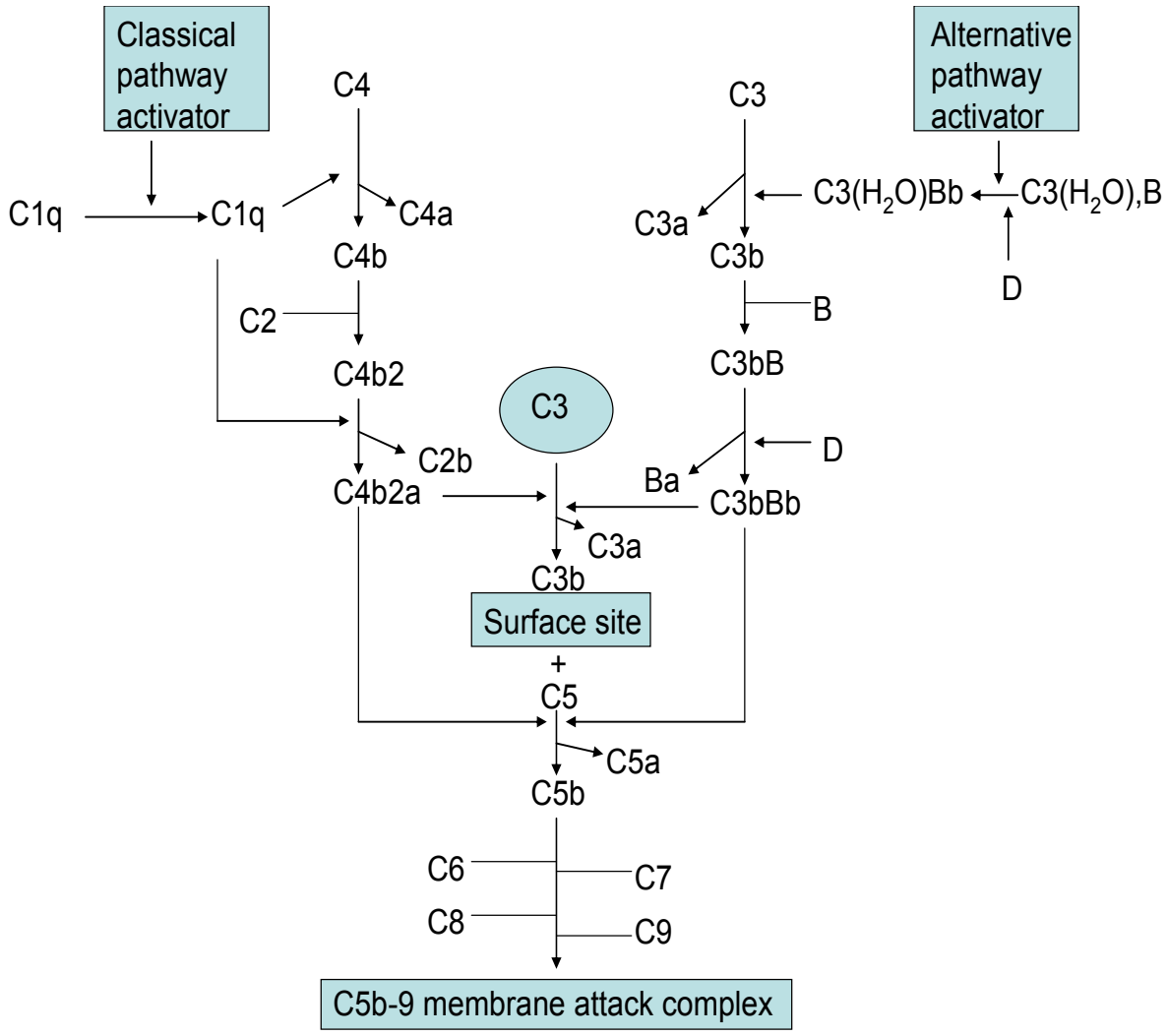

Figure 1.1. The activation steps of the classical pathway (left) are triggered by immune complexes, while the alternative pathway (right) is amplified by a wide variety of cell surfaces and compounds. Adapted from (Law, 1988).

Table 1.1 Proteins of the human complement system

Prevalent form	Functional group		
	Participate in activation	Regulatory	Receptors
Serum/soluble	C1q, C1r, C1s, C4, C2, C3, B, D, C5, C6, C7, C8, C9	C1 INH, C4bp, H, I, P, C3a/C5a INA, S protein	
Membrane-associated		CR1, MCP, DAF, HRF	C1qR, CR1, CR2, CR3, CR4, CR5, C3a/C4aR, C5aR

Abbreviations: INH, Inhibitor; C4bp, C4b-binding protein; INA, inactivator; R, receptor, e.g. CR2 complement receptor 2; DAF, decay-accelerating factor; MCP, membrane cofactor protein; HRF, homologous restriction factor. Adapted from (Volanakis, 1990).

1.2.1.1.2. The classical complement pathway

Activation of classical complement pathway needs antigen-antibody complexes. These antibodies in human could be from IgM class, IgG1, IgG2 and IgG3 subclasses. Complement components C1, C4 and C2 are responsible for the assembly of C3 convertase. C1 is a calcium-dependent macro-molecular complex of three distinct sub components: Clq, Clr and Cls (Lepow et al., 1963). Clq is a glycoprotein and consists of 18 polypeptide chains linked together by disulphide bonds. Clr and Cls are single chain serine proteinases, connected together with Clq via calcium (Cole, 1988). Interaction of the Cl complex with antigen-antibody complex via C1q activates C1r and C1s proenzymes. C4 binds C2 in a magnesium-dependent manner after releasing fragment C4a with the influence of activated C1. The bound C2 is also cleaved by the C1, resulting in formation of active C3 convertase, C4b2a. This C4b2a cleaves C3 by releasing a small peptide C3a. The large remaining fragment C3b contains a labile binding site through which C3b binds covalently with a nearby target surface. Then, C5 combines with the attached C3b and as a result, modifications take place in C5 to make it susceptible to get cleaved by the neighboring C4b2a enzyme. Activation of C5 is followed by the formation of C5b-9 cytolytic complex (Vogt et al., 1978; N.C., 1986).

C1 inhibitor is a control protein that can bind with C1r and C1s to remove them from the C1-antibody-antigen complex and inactivate the C1 enzymatic activity. C4b binding protein is another control protein which regulates the classical pathway C3 convertase as well as fluid-phase and cell bound C4b by binding to C4b and making it prone to get cleaved by factor I (Hourcade et al., 1989). EDTA can inhibit the activation of classical complement pathway by chelating Ca^{2+} and Mg^{2+} to prevent the assembly of C4b2a.

1. 2.1.1.3. The alternative complement pathway

The major difference between the classical and alternative pathways is that the alternative pathway can be activated in the absence of antibodies and can neutralize a wide variety of microorganisms in the absence of specific antibodies. The process of activation of the alternative pathway can be divided into four phases: initiation, deposition of C3b, recognition and amplification (Pangburn, 1986).

Under the normal circumstances, the initiation of alternative pathway occurs by hydrolysis of the intra-molecular thiolester bond of C3. This leads to the formation of C3 (H_2O). C3 with a hydrolyzed thiolester, without the loss of its C3a fragment, is called C3i or C3 (H_2O). This is continuously produced under normal conditions at a very low rate in aqueous phase. C3 (H_2O) has the same functional properties of C3b and it forms a complex with factor B in an Mg^{2+}-dependent manner. The cleavage of B into Bb by factor D is followed by the formation of C3 convertase with the composition C3 (H_2O) Bb (Pangburn and Muller-Eberhard, 1980). This convertase enzyme is capable of forming a small number of metastable C3b molecules in the fluid phase but most of these metastable C3b molecules rapidly get inactivated by factor H and I. Some of the C3b molecules non-specifically bind with any particles including host cells. The C3b bound to the host cells are eliminated and inactivated by factor I and membrane associated regulators. C3b bound to foreign particles are recognized as the proper sites of activation and serve as anchors for the assembly of the surface-associated convertase enzymes of the alternative complement pathway.

Recognition of activator or non-activator surfaces of alternative pathway depends on the nature of the surface to which C3b is bound (Pangburn et al., 1980; Meri and Pangburn, 1990). The alternative pathway amplification loop is restricted to certain surfaces as a result of the balance of interactions between complement proteins. Non-activating surfaces permit the

control proteins to prevent the formation of C3bBb complex either by displacement of Bb from C3b or by cleavage of C3b into iC3b (figure 1.3). Activation surfaces have the common but unidentified property of providing the action of control proteins on C3bBb, allowing the positive feedback C3 activation loop to operate on the surface as shown in figure 1.3 (Law, 1988).

1.2.1.1.4. Central role of C3

C3 is the central protein in the complement system since the classical and alternative pathways merge at the C3 activation step. C3 is a β-globulin composed of an α chain and a β chain. The molecular weight of C3 is 185 kD. In the classical pathway, C3 is activated by the proteolytic cleavage of C3 into C3a and C3b. The removal of C3a induces conformational changes in C3b which leads to the exposure of internal thiolester which is quite inaccessible in native C3. The exposed thiolester is very reactive and subjected to nucleophilic attack by water or the amino or hydroxyl groups of other molecules. If these hydroxyl or amino groups belong to the cell surface molecules, C3b become covalently bound to the cell by an amide or an ester bond (Law, 1988). Surface-bound C3b, generated by the classical pathway has the same convertase activity as C3b generated by the alternative pathway and both pathways share the effector mechanisms such as the generation of anaphylotoxin C5a, engulfment of C3b-coated particles by phagocytotic cells having C3b receptors and the generation of C5b which mobilizes C6, C7, C8 and C9 to form the membrane attack complex.

1.2.1.1.5. Breakdown products of C3

Membrane bound C3b (185 kd) release C3f (3kd) once acted on by factor I and cofactors leaving iC3b attached to the membrane. Serum proteases or factor I in association with CR1 react with iC3b to release C3c and leave C3dg (40kd) attached to the membrane. Exogenous proteases release C3g (5kd) to leave C3d (35 kd) attached to the membrane as

shown in Figure 1.3. Complement's physiological effects, except the lytic event mediated by membrane attack complex, are generated by the interaction of the complement fragments with their specific receptors present on specialized cells. The major outcomes of the complement fragment-receptor interaction are the uptake and phagocytosis of opsonised particles and the activation of the cell type bearing the specific receptor for each complement fragment.

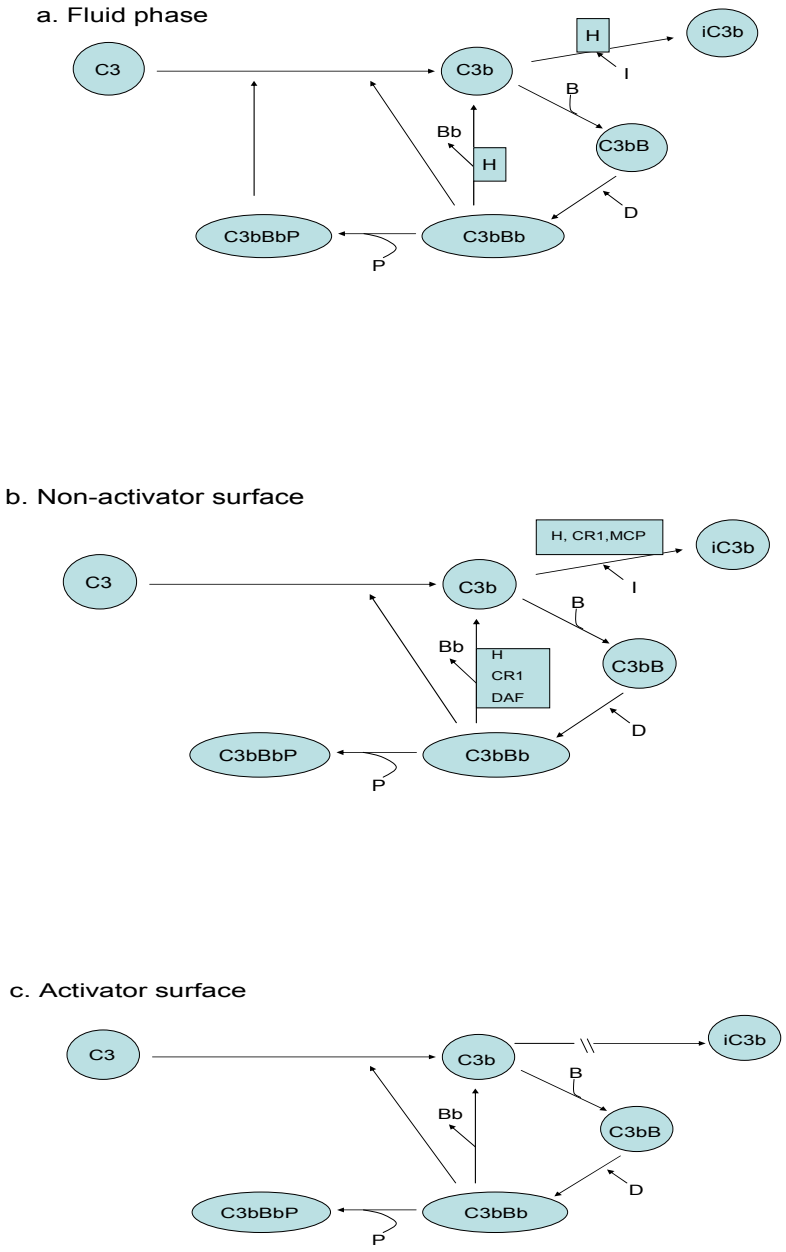

Figure 1.2. Amplification loop is restricted to certain surfaces by a balance of interactions between complement proteins. (a) In the fluid phase activation is curtailed by efficient cleavage of C3b by factor I and cofactors. **(b)** Surface bound C3b on non-activators is regulated similarly and in addition C3bBb is actively dissociated by factor H, CR1 and DAF. **(c)** On activator surfaces these two regulatory pathways are inhibited and rapid deposition of C3b on the surface ensues. Adapted from Law and Reid, 1988).

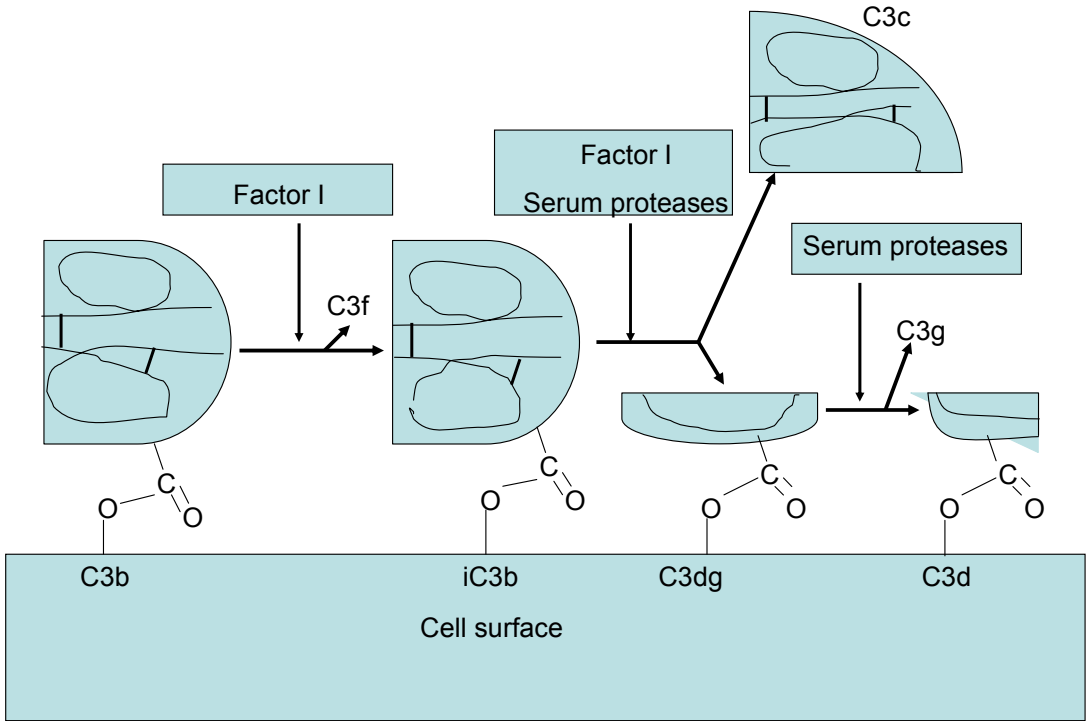

Figure 1.3. Breakdown of C3b**.** Adapted from Law and Reid, 1988)

1.2.1.1.6. Receptors for breakdown products of C3

1.2.1.1.6.1. CR1

CR 1 (CD35) is a single chain transmembrane protein that binds C3b. CR1 is highly expressed on monocytes, B cells and eosinophils while it is expressed in low amounts on erythrocytes and T cells. Human CR1 is genetically polymorphic and occurs in four different molecular weights ranging from 190-280 kD (Wong et al., 1983; Ross and Medof, 1985; Ross, 1989). Binding of immune complexes to phagocytes via CR1 mediates phagocytosis as well as cell activation. CR1 on human erythrocytes allows immune complexes in the circulation to attach to erythrocytes for transport to phagocytes in the spleen and liver, leading to their clearance. Rodent platelets express CR1, which performs a similar transport function (Roitt,

2001). CR1 promote dissociation of C3bBb and also act as a cofactor for factor I which cleaves C3b into small fragments thereby act as a complement regulatory protein (Roitt, 2001). Soluble form of CR1 has been used in human clinical trials in acute respiratory distress syndrome, and to reduce tissue damage in lung transplantation and myocardial infarction (Zamora et al., 1999; Zimmerman et al., 2000).

1.2.1.1.6.2. CR2

CR2 (CD21) is a 145 kD transmembrane protein which acts as the receptor for C3d, the final degraded product of C3. CR2 primarily acts as a B cell co-receptor for antigen receptor-mediated signal transduction (Holers, 2005). CR2 is composed of 15 or 16 short consensus repeats followed by a 28 amino acid transmembrane domain and a relatively short intra cytoplasmic tail (Holers, 2005). In mice, CR1 and CR2 are transcribed from the alternative splicing of a single gene located on chromosome 1 while in humans they are derived from closely linked two distinct genes located on chromosome 1(Boackle, 2005). No activation signal has been discovered that induces the alternative splicing of one form over the other (Zabel and Weis, 2001).

In mice, CR2 is predominantly expressed on B cells, follicular dendritic cells and activated granulocytes (Kinoshita et al., 1988; Carroll, 1998). Mouse CR2 is also expressed on peritoneal and splenic B-1 cells. Human CR2-expressing cells include mature B cells, double-negative thymocytes, a subset of CD4 and CD8 thymic and peripheral T cells, follicular dendritic cells, basophils, mast cells, keratinocytes and epithelial cells (Holers and Boackle, 2004). In human and mice, expression of CR2 first appears during $IgM^{high}IgD^{low}$ immature B cells. This stage specific expression of CR2 is controlled by an intronic silencer in both human and mice (Holers, 2005). The expression of this receptor on mouse T cells has been less well documented (Qian et al., 2005). CR2 was expressed on activated T cells in A/J mice (Chen et

al., 2005). A subset of $CD44^{hi}CD62L^{lo}$ T cells, but not naïve $CD44^{lo}CD62L^{hi}$ T cells, expressed CR2 suggesting that these receptors were directly or indirectly involved in the expression of activation markers on B and T cells and T cell proliferation (Kaya et al., 2001). CR2 was detectable on both $CD4^+$ and $CD8^+$ subsets of T cells (Kaya et al., 2005). In T cells, CR2 may act either as an activating receptor or, more likely, as an adhesion molecule to promote the recruitment or retention of tissue-infiltrating cells (Holers, 2005).

Human CR2 has at least four unique classes of ligands including, cleaved C3 activation fragments (Iida et al., 1983; Weis et al., 1984), Epstein-Barr virus via the surface protein gp350, 220 (Fingeroth et al., 1984; Nemerow et al., 1989), the immunomodulatory protein CD23 (Aubry et al., 1992) and interferon-α (Delcayre et al., 1991). Mouse CR2 demonstrates an altered ligand repertoire because, while it binds C3 activation fragments identically to the human receptor, it does not bind gp350/220 of Epstein Barr Virus or mouse CD23. It is not yet known whether mouse CR2 binds IFN-α (Holers, 2005). Ziya Kaya et al (2001) suggested that CR2 is necessary for optimal activation of B and T cells because $CR^{-/-}$ mice showed lower expression of activation markers on both B and T cells and decreased amounts of T cell cytokines, such as IL-2, IL-4 and IFN- γ, after antigen stimulation.

CR2 has been shown to have multiple effects on B and T cell responses, including the amplification of antigen-induced B cell activation through surface IgM, the rescue of peripheral B cells from IgM-mediated apoptosis (Kozono et al., 1995), the promotion of antigen processing and presentation of C3d-bound targets, modulation of the expression of costimulatory molecules and targeting of immune complexes to germinal centers in secondary lymphoid organs. It is likely that many of these functions occur through interactions of CR2 with CD19 and CD81 on B cell surface, where these receptors form a multimolecular signal transduction complex. CR2 on both B cells and follicular dendritic cells are required for the

generation of normal T-dependent antibody responses (Boackle, 2005). B cell lines expressing higher levels of CR2 were more efficient in processing antigen than those with lower levels. These findings suggest that presentation of antigen by B cells in immune individuals is dependent on the binding of complement-antigen immune complexes to CR2 (Boackle et al., 1997). Although not investigated, the complexes of cleavage products of complement component C3 (C3b, iC3b and C3d) and soluble or particulate trypanosomal antigens might have a profound effect upon binding to cells bearing receptors for C3b, iC3b and/or C3d (Tabel et al., 2000).

Mesenteric lymph nodes of wild type C57BL/6 mice contain about 4-5% of $CR2^+$ $CD4^+$ T cells while this population is not detected in CR2 deficient mice (Molnar et al., 2008). Elevated expression of CR2 is found on apoptotic T cells. Binding of 7G6 monoclonal anti-CR2 antibody to T cells induced NF-κB translocation to the nucleus (Molnar et al., 2008).

1.2.1.1.6.3. CR3

CR3 is the receptor for C3bi and this receptor is located on phagocytic cells and natural killer cells. CR3 is composed of two non-covalently linked glycoprotein chains of an α subunit named CD11b and a β subunit named CD18 (Sanchez-Madrid et al., 1983). CR3 expresses two binding sites, one for Arg-Gly-Asp-containing peptides and the other one is for bacterial LPS (Wright et al., 1989).

CR3 is the major, but not the only, receptor involved in IgM anti-VSG-mediated phagocytosis of *T. congolense* by macrophages and IgM anti-VSG-mediated phagocytosis of *T. congolense* enhances the synthesis of TNF-α but inhibits the synthesis of trypanocidal NO. It has been suggested that signaling of inhibition of NO synthesis might be mediated via CR3 (Pan et al., 2006).

1.2.1.1.6.4. CR4

CR4 is the predominant type of C3 receptor expressed on tissue macrophages that frequently lack detectable levels of CR1 and CR3, and plays the same role as CR3. CR4 is composed of two subunits named as CD11c and CD18 (Taborda and Casadevall, 2002). CR4 can act as another C3bi and C3d receptor of phagocytic cells and it is functionally distinct from CR2 but similar to CR3 (Myones et al., 1988). The binding site on CR4 is calcium dependent (Corbi et al., 1988).

1.2.1.1.7. Complement interaction with parasites

It is difficult to form a general overview on the possible role of complement system against the numerous different types of parasite infections. Many parasites appear to activate the alternative complement pathway, and this would be amplified by the presence of specific antibodies against the parasites. In some cases, complement may provide beneficial effects to the parasites. One such example is by allowing entry into host cells of C3b-and/or iC3b-coated parasites (Leishmania) via CR1 and/or CR3 on host cells. Side effects due to activation of complement by immune complexes can lead to nephritis in malaria (Law, 1988).

1.2.1.1.8. Complement interaction with trypanosomes

Complement mediated lysis of trypanosomes was observed *in vivo* and in vitro (Vickerman, 1982). In cattle, trypanosomal infections are accompanied with severe depression of hemolytic complement activity (Tabel et al., 1980). Depression of hemolytic complement activity is more prominent in susceptible cattle than in trypanotolerant cattle (Authie and Pobel, 1990). Complement depletion in the serum probably contributes to increased susceptibility of secondary infections in trypanosome-infected animals (Maxie et al., 1979).

Homogenates of *T. congolense* or trypanosomes lacking their glycoprotein coat could activate alternative pathway of complement (Tabel, 1982; Ferrante and Allison, 1983).

Both the classical pathway and alternative pathways of complement are activated during African trypanosomiasis (Greenwood and Whittle, 1980). Even though the clearance of trypanosomes by phagocytic cells occur in the absence of complement (Ngaira et al., 1983), the efficiency of immune complex removal and parasite clearance is enhanced by complement (Stevens and Moulton, 1978). Severe impairment of hepatic uptake of opsonized *T. brucei brucei* has been observed in mice depleted of C3 by treatment with cobra venom factor (Macaskill et al., 1980). But, partial depletion of C3 in *T. brucei brucei* -infected mice (Shirazi, 1980) or *T. brucei rhodesiense* (Dempsey and Mansfield, 1983) had no effect on controlling parasitemia or phagocytosis.

Hypocomplementemia is a marked feature of African trypanosomiasis in cattle (Tabel, 1982), sheep (Malu and Tabel, 1986) and mice (Otesile et al., 1991). Total hemolytic complement activity and C3 are more reduced in trypanosusceptible Zebu than in trypanotolerant Baoule cattle (Authie and Pobel, 1990). Preinfection levels of factor H was significantly higher in resistant C57BL/6 mice than in susceptible BALB/c mice and the amount of factor B in plasma of infected mice during the later stages of infection showed a positive correlation with survival time and was higher in trypanotolerant C57BL/6 mice (Otesile et al., 1991). The activity of alternative pathway of complement amplification measured by deposition of complement protein C3b demonstrated that deposition of C3b was significantly higher in the plasma of resistant C57BL/6 mice than in BALB/c mice infected with *T. congolense* and the degradation of C3b to C3d during the amplification of the alternative pathway of complement proceeds faster in BALB/c than in C57BL/6 (Ogunremi et al., 1993). C5-sufficient and C5-deficient mice did not show significant differences in either

hepatic uptake of parasites (Macaskill et al., 1980) or in their survival periods (Jones and Hancock, 1983), indicating that complement mediated lysis does not play a major role in resistant to trypanosomiasis.

1.2.1.2. Role of nitric oxide (NO)

NO is a free radical gas which acts as a nonspecific cytotoxin in the host immune system (Moilanen and Vapaatalo, 1995).The small and lipophilic nature of NO facilitates its entrance into microbes. The primary targets of NO are sulfhydryls and iron which is central to the biochemistry of microbes (MacMicking et al., 1997). NO produced by activated macrophages is considered to be an important arm of non-specific immunity (May and Machesky, 2001).

Different pathogens or stimuli use different signaling pathways for NO production. It has been shown that Fc receptors (Bayon et al., 1997), mannose receptors (Karaca et al., 1995) and Toll-like receptors (Mizel et al., 2003) can all affect NO synthesis. It has been shown that NO is trypanostatic for *T. congolense*, *T. musculi*, *T. gambiense* and *T. brucei* in vitro (Vincendeau et al., 1992; Kaushik et al., 1999a). Bone marrow derived macrophages and peritoneal macrophages of resistant C57BL/6 mice produce more NO than the macrophages of susceptible BALB/c mice in *T. congolense* infections (Kaushik et al., 1999a). In *T. brucei*-infected highly susceptible mice, NO has been reported to be partially responsible for macrophage-mediated splenic immunosuppression (Mabbott et al., 1995) but this is not true for *T. congolense*-infected mice (Uzonna et al., 1998b). The upregulation of NO is not found in cattle during *T. congolense* infection and was not responsible for the reduction of T cell proliferation (Taylor et al., 1998). *T. congolense*–infected $iNOS^{-/-}$ C57BL/6 mice were considerably more susceptible than wild-type C57BL/6 mice, suggesting a role for NO in parasitemia control in vivo. Interestingly, the $iNOS^{-/-}$ mice were not as susceptible to *T.*

congolense infections as IFN-γ-$R^{-/-}$ mice were, suggesting that the protective role of IFN-γ signaling would extend beyond its function in iNOS activation (Magez et al., 2006). IgM anti-VSG-mediated phagocytosis of *T. congolense* enhances the synthesis of TNF-α but inhibits synthesis of parasite-controlling NO (Pan et al., 2006).

1.2.1.3. Role of macrophages

Clearance of the trypanosomes from the circulation is predominantly achieved by phagocytosis of the opsonized parasites by macrophages in the liver (Kupffer cells) and to some extent by macrophages of the spleen (Holmes et al., 1979; Macaskill et al., 1980; Macaskill et al., 1981; Dempsey and Mansfield, 1983). After phagocytosis, trypanosomes are rapidly digested in the phagolysosomes of the Kupffer cells (Mansfield, 1990). In trypanosome infections, the numbers of macrophages are increased in liver, spleen and lymph nodes (Clayton et al., 1980; Murray and Dexter, 1988).

It has been suggested that macrophage membrane Fc receptors for IgG and complement receptors play a role in mediating phagocytosis of trypanosomes. In *in vitro* studies, procyclic forms of *T. brucei* adhered readily to mouse peritoneal macrophages in the absence of antibodies and this attachment was mediated by a non-Fc, non-C3 receptor on the plasma membrane of macrophages suggesting that ligands for macrophage receptors may be present on trypanosomal plasma membranes (Mosser and Roberts, 1982). *T. brucei* is taken up by monocytes *in vitro*, in the presence of IgM or IgG anti-VSG antibodies in the absence of complement (Ngaira et al., 1983; Rurangirwa et al., 1986). This indicates that both Fc receptor-dependent and independent mechanisms might play a role in phagocytosis. It has been suggested that binding of IgM antibodies to the trypanosome surface causes activation of complement and due to the deposition of C3 cleavage products on trypanosomes results in phagocytosis of trypanosomes via the macrophage receptors for C3 cleavage products (Devine

et al., 1986; Mansfield, 1990). This has been shown to occur when IgM antibodies bind to VSG of *T. congolense* (Pan et al., 2006). It has been shown that CR3 (CD11b/CD18) is the major receptor for IgM antibody-mediated phagocytosis of African trypanosomes and this phagocytosis is complement dependent (Pan et al., 2006). IgM anti-VSG-mediated phagocytosis of *T. congolense* enhances synthesis of disease-producing TNF-α but inhibits synthesis of parasite-controlling NO. This inhibition of synthesis of NO is dependent on CR3 but is independent of complement (Pan et al., 2006).

There were no significant differences observed in phagocytosis of trypanosomes by macrophages and monocytes of *T. congolense*-infected N'Dama and Boran cattle suggesting that even though phagocytosis is the primary mechanism involved in the control of parasites, factors other than phagocytosis dictate or contribute to the differences between resistant and susceptible animals (Sileghem, 1991). During experimental murine trypanosomiasis, the induction of costimulatory cytokines such as IL-1 and IL-2 were found to be depressed in both susceptible and resistant mouse strains (Mitchell et al., 1986). In another study, the induction of costimulatory cytokines was increased from monocytes and macrophages of *T. congolense*-infected cattle (Sileghem et al., 1993). Trypanosome-mediated early activation of macrophages may lead to stimulate trypanosome-specific helper T cells and non-specific stimulation of T cells as a result of elevated costimulatory cytokines (Mansfield, 1990). Continued stimulation of macrophages by T cell-derived cytokines (IFN-γ) or autoregulatory factors like TNF-α, IL-10 and prostaglandins could interfere with further stimulation of T cells (Mansfield, 1990; Sileghem et al., 1994a; Uzonna et al., 1998b). Activation of macrophages and down regulation of macrophages in trypanosomiasis may occur in a T cell-independent manner since these are observed in infected congenitally T cell deficient athymic mice (Mansfield, 1990).

Susceptibility of mice to *T. congolense* and *T. brucei* infections can be suppressed by treatment with macrophage stimulants such as LPS, BCG, *Bordetella pertussis* and *Corynebacterium parvum* before the infection suggesting that the trypanosomes may be controlled by non-specific mechanisms of activated macrophages (Singer et al., 1964; Murray and Morrison, 1979). Upon activation, macrophages produce certain molecules which have microbicidal properties such as reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs) and TNF-α (Adams and Hamilton, 1984; Auger, 1992). In C57BL/6 mice, IL-10 produced by $FoxP3^+$ Tregs regulate classical activation of the macrophages resulting in reduced TNF-α production and this was beneficial for the host survival by limiting the tissue damage (Guilliams et al., 2007).

Phagocytosis of *T. congolense* by Kupffer cells of the liver leads to rapid activation of the Kupffer cells with release of monokines and enlargement of Kupffer cells (Shi et al., 2004). There is very little trypanosomal antigen detectable in the liver and the Kupffer cells are not visibly enlarged up to day 5 post-infection. However, at day 6, there is a prominent accumulation of parasite antigen in Kupffer cells and the Kupffer cells are enlarged (Fig 1.4). There was a 5-fold increase in size of the Kupffer cells towards the terminal stage of infection and about 10% of the Kupffer cells undergo apoptosis (Shi et al., 2005).

1.2.2. Adaptive immune responses

Adaptive immunity or acquired immunity is an antigen-specific defense mechanism consists of two major categories; humoral immunity and cell mediated immunity.

1.2.2.1 Humoral immune responses

Humoral immunity is mediated by antibody molecules produced by B lymphocytes in response to antigens. Specific B cell responses against VSG surface epitopes represent composite T cell-dependent and T cell-independent processes (Reinitz and Mansfield, 1990). The T cell-independent B cell responses are associated with temporary immunity to the variant antigenic types of trypanosomes arising during acute and chronic infections (Campbell and Phillips, 1976; Pinder et al., 1986; Reinitz and Mansfield, 1990). The contribution of T cell-independent and T cell-dependent processes to the total antibody production during infections is variable (Reinitz and Mansfield, 1990). Nude BALB/c and C57BL/6 mice, which lack T cells, are able to control the first wave of parasitemia (Campbell and Phillips, 1976; Pinder et al., 1986).

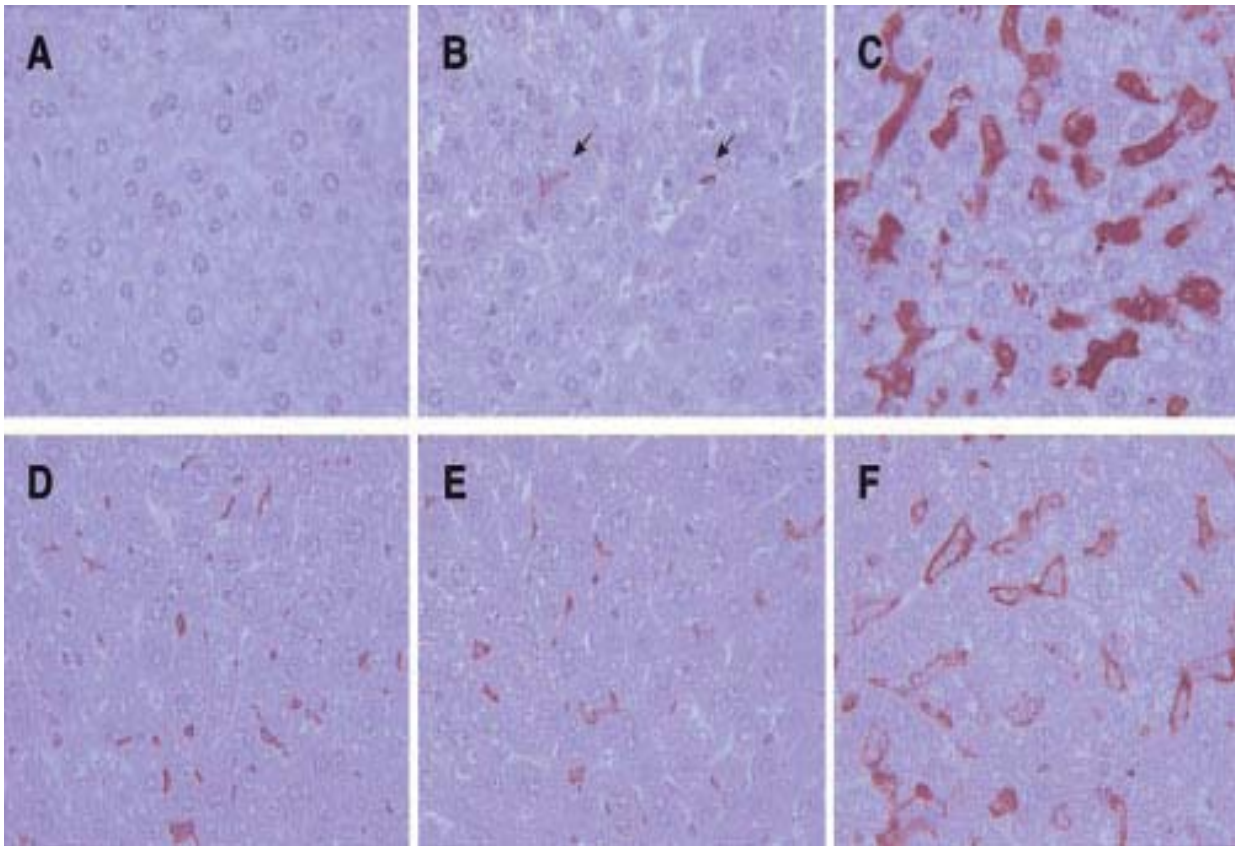

Fig. 1.4. Accumulation of trypanosomal antigens in Kupffer cells of the liver. BALB/c mice were infected with 10^3 *T. congolense* VAT TC13 i.p. Mice were killed on days 0–7 post-infection. Peroxidase stain for trypanosomes (A–C): No trypanosomal antigen was detected on day 0 post-infection (A). The earliest time of detection of trypanosomal antigens was on day 5 (B). Accumulations of parasite antigens significantly increased on day 6 (C). Peroxidase stain (anti-F4/80) for Kupffer cells (D–F): Kupffer cells appeared not visibly enlarged up to day 5 (D, E). In contrast, Kupffer cells were markedly enlarged on day 6 (F). Original magnification, X 400 (Shi et al., 2004).

The B cell specific responses to VSG result in elimination of trypanosomes expressing the target surface antigen and control parasitemia. In the presence of specific antibody, trypanosomes are rapidly eliminated from the circulation (Mansfield, 1990; Shi et al., 2004). The resistant mice exhibit marked production of VSG-specific antibodies during an infection (Levine and Mansfield, 1984; Newson et al., 1990), while mice highly susceptible for trypanosomiasis were found to have little or no detectable antibodies against VSG (Black et al., 1983; Morrison and Murray, 1985) even though their B lymphocytes are extensively activated. The primary immune responses to VSG consists of both IgG and IgM classes of antibodies and reaches the maximum in 7-14 days following challenge (Roelants and Pinder, 1984). During the initial parasitemic wave, IgM was the only detectable class of antibody (Dempsey and Mansfield, 1983). Highly immunogenic VSG can induce high amounts of IgM in infected mice and cattle (Radwanska et al., 2000). The massive IgM production is not accompanied by a concomitant increase in production of IgG antibody in susceptible BALB/c mice (Uzonna et al., 1999).

During early onset of *T. brucei* infection in mice, spleen remodeling results in a rapid loss of IgM^{+} marginal zone B cell population characterized as $B220^{+}IgM^{high}IgD^{int}CD21^{high}CD23^{low}CD1d^{+}CD138^{-}$ (Radwanska et al., 2008). When these cells were isolated during the first peak of infection, they were stained positive for Annexin V and had increased caspase-3 enzyme activity. Increased caspase-3 mRNA levels coincided with decreased levels of anti-apoptotic Bcl-2 mRNA and BAFF receptor, indicating the onset of apoptosis. Also, affected B cells become unresponsive to stimulation by B cell receptor cross-linking with anti-IgM Fab fragments and infection-induced loss of IgM^{+} B cells coincided with the absence of protective variant-specific T-independent IgM responses,

rendering the host susceptible to re-challenge with previously encountered parasites (Radwanska et al., 2008).

1.2.2.1.1. Role of antibodies

The production of antibodies against various predominant VSGs provides protective immunity in infected animals (Naessens, 2000). VSG-specific antibodies mediate complement-mediated lysis (Flemmings and Diggs, 1978; Crowe et al., 1984) and increase the uptake of trypanosomes by macrophages (Ngaira et al., 1983; Shi et al., 2004). The clearance of the parasites is an immune-mediated mechanism and needs VSG-specific antibodies (Pinder et al., 1986). Blood stream trypanosomes are protected against phagocytosis in the absence of antibodies (Mosser and Roberts, 1982). Antibodies against non-variant antigens may neutralize toxic or pathogenic effects of certain trypanosomal molecules and may prevent anemia after repeated infections (Paling et al., 1991). In a study of *T. congolense* infections in BALB/c mice, Otesile and Tabel (1987) concluded that variant-specific antibody is necessary but not enough to control the infection.

Highly susceptible BALB/c mice produced IgM antibodies to common trypanosomal epitopes earlier than the resistant C57BL/6 mice. But BALB/c mice failed to switch to produce IgG2a and IgG3 antibodies (Uzonna et al., 1999). When infected mice were treated with Berenil, they produced IgG3 and IgG2a antibodies against trypanosomal common epitopes. Mice that underwent self cure had the highest levels of IgG3 and IgG2a antibodies against common trypanosomal epitopes (Uzonna et al, unpublished). When *T. congolense* and IgG2a anti-VSG were added to macrophage cultures, the induction of NO production by the macrophages was 2 to 9 fold higher than the production of NO by the macrophages added with *T. congolense* and IgM anti-VSG antibodies (Kaushik et al., 1999a). IgM is more efficient than IgG in complement-mediated lysis of *T. brucei* (Seed, 1977). In *in vitro* studies, IgG and

IgM had equivalent effects on phagocytosis but had different effects on macrophage activation (Kaushik et al., 1999b).

When mice were infected with purified VSG or non-multiplying trypanosomes, resistant and susceptible mice produced almost similar amounts of anti-VSG antibodies with identical kinetics (Morrison and Murray, 1985; Pinder et al., 1986). Resistant cattle produce more IgG antibodies against VSG and invariant antigens than susceptible cattle (Authie et al., 1993; Taylor et al., 1996) while susceptible mice made higher IgM antibody responses (Taylor et al., 1996; Williams et al., 1996). Superior IgG responses to invariant antigens are associated with enhanced resistance in trypanotolerant cattle breeds (Authie et al., 1993; Agur and Mehr, 1997).

1.2.2.2 Cellular immune responses

1.2.2.2.1. T cells

T cells are central to the regulation and activation of immune responses. Mature T cells show heterogeneity functionally as well as by the expression of CD4 and CD8 molecules (Reeves G., 1996). The T cells which cooperate with B cells, helping them to respond to the antigens, resulting in differentiation of B cells into antibody-secreting plasma cells are termed helper T cells. Distinct sub populations of T helper cells preferentially promote T cell immunity (T_H1) or stimulate antibody production (T_H2) based on the cytokines they produce (Reeves G., 1996).

Some T cells are able to suppress immune responses and therefore are designated suppressor T cells. Some other T cells are able to kill the cells expressing foreign determinants on their surfaces and to kill virus-infected cells, and are named cytotoxic T cells (Reeves G., 1996).

1.2.2.2.2. Role of T cells in protection against African trypanosomiasis

The role of T cells in protection against African trypanosomiasis is poorly investigated. The VSG surface epitope-specific B-cell responses in mice infected with trypanosomes represent composite T-cell-dependent and T-cell-independent processes, and a significantly stronger response is made in the presence of T cells (Reinitz and Mansfield, 1990). The synthesis of IgG2a but not IgM anti-parasitic antibodies were reduced in $CD4^{-/-}$ BALB/c mice infected with *T. congolense* (Shi et al., 2006a) supporting the idea that IgM production is predominantly $CD4^+$ T cell independent and the switch from IgM to IgG2a requires help from $CD4^+$ T cells.

IFN-γ in *T. congolense* infections of mice is mainly produced by matrix-adherent splenic $CD4^+$ T cells (Uzonna et al., 1998c). Experiments carried out using IFN-γ knock-out mice showed that IFN-γ is required for the survival of relatively resistant C57Bl/6 mice infected with *T. brucei* (Hertz et al., 1998) or *T. congolense* (Magez et al., 2006). Wei and Tabel (2008) reported that $CD8^+$ NKT cells have a potential for protection against *T. congolense* infection induced by sub cutaneous injection of trypanosomes, presumably by inducing NO, but under normal conditions of infections, the $CD8^+$ NKT cells appear to be suppressed by $CD4^+FoxP3^+$ Tregs.

1.2.2.2.3. Role of T cells in the inflammatory response in *T. congolense* infections

The cytokine pattern observed in *T. congolense* infections does not fit the T_H1/T_H2 paradigm since T. congolense-infected BALB/c mice have high serum levels of IFN-γ and IL-10 (Mosmann and Coffman, 1989). IFN-γ and IL-10 were produced by matrix-adherent spleen cells (Uzonna et al., 1998c). The production of IFN-γ and IL-10 required the absolute synergy of the Thy 1.2^+ and Thy1.2^- cell populations, since neither the Thy 1.2^+ cells nor the Thy1.2^- cells alone produced significant amounts of these cytokines (Uzonna et al., 1998c).

The early mortality in infected BALB/c mice occurs due to systemic inflammatory response syndrome mediated by IFN-γ produced by MHC class II-restricted $CD4^+$ T cells (Shi et al., 2003). Studies with monoclonal antibodies which block IL-10 receptor showed that the excessive action of these pathogenic T cells and excessive activation of the macrophages are down-regulated in infected C57BL/6 mice via the action of IL-10 (Shi et al., 2003; Shi et al., 2007). In *T. congolense*-infected C57BL/6 mice, $CD4^+CD25^+FoxP3^+$ Tregs producing IL-10 are required for controlling the excessive activation of macrophages (Guilliams et al., 2007). Tabel et al., (2008) discussed the *T. congolense* infection of mice in relation to dysregulation of macrophages and their interaction with T cells with the use of a simplistic diagram (Fig. 1.5).

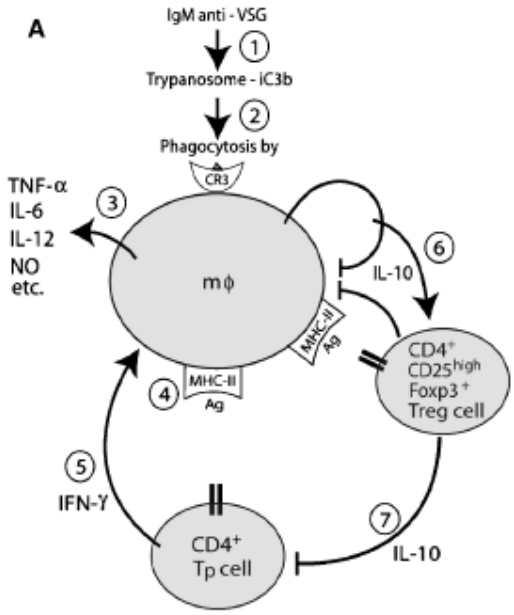

Fig.1.5. (A). Model for relatively resistant C57BL/6 mouse infected with *T. congolense***.** (Tabel et al., 2008).

T cell-independently produced (Campbell et al., 1978; Pinder et al., 1986) IgM anti-VSG will bind to the circulating trypanosomes (**step 1**), activate complement and mediate deposition of iC3b (Pan et al., 2006). Macrophages phagocytose the opsonized parasites predominantly via macrophage receptor CR3 (**step 2**) (Pan et al., 2006). Pulsed macrophages produce moderate amounts of monokines (**step 3**) (Kaushik et al., 1999b; Kaushik et al., 2000). The macrophage will process the engulfed trypanosomes and present trypanosomal antigens via MHC class II to the $CD4^+$ T cell (**step 4**). Activated $CD4^+$ pathogenic T cell will produce IFN-γ (**step 5**), which will induce the macrophage to produce enhanced amounts of monokines and NO (**step 3**) (Kaushik et al., 1999a; Kaushik et al., 2000). The macrophages also produce IL-10, which down-regulates the activation produced by IFN-γ in a negative feed back loop via an autocrine pathway (**step 6**). IL-10 has an effect on maturation of $CD4^+CD25^+FoxP3^+$ Tregs (Papiernik, 2001). Tregs will produce IL-10 which down-regulate the effects of pathogenic T cells and macrophages (**step 7**) (Shi et al., 2006a; Guilliams et al., 2007). Steps 1, 2, 3 and 5 have been well documented. Whether the pathogenic/suppressor T cells are specific for one or several different trypanosomal antigens remains to be determined.

In trypanosome infections, T lymphocyte proliferation occurs but is decreased with the progress of infection (Mayor-Withey et al., 1978). Analysis of VSG-specific antibodies in trypanosome infected BALB/c nude (nu/nu) and Thymus intact (nu/+) mice demonstrated that in the absence of T cells there is a significant B cell response to the exposed VSG epitopes. However, these responses are greatly enhanced in the presence of T cells. Immunization with soluble VSG in the absence of infection produced only T cell-dependent responses (Reinitz and Mansfield, 1990). VSG-specific $CD4^+$ T helper cells were predominantly found in the peritoneal cavities of relatively resistant mice (Schleifer and Mansfield, 1993). Mice which lack B cells, but not T cells, were unable to mount an effective response (Campbell et al.,

1977). Reduced T and B cell responsiveness has been credited to T cell mediated immmunosuppression (Eardley and Jayawardena, 1977; Jayawardena et al., 1978; Pearson et al., 1978; Pearson et al., 1979). It has been shown that there are suppressor (Uzonna et al., 1998c) and pathogenic T cell populations (Shi et al., 2003) in *T. congolense*-infected BALB/c mice. They are the main producers of IFN-γ, which influences the suppression of splenocyte proliferation responses to Con A in mice (Uzonna et al., 1998a; Uzonna et al., 1998c). These cells are also involved in the suppression of T cell proliferative responses to Con A as well as suppression of B cell responses to T cell-dependent antigens *in vitro*. It seems that these IFN-γ-producing T cells, that show disease enhancing effects and show suppressor activities, are predominantly $CD4^+$ (Uzonna et al., 1998c; Shi et al., 2003) and MHC-II restricted (Shi et al., 2006a). Although Schofield et al (1999) have shown that immunization with purified membrane VSG induces CD1d-restricted NKT cells that facilitate IgG antibody responses, CD1d-restricted immune responses do not play an important role in susceptibility and resistance of mice infected with *T. congolense* intraperitoneally (Shi et al., 2006b). In contrast, Wei and Tabel (2008) reported that there is an early protective response mediated by $CD8^+$ NKT cell dependent activation of macrophages to kill parasites by production of NO in susceptible BALB/c mice treated with anti-CD25 antibody prior to subcutaneous infections.

1.2.2.3 Cytokines in African trypanosomiasis

In murine *T. brucei* infections, suppression of lymph node T cells results from suppression of IL-2 secretion and IL-2 receptor expression (Sileghem et al., 1987; Sileghem et al., 1989b; Sileghem and Flynn, 1992). Suppression of IL-2 secretion has been shown to be due to production of prostaglandins by suppressor macrophages (Sileghem et al., 1989a) and IFN-γ might be actively involved in the inhibition of IL-2 receptor expression (Darji et al., 1993; Darji et al., 1996).

IFN-γ can play a beneficial or detrimental role in animal trypanosomiasis. The role of IFN-γ may depend on certain factors such as virulence of infecting strain, trypanosome species and genetic make up of the host. IFN-γ has been claimed to act as a growth factor for *T. brucei* (Olsson et al., 1991). But it has not been possible to demonstrate growth stimulatory effects of IFN-γ on *T. congolense* (Kaushik et al., 1997). *T. brucei*-infected mice which have disrupted IFN-γ genes showed reduced parasitemia and increased survival time (Bakhiet et al., 1996). Opposite outcomes were observed in another study (Namangala et al., 2001). However, it has also been convincingly shown, using IFN-γ knock-out mice, that IFN-γ is required for the survival of relatively resistant C57BL/6 mice infected with *T. brucei* (Hertz et al., 1998) or *T. congolense* (Magez et al., 2006). IFN-γ-receptor deficient mice were used to show that IFN-γ-mediated immune activation is crucial for parasitemia control (Magez et al., 2006). In contrast, excessive secretion of IFN-γ during *T. congolense* infections in highly susceptible BALB/c mice leads to early mortality (Uzonna et al., 1998c; Shi et al., 2003). Anti-IFN-γ treatment of *T. congolense*-infected susceptible BALB/c mice prevents the early death of the infected mice (Uzonna et al., 1998a). IFN-γ plays a dual role in African trypanosomiasis (Tabel et al., 2008) by being a requirement for the survival of relatively resistant C57Bl/6 mice infected with *T. brucei* (Hertz et al., 1998) or *T. congolense* (Magez et al., 2006) and being detrimental by mediating the early death of susceptible BALB/c mice infected with *T. congolense* (Shi et al., 2003).

IFN-γ and IL-10 were produced by matrix-adherent spleen cells (Uzonna et al., 1998c). The production of IFN-γ as well as IL-10 required the absolute synergy of the Thy 1.2^+ and Thy 1.2^- cell populations, since neither the Thy 1.2^+ nor the Thy 1.2^- population alone produced significant amounts of above cytokines (Uzonna et al., 1998c). Most of the adherent spleen cells were mφs which were Thy 1.2^-. Deletion experiments and immunocytochemistry

showed that a small proportion of the adherent cell population was T cells: Thy 1.2^+ $CD3^+$ $TCR\beta^+/\gamma\delta^-$ and predominantly $CD4^+$ (Uzonna et al., 1998c; Shi et al., 2006a).

IL-10 is a known suppressor of the production of other cytokines as well as certain regulatory molecules such as NO (Taylor et al., 1998). Cattle infected with *T. congolense* had elevated IL-10 mRNA in their spleen, blood and lymph nodes suggesting that IL-10 might have an effect on the apparent failure of bovine monocytes to produce inflammatory molecules (Taylor et al., 1998). IL-10 level is found to be increased in both infected tolerant N'Dama cattle and susceptible Boran cattle (Naessens, 2000). Higher levels of IL-10 mRNA transcripts were detected in the spleens, peripheral blood mononuclear cells and lymph nodes of susceptible than in the resistant breeds of cattle infected with *T. congolense* (Taylor et al., 1996). It has been suggested that higher levels of IL-10 might be associated with the observed higher levels of immunosuppression in susceptible breeds (Taylor, 1998). Plasma of highly susceptible BALB/c mice contains significantly higher levels of IL-10 than relatively resistant C57BL/6 mice following infection. Highly susceptible BALB/c mice, when treated with anti-IL-10 antibody during the infection showed a reduction of parasitemia and a moderate increase in survival (Uzonna et al., 1998b). Infected resistant C57BL/6 mice, when treated with antibodies to the IL-10 receptor (IL-10R) die early suggesting that IL-10 is crucial in controlling the detrimental effects mediated by IFN-γ (Shi et al., 2003). When IL-10 function is impaired, MHC class II-restricted immune responses mediate early mortality in resistant C57BL/6 mice. Therefore, in *T. congolense* infections, MHC class II-restricted immune responses mediated either disease or protection, depending on IL-10 function (Shi et al., 2007). IL-10 plays a dual role in African trypanosomiasis by exerting a detrimental role in mediating immunosuppression (Uzonna et al., 1998b) but being beneficial by controlling

excessive production of lethal cytokines by macrophages (Shi et al., 2003; Shi et al., 2006a; Guilliams et al., 2007).

Tumour necrosis factor (TNF-α) is an inflammatory cytokine which has a wide range of biological activities including apoptosis, cytotoxicity, immunomodulation, inflammation and cellular proliferation (Aggarwal and Natarajan, 1996). Induction of TNF-α, and the role of TNF-α in immunopathology is well documented in African trypanosomiasis (Sileghem et al., 1994a; Sileghem et al., 1994b). Increased expression of TNF-α in the brains of *T. brucei*-infected mice has been documented (Hunter et al., 1991). Continuous release of TNF-α from the activated macrophages of infected animals is a characteristic feature in chronic wasting disease associated with trypanosomiasis (Beutler and Cerami, 1988). It has been found that there is an association between TNF-α production by monocytes and the severity of anemia in infected cattle (Sileghem et al., 1994a), trypanosome-elicited immunsuppression and morbidity (Magez et al., 1999) and neuropathological symptoms in human sleeping sickness (Okomo-Assoumou et al., 1995). Treatment of *T. brucei*-infected mice with anti-TNF-α antibodies showed a significant increase in parasitemia suggesting that TNF-α also is important in the control of trypanosomal growth (Magez et al., 1993; Magez et al., 1997). It has been claimed that TNF-α is both trypanolytic and trypanostatic for *T. brucei brucei* and *T. brucei rhodesiense in vitro* (Lucas et al., 1994). It has been found that the control of *T. congolense* infection depends on macrophage/neutrophil derived soluble TNF and intact TNF receptor 1 (TNFp55) signaling, which induces trypanolytic NO (Magez et al., 2007). TNF-α plays a dual role in African trypanosomiasis by exerting a detrimental role in immunopathology and being beneficial by acting as a regulator of trypanosomal growth (Magez et al., 1993; Magez et al., 1997).

Knowledge of the roles of other cytokines in trypanosomiasis is limited. Following infections with *T. brucei*, higher levels of TGF-β and lower levels of TNF-β mRNA was detected in spleens of resistant and susceptible mice respectively (Bakhiet et al., 1996). During, experimental infection with *T. congolense*, the induction of costimulatory cytokines such as IL-1 and IL-2 were depressed in both susceptible AJ and resistant C57BL/6 mouse strains at the early infection (Mitchell et al., 1986).

1.2.3. Immunomodulation

1.2.3.1. Polyclonal B cell activation

Hypergammaglobulinemia with mainly IgM antibody and marked B cell expansion is consistently found in the spleen and lymph nodes in trypanosome infections (Murray et al., 1974). This elevated serum IgM level is used as a screening test in diagnosing sleeping sickness of humans in endemic areas of sub-Saharan Africa (Cunningham et al., 1967).

The increased levels of immunoglobulins consists of antibodies against both trypanosome-related and unrelated antigens, including autoantibodies (Hudson et al., 1976). Trypanosome-unrelated antibodies produced in *T. brucei*-infected mice are antibodies to sheep red blood cells, pneumococcal polysaccharides and haptens (Hudson et al., 1976; Kobayakawa et al., 1979). The above observations led to the suggestion that African trypanosomes possess B cell mitogens which cause non-specific activation of B cells (polyclonal activation).

It has been demonstrated that the trypanosomal membranes were mitogenic for spleen cells of normal athymic and cyclophosphamide-treated mice *in vitro* (Esuruoso, 1976). Also, whole or fractionated homogenates of trypanosomes have been reported to induce polyclonal activation of lymphocytes (Mansfield, Craig et al. 1976; Greenwood and Oduloju 1978). It has been reported that the purified soluble VSG molecules are mitogenic for B cells (Diffley, 1983). Mice immunized with purified VSG showed marked enlargement of B cell

compartments in the spleen and an increase in serum IgG levels mostly due to the production of polyclonal antibodies (Diffley, 1983). The mechanism of the polyclonal B cell activation is, however, unknown.

It is known that binding of an antigen by the B cell receptor and cross-linking of the complement receptor 2 (CR2) simultaneously has a synergistic effect on B cell activation (Klaus, 1986; Tedder et al., 1997). 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 (Liu, 1991; Liu et al., 1993).

The membranes of insect stages of trypanosomes which do not possess VSG, do not cause polyclonal activation suggesting that bloodstream forms of trypanosomes may induce polyclonal B cell activation as an evasion mechanism (Oka et al., 1988). Because the affinity maturation does not occur during polyclonal B cell activation, selective proliferation and the production of high affinity antibodies against the trypanosomes might be prevented (Roitt, 1997).

1.2.3.2. Immune suppression

Immunosuppression is a remarkable feature of trypanosomiasis in cattle, mice and humans (Roelants and Pinder, 1984; Askonas, 1985; Sileghem et al., 1994b; Taylor, 1998). B and T cell responses to trypanosome and non-trypanosome antigens have been suppressed in most hosts, with the exception of trypanoresistant wildlife (Mulla and Rickman, 1988). It was proposed that the major cause of increased susceptibility of trypanosome-bearing individuals to opportunistic infections is generalized immunosuppression observed in patients (Greenwood et al., 1973). Infections of cattle with *T. congolense* and *T. vivax* cause suppression of antibody responses to some vaccines (Rurangirwa et al., 1978; Ilemobade et al., 1982). Suppressed antibody response to *Brucella abortus* was observed in sheep infected with

T. congolense (Malu and Tabel, 1986). In dogs, infections with *T. congolense* have been shown to suppress antibody response to *Brucella abortus* vaccine (Anene, 1989). Water buffaloes infected with *T. evansi* had an increased prevalence of brucellosis (Bajyana Songa et al., 1987).

Suppression of T cell and B cell responses has been studied in laboratory rodent models. Various T cell responses are severely affected including allogeneic graft rejection (Pearson et al., 1978), mixed lymphocyte reaction (Pearson et al., 1978; Roelants et al., 1979), mitogen-induced proliferation (Jayawardena and Waksman, 1977; Morrison et al., 1978; Sileghem et al., 1989b) and delayed type hypersensitivity (Mansfield and Wallace, 1974).

A progressive depletion or exhaustion of antigen-reactive B cells due to polyclonal activation could later result in immunosuppression. Polyclonal activation and immunosuppression were induced *in vivo* by the administration of trypanosome membrane fragments (Clayton et al., 1979b; Sacks et al., 1982). It has been suggested that parasite-unrelated immune responses are depressed in trypanosomiasis while the variant-specific immune responses are not affected (Mansfield, 1981) and the recovery of B cell function is very rapid after chemotherapy (Clayton et al., 1980). There is evidence that antigen nonspecific suppressor cells present in the lymphoid compartments of trypanosome-infected animals are involved in immunosuppression (Corsini et al., 1977; Jayawardena and Waksman, 1977; Pearson et al., 1978; Pearson et al., 1979; Roelants et al., 1979; Wellhausen and Mansfield, 1979; Pearson et al., 1979a; Roelants et al., 1979b; Askonas, 1985; Schleifer and Mansfield, 1993; Sileghem, 1994; Sileghem et al., 1994b). Peritoneal macrophages of *T. brucei*-infected mice were also capable of passively transferring suppression to normal spleen cells (Corsini et al., 1977; Clayton et al., 1979a).

Suppression of T cells in lymph nodes in *T. brucei*-infected mice results from suppression of IL-2 secretion and IL-2 receptor expression (Sileghem et al., 1987; Sileghem et al., 1989b; Sileghem and Flynn, 1992). Suppression of IL-2 receptor expression was associated with endogenously produced IFN-γ (Darji et al., 1993; Darji et al., 1996). It has been suggested that IFN-γ acts on macrophages, leading to the release of a soluble factor associated with the suppression of lymph node proliferative responses (Darji et al., 1996). TNF-α has also been implicated in suppression of lymph node cells in mice infected with *T. brucei* (Lucas et al., 1993; Darji et al., 1996). IL-10 and IFN-γ contributed to the suppression of splenocyte proliferative responses to ConA in infected BALB/c mice (Uzonna et al., 1998b; Uzonna et al., 1998c), identifying IL-10 and IFN-γ as mediators of immunosuppression. The induction of IL-10 and IFN-γ synthesis was trypanosome-specific and required antigen-presenting cells (Uzonna et al., 1998c).

It has been shown that NO mediates suppression of splenic T cell responses in *T. brucei* (Sternberg and McGuigan, 1992; Sternberg and McGuigan, 1994; Mabbott et al., 1995) and *T. rhodesiense*-infected mice (Schleifer and Mansfield, 1993). It has been shown that IL-10 and IFN-γ but not NO are responsible for the observed immunosuppression in *T. congolense*-infected mice (Uzonna et al., 1998b). Therefore, the role of NO in mediating immunosuppression in trypanosomiasis depends upon various factors such as the strain of mice and the strain of the parasite as well as the host.

1.2.3.3. Role of T cells in immunosuppression in African trypanosomiasis

General immunosuppression has been known in *T. brucei*- and *T. congolense*- infected cattle and mice (Hudson et al., 1976; Roelants et al., 1979; Rurangirwa et al., 1979; Askonas, 1985). Roelants and Pinder (1984) concluded that immunosuppression might be mediated by both the suppressor macrophages and suppressor T cells. IL-10 as well as IFN-γ contributes to

the suppression of proliferative responses of the splenocytes to ConA in infected BALB/c mice (Uzonna et al., 1998b; Uzonna et al., 1998c). Thy 1.2^- adherent spleen cells from uninfected mice could provide the suppressive effects only when pulsed with *T. congolense* and coculture with Thy 1.2^+ cells obtained from infected mice but not with Thy 1.2^+ cells from normal mice (Uzonna et al., 1998c).

1.2.3.4. Regulatory T cells (Tregs)

Tregs are a specialized subpopulation of T cells which act to control activation of other immune cells and thereby maintain the homeostasis of the immune system, self-tolerance and control excessive immune responses to foreign antigens (Le and Chao, 2007). Regulatory T cells first described in 1995, are known to suppress autoimmunity (Fehervari and Sakaguchi, 2004). They can suppress immune responses to infectious agents and control immunopathology (Mills, 2004; Belkaid and Rouse, 2005). The best described Treg population is the natural Tregs which arise during T cell development in the thymus and highly express the α chain of the IL-2 receptor (CD25). The other types of Tregs can be induced (inducible Tregs) from naïve T cells in the periphery during normal immune responses.

1.2.3.5. Natural T regs

Naturally occurring $CD4^+CD25^+$ T cells in mice have regulatory functions and constitute about 5-10 % of all T helper cells were first described in 1995 (Sakaguchi et al., 1995). Naturally occurring Tregs are thymus-derived (Sakaguchi et al., 1995). Natural $CD4^+CD25^+$ Tregs were found to express high levels of transcription factor FoxP3 (Hori et al., 2003). Natural $CD4^+CD25^+$ T cells could not be detected in $FoxP3^{-/-}$ mice (Fontenot et al., 2003). It is assumed that only a part of $CD4^+CD25^+$ T cell population exerts regulatory

activity, such as $CD4^{+}CD25^{high}$ cells. Also it is clear that not all T cells that exert suppressive activity express CD25 (Beissert et al., 2006).

An increased pool of $CD4^{+}CD25^{+}$ regulatory T cells was found in the peripheral blood of cancer patients with potent immunosuppressive features (Wolf et al., 2003). Tregs in the tumor sites prevent the development of CD4 helper cells and subsequent development of efficient CD8 T cell activity required for the control of tumor growth (Chaput et al., 2007). Although natural Tregs require activation with specific antigen to attain their suppressive phenotype, once activated they execute inhibition in an antigen specific as well as non-specific fashion.

Although most studies have investigated the regulatory properties of Tregs and natural killer T cells independently of each other, recent reports have provided evidence for cross-talk between Tregs and NKT cells. Activated NKT cells seem to modulate Treg function through IL-2-dependent mechanisms, whereas Tregs can suppress the proliferation, cytokine release and cytotoxic activity of NKT cells by cell-contact-dependent mechanisms (La Cava et al., 2006).

Natural Tregs could control allergic airway inflammation in an IL-10 independent manner in a murine model (Leech et al., 2007). Tregs facilitate early protective responses to local viral infection by allowing a timely entry of immune cells into infected tissue during mucosal herpes simplex virus infection in mice (Lund et al., 2008). In murine models of Leishmania infection, following resolution of infection in healed mice, $CD25^{+}Foxp3^{+}$ Tregs function in an IL-10-dependent manner to prevent sterile cure and to establish a long-term state of functional immune privilege in the skin (Peters and Sacks, 2006). Further, the majority of natural Treg cells at the infected site are Leishmania-specific and parasite-specific natural

Treg cells are restricted to sites of infection and that their survival is strictly dependent on parasite persistence (Suffia et al., 2006).

1.2.3.6. Inducible T regs

The inducible Tregs are induced from the naïve T cells of the periphery by the immunosuppressive cytokine TGF-β (Chen et al., 2003) or by low dose antigenic stimulation by peptides (Apostolou and von Boehmer, 2004). Inducible Tregs do not have unique surface markers to distinguish them from other T cell subsets (Table 1.2). Also, it is not clear whether FoxP3 regulate the development of Th3 or Tr1 cells. However, TGF-β has been shown to convert peripheral $CD4^+CD25^-$ naïve T cells into $CD4^+CD25^+$ Tregs, probably through the induction of FoxP3 expression (Chen et al., 2003). Unlike natural Tregs, both Th3 or Tr1 are inducible Tregs and appear to function independently of cell-cell contact and suppress immune responses through the secretion of cytokines such as TGF-β (Josien et al., 1998) and IL-10 (Groux et al., 1997).

It has been shown that activation of human $CD4^+$ T cells with anti-CD3 and anti-CD46 induced a T-regulatory cell 1 phenotype which produced enhanced amounts of IL-10 (Kemper et al., 2003). Thus, cross-linking of Crry or CD46 by antigens coated with complement fragments during antigen presentation can modulate the T reg cell response (Kemper et al., 2005).

1.2.3.7. Roles of regulatory T cells during infections

In Tuberculosis, higher levels of FoxP3 mRNA and slightly lowered expression of IL-10 was observed in blood of TB patients while a significantly lower FoxP3 mRNA expression with no difference in IL-10 expression was observed in recently infected contacts. It is not clear whether the Tregs play a beneficial or a harmful effect in tuberculosis (Burl et al., 2007).

Table 1.2. Subsets of natural and induced regulatory T cells

Treg subset	Regulatory mechanisms	Transcription factor expressed	Target cells	Function
$CD4^+CD25^+$ Tregs	Cell contact-dependent, Cytokines (IL-10) ?	FoxP3	T cells, APCs	Suppression of autoimmunity, inhibition of allograft rejection and of immune responses induced by microbial infection
$CD4^+CD25^-$ Tregs	Mostly by cytokines	FoxP3?	T/B cells, APCs	Suppression of autoimmunity
Tr1 cells	Mediated by IL-10	FoxP3?	T cells	Suppression of autoimmunity
Th3 cells	Mediated by TGF-β	?	T cells	Suppression of autoimmunity
NK Tregs	IL-4, IL-10, TGF-β, cytotoxicity	?	T cells, APCs, tumor cells	Elimination of tumors and pathogens, Suppression of autoimmunity
$CD8^+$ Tregs	Cell contact-dependent	FoxP3?	T cells	Suppression of autoimmunity, regulation of peripheral TCR repertoire
$CD8^+CD28^-$ Tregs	Induction of ILT3/ILT4 in dendritic cells	FoxP3?	Dendritic cells/APCs	Regulation of autoimmunity

?Issue uncertain, not yet clear or not yet investigated. Abbreviations: APC, antigen-presenting cell; ILT, immunoglobulin transcript; NK Treg, regulatory cells of natural killer T cell phenotype; Th3, T helper type 3; Tr1 cell, type 1 regulatory T cell; Treg, regulatory T cell. (Adapted from Beissert et al., 2006)

The frequency and the absolute count of duodenal mucosal Tregs were highly increased in untreated HIV patients but were normal in treated HIV patients. However, in peripheral blood of HIV patients, the absolute number of Tregs was not increased, and their frequency was only slightly elevated. The high increase in count and frequency of mucosal Tregs observed in untreated HIV infection, suggest a significant contribution of Tregs to the pathogenesis of HIV disease (Epple et al., 2006).

The increased proportion of Tregs in aged mice was associated with the spontaneous reactivation of chronic Leishmania major infection, likely because Tregs efficiently suppressed the production of IFN-γ by effector T cells. In vivo depletion of Tregs in old mice attenuated the severity of the disease. Accumulation of functional Tregs in aged hosts could therefore play an important role in the frequent reactivation of chronic infections that occurs in the elderly (Lages et al., 2008).

It has been demonstrated in Hepatitis C virus (HCV) infection in chimpanzees that the frequency of $Foxp3^{+}CD4^{+}CD25^{+}$ Tregs and the extent of suppression was as high in spontaneously recovered chimpanzees as in persistently infected chimpanzees. Thus, Tregs control HCV-specific T cells not only in persistent infection but also after recovery, where they may regulate memory T-cell responses by controlling their activation and preventing apoptosis (Manigold et al., 2006). In human T-lymphotropic virus type 1 infection, there was a strong negative corelation between the frequency of $CD4^{+}FoxP3^{+}$ Tregs in the circulation and the rate of cytotoxic T lymphocyte-mediated lysis of autologous HTLV-1-infected cells ex vivo (Toulza et al., 2008).

1.2.3.8. Impact of pathogens on regulatory T cells

Epstein-Barr virus can recruit Tregs to the microenvironment of Hodgkin's lymphoma by inducing the expression of chemokine ligand 20 (CCL20) in the tumor cells and, by doing

so, prevent immune responses against the virus-infected tumor population (Baumforth et al., 2008). Functionally active and virus-specific FoxP3$^+$ Tregs are induced in hepatitis C virus infection, thereby providing targeted immune regulation in vivo (Ebinuma et al., 2008). Suppression assays carried out using Tregs purified from WT and IL-6$^{-/-}$ C57BL/6 mice infected with influenza virus (H17) showed that IL-6 induced by influenza virus (H17) limits the activity of virus-specific Tregs, thereby facilitating the activity of virus-specific memory CD4$^+$ T cells (Longhi et al., 2008). Intrathymic injection of the lentiviral vector resulted in an enrichment of hemagglutinin antigen-specific Tregs in peripheral lymphoid organs in mice (Marodon et al., 2006). In humans, M. tuberculosis mannose-capped lipoarabinomannan (ManLAM) resulted in regulatory T cell expansion, whereas the M. tuberculosis 19-kDa protein and heat shock protein 65 had no effect (Garg et al., 2008).

1.2.3.9. Tregs in trypanosomal infections

In relatively resistant C57BL/6 mice infected with *T. congolense*, Foxp3$^+$ Tregs originating from the naturally occurring Treg pool expanded in the spleen and the liver. These Tregs produced IL-10 and limited the production of IFN-γ by CD4$^+$ and CD8$^+$ effector T cells (Guilliams et al., 2007). Also, Tregs down-regulated classical activation of macrophages resulting in reduced TNF-α production. These observations suggest a cardinal role for naturally occurring Tregs in the development of a trypanotolerant phenotype during African trypanosomiasis (Guilliams et al., 2007).

When susceptible BALB/c mice were injected with optimal amounts of a depleting mAb specific for CD25 two days prior to the infection, the infected mice did not develop parasitemia and eliminated all parasites and showed no signs of disease. There was a 100% reduction of CD4$^+$CD25^{high} T cells and a 70% reduction of CD4$^+$CD25$^+$Foxp3$^+$ T cells in the spleen 7 days post infection. Administration of L-N6-(1-imminoethyl) lysine, a specific

inhibitor of inducible NO synthase or administration of anti-CD8 Ab could reverse the protective effect caused by treatment of anti-CD25. Most of the anti-CD25 antibody treated $CD1d^{-/-}$ mice developed parasitemia but could control subsequent waves of parasitemia (Wei and Tabel, 2008). Further, when treated BALB/c mice with anti-CD25 mAb, there was an increase of early cytokine release (at day 5 post infection) and an early elevation (at day 3 post infection) of $CD8^{+}CD122^{+}$ cells in the spleen was detected. Collectively these results suggest that in normal trypanosomal infections of BALB/c mice, Tregs prevent the activation of $CD8^{+}$ NKT cells which could activate macrophages to produce trypanocidal NO (Wei and Tabel, 2008). It has been proposed that there is a cross regulation of NKT cells and $CD4^{+}CD25^{high}FoxP3^{+}$ Tregs in experimental *T. congolense* infections (Tabel et al., 2008).

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Introduction

Rapid death observed in highly susceptible BALB/c mice infected with virulent strains of *Trypanosoma congolense* or *Trypanosoma brucei* is due to a systemic inflammatory response syndrome (SIRS) (Shi et al. 2003). A small subset of pathogenic, MHC class II-restricted $CD4^+$ T cells, activated during the course of *T. congolense* infection, mediates early mortality in susceptible BALB/c mice via excessive synthesis of IFN-γ (Shi et al. 2006; Uzonna et al. 1998a & c). Since these pathogenic T cells are matrix-adherent (Shi et al. 2006; Uzonna et al. 1998c), they can be distinguished from the conventional Th1 cells. There is a possibility for this subset of pathogenic $CD4^+$ T cells to be a population of T cells with unique surface markers.

Both the classical pathway and alternative pathways of complement are activated during African trypanosomiasis (Greenwood and Whittle, 1980). Hypocomplementemia is a prominent feature of experimental African trypanosomiasis in cattle (Tabel et al., 1980), sheep (Malu and Tabel, 1986) and mice (Otesile et al., 1991). During the activation of the complement system, the final degradation product of complement protein C3 is complement component C3d. The amplification of the alternative pathway of complement and the degradation of complement C3b to C3d proceeds faster in BALB/c mice than in C57BL/6 (Ogunremi et al., 1993). Complement receptor CR2 is a 145 kD transmembrane protein which acts as the receptor for C3d. The expression of CR2 on the cell surface of a subpopulation of mouse T cells has been documented (Qian et al., 2005). CR2 is expressed on activated T cells in AJ mice (Chen et al., 2005).

T. congolense-infected BALB/c mice have more VSG-containing immune complexes in their plasma than infected C57BL/6 mice (Pan & Tabel, unpublished). Most of the immune complexes might contain VSG-C3d complexes in *T. congolense*-infected BALB/c mice. The presence of VSG-C3d immune complexes in *T. congolense*-infected BALB/c mice might play an important role in the pathogenesis of the infection: costimulation of $CR2^+$ T cells by VSG-C3d immune complexes might induce a subpopulation of T cells that express CR2 to produce excessive amounts of IFN-γ.

2.2 Hypothesis

In *T. congolense*-infected BALB/c mice, most of the IFN-γ-producing $CD4^+$ T cells are $CR2^+$ and the numbers of $CR2^+$ T cells increase with progression of infection.

2.3 Objectives

1. Study the kinetics of $CR2^+$ T cells in *T. congolense* infections in BALB/c mice
2. Test the subpopulations of T cells that produce IFN-γ in experimental *T. congolense* infections in BALB/c mice

3.0 KINETICS OF $CR2^+$ T CELLS IN EXPERIMENTAL MURINE *T. CONGOLENSE* INFECTIONS

3.1. Abstract

In African trypanosomiasis, the complement system is highly activated, leading to covalent complexes of variant surface glycoprotein (VSG) and degradation products of complement component C3. The final degradation product of C3 is C3d. Complement receptor CR2 is the cell surface receptor for C3d.

I investigated the kinetic pattern of T cells ($CD3^+$) expressing CR2 in BALB/c mice infected with *T. congolense.* In the first set of experiments, spleen cells of normal and infected BALB/c mice were stained with $^{PE\text{-}Cy5}$anti-CD3, ^{PE}anti-CR2 and ^{FITC}anti-CD25 at days 3, 4, 5, 6 and 7 post infection and were analyzed by FACS. Total numbers of spleen cells were increased 5-fold with progressive infection. Total numbers of $CD3^+$ cells, $CR2^+$ cells and $CD25^+$ cells were increased 4 to 5-fold, 3-fold and 6-fold, respectively, with progressive infection. The numbers of $CD3^+CR2^+$ cells, $CD3^+CD25^+$ cells and $CD25^+CR2^+$ cells were increased 3-fold, 2 to 3-fold and 3 to 3.5-fold, respectively, with the progression of infection. Absolute numbers of B cells ($CD19^+$) showed a 5 to 6 fold increase and the numbers of $CR2^+$ B cells showed a 4-fold increase at day 7 post infection. In a second set of experiments spleen cells of normal and infected BALB/c mice were stained with ^{FITC}anti-CD3 and ^{PE}anti-CR2 antibodies. The pattern of $CR2^+$ cells was similar to the one of the previous study. However, the total numbers of $CR2^+$ T cells in the spleen of normal mice was found to be considerably less, about 1.5×10^6 (instead of 3×10^6) per spleen. The numbers of $CD3^+$ cells and $CD3^+CR2^+$ cells showed 5-fold and 7-fold increases, respectively. While the total numbers of $CR2^+$ cells (including B cells) showed a 4-fold increase, the mean numbers of CR2 per cell showed a 50% reduction with progressive infection.

In summary, the study shows that a small subpopulation of $CR2^+$ T cells progressively increases 3 to 7-fold in BALB/c mice infected with *T. congolense*.

3.2. Introduction

In experimental infections with African trypanosomes, T lymphocyte proliferation occurs but is decreased with the progress of infection (Mayor-Withey et al., 1978). Analysis of VSG-specific antibodies in trypanosome-infected nude (nu/nu) BALB/c and thymus intact (nu/+) mice showed that in nude mice there is a significant B cell response to the exposed VSG epitopes, indicating that most of the early antibody response to VSG is T cell-independent (Campbell et al., 1978). In *T. congolense*-infected BALB/c mice, there is a relatively small subpopulation of T cells that have an immunosuppressive (Uzonna et al., 1998c) and a general pathogenic (Shi et al., 2003; Shi et al., 2006a) function. They are the main producers of IFN-γ (Uzonna et al., 1998a; Uzonna et al., 1998c). It seems that these IFN-γ-producing T cells, that show disease enhancing effects and suppressor activities, are predominantly $CD4^+$ (Uzonna et al., 1998c; Shi et al., 2003) and MHC-II-restricted (Shi et al., 2006a). This subpopulation of T cells differs from the majority of Th1 cells in that they are matrix-adherent (Uzonna et al. 1998c; Shi et al. 2006).

In mice, CR2 is predominantly expressed on B cells, follicular dendritic cells and activated granulocytes. Mouse CR2 is highly expressed on peritoneal and splenic B-1 B lymphocytes (Kinoshita et al., 1988; Carroll, 1998). It is known that binding of an antigen by the B cell receptor and cross-linking of the complement receptor 2 (CR2) simultaneously has a synergistic effect on B cell activation (Klaus, 1986; Tedder et al., 1997). In T cells, CR2 may act either as an activating receptor or as an adhesion molecule to promote the recruitment or retention of tissue-infiltrating cells (Holers, 2005). Kaya et al. (2001) have provided evidence

that $CR2^+$ T cells participate in the development of experimental autoimmune myocarditis. CR2 has been found to be expressed on activated T cells in A/J mice (Chen et al., 2005). CR2 was detectable on both $CD4^+$ and $CD8^+$ subsets of T cells (Kaya et al., 2005).

Although not investigated, the complexes of cleavage products of complement component C3 (C3b, iC3b and C3d) and soluble or particulate trypanosomal antigens might have a profound effect upon binding to cells bearing receptors for C3b, iC3b and/or C3d (Tabel et al., 2000). *T. congolense*-infected BALB/c mice have more immune complexes containing trypanosomal variant surface glycoprotein than infected C57BL/6 mice (Pan & Tabel, unpublished data). The degradation of C3b to C3d is very fast in BALB/c mice (Ogunremi et al. 1993). We speculate that the majority of the immune complexes in *T. congolense*-infected BALB/c mice might be VSG-C3d immune complexes and that binding of these complexes to $CR2^+$ T cells might costimulate these T cells to produce IFN-γ.

Experiments discussed in this chapter describe the kinetics of $CR2^+$ T cells and B cells in the spleen of *T. congolense*-infected BALB/c mice.

3.3. Materials and Methods

3.3.1. Mice

Female BALB/c mice and CD1 mice were obtained from the Animal Resource Center of the University of Saskatchewan. BALB/c mice were 8 to 10 weeks old and CD1 mice were 6 weeks old. All mice were maintained according to the recommendations of the Canadian Council of Animal Care.

3.3.2. Parasites

T. congolense, variant antigenic type (VAT) TC13 (Tabel, 1982) was used in this experiment. TC13 was passaged in immunosuppressed CD1 mice as described (Tabel, 1982). The parasites for the infection of BALB/c mice were obtained by isolating the parasites from the blood of

infected CD1 mice using DEAE-cellulose chromatography columns as described (Lanham and Godfrey, 1970).

3.3.3. Antibodies

The following principle antibodies were used to stain the spleen cells for FACS analysis: $^{PE\text{-}Cy5}$ Armenian hamster anti-mouse CD3 (BD Pharmingen), ^{PE} rat anti-mouse CR2 (eBioscience), ^{FITC} Rat anti-mouse CD25 (eBioscience), ^{FITC} rat anti-mouse CD19 (eBioscience), and ^{FITC} hamster anti-mouse CD3 (Cedarlane Laboratories). The following antibodies were used as isotype control antibodies: ^{PECy5} Armenian hamster IgG (eBioscience), ^{PE}rat IgG2a (eBioscience), ^{FITC} rat IgG1 (eBioscience), , ^{FITC}mouse IgA (eBioscience) and ^{FITC} hamster IgG (eBioscience).

3.3.4. Experimental design

BALB/c mice were infected intraperitoneally with 10^3 organisms of trypanosomes. Four normal BALB/c mice and four infected BALB/c mice were euthanized each day using CO_2 and single cell suspensions were prepared from the collected spleens at days 3, 4, 5, 6, 7 and 8 post infection. Red blood cells were lysed using ACK lysing buffer as described (Coico, 2005). Spleen cells were resuspended at 10^7 cells/ml in FACS buffer to use these cells for staining and FACS analysis.

3.3.5. Estimation of parasitemia

To estimate the circulating parasite numbers, a drop of blood from the tail vein of each mouse was examined at 400x magnification by phase contrast microscopy. Parasitemia was estimated by counting the number of parasites present in 20 fields at the early infection. Heavy parasite loads were quantified according to Herbert and Lumsden (Herbert and Lumsden, 1976).

3.3.6. FACS analysis

One hundred micro liter aliquots of 10^7 cells/ml in FACS buffer were incubated for 10 minutes on ice with 4 µl of rat anti-mouse CD16/32 (Fcγ II/III receptor, Fc Block, eBioscience) monoclonal antibody. Appropriate volumes of each antibody specific for a particular cell surface marker were added and incubated on ice in the dark for 30 minutes. The cells were washed 2 times with FACS buffer and resuspended in FACS buffer containing 1% formalin. Fixed cells were analyzed using a FACScan flow cytometer using Flowjo software (Tree Star Inc.).

3.3.7. Statistical analysis

Multiple comparisons were performed by one way analysis of variance (ANOVA) using SPSS software. Differences between infected groups and respective normal groups were tested by paired T test. A statistical probability of $P < 0.05$ was considered statistically significant.

3.4. Results

3.4.1. Kinetics of T and B cells in the spleen of infected BALB/c mice

3.4.1.1. Parasitemia and total numbers of spleen cells

Infected mice did not have any visible parasites up to day 4 following infection. Parasites started to appear in blood at day 5 post infection and this was followed by an increase of parasitemia up to day 7 until the experiment was terminated (Fig.3.1.A). The parasitemia pattern was similar in all kinetic studies performed.

Total number of spleen cells in normal mice was about 10^8 in all cases. This number showed a 3-fold increase at day 5 post infection and up to 5-fold increases were observed at day 7 post infection (Fig.3.1.B).

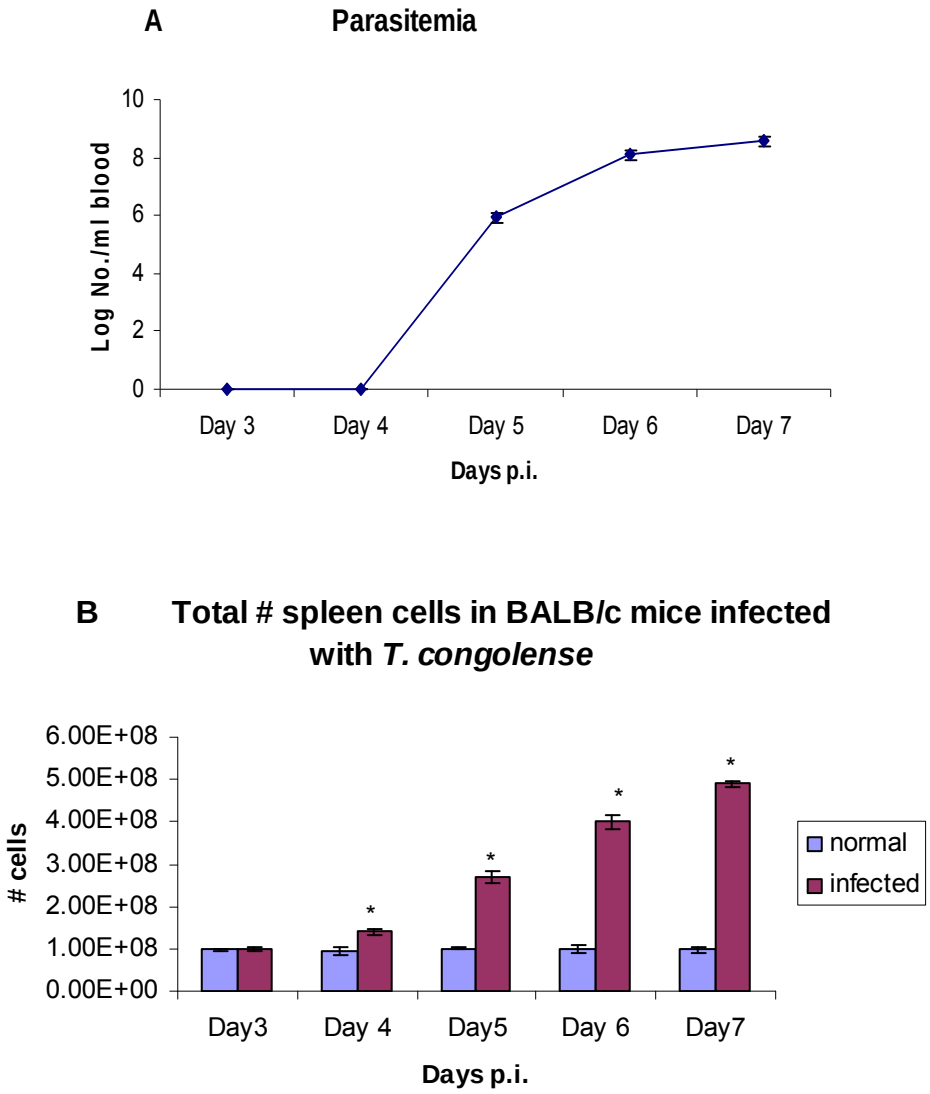

Fig. 3.1. Parasitemia (A) and total number of spleen cells (B) of BALB/c mice infected intraperitoneally with 10^3 *T. congolense*. (The data shown are representative of mean±SE of four similar experiments, n= 4). * $P < 0.05$ between infected group and respective control group.

3.4.1.2. Kinetics of $CD3^+$ cells, $CR2^+$ cells, and $CD25^+$ cells in the spleen

In the results of these experiments, absolute and relative numbers of each cell population are presented. The relative number of a particular cell population is presented as the percentage of the total spleen cell population. The total numbers of spleen cells increase with progress of the infection. Since the dynamics of a subpopulation may change at a different rate than that of the total spleen cell numbers, the profile of relative numbers from day 0 to day 8 might be quite different from the profile of the absolute numbers.

Absolute numbers of T cells showed a 4 to 5-fold increase with progressive infection (Fig.3.2.A) while the relative numbers of T cells were lower than in normal mice at early infection, but increased up to day 7 post infection (Fig.3.2.B).

There was a 2 to 3-fold increase in absolute numbers of $CR2^+$ cells in the spleen of BALB/c mice during the infection (Fig.3.3.A) while the relative numbers showed a decline at days 5, 6 and 7 post infection (Fig.3.3.B). It is known that the majority of $CR2^+$ cells in the spleen are B cells and follicular dendritic cells (Carroll, 1998).

There was a progressive increase in the absolute numbers of $CD25^+$ cells in the spleen of infected BALB/c mice which reached a 6-fold increase at day 7 post infection compared to those of normal mice (Fig.3.4.A) while the relative numbers showed a 2-fold increase at day 7 post infection (Fig.3.4.B). This population of cells would include activated T cells and Tregs.

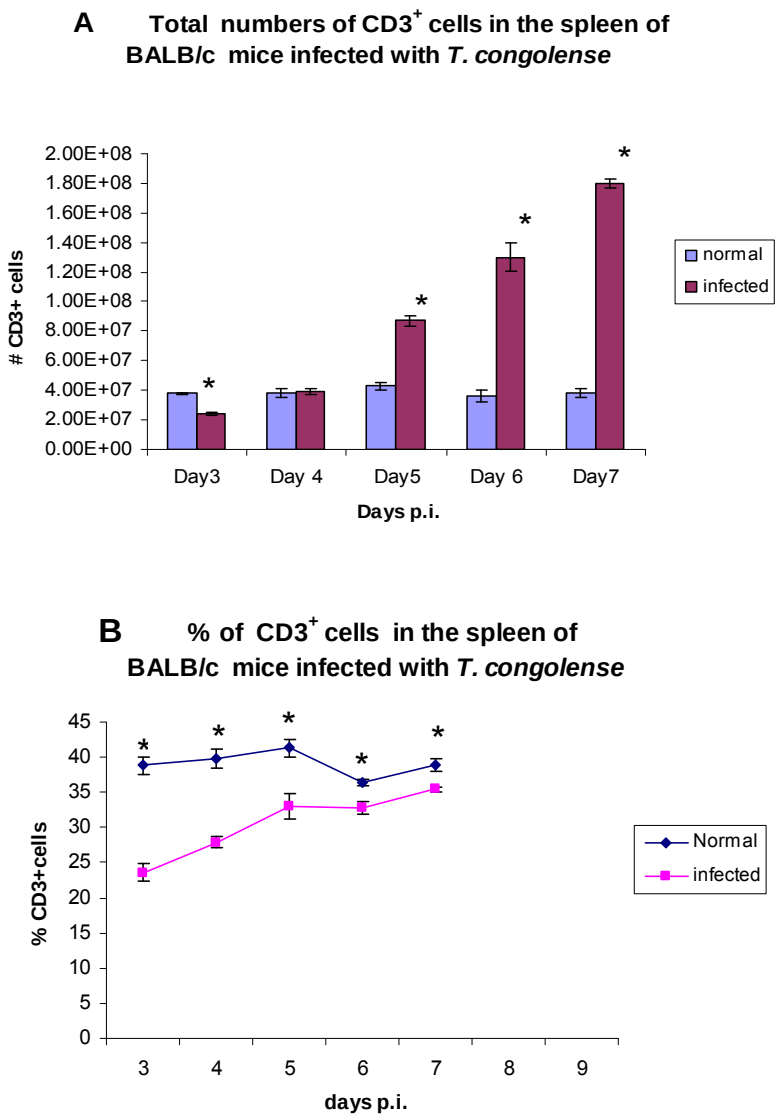

Fig.3.2. Absolute numbers of $CD3^+$ cells (A) and the relative numbers of $CD3^+$ cells (B) in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. ^{PECy5}anti-CD3 antibody was used to stain the spleen cells. Values represent mean±SE, n= 4. (The data shown are representative of four similar experiments). * $P < 0.05$ between infected group and respective control group.

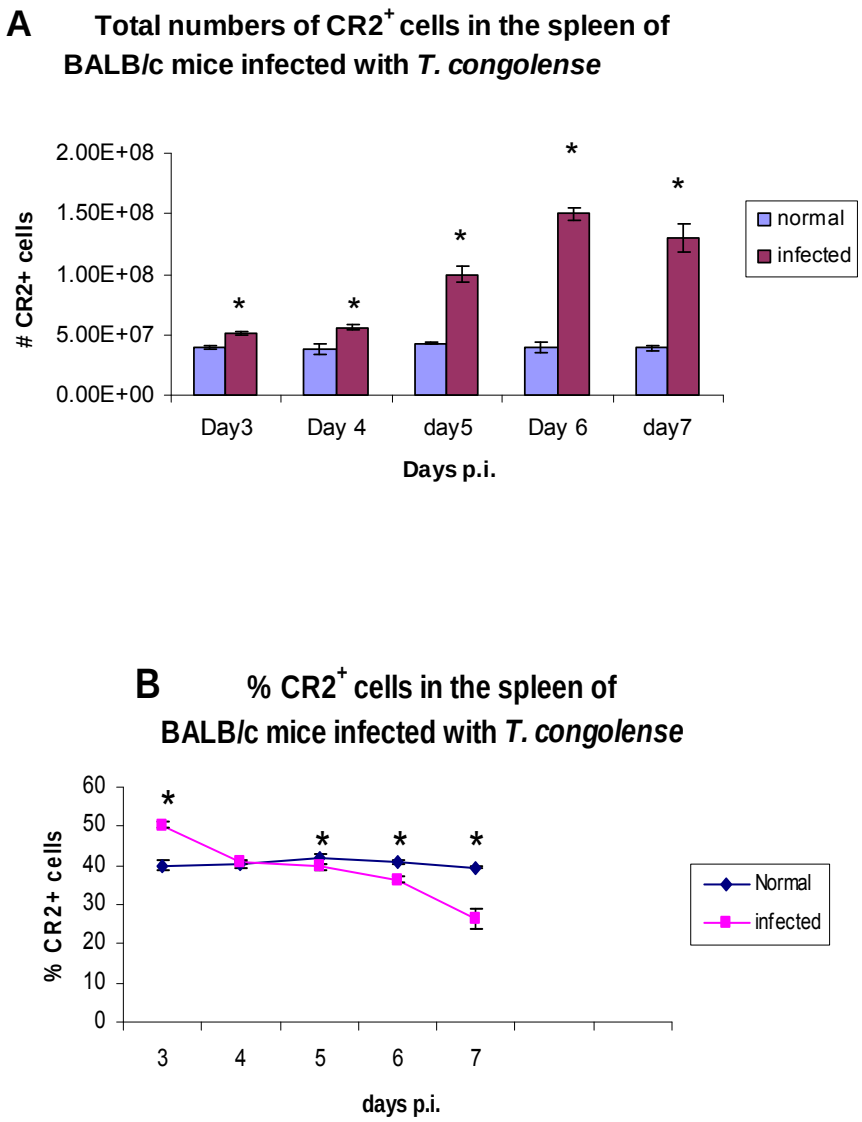

Fig.3.3. Absolute numbers of $CR2^+$ cells (A) and the relative numbers of $CR2^+$ cells (B), in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. ^{PE}anti-CR2 antibody was used to stain the spleen cells. Values represent mean±SE, n= 4. (The data shown are representative of four similar experiments). * $P < 0.05$ between infected group and respective control group.

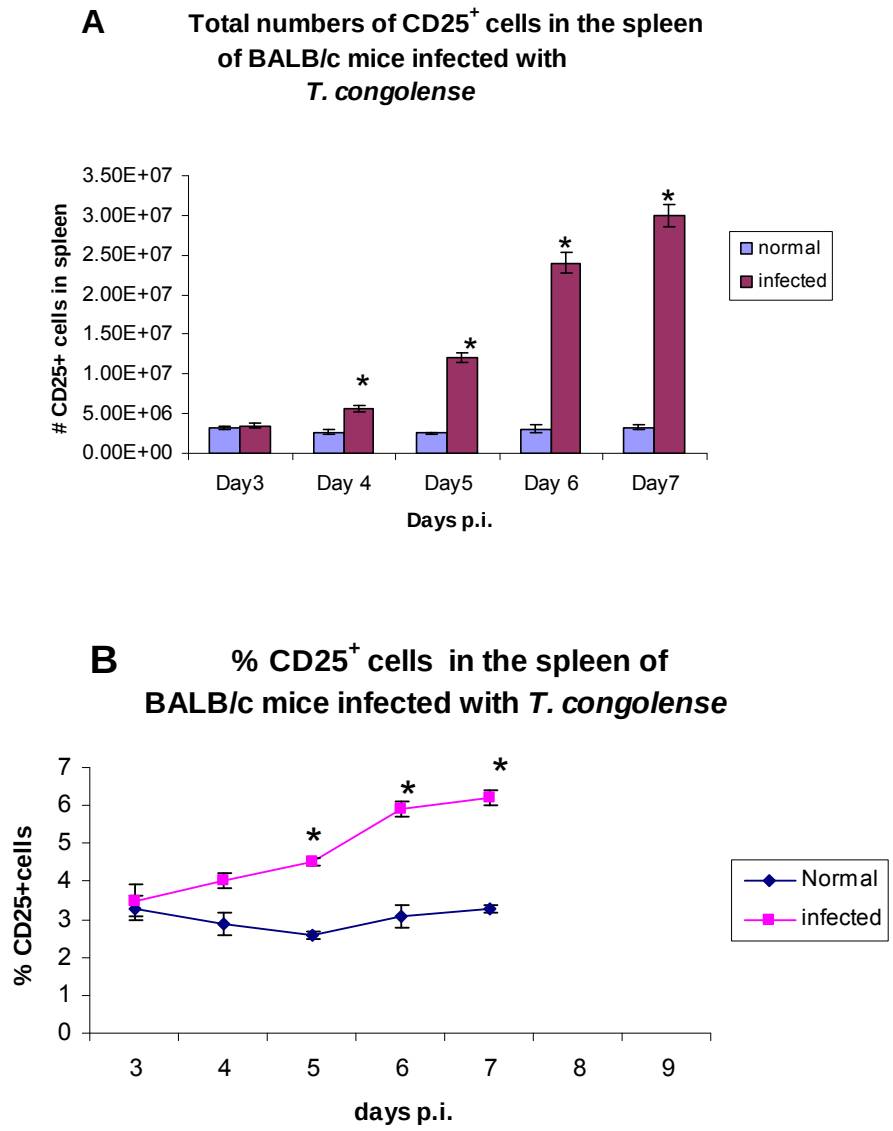

Fig.3.4. Absolute numbers of $CD25^+$ cells (A) and the relative numbers of $CD25^+$ cells (B), in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. ^{FITC}anti-CD25 antibody was used to stain the spleen cells. Values represent mean±SE, n= 4. (The data shown are representative of four similar experiments). * $P < 0.05$ between infected group and respective control group.

3.4.1.3. $CD3^+CR2^+$ cells in the spleen of normal BALB/c and C57BL/6 mice

The kinetics of complement activation in highly susceptible BALB/c mice is different from that of relatively resistant C57BL/6 mice. The degradation of C3b to C3d during the amplification of the alternative pathway of complement proceeds faster in BALB/c than in C57BL/6 (Ogunremi et al., 1993). Therefore we speculated that the expression of CR2, the receptor for C3d, in T cells might be different between the susceptible and resistant mice. Spleen cells of normal BALB/c and C57BL/6 mice were stained for CD3 and CR2 to find out whether the numbers of $CR2^+$ T cells show a difference between the two strains of mice. There was no significant difference between the numbers of $CR2^+$ T cells in normal BALB/c and C57BL/6 mice according to the results (Fig.3.5) obtained by FACS analysis.

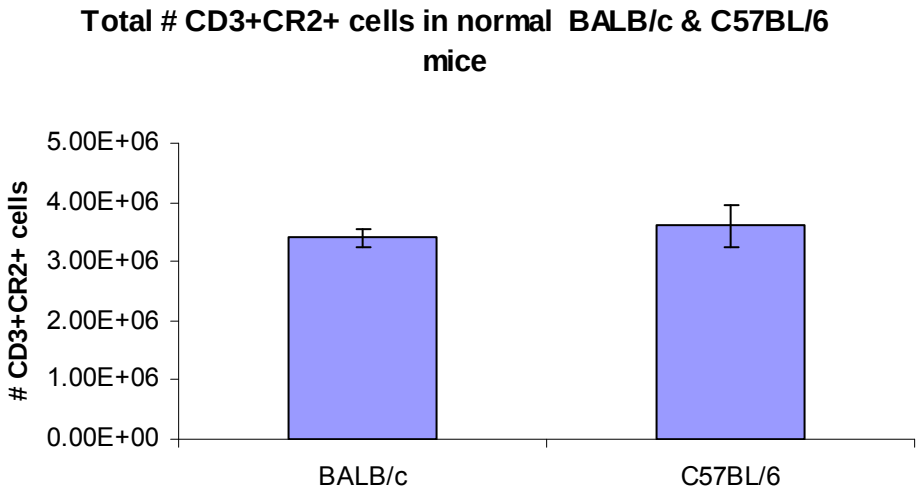

Fig.3.5. Absolute numbers of $CD3^+CR2^+$ cells in the spleen of susceptible BALB/c mice and relatively resistant C57BL/6 mice. The cells were stained with ^{PECy5} Armenian hamster anti-mouse CD3 and ^{PE} rat anti-mouse CR2 and analyzed by FACS. (Values represent mean±SE, n= 8).

3.4.1.4. Kinetics of $CD3^+CR2^+$ cells in the spleen

The absolute numbers of $CD3^+CR2^+$ cells ($CR2^+$ T cells) in the spleen of a normal BALB/c mouse was around 3 x 10^6, when the cells were stained with ^{PECy5} Armenian hamster anti-mouse CD3 and ^{PE} rat anti-mouse CR2 and analyzed by FACS. There was no increase in the number of $CD3^+CR2^+$ cells up to day 3 post infection, but there was a 3-fold increase achieved at day 5 and remained at that level until the experiment was terminated at day 7 post infection (Fig.3.6.A). The relative numbers of $CD3^+CR2^+$ cells did not show much difference at early infection but showed a slight decrease at day 6 and 7 post infection (Fig.3.6.B). The original FACS pictures of representative normal and infected spleen cells of BALB/c mice, collected at day 6 post infection, are shown in Fig. 3.7.

3.4.1.5. Kinetics of $CD3^+CD25^+$ cells and $CD25^+CR2^+$ cells in the spleen

Absolute numbers of $CD3^+CD25^+$ T cells showed a 2 to 3-fold increase at day 5, followed by a 10-fold increase at day 7 post infection (Fig.3.8.A), indicating a progressive proliferation of $CD25^+$ T cells during the infection. Relative numbers of $CD3^+CD25^+$ T cells also increased with progressive infection (Fig.3.8.B). The original FACS pictures of representative normal and infected spleen cells of BALB/c mice, collected at day 7 post infection, are shown in Fig. 3.9.

Absolute numbers of $CD25^+CR2^+$ cells showed a 3 to 3.5-fold increase at day 7 post infection compared to the normal mice (Fig.3.10.A) but the relative numbers of $CD25^+CR2^+$ cells remained fairly constant at about 1% during the infection (Fig.3.10.B). The original FACS pictures of representative normal and infected spleen cells of BALB/c mice, collected at day 7 post infection, are shown in Fig. 3.11.

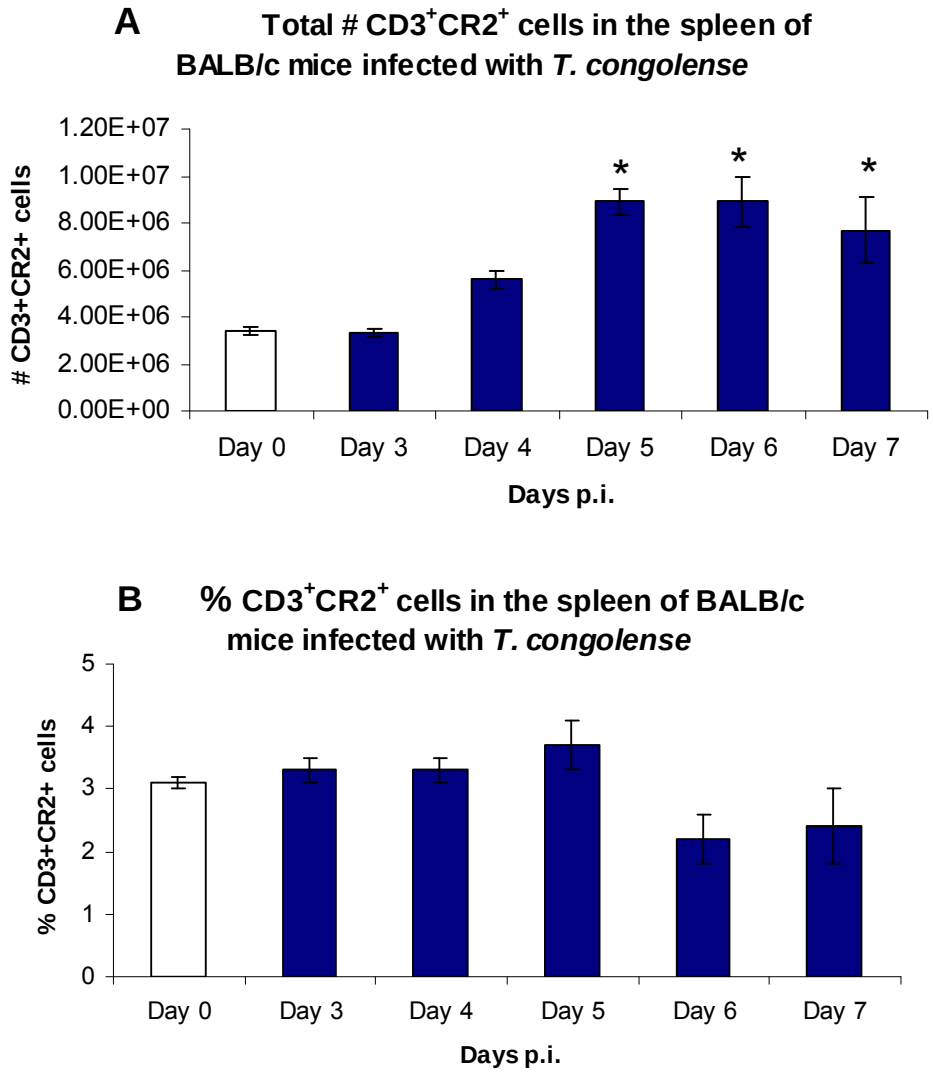

Fig.3.6. Absolute numbers of $CD3^+CR2^+$ cells in the spleen (A) and relative numbers of $CD3^+CR2^+$ cells (B) in the spleen of BALB /c mice infected with 10^3 organisms of *T. congolense* TC13. The spleen cells were stained with ^{PECy5} Armenian hamster anti-mouse CD3 and ^{PE} rat anti-mouse CR2 and analyzed by FACS. (Mean±SE values of four experiments.)

*P < 0.05 between control group and infected groups.

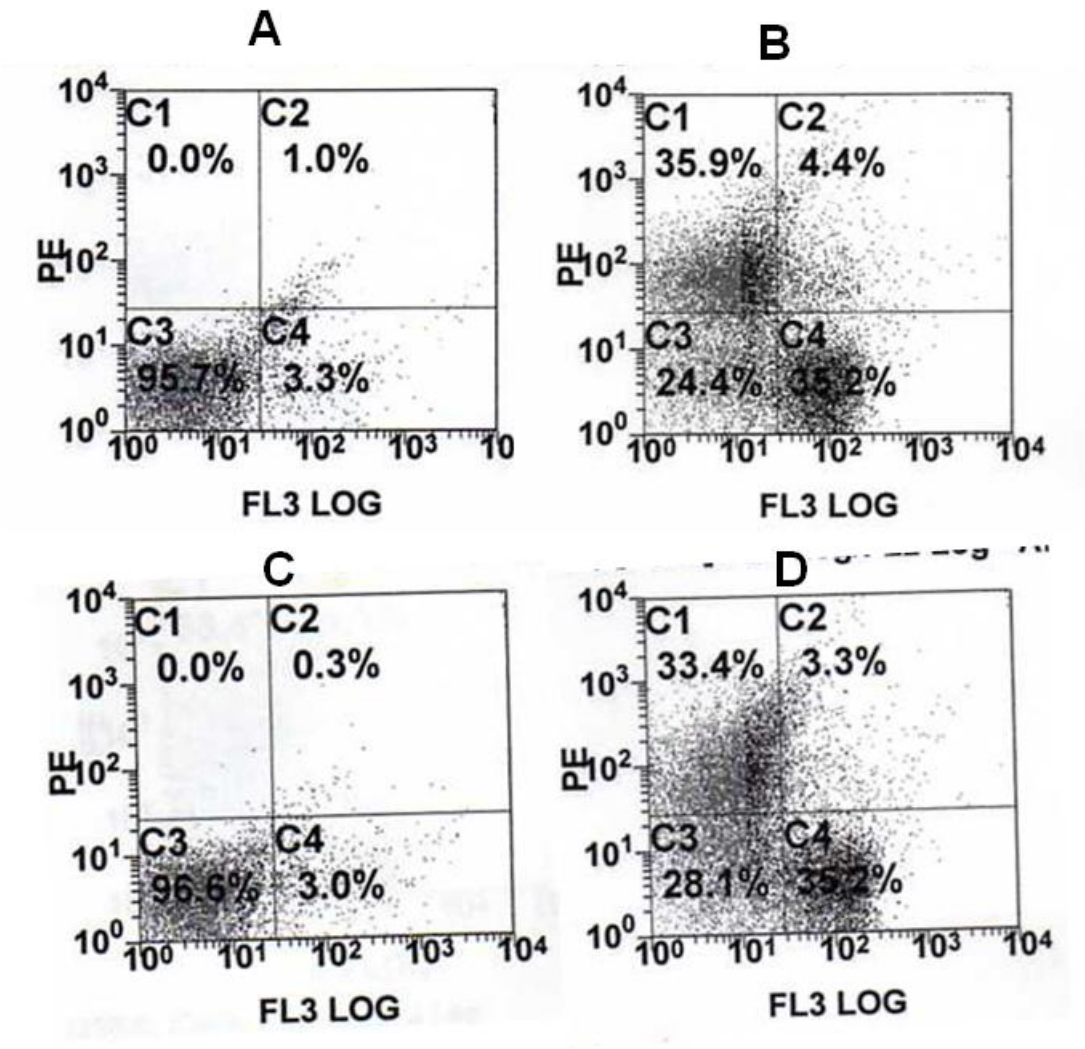

Fig.3.7. FACS analysis to detect $CD3^+CR2^+$ cells in the spleen. Spleen cells collected from normal BALB/c mice and *T. congolense*-infected BALB/c mice at day 6 post infection were stained with ^{PECy5}anti-CD3, ^{PE}anti-CR2 and isotype control antibodies and analyzed by FACS. A: isotype control of normal BALB/c spleen cells, B: normal BALB/c spleen cells stained with ^{PECy5}anti-CD3 and ^{PE}anti-CR2, C: isotype control of infected BALB/c spleen cells collected at day 6 post infection, D: spleen cells collected at day 6 post infection stained with ^{PECy5}anti-CD3 and ^{PE}anti-CR2.

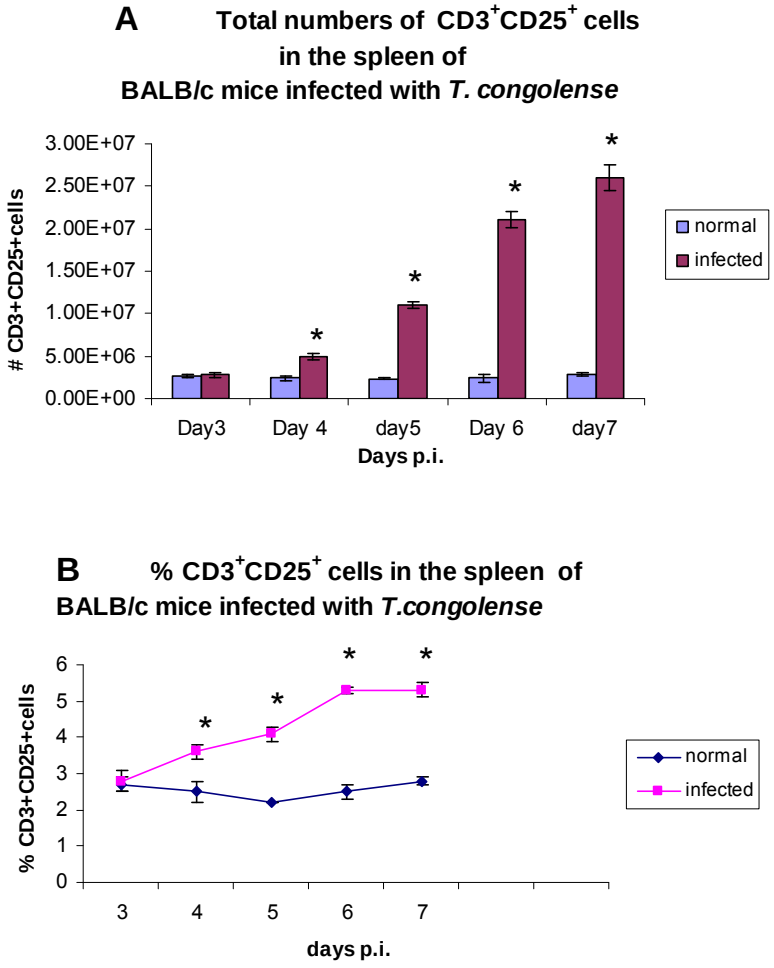

Fig. 3.8. Absolute numbers of $CD3^+CD25^+$ cells (A) and relative numbers of $CD3^+CD25^+$ cells (B) in the spleens of BALB/c mice infected intraperitoneally with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells were stained with ^{FITC}anti-CD25 and ^{PECy5}anti-CD3 antibodies and analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are representative of four similar experiments). * $P < 0.05$ between infected group and respective control group.

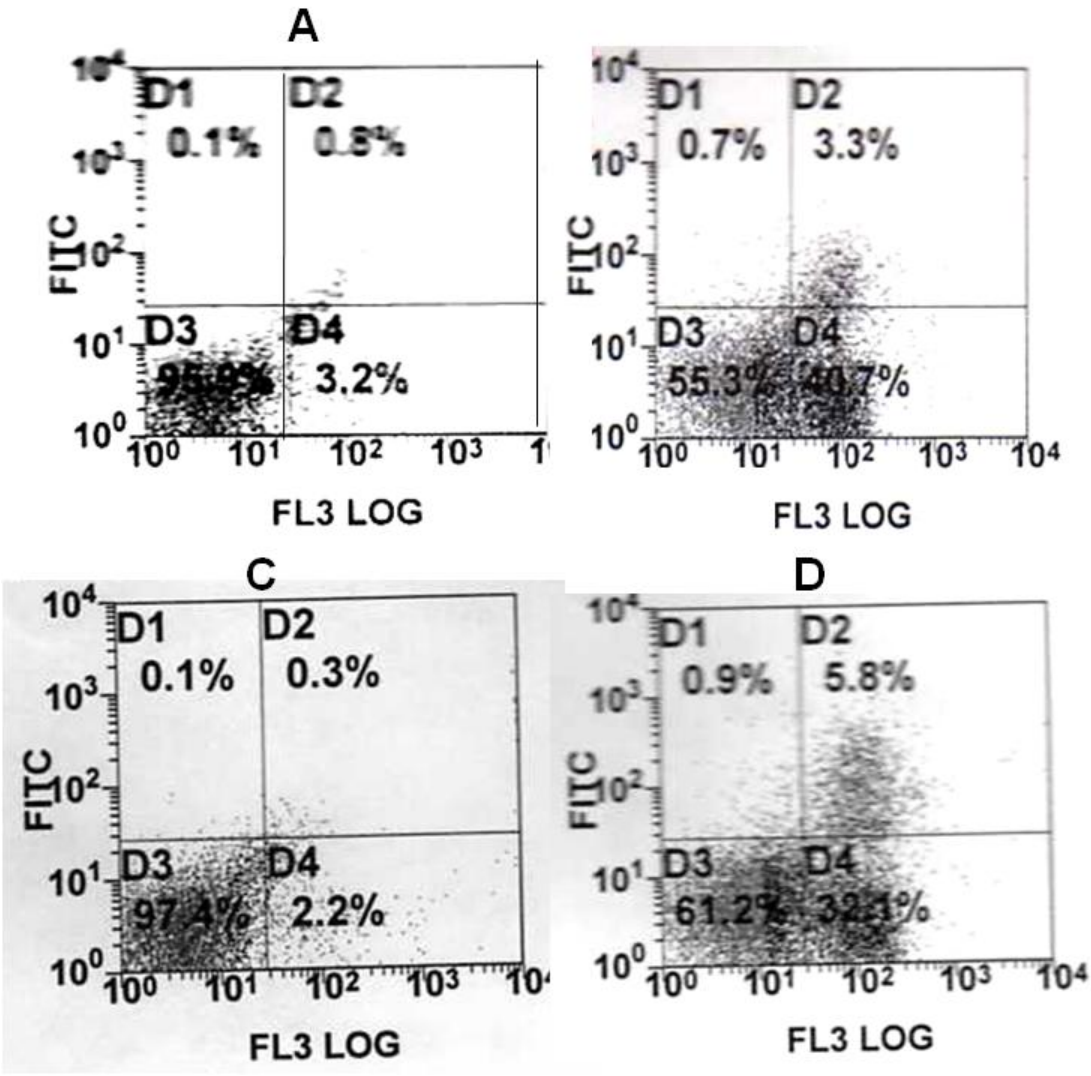

Fig.3.9. FACS analysis to detect $CD3^+CD25^+$ cells in the spleen. Spleen cells collected from normal BALB/c mice and *T. congolense*-infected BALB/c mice at day 7 post infection were stained with ^{PECy5}anti-CD3, ^{FITC}anti-CD25 and isotype control antibodies and analyzed by FACS. A: isotype control of normal BALB/c spleen cells, B: normal BALB/c spleen cells stained with ^{PECy5}anti-CD3 and ^{FITC}anti-CD25, C: isotype control of infected BALB/c spleen cells collected at day 7 post infection, D: spleen cells collected at day 7 post infection stained with ^{PECy5}anti-CD3 and ^{FITC}anti-CD25.

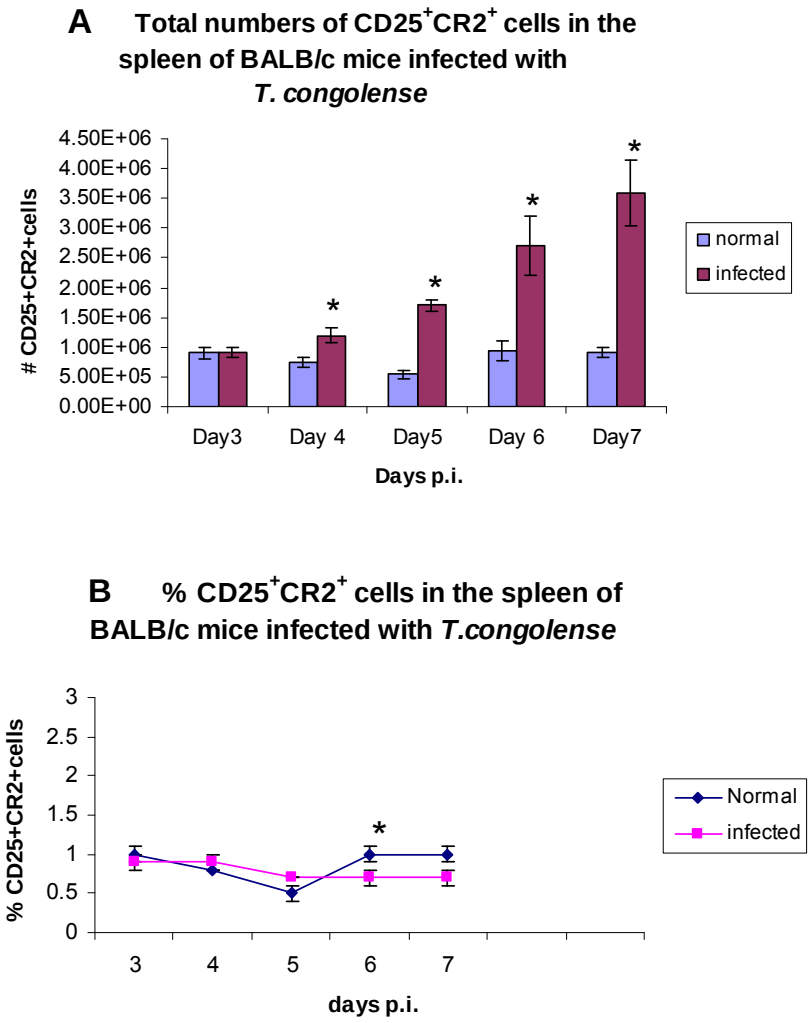

Fig. 3.10. Absolute numbers of $CD25^+CR2^+$ cells (A) and relative numbers of $CD25^+CR2^+$ cells (B) in the spleens of BALB/c mice infected intraperitoneally with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells were stained with ^{FITCanti}-CD25 and ^{PE}anti-CR2 antibodies and analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are representative of four similar experiments). * $P < 0.05$ between infected group and respective control group.

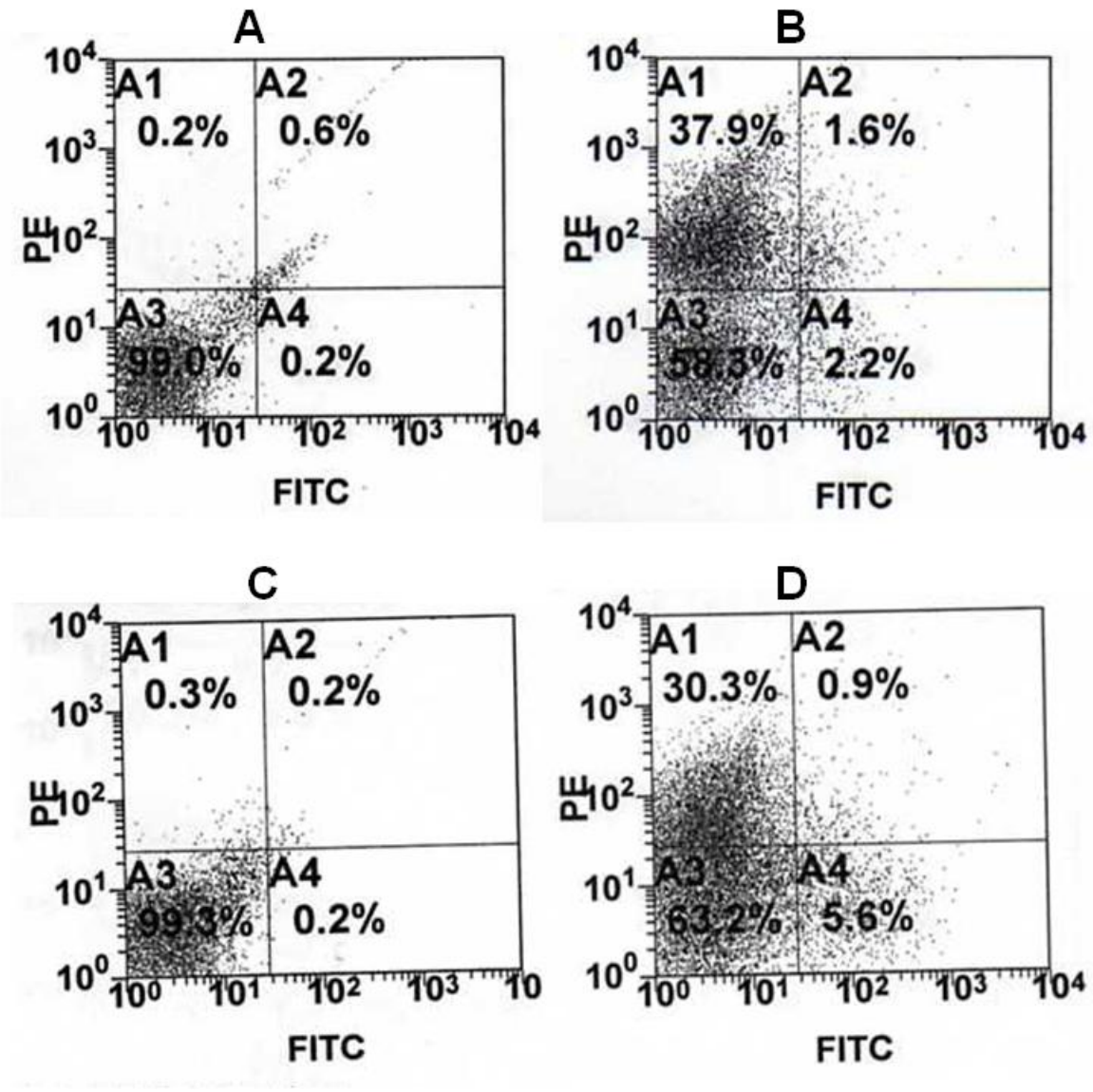

Fig.3.11. FACS analysis to detect $CD25^+$ $CR2^+$cells in the spleen. Spleen cells collected from normal BALB/c mice and *T. congolense*-infected BALB/c mice at day 7 post infection were stained with ^{PE}anti-CR2, ^{FITC}anti-CD25 and isotype control antibodies and analyzed by FACS. A: isotype control of normal BALB/c spleen cells, B: normal BALB/c spleen cells stained with ^{PE}anti-CR2 and ^{FITC}anti-CD25, C: isotype control of infected BALB/c spleen cells collected at day 7 post infection, D: spleen cells collected at day 7 post infection stained with ^{PE}anti-CR2 and ^{FITC}anti-CD25.

3.4.1.6. Mean intensity of CR2 in the spleen cells during *T. congolense* infections

In these kinetic studies, the mean expression of numbers of CR2 per cell decreased with the progressive *T. congolense* infection in BALB/c mice (Fig. 3.12). It is conceivable that continued binding and endocytosis of VSG-C3d complexes might lead to a decrease of $CR2^+$ intensity in B cells with the progressive infection.

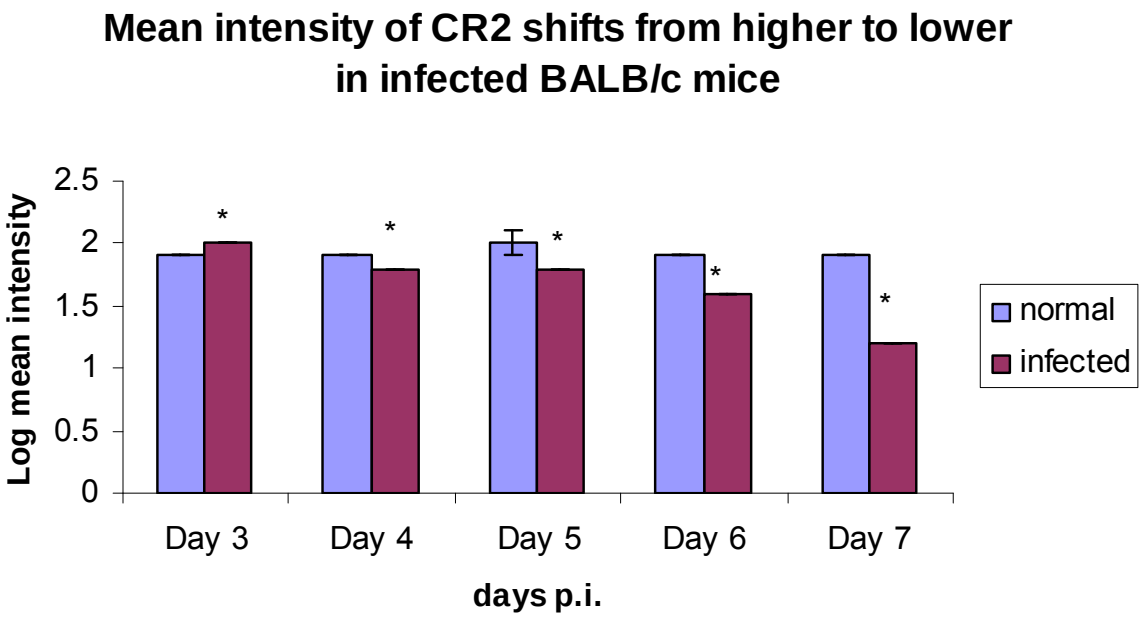

Fig.3.12. Mean intensity ± SE of CR2 on all $CR2^+$ cells in the spleen cells of normal and *T. congolense*-infected BALB/c mice. ^{PE}anti-CR2 antibody was used to stain the spleen cells. The fluorescent intensity of the spleen cells were measured by FACS. Values represent mean±SE, n= 4. (The data shown are representative of three similar experiments). * $P < 0.05$ between infected group and respective control group.

3.4.1.7. Kinetics of $CD19^+$ cells and $CD19^+CR2^+$ cells in the spleen

B cells express CD19 and most of the B cells express CR2 while the expression of CR2 in T cells is limited to a small subpopulation of T cells. Therefore it is worthwhile to find out the kinetics of $CR2^+$ B cells in *T. congolense*-infected BALB/c mice in parallel to the kinetics of $CR2^+$ T cells. In order to find out the kinetics of B cells and CR2-expressing B cells, spleen cells of normal and infected mice were stained for CD19 and CR2.

Absolute numbers of B cells showed a 5 to 6-fold increase by day 7 post infection (Fig.3.13.A) indicating enhanced B cell proliferation or decreased apoptosis. The relative numbers of B cells remained similar to that of normal BALB/c mice throughout the experimental period (Fig.3.13.B), There was a 4-fold increase in the absolute numbers of $CR2^+$ B cells at day 7 post infection compared to the numbers in normal spleen (Fig.3.14.A). Relative numbers of $CR2^+$ B cells did not change significantly throughout the experimental period (Fig.3.14.B).

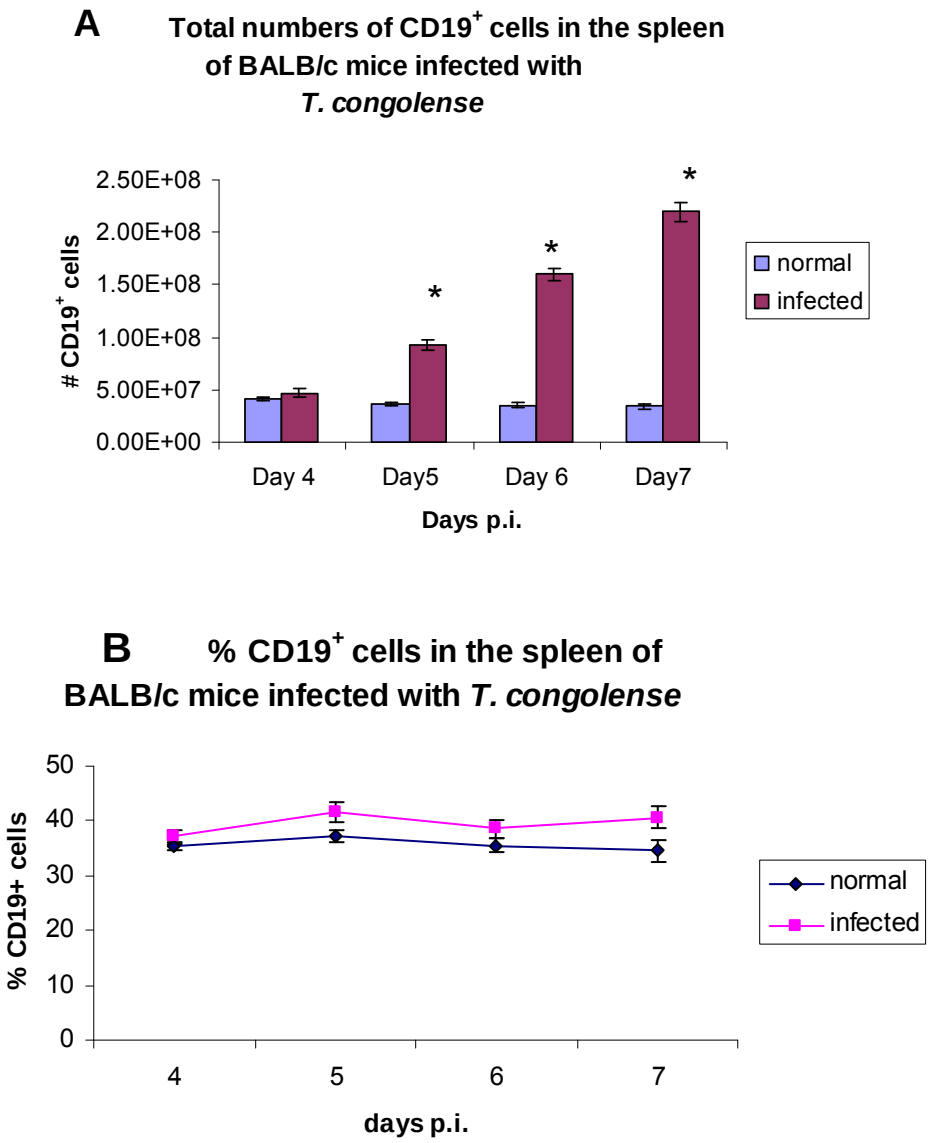

Fig. 3.13. Absolute numbers of $CD19^+$ cells (A) and relative numbers of $CD19^+$ cells (B), in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells stained with ^{FITC}anti-CD19 were analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are representative of two similar experiments). * $P < 0.05$ between infected group and respective control group.

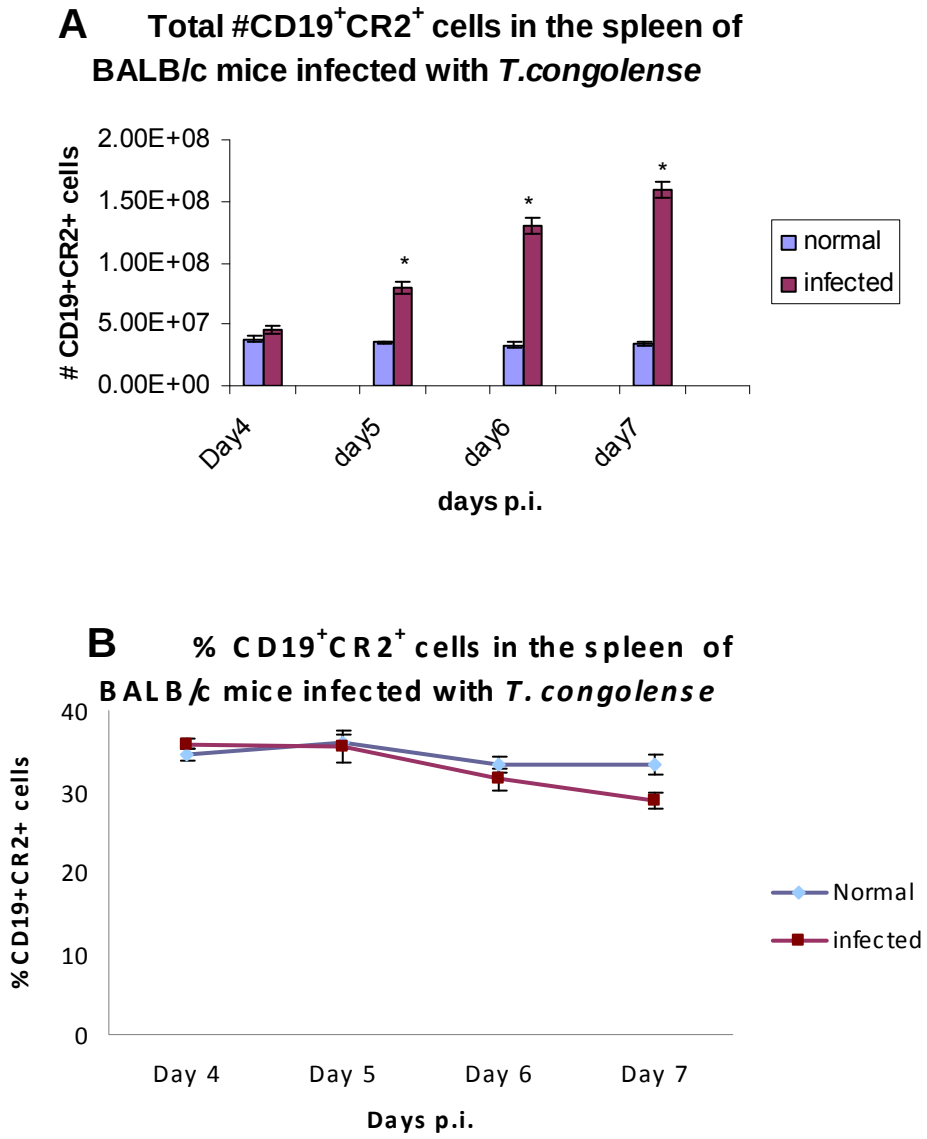

Fig. 3.14. Absolute numbers of $CD19^{+}CR2^{+}$ cells (A) and relative numbers of $CD19^{+}CR2^{+}$ cells (B) in the spleen of BALB/c mice infected with 10^{3} organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells were stained with ^{FITC}anti-CD19 and ^{PE}anti-CR2 antibodies and analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are representative of two similar experiments). * $P < 0.05$ between infected group and respective control group.

3.4.1.8. Preliminary discussion of the first set of experiments: technical problems

In the first set of kinetic studies ^{FITC}anti-CD25, ^{PE}anti-CR2 and ^{PECy5}anti-CD3 were used to stain spleen cells for FACS analysis. FACS analysis of the spleen cells stained with ^{PECy5} anti-CD3 antibody indicated a right shift of the negative cell population stained with a PE antibody specific for an unrelated antigen. The use of ^{PE}anti-CR2 and ^{PECy5}anti-CD3 in the (triple stain) kinetic study was unsatisfactory, because there is a problem with an overlap of PE and PECy5 stains in analysis of FACS (Fig.3.20). These technical problems might affect the results especially when working with very low numbers of cells, i.e. less than 2% of total cells. Thus, spleen cells stained with ^{PECy5}anti-CD3 and ^{PE}anti-CR2 yield higher numbers of $CD3^+CR2^+$ cells, as detected by FACS analysis, than spleen cells stained with ^{FITC}anti-CD3 and ^{PE}anti-CR2. We had not been aware of this problem before. This was the reason for carrying out the second set of kinetic study in normal and infected BALB/c mice using ^{FITC}anti-CD3 and ^{PE}anti-CR2 antibodies and suitable isotype control antibodies.

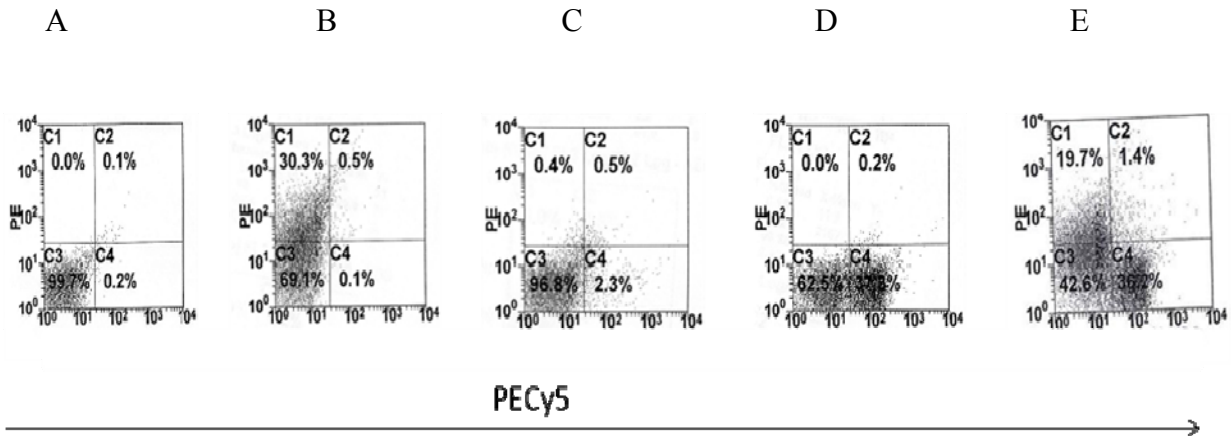

Fig.3.15. Histograms showing PECy5 intensity of unstained spleen cells (A) and the spleen cells stained with ^{PE} anti-CR2 **(**B**),** isotype control antibodies for PECy5 and PE (C), ^{PECy5}anti-CD3 (D), and spleen cells stained with ^{PE}anti-CR2 and ^{PECy5}anti-CD3 (E) as detected by FACS analysis. Spleen cells were obtained from normal BALB/c mice.

3.4.2. Kinetics of $CD3^+CR2^+$ cells in the spleen using a different set of antibodies

In a second set of experiments spleen cells of normal and infected BALB/c mice at days 3, 5, 6, 7 and 8 post infection were stained with ^{FITC}anti-CD3 and ^{PE}anti-CR2 antibodies.

3.4.2.1. Parasitemia and total numbers of spleen cells

Parasitemia and the total numbers of spleen cells showed a similar pattern as observed in previous kinetics experiments described in Fig.3.1. Infected mice did not have any visible parasites up to day 4 following infection. Parasites started to appear in blood at day 5 post infection and this was followed by an increase of parasitemia up to day 8 when the experiment was terminated (Fig.3.16).

Total number of spleen cells in normal mice was about 10^8 in all cases. The number of spleen cells showed a 3-fold increase at day 5 post infection and an up to 5-fold increase has been observed at day 7 and 8 post infection (Fig.3.16). This increase might be due to the proliferation of different cell types of the spleen as a response to the ongoing infection.

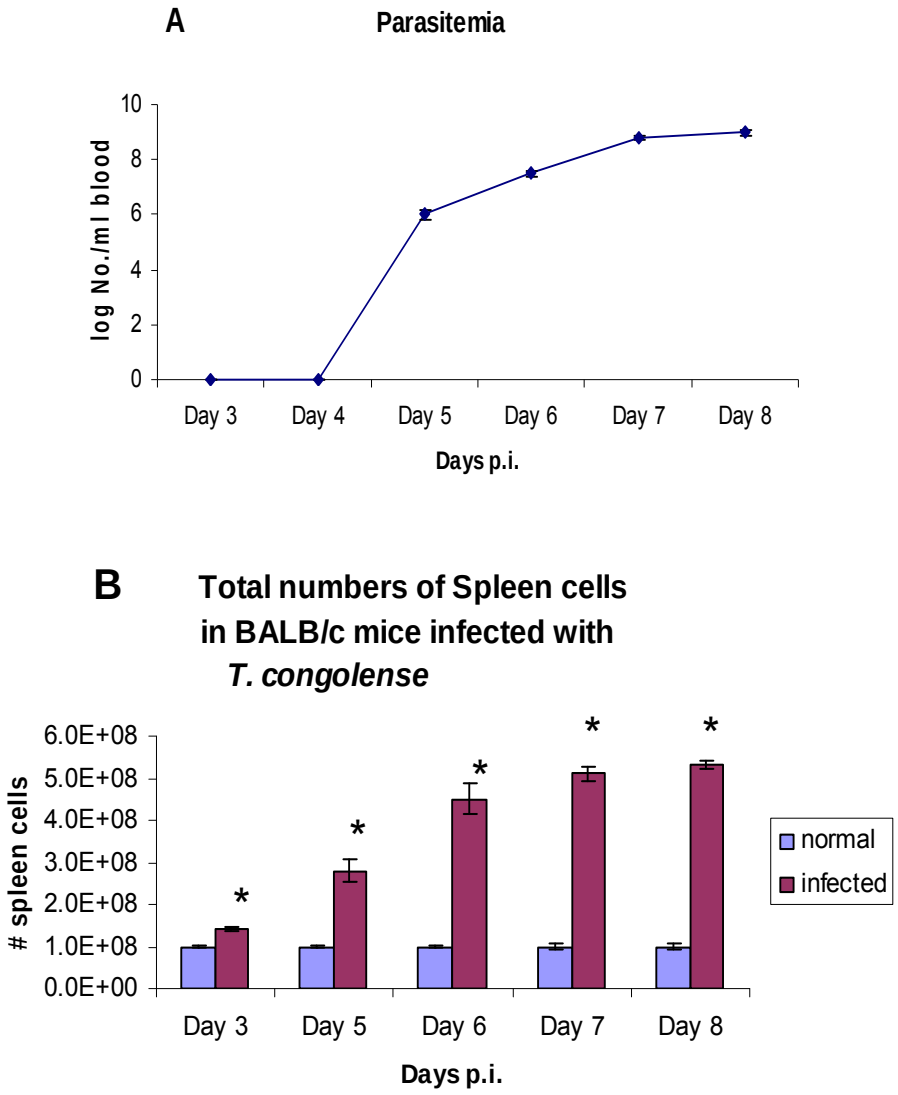

Fig. 3.16. Parasitemia (A) and total number of spleen cells (B) of BALB/c mice infected intraperitoneally with 10^3 *T. congolense* organisms. Values represent mean±SE, n= 4. (The data shown are from a single experiment). * $P < 0.05$ between infected group and respective control group.

3.4.2.2. Kinetics of $CD3^+$ cells and $CD3^+CR2^+$ cells in the spleen

The absolute numbers of $CD3^+$ cells showed a 5-fold increase with progressive infection (Fig. 3.17.A). Relative numbers of $CD3^+$ cells decreased at day 3 and 5 and then came back to normal at day 6, 7 and 8 post infection (Fig.3.17.B). The absolute numbers of $CD3^+CR2^+$ cells increased with the infection up to 7-fold (Fig.3.18.A). There was an increase of relative numbers of $CD3^+CR2^+$ cells at day 7 and 8 post infection, resulting in a 2-fold increase at day 8 (Fig. 3.18.B). The original FACS pictures of representative normal and infected spleen cells of BALB/c mice, collected at day 8 post infection, are shown in Fig. 3.19.

3.4.2.3. Kinetics of $CR2^+$ cells in the spleen

The numbers of all $CR2^+$ cells showed an increase up to day 6 and then showed a slight decrease at day 7 and 8 (Fig.3.20.A) while the relative numbers of $CR2^+$ cells decreased with the progression of infection (Fig.3.20.B). There was a significantly reduced mean expression of numbers of CR2 per cell in all $CR2^+$ cell population with progression of the infection (Fig.3.20. C, D & E).

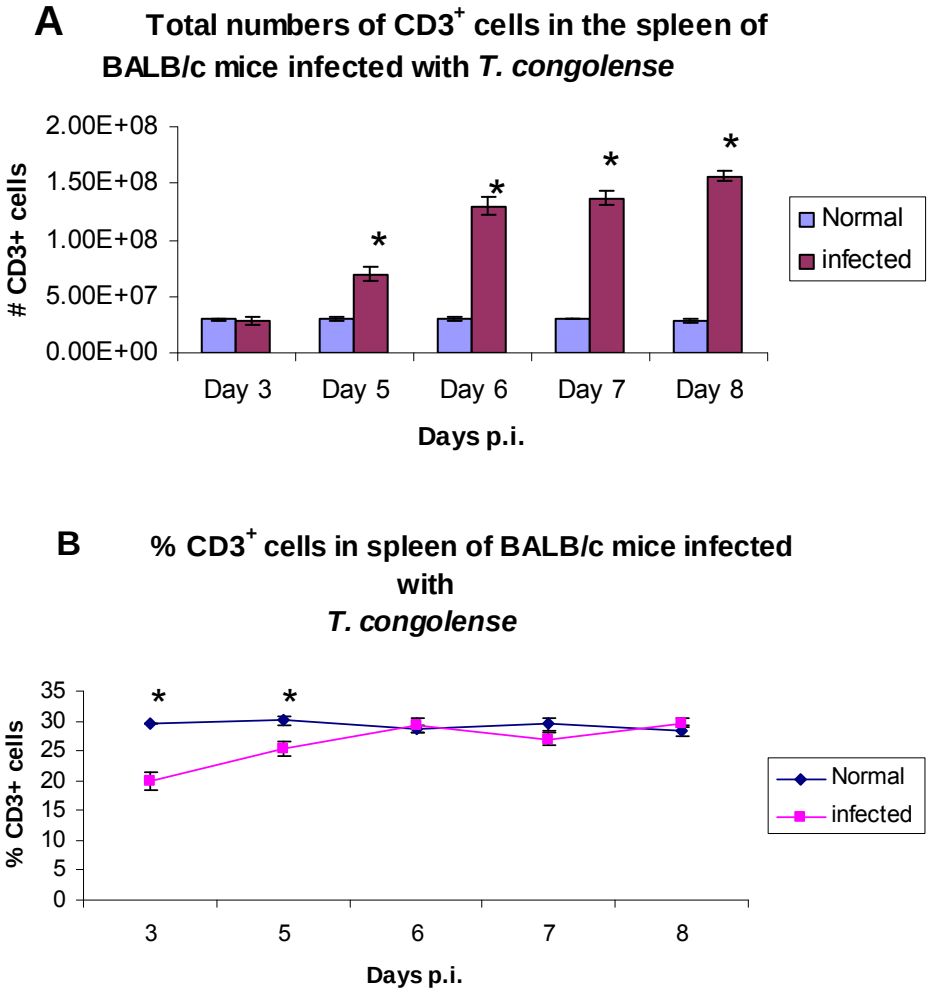

Fig. 3.17. Absolute numbers of $CD3^+$ cells (A) and relative numbers of $CD3^+$ cells (B) in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells were stained with ^{FITC}anti-CD3 and analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are from a single experiment). * $P < 0.05$ between infected group and respective control group.

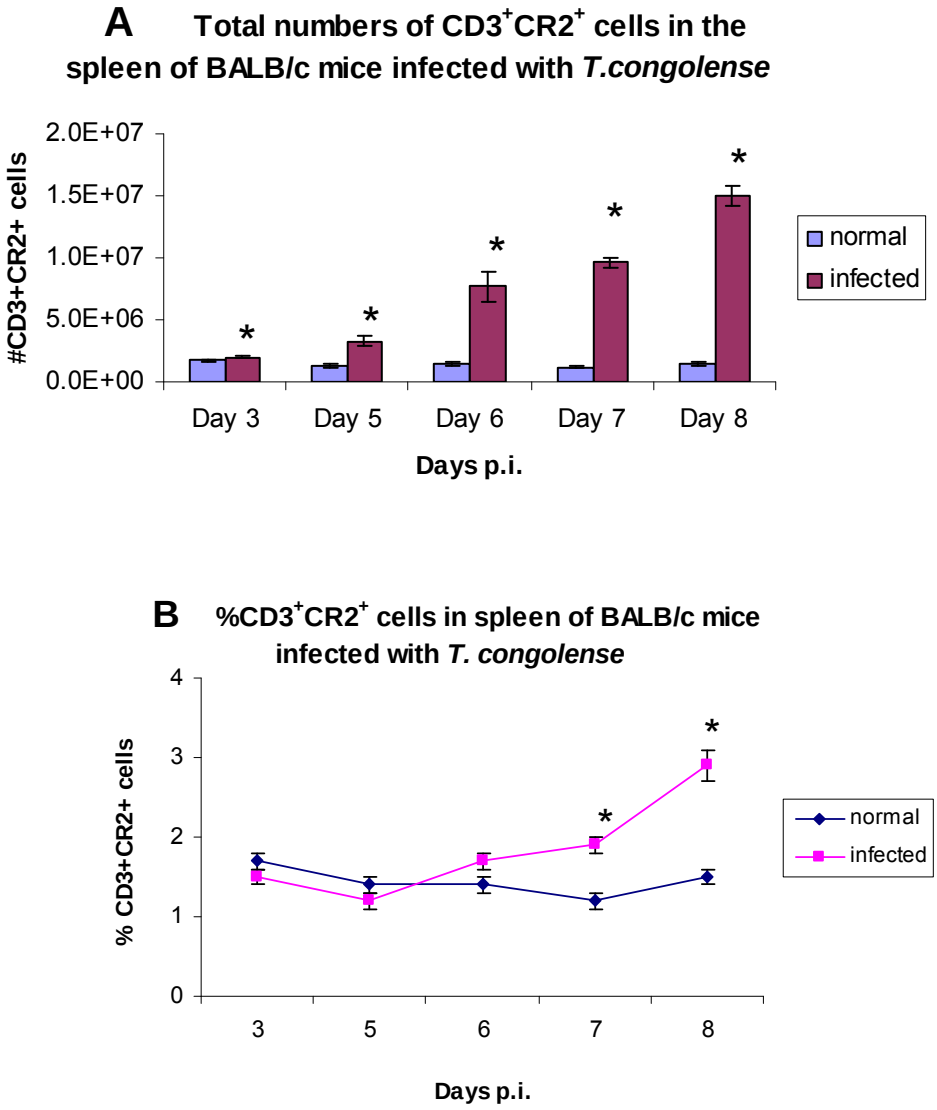

Fig. 3.18. Absolute numbers of $CD3^+CR2^+$ cells (A) and relative numbers of $CD3^+CR2^+$ cells (B) in the spleen of BALB/c mice infected with 10^3 organisms of *T. congolense* TC13 compared to those of normal BALB/c mice. The spleen cells were stained with ^{FITC}anti-CD3 and ^{PE}anti-CR2 antibodies and analyzed by FACS. Values represent mean±SE, n= 4. (The data shown are from a single experiment). * $P < 0.05$ between infected group and respective control group.

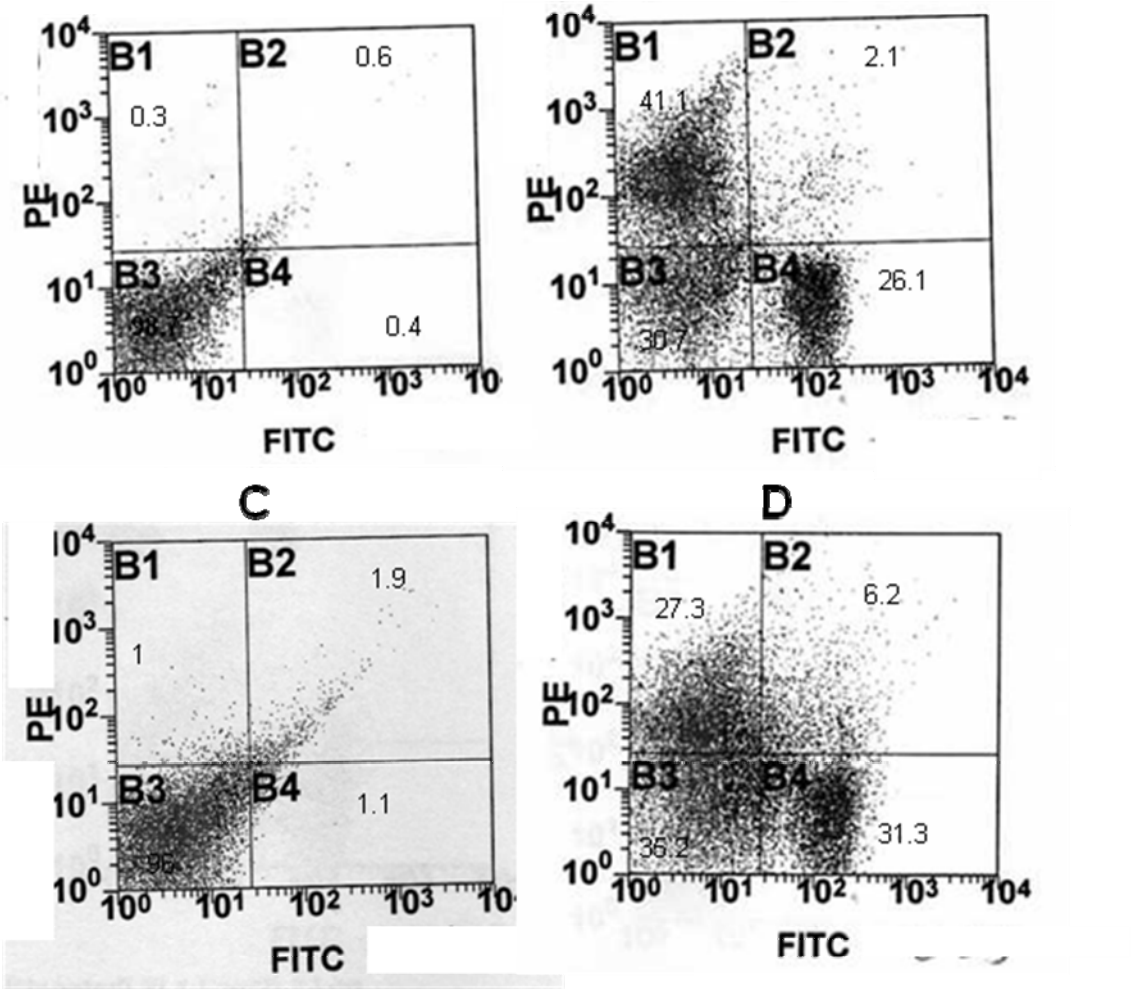

Fig.3.19. FACS analysis to detect $CD3^+CR2^+$ cells in the spleen. Spleen cells collected from normal BALB/c mice and *T. congolense*-infected BALB/c mice at day 8 post infection were stained with ^{PE}anti-CR2, ^{FITC}anti-CD3 and isotype control antibodies and analyzed by FACS. A: isotype control of normal BALB/c spleen cells, B: normal BALB/c spleen cells stained with ^{PE}anti-CR2 and ^{FITC}anti-CD3, C: isotype control of infected BALB/c spleen cells collected at day 8 post infection, D: spleen cells collected at day 8 post infection stained with ^{PE}anti-CR2 and ^{FITC}anti-CD3.

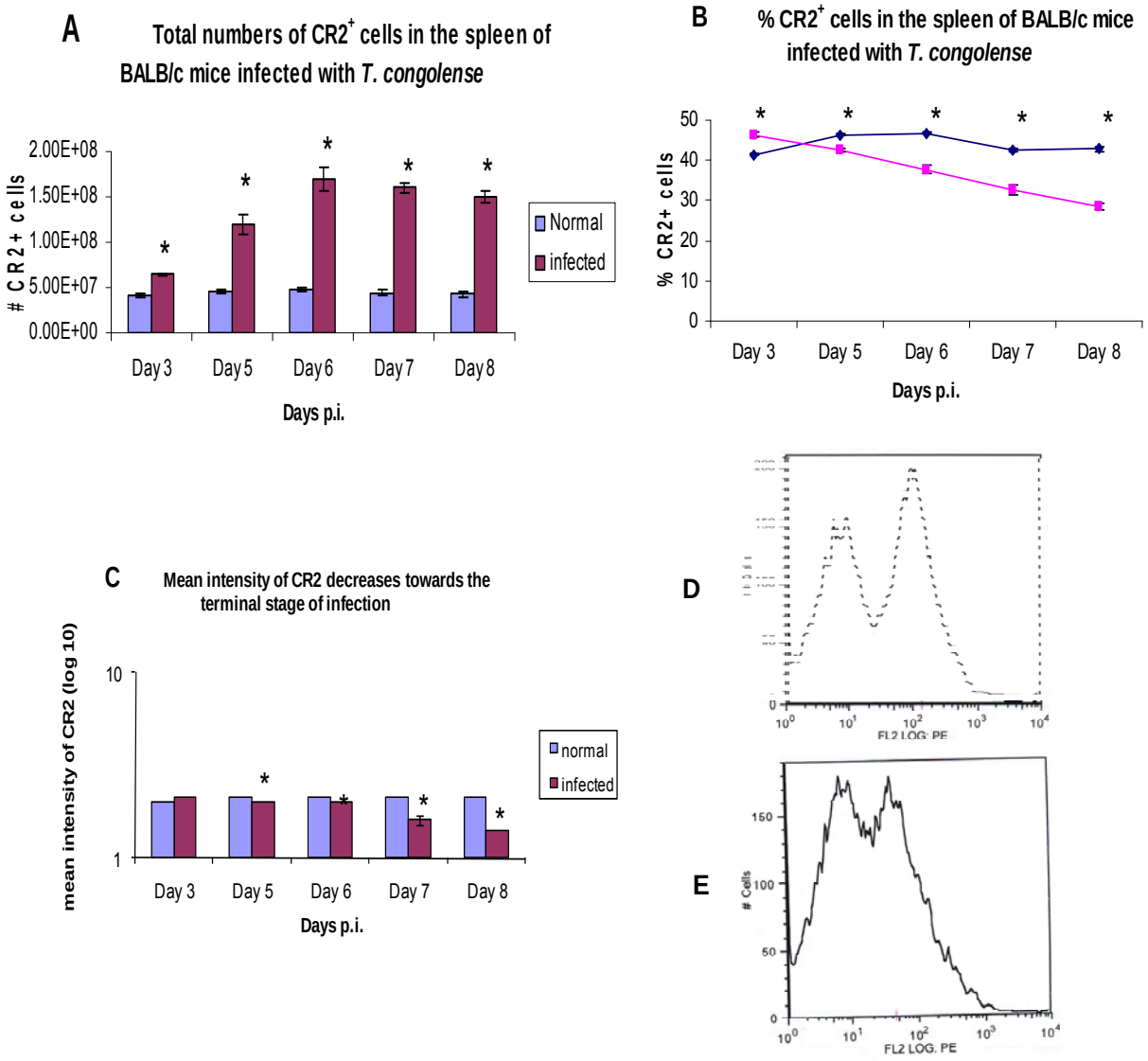

Fig.3.20. Absolute numbers of $CR2^+$ cells (A) and relative numbers of total $CR2^+$ cells (B). Mean intensity ± SE of CR2 in the splenic $CR2^+$ cell population of normal and *T. congolense*-infected BALB/c mice (C) and histograms of PE intensity of $CR2^+$ cell population of normal (D) and *T. congolense*-infected BALB/c mice at day 8 post infection (E). Values represent mean±SE, n= 4. (The data shown are from a single experiment). * $P < 0.05$ between infected group and respective control group.

3.5. Discussion

The patterns of parasitemia were similar in both kinetic studies, whether triple staining or double staining were used (Fig.3.1 & 3.16), indicating that the levels of infections were similar throughout the experiments. Similar increases in numbers of total spleen cells (Fig.3.1 & 3.16) further suggest that the status of infections was fairly consistent throughout these kinetic studies. The observed increase in total numbers of spleen cells could mean increased proliferation and/or decreased apoptosis and/or increased influx but decreased outflow of the cells.

Even though CR2 is predominantly expressed on B cells, follicular dendritic cells and activated granulocytes (Carrol, 1998), there are only few reports about the presence of CR2 on murine T cells. The results of these experiments showed that there are $CR2^+$ T cells present in normal BALB/c and C57BL/6 mice (Fig.3.5) and the numbers of these cells increased in *T. congolense* infections (Fig.3.6 & 3.18). But the available data are insufficient to make comments on the possible role of CR2 in T cells in *T. congolense* infections of mice.

Absolute numbers of $CD25^+$ T cells showed a 2 to 3-fold increase at day 5, followed by a 10-fold increase at day 7 post infection (Fig.3.8). These $CD25^+$ T cells may contain activated T cells and/or T regulatory cells. To confirm this it would be necessary to include an additional stain to detect FoxP3, a marker of Tregs which is not expressed on activated T helper cells.

The majority of $CR2^+$ cells would be B cells while only a few of them are T cells (Kaya et al., 2005). $CD25^+$ $CR2^+$ cells could be either $CD25^+$ $CR2^+$ T cells or $CD25^+$ $CR2^+$ B cells and these two cell types cannot be distinguished without additional markers. Therefore it is not possible to come to any conclusions about these $CD25^+$ $CR2^+$ cells except that they increase in numbers in *T. congolense* infections of mice.

There was an increase in absolute numbers of $CR2^+$ cells up to day 5 followed by a slight decrease as observed in these kinetic studies (Fig.3.3 & 3.20). According to the results of these experiments, the numbers of both $CR2^+$ T cells and B cells increased with the progression of infection. Spleen remodeling during *T. brucei*- infection in mice, results in the rapid loss of IgM^+ marginal zone B cell population characterized as $B220^+IgM^{high}IgD^{int}$ $CR2^{high}CD23^{low}CD1d^+CD138^-$ (Radwanska et al., 2008). This trypanosomiasis-induced B cell apoptosis could be common to *T. congolense*-infections. Continued binding and endocytosis of VSG-C3d complexes might lead to a decrease of $CR2^+$ intensity in B cells with progressive infection (Fig. 3.12 & 3.20).

IFN-γ producing T cells play an important role in enhancing the immunopathology in African trypanosomiasis. Whereas IL-10-producing T cells dampen the excessive activation of macrophages (Tabel et al., 2008).

There is now good evidence that the numbers of $CR2^+$ T cells significantly increase with progression of infection by *T. congolense*. Whether the presence of $CR2^+$ T cells can be causatively linked to the capacity to produce IFN-γ remains to be established.

4.0. GENERAL DISCUSSION

The primary goals of the present thesis work were to find out the kinetics of CR2 expressing T cells and the role of these $CR2^+$ T cells in experimental murine *T. congolense* infections. This study has shown that total numbers of $CR2^+$ T cells increase in the spleen of *T. congolense*-infected BALB/c mice with progressive infection (Fig.3.6 & 3.14).

Splenomegaly observed in murine trypanosomiasis is a result of proliferation of T and/or B cells and also could be due to reduced apoptosis. Proliferation of "null cells" in the spleen could also contribute to the observed splenomegaly (Roelants, 1982). Lymphocytic hyperplasia in *T. musculi* infections was observed only in immunologically intact mice but not in nude mice indicating that splenomegaly in murine trypanosomiasis is T cell dependent (Robinett and Rank, 1979). The present study showed that there is an increase of the total number of spleen cells with progressive *T. congolense* infections in BALB/c mice (Fig. 3.1 and Fig. 3.12). This observed increase of spleen cell number might be due to the proliferation of lymphocytes or due to the reduction of apoptosis of cells. It might also be due to increased cell influx to the spleen or reduced outflow from the spleen.

CR2 in mice is predominantly expressed on B cells, follicular dendritic cells and activated granulocytes(Carrol, 1998). Expression of CR2 on mouse T cells has also been documented. CR2 has been detected on subsets of activated T cells (Kaya et al., 2001; Chen et al., 2005). The presence of CR2 in $CD25^+$ cells and the increase of $CD25^+CR2^+$ cells with the progressive infection (Fig.3.8) suggest that there is an increased expression of CR2 in activated lymphocytes in the spleen of *T. congolense*-infected BALB/c mice. CR2 was also detectable on both $CD4^+$ and $CD8^+$ subsets of T cells (Kaya et al., 2005). Mesenteric lymph nodes of wildtype C57BL/6 mice contain about 4.5% $CD4^+CR2^+$ cells (Molnar et al., 2008).

Expression of CR2 on splenic T cells of normal or *T. congolense*-infected BALB/c mice has been unknown.

The present study showed that uninfected susceptible BALB/c mice and relatively resistant C57BL/6 mice express CR2 on subsets of T cells in the spleen (Fig.3.5). The kinetics of complement activation is different in highly susceptible BALB/c and relatively resistant C57BL/6 mice. The degradation of C3b to C3d during the amplification of the alternative pathway of complement proceeds faster in BALB/c than in C57BL/6 mice (Ogunremi et al., 1993). This would result in more VSG-C3d immune complexes to be present in BALB/c mice than in C57BL/6 mice. The present study showed that there is no difference in the numbers of $CR2^+$ T cells between normal BALB/c and normal C57BL/6 mice (Fig.3.5). Whether the numbers of $CR2^+$ T cells differ in *T. congolense*-infected BALB/c and C57BL/6 mice has not been tested in the present study.

An increase in the numbers of $CR2^+$ T cells in the spleen of *T. congolense*-infected BALB/c mice has been observed in the present kinetic studies. The total numbers of $CR2^+$ cells increase up to day 6 post infection followed by a decline at day 7 and 8 post infection (Fig.3.3 and Fig.3.20). Most of these $CR2^+$ cells might be B cells. The numbers of both $CD19^+$ B cells and $CR2^+$ B cells increase with the progressive infection indicating proliferation of B cells.

Interestingly, the expression of CR2 molecules per cell showed a reduction with progressive *T. congolense* infection in BALB/c mice (Fig. 3.9 and Fig.3.20). There is evidence that spleen remodeling during *T. brucei*-infections in mice results in the rapid loss of IgM^+ marginal zone B cell population characterized as $B220^+IgM^{high}IgD^{int}$ $CR2^{high}CD23^{low}CD1d^+CD138^-$ (Radwanska et al., 2008). We suggest that enhanced B cell apoptosis, observed in *T. brucei* infections, might also occur in *T. congolense* infections.

Continued binding and endocytosis of VSG-C3d complexes might lead to a decrease of CR2 intensity in B cells with progressive infection.

In conclusion, the results of the present study showed that the total numbers of $CR2^+$ T cells in the spleen increase with progressive *T. congolense* infections in susceptible BALB/c mice. Analysis of spleen cells from *T. congolense*-infected BALB/c mice for CR2 and intra cellular IFN-γ would indicate whether the IFN-γ producing cells express CR2. CR2 in T cells might play a role in the pathogenesis of *T. congolense* infections. Further studies are necessary to find out the potential role of $CR2^+$ T cells in *T. congolense* infections in susceptible BALB/c mice.

5.0. CONCLUSION

The major objectives of the present work were to find out the kinetics of CR2 expressing T cells and the role of these $CR2^+$ T cells in experimental murine *T. congolense* infections.

The present study showed that both uninfected BALB/c mice and uninfected C57BL/6 mice express CR2 on subsets of the splenic T cells. The total numbers of $CR2^+$ T cells in the spleen of *T. congolense*-infected BALB/c mice increased with progressive infection. Total numbers $CR2^+$ B cells also increased in the spleen of *T. congolense*-infected BALB/c mice with progressive infection.

The mean numbers of CR2 molecules per cell in $CR2^+$ cell population of the spleen significantly declined with progressive *T. congolense* infection in BALB/c mice.

Overall, the results of the present study showed that total numbers of $CR2^+$ T cells in the spleen increase with progressive *T. congolense* infections in susceptible BALB/c mice. But whether they require cross-linking of CR2 by complement C3d as a costimulation for the production of maximal amounts of IFN-γ is not addressed in the present study. Further studies are necessary to find out the potential role of $CR2^+$ T cells in *T. congolense* infections in susceptible BALB/c mice.

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7.0. APPENDIX: SUBPOPULATIONS OF T CELLS PRODUCING IFN-γ AND/OR IL-10 IN EXPERIMENTAL *TRYPANOSOMA CONGOLENSE* INFECTIONS

7.1. Abstract

The experiments described in this chapter were carried out to find out the subpopulations of T cells producing IFN-γ and/or IL-10 in *T. congolense*-infected BALB/c mice. $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ cells were sorted by FACS from the spleen cells collected from normal and *T. congolense*-infected BALB/c mice at day 6 post infection. These sorted cells were cocultured with trypanosome-loaded peritoneal macrophages obtained from normal syngeneic BALB/c mice to find out the production levels of IFN-γ in these cocultures. The majority of IFN-γ was produced by $CD4^+CD25^{high}$ cells cocultured with trypanosome-loaded peritoneal macrophages. In titration studies, detectable levels of IFN-γ and IL-10 were produced by adding $5x10^3$ to $5x10^4$ sorted T cells to the peritoneal macrophage cultures. The maximal production was observed with $5x10^4$ sorted T cells when cocultured with macrophages. Equal levels of IFN-γ were observed when $5x10^4$ $CD4^+CD25^{low}$ or $5x10^4$ $CD4^+CD25^{high}$ T cells cocultured with macrophages. Trypanosome-loaded macrophages when cultured without sorted T cells did not show IL-10 production. There were no detectable amounts of IL-6 production when the macrophages were cultured alone. IL-6 production did not show much difference between the different cocultures but showed a slight dose-dependent increase with the increased numbers of T cells.

10^5 APC when cocultured with $5x10^4$ $CD4^+CD25^+$ cells produced the highest amount of IFN-γ and IL-10. The production of IFN-γ and IL-10 was also well within the measurable range when 10^5 APC were cocultured with $1.7x10^4$ $CD4^+CD25^+$ cells in 96-well flat bottom tissue culture plates. None of the measured cytokines were produced in detectable levels when

the MACS-sorted $CD4^+CD25^+$ cells were cultured alone. The coculture experiments indicated that the majority of IL-6 was produced by APC. IL-6 production did not show a strong correlation with the numbers of T cells in the cocultures suggesting that IL-6 production is only weakly T cell-dependent. The amounts of IFN-γ produced in the cocultures of 10^5 APC with either $5x10^4$ $CD4^+CD25^+$ cells or $5x10^4$ whole spleen cells were similar. This suggests that there might be cofactors present in the whole spleen cell population which might not be present or present only at lower levels in T cell enriched populations. We speculate these cofactors might influence the production of IFN-γ.

7.2. Introduction

I tried to further characterize the small T cell population that is involved in the production of IFN-γ and IL-10. $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ T cells isolated by FACS from the spleen cells of normal and *T. congolense*-infected BALB/c mice were co-cultured with trypanosome-loaded peritoneal macrophages. In preliminary experiments, the majority of IFN-γ was shown to be produced by $CD4^+CD25^{high}$ T cells. But I was unable to establish an optimal T cell number to be added onto the cocultures due to practical difficulties encountered during FACS sorting. We speculate $CD4^+CD25^+$ T cells to be the major IFN-γ producers. Attempts to sort the $CD4^+CD25^+$ T cells, using mouse Treg isolation kits (Milteny Biotec), were not successful since this protocol failed to enrich the major IFN-γ producing cells.

7.3. Materials and Methods

7.3.1. Mice

Female BALB/c mice and CD1 mice were obtained from the Animal Resource Center of the University of Saskatchewan. BALB/c mice were 8 to 10 weeks old and CD1 mice were 6 weeks old. All mice were maintained according to the recommendations of the Canadian Council of Animal Care.

7.3.2. Parasites

T. congolense, variant antigenic type (VAT) TC13 (Tabel, 1982) was used in this experiment. TC13 was passaged in immunosuppressed CD1 mice as described (Tabel, 1982). The parasites for the infection of BALB/c mice were obtained by isolating the parasites from the blood of infected CD1 mice using DEAE-cellulose chromatography column (Lanham and Godfrey, 1970).

7.3.3. Trypanosome homogenate

Trypanosomes isolated from the blood of infected CD1 mice using DEAE-cellulose chromatography column were washed and resuspended in RPMI-1640 complete medium supplemented with 10% FBS at 10^7 trypanosomes/ 100 µl. The trypanosome suspension was sonicated 10 times at 5 seconds each time with 10 seconds intervals while keeping the suspension on ice. The homogenate was divided into aliquots of 100 µl portions and stored at -80^0C until use.

7.3.4. Antibodies

The following principle antibodies were used for FACS analysis: ^{FITC} rat anti-mouse CD4 (Cedarlane Laboratories), ^{PE} rat anti-mouse CD25 (eBioscience), ^{PECy5} rat anti-mouse CD25 (eBioscience) and ^{PECy5} Armenian hamster anti-mouse CD3 (BD Pharmingen). The following

antibodies were used as isotype control antibodies: ^{FITC} rat IgG2a (eBioscience), ^{PE}rat IgG2a (eBioscience), ^{PECy5}rat IgG1 (eBioscience) and ^{PECy5} Armenian hamster IgG (eBioscience).

7.3.5. Preparation of Antigen Presenting Cells (APC)

Peritoneal cells were collected from 8-10 weeks old normal female BALB/c mice, using filter-sterilized 0.34 M sucrose solution. The cells spun at 1000 rpm for 10 minutes were resuspended in FACS staining buffer (PBS pH 7.2, 0.5% BSA and 2 mM EDTA) and stained with MACS anti-Thy 1.2 micro beads. Thy1.2 negative cells were purified by MACS separation using a MS column according to the manufacturer's protocol. Cells in the negative fraction were counted and resuspended in RPMI-1640 complete medium supplemented with 10% FBS.

7.3.6. Sorting of $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ cells from the spleen cells by FACS

First, $CD4^+$ cells were isolated by positive selection from spleen cells collected from normal and *T. congolense*-infected BALB/c mice at day 6 post infection, using MACS anti-CD4 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. The purity of the enriched $CD4^+$ cell population was 87% as detected by FACS. The enriched $CD4^+$ cells were then stained with ^{FITC}anti-CD4 and ^{PECy5}anti-CD25. The stained cells were sorted into $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ cells by FACS. Sorted cells were counted, resuspended in appropriate volumes of RPMI-1640 complete medium supplemented with 10% FBS cocultured with $5x10^5$ APC per well in the presence of a trypanosome homogenate (equivalent to 10^6 organisms) in 96 well flat bottom tissue culture plates.

7.3.7. Isolation of $CD4^+CD25^+$ Tregs from the spleen cells by MACS

Spleen cells collected from four *T. congolense*-infected BALB/c mice at day 7 post infection were subjected to lysis of red blood cells and trypanosomes by mixing the cell suspension in

sterile distilled water for 20 seconds followed by addition of 10X PBS. These cells were used for the isolation of $CD4^+CD25^+$ Tregs using a Mouse Treg Isolation Kit (Miltenyi Biotec) according the manufacturer's protocol. The cells were resuspended in RPMI-1640 complete medium supplemented with 10% FBS and seeded in 96 well flat bottom tissue culture plates at either 5 X 10^4 cells/well, 1.7 X 10^4 cells/well, 5 X 10^3 cells/well or 1.7 X 10^3 cells/well. Aliquots of cells from spleen cells, enriched for $CD4^+$ cells or $CD4^+CD25^+$ cells were stained with ^{FITC}anti-CD4 and ^{PE}anti-CD25 to analyze by FACS. The enriched $CD4^+CD25^+$ group had 70% purity as detected by FACS (Fig.7.7).

7.3.8. Block titration of APC and $CD4^+CD25^+$ T cells

APC and $CD4^+CD25^+$ T cells were cocultured in order to do a block titration to find out the optimal numbers of each cell type to be used in future coculture studies. The APC were seeded in 96 well flat bottom tissue culture plates at either 10^4 cells/well, 3.3 X 10^4 cells/well or 10^5 cells/well. Aliquots of cells before and after MACS separation were stained with ^{PECy5}anti-CD3 for FACS analysis. A homogenate equivalent to 10^6 trypanosomes was added to each well and the cells were incubated for 48 hours at 37^0C and 5%CO_2. APC alone, $CD4^+CD25^+$ T cells alone and 10^5 APC with 5 X 10^4 whole spleen cells were included as control groups. The culture supernatants collected after 48 hours were used to measure the production of IFN-γ, IL-10 and IL-6 by routine sandwich ELISA according to the manufacturer's protocols.

7.3.9. Cytokine assays

The levels of IL-6, IL-10 and IFN-γ in the culture supernatants were determined by routine sandwich ELISA, according to the manufacturer's protocols. The ELISA kits were purchased from BD Pharmingen. The limits of detection of the ELISA assays were 62 pg/ml for IFN-γ and 15 pg/ml for IL-6 and IL-10.

7.3.10. FACS analysis

One hundred microliter aliquots of 10^7 cells/ml in FACS buffer were incubated for 20 minutes on ice with 4 µl of rat anti-mouse CD16/32 (Fcγ II/III receptor, Fc Block, eBioscience) monoclonal antibody. Appropriate volumes of each antibody were added and incubated on ice in the dark for 30 minutes. The cells were washed 2 times with FACS buffer, and then resuspended in FACS buffer containing 1% formalin. Fixed cells were analyzed by a FACScan flow cytometer using Flowjo software (Tree Star Inc.).

7.4. Results

7.4.1. $CD4^+CD25^{high}$ T cells cocultivated with *T. congolense*-loaded macrophages produce IFN-γ

In preliminary experiments, $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ T cells were sorted by a combination of MACS and FACS from the spleen cells of normal and *T. congolense*-infected BALB/c mice and were co-cultured with trypanosome-loaded peritoneal macrophages obtained from normal syngeneic BALB/c mice. The majority of IFN-γ was shown to be produced by $CD4^+CD25^{high}$ T cells (Fig.7.1). None of the other cocultures produced high amounts of IFN-γ. There were no distinguishable $CD25^{low}$ and $CD25^{high}$ cell populations in normal mouse spleen cells when analyzed by FACS. Therefore, from normal mouse spleen cells, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were sorted and cocultured with trypanosome-loaded syngeneic peritoneal macrophages. Culture supernatants were used in 2-fold dilutions in ELISA in order to make sure that the cytokine levels to fit within a measurable range. Gates used for FACS cell sorting is shown in Fig. 7.2.

$CD4^+CD25^{high}$ T cells cocultivated with *T.congolense*- loaded macrophages produce IFN-γ

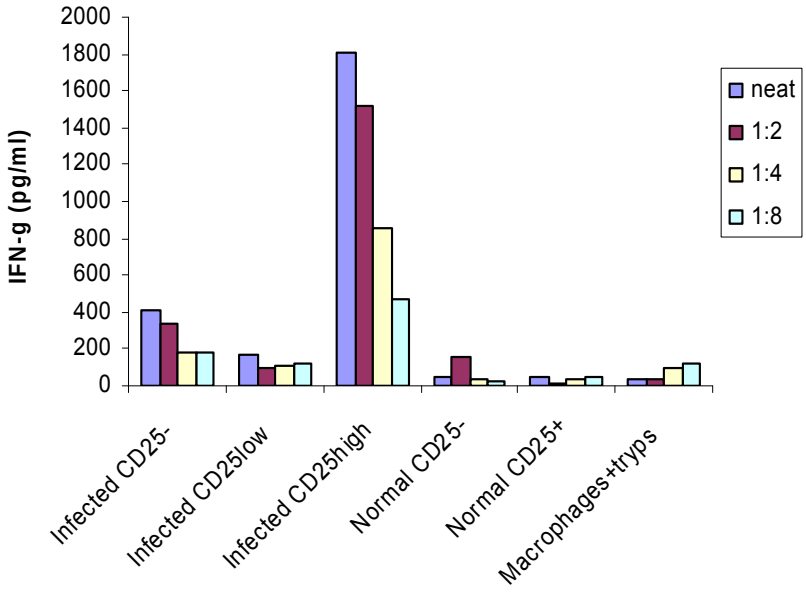

Fig 7.1. Peritoneal macrophages ($5x10^5$/well) loaded with *T. congolense* were cocultured with $2x10^4$ $CD4^+CD25^-$, $CD4^+CD25^{low}$ or $CD4^+CD25^{high}$ T cells. The cells were isolated from spleen cells of normal BALB/ mice and from infected BALB/C mice 6 days post infection. The T cells were sorted by a combination of MACS and FACS from the spleen cells. Culture supernatants collected after 48 hrs were used in undiluted form (neat) and in 3 different dilutions (1:2, 1:4, and 1:8) in ELISA to measure the production of IFN-γ.

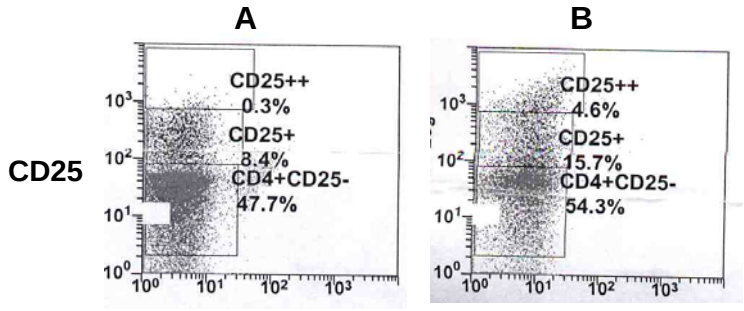

Fig.7.2. FACS sorting of CD25$^-$, CD25^{low} and CD25^{high} cells. CD4$^+$ cells were positively selected from the spleen cells of normal (A) and *T. congolense*-infected BALB/c mice (B), by MACS. CD4$^+$ cells were stained with ^{PECy5} rat anti-mouse CD25 to sort by FACS. The purity of the enriched CD4$^+$ cell population was 87% as detected by FACS.

7.4.2. What are the optimal numbers of T cells to be used in cocultures?

The next aim was to find out the optimal number of sorted cells to be cocultured with $5x10^5$ peritoneal macrophages in order to obtain detectable levels of the cytokine production. Therefore $5x10^4$, $1.7x10^4$, $5x10^3$ and $1.7x10^3$ of FACS-sorted CD4$^+$CD25$^-$, CD4$^+$CD25^{low} and CD4$^+$CD25^{high} T cells were co-cultured with $5x10^5$ trypanosome-loaded, peritoneal macrophages. The culture supernatant fluids were tested for IL-6, IL-10 and IFN-γ by ELISA. Only the CD4$^+$CD25$^-$, CD4$^+$CD25^{low} and CD4$^+$CD25^{high} T cells sorted from the spleen cells of *T. congolense*-infected BALB/c mice were co-cultured with trypanosome-loaded peritoneal macrophages obtained from normal syngeneic BALB/c mice in this experiment. Since the CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells sorted from normal BALB/c mice had previously shown negligible amounts of IFN-γ production in the cocultures, I did not include these cells in this experiment.

Detectable levels of IFN-γ and IL-10 were produced by adding $5x10^3$ to $5x10^4$ sorted T cells to the macrophage cultures. The maximal production was observed with $5x10^4$ sorted T cells when cocultured with macrophages (Fig. 7.3.A and B). It seemed that the production of almost equal levels of IFN-γ was observed when $5x10^4$ $CD4^+CD25^{low}$ and $5x10^4$ $CD4^+CD25^{high}$ T cells were cocultured with macrophages (Fig.7.3.B). I speculate that the measurement of equal levels of IFN-γ in these cases were not quite accurate due to a technical problem of the ELISA assays, since the IFN-γ values were higher than the highest IFN-γ value (2000 pg/ml) of the standard curve. $1.7x10^4$ or lower numbers of $CD4^+CD25^{high}$ T cells produced higher amounts of IFN-γ than equivalent numbers of $CD4^+CD25^{low}$ cells (Fig. 7.3. B).

Macrophages alone did not produce detectable levels of any of the cytokines measured. Trypanosome-loaded macrophages when cultured without T cells did not produce detectable amounts of IFN-γ. This is not surprising, since T cells are known to be the major producers of IFN-γ (Uzonna et al., 1998c). Trypanosome-loaded macrophages when cultured without T cells did not show IL-10 production, suggesting that T cell interaction with macrophages might be necessary for the production of IL-10 by macrophages and/or T cells. There were no detectable amounts of IL-6 production when the macrophages were cultured alone. IL-6 production did not show much difference between the different cocultures. The production of IL-6 was less in trypanosome-loaded macrophages when cultured without adding sorted T cells (Fig. 7.3.C). IL-6 can be secreted by T cells as well as macrophages. The observed pattern of IL-6 levels in these cocultures would indicate that most of the IL-6 was produced by macrophages.

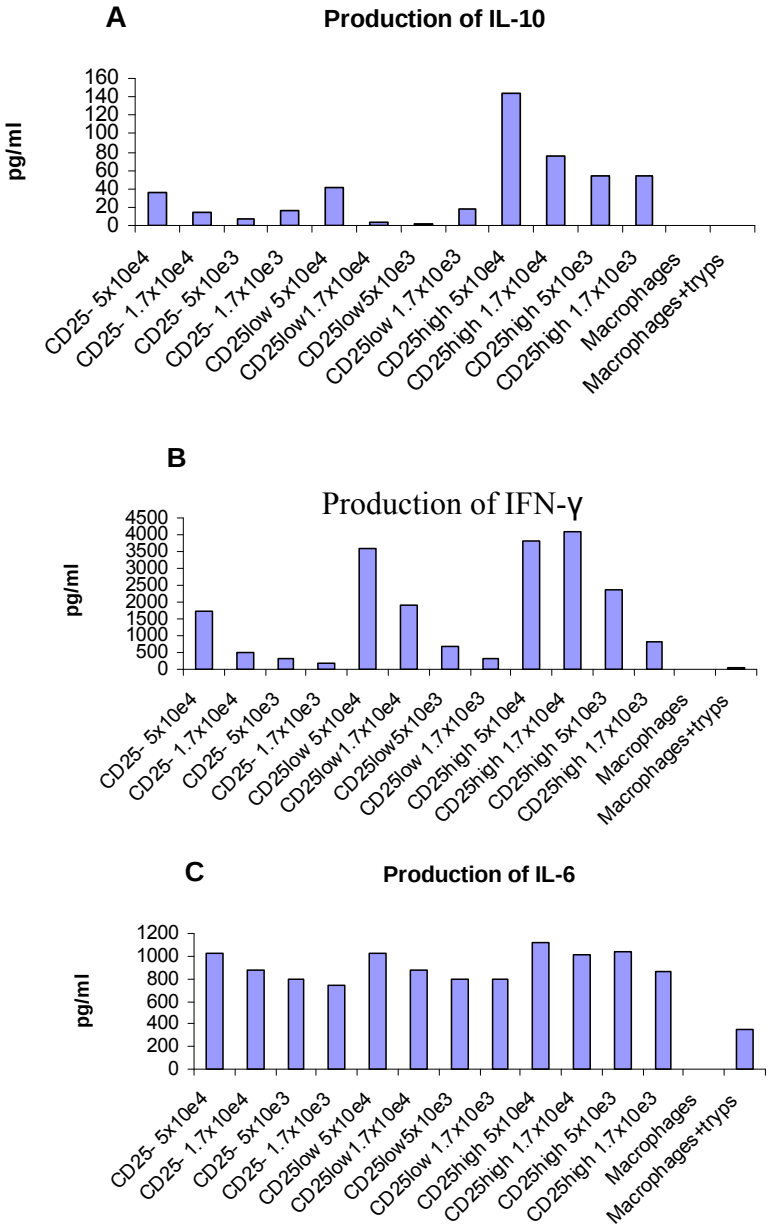

Fig. 7.3. Peritoneal macrophages ($5x10^5$/well) loaded with *T. congolense* were cocultured with $5x10^4$,$1.7x10^4$, $5x10^3$ and $1.7x10^3$ $CD4^+CD25^-$,$CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ T cells sorted from spleen cells of infected BALB/C mice 6 days post infection. Supernatants collected after 48 hrs of culture were used in undiluted form to measure the production of IL-10 (A) and 1:2 dilutions to measure IFN-γ (B) and IL-6 (C) in ELISA.

7.4.3. Block titration of APC and $CD4^+CD25^+$ T cells

There were difficulties in sorting sufficient numbers of cells from a group of mice to have enough replicates to continue the above experiment (Fig.7.3). The cell sorting produced variable results. It is a time consuming and expensive procedure. Therefore, a block titration was done using $CD4^+CD25^+$ cells enriched from spleen cells of *T. congolense*-infected BALB/c mice at day 6 post infection by MACS using a mouse Treg isolation kit. $CD4^+CD25^-$ cells were not included for this block titration since none of the previous preliminary experiments showed a clear production of IFN-γ or IL-10 in the cocultures of $CD4^+CD25^-$ cells and peritoneal macrophages. The enriched $CD4^+CD25^+$ cell population was 70% pure as detected by FACS (Fig.7.7).

According to the results of this experiment, 10^5 APC when cocultured with $5x10^4$ $CD4^+CD25^+$ cells produced the highest amount of IFN-γ and IL-10. The production of IFN-γ and IL-10 was also well within the measurable range when 10^5 APC were cocultured with $1.7x10^4$ $CD4^+CD25^+$ cells (Fig.7.4 and Fig.7.5). None of the measured cytokines were produced in detectable levels when the MACS-sorted $CD4^+CD25^+$ cells were cultured alone suggesting that the synergy of both T cells and macrophages are required for the production of these cytokines. It has been known that IFN-γ is mainly produced by T cells while both macrophages and T cells produce IL-6 and IL-10. IL-6 production increased more with the increase of APC numbers than with the increase of T cell numbers in the cocultures (Fig.7.6). This would indicate that the majority of IL-6 in these cocultures was produced by APC.

The number of $CD4^+CD25^+$ cells in $5x10^4$ cells of the enriched cell group should be higher than the number of $CD4^+CD25^+$ cells in $5x10^4$ whole spleen cells. Therefore, IFN-γ production would be expected to be higher in cocultures of APC with enriched $CD4^+CD25^+$cells. But the productions of IFN-γ were almost similar in cultures of 10^5 APC

when cocultured with $5x10^4$ $CD4^+CD25^+$ cells as when cocultured with $5x10^4$ whole spleen cells. Thus, there might be cofactors in the whole spleen cell population that enhance the production of IFN-γ. The enriched cells might contain less cofactors compared to those of whole spleen cells.

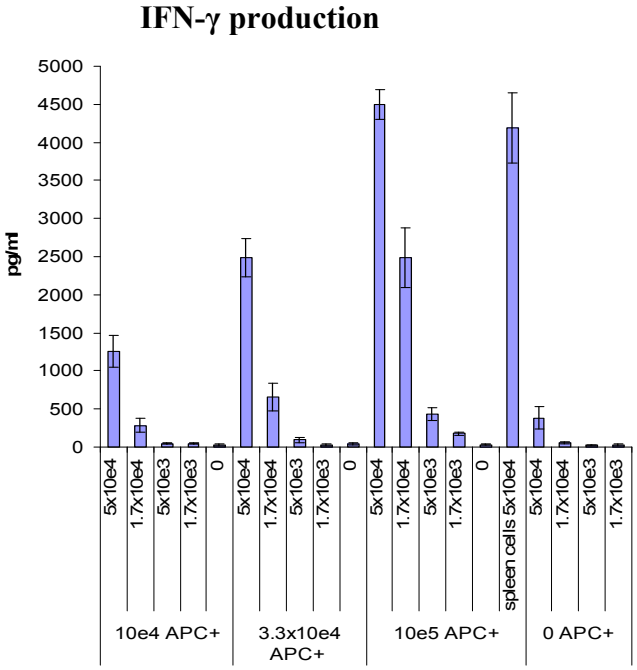

Fig.7.4. Block titration to determine cell concentrations and ratios for optimal synthesis of IFN-γ *in vitro*. Antigen presenting cells (APC), obtained from the peritoneal cells **(**depleted of $Thy1.2^+$ cells), were loaded with 10^6 equivalents of trypanosome homogenate per well and cocultured with different numbers of $CD4^+CD25^+$ cells from BALB/c mice infected with *T. congolense* for 6 days, enriched by using Mouse Treg Isolation Kit (Miltenyi Biotec).The culture supernatants (1:2 dilution) collected after 48 hours were used to measure the production of IFN-γ by routine sandwich ELISA. Values represent mean ± standard error of the samples obtained from 4 mice. **(Representative results of two experiments).**

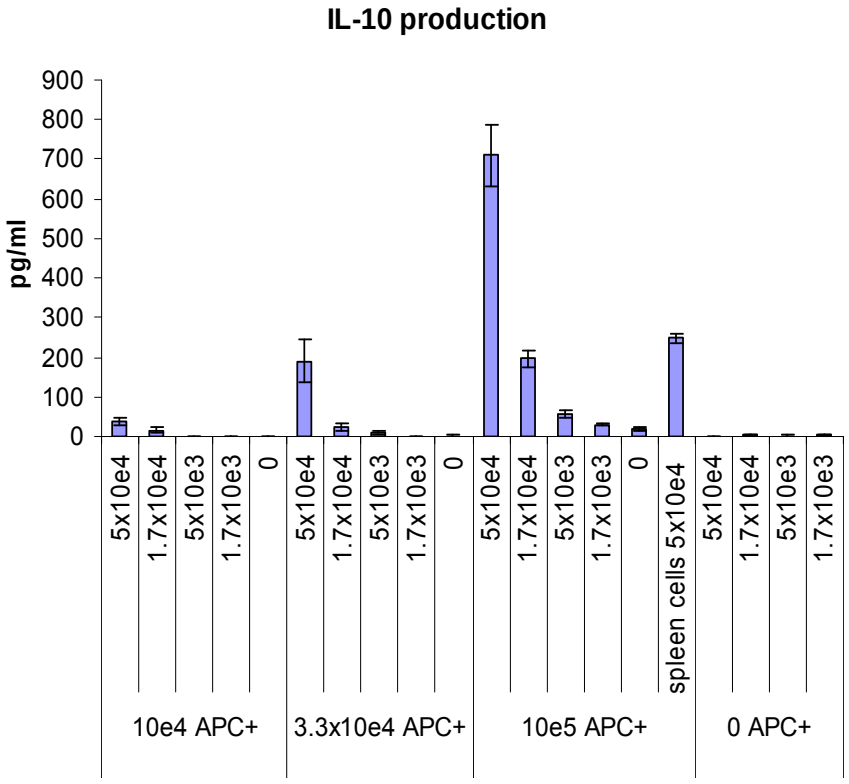

Fig. 7.5. Block titration to determine cell concentrations and ratios for optimal synthesis of IL-10 *in vitro*. Antigen presenting cells (APC), obtained from the peritoneal cells **(**depleted of Thy1.2^{+} cells), were loaded with 10^{6} equivalents of trypanosome homogenate per well and cocultured with different numbers of CD4^{+}CD25^{+} cells from BALB/c mice infected with *T. congolense* for 6 days, enriched by using Mouse Treg Isolation Kit (Miltenyi Biotec).The culture supernatants collected after 48 hours were used to measure the production of IL-10 by routine sandwich ELISA. Values represent mean ± standard error of the samples obtained from 4 mice. **(Representative results of two experiments).**

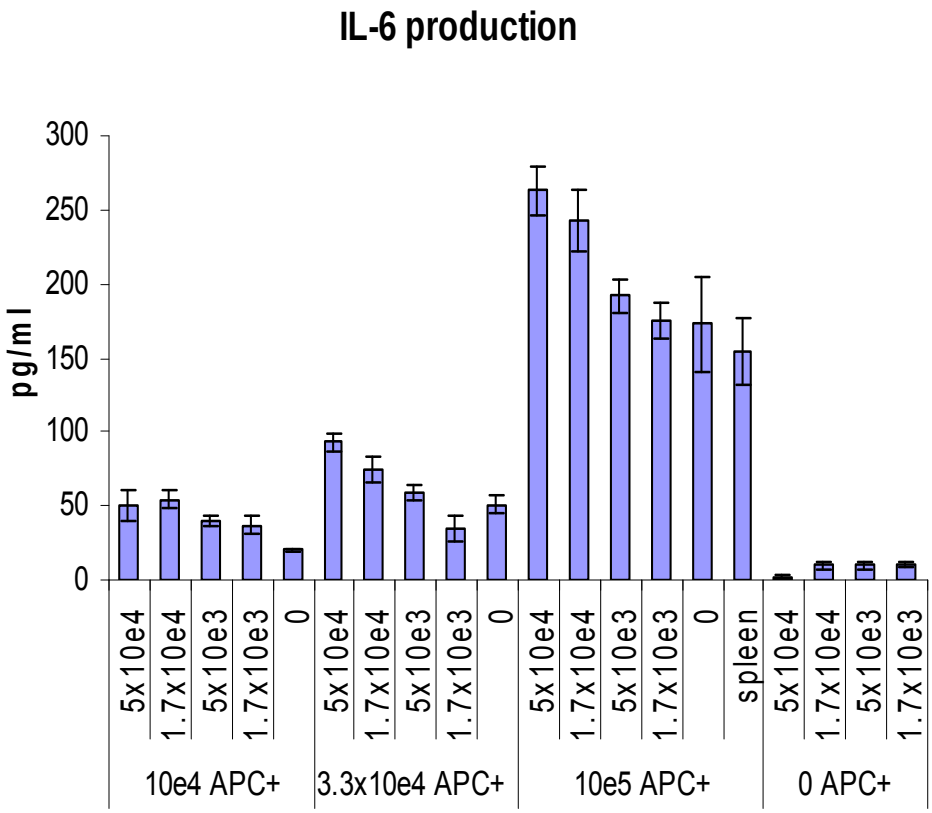

Fig.7.6. Block titration to determine cell concentrations and ratios for optimal synthesis of IL-6 *in vitro*. Antigen presenting cells (APC), obtained from the peritoneal cells **(**depleted of Thy1.2^{+} cells), were loaded with 10^{6} equivalents of trypanosome homogenate per well and cocultured with different numbers of CD4^{+}CD25^{+} cells from BALB/c mice infected with *T. congolense* for 6 days, enriched by using Mouse Treg Isolation Kit (Miltenyi Biotec).The culture supernatants (1:2 dilution) collected after 48 hours were used to measure the production of IL-6 by routine sandwich ELISA. Values represent mean ± standard error of the samples obtained from 4 mice. **(Representative results of two experiments).**

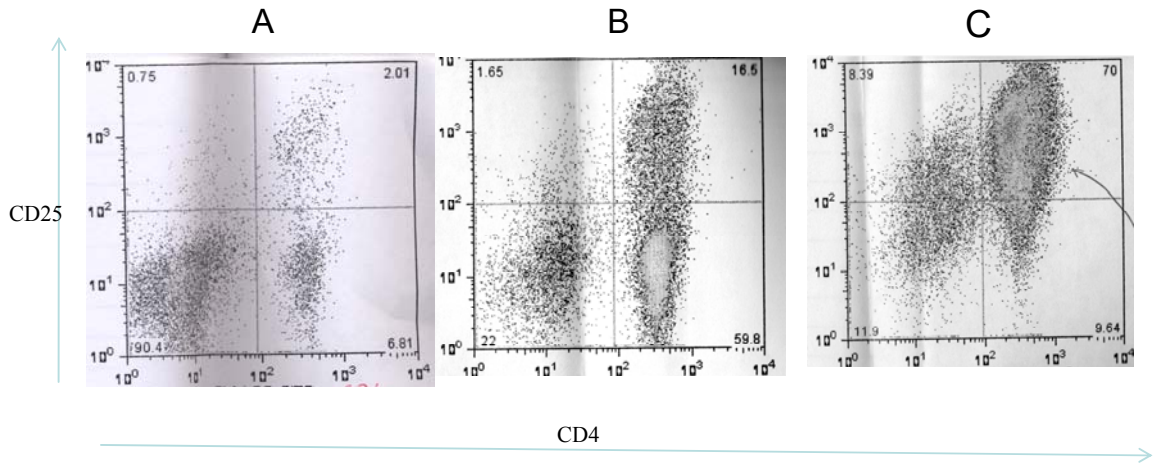

Fig. 7.7. FACS analysis of the enriched $CD4^+$ and $CD4^+CD25^+$ cell populations of the spleen of BALB/c mice 6 days after *T. congolense* infection. Whole spleen cells stained with ^{FITC} rat anti-CD4 and ^{PE} rat anti-CD25 (A), Enriched $CD4^+$ cell fraction stained with ^{FITC} rat anti-CD4 and ^{PE} rat anti-CD25 (B) and the enriched $CD4^+CD25^+$ cell population stained with ^{FITC} rat anti-CD4 and ^{PE} rat anti-CD25 (C) as detected by FACS analysis.

7.5. Discussion

In preliminary experiments, the $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ T cells, sorted by FACS from the spleen cells of normal and *T. congolense*-infected BALB/c mice, were co-cultured with trypanosome-loaded peritoneal macrophages. The majority of IFN-γ was shown to be produced by $CD4^+CD25^{high}$ T cells co-cultured with trypanosome-loaded peritoneal macrophages (Fig. 7.1). Although presently there is no direct experimental evidence, I speculate that these $CD4^+CD25^{high}$ cells might be a small subpopulation of highly activated $CD4^+CR2^+$ T cells. Whether the IFN- γ and IL-10 was produced by different cell populations or the same T cells is unknown. There is a body of evidence that T cells with regulatory functions can be double producers, i.e., produce both IL-10 and IFN-γ (Trinchieri,

2001). Such T cells have been found in humans (Pohl-Koppe et al., 1998; Winkler et al., 1998; Gerosa et al., 1999; Cella et al., 2000; Kadowaki et al., 2000; Levings et al., 2001) and mice (Ganapamo et al., 2000; Belkaid et al., 2001) infected with viruses, bacteria or protozoa.

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells sorted from normal BALB/c mice showed negligible amounts of IFN-γ production in the cocultures suggesting that IFN-γ production is trypanosome-specific (Fig.7.1). Detectable levels of IFN-γ and IL-10 were produced by adding $5x10^3$ to $5x10^4$ sorted T cells to the macrophage cultures while the maximal production was observed with $5x10^4$ sorted T cells when cocultured with macrophages (Fig.7.2). In subsequent experiments, production of almost equal levels of IFN-γ was observed in titration studies when $5x10^4$ $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ T cells were cocultured with macrophages (Fig.7.3). Trypanosome-loaded macrophages, when cultured without sorted T cells, did not produce detectable amounts of IFN-γ. This would confirm that the T cells are the major producers of IFN-γ. Macrophages alone or trypanosome-loaded macrophages when cultured without sorted T cells did not show IL-10 production (Fig.7.3), suggesting that T cell interaction might be necessary for the production of IL-10 by the macrophages and/or T cells.

IL-6 is secreted by T cells and macrophages. BMDM from BALB/c mice produced significantly more IL-6 and IL-10 than those from C57BL/6 mice indicating that cytokine genes are differentially regulated in macrophages from trypanosome-susceptible and -resistant mice (Kaushik et al., 2000).The observed increase of IL-6 levels in trypanosome-loaded macrophages and sorted T cells cocultures (Fig.7.6) could be due to either the production of IL-6 by the added T cells and/or by macrophages when they get T cell stimulation. The production of IL-6 did not show a strong correlation with the numbers of added T cells. This

suggests that IL-6 production is somewhat T cell interaction-dependent but IL-6 is mostly synthesized by the macrophages.

According to the results of block titration experiment, cocultures of 10^5 APC and $5x10^4$ $CD4^+CD25^+$ cells produced the highest amount of IFN-γ and IL-10 (Fig.7.4 and 7.5.). The production of IFN-γ and IL-10 was also well within the measurable range when 10^5 APC were cocultured with $1.7x10^4$ $CD4^+CD25^+$ cells. None of the measured cytokines were produced in detectable levels when the MACS-sorted $CD4^+CD25^+$ cells were cultured alone, suggesting that the synergy of both T cells and macrophages are required for the production of these cytokines. IFN-γ is mainly produced by T cells while both macrophages and T cells produce IL-6 and IL-10. Increase of IL-6 production with the increase of APC number (Fig.7.6) indicates that the majority of IL-6 in these cocultures was produced by APC. The production of IFN-γ was similar in 10^5 APC when cocultured with $5x10^4$ $CD4^+CD25^+$ cells and 10^5 APC when cocultured with $5x10^4$ whole spleen cells (Fig.7.4). This result suggests that there might be certain cofactors present in the whole spleen cells which can enhance production of IFN-γ, but there might have been less cofactors in the enriched cell populations.