CHARACTERIZATION OF *TRYPANOSOMA CONGOLENSE*-INDUCED ENDOTOXIC SHOCK-LIKE RESPONSE IN MOUSE MODELS

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DECLARATION

I confirm that the work in this thesis is the product of my own research and authorship and does not contain the work or writing of another except where appropriately cited and referenced. I confirm that the work in this thesis has not been submitted for another award at Universita degli Studi della Tuscia or elsewhere.

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DEDICATION

To my loving mum Theresiah Mukiki Wambua, the inspiration and cornerstone of my education. My late brother Lawrence – I really wish you lived to share this moment with me. To my siblings, Ronald and Caroline and my loving husband Kiio and kids Alice and Mwendwa – your constant encouragement, smiles, calls and prayers have been the wind beneath my wings.

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LIST OF ABBREVIATIONS

aaMφ: Alternatively activated macrophage

ANOVA: Analysis of variance

BMDM: Bone marrow:derived macrophage

caMφ : Classically activated macrophage

CD14: cluster of differentiation 14

DNA: Deoxyribonucleic acid

ELISA: Enzyme:linked immunosorbent assay

GPI: Glycophosphatidylinositol

IFN: interferon

IL: Interleukin

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MHC: Major histocompatibility complex

MIAME: minimum information on a microarray experiment

PLC: Phospholipase C

QTL: Quantitative trait loci

RMA: Robust multiarray average

RNA: Ribonucleic acid

TcDNA: Trypanosoma congolense Deoxyribonucleic acid

TcVSG: Trypanosoma congolense variant surface glycoprotein

TGF: Transforming growth factor

TLR: Toll-like receptor

VSG: Variant surface glycoprotein

DEAE: Diethylaminoethyl

EDTA: Ethylenediaminetetraacetic acid

TE: Tris Ethylenediaminetetraacetic acid

FBS: Foetal bovine serum

RPMI: Roswell Park Memorial Institute Medium

SOP: Standard Operating Procedures

SUMMARY (ENGLISH VERSION)

African Bovine Trypanosomiasis caused by *Trypanosoma congolense* is a serious disease that threatens the health, productivity and diversity of cattle in Sub-Saharan Africa. The acute phase of this disease is marked by mounting of aggressive host immune response which coincides with peak levels of the parasite in host blood. The acute phase is beneficial to the host for parasite clearance but also precipitate severe immune dysregulation, exacerbated pathology and death of the host if uncontrolled. The objective of this study was therefore to characterize host-pathogen interactions during acute *T.congolense* infection in A/J, Balb/c and C57BL/6 mouse models which vary in susceptibility to *T.congolense*.

Transcriptional profiles of liver tissues of uninfected mice were compared against mice infected for 9 days when peak parasite levels are observed. The results showed that inflammation was the fundamental host response to infection in acute disease and that this inflammation mirrored bacterial endotoxic shock as depicted by similar gene expression signatures. The results also revealed the inflammation was signaled via toll-like receptors 1, 2 and 4 leading to increased transcription of pro-inflammation cytokines and chemokines. Follow-up investigations negated the involvement of bacterial endotoxins in infected mice making it apparent that observed inflammatory response was *T.congolense*-specific.

An *in vitro* system of bone-marrow derived macrophages obtained from A/J and C57BL/6 mice was subsequently developed. This system provided a clean and immunologically naïve set-up to validate and characterize *T.congolense* induced inflammation. Using this system, the variant surface glycoprotein was characterized as the main parasite factor eliciting *T.congolense* inflammation. *T.congolense* DNA also contributed towards the response although to a lesser extent. Both *T.congolense* VSG and DNA, in the absence of IFN-γ, activated macrophages a classical phenotype exhibiting increased levels of pro-inflammatory cytokines TNF-α, IL-1α, IL-6 and IL-12p40. Chemokines CCL3, CCL5, CXCL1 and CXCL10 were also significantly elevated. These cytokine and chemokine profiles were confirmative of T-helper 1

polarization of early responses *T.congolense*. Mouse macrophages of tolerant C57BL/6 mice secreted higher levels of the inflammatory mediators than A/J showing a role of the early inflammatory response in tolerance to *T.congolense*.

These findings demonstrate for the first time a comprehensive characterization of inflammatory responses in acute *T.congolense* infection. Confirmation of *T.congolense* VSG as the causative factor presents new opportunities for the exploration of GPI-based therapy for the control of inflammation and infection-associated pathologies. Additionally, further characterization of the conserved GPI anchor of *T.congolense* VSG coupled with advances on carbohydrate-based vaccines presents a fresh perspective in the quest for a vaccine for African Trypanosomiasis. Further research into these, will yield better interventions for the control of Bovine Trypanosomiasis, thereby improving health and conserving the diversity of African cattle.

SUMMARY (ITALIAN VERSION)

La tripanosomiasi africana bovina causata da *Trypanosoma congolense* è una malattia grave che costituisce una minaccia per la salute, la produttività e la diversità del bestiame nell'africa Africa sub-sahariana. La fase acuta di questa malattia è caratterizzata da una aggressiva risposta immunitaria che coincide con il picco del livello del parassita nel sangue dell'ospite. La fase acuta permette l'eliminazione dei parassiti, ma se non controllata può causare una grave disregolazione immunitaria, l'aggravarsi della patologia e potenzialmente la morte dell'ospite.L'obiettivo di questo studio è stato quello di caratterizzare le interazioni ospite-patogeno durante l'infezione acuta *T.congolense* nei modelli murini A / J, Balb / C e C57BL / 6, che mostrano divcersi livelli di suscettibilità a *T. congolese*.

I profili di trascrizione del fegato di topi sani sono stati confrontati con lo stesso tessuto di topi infettati da 9 giorni, nel momento del picco di diffusione del parassita. I risultati hanno dimostrato che la principale risposta dell'ospite all'infezione è stata l'infiammazione e che questa infiammazione batterica riflette lo shock endotossico come previsto in base ai modelli di espressione genica. I risultati hanno anche rivelato che l'infiammazione viene trasmessa attraverso i recettori Toll-like 1, 2 e 4, che causano un aumento di trascrizione di citochine e chemochine. Indagini succesive hanno escluso il coinvolgimento di endotossine batteriche nei topi infettati rendendo evidente che la risposta infiammatoria è causata in maniera specifica da *T. congolense*.

Successivamente è stato sviluppato un siatema *in vitro* basato su macrofagi di midollo osseo ottenuti da A / J e topi C57BL / 6. Questo sistema ha fornito un set-up per confermare e caratterizzare l'infiammazione indotta da *T. congolense*. Utilizzando questo sistema, è stata identificata una glicoproteina di superficie che rappresenta il principale fattore che provoca l'infiammazione da *T. congolense*. Anche l'analisi di DNA di *T. congolense* ha contribuito allo studio del processo infiammatorio, anche se in misura minore.. Sia il VSG che il DNA di *T. congolense*, in assenza di IFN-γ, attivano un tipico fenotipo nei macrofagi caratterizzato dall'aumento dei livelli di citochine TNF-α, IL-1α, IL-6 e IL-12p40. Anche le chemochine CCL3, CCL5, CXCL1 e CXCL10 sono

aumentate in maniera significativa. Questi profili di citochine e chemochine hanno confermato la polarizzazione T-helper 1 della risposta iniziale a *T. congolense*. Macrofagi murini provenienti dalla linea tolleranti C57BL/6 hanno secreto livelli più elevati di mediatori infiammatori rispetto a quelli di A/J, confermando il ruolo della risposta infiammatoria precoce nella resistenza a *T. congolense*.

Questi risultati costituiscono la prima caratterizzazione completa della risposta infiammatoria nelle infezioni acute da *T. congolense*. La conferma che il VSG di *T. congolense* rappresenta il fattore scatenante presenta nuove opportunità per lo studio di terapie basate su GPI che possano controllare l'infiammazione e le patologie associate all'infezione. Inoltre, un'ulteriore caratterizzazione delle GPI delle VSG di T. congolese, unite ai progressi in materia di vaccini a base di carboidrati, rappresenta una nuova prospettiva nella ricerca di un vaccino per la tripanosomiasi africana. Ulteriori ricerche in questo campo porteranno ad una migliore riuscita degli interventi per il controllo della tripanosomiasi bovina, migliorando così la salute e la conservazione della diversità dewi bovini africani.

1 INTRODUCTION

African livestock are plagued by a myriad of diseases which jeopardize their health and productivity indices, posing great threats to the livelihoods of small-scale farmers in the region. More importantly, these diseases influence the livestock genetic pool through continuous selection of traits and alleles that bear on favourable disease outcomes under challenge. This selection process has yielded locally adapted animals with significant diversity and desirable traits that confer the versatility to survive under harsh environmental and prevailing disease challenges. As such, investigation of genetic responses of livestock to diseases is imperative in the quest to understand, characterize and hence conserve desirable genetic traits in indigenous livestock populations. Towards this end, mice have emerged as invaluable laboratory models for study of these diseases. The advent of high throughput technologies in genetics and genomics in the recent decades, coupled with the availability of appropriate mouse models has accelerated the efforts towards genetic characterization of livestock for disease tolerance traits.

African Bovine Trypanosomiasis is an important, albeit neglected debilitating disease of livestock, particularly cattle, in Sub-Saharan Africa. It is a tsetse-transmitted disease caused by three *Trypanosoma* species: *T.brucei*, *T.vivax* and *T.congolense*. *T.brucei.* The greatest burden and fatalities are attributed to *T.congolense* due to its high levels of pathogenicity. Bovine Trypanosomiasis impacts massively on agriculture in Africa killing up to seven million cattle per year in Sub-Saharan Africa and causing insurmountable economic losses to poor small-scale livestock farmers through reduced productivity in milk, meat and draught power. The cost of treatment and prophylactic control of bovine Trypanosomiasis is estimated at about four billion dollars annually. The disease has been further compounded by development of drug resistance to the current regime of drugs which also elicit high levels of toxicity in the animals. The quest for a vaccine against African bovine Trypanosomiasis has been thwarted by the parasite's ability to alter its antigenic surface membrane (Cross et al, 1998). However, hope lies in the exploitation of natural tolerance in some cattle breeds of West Africa, notably the N'dama cattle, which have acquired relative resistance to Trypanosomiasis

as a result of strong natural selection forces over several centuries, thereby affording the animals capacity to maintain productivity under intense disease pressure. This relative resistance to Trypanosomiasis has been termed trypanotolerance (Murray et al, 1984).

Considerable efforts have been directed discovering the genetic mechanisms underlying trypanotolerance. To this effect, mice have proved to be valuable experimental models for the study of Trypanosomiasis due to their analogous genetic make-up, establishment of comparable course of disease, short generation time and ease of genetic manipulation. With the use of inbred and congenic mouse models, three quantitative trait loci (QTLs) controlling tolerance and susceptibility to *T.congolense* infection have been mapped on chromosomes 1, 5 and 17. These QTLs have been named as Tir 1, Tir2, Tir3 respectively (Kemp et al, 1997).

Severe anemia, wasting, intermittent fever, general loss of condition and death are hallmarks of *T.congolense* infection in cattle and murine models of *T.congolense* infection. Pathogenesis usually begins with an acute, short-lived stage during which strong immune responses are invoked in the host to control parasite invasion. Individuals that survive death develop chronic disease highlighted by persistent parasitemia and immune suppression culminating in secondary infections and death. Host immune responses in the acute phase of infection appear to be crucial indicators of disease prognosis. A prompt induction of appropriate immune responses has been associated with parasite clearance, recovery and survival; aggressive responses have been shown to precipitate infection-associated immunopathologies as anaemia and wasting; whereas delayed immune responses may spell immediate death for the affected animals (Maxie et al, 1979). There remains a need for thorough understanding of early host responses to *T.congolense* and their bearing on the course and consequence of infection.

The acute phase of infection in Trypanosomiasis due to *T.congolense* and other African trypanosomes is marked by relentless struggle for survival for both the host and the trypanosome. In an effort to replicate to large numbers, the parasites exert incessant efforts to evade host immune responses by continuous switching of their protective variant surface glycoproteins (VSG). The fact that each trypanosome expresses about

10^7 molecules of VSG and each wave of parasitemia presents $>10^8$ parasites in the experimental murine model effectively implies that the host immune system is accosted with about 2μg of VSG antigens. This VSG load is deemed significant, given that similar amounts of bacterial endotoxin in mice is adequate to cause marked immunological changes and death (Mahieu et al, 2006; Zorzeto et al, 2009). This therefore implies that significant host-pathogen interactions are centered around trypanosome VSG. Nonetheless, how the infected host deals with this enormous antigenic load remains largely unexplored in *T.congolense* infection and dedicated studies towards this are warranted.

Innate responses play major roles in the control of early infection with trypanosomes. These responses are complex and not fully understood. However, an increasing body of evidence indicates inflammation mediated by activated host macrophages to be a key innate response in acute phase of infection with *T.brucei*, *T.vivax* and *T.congolense* parasites. Increased levels of proinflammatory mediators as TNF-α, IL-1, IL-6, IL-12 and nitric oxide host are observed in infected host. This inflammation is deemed beneficial to the host in limiting the parasites numbers through direct lysis, stasis in parasite replication, induction of antibody responses and T-lymphocyte responses (Murray and Morrison, 1979; Tabel et al, 2000). A destructive state of hyperinflammation develops if initial inflammation is not promptly modulated setting stage for immunopathologies.

In *T.brucei* infections, the variant surface glycoprotein (VSG) and glycophasphatidylinositol (GPI) moieties of parasites have been implicated as the triggers behind an early inflammatory response in mice. *T.brucei* VSG has been demonstrated to activate macrophages to a classical phenotype (caMΦ) exhibiting secretion of proinflammatory cytokines as TNF-α and IL-1 (Tachado and Schofield, 1994; Magez et al, 1998). Similar early inflammatory responses have been noted in monocytic cells of cattle and mice infected with *T.congolense* (O'Gorman et al. 2006; Shi et al. 2004). Gene expression analysis of peripheral blood mononuclear cells (PBMCs) from cattle infected with *T.congolense* revealed a marked increase in the transcripts of genes encoding for proinflammatory cytokines within the first 14 days of

infection. *T.congolense*-infected mice secreted significantly elevated levels of IL-1β, IL-12p40 and IL-10 by 6hrs of exposure. The role of *T.congolense* VSG in eliciting early inflammatory responses has so far not been explored. Furthermore, activation of macrophages by *T.congolense* DNA has not been elucidated.

It has been postulated that loss of membrane integrity in dead and dying trypanosomes may expose the host immune system to Trypanosomal factors as parasite DNA that would otherwise not be recognized by the host immune system. Indeed, evidence collated from studies *T.brucei* infections points to the involvement of parasite DNA in inflammatory response in host macrophages. *T.brucei* DNA has been shown to activate macrophage cell lines to a classical phenotype whose gene expression and cytokine profiles depicted an inflammatory response. The macrophage stimulatory capacity of *T.brucei* DNA was attributed to presence of bacterial-like unmethylated CpG motifs (Shoda et al, 2001). The immunostimulatory capacity of *T.congolense* DNA has not been explored to date as no studies have been undertaken to this effect.

A case has also been made for contribution of bacterial endotoxins to the inflammatory response as a concomitant increase in levels of circulating endotoxins has been demonstrated in *T.brucei*-infected mice, humans and rats. To this effect, it has been hypothesized that inflammatory responses due to *T.brucei* infection compromise the integrity of the gut epithelium causing leakage of gut flora into the blood subsequently leading to elevation of circulating endotoxins which are implicated in an aggravated inflammatory response. It has been shown that administration of exogenous bacterial endotoxin to mice prior to infection with *T.congolense* parasites conferred tolerance to trypanosome infection. However, administration of endotoxin in mice with established *T.congolense* infection led to sudden death, implying that inflammatory responses against trypanosome VSG renders the host to be hyper-responsive to exogenous bacterial endotoxins (Singer et al. 1964). Endogenous sources of bacterial endotoxins during *T.congolense* infection have however not been demonstrated and the involvement of bacterial endotoxins in natural *T.congolense* infections remains speculative.

It is clear from this background that host immune responses to infection during the acute phase of Trypanosomiasis dictate major stakes in i) control of parasite loads ii) survival of the host iii) development of infection-associated immunopathologies and iv) progression to chronic disease. Inflammation has emerged as a key response in early infection with trypanosomes. However, due to the paucity of data on comprehensive analysis of gene responses to infection, the trypanosome factors and the host cells involved in response to the major livestock trypanosome, *T.congolense* have so far been inadequate. It is currently not clear if inflammation is the major response at the early stage of *T.congolense* infection or if perhaps there exist other complementary immune mechanisms. As such intensive investigations of early-stage host responses to *T.congolense* infection would provide in-depth clues to the complex innate mechanisms behind parasite clearance, development of pathology and possibly, tolerance to disease.

Present-day technologies for high throughput for gene expression profiling and powerful analytical tools in bioinformatics provide means for such studies to be accomplished within shorter intervals and with great scope. The application of these technologies in appropriate mouse models for *T.congolense* infection will provide an ideal biological system to screen for host-pathogen interactions during acute disease. Towards this end, this study explored gene expression profiles of highly susceptible A/J, moderately tolerant Balb/c and tolerance C57BL/6 mice infected with *T.congolense* parasites over a 17-day timecourse. Host-pathogen interactions occurring in the acute phase of *T.congolense* infection were deduced by analyzing transcriptional profiles of uninfected mice with those infected for nine days which coincides with peak parasite loads in host blood.

Activated macrophages have emerged as central effectors of parasite clearance and activation of both innate and adaptive immune response to *T.congolense* infection. Due to their role in secretion of pro-inflammatory and anti-inflammatory mediators, macrophages may also be pertinent players and modulators of *T.congolense*-induced immune dysregulation processes as hyperinflammation and immunosuppression. It is therefore plausible that a standardized *in vitro system* of host macrophages would be

invaluable in characterization and validation host responses to *T.congolense* infection. In this context, bone marrow-derived macrophages of trypanosusceptible A/J and tolerant C57BL/6 mice were isolated and established in culture to provide a clean immunologically unperturbed host macrophage system. This system was utilized to validate the role of *T.congolense* VSG and DNA and bacterial endotoxins in inducing inflammatory responses. The system was also employed in comprehensive characterization of macrophage activation in response to *T.congolense* VSG and DNA as portrayed by their chemokine and cytokine milieu.

It is anticipated that the output of these investigations will make significant contributions to the understanding of the acute stage of *T.congolense* infection by providing a comprehensive perspective of the genes, gene networks and signaling pathways unfolding at this stage of this disease. The *in vitro* investigations are envisaged to investigate the involvement of host macrophages, *T.congolense* VSG and DNA in inducing early inflammatory responses. Chemokine and cytokine profiles of treated macrophages will give insights into macrophage activation by *T.congolense* structural components and polarization of immune responses. It is envisioned that knowledge drawn from these experiments may be interpreted to provide evidence of genetic mechanisms underlying *T.congolense* pathogenesis and tolerance.

2 LITERATURE REVIEW

2.1 Trypanosomiasis

Trypanosomiasis is a generic term for a range of tropical diseases caused by various species of the trypanosomes. It affects humans and livestock and like other protozoan diseases like malaria, is transmitted by arthropod vectors of the *Glossina* and *Triatoma* species. Trypanosomiasis encompasses three main diseases depending on the host affected: African human Trypanosomiasis also called sleeping sickness, African animal Trypanosomiasis also called nagana and American human Trypanosomiasis also referred to as Chaga's disease. The focus of this research is African animal Trypanosomiasis that affects livestock in the African continent.

2.2 Bovine Trypanosomiasis

African animal Trypanosomiasis (AAT) which affects goats, sheep and cattle in Sub-Saharan Africa is caused by three trypanosome species namely: *T.congolense*, *T.vivax* and quite rarely, *T.brucei* which are transmitted by tsetse fly vector of the Glossina species (Murray et al, 1982). Bovine Trypanosomiasis, afflicts cattle is attributed mainly to *T.congolense* infection and is the focus of this study. This report will discuss Bovine Trypanosomiasis in the context of and with reference to *T.congolense* infection.

In areas of high tsetse infestation in Africa, all the three trypanosome species may be present in animals causing mixed infections. The infected animals exhibit clinical symptoms of Trypanosomiasis characterized by intermittent fever, initial enlargement of the superficial lymph nodes, anaemia and gradual deterioration of general body condition of the animal. African trypanosomes are capable of establishing chronic disease lasting over three months in cattle. Eventual fatalities are associated with slow, progressive anaemia that finally culminates to heart failure.

2.3 The Problem

African animal Trypanosomiasis remains the most important ruminant disease in Sub-Saharan Africa impacting negatively on animal genetic resources, food production and

economic growth. In the tsetse-zones, which span 30% of the African continent, Trypanosomiasis remains a threat to livestock diversity and food security in Sub-Saharan Africa by causing economic losses estimated at $5 million as a direct effect of infection and death of the animals. This disease further causes animal loss due to decreased fertility in affected animals with reports of abortions (Morrison and Murray 1979) and in cases of chronic infection, Trypanosomiasis reduces productivity in milk and meat production in the infected livestock due to retardation of growth and wasting of the animals.

According to the Food and Agriculture Organization (FAO) estimates, there are 120 million pastoralists in the world whose livelihoods are entirely dependent on pastoralism and agro-pastoralism and hence constitute the world's poorest population. 50% of these individuals reside in Sub-Saharan Africa the face challenges as irregular rainfall patterns, droughts, floods and overgrazing. Their meagre economic capacities are further crippled by livestock diseases as Trypanosomiasis (Food and Agriculture Organization of the United Nations 2006).

More research by FAO indicates that sub-Saharan Africa registers the highest prevalence of undernourished individuals where 33% of the total population is undernourished. This region also records the lowest dietary protein consumption levels (Food and Agriculture Organization of the United Nations 2006). With the African population growing at 2% annually, and food production capacity in Africa falling below the limits in comparison to other developing countries, Trypanosomiasis only serves to complex the problems and frustrates the efforts towards achievement of food security and economic advancement in the African continent.

Current control strategies include the chemotherapy and vector control. The hope for a vaccine against animal trypanosomes based on their variant surface glycoprotein (VSG) coat has been thwarted by the complexity of the parasite's antigenic repertoire (Borst et al. 1998). The trypanosome achieves this by alternately activating different expression sites on the VSG gene, or by effecting DNA rearrangements within a given site. This results to antigenic variants occurring hierarchically in the hosts' blood with a mixture of

order or randomness, and these acts as a defence for the parasite against the hosts' innate and acquired immune response (Pays 1995). In effect, VSG is the only antigen that the animal host can respond to. Hence, attempts to develop vaccines based on other invariant trypanosome proteins such as tubulin and cysteine proteins have not been successful as they do not elicit an immune response significant enough to confer protective against infection (Barry and McCulloch 2001; Taylor et al. 1998). Consequently, there is no vaccine to date.

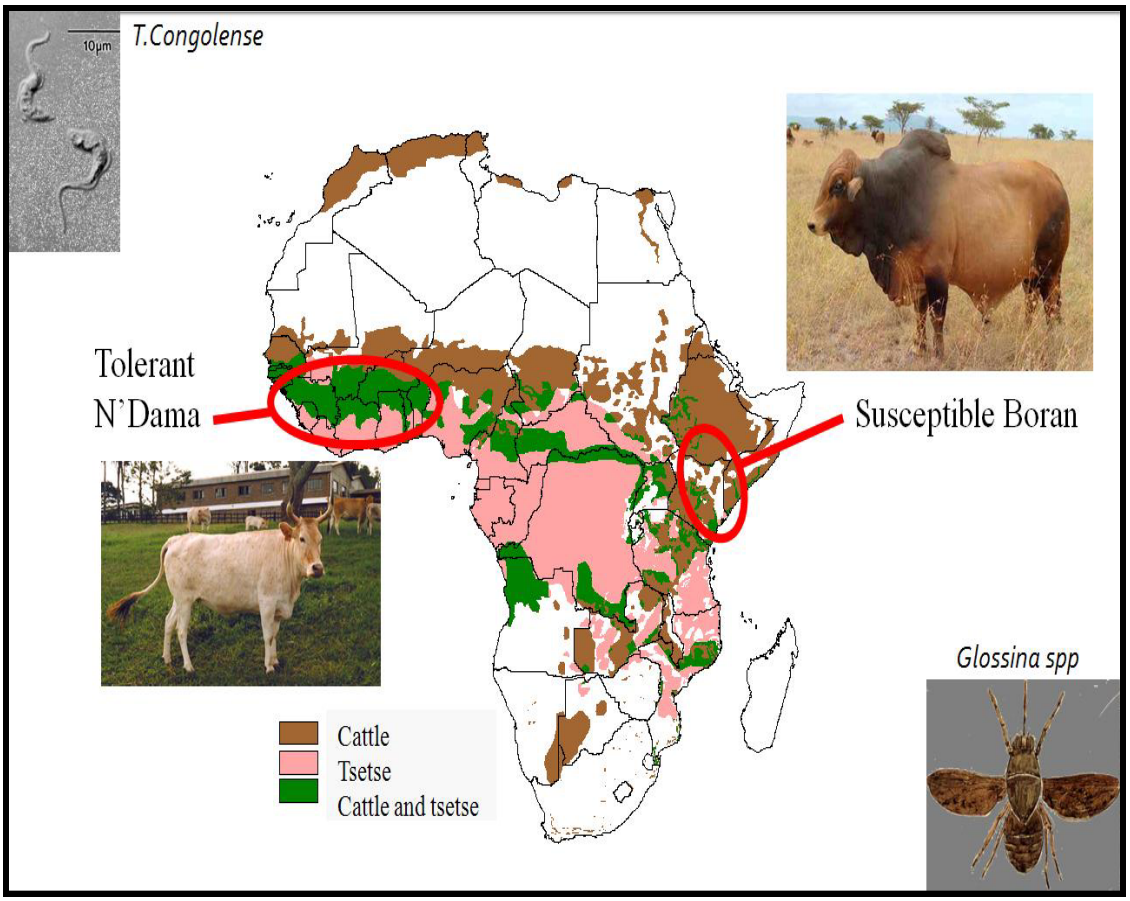


Fig 1.1: Distribution of African Bovine Trypanosomiasis in Sub-Saharan. The disease occurs in areas where there is presence of cattle and the tick vector. The tolerant N'dama cattle are found in West Africa where there is intensive disease whereas the susceptible Boran cattle are found in the Eastern Africa region.

2.4 **Control strategies against bovine Trypanosomiasis**

The current mainstay of Trypanosomiasis control is chemotherapy and chemoprophylaxis with trypanocides and exploitation of natural host tolerance. For chemotherapy and prophylaxis isometamidium and homidium which are both therapeutic and prophylactic and diminazene which is only therapeutic (Geerts et al. 2001) make the current regime. These drugs have been widely utilized over the last 40 years with 35 million doses being administered annually to approximately 50 – 70 million animals at risk and as a consequence drug resistant trypanosomes have developed (Diarra et al. 1998). In East Africa alone, drug resistance is estimated at over 50% and further research has shown that prolonged exposure of the trypanosomes to the drugs has diminished their trypanocidal effect. Hence, they rarely cause death of the parasites, but only restrict their replication thereby allowing their clearance by the immune system (Eisler et al. 2001; Osman et al. 1992)

Other than the emergence of the drug resistance parasite strains, chemotherapy against African trypanosomes is still wanting due to the high toxicity of the current trypanocidal regimens to the host, the relative paucity of these expensive drugs in the regions where they are required, compounded by lack of funds and trained personnel (Nok et al. 2005). There is a dire need to enhance control of trypanosomes by chemotherapy and prophylaxis through intensified research for novel drugs that will provide a solution to drug resistance. Such research efforts are however minimal and prospects for new inexpensive and less toxic drugs remains unrealized. Indeed, Trypanosomiasis is largely an "orphan disease" and therefore not a priority research area for the multinational pharmaceutical conglomerates. In comparison to other protozoan diseases as malaria where there have been massive investments for vaccine and drug development, the situation in Trypanosomiasis has been deplorable, attracting only nominal efforts from the pharmaceutical and scientific community in this regard (Fairlamb 2003).

Vector control has also been practiced to curb Animal Trypanosomiasis in Africa. This method has entailed spraying the tsetse zones with insecticides to eliminate the vector and reduce the rates of transmission of disease. This method however has posed a

number of challenges as there exists twenty two different kinds of species of tsetse flies across the continent, each adapted the varying climatic and ecological conditions (Ford and Blaser 1971). In addition, massive insecticide spraying has had undesirable environmental effects by eliminating non-target organisms thereby disrupting the balance of the ecosystem (Everts et al. 1985). Re-invasion of the cleared areas by tsetse flies occurs often, thereby necessitating constant surveillance efforts against recurrent infestations, and the high costs associated with this in effect make spraying of tsetse zones an inappropriate mode of trypanosome control. The use of tsetse traps and targets has also been explored for Trypanosomiasis control mainly in central and southern Africa. The targets and traps are insecticide-impregnated baits that function by attracting the flies to the device where they are killed by the insecticide. They have however not been useful as large-scale systems for vector control due to the short-lived activity of the insecticide, the high cost of production and maintenance of the devices as well as their differential efficiency in capturing the varying species of tsetse flies across the continent (Kuzoe and Schofield 2004). As a result, the use of traps and targets is limited only to entomological surveillance and maintenance of temporary barriers between insecticide-treated and untreated regions to discourage tsetse re-invasion.

A biological method of tsetse control by use of the sterile male release was developed and tested as an alternative to the use of insecticide (Dame and Schmidt, 1970). This involved rearing large number of highly competitive tsetse males and rendering them artificially sterile using ionizing radiation, followed by sustained release of these sterile populations into the target areas to compete with wild tsetse males for mating with the females to eventually diminish the tsetse population in the area. The applicability of this method was however limited by the high cost associated with large-scale production of sterile males and has also been linked with increased risk of parasite transmission as the sterile males as equally competitive in transmitting the trypanosomes as the wild populations (Moloo 1982; Roelants and Williams 1982).

2.5 **Natural tolerance to *T.congolense* infection in African bovines**

The compounding limitation in the control of African Trypanosomiasis is that the livestock are reared under highly controlled agricultural systems maintained by intensive artificial interventions in the form of chemotherapy against the parasite and vector. This scenario creates an unstable ecosystem whose balance if disturbed by a breakdown in infrastructure, drug-resistant parasites or adverse climatic changes deals catastrophic losses to the livestock and livelihoods (Kemp and Teale 1998). This fact coupled with the elusive Trypanosomiasis vaccine can however be circumvented by exploiting the heritable natural immunity elicited by some indigenous cattle breeds living in west and central Africa, presumably due to natural selection resulting from disease pressure exerted on the animals in these hyper-endemic regions (Taylor and Mertens 1999). The taurine breeds such as N'dama and the West African Shorthorn breeds have developed relative tolerance to the disease and are able to maintain normal weight, limit parasitemia, maintain a normal reproductive capacity and most importantly control infection-associated anaemia which is the primary cause of death in infected animals (Ellis et al. 1987). The converse is true for indicine cattle breeds such as the Kenyan Boran largely present in East and Southern Africa that cannot handle the infection and often succumb to disease, exhibiting loss of weight, reduced yield and loss of fertility, reduced capacity to work and anaemia finally culminating in death (Murray et al. 1984).

The acquisition of natural tolerance to Trypanosomiasis in African bovidae has been largely attributed to co-evolutionary contacts between the animals, the vector and the trypanosomes (Hanotte et al. 2000; Mattioli and Wilson 1996). This has its basis on the historical occurrence of trypanosomes, tsetse flies and the cattle in the Nile Delta in Egypt - primary centre of agriculture and civilization. Trypanosomes in Nile Delta are known of considerable geological age and have been infected several tsetse fly species which were already resident in the same area more than 40 million years ago (Lambrecht, 1964). Bovine species then started to arrive and settle in the Nile Delta about 20 million years ago, followed by the taurine ancestor, Bos taurus in 5000 B.C and the taurine cattle in 2750 – 2500 B.C. Indicine cattle breeds were not present in the Nile Delta until after the Arabian invasion in 699 A.D. (Murray et al. 1982; Paling et al.

1991). The length of evolutionary contact therefore becomes proportional to present day trypanotolerance observed in bovidae with the wild bovidae as the African buffalo being highly resistant, followed by the pure taurine cattle breeds as N'dama, whereas the indicine breeds as the Kenyan Boran are susceptible (Hanotte et al. 2000).

Recent decades have however seen cross-breeding between the pure trypanotolerant taurine breeds of West Africa e.g. N'dama and the trypanosusceptible indicine breeds as the Boran, in favour of the high productivity traits of the indicine breeds leading to the depletion of trypanotolerance due large-scale indicine introgression into the pure trypanotolerant breeds (Hanotte et al. 2000; Loftus et al. 1994). As a consequence, it is estimated that only 6% of the total present day cattle are pure trypanotolerant populations, and 24% are related to trypanotolerant breeds, and the rest are trypanosusceptible. Considerable efforts are now being channelled towards identifying physiological and genetic mechanisms underlying bovine trypanotolerance with the overall aim of exploiting this information to conserve this desirable trait and improve the natural immunity, survival and productivity of the numerous trypanosusceptible cattle populations living in high transmission tsetse infested zones in sub-Saharan Africa through strategic breeding programmes. This need to conserve trypanotolerant breeds has necessitated the mapping of genetic loci associated with trypanotolerance.

The West African N'dama cattle population are so far the best characterized trypanotolerant livestock. They are the African representatives of pure humpless taurines, although molecular characterization studies have indicated indicine admixture (Loftus et al. 1994). Although trypanotolerance has largely been described as a West African taurine-associated trait i.e. in pure taurines or due to taurine introgression, one unique indicine cattle population, the Orma boran of East African has been shown to have superior response to tsetse and trypanosome challenge. These cattle are thought to have independently acquired tolerance from tsetse exposure compared to the other indicine population as improved Kenya boran that live in a similar habitat (Njogu et al. 1985). Breeding and rearing of trypanotolerant cattle has been advanced as the most favourable means to realize sustainable livestock rearing in tsetse-infested regions of

Sub-Saharan Africa. This however calls for a thorough understanding of the mechanisms underlying trypanotolerance.

Trypanotolerance has been described as a complex trait involving intricate interplay of both physiological and genetic components (Murray et al. 1982). The mechanisms behind bovine trypanotolerance have been extensively explored over the years. Trypanotolerant animals are known to exhibit superior innate systems to control pathology while maintaining productivity. Naessens viewed bovine trypanotolerance to be attributed in part to a natural ability to prevent severe anaemia and haemophagocytic syndrome, implying that tolerant animals possessed an inherent ability to maintain their packed cell volume (PCV) and to limit destruction of their red blood cells by hyperactivated phagocytosing macrophages (Naessens 2006). This observation was strongly supported by previous experiments that involved generation of haemapoietic N'dama and Boran twin chimeras through embryo transfer and placental fusion (Naessens et al. 2003). When the chimeric twins were compared against their pure N'dama and Boran parents upon challenge with *T.congolense*, the N'dama chimeras lost the resistance phenotype and exhibited high anaemia as the native Borans, while the native N'dama animals maintained their PCV. Interestingly though, the N'dama chimeras, albeit loosing capacity to control anaemia, controlled their parasite load well in a similar pattern as their native parents, while the Boran chimeras and their native parents displayed high parasitemia. These observations led to the conclusion that the haemopoietic system played a crucial role in trypanotolerance by controlling anaemia, a significant marker of pathology in animal Trypanosomiasis. However, the haematopoietic system had little role in parasite burden, which was likely to be under the control of other factors of the innate and adaptive immunity

2.6 **Mouse models for the study of bovine trypanotolerance**

Variations in response to trypanosome infection was demonstrated in inbred laboratory mice by Morrison who showed response of mice to *T.congolense* varied by strain as evidenced by discrepancies in their spleen lymphocyte populations during the course of infection (Morrison et al. 1978). This relative susceptibility and tolerance to

trypanosome infection was proved to be a heritable trait. C57BL/6 mice were the most tolerant strain with a mean survival time (MST) of 110 days post infection (p.i) with *T.congolense*, whereas Balb/c mice were moderately susceptible at MST 50 days and A/J mice were highly susceptible succumbing to the infection at MST 16 days (Morrison and Murray 1979).

Trypanotolerance in mice is however, not completely analogous to that in cattle in that there as some fundamental differences. Firstly, in mice, the tolerant C57BL/6 mice have been showed to have lower parasite levels but higher anaemia levels than the susceptible A/J counterparts during the course of infection, whereas in cattle, the tolerant N'dama animals exhibit lower parasitemia as well as lower anaemia levels than their susceptible Boran equivalents (Nakamura et al. 2003). This observation implied that trypanotolerant in murine models depended largely on capacity to control parasitemia, whereas in cattle, it was attributable to both parasite and anaemia control but largely to control of anaemia (Naessens 2006).

A second fundamental difference between murine and bovine trypanotolerance is reflected in the mechanisms employed to control parasitemia levels. Mice rely heavily on antibodies particularly IgG antibodies to control parasite burden, whereas in bovines parasite control is not dependent on antibodies, but rather on innate immune responses (Campbell et al. 1977). Trypanotolerant cattle are by-products of natural selection over many decades, which is not the case for mouse models. Indeed, mechanisms that seem critical for murine trypanotolerance as the antibody responses may be quite irrelevant in bovines. Caution needs to be exercised in choice of animal models for trypanotolerance studies and also in the interpretation of results from such studies (Naessens 2006). Nevertheless, owing to their short generation time and relative ease in physical and genetic manipulation as well as availability of various genetic combinations in the form of mutants and knock-outs, laboratory mice strains remain the preferred models for characterization of trypanotolerance in bovines (Iraqi et al. 2000; Kemp et al. 1997; Kemp and Teale 1998).

2.7 **Mapping of quantitative trait loci for trypanotolerance**

Having appreciated trypanotolerance to be a complex polygenic trait, Kemp et al explored the genetic basis of this trait in the mouse model by applying a high resolution murine linkage map based polymormophic microsatellite markers to perform a genome-wide scan for genetic loci controlling resistance (Kemp et al. 1997). F2 progeny derived from crossing the susceptible A/J and Balb/c mice with the resistant C57BL/6 mice were generated and challenged with *T.congolense* parasites. The long and short survivors were then genotyped with microsatellites spanning all the 19 autosomes and the X chromosome. The study revealed three regions in chromosomes 17, 5 and 1 to have a logarithm of odds (LOD) of greater than 2. The LOD score is useful in genetic linkage analysis as a measure of the strength of association between a marker(s) and a given trait (Ott et al. 1999). An LOD score greater than 3 is considered significant and denotes evidence of linkage of presence of a quantitative trait locus (QTL). Therefore, based on a confidence interval of 10-40cM, Kemp et al deduced that the regions on chromosomes 17, 5 and 1 exhibiting high LOD scores were associated with trypanotolerance and were putative QTLs. These QTLs were hence designated as Tir1, Tir2 and Tir3 respectively. On further analyses they showed that Tir1 and Tir2 (on chromosomes 17 and 5) were highly correlated with longer survival time. Tir1 and its association with longer survival under *T.congolense* challenge, was further confirmed with an F3 population of AJ and C57BL/6 cross where the individuals were homozygous for either resistant loci derived from the C57BL/6 parent or the susceptible loci derived from the A/J parent (Kemp and Teale 1998).

Subsequent studies thereafter undertook to confirm the QTLs and map them at a finer scale with lower confidence intervals. Previous studies had utilized F2 intercrosses which had low recombinant and therefore restricted the QTL mapping to large confidence intervals. However, the advanced intercross design had been shown to have the potential to increase the recombination between loci thereby amplifying the confidence interval and resolution of the resultant QTL map for positional cloning and positional candidate gene identification (Darvasi and Soller 1995). In this design, Advanced Intercross Lines (AIL) of mice were obtained by random and sequential

intercrossing of F2 mice for several generations until the desired genotype was achieved. This approach was applied by Iraqi et al to fine map the trypanotolerance QTLs. Using this approach the 3 trypanotolerance QTLS were confirmed and fine-mapped with a confidence interval of 15cM (Iraqi et al. 2000).

2.8 Host-pathogen interactions and Immune responses during *T.congolense* infection

Consequences of interactions between the bovine host and the *T.congolense* parasites are the precinct of host immune responses and pathologies associated with bovine Trypanosomiasis. Innate and adaptive host immune responses are elicited by presence of the parasite or immunogenic structural components of the parasites in the host's circulation. A much desired consequence of these immune responses is protection of the host through parasite clearance, although these mechanisms may indirectly set stage for severe host pathologies. Trypanosomes have been shown to release a range of biologically active products including surface glycoproteins, toxic metabolites and enzymes which effectively activate host immune responses (Tizard et al. 1978). However, significant host response are direct against the variant surface glycoproteins (VSGs) released by metacyclic forms of the parasites which are the infective stages in the mammalian host.

2.8.1 Synthesis and structure of the Trypanosome VSG

The variant surface glycoprotein (VSG) is the principal antigenic component of *T.congolense* and other African trypanosomes. VSGs are encoded by a repertoire of more than 1000 transcriptionally inactive genes comprising >10% of the parasite's total genome (Cross 1975). Antigenic variation is occurs as a consequence of continuous stochastic switching and transcription of the VSG genes at the rate of 10^{-3} and 10^{-4} per cell division on a single transcriptionally active site (Cross et al. 1998; Donelson 2003). VSG synthesis occurs in the endoplasmic reticulum and newly synthesized molecules are modified by N-linked glycosylation and addition of glycophosphoinositol (GPI) anchors, after which they are exocytosed to the cell surface with concomitant addition of oligosaccharide chains and incorporated into the parasite's plasma membrane (Bangs et

al. 1997; Duszenko et al. 1988). The VSG molecules dimerize and form a dense monolayer surface coat of about 12-15nm that completely envelopes the parasite. In *T.congolense* parasites it is estimated that each parasite is coated with about 10^7 molecules of VSG accounting for 10-20% of the total protein content of the parasite (Vickerman 1969).

The VSG structure of trypanosomes is organized in two domains: the C-terminal domain and the N-terminal domain. The C-terminal domain consists of about 100 amino acids and is quite conserved, whereas the N-terminal consists of 300-500 amino acids and is highly polymorphic. In the secondary structure, the C-terminal forms a core of four α-helices and the N-terminal forms a scaffold around the C-terminal core structure. Interestingly, different VSG molecules have quite divergent primary structures with <16% sequence similarity largely due to the variations in the composition of their N-terminals, although they maintain quite conserved secondary and tertiary structures (Blum et al. 1993; Chattopadhyay et al. 2005).

VSG molecules occur in both membrane-bound and soluble forms. The membrane form of VSG (mfVSG) is tethered to the parasite membrane via the GPI anchor whereas the soluble form of VSG (sVSG) is released into the host bloodstream (Cardoso de Almeida et al. 1984). In mfVSG, the C-terminal of the VSG is linked via a phosphodiester bond to the parasite's cell membrane through a glycosylphosphatidylinositol (GPI) anchor. The GPI anchor consists of hydrophilic carbohydrate and hydrophobic lipid moieties. The hydrophilic moiety is comprised of ethanolamine, a mannose chain and glucosamine in a conserved motif whereas the hydrophobic moiety is comprised of dimyristoyl-phosphatidylinositol (Cross 1984b). Release of the mfVSG is orchestrated by a GPI-specific phospholipase C (GPI-PLC), which is expressed in numerous copies (> 30,000) by each trypanosome (Bulow and Overath 1986; Cross 1984a). This endogenous GPI-PLC selectively cleaves the VSG at the phosphodiester bond on the lipid moiety, effectively releasing the sVSG and leaving dimyristoylglycerol anchored to the membrane.

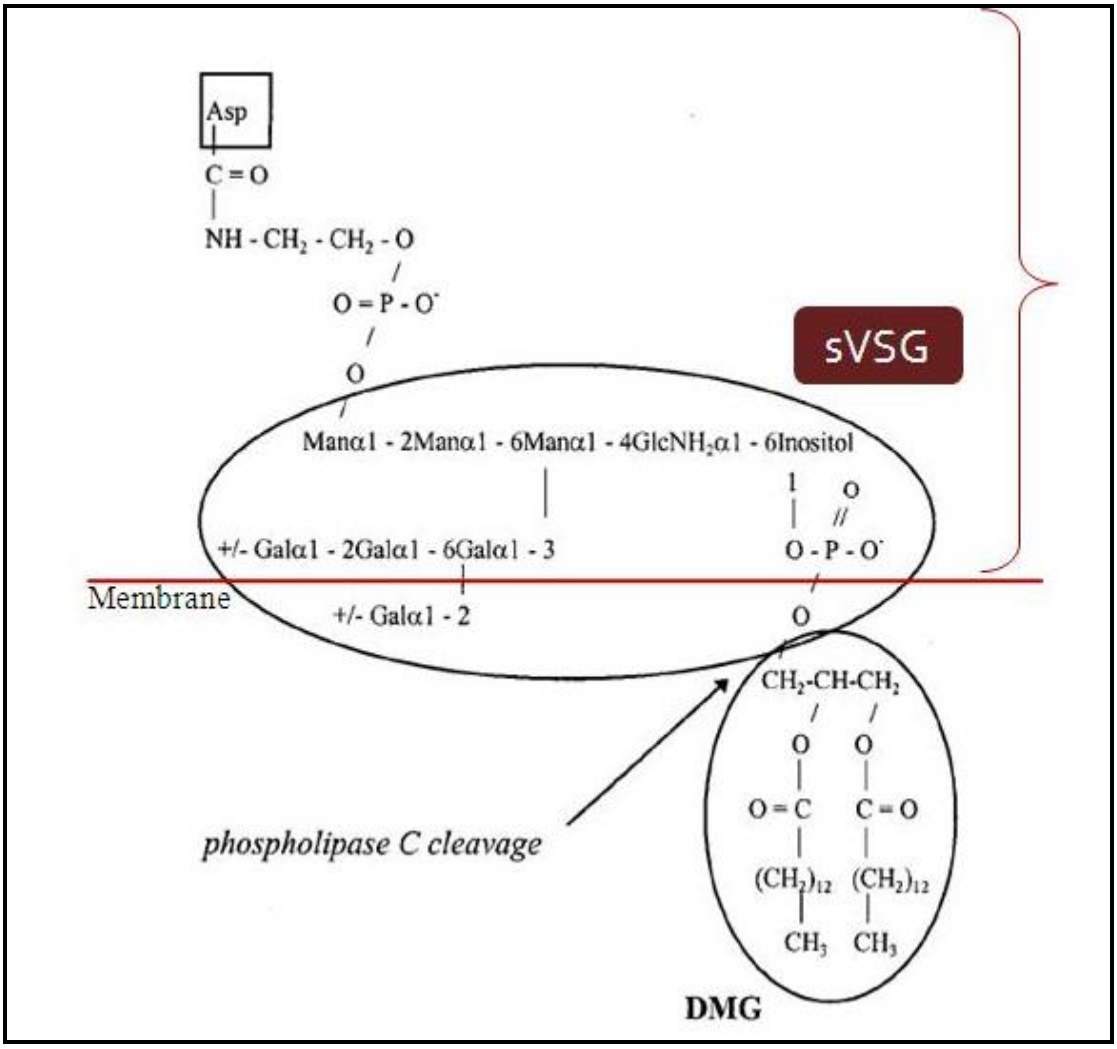


Fig 1.2: The structure of trypanosome variant surface glycoprotein (VSG). VSG is anchored to the plasma membrane via the GPI anchor. Cleavage of the VSG by phospholipase C enzyme releases soluble VSG (sVSG). Figure is adapted from Magez et al, 1998.

2.8.2 Immune responses to *T.congolense* VSG

Immune responses to the bloodstream form of *T.congolense* are primarily directed against parasite variant surface glycoprotein (VSG). Each wave of parasitemia presents parasite colonies with unique VSG signatures (Sternberg 2004). The VSG molecules in successive waves of parasitemia are usually antigenically distinct and non-cross-reactive affording the parasite novel antigenic coats in successive generations to elicit a fresh host immune response. At peak blood levels of 10^8 parasites/ml in the mouse model, the total estimate of VSG load in the host is substantial at about 2µg (Magez et al. 2002; Paulnock and Coller 2001). These high VSG levels, coupled with unique molecular signatures mean that *T.congolense* VSG plays a pivotal role in host immune response as immunodominant antigen. Substantial knowledge has been accumulated on T-cell, B-cell and innate responses to trypanosome VSG and is further reviewed in the following sections.

2.8.2.1 T-lymphocyte responses

T-lymphocytes drive essential immune processes through secretion of cytokines, parasite killing through cytotoxicity and modulation of other immune effectors. As such, investigations have been conducted to delineate to role of T-lymphocytes in Trypanosomiasis. In experimental murine Trypanosomiasis *T.brucei* VSG did not elicit clonal proliferation of T-cell populations in infected mice although it induced secretion of IFN-γ and IL-2 (Schleifer et al. 1993). These VSG-specific T-cells lacked cytotoxic properties and were MHC class II restricted. The above observations were echoed in bovine hosts where T-cell proliferation was not observed in a variety of cells in response to *T.congolense* VSG stimulation, although an INF-γ response was induced in lymph node cells that was more pronounced in cells from susceptible Boran cattle than tolerant N'dama cattle (Lutje et al. 1996). No differences in T-cell responses to *T.congolense* VSG between tolerant and susceptible cattle were noted implying that T-cell responses do not play a significant role in disease tolerance or susceptibility.

2.8.2.2 Antibody responses

Humoral responses through B-cell activation and production of antibodies against parasite VSG is one of the chief host control strategies against *T.congolense*. Effective antibody responses have been demonstrated to correlate with tolerance to *T.congolense* infections in murine models where the tolerant model C57BL/6 mounts high VSG-specific IgM titres upon primary challenge whereas the susceptible A/J model responds with little or no antibodies (Mitchell and Pearson 1986). In trypanosome infections, VSG-specific antibodies opsonise the VSG-expressing trypanosomes and target them for destruction by complement lysis or phagocytosis. A correlation has been demonstrated between the antibody isotype and tolerance to *T.congolense* infections where a prompt anti-VSG IgM response is required for clearance of the first parasiteamia peak and survival was dependent on a timely IgM-IgG switch (Uzonna et al. 1999).

Complete immunity to infection is however, not achievable with anti-VSG antibodies as effective responses require presence of consistently high titres of antibodies, which are not achievable due to the parasite's genius mechanism of rapid synthesis and turnover of new VSG molecules. It is indeed demonstrated that trypanosomes are able to endocytose VSG-antibody complexes from their cell surfaces, render them for intracellular destruction in their lysosomes and effectively replace them with a fresh pool of VSG molecules within 12mins (Engstler et al. 2004). This rapid turnover rate of VSG is not comparable to the time in days required by the host to mount a VSG-specific antibody response implying that the antibody response although important may not be a primary contributing factor to resistance to *T.congolense* infections (Mitchell and Pearson 1986).

Antibody responses are known to be activated through either T-cell-dependent T-cell-independent mechanisms (Snow 1991). Characterization of the antibody responses to *T.congolense* VSG have shown that B-cell activation in this instance is T-cell independent as athymic mice were demonstrated to develop similar anti-VSG antibody titres as normal mice (Pinder et al. 1986). Other investigations have confirmed that T-cell independent polyclonal B-cell activation occurs in *T.congolense*-infected mice and

cattle effectively increasing the B-cell population leading to heightened antibody responses (Morrison et al. 1978; Naessens and Williams 1992).

2.8.2.3 Complement-mediated lysis

The complement system comprises several soluble factors present in serum which have an inherent capacity to effect non-specific lysis of parasite (Osler 1976). During *T.congolense* infection in mice and cattle the complement system is mainly activated by VSG-antibody immune complexes (Nielsen et al. 1978) although dead parasites are also capable of the same (Nielsen and Sheppard 1977). In a twist of expectations, the VSG coat is shown to be protective to the parasite effectively shielding it from destruction by complement where parasites without a VSG coat were more susceptible to complement lysis (Ferrante and Allison 1983). *T.congolense* infection triggers activation of both classical and alternative pathways of complement (Nielsen et al. 1978) and excessive activation of complement by VSG-antibody immune complexes has been linked to development of pathologies as adverse tissue damage (Uche and Jones 1992) and susceptibility to secondary bacterial infections (Tabel et al. 2000). There however, appears to be no correlation between complement mediated lysis and tolerance experimental *T.congolense* infection as no significant differences are noted in survival between normal mice and those with a defective complement system (Tabel et al. 2000).

2.8.2.4 Cytotoxic activity by natural killer cells

Natural killer (NK) cells participate in the clearance of trypanosomes and other extracellular parasites through non-specific cytotoxicity. Although their role in *T.congolense* infection remains poorly understood, their activity in lysis of *T.cruzi* and *T.brucei* parasites has been demonstrated in mice (Hatcher and Kuhn 1982). Albright and colleagues also showed that the activity of natural killer cells varied across different trypanosome species (Albright et al. 1984). The most significant role of natural killer cells is however the provision of an early source of IFN-γ, TNF-α and chemokines which facilitate development of inflammatory responses in infected animals (Cardillo et al. 1996).

2.8.2.5 Macrophage responses

Macrophages play a central role in immunity against *T.congolense* infections by participating in a series of processes including phagocytosis, production of reactive nitrogen species and secretion of cytokines. Destruction of *T.congolense* VSG-antibody complexes by phagocytosis is proposed to be the most important clearance mechanism (Shi et al. 2004). During this process, opsonisation of trypanosomes by VSG-specific antibodies preceded their destruction by lytic activity in macrophage phagosomes (Dempsey and Mansfield 1983). Optimal parasite clearance by phagocytosis is dependent on complement activity (Macaskill et al. 1980). The liver has been demonstrated to be the major site for phagocytosis where destruction of *T.congolense* parasites by Kupffer cells has been associated with development of a systemic inflammatory response syndrome that caused early mortality in experimental murine models (Shi et al. 2003; Shi et al. 2004).

T.congolense VSG infection leads to secretion of IFN-γ by spleen cells and natural killer cells (Kaushik et al. 1999). IFN-γ-dependent priming of macrophages results in the secretion of nitric oxide (NO) and a range of proinflammatory cytokines as TNF-α, IL-1α, IL-1β, IL-6 and IL12p40 (Kaushik et al. 2000). NO is a product of the inducible nitric oxide synthase (iNOS) gene and its secretion by activated macrophages during *T.congolense* infection has been shown to exert trypanostatic effects on the parasites. The expression of the iNOS gene is influenced by host genetic susceptibility to *T.congolense* infections, where tolerant N'dama cattle and C57BL/6 mice secrete high levels of NO (Kaushik et al. 1999; Taylor et al. 1998).

TNF-α secreted by activated macrophages is known to synergize with IFN-γ to amplify NO secretion (Kamijo et al. 1993). In *T.brucei* infections, TNF-α was demonstrated to lyse the parasites (Magez et al. 1997), although these trypanolytic effects of TNF-α were not observed with *T.congolense* parasites (Kitani et al. 2002). Nevertheless, TNF-α is crucial for parasite control in *T.congolense* infections as TNF knockout mice are predisposed to infection and high parasitemia levels (Iraqi et al. 2001; Naessens et al. 2004). It is postulated that protective host immunity in murine *T.congolense* infections is

dependent on a combination of an early inflammatory response and antibody responses where inflammatory effectors as nitric oxide compromising parasite fitness and viability effectively rendering them more prone to opsonisation by antibodies and clearance by phagocytosis (Magez et al. 2006).

Noteworthy is the fact that macrophages act as a crucial link to innate and adaptive responses to *T.congolense* infections. Macrophages participate in activation of adaptive responses by processing and presenting *T.congolense* antigens to T-lymphocytes (Dagenais et al. 2009). Additionally, depending on their activation status macrophages secrete inflammatory and anti-inflammatory cytokines as well as type I and type II chemokines which are capable of modulating the T-lymphocyte responses to either Th1 or Th2 (Noel et al. 2002). Inflammation in the acute phase of infection has been associated with a predominant macrophage-derived type I cytokine milieu consisting of but not limited to TNF-α, IL-1, IL-6 and IL-12 whereas a type II cytokine repertoire consisting of IL-4, IL-2, IL-10 and TGF-β has been associated with immune suppression and chronic disease (Mertens et al. 1999; O'Gorman et al. 2006; Uzonna et al. 1998). Cytokines secreted by macrophages have also been implicated in the development of pathology during *T.congolense* infection including anaemia (Noyes et al. 2009), cachexia (Korner et al. 2010), pyrexia (Mutayoba et al. 1995), hepatomegaly (Shi et al. 2005) and spleenomegaly (Morrison et al. 2010). Minimal information has been availed on the cytokine response to the parasite's immudominant antigen VSG, although studies in the human trypanosome *T.brucei* have shown VSG as a potent inducer of cytokine secretion (Coller et al. 2003; Magez et al. 1998). Additionally, the response and role of chemokines in *T.congolense* infection is poorly understood.

These facts suggest that macrophage responses have a heavy influence on the polarization of immune responses, pathology and pathogenesis of *T.congolense* infections. However, much of the knowledge available on macrophage responses to African Trypanosomes is mostly based on *T.brucei* which infects humans. The interactions of host macrophages with the most important African livestock trypanosome *T.congolense* remain largely unexplored and extensive studies towards this end are required to add onto existing knowledge. For instance, there is need to characterize

macrophage responses to *T.congolense* VSG and other antigens as shown by their cytokine and chemokine profiles and their activation status. There also remains a need for a comprehensive analysis of gene expression profiles elicited by macrophages in response to *T.congolense* parasites and *T.congolense* antigens most notably, VSG. Such investigations will give invaluable insights to significant host-pathogen interactions taking place during *T.congolense* infection. They would also provide clues into inherent mechanisms underlying pathology and tolerance or susceptibility to disease.

2.9 **Characterization of host-pathogen interactions**

2.9.1 Gene expression profiling by microarray technology

Microarrays have been utilized to quantify gene expression patterns in cells and tissues in response to various biological stimuli. In medical and veterinary medicine, DNA microarrays have been widely used to provide insights on gene expression differences between disease and healthy cells and to discover useful biomarkers that predict presence or absence of a disease.

DNA microarrays are anchored on the principle of complementary hybridization, where thousands of known genes within the genome of interest are represented by probes which are immobilized onto the array chip. In Genechip platforms as Affymetrix, the abundance of a particular transcript is estimated based on the signal generated by a set of 11-20 probe pairs. Each probe pair consists of a perfect match (PM) probe which has 100% sequence complementarity to a locus on the target transcript and a mismatch (MM) probe which differs from the PM probe at the 13^{th} nucleotide due to the introduction of a reverse complement nucleotide. Messenger RNA from the target cells or tissues is labeled with fluorescent dye and allowed to hybridize onto complementary probes on the array. Signals are generated as fluorescent intensities by laser scanning of the array and an image of the intensities is rendered where each probe is seen as a spot on the image whose intensity is proportional to the degree of hybridization and the level of expression for the target gene transcript. However, in order to draw meaningful biological interpretations from microarray experiments, the raw intensity values need to be adjusted for technical variations in order to retain biological variations only. Several

data processing steps are undertaken to achieve this which include: Background correction, normalization, probe summarization, classification, statistical identification of differentially regulated genes and interpretation of the results in the biological context (Muller and Nicolau 2005).

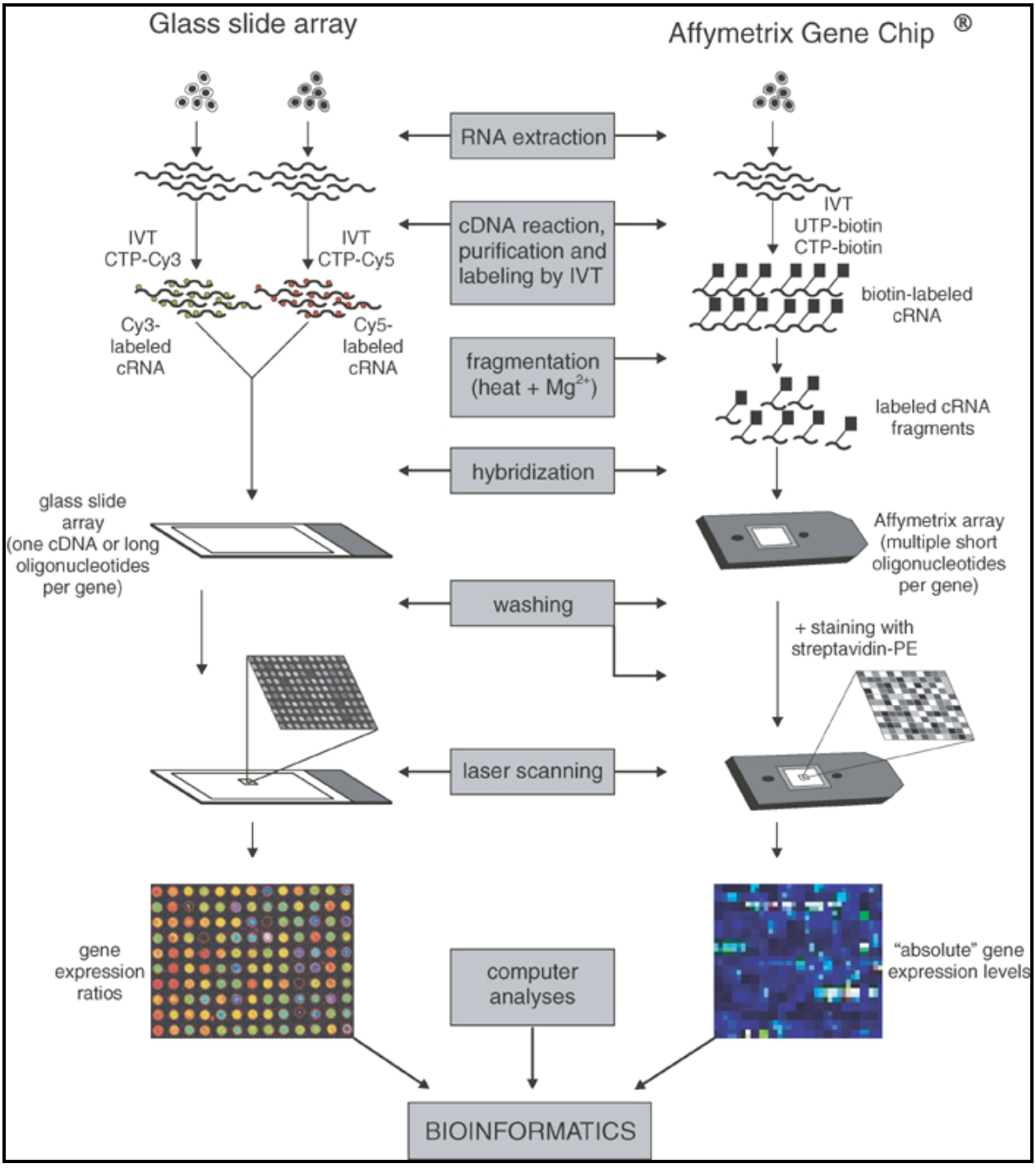


Fig 1.3: Summary of the microarray workflow for two-channel and single channel cDNA arrays (Staal et al, 2003). Fundamental principles of the technique involve extraction of total RNA and conversion to cDNA, followed by hybridization of gragmented cDNA onto array. After washing off unhybrized cDNA, the arrays are scanned to give a readout in the form of intensities, which are subjected to further computational analyses.

2.9.1.1 Background correction

Fluorescence intensities on an array result from specific binding of labeled transcripts to probes or they may be contributed by dye bias in two-channel experiments, non-specific hybridization, cross-hybridization, systematic variation or autoflourescence from the chip surface with the latter four being the main sources of background noise in single-channel Genechip arrays. In order to accurately estimate the transcript abundance, background correction is undertaken to subtract noise from the raw intensities. This can be simplistically done by subtracting the MM from the PM probe intensities, although studies have indicated that hybridization of MM probes contributes significantly to the signal and that subtracting MM probe intensities may fundamentally bias the results (Chudin et al. 2002). Background adjustment may also be achieved by subtracting the average intensity of the areas between the spots on the array (Yang et al. 2002). This approach is however not without challenges as it may give rise to spots with negative signal intensities if the background intensity is higher than the spot intensity. Other studies have indicated that skipping the background correction altogether had no effect on downstream estimation of differentially expressed genes (Tran et al. 2002; Yang et al. 2001).

More sophisticated methods of background correction that solve the problem of negative intensities have been developed. The exponential-normal convoluted model and variance stabilization are two such methods. The convolution model is based on the fact that true signal intensities have an exponential distribution whereas the ubiquitous noise is normally distributed and truncated at zero to avoid negative values (Chen et al. 2009; McGee and Chen 2006). This method is intensive on information required to implement it and is restricted to GenePix data only and cannot be applied on Affymetrix data. Variance stabilization is another method for background adjustment, where intensities are transformed by a differential statistic whose variance is constant along the whole intensity range (Huber et al. 2002). This correction which is utilized for background adjustment in this study is rational for multivariate analysis, can be applied on data from different platforms and provides a basis for further statistical inference of the data.

2.9.1.2 Normalization and probe summarization

Normalization is performed on background adjusted intensities to remove non-biological variation and facilitate comparisons between different arrays. It compensates for differences in labeling, and detection methods. Normalized probe level intensities are converted to probeset expression values by probe summarization algorithms resulting in an expression matrix. Numerous methods for normalization and probe summarization are available, they may be global or intensity-dependent, based on linear or non-linear models and usage of a particular approach is typically determined by type of array and the assumptions made about biases in the data. Normalization can be performed based on all genes in the array as in global normalizations based on the assumption of uniformity is initial RNA concentrations and hence equal levels of hybridization signal in all arrays, or may be based on non-variable genes as house-keeping genes or "spiked" internal control genes (Kepler et al. 2002; Quackenbush 2002). Caution has been advanced when normalizing against house-keeping genes as they have been demonstrated to vary significantly hence potentially introducing more errors (Kim et al. 2002). A method for normalizing one-channel array data based on an *ad-hoc* selected invariant set of genes has been developed by Li and Wong on the assumption that there would always be a group of genes in any given experiment whose expression does not change (Li and Wong 2001). The limitation however is that each experiment presents a unique set of invariant genes.

Linear and non-linear regression methods exist for normalization. Linear approaches for normalization assume similar labeling and detection efficiencies for all samples, in which case signal intensities are adjusted to and distributed around a line of best fit whose slope is one (Finkelstein et al. 2002). The MAS5 algorithm that is commonly used in one-channel arrays as Affymetrix Genechip is based on linear regression where 1% of extremely high and low probes intensities are eliminated and a regression line subsequently fitted for 98% of the remaining probe intensities (Hubbell et al. 2002). In instances where the general distribution of signal intensities is non-linear, regression approaches may be utilized for normalization. A popular regression normalization method which is utilized in two-channel is the LOcally WEighted Scatterplot Smoothing

(LOWESS), which adjusts the signal intensities by fitting a smoothing curve (Cleveland and Devlin 1988).

Quantile methods adjust probe intensities in experimental arrays by normalizing them against a reference chip. In this approach, the distribution of each of the probes is pooled across all arrays and a quantile value of the intensities of each probe's distribution computed. The original probe intensities are transformed to the quantile value on the reference chip. This transformation is robust enough to cope with both linear and non-linear distributions and is computationally non-intensive (Bolstad et al. 2003). The Robust Multi-array Average (RMA) employed in this study relies on quantile normalization (Irizarry et al. 2003). A variant of the RMA method which factors in differential hybridization affinity of the probes due to their GC content has also been developed (Wu et al. 2003).

Comparative investigations of the effect of different normalization methods on data analysis and gene expression outputs have been done (Harr and Schlotterer 2006; Lim et al. 2007; Williams et al. 2006). Lim et al benchmarked on a dataset the performance of MAS5, RMA, GCRMA and Invariant set which are popularly used normalization methods and concluded that no single approach was without flaw and that the selected method employed could significantly alter the gene expression outcome. Harr and Schlotterer recommend that the ultimate choice of the normalization approach should be driven by the objective of the study. A positive observation however is that utilizing different normalization approaches on the same dataset will result in different though largely overlapping (>90%) lists of differentially expressed genes (Quackenbush 2006).

2.9.1.3 Pattern recognition by gene clustering

The level of expression for each gene is visually represented by an expression matrix obtained after normalization and probeset summarization algorithms. The expression values are often converted to log ratios and rendered in varying intensities of black, green or red representing no change, downregulation or upregulation respectively, relative to identical probesets in a reference or control array. The expression matrix generally has no pattern or order and interpretation usually relies on classification by

gene clustering to identify biological patterns e.g. which genes are differentially regulated, which genes share similar expression profiles and which genes are co-regulated.

Gene clustering is accomplished primarily through supervised or unsupervised learning. In supervised learning, clustering algorithms are informed by pre-existing information on the biological states (e.g. normal and diseased), processes or functions of the genes in question, whereas unsupervised learning assumes no pre-existing information on the genes but rather classifies the probesets according to their similarities in expression profiles and are therefore ideal for exploratory analyses.

Supervised support vector machines (Brown et al. 2000), Decision trees (Cios et al. 2007), Naïve bayes (Demichelis et al. 2006) and Neural networks (Lancashire et al. 2009) are some commonly utilized supervised algorithms for gene clustering. Supervised methods are advantageous in that they incorporate biological knowledge which is useful in inferring the output but their greatest pitfall is their paucity to discover novel gene clusters and associations.

Hierarchical clustering (Sturn et al. 2002) and K-means clustering (Tavazoie et al. 1999) are unsupervised approaches for gene clustering. K-means clustering partitions observations (i.e. probe intensities) into clusters through iterative assignment of probesets to the cluster with the nearest mean based on a Euclidean distance until convergence is achieved (Hartigan and Wong 1979). Although K-means clustering is popularly employed in clustering microarray data, it has several limitations in that i) the algorithm relies on a pre-defined number of cluster (k) which if not optimal compromises the results ii) it is computationally memory-intensive and time-consuming as the observations have to be iteratively assigned to different clusters until convergence is achieved iii) the algorithms aims to produce clusters of a similar size resulting in erroneous assignment of genes to clusters which may not be of any biological significance (Peña et al. 1999).

Hierarchical clustering on the other hand does not rely on a pre-set number of clusters but rather groups the observations (i.e. probeset intensities) based on either

agglomerative or divisive hierarchical algorithms. Agglomerative hierarchical algorithms are more commonly employed and assume that each observation is a cluster and successively builds up hierarchical clusters by aggregating individual observations according to any valid measure of distance including Eucledian, maximum, manhattan distances (Day and Edelsbrunner 1984). Divisive algorithms assume a single cluster for the whole dataset and successively split the cluster into sub-clusters based on the distance relatedness of the individual observations (Guénoche et al. 1991). Agglomerative hierarchical clustering was employed in this study the method of choice for exploratory analysis of the microarray data due to its effectiveness in discovery of small clusters. It is also comparatively faster to run than k-means and the results can be viewed as a hierarchical dendogram.

2.9.1.4 Analysis of perturbed genes

Differential regulation of gene expression in a microarray experiment can be analyzed on at least three levels in increasing complexity; first of the single gene level where differences in gene expression of specific genes is quantified, second is regulation of gene groups of clusters with similar expression patterns and third is regulation of complex gene networks. Ascertaining differential regulation at any of the above levels however requires appropriate statistical methods.

The objective of any microarray experiment is to quantify gene expression between a control sample and test sample. A simplistic approach to decipher this is to compute of fold changes where a gene is said to have significant variation if its average log expression values in the control and test sample is more that an arbitrary constant factor, usually two (DeRisi et al. 1997). This approach has no statistical background and has limited biological foundation as a fold change of two may have a profound or insignificant effect in the biological context. A better tool to overcome these limitations is the t test, which employs the empirical means and variances between two samples to compute a normalized distance between the two samples (Student 1908). Although superior to the fold change approach in statistical inference, the t test is confined to differential expression between two treatments only and cannot be used in comparison

of more than two conditions. Robust t tests also require a high number of replicates which presents a limitation as microarray experiments usually have limited replicates due to sampling and cost implications. Furthermore, t tests are limited by the multiple testing challenges posed by the high number of genes presented by microarrays experiments.

In instances where differential gene expression is being computed for more than two conditions, analysis of variance (ANOVA) is the preferred statistical method (Kerr et al, 2000). Here, the expression of gene A is computed relative to the weighted average of the gene in all conditions in the experiment. This yields the *F* statistic which detects variations among sample replicates and between conditions. Where multiple sources of variation are anticipated, mixed ANOVA models are recommended (Cui and Churchill 2003).

Classical statistics as Students t and ANOVA tests infer differential expression of genes by testing against a null hypothesis that assumes no differences between the test samples. Conclusions are made by applying a user-defined confidence interval and minimizing type I error. However, biological systems are known to have a high degree of dimensionality which cannot be adequately simulated by microarray experiments. Even a large multifactorial microarray experiment has limited capacity to measure all possible variables. Therefore majority of the hidden variables can only be inferred by incorporating probability in statistical methods used. The Bayesian statistics therefore presents a powerful way of combining probabilistic methods in statistical analysis of microarray data (Baldi and Long 2001). This framework is founded on the Bayes theorem and differential expression is computed as a posterior distribution which the condition distribution of the genes as inferred from a prior distribution and actual observations (Shoemaker et al. 1999). Therefore for a list of differentially expressed genes in a Bayesian framework, confidence is assessed directly by calculating the probability that each of the genes is truly differentially expressed as opposed to classical statistics where confidence is based on a user-specified cut-off. Bayesian statistics have been incorporated successfully with classical ANOVA and t tests to infer differential expression (Gottardo et al. 2003; Smyth 2004).

2.9.1.5 Analysis of gene networks and pathways

In the biological context genes do not occur or function in isolation, but rather in gene networks and biological pathways. Therefore, biological processes and phenotypes are a cumulative effect of interactions between several genes. On the basis of this, there is need to understand the effects that differential gene expression bear upon biological networks and pathways.

The interactions of genes within networks and pathways can be computationally modeled to simulate the biological context. Two main approaches exist for this: Boolean and continuous modeling. Boolean modeling relates gene interactions based on logical functions within a directed graph/network where the expression status of a gene is denoted as either ON or OFF (Kauffman and Glass 1973; Shmulevich et al. 2002). This model is more suited for prokaryotic genomes and presents limitations in eukaryotic and metazoan genomes where gene expression is dependent on transcript abundance whose levels are dynamic. Continuous models are based on similar concepts as Boolean models but attempt to overcome the binary approach of Boolean models by incorporating changes in transcript abundance over time. Since gene expression in biological systems is stochastic in nature, continuous models present a powerful approach to delineate the dynamics that depend on finer timing and exact molecular concentrations of RNA transcripts (Hatzimanikatis and Lee 1999; Neidhart and Savageau 1996).

A formidable challenge in the presentation of gene networks is need for standardized ontologies because genes have specific functions which feed into a pipeline of a process occurring in a specialized biological compartment. This challenge has been addressed by the gene ontology consortium which has developed the Gene Ontology (GO) resource for eukaryotes, where genes and their relationships in the gene networks have been described using controlled vocabulary (Ashburner et al. 2000). This resource draws on the high degree of sequence and functional similarities in eukaryotic genes and defines each gene by three attributes i) its molecular function ii) the biological process it participates in and iii) the cellular compartment where process occurs. Information on the biological functions and processes for a given set of differentially regulated genes for

instance can be performed by querying the GO database, essentially by performing an over-representation or enrichment analysis of functional categories, thereby enabling one to deduce functional categories associated with a given condition. GO analysis can also be used to annotate previously unannotated genes enabling researchers to turn data into knowledge (Harris et al. 2004).

Although GO analysis is very useful in interpretation of gene expression data, it is not without limitations. Firstly, the GO database is dependent on existing annotation databases which may be incomplete, not updated or have a degree of incorrect information (King et al. 2003; Wang et al. 2004). Secondly, GO analysis of microarray data does not incorporate experimental evidence of the levels of expression for the given genes and therefore assigns all the represented functional categories an equal weight, which is not useful in inference of the most significant biological processes under a given condition. Thirdly, GO analysis and other over-representation approaches do not consider the position of differentially regulated genes in the network, or whether the gene has multiple functions in different signaling pathways (Khatri and Draghici 2005). The above limitations have been addressed by development of Pathway Express, a tool for impact analysis in a systems biology context which computes the impact of differential regulation of a gene based on the cumulative effect of the magnitude of transcriptional change, position in the pathway, function and interactions with other genes (Draghici et al. 2007). This tool that has been employed for pathway analysis in this study combines statistical and biological evidence to extract the most relevant pathways and gene networks from high-throughput expression experiments for any given cellular environment.

2.9.2 Cytokine and chemokine profiling

Intercellular signaling during immune reactions is effected by cytokines and chemokines which are mainly secreted by leukocytes. Cytokines are soluble proteins secreted in response to infection and function by mediating cell communication during an immune response through stimulating proliferation and differentiation of lymphocytes and haematopoietic cells and activating different effector cells to eliminate microbes and

antigens (Plotnikoff 2007). Chemokines on the other hand are chemotactic cytokines which exhibit high levels of structural homology and whose primary function is promote movement of leukocytes to sites of injury during an immune response (Rossi and Zlotnik 2000). Due to their central importance in host defense, cytokines and chemokines have been explored greatly as therapeutic agents (Koon and Atkins 2008) and as targets for immune regulation through selected agonists (Osman et al, 1998) and antagonists (Alexander and Hilton 2004). This trend has seen them emerge as critical interventions for extreme immunological dysfunctions as inflammatory (Kavanaugh 2002), cancer (Tagawa 2000), autoimmune (Smolen et al, 2005) and immunosuppressive (Parienti 2002) conditions.

Several approaches for detecting cytokines and chemokines in biological samples have been developed. Although there are direct methods for detection of nucleic acids of target cytokines through PCR-based methods, most popular approaches exploit indirect methods through use of immunoassays which detect the cytokine proteins through use of specific antibodies by capitalizing on the simple antigen-antibody binding reaction (Abbas and Lichtman 2004). The western blot technique is one such indirect method, which has been in use for many years. It relies on the basic principles of electrophoresis and immunofixation, where the proteins in a biological sample e.g. serum are separated on a denaturing gel, transferred and immobilized onto an adhesive membrane as nitrocellulose and probed for cytokines or chemokines of interest using specific antibodies (Towbin et al. 1979). However western blots are disadvantageous in that they are not quantitative, require many optimization steps and are time and labour-intensive.

The Enzyme-Linked Immmunosorbent Assay (ELISA) is arguably the standard technique for detection of cytokines and chemokines. In this assay binding of the target cytokine to the complementary antibody is detected as a color change due to the reaction of a coupled enzyme with an appropriate substrate, in which case the colour change is relatively proportional to the concentration of the target cytokine (Wardley and Crowther 1982). ELISA is a versatile immunoassay for which required reagents and equipment are readily available. It is highly specific, sensitive and is preferred over radioimmunoassays as it does not require use of radioisotopes. ELISA yield both

qualitative and quantitative results, is performed with relative ease and speed although it is comparatively most costly than western blots (Crowther 2001).

Several variations of ELISA exist including direct, indirect, competitive and sandwich ELISA although the latter is most commonly employed (Hornbeck 1994). The sandwich ELISA uses two antibodies – a primary antibody normally a monoclonal antibody raised against a specific site of the target cytokine and secondary antibody raised against the primary antibody. A fixed concentration of the primary antibody is immobilized onto a microtitre plate, after which the test sample is added and the binding of the immobilized primary antibody and target cytokine occurs. Unbound antigen is washed off and unbound sites blocked after which the secondary antibody and covalently coupled to an enzyme is added. Finally the color reaction is generated by addition of a specific substrate.

The conventional sandwich ELISA is designed to assay only a single target and requires a considerable amount of starting sample - at least 1ml or more to allow for dilutions and replicates. This becomes a challenge when assaying for multiple cytokines in cell culture experiments where sample volumes are limited. However, this challenge has been overcome by the development of multiplex systems where several cytokines can be analyzed within a single sample. The x-MAP technology developed by the Luminex Corporation enables multi-analyte profiling of cytokines within biological samples including serum, plasma and cell culture supernatants where up to 100 cytokines and chemokines may be assayed simultaneously in a 25µl sample (www.luminexcorp.com).

Cytokine profiling with the x-MAP technology employs polystyrene microspheres which are impregnated with red and infrared flourophores whereby the incorporation of varying intensities of the two flourophores in the microspheres yields bead-sets with up to 100 unique spectral signatures. The spheres are designed with a surface chemistry that allows for the binding of antibodies for capture of specific target cytokines. The reporter system consists of the secondary antibody (IgG) tagged to streptavidin-PE. Detection is achieved through an effective fluidics system employing fluorescent cell sorting (FACS) where laser excitation and fluorescence intensity for each sphere is performed. Although

the beads are pooled during the assay, each microsphere is re-assigned to its original bead-set on the basis of its spectral signature. This technology can be utilized to assay up to 100 different analytes per well (i.e. cytokines and chemokines) in a 96-well plate, effectively yielding high-throughput data, with minimal volumes of starting samples and with great savings on time and cost. This technology has successfully been employed in other investigations (Bremer et al. 2010; Cocco et al. 2009) and was utilized in quantitative analysis of cytokine and chemokines in this study.

3 TRANSCRIPTIONAL PROFILES OF *TRYPANOSOMA CONGOLENSE*-INFECTED MICE REVEAL A BACTERIAL ENDOTOXIC SHOCK EXPRESSION SIGNATURE

3.1 ABSTRACT

A large profile gene expression study was carried out to elucidate gene expression profiles of mice infected with the African animal trypanosome, *T.congolense*. Infections were carried out over 0, 3, 5, 7, 9, 13 and 17 days timecourse and tissue-specific responses of A/J, Balb/c and C57BL/6 mouse strains ascertained to vary in susceptibility to *T.congolense* infection was investigated. This study focused on differential gene expression during the early phase of infection in the liver tissue by comparing uninfected mice (day 0) with those infected for 9 days when peak parasite levels are achieved. The results showed that the early response to *T.congolense* infection was characterized by up-regulation of inflammation genes with a gene expression signature was akin to the endotoxic shock response elicited by the bacterial endotoxin, lipopolysaccharide (LPS). Confirmatory investigations within a standardized and reproducible *in vitro system* are recommended to validate and characterize this response.

3.2 INTRODUCTION

3.2.1 Murine models for infectious diseases

The application of mice as model organisms for infectious and genetic diseases is occasioned by the similarities in the genetic make-up and physiological response between mice and mammalian definitive hosts. This is thought to be the reason why most mammalian diseases and physiological conditions are easily induced and studied in the mouse (Sande, 1999). Additionally, the relative ease of genetic manipulation, lower cost of maintenance and short generation time of the mouse have contributed to the mouse being the premier model in medical and veterinary research (Chow, 2008) .

3.2.2 Mouse models for study of bovine Trypanosomiasis

Mice have emerged as useful tools for the characterization of bovine Trypanosomiasis. They are enlisted as appropriate model organisms due to their ability to establish a comparable course of disease as that observed in bovine infections characterized by initial acute stages coinciding with the peak parasitemia followed by subsequent chronic disease (Morrison et al, 1978; Antoine-Moussiaux et al, 2008). Experimental murine infections demonstrate similar pathological consequences as those of bovine Trypanosomiasis including anaemia (Koyabashi, 1976; Naessens, 2005; Noyes et al, 2009), cachexia (Truyens et al, 2005) and immunosuppression (Dempsey and Mansfield, 1983; Clayton et al, 2007). Moreover, trypanosome infections in murine models present analogous macro-histological effects to those observed in bovine infections including hepatomegaly and spleenomegaly (Takeya et al, 1987).

Advances in the use of mouse models for Trypanosomiasis research in the post-genomics era have led to the identification and use of specific mouse models for the study of the genetic mechanisms of underlying bovine trypanotolerance. Under challenge with *T.congolense* C57BL/6 mice are observed to have a mean survival time of 110 days compared to 50 days in Balb/c and 16 days in A/J mice (Morrison and Murray, 1979; Uzonna et al, 1998). These three mouse strains therefore provide

attractive murine models to study tolerance and susceptibility in bovine Trypanosomiasis. It was further confirmed using the above mouse models that tolerance or susceptibility to *T.congolense* infection was a heritable trait following successful mapping of three quantitative trait loci (QTL) on chromosome 17, 5 and 1 currently designated as Tir1, Tir2 and Tir3 respectively (Kemp et al, 1996; Kemp et al, 1997; Iraqi et al, 2000).

3.2.3 Experimental murine Vs Bovine Trypanosomiasis

There exists significant differences in the course of disease and pathology between experimental murine Trypanosomiasis and bovine Trypanosomiasis and therefore, over-reliance on these mouse models for studies of bovine trypanotolerance is a point of caution. The trypanotolerant murine representative, C57BL/6 exhibits relatively lower parasitemia levels, longer survivability and a marked decrease in packed cell volume when compared against the susceptible A/J and Balb/C mice. The scenario in bovine Trypanosomiasis is different with tolerant N'dama showing long-term survival, better parasitemia and anaemia control under trypanosome infection than their Boran counterparts. These observations therefore suggest that trypanotolerance as defined by long-term survival under trypanosome infection correlates with parasitemia control and not anaemia control in the murine model, whereas in bovine Trypanosomiasis, tolerance is primarily correlated with anaemia control and parasitemia control to a lesser extent (Ogunremi and Tabel, 1995; Naessens et al, 2006).

High levels of parasitemia and high death rates are a hallmark of experimental murine Trypanosomiasis. In infected mice parasite counts exceed 1 x 10^8 parasites/ml at peak parasitemia in both susceptible and tolerant strains. In bovine parasitemia however, the peak levels attained rarely surpass 1 X 10^6 parasites/ml. These differences may be explained by the number of parasites required to establish an infection in both species with mice requiring an infective dose of 10^4 parasites/ml and cattle 10^3 parasites/ml. Nevertheless, the parasite load in murine Trypanosomiasis is significant when taken in the context of the comparative body weight between mice and cattle. It is therefore plausible that such a high parasite load would account for the pronounced pathology and

high rates of death in observed in murine Trypanosomiasis in comparison to bovine infections. For this reason, clearance of the high parasitemia levels in the mouse model is accompanied by enhanced lesions, in contrast with bovines which exhibit a more efficient parasite clearance mechanism with reduced lesions (Antoine-Moussiaux et al, 2008).

3.2.4 Use of mouse models for gene discovery in Trypanosomiasis

Despite the above inconsistencies, mice still remain the choice organisms for the study of bovine Trypanosomiasis. The availability of tolerant and susceptible mouse models for bovine Trypanosomiasis and high-throughput technologies for functional genomics offers us the opportunity to discover associations between phenotype and genotype and to unlock understanding into the interplay between various biological components during the course of disease. Specialized technologies emerging in the recent past as a result of large-scale genome sequencing efforts have made the study of host-parasite interactions achievable. The microarray technology in particular has made it possible to scrutinize perturbations of the genome in response to alterations in normal physiological state (Schena et al, 1998; Schulze and Downward, 2001).

A typical microarray expression experiment involves exposure of messenger RNA (mRNA) from normal and perturbed cells/tissues/organisms to an array of immobilized DNA motifs of known identity representative of various genes of the organism under study. Fluorescence signals emitted as a result of hybridization of mRNA molecules to complementary DNA motifs are directly proportional to the expression level of the genes. Analysis of the differential gene expression between the normal and perturbed arrays leads to greater understanding of the response and function of different genes under various physiological conditions. Microarray technology has been widely applied in biomedical research for gene discovery studies, disease diagnosis, drug discovery and toxicology studies (Xiang and Chen, 2000; Blohm and Guiseppi-Elie, 2001; Letieri, 2006).

In the case of bovine Trypanosomiasis due to *T.congolense* infection, the dissection of genomic sections that control host susceptibility and tolerance with the use of selected strains of inbred mice was a notable milestone. However, the prevailing mystery that remains enigmatic to scientists is to unravel the intricate details of the host genome response to trypanosome infection. This entails moving beyond QTL studies towards a deeper understanding of host-parasite interactions using functional genomics approaches in order to answer fundamental questions like: How does the host genome interact with trypanosome antigenic factors? Which genes respond to infection? What are the functions of the responding genes? How do the host genes interact with each other during infection? Finally, what are the physiological consequences of the gene interactions and their influence on host pathology and disease?

3.3 MATERIALS AND METHODS

3.3.1 Experimental design

To understand the gene dynamics during *T.congolense* infection, we performed a large-scale transcriptional profiling of tissue-specific gene responses in tolerant (C57BL/6), moderately susceptible (Balb/c) and susceptible (A/J) mice to infection over a 17-day time-course. We employed a 3 x 7 factorial experimental design, where mouse strain and time-point were factors. A total of 630 mice were allocated to three groups by strain, resulting in 210 mice per strain. Within each strain, mice were randomly assigned to seven groups of 30 mice each (Table 1). The seven groups formed the basis for the duration for which the mice would be subjected to *T.congolense* infection i.e. 0 days, 3 days, 5 days, 7 days, 9 days, 13 days and 17 days. A balanced sex ratio was ensured for each group. Microarray analysis was performed to assay for differential gene expression associated with early infection i.e. between day 0 and day 9. This experimental design provided >90% statistical power to detect differential expression at a confidence level of 5%.

3.3.2 Animal management

All mice were imported from Harlan (UK) and were maintained in a trypanosome-free environment at the International Livestock Research Institute (Kenya) in the small animal unit. They were housed standard mouse cages five mice per cage. They were supplied ad *libitum* with mouse pellets (Harlan, UK) and water and maintained in accordance with ILRI's regulations of animal care and use (IACUC).

3.3.3 T.congolense challenge

At approximately 12 weeks of age, mice were subjected to challenge with *T.congolense* clone IL1180 a derivative of STIB 212 which was maintained by regular passaging in mice (Geigy and Kaufmann, 1973; Nantulya et al, 1984). All mice except those allocated to Day 0 groups received an intraperitoneal infective dose of 1 X 10^4 bloodstream parasites per animal. Mice in all groups were subsequently monitored daily

for parasitemia levels by microscopic examination of a thick blood smear obtained from their tail tips.

3.3.4 Tissue harvesting and RNA extraction

Mice were promptly sacrificed to harvest specific tissues at the designated time-points post-infection i.e. at days 0, 3, 5, 7, 9, 13, 17 and 35. On the material days, mice were sacrificed by cervical dislocation and aseptically dissected to collect liver, spleen and kidney tissues. The tissues were saved for subsequent RNA extractions in pre-labeled tubes containing RNAlater® solution (Invitrogen) and immediately flash-frozen in liquid nitrogen to ensure apt stabilization and preservation of RNA integrity.

Total RNA extraction was subsequently done using TriZol reagent (Invitrogen) according to the manufacturer's recommended protocol. DNAse 1 (Invitrogen) digestion, qualitative and quantitative analysis of the RNA 6000 nano chip kit (Agilent technologies) were also performed according to manufacturer recommendations. All experiments pertaining to animal handling, *T.congolense* challenge, tissue harvesting and RNA extraction, RNA quality and quantity analysis and storage were performed at ILRI, Kenya. Subsequent experiments for microarray hybridization were performed by project collaborators at the Roslin Institute, Edinburgh UK.

3.3.5 Pooling schedules

RNA samples were extracted from thirty individual mice from each strain, tissue and time-point. Prior to hybridization, these RNA samples were subjected to a pooling procedure, where RNA preparations from 5 mice were pooled into one group thereby resulting into 6 pools per strain per time-point for each tissue. This kind of pooling procedure was necessary to minimize within-group variations while effectively decreasing the number of arrays used for the experiment without undue loss of precision (Kendziorski, 2003).

3.3.6 Microarray hybridizations

The total RNA pools above was labeled and hybridized to GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). These arrays consisted of 45,000 probe sets representative of over 39,000 mouse transcripts and variants from over 34,000 well characterized mouse genes thereby providing a comprehensive coverage of the mouse genome.

For each of the liver, spleen and kidney tissues obtained from each of the three mouse strains, we hybridized total RNA obtained from days 0, 3, 7, 9, and 17 only. RNA obtained from days 5, 13 and 35 were not hybridized. The former time-points were prioritized over the latter based on the knowledge of *T.congolense* replication patterns in murine Trypanosomiasis as well as immunological and pathological responses to infection at early, mid and late stages.

3.3.7 Miame compliance

Guidelines for minimum information about a microarray experiment (MIAME) were adhered to in order to allow for the unambiguous and reproducible interpretation of the results (Brazma et al, 2001). MIAME compliant data comprising raw intensity files (.CEL files), normalized data, experimental design, annotations and standard operating procedures have been deposited in ArrayExpress http://www.ebi.ac.uk/arrayexpress/ with the accession number E-MEXP-1190 as well as on the project website http://www.genomics.liv.ac.uk/tryps/resources.html.

3.3.8 Data analysis

Gene expression read-outs in the form of .CEL files obtained from the hybridizations were subjected to stepwise analyses to deduce perturbed genes and biological pathways. The entire experimental set-up entailed several biological variables that could potentially affect expression profiles including i) strain ii) tissue and iii) time-point in addition to combinations of these effects. A large-scale analysis was therefore performed to identify differentially expressed genes based on these criteria (Fisher et al, 2009). However, due

to the complexity of the dataset this document does not describe the analyses and results of the full dataset but rather focuses on the differentially-regulated genes and gene networks in liver tissue between Day 0 and Day 9 across the three mouse strains. This sub-set of data comprised 30 output files (.CEL files) derived from 30 arrays representing five replicates of liver tissue from the three mouse strains A/J, Balb/c and C57BL/6 mice, across the two time-points i.e. Day 0 and Day 9

3.3.8.1 Background correction, normalization and summary of probe intensities

The raw intensity files above were subjected to background correction, quantile normalization and summary of probe intensities to obtain actual measures of expression levels for individual genes. This was achieved using the robust multi-array analysis (RMA), an algorithm developed for Affymetrix Genechip data which has been proven to give better precision and sensitivity in quantification of gene expression than standard algorithms as MAS 5.0 and dChip (Irizarry et al, 2003). This algorithm is dependent upon an additive log scale linear additive model incorporating intensities of constituent arrays, affinity effects of the probes and a cumulative error term for the arrays and probes to produce a summary statistic of the effective expression measurements. The model is represented by:

$\mathbf{T(PM_{ij}) = e_i + a_j + \varepsilon_{ij}}$ **and** $\mathbf{_j = 1…..J}$ where,

- **T** represents the transformation that background corrects, normalizes, and logs the perfect match (PM) intensities,
- $\mathbf{e_i}$ represents the log2 scale expression value found on arrays$_i$ = 1….I
- $\mathbf{a_j}$ represents the log scale affinity effects for probes $_j$ = 1…..J and
- $\boldsymbol{\varepsilon_{ij}}$ represents error as above.

This algorithm runs in the R environment and is incorporated within the Bioconductor project as the *Affy* package (Bioconductor core, 2002; R language definition, 2010). On running our raw intensity files, we obtained an expression output file, which we analyzed further for differential gene expression.

3.3.8.2 Cluster analysis

It was necessary to ascertain the success of the background correction and normalization procedures and we achieved this by performing hierarchical cluster analysis of the post normalization data. Because our dataset comprised within-array replicates, carrying out a cluster analysis provided us with a quality assessment of our normalized data and made it easier to spot obvious outliers. We employed *PVClust*, a method that calculates probability values for each cluster using bootstrap resampling techniques (Suzuri and Shimodaira, 2006).

Each of the resultant clusters was denoted by two kinds of p-values i.e. an approximately unbiased p-value (AU) and a bootstrap probability value (BP). The AU p-value was derived from multiscale bootstrap resampling whereas the BP value was derived from ordinary bootstrap resampling, thereby making the AU p-value superior in bias over the AU value. Cluster analysis using this approach was computed for our data with 100 bootstrap replications. The PVClust algorithm was a constituent of the Bioconductor project and ran in the R environment.

3.3.8.3 Identification of DE genes

The expression values from the above procedure were then subjected to statistical analysis to discern genes that were differentially expressed (DE). This was achieved by implementation of the empirical Bayes linear approach which allowed multiple testing of data from a relatively complex microarray experiment with arbitrary number of treatments and samples (Smyth, 2004). This method referred to as Linear Models for Microarray Analysis (*Limma*) also ran in the R environment as a constituent of the Bioconductor project (www.bioconductor.org). Limma was an attractive choice as it uses a pooled correlation scheme for experiments with within-array replicates as was the case with our dataset, thereby resulting in improved power and a more stable inference (Smyth et al, 2005).

To fit a linear model on our data using this approach, we began by defining a design matrix outlining the RNA sources and treatments in each array using appropriate coefficients. This was followed by specification of a contrast matrix to delineate desired comparisons to give an output of target DE genes. We defined a contrast matrix to compare gene expression between the two time-points i.e. Day 0 versus Day 9. The output file outlined summary statistics ranking the DE genes according to log fold changes, standard errors, t-statistics and p-values. The genes in the output file were annotated using the corresponding probe database of the array platform used i.e. mouse4302.db.

3.3.8.4 Pathway analysis

To assess the biological pathways that were responding to *T.congolense* infection in the mouse livers between Day 0 and Day 9 we performed a pathway analysis using the list of differentially expressed genes that were asserted to have > 2-fold change with a confidence level of 95% between Day 0 and Day 9. Whereas most studies achieve this by use of over-representation analyses (ORA) or functional class scoring (FCS) approaches, we chose to perform this using impact analysis - a relatively new systems biology approach (Draghici et al, 2007; Tarca, 2009). This direction was motivated by the fact that gene functions primarily occur in a system context and ORA and FCS approaches do not factor in dependencies and interactions of perturbed genes within the signaling pathways. The impact analysis approach that we employed had the advantage of outlining and ranking perturbed signaling pathways based on the magnitude of change of the input genes, their functional significance and position in the pathway as well as their interactions with other genes within the pathway framework.

The workflow behind the impact analysis fundamentally depended on computation of two parameters i.e. i) a perturbation factor (PF) calculated for each gene and ii) an impact factor (IF) calculated for the given pathway.

PF took into account the additive effects of the magnitude of change in expression of each given gene and its interactions with related genes in the context of the pathway topology. PF for gene g was calculated as:

$$\mathbf{PF_g = FC_g + \sum \Delta U_g / \Delta D_g \, . \, \beta}$$

Where $\mathbf{PF_g}$ is the perturbation factor of gene g, $\mathbf{FC_g}$ is the fold change in expression of gene g, $\sum \Delta \mathbf{U_g}/\Delta \mathbf{D_g}$ is the summative effect of the change in expression of all genes upstream of gene g normalized the change in expression of all gene downstream of gene g. $\boldsymbol{\beta}$ is a constant depicting the type of interaction i.e. induction or repression

The Impact factor (IF) was computed for each pathway and gave the ranking for all the pathways that were significantly perturbed under the experimental conditions. Impact factor for each pathway was calculated as:

$$\mathbf{IF_X = P_x + \sum PF_a + PF_b \ldots\ldots\ldots PF_n}$$

Where the $\mathbf{IF_x}$ is the impact factor of pathway X, $\mathbf{P_x}$ is the probability of obtaining the observed number of differentially expressed genes for pathway X just by chance and $\sum \mathbf{PF_a + PF_b \ldots\ldots\ldots PF_n}$ is the absolute perturbation measure for pathway X based on the summation of perturbation factors for all constituent genes (PF) in the pathway.

3.4 RESULTS

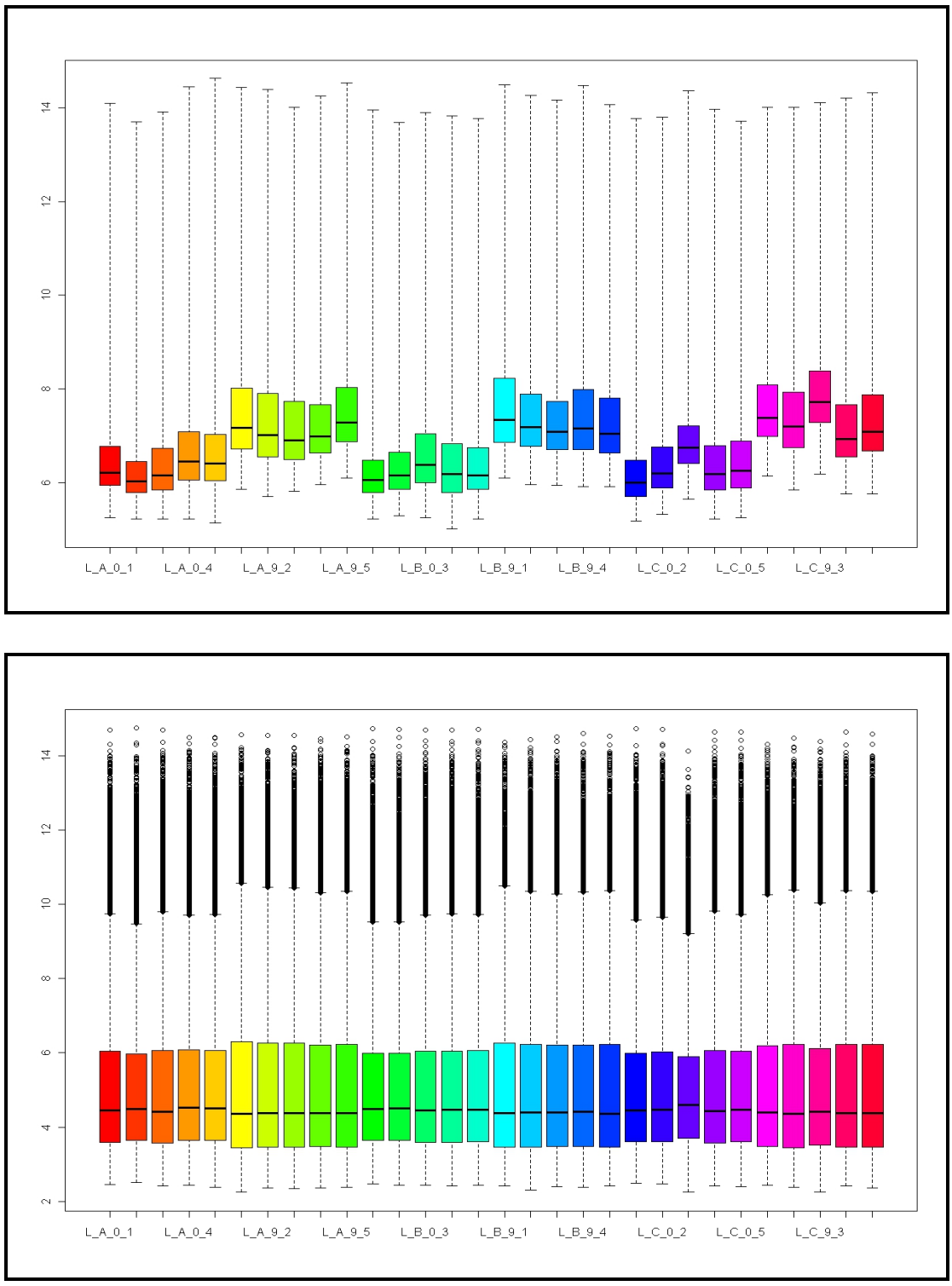
3.4.1 Quality control and Normalization

Exploratory analyses of the raw expression data was necessary to discern quality of the data output from each of the arrays, display the general distribution of the data values, identify outliers and ascertain the success of the normalization procedure. This was achieved use of box plots, cluster analysis and principal component analysis.

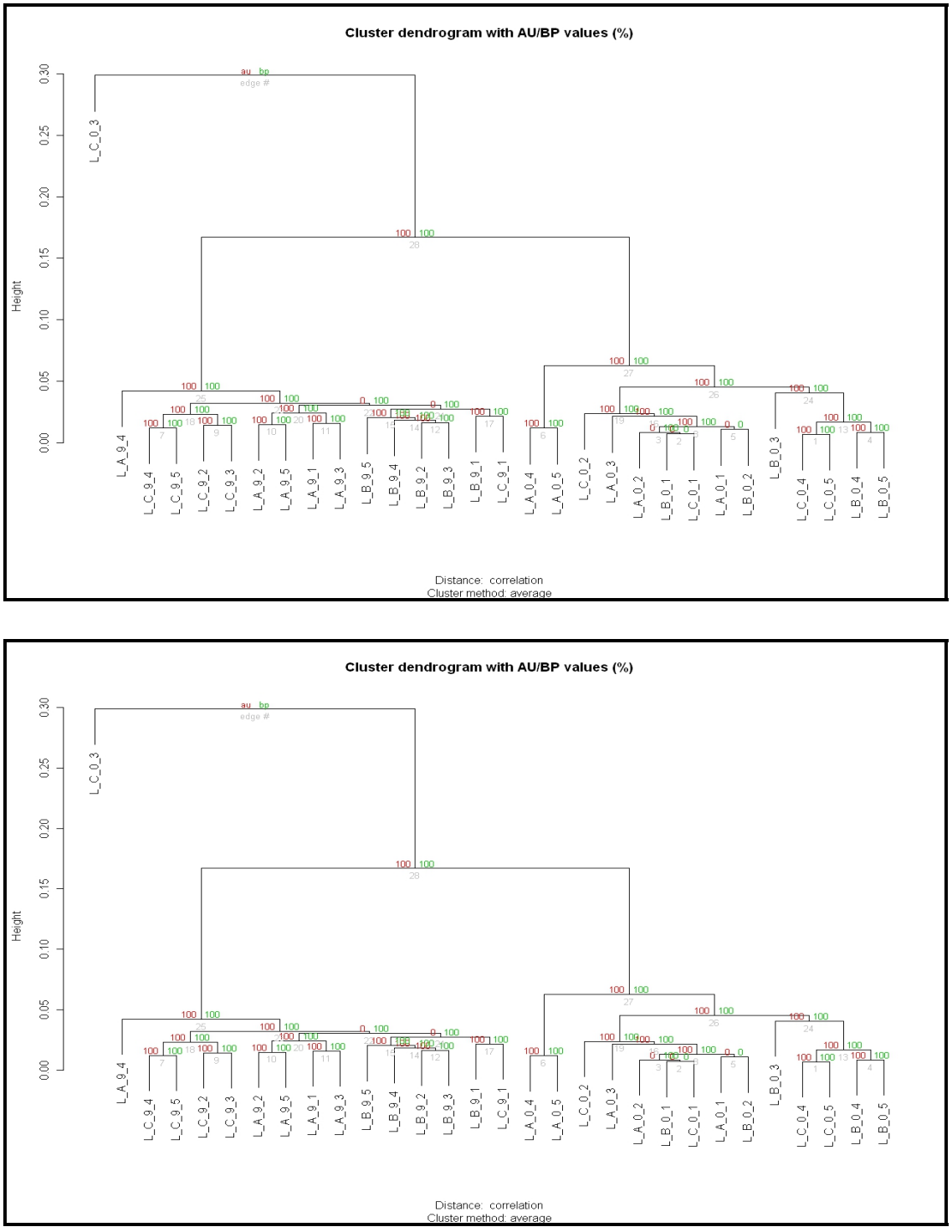
Visual inspection of raw intensity value files generated by the above mentioned approaches was performed before and after normalization. There was marked variation in expression among the samples obtained at different time points and strains. However, variation among replicates of each sample was minimal as depicted by assessing the box plots before normalization (Fig 1a). It was not clear from this figure if the presumed variation was biological or technical in nature. However, correcting for technical variation by RMA normalization resulted in a more centralized and evenly distributed dataset as depicted by the post-normalization box plots (Fig 1b).

3.4.2 Cluster analysis

Clustering patterns of pre-normalized and post-normalized data were similar indicating that normalization had no significant effect on the hierarchical grouping of the samples (Fig 2a and 2b). According to the cluster analysis, the dendogram segregated into two main branches i.e. infected samples (Day 0) and un-infected samples (Day 9). Analysis of the sub-groups within the infected category revealed strain-specific clustering implying gene expression variations between A/J, Balb/c and C57BL/6 due to infection. Random clustering was however observed within the un-infected group where the clusters were of mixed strains, implying that the baseline expression profiles in the un-infected mice strains were generally similar across the three mouse strains.



Figures 3.1a and 3.2b: Boxplots of the raw intensity files before and after normalization. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Exploratory analysis of the raw intensity files (.CEL files) by boxplots was performed on before and after Robust MultiArray normalization.



Figures 3.2a and 3.2b: Hierarchical cluster dendograms of raw intensity files before and after normalization. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Exploratory analysis of the raw intensity files (.CEL files) by hierarchical clustering was performed on before and after Robust MultiArray normalization.

3.4.3 Differential gene expression

Differential gene expression across the uninfected (Day 0) and infected (Day 9) mouse strains was analyzed. As the objective was to nail the genes responsible for the severe immune dysregulation and pathology in the acute phase of infection, the criterion for upregulation of downregulation was set at >3-fold change asserted at 5% confidence level based on an adjusted P-value. A total of 1050 genes were confirmed to be differentially expressed under this criterion. Of these, 717 genes were differentially regulated across all three strains, whereas 111 genes were differentially regulated in A/J mice only, 159 in Balb/c and 94 in C57BL/6 mice only as depicted in the Venn diagram (Fig 3).

3.4.4 GO enrichment analysis

We analyzed for enrichment of gene ontology terms over-represented in the list of differentially expressed genes. We achieved this by performing heuristic clustering using a grouping algorithm that partitioned the list of genes into clusters based on the degree of similarity in their annotations (Dennis et al, 2003; Huang et al, 2009). The result was a ranked functional annotation clustering report with over-represented annotation terms and associated p-values. We generated functional annotation clusters for the genes that were differentially regulated by all three mouse strains (Fig 4).

The results generally revealed over-representation of terms associated with positive regulation of immune response or activity. Inflammation ranked highly in the analysis of DE genes shared across all strains (Fig 4) implying that inflammatory response was fundamental in *T.congolense* pathogenesis. This observation was further augmented by the significant ranking of GO terms as systemic lupus erythematosus which are characterized by excessive inflammation. Analysis of gene composition of the topmost cluster revealed enrichment of chemokine, toll-like receptor, complement and MHC genes. With respect to individual mouse strains, the highest-ranking annotation terms in A/J and C57BL/6 were immune response and inflammation. However in Balb/c mice, carbohydrate metabolism ranked highest (Fig 4d).

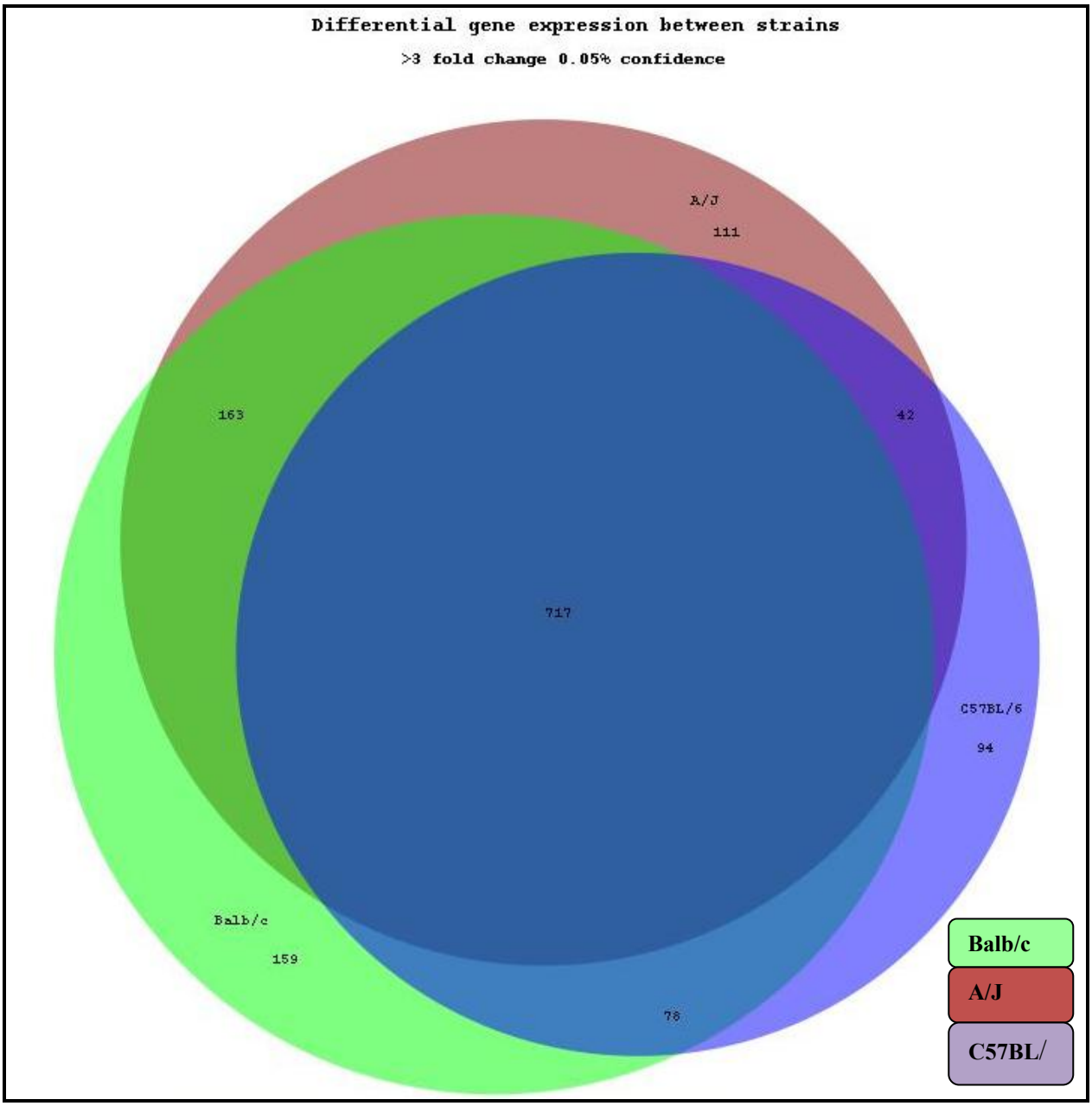


Figure 3.3: Venn diagram of differentially expressed genes in early infection with T.congolense in mice. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Differential gene expression was ascertained by linear modeling and empirical Bayes statistics. 1050 genes were asserted at an alpha value of 0.05 to be differentially expressed with >3 fold change.

Table 3.1: Gene ontology enrichment analysis for genes differentially expressed by all mouse strains in response to early infection to T.congolense. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Differential gene expression was ascertained by linear modeling and empirical Bayes statistics. The inflammatory response was asserted with a high enrichment score for all strains in response to the infection. Enrichment of genes responsive to bacterial infection was observed.

GO analysis for differentially expressed genes in response to *T.congolense* infection					
Term	**Count**	**%**	**P-Value**	**Fold Enrichment**	**Benjamini corr**
defense response	65	11.6487455	1.75E-25	4.682834238	1.90E-22
response to wounding	53	9.49820789	9.28E-22	4.92969258	6.73E-19
inflammatory response	42	7.52688172	1.54E-20	6.024766429	8.39E-18
response to molecule of bacterial origin	14	2.50896057	1.87E-09	9.221581269	1.76E-07
response to bacterium	22	3.94265233	1.56E-08	4.522686355	1.13E-06
response to lipopolysaccharide	8	1.43369176	1.41E-04	6.794849356	0.002
Taxis	24	4.30107527	2.06E-13	7.106539694	3.73E-11
chemotaxis	24	4.30107527	2.06E-13	7.106539694	3.73E-11
locomotory behavior	29	5.19713262	1.34E-09	3.916278238	1.33E-07
chemokine signaling pathway	27	4.83870968	3.01E-08	3.503052503	2.05E-06
chemokine activity	11	1.97132616	8.92E-08	9.913727618	2.42E-05
chemokine receptor binding	11	1.97132616	1.17E-07	9.659529474	2.12E-05
Small chemokine, interleukin-8-like	10	1.7921147	6.92E-07	9.487768401	1.95E-04
Cytokine	17	3.04659498	4.88E-05	3.362335217	8.80E-04
Cytokine-cytokine receptor interaction	25	4.48028674	8.06E-05	2.419382041	0.001
cytokine activity	15	2.68817204	8.01E-04	2.85395189	0.02

An interesting observation was the significant enrichment of bacterial response genes as depicted by observation of the GO terms "response to bacterium" or "response to molecule of bacterial origin." This result was further corroborated by upregulation of genes as CD14, LBP, and Ly96 typically known to respond to the classical endotoxin – lipopolysaccharide (LPS) from gram negative bacteria (see Fig 4). This was an unexpected output as *T.congolense* which was used to infect the mice is a protozoan and not a bacterium and is not known to express lipopolysaccharide on its surface membrane.

3.4.5 Pathway analysis

Pathway analysis was performed on the list of differentially expressed genes in all three strains to investigate perturbation of biological processes in the mice due to *T.congolense* infection. Perturbed pathways were ranked according to their impact factors and p-values with the highly perturbed pathways ranking top on the table (Table 2). Antigen processing and presentation was the highest ranking perturbed pathway. *T.congolense* infection was seen to invoke activity of both MHC class I and MHC class II pathways (Fig 5a). Consistent with the findings from GO enrichment, the pathway analysis output also showed significant perturbation of pathways associated with excessive inflammation i.e. graft versus host disease, cytokine-cytokine interaction, toll-like receptor signalling and systemic lupus erythematosus. There was increased transcription of toll-like receptors TLR1, TLR2 and TLR4 in all mouse strains in addition to the proinflammatory chemokines as CXCL1, CXCL9, CXCL10 and activation of the NFkb and MAPK signalling cascades (Fig 5b and 5c).

3.4.6 Incidence of DE genes in *T.congolense* QTLs

QTLs for Trypanosomiasis tolerance or susceptibility are well characterized and so we explored the list of differentially expressed genes in all three mouse strains for occurrence in known QTLs. All of the 1050 DE genes were mapped to their respective

genomic locations according to the mouse genome database (MGI) and subsequently queried against the genes in known QTLs using a QTL viewing tool (Irvine, 2005).

A total of 39 differentially expressed genes were found to occur in known Trypanosomiasis QTLs. Twenty two of these genes were in Tir1, Three in Tir2, two in Tir3a, four in Tir3b and eight in Tir3c. Majority of these genes were MHC genes and interferon-regulated genes (Table 3).

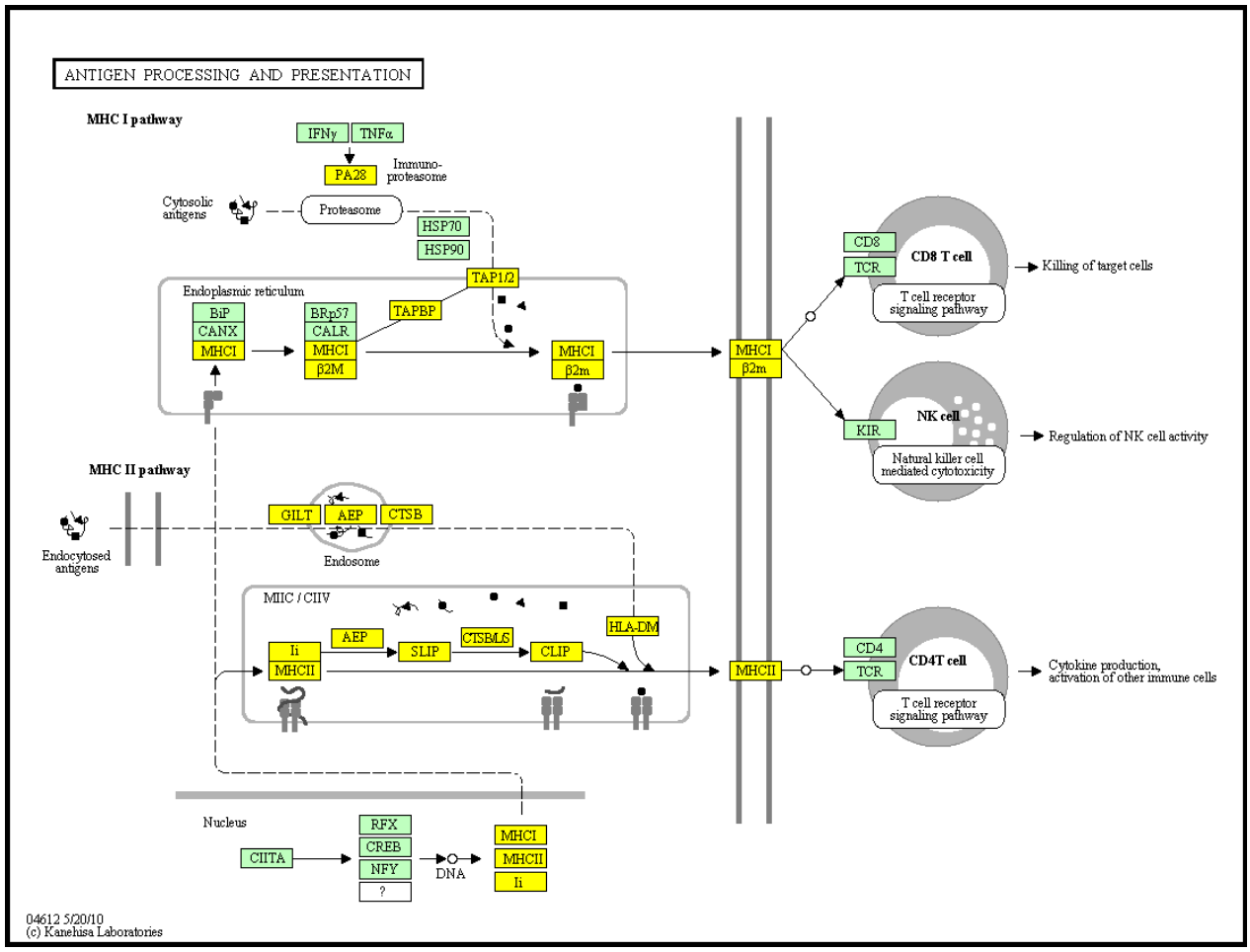


Figure 3.4: Analysis of the perturbation of genes in the antigen processing and presentation pathway as a result of T.congolense infection. Perturbed genes are highlighted in yellow. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Pathway analysis was performed on the list of differentially expressed genes. The antigen processing and presentation pathway was the most perturbed pathway.

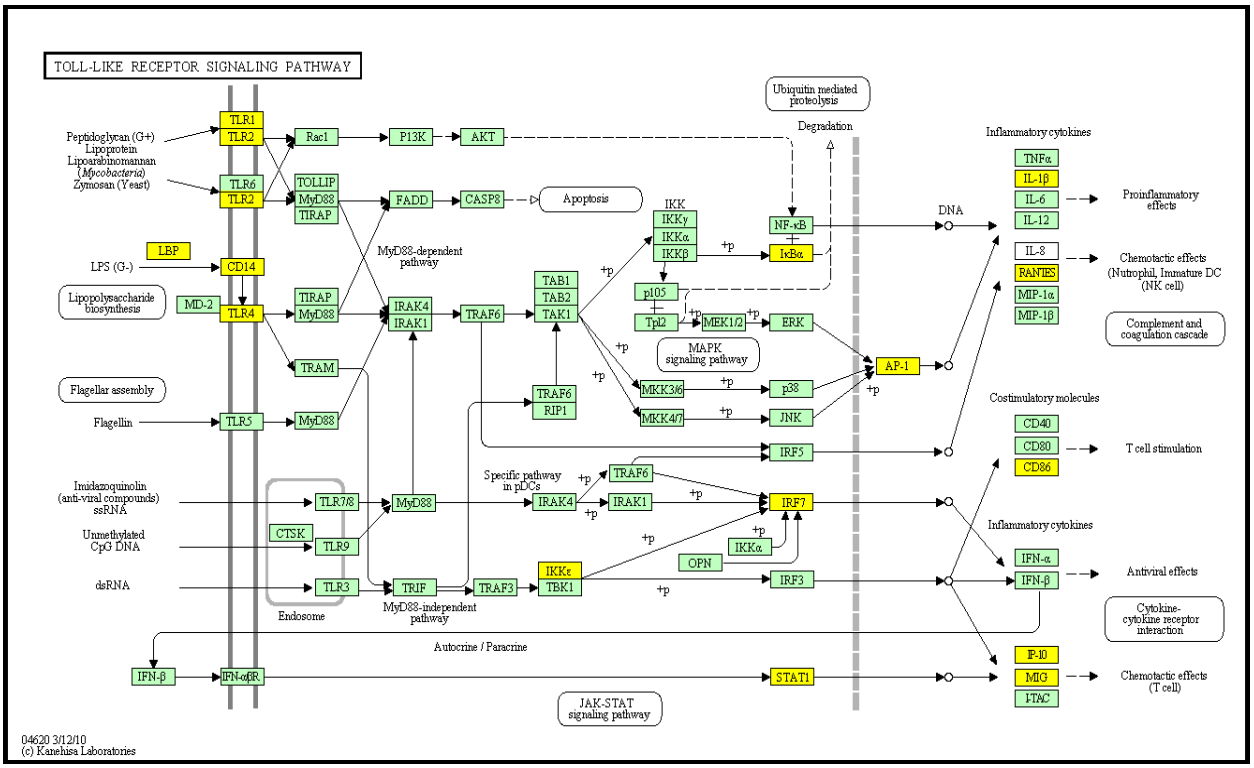


Figure 3.5: Analysis of the perturbation of genes in the toll-like receptor signaling pathway as a result of T.congolense infection. Perturbed genes are highlighted in yellow. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Pathway analysis was performed on the list of differentially expressed genes. The involvement of TLR1, TLR2 and TLR4 is noted. Also noteworthy was the up-regulation of genes in the bacterial response complex LBP/CD14/TLR4.

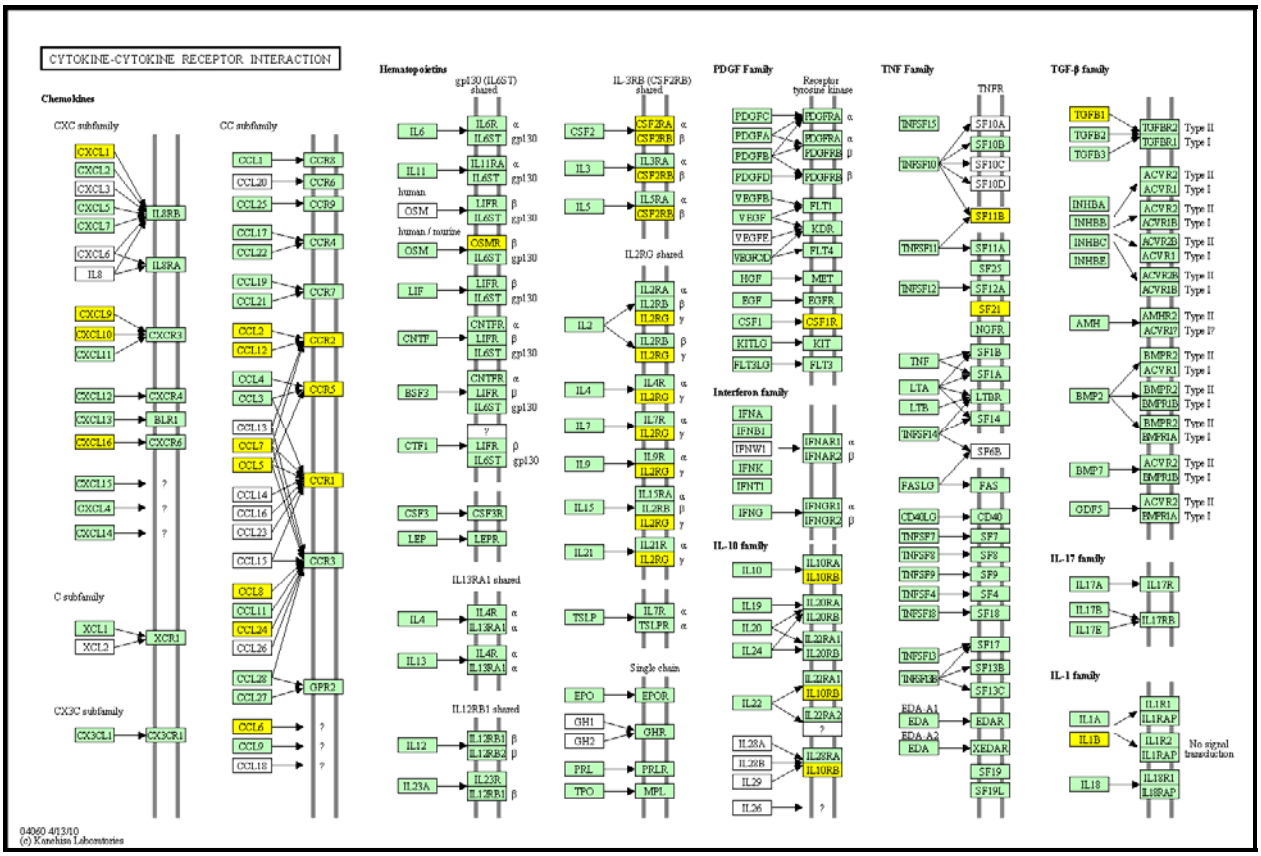


Figure 3.6: Analysis of perturbation of genes in the cytokine and chemokine signaling pathways as a result of early T.congolense infection. Differentially expressed genes are highlighted in yellow. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Pathway analysis was performed on the list of differentially expressed genes. Perturbation of cytokines and chemokines was observed.

Table 3.2: Experimental design of the complete timecourse investigation of gene expression profiles in mice infected with T.congolense. A/J, Balb/c and C57BL/6 mice which vary in degree of susceptibility to T.congolense were infected over a 17-day timecourse. For each strain and time point, mice were randomly allocated to groups of 30 and infected with T.congolense parasites. Total RNA was extracted on the designated days and hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix).

Experimental design for gene expression profiling in *T.congolense*-infected mice								
	Day 0	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 13**	**Day 17**	Total
A/J mice	30	30	30	30	30	30	30	210
Balb/c mice	30	30	30	30	30	30	30	210
C57BL/6 mice	30	30	30	30	30	30	30	210
							Grand total	630

Table 3.3: Ranking of pathways that are significantly perturbed during early infection with T.congolense infection in all mouse strains. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). Pathway analysis was performed on the list of differentially expressed genes. Pathways were ranked from the highly perturbed to least perturbed according to the respective impact factors and p-values.

Rank	Database	Pathway Name	Impact	#Genes in	#Input Genes in	p-
1	KEGG	Antigen processing and presentation	26.57	100	20	2.89E-
2	KEGG	Cell adhesion molecules (CAMs)	25.46	159	23	8.78E-
3	KEGG	Graft-versus-host disease	22.11	66	12	2.51E-
4	KEGG	Cytokine-cytokine receptor interaction	20.28	249	25	1.56E-
5	KEGG	Type I diabetes mellitus	20.28	71	12	1.56E-
6	KEGG	Toll-like receptor signaling pathway	19.39	101	16	3.78E-
7	KEGG	Allograft rejection	19.06	66	11	5.28E-
8	KEGG	Leukocyte transendothelial migration	16.68	119	16	5.71E-
9	KEGG	Autoimmune thyroid disease	16.52	81	11	6.69E-
10	KEGG	Systemic lupus erythematosus	15.61	181	13	1.67E-
11	KEGG	Hematopoietic cell lineage	15.31	86	13	2.26E-
12	KEGG	Natural killer cell mediated cytotoxicity	14.75	109	14	3.91E-
13	KEGG	Cell cycle	13.34	124	14	1.61E-
14	KEGG	B cell receptor signaling pathway	11.41	71	10	1.10E-
15	KEGG	DNA replication	10.31	36	7	3.34E-
16	KEGG	Asthma	9.86	35	6	5.24E-
17	KEGG	p53 signaling pathway	9.79	74	9	5.60E-
18	KEGG	Regulation of actin cytoskeleton	9.13	213	16	1.09E-
19	KEGG	ECM-receptor interaction	8.46	81	9	2.13E-
20	KEGG	Fc epsilon RI signaling pathway	8.36	80	9	2.35E-
21	KEGG	Focal adhesion	7.37	199	14	6.28E-
22	KEGG	T cell receptor signaling pathway	7.34	124	10	6.48E-
23	KEGG	Jak-STAT signaling pathway	6.75	157	11	0.001
24	KEGG	Primary immunodeficiency	6.09	36	5	0.002
25	KEGG	Adherens junction	4.18	77	6	0.015
26	KEGG	Pathways in cancer	3.59	339	15	0.028
27	KEGG	Proteasome	3.52	51	4	0.03
28	KEGG	Complement and coagulation cascades	3.34	74	5	0.036

Table 3.4: Analysis of incidence of differentially expressed genes in known Trypanosomiasis QTLs. Total RNA from livers of A/J, Balb/c and C57BL/6 mice infected with T.congolense parasites over zero and nine days was hybridized onto GeneChip® Mouse Genome 430 2.0 Oligonucleotide Arrays (Affymetrix). The list of differentially expressed genes was analyzed for genes in known QTLs. 39 genes that were differentially expressed genes fell within QTLs known to be associated with T.congolense infection.

QTL	Symbol	Gene name
	Aif1	allograft inflammatory factor 1
	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)
	Clic1	chloride intracellular channel 1
	Cyp4f16	cytochrome P450, family 4, subfamily f, polypeptide 16
	H2-Aa	histocompatibility 2, class II antigen A, alpha
	H2-Ab1	histocompatibility 2, class II antigen A, beta 1
	H2-D1	histocompatibility 2, D region locus 1
	H2-DMb1	histocompatibility 2, class II, locus Mb1
	H2-Eb1	histocompatibility 2, class II antigen E beta
	H2-M3	histocompatibility 2, M region locus 3
	H2-T10	histocompatibility 2, T region locus 10
	Myo1f	myosin IF
	Nfkbie	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
	Pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
	Psmb8	proteasome subunit, beta type 8
	Psmb9	proteasome subunit, beta type 9
	Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
	Tapbp	TAP binding protein
	Tnfrsf21	tumor necrosis factor receptor superfamily, member 21
	Tubb5	tubulin, beta 5
	Ubd	ubiquitin D
Tir 1	Cxcl1	chemokine (C-X-C motif) ligand 1
	Cxcl10	chemokine (C-X-C motif) ligand 10
	Igj	immunoglobulin joining chain
Tir2	Actr3	ARP3 actin-related protein 3 homolog (yeast)
	Marco	macrophage receptor with collagenous structure
Tir3a	Btg2	B-cell translocation gene 2, anti-proliferative
	Ikbke	inhibitor of kappaB kinase epsilon
	Mcm6	minichromosome maintenance deficient 6
	Ptprc	protein tyrosine phosphatase, receptor type, C
Tir3b	Cd48	CD48 antigen
Tir3c	Cd84	CD84 antigen
	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide
	Fcgr3	Fc receptor, IgG, low affinity III
	Ifi204	interferon activated gene 204
	Refbp2	RNA and export factor binding protein 2
	Sell	selectin, lymphocyte
	Slamf8	SLAM family member 8

3.5 **DISCUSSION**

Complex genetic traits as susceptibility or tolerance to disease are intriguing biological phenomena that often require intricate approaches to unravel. High-throughput expression profiling through the use of microarray technology however, provide a means to understand these traits through scrutiny of gene expression signatures between different phenotypic variants. In this study, we aimed characterize the genetic basis underlying susceptibility or resistance to disease in experimental murine Trypanosomiasis due to *T.congolense* infection. We achieved this by performing a large-scale gene expression profiling of three murine models - A/J, Balb/c and C57BL/6 mice, previously described to vary in their susceptibility to *T.congolense* infection, with the latter strain been relatively resistant than the former two strains (Kemp et al, 1997).

To shed light into the host–pathogen interactions taking place in the acute phase of infection, this report will discuss results drawn from analysis of a sub-set of the data i.e. gene expression in liver tissue across the mouse strains between days 0 and Day 9. The choice to analyze gene expression in this specific dataset was motivated by the central significance of the liver in host pathology and immune response of *T.congolense* infection. The liver cells are known to engage in immunological responses to facilitate parasite clearance but this process is often accompanied by marked pathological consequences including hepatomegaly and thickening of liver ducts (Takeya et al, 1987). Further, the *T.congolense* parasitemia in A/J, Balb/c and C57BL/6 mice attain peak levels about 9 days post infection and this coincides with rapid increase in hepatic and splenic cell populations (Morrison et al, 1978). Analysis of this particular dataset was therefore considered critical in providing useful insights into the host responses to *T.congolense* in early stages of infection.

We utilized a systematic workflow to achieve this objective by applying a pathway-based analysis to attain a comprehensive perspective of the consequences of *T.congolense* infection on the host's biological systems. 1,050 differentially expressed probesets were identified, 717 of which were differentially expressed in all the three

mouse strains, 111 were unique to A/J mice, 159 to Balb/c mice and 94 to C57BL/6 mice. These were interrogated for enrichment for gene ontology terms of known biological processes, involvement in biological signalling pathways as well as location in known quantitative trait loci. This was deemed superior to the gene-based analysis which only stops at a list of differentially-expressed genes. This workflow has previously been utilized on a similar complex dataset with desirable outcomes (Fisher et al, 2009).

Gene ontology and pathway analysis of the differentially expressed genes revealed significant perturbation of immune processes characterized by elevated antigen processing and presentation activity, indicating that the mice were responding to the *T.congolense* infection. Inflammation in particular stood out of our analysis as a fundamental response to *T.congolense* infection in mice as revealed by its high enrichment score across all strains. This response was characterized by the increased expression of proinflammatory cytokines, chemokines and toll-like receptors. Activation of adaptive immune response through T-lymphocyte and antibody-mediated responses was also evident from the findings.

In trypanosome infections, the time taken for clearance of the first wave of parasitemia is inversely correlated with survival and therefore susceptibility or resistance to disease (Ogunremi and Tabel, 1995). The inflammatory response as depicted by our findings is thought to play a central role in the control of these initial aggressive waves of parasitemia through action of classically-activated macrophages that are characterized by the secretion of proinflammatory mediators including TNF-α, IL-1, IL-6 and IL-12 (Namangala et al, 2001). This is particularly true for *T.cruzi* infections where clearance is highly dependent on the inflammatory environment occasioned by the endogenous synthesis of IL-12, TNF-α and NO by activated macrophages (Camargo et al, 1997; Almeida et al, 2000). *T.brucei* clearance is primarily directed via the adaptive immune response through antibody-mediated phagocytosis by liver Kupffer cells, although inflammatory responses by resident macrophages also participate in parasite clearance (MacAskill et al, 1980; MacAskill et al, 1981; Namangala et al, 2001). In murine

T.congolense infection, effective parasite clearance is also increasingly correlated with an inflammatory host phenotype (Murray and Morrison, 1979; Tabel et al, 2000).

An interesting albeit unexpected observation from our gene ontology analysis was the significant enrichment of genes that respond to bacteria invasion. Concurrent with this observation was the up-regulation of CD14, LBP and TLR4 genes which are characteristic of response to the classical endotoxin, lipopolysaccharide (LPS). LPS is the major structural component of the outer membrane of gram negative bacteria where it plays a crucial role in maintaining an intact membrane. Upon host invasion, the membrane integrity may be lost thereby exposing LPS molecules the host's immune system. Under these circumstances LPS is bound onto the CD14/TLR4/LBP receptor complex, subsequently triggering a cascade of signalling events which culminate in the secretion of proinflammatory cytokines as TNF-α and IL-1α, by the host cells. These inflammatory mediators in turn activate antibody and T cell-mediated responses which synergize to clear the local infection. Unchecked secretion of these proinflammatory cytokines however, capitulate systemic consequences of endotoxic shock by causing massive vasodilatation, decreased blood pressure, decreased tissue perfusion and reduced oxygen availability in tissues, fever, myocardial dysfunction, multiple organ failure and eventual death (Todd et al, 1978; Tracey and Cerami, 1993; Karima et al, 1999).

Our data analysis clearly depicts marked inflammation as a key response in the infected mice. However, the causative factor of this inflammatory response in our experimental analysis is ambiguous at this point. It is not clearly deducible if the inflammation is due bacterial LPS or is a direct result of *T.congolense* infection. LPS even in picomolar levels can cause significant inflammatory responses (Taramelli et al, 1980). It is possible that LPS contamination could have occurred due to undetected bacterial co-infection in our experimental animals or may have been as a result of direct LPS contamination of the harvested biological tissues during the experimental procedure. Furthermore, elevated levels of circulating endotoxins have been described as a feature of experimental human Trypanosomiasis (Nyakundi et al, 2002). In this case, *T.brucei*

infection in rats was confirmed to compromise gut integrity and increased intestinal permeability thereby causing leakage of gut bacterial endotoxins into the circulation, subsequently increasing secretion of inflammatory cytokines and nitric oxide. Indeed, intestinal mucosal damage is a pathological element of tissue invasive trypanosomes including *T.brucei*, *T.vivax* and *T.cruzi* (Anosa, 1983; Ben-Rashed et al, 2003; Ny et al, 2008). An investigation of possible bacterial and endotoxin contamination in blood of *T.congolense*-infected mice by culture tests and limulus amoebocyte assays respectively yielded negative results.

Trypanosome structural components known to be potent triggers of inflammation may be possible sources of the observed inflammatory response. Variant surface glycoprotein (VSG) which is the immunodominant antigen expressed on the surface of all trypanosomes may be one such compound. Each trypanosome expresses about 10^7 VSG molecules on its surface, forming a protective shield from effective host immune responses through programmed antigenic variation (Pays et al, 1983). At peak parasitemia levels of 10^8 parasites/ml in the murine model (as was the case in our set-up), the host is confronted with >200 μg of parasite-derived VSG, which are significantly adequate to cause marked inflammation and associated immunopathology (Magez et al, 2002; Drennan et al, 2005). *T.brucei* VSG molecules have been shown to possess capacity to trigger macrophage activation leading to increased secretion of inflammatory cytokines as TNF-α and IL-1α (Magez et al, 1998). Proinflammatory potential of the GPI anchors of *Trypanosoma cruzi*, *Plasmodium falciparum* and *Leishmania mexicana* VSGs have also been reported (Tochada et al, 1997; Almeida and Gazzinelli, 2001). However, the immune-activating capacity of *T.congolense* VSG remains largely unexplored.

Another potential proinflammatory molecule is parasite DNA. Bacterial DNA of gram positive bacteria (which do not express LPS) is documented to cause septic shock by stimulating elevated serum levels of TNF-α, although in our case bacterial contamination was already ruled out by negative bacterial culture tests. The immunostimulatory activity of bacterial DNA is attributed to presence of high frequency

of unmethylated CpG dinucleotides which stimulate inflammation through the toll-like receptor signaling cascade (Medzhitov, 2001). There is increasing evidence of mitogenic activity of protozoan DNA (Shoda et al, 2001; Harris et al, 2007). Genomic DNA from *Babesia bovis*, *T.cruzi* and *T.brucei* have been reported to stimulate macrophages to secrete inflammatory mediators. This immunostimulatory capacity was positively correlated with the content of unmethylated CpG motifs in the DNA (Harris et al, 2006). No reports exist to our knowledge on the effect of *T.congolense* DNA on host immune responses.

3.6 **CONCLUSION AN RECOMMENDATIONS**

Our results indicate that the acute phase of experimental murine Trypanosomiasis due to *T.congolense* triggers host inflammatory response pathways similar those prompted by the prototype endotoxin, bacterial LPS. As it is not clear cut whether this endotoxic shock-like response is bacterial or *T.congolense*-induced, it is increasingly important to validate the role of bacterial endotoxins, *T.congolense*-derived VSG and DNA in the pathology of *T.congolense* infection. It is also of fundamental significance to understand the effects of these antigens on host gene expression profiles and to characterize the signalling cascades triggered as a result of the stimulation. There is therefore propose a comprehensive investigation within a well-designed *in vitro system* to provide a controlled mechanism for such a study with minimal interfering factors by implementing tight exclusion of contamination by exogenous bacteria and LPS and inclusion of appropriate experimental controls.

4 MACROPHAGE ACTIVATION BY *TRYPANOSOMA CONGOLENSE* VARIANT SURFACE GLYCOPROTEIN AND DNA

4.1 ABSTRACT

Macrophages are immune cells with profound elasticity that allows them the capability to markedly alter their phenotypic and physiological aspects in response to environmental and inherent stimuli. The capacity of *TcVSG and TcDNA* to activate macrophages was explored. Immunologically-naive bone marrow-derived macrophages (BMDMs) obtained from A/J and C57BL/6 mice which are known to differ in their genetic susceptibility to *T.congolense* infection. The cells were maintained in an IFN-γ-free system were co-cultured with 100ng/ul either *T.congolense* VSG (TcVSG), 100ng/ul *T.congolense* DNA (TcDNA), 100ng/ul *T.congolense* total lysate, 100ng/µl homologous mouse DNA, 100pg/ul *E.coli* lipopolysaccharide (LPS) or culture medium where the latter two represented the positive and negative controls respectively. Cell supernatants were harvested after 6hrs or 24 hrs of exposure to the above treatments and assayed for a panel of 12 cytokines and over 6hr and 24hr timecourse and the cell supernatants were aseptically harvested and assayed for a panel of twelve cytokines (TNF-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL12p70, TGF-β and leptin) and four chemokines(CCL3, CCL5, CXCL1 and CXCL10). The effect of time, host genotype and type of treatment on the levels of cytokines and chemokines were analyzed.

The results showed that macrophage activation by TcVSG and TcDNA was comparable to that elicited by bacterial LPS. Macrophages were activated to a classical phenotype by bacterial LPS, TcVSG and TcDNA as characterized by increased secretion of proinflammatory mediators as TNF-α, IL-1α, IL-6, IL12p40, CCL3, CCL5, CXCL1 and CXCL10 with parallel elevation of the anti-inflammatory cytokine, IL-10. These secretion profiles were indicative of active inflammation which occurred as an early response i.e. by 6hrs of exposure to bacterial LPS, TcVSG and TcDNA and was markedly diminished by 24hrs. Homologous mouse DNA and *T.congolense* lysate caused no significant difference in secretion profiles when compared against the

untreated controls. Significant variations were noted in the secretion levels of TGF-β, IL12p40 and IL-6 between A/J and C57BL/6 macrophages. This provides a basis for the role of the above cytokines in tolerance and susceptibility to *T.congolense* infection.

4.2 INTRODUCTION

4.2.1 Macrophages

Macrophages are white blood cells that arise from differentiated monocytes. They are found in blood circulation of mammalian vertebrates and are located strategically in various tissues where they are adapted to perform localized defense functions.

Macrophages are activated on encounter with pathogens effectively forming a valuable first line defense against microbial invasion by applying multiple strategies. One strategy for parasite clearance is through phagocytosis where the macrophages engulf and destroy the parasites by enzymatic digestion or process for presentation to specific T-lymphocytes (Taylor et al. 1998). Activated macrophages may also secrete a wide range of bioactive molecules including cytokines, chemokines and reactive oxygen species. Cytokines positively regulate the macrophages to secrete more cytokines or activate lymphocytes to destroy the pathogen through other specific mechanisms as cytotocixity and antibody production (Vincendeau and Bouteille 2006). Chemokines on the other hand recruit neutrophils and monocytes to the site of injury in response to the infection and act in synergy with either type I cytokines to promote inflammation or with type II cytokines in restoration of homeostasis and tissue maintenance (Hillyer et al. 2003). Reactive oxygen species as nitric oxide inhibit replication of the parasites (Hillyer et al. 2003; MacMicking et al. 1997).

4.2.2 Macrophage activation

Macrophages respond to pathogens by altering their morphology and functional activities through a process referred to as macrophage activation. Indeed, macrophage activation is now appreciated to be a generic term that defines the heterogeneous physiological and immunological status of macrophages when exposed to various stimuli. The activation status of macrophages may vary as determined by the stimulant and the prevailing cytokine and chemokine milieu. Indeed, both cytokines and chemokines cooperate as co-activators of macrophages thereby influencing the polarization of immune responses (Dorner et al. 2002). To this effect, three facets of

macrophage activation have been described; classically activated macrophages (caMΦ), alternatively activated macrophages (aaMφ) and type II activated macrophages (tIIaMφ) (Mosser 2003; Mosser and Edwards 2008).

Macrophages become classically activated on exposure to two inducers. The first is exposure to IFN- γ which has been shown not to activate the macrophages *per se* but instead prime them to elicit a caMΦ phenotype, on stimulation with exogenous compounds (Nathan 1991). The primary, albeit transient source of IFN- γ during the early onset of infection is natural killer cells, following which T helper cells provide a sustained production of IFN-γ (O'Shea J.J and Murray 2008). The second inducer of caMΦ is exogenous TNF or exogenous inducers of TNF. The classical example of the latter is lipopolysaccharide or endotoxin from gram negative bacteria which involves toll-like receptor activation resulting in secretion of TNF by the macrophage itself. In the standard model of macrophage activation by *E.coli* endotoxin, the predominant caMΦ secrete type I proinflammatory cytokines including IL-1, IL-6, and IL-12. Additionally, the chemotactic ability of these caMΦ is enhanced by their secretion of chemokines as CCL3, CCL4, CXCL1, CXCL9, CXCL10 and CXCL11. The secretion of TNF-α, NO and other superoxide anions further increases the killing capacity of caMΦ (Dale et al. 2008).

Alternatively activated macrophages (aaMφ) also referred to as wound-healing macrophages, were first described by Stein and colleagues in 1992 when they showed that treating macrophages with IL-4 caused the macrophages to assume a counter-inflammatory phenotype (Stein et al. 1992). Extensive investigations have since been done to characterize the aaMφ. These macrophages are functionally distinct from caMΦ in that they do not produce NO and secrete less of the type I inflammatory cytokines and for this reason these cells are less effective in killing microbial invaders (Modolell et al. 1995). Instead, aaMφ are more primed to modulate the inflammatory response by secreting anti-inflammatory (type II) cytokines, primarily IL-10, TGF-β and IL-1 receptor antagonist (Anderson and Mosser 2002). aaMφ also secrete high levels of fibronectin and arginase which promote fibrogenesis, cell growth and tissue repair

(Gratchev et al. 2001; Hesse et al. 2001; Song et al. 2000). The anti-inflammatory and reparative properties of aaMφ are congruent with their appearance in the system where they are observed following either an acute inflammation due to innate response or during the later stages of a TH1 response (Goerdt and Orfanos 1999).

A third macrophage phenotype has also been described as type II activated macrophage (tIIaMφ). This phenotype occurred on ligation of the macrophage Fcγ receptors resulting in dampening of IL-12 secretion and heightening of IL-10 (Anderson and Mosser 2002; Sutterwala et al. 1997). The tIIaMφ appear to arise at the interphase of caMΦ and aaMφ eliciting a hybrid response with the typical secretion patterns of TNF-α, IL-1 and IL-6 as caMΦ and increased IL-10 levels of aaMφ (Gerber and Mosser 2001; Sutterwala et al. 1998). This characteristic repertoire of tIIaMφ gives these macrophages a regulatory role in inflammation.

4.2.3 Macrophage activation during trypanosome infection

Macrophage activation is a major characteristic of trypanosome infection and has been extensively studied in *T.brucei* and to a lesser extent in *T.congolense*. Early studies have shown that infection with *T.brucei* elicits a macrophage activation cascade similar to that elicited by Mycobacterium bovis Bacillus Calmette-Guerin (BCG) antigen (Askonas et al, 1979). This observation was further confirmed when Mycobacterium BCG and *T.brucei* infection in mice were shown to act on macrophages by inhibiting the uptake of mannose-terminal glycoproteins and macrophage-specific markers e.g. F4/80 and Mac-1 (Grossinsky et. al, 1983). This inhibition of macrophage markers was the basis for functional depletion of B and T cell populations and thereby leading to Trypanosomiasis-associated immunosuppression (Askonas et al, 1979).

During trypanosome infection, macrophages participate primarily in parasite killing events. Macrophages mediate the most important clearance mechanisms for trypanosomes, through nitric oxide production, cytokine secretion and phagocytosis. The trypanostatic properties of macrophage-secreted NO have been described (Vincendeau et al, 1992; Mabott et al, 1994; Gobaert et al, 1998). Differential NO production in

trypanosusceptible and trypanotolerant murine models has also been advanced as a basis for innate resistance to *T.congolense* infection (Kaushik et al, 1999).

Both caMΦ and aaMφ phenotypes are observed in trypanosome infections and are crucial in the control of trypanosome parasites. CaMΦ are predominantly found to develop in early stages of infection due to a prevailing type I cytokine environment whereas aaMφ are observed to occur in late stages of infection. An early response inflammatory response mediated by type I cytokines and caMΦ is essential for control of the initial aggressive waves of parasitemia which in turn influences survival of the host (Lucas et al, 1993; Seed and Sechelski, 1995). TNF-α, one of the cytokines secreted by caMΦ has been shown to be trypanolytic (Magez et al, 1997; Kitani et al 2002). However, a persistent inflammatory environment due to caMΦ may turn detrimental to the host owing to development of pyrexia, cachexia, anaemia and hypertryglyceridemia mediated by proinflammatory cytokines (Rouzer and Cerami, 1980; Okomo-Assoumou et al, 1995; Quan et al, 2003). Therefore, control of excessive inflammation and successive waves of parasitemia leading to prolonged host survival is pegged on induction of an anti-inflammatory environment mitigated by type II cytokines and aaMφ (Namangala et al, 2001; Vincendeau and Bouteille, 2006).

Specific structural component of the trypanosome seem to have inherent capacity to activate macrophages. The VSG moieties of *T.brucei* and *T.cruzi* are potent proinflammatory activators resulting in development of caMΦ which secrete TNF-α, IL-1α, IL-6 and IL-12 (Magez et al, 1998; Almeida et al, 2001; Coller et al, 2003). VSG molecules are expressed on the cell surface of all trypanosomes and are cyclically released into the host's blood where they aid in host immune evasion through antigenic variation. It is estimated that each trypanosome is covered with about 10^8 molecules of VSG and that at peak parasitemia of 10^7 parasites/ml of blood in the mouse model, the total estimate of VSG load in the host is about 2μg (Paulnock and Coller, 2001). The occurrence of aaMφ however, is correlated with attenuated parasites that lack the capacity to release VSG molecules (Namangala et al, 2001; De Baetselier et al, 2001). Further, the genomic DNA molecules of *T.cruzi* and *T.brucei* have also been reported to

stimulate macrophages to secrete inflammatory mediators. This activation was positively correlated with the content of unmethylated CpG motifs in the DNA (Harris et al, 2006). The effect of *TcVSG and TcDNA* on macrophage activation remains unexplored.

4.2.4 Exploring macrophage response to *TcVSG and TcDNA*

The time taken for clearance of the initial aggressive waves of parasitemia in *T.congolense* infections is inversely correlated with survival and therefore susceptibility or resistance to disease (Ogunremi and Tabel, 1995). The inflammatory response stimulated by parasite VSG and DNA in the *T.brucei* model is orchestrated by action of classically-activated macrophages and plays a central role in the control of infection (Namangala et al, 2001; Coller, 2003; Harris, 2006). Although it is appreciated that *T.congolense* infection triggers a predominantly inflammatory response in the host as *T.brucei*, little has been done to delineate the role of various structural components of *T.congolense* in macrophage activation and subsequent generation of the inflammatory response (Noel et al, 2002). There is need for a more comprehensive investigation of macrophage activation due to *T.congolense* infection in appropriate mouse models as this may also provide useful clues into the innate mechanisms underlying tolerance in African Animal Trypanosomiasis.

Prior studies on macrophage activation during trypanosome infection have been in vivo or ex vivo studies involving multiple cell types (Namangala et al, 2001; Donner et al, 2002). For this reason, it has been difficult to home in onto the specific cell populations involved in generation of the responses. *In vitro*studies have employed macrophage cells lines that are generated through various cell manipulations as immortalization (Harris et al, 2006). Although such systems have provided excellent tools to investigate macrophage activation during trypanosome infection, there remains a need for a standard macrophage cell system to validate these responses.

This work explores the role of *TcVSG and TcDNA* in macrophage activation as depicted by the cytokine and chemokine profiles of macrophages from trypanosusceptible and trypanotolerant mouse models. Bone marrow-derived macrophages of A/J and C57BL/6

mice are utilized in a standardized cell culture system to provide a “clean and immunologically naïve” environment for the study.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

A 2 x 2 x 6 factorial design was employed where bone marrow-derived macrophages from two mouse strains A/J and C57BL/6 were incubated for 6hrs or 24 hrs with any one of the following treatments i.e. i) 100 ng/µl *T.congolense* VSG ii) 100 ng/µl *T.congolense* DNA iii) 100 ng/µl *T.congolense* total lysate iv) 100ng/µl homologous mouse DNA v) 10ng/µl *E.coli* LPS and vi) No treatment. The cell supernatants were harvested following the aforementioned time points and assayed for the levels of 12 cytokines and 4 chemokines. A detailed experimental outline is described below.

4.3.2 Animals

Trypanosusceptible A/J mice and trypanotolerant C57BL/6 mouse models were used in the study for isolation of bone marrow-derived macrophages. All mice were between 8-12 weeks old, males and females in equal proportion, free from any infections and maintained under sterile conditions in the ILRI small animal unit (Nairobi, Kenya). Sprague Dawley rats were also obtained for expansion of *T.congolense* parasites. All animal were given food and water ad *libitum* and handled in compliance to ILRI's recommended institutional animal care and use committee (IACUC) regulations.

4.3.3 Isolation of bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared according to established methods (Stanley, 1997). Briefly, mice were sacrificed by cervical dislocation and their hind legs sterilized with 70% ethanol. An incision was made to expose the femur and tibia and these were de-fleshed as much as possible using sterile scissors and forceps. The femurs were then isolated without puncturing the ends and placed in RPMI medium supplemented with 10% foetal bovine serum (FBS), penicillin/streptomycin and gentamycin. The femur bones were then opened up under sterile environment by clipping both ends of the bones with sterile scissors, after which the bone marrow was flushed out of the bone with RPMI1640 medium (GIBCO®) into sterile Petri dishes and

dissociated using needle and syringe to obtain a cell suspension. A/J and C57BL/6 mice bone marrows were processed separately.

The cell suspensions were spun down at 1500 x g and the cell pellets were resuspended in RPMI1640, supplemented with 10% FBS (GIBCO®) 30% L929-conditioned medium (See appendix), penicillin/streptomycin and gentamycin. The cultures were incubated at 37°C and 5% CO2 continuously for three days and on the fourth day the non-adherent cell populations were discarded while the adherent cells were washed and cultured again in the same medium for a further two days. On day seven, the cells were dislodged by trypsinization, counted by trypan blue exclusion on a haemocytometer, and plated out in 96 well culture plates at a density of 10^5 cells/well.

4.3.4 Preparation of Trypanosomal factors

T.congolense clone IL1180 was obtained as a stabilate from the biological services unit in ILRI, Kenya, following which they were multiplied by expansion in mice and rats. Parasites were harvested and used to prepare *T.congolense* VSG, DNA and total lysate (see appendix for SOPs).

4.3.5 Co-culture of murine BMDM cells with *T.congolense* parasite preparations

The bone marrow-derived macrophages were subjected to treatment with the following i) 100 ng/µl *T.congolense* VSG ii) 100 ng/µl *T.congolense* DNA iii) 100 ng/µl *T.congolense* total lysate iv) 100ng/µl homologous mouse DNA v) 10ng/µl *E.coli* LPS (Lonza) and vi) No treatment. Experimental controls included *E.coli* LPS as a positive control and untreated cells as a negative control.

The plates were then incubated at 37°C with 5% CO_2 for two time points i.e. 6hrs and 24hrs. Each of the six treatments was performed in triplicate for each strain of mouse for each of the time points. On elapse of each of the time points, the cell supernatants were aseptically aspirated into sterile labelled tubes and stored at -80°C for subsequent cytokine assays.

4.3.6 Cytokine and chemokine assay using Luminex technology

The cell supernatants obtained above were assayed for 12 cytokines and 4 chemokines within a dynamic range of 2.44– 40,000 pg/ml. The cytokines included TNF-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL12p70, TGF-β and leptin whereas the chemokines included CCL3 (MIP-1α), CCL5 (RANTES), CXCL10 (IP-10) and CXCL1 (KC).

The assays were performed by the Procarta® Cytokine Assay Service using the Luminex Technology (Affymetrix, Freemont USA). This technology employs basic ELISA principles and entails the use of microsphere beads impregnated with a combination of two fluorescent dyes in unique ratios, thereby according each bead a distinct spectral signature. Each of the colour-coded beads is coated with a bioanalyte (in this case, an antibody to a specific cytokine or chemokine), which is used in the capture of the complementary cytokine in the test sample. This is a highly robust technology where up to 100 analytes can be assayed in a multiplex reaction within a single sample.

All assay reactions were performed according to manufacturer's instructions. Firstly, the beads coated with the primary antibody were added to the test samples in addition to a set of calibrated control antigens, and a detection antibody in this case an anti-mouse IgG was added and finally the substrate (streptavidin-PE) was incorporated into the reaction to prompt a luminescence on exposure to laser beams. Real-time digital signal processing was then performed by the Luminex reader utilizing a flow cytometry approach, where the beads arraigned in a flow cell were singly interrogated by bead colour and fluorescence intensity. The signal intensity attained was therefore specific to the abundance of a given cytokine within the test sample. The workflow of the assay is illustrated in Fig 6.

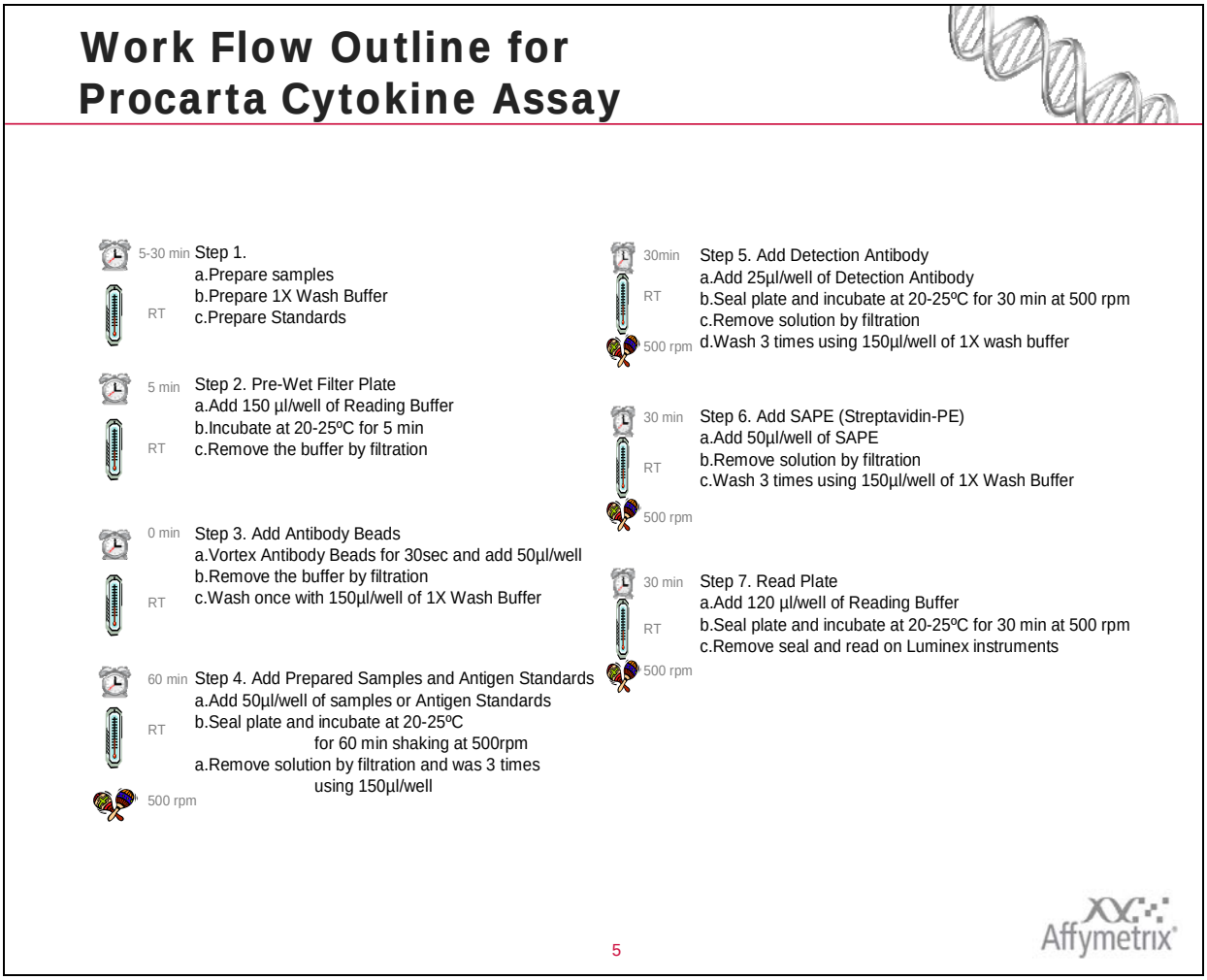


Figure 4.1: Workflow for cytokine and chemokine assay using the Luminex technology (Procarta/Affymetrix). This workflow was utilized to quantitate the concentration levels of twelve cytokines and four chemokines in cell supernatants of A/J and C57BL/6 bone marrow-derived macrophages exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium over 6hrs and 24hrs.

4.3.7 General trends of cytokine and chemokine secretion

The average secretion of all cytokines and chemokines was analyzed across all time points, strains and treatments. The cytokines with the highest levels were TNF-α, IL-1α, IL-10, IL-6 and IL12p40 all of which are proinflammatory cytokines. All the four chemokines assayed i.e. CCL3, CCL5, CXCL1 and CXCL10 also had high secretions levels ranging between 1000-11000 pg/ml (Fig 1). IL-2, IL-4 IFN-γ, IL12p70 and leptin had average secretion levels less than the lower limit of the dynamic range for the assay. These five cytokines were subsequently excluded from further analyses. There was a response pattern with respect to time whereby cytokines and chemokines were attained by 6hrs markedly reduced by 24hrs except for IL12p40 and CCL5 whose levels continued to be elevated at 24hrs (Fig 2).

C57BL/6 BMDMs secreted higher levels of cytokines and chemokines than A/J BMDMs. These levels were particularly heightened in response to *E.coli* LPS and *T.congolense* VSG. *T.congolense* DNA also elicited cytokine and chemokine secretion albeit to a lesser magnitude in comparison to *T.congolense* VSG (Fig 3). *T.congolense* total parasite lysate elicited a very low macrophage response as depicted by the low levels of cytokines and chemokines and the homologous mouse DNA-treated macrophages elicited a comparable response to that observed in untreated negative controls.

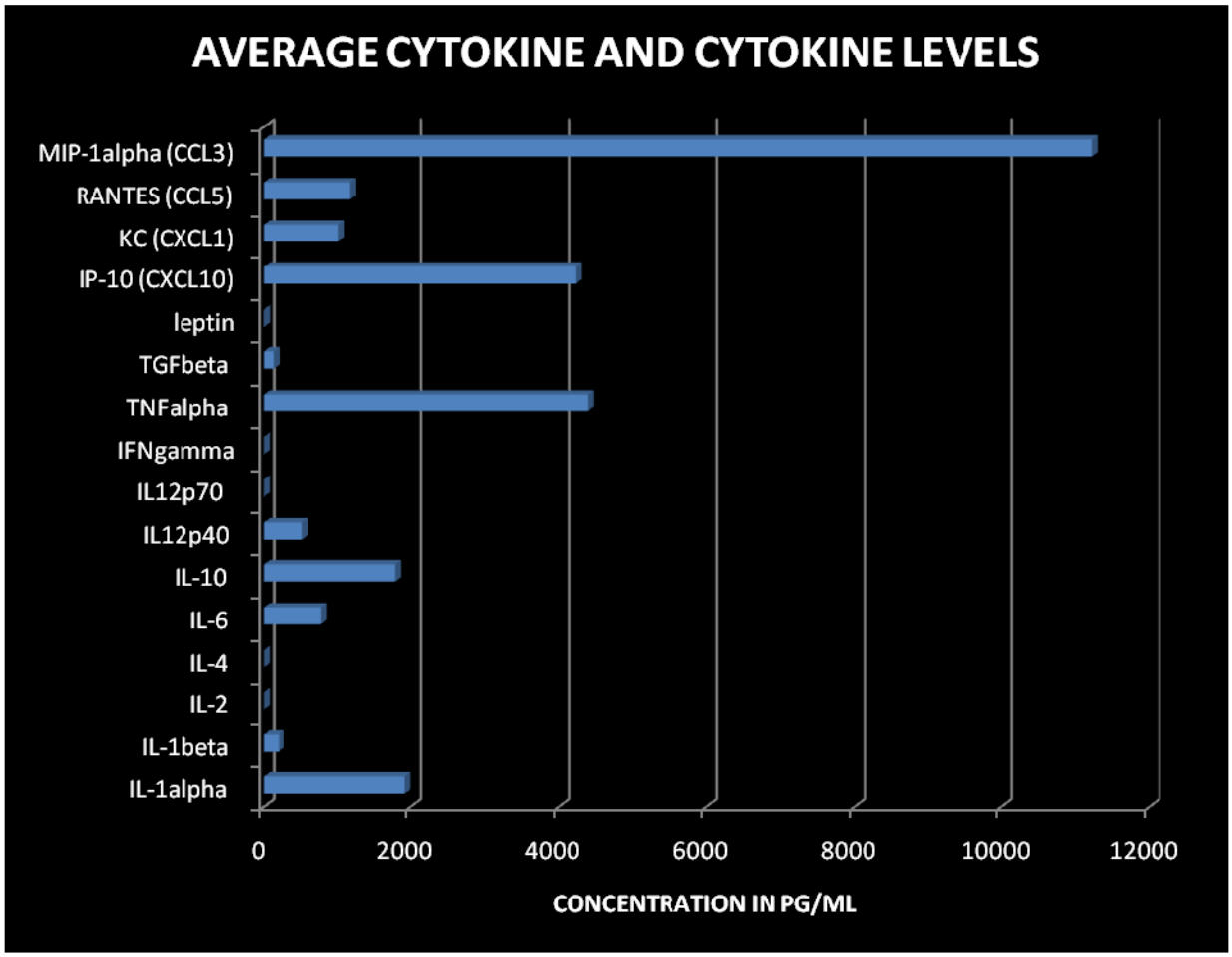


Figure 4.2: General trends of cytokine and chemokine secretion by bone-marrow derived macrophages obtained from A/J and C57BL/6 mice. Macrophages were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium. Cell supernatants were harvested after 6hrs or 24hrs of treatment and assayed for cytokine and chemokine concentrations. Average concentration for each of the cytokines and chemokines was computed. CCL3, TNF-α, CXCL10, IL-1α, IL-10, CCL5, CXCL1, IL-6 and Il12p40 recorded average concentration of >500pg/ml.

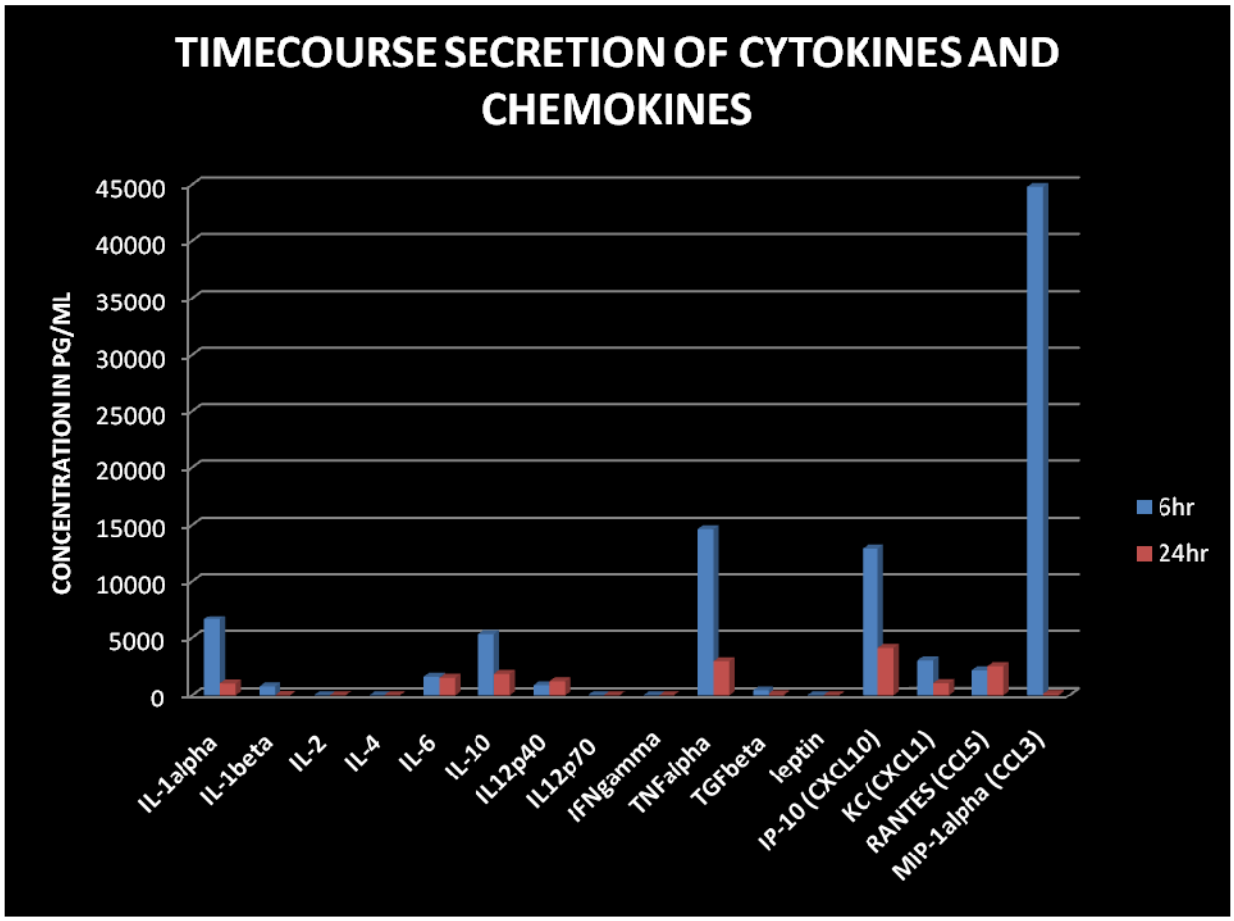


Figure 4.3: Temporal patterns in cytokine and chemokine secretion by A/J and C57BL/6 mouse bone marrow-derived macrophages in response to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium over 6hrs and 24hrs. Average concentration of each of the cytokines and chemokines computed at 6hrs and at 24hrs. The general trend was elevated by 6hrs followed by marked decrease in cytokine and chemokine levels by 24hrs of treatment.

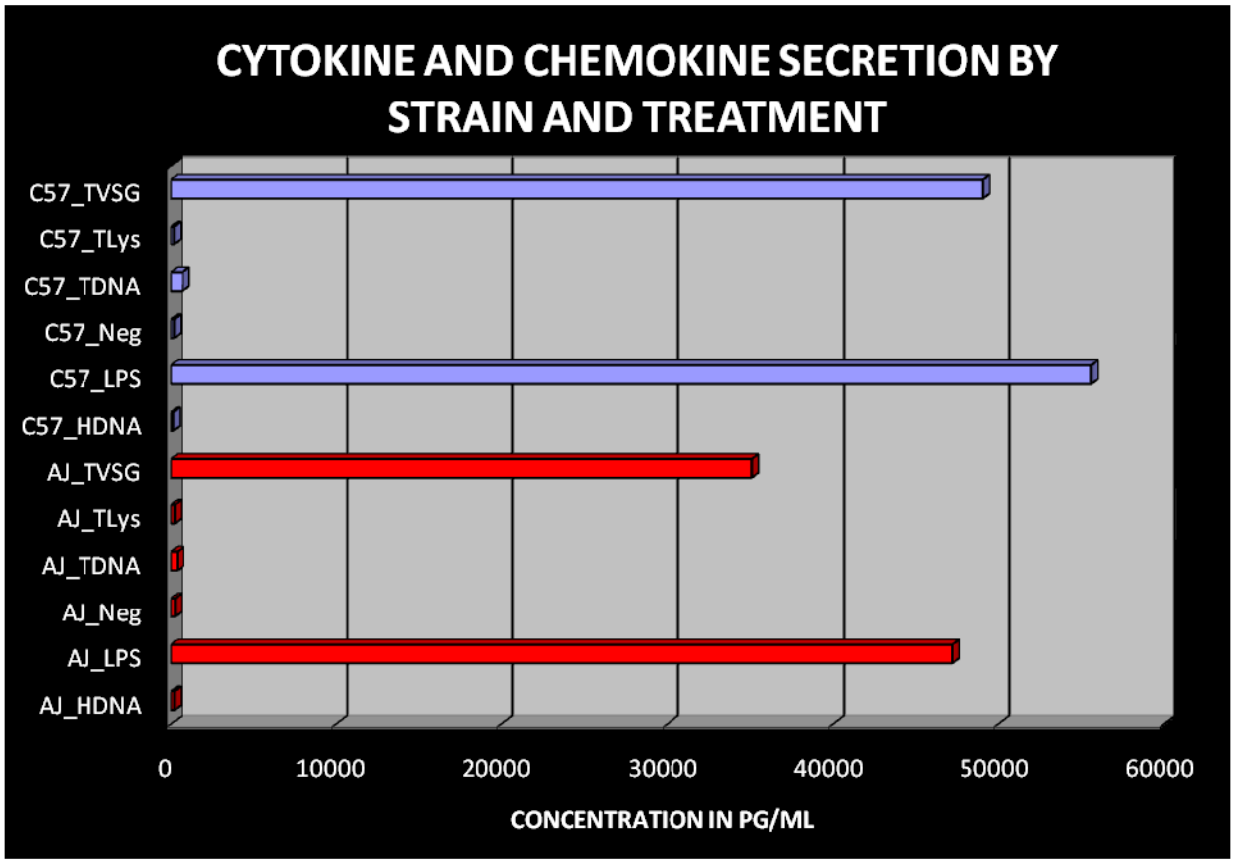


Figure 4.4: Effect of host genotype and type of treatment on secretion of cytokines and chemokines by mouse bone marrow-derived macrophages. Bone marrow-derived macrophages from A/J and C57BL/6 mice which vary in their genetic susceptibility to T.congolense were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium over 6hrs and 24hrs. Average concentration of cytokines and chemokines was calculated for each mouse strain and treatment. Highest cytokine and chemokine levels were observed for C57BL/6 mice in response to E.coli LPS.

4.3.8 Effect of treatment, time and host genotype on secretion of cytokines

The effect of time of exposure to antigen, type of antigen and host genotype as depicted by the strain of mouse model from which the macrophage cells were derived on secretion of each of 7 cytokines and 4 chemokines was achieved through general analysis of variance (ANOVA) using GenStat (Discovery edition 3) by applying the model below:-

$$\mathbf{T_s = a_{rx} + b_t + c_{st} + e}$$

Where,

$\mathbf{T_s}$ is the total secretion level of each cytokine

$\mathbf{A_{rx}}$ is the effect of treatment (i.e. LPS, TcVSG, TcDNA, TcLysate, MusHDNA and Untreated),

$\mathbf{b_t}$ is the effect of time of exposure to treatment (i.e. 6hrs and 24hrs),

$\mathbf{c_{st}}$ is effect of host genotype i.e. strain of mouse from which the BMDMs were derived (i.e. A/J and C57BL/6) and,

e was the error

The analysis showed that treatment and time affected secretion of majority of cytokines whereas genotype had selected effect on secretion of three cytokines only. To this effect, IL-1α and TGF-β were not significantly affected by type of treatment whereas; IL-6 and IL12p40 did not vary significantly with time of exposure to treatments. The chemokine CCL5 did not respond significantly to both treatment and time. There was significant effect of host genotype on secretion of IL-6, IL-12p40 and TGF-β (Table 1). Specific effects of treatment, time and strain on secretion profiles of the chemokines and cytokine were explored in detail.

4.3.8.1 Effect of treatment on cytokine and chemokine levels

Of the eleven cytokines assayed, there were significant changes relative to the control in eight of the cytokines in response to treatments with the exception of IL-1α, TGF-β and CCL5 (Table 1). IL-6, IL-10, CXCL1 and CXCL10 showed marked response to

treatment ($p<0.001$) while TNF-α, IL-1β, IL12p40 and CCL3 also responded significantly to treatment ($p<0.05$).

Table 4.1: Effect of time, treatment and host genotype (strain) on secretion of cytokines and chemokines by A/J and C57BL/6 bone marrow-derived macrophages. Macrophages were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium. Cell supernatants were harvested after 6hrs or 24hrs of treatment and assayed for cytokine and chemokine concentrations. Differential secretion of each of the cytokines and chemokines due to type of treatment, time of exposure to treatment and host genotype was calculated by ANOVA. Significant variation in cytokine and chemokine concentrations are highlighted in red ($p<0.05$).

Classification	Description	Treatment	Timepoint	Strain
Cytokines	TNF-a	0.003	0.02	0.848
	IL-1a	0.848	0.035	0.26
	Il-1b	0.019	0.018	0.975
	IL-6	<.001	0.781	0.044
	IL-10	<.001	0.044	0.269
	IL12p40	0.006	0.546	0.01
	TGF-b	0.242	<.001	0.02
Chemokines	CCL3	0.047	0.017	0.62
	CCL5	0.121	0.138	0.192
	CXCL1	<.001	0.023	0.167
	CXCL10	<.001	0.016	0.936

Table 4.2: Effect of treatment on secretion of cytokines and chemokines by A/J and C57BL/6 bone marrow-derived macrophages. Macrophages were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium. Cell supernatants were harvested after 6hrs or 24hrs of treatment and assayed for cytokine and chemokine concentrations. Differential secretion of each of the cytokines and chemokines between treatments was computed by ANOVA. Significant effects are denoted by "+" sign ($p<0.05$) whereas a minus "-"sign denotes no significant difference ($p>0.05$). E.coli LPS and T.congolense VSG had the highest effect on cytokine and chemokine secretion.

Classification	Description	Untreated	E.coli LPS	TcVSG	TcLysate	TcDNA	MusHDNA
Cytokines	TNF-a	-	+	+	-	-	-
	IL-1a	-	+	-	-	-	-
	Il-1b	-	+	-	-	-	-
	IL-6	-	+	+	-	-	-
	IL-10	-	+	+	-	-	-
	IL12p40	-	+	+	-	-	-
	TGF-b	-	-	-	-	-	-
Chemokines	CCL3	-	+	-	-	-	-
	CCL5	-	+	-	-	-	-
	CXCL1	-	+	+	-	-	-
	CXCL10	-	+	+	-	-	-

The role of each treatment on each of the seven cytokines and four chemokines was explored in detail. *E.coli* LPS and *T.congolense* VSG triggered the highest effect on cytokine and chemokine levels (Table 2). All analytes except TGF-β responded significantly to LPS treatment whereas *T.congolense* VSG, elicited significant response in TNF-α, IL-6, IL-10, IL-12p40, CXCL1 and CXCL10 only. TcDNA elicited 200-fold increase in CXCL10, >30 fold increase in TNF-α, >100-fold increase in CXCL1 and IL12p40 and IL-10 and 3-fold increase in IL-1α and CCL3 by 6hrs relative to untreated cells. These increases were however not statistically significant. Cells exposed to *T.congolense* lysate, homologous mouse DNA and untreated cells showed no significant variations in cytokine secretion for all 11 cytokines.

An interesting observation was the time-strain relationship in response to *T.congolense* DNA, where A/J macrophages secreted highest cytokine levels at 6hrs whereas C57BL/6 levels peaked at 24hrs after treatment (Fig 4). Cytokine levels of TcDNA-treated C57BL/6 macrophages however remained low by 6hrs and peaked at 24hrs, contrary to the A/J observation of an early response.

Pairwise comparisons between treatments based on the collective cytokine and chemokine expression elicited by each treatment were performed by computing differences between treatments using student t-tests (Gosset, 1908). Cytokine secretion of cells treated with LPS, *T.congolense* VSG and *T.congolense* DNA differed significantly from each other and all other groups i.e. they had unique cytokine secretion profiles ($p<0.05$). There were no significant differences between untreated cells and cells treated with homologous mouse DNA or *T.congolense* total lysate (Table 3).

4.3.8.2 Effect of time on cytokine and chemokine secretion

Cytokine and chemokine secretion by the mouse macrophages in response to the treatments was investigated over a 6hr and 24hr timecourse. The general trend depicted a heightened cytokine response by 6hrs followed by a marked reduction in secretion by 24hrs of treatment (Figure 2). ANOVA analysis of individual cytokines and chemokines corroborated this trend, with significant variation in secretion across the time intervals

($p<0.05$) in nine out of the eleven cytokines and chemokines except for IL-6 and IL12p40 which persisted in high titres across the time points, as well as the chemokine CCL5 whose secretion did not vary significantly (Table 1). TGF-β recorded the most significant variation in secretion over time ($p<0.001$). These observations were confirmed by a series of t-tests between 6hr responses and 24hr responses (Table 4).
Effect of host genotype on cytokine and chemokine secretion

Differential responses between macrophages obtained from trypanosusceptible (A/J) and the trypanotolerant (C57BL/6) mouse models as analyzed by t-test indicated that macrophages from trypanosusceptible and trypanotolerant mouse models differed significantly ($p<0.05$) in their cytokine secretion (Table 4). C57BL/6 macrophages consistently secreted higher levels of the cytokines and chemokines relative to A/J macrophages in response to treatment with LPS, TcVSG and TcDNA (Figure 3). An interesting observation however, was that macrophages of the trypanosusceptible A/J mice secreted significantly higher levels ($p<0.05$) of the anti-inflammatory cytokine TGF-β than the trypanotolerant C57BL/6 macrophages (Fig 5)**.** In addition, A/J macrophages secreted higher levels albeit not statistically significant ($p>0.05$) of the CXC chemokines i.e. CXCL1 and CXCL10 than C57BL/6 macrophages (Table 4). Secretion of two other cytokines i.e. IL12p40 and IL-6 was influenced by host genotype with C57BL/6 macrophages producing significantly higher levels ($p<0.05$) of these cytokines relative to A/J macrophages (Fig 5).

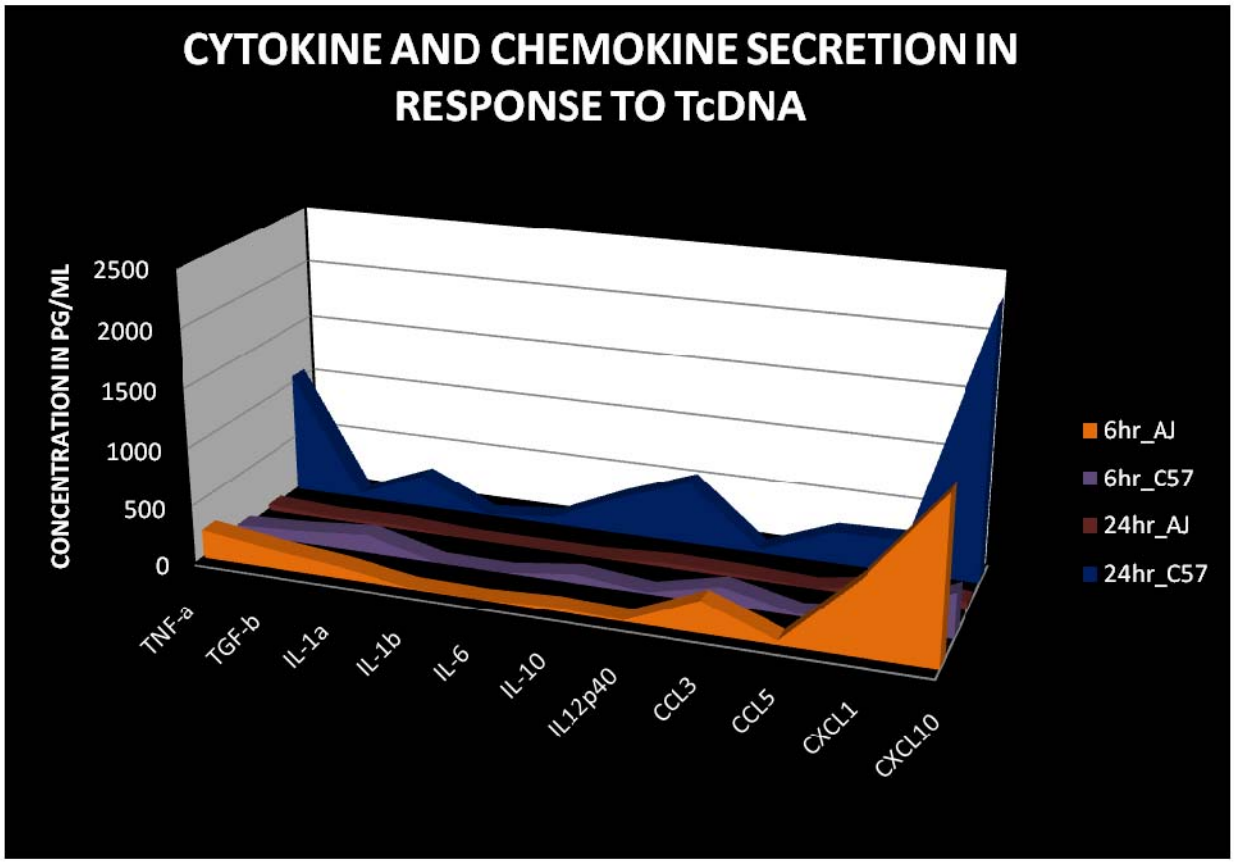


Figure 4.5: Effect of *T.congolense* DNA on cytokine and chemokine secretion by A/J and C57BL/6 bone marrow-derived macrophages over 6hr and 24hr timecourse. Bone marrow-derived macrophages from A/J and C57BL/6 mice which vary in their genetic susceptibility to *T.congolense* were exposed to 100ng/µl *T.congolense* DNA. Cell supernatants were harvested after 6hrs or 24hrs of treatment and assayed for cytokine and chemokine levels. A/J macrophages respond by 6hrs with increased levels of cytokines and chemokines whereas C57BL/6 macrophages are more responsive at 24hrs.

Table 4.3: Pairwise comparison of cytokine and chemokine secretion between treatments in bone marrow-derived macrophages from A/J and C57BL/6 mice were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium over 6hrs and 24hrs. Differences in cytokine and chemokine secretion levels between treatments were calculated by ANOVA. Significant differences between treatment groups are denoted by a plus "+" sign ($p<0.05$) whereas a minus "-"sign means there is no significant difference between treatments ($p>0.05$). Cytokine and chemokine secretion profiles for macrophages treated with E.coli LPS, T.congolense VSG and T.congolense DNA significantly varied from untreated macrophages. There were no significant differences in macrophages treated with T.congolense lysate, homologous mouse DNA and no treatment.

	Untreated	E.coli LPS	TcVSG	TcLysate	TcDNA	MusHDNA
Untreated	-	+	+	-	+	-
E.coli LPS	+	-	+	+	+	+
TcVSG	+	+	-	+	+	+
TcLysate	-	+	+	-	+	+
TcDNA	+	+	+	+	-	+
MusHDNA	-	+	+	+	+	-

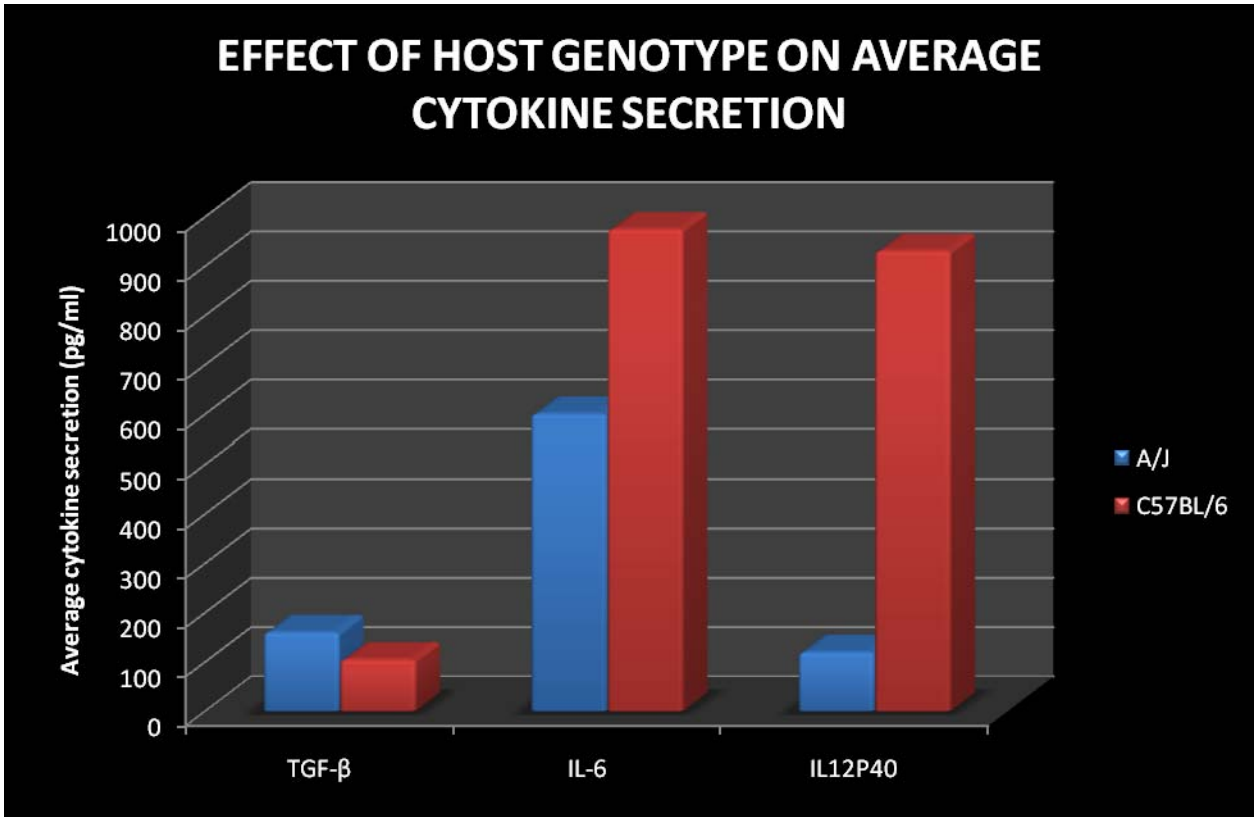


Figure 4.6: Effect of host genotype on secretion of TGF-β, IL-6 and IL12p40 by A/J and C57BL/6 bone marrow-derived macrophages. Bone marrow-derived macrophages from A/J and C57BL/6 mice which vary in their genetic susceptibility to T.congolense were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium over 6hrs and 24hrs. TGF-β, IL-6 and IL-12p40 are significantly differentially secreted by A/J and C57BL/6 macrophages ($p<0.05$). C57BL/6 macrophages secrete significantly higher levels of IL-6 and IL-12p40 whereas A/J macrophages secrete significantly higher levels of TGF-β.

Table 4.4: Effect of time of exposure to treatments on secretion of cytokines and chemokines by A/J and C57BL/6 bone marrow-derived macrophages. Macrophages were exposed to 100pg/µl E.coli LPS, 100ng/µl T.congolense VSG, 100ng/µl T.congolense DNA, 100ng/µl T.congolense total lysate, 100ng/µl homologous mouse DNA and culture medium. Cell supernatants were harvested after 6hrs or 24hrs of treatment and measured for cytokine and chemokine concentration (in pg/ml). Differences in cytokine and chemokine secretion between 6hrs and 24hrs of exposure to treatments were computed by student t-test. Cytokine and chemokine levels varied significantly ($p<0.05$) between 6hrs and 24hrs of treatment.

		6hr all Rxs		24hr all Rxs	
Classification	**Description**	**A/J**	**C57BL/6**	**A/J**	**C57BL/7**
	TNF-a	49508.72	38363.14	5981.28	11810.5
	IL-1a	12975.06	26841.43	1520.21	4725.13
	Il-1b	2324.81	2331.79	83.26	129.55
	IL-6	3725.17	6013.22	3476.61	5679.33
	IL-10	11950.28	19939.45	4142.76	7033
	IL12p40	576.17	4697.55	853.93	6466.83
Cytokines	TGF-b	1633.8	974.46	282.7	278.18
	CCL3	108778.56	160016.77	409.05	296.94
	CCL5	12990.56	86745.58	7562.12	7598.86
	CXCL1	11556.15	6631.31	4117.07	2192.84
Chemokines	CXCL10	39562.97	37531.33	12132.31	12572.57
t-test 6hr A/J and C57BL/6 Vs 24hr A/J and C57BL/6				p= 0.00471541*	
t-test 6hr A/J Vs 24hrA/J				p= 0.036485582*	
t-test 6hr C57BL/6 Vs 24hr C57BL/6				p= 0.033911737*	

4.4 **DISCUSSION**

Macrophage cells are central in control of *T.congolense* infection and constitute the first line defence against the parasites through secretion of cytokines, chemokines and reactive oxygen and nitrogen species all of which either influence the direct killing of the parasites or activate other immune components. The activation status of the macrophages as defined by their cytokine and chemokine repertoire influences the course of immune direction and disease, where caMΦ favour an inflammatory response whereas aaMφ are inclined to an anti-inflammatory response. While previous investigations have shown involvement of both caMΦ and aaMφ in *T.brucei* and *T.cruzi* infections (Almeida and Gazzinelli, 2001; Stijlemans et al, 2007), the role of *T.congolense* in triggering macrophage activation during the course of disease has not been fully explored.

This study therefore explored activation of naive macrophage populations derived from two mouse strains; AJ and C57BL/6 which differ in genetic susceptibility to *T.congolense* infection. Macrophage activation was characterized by cytokine and chemokine secretion profiles in response to two *T.congolense* PAMPS - VSG and DNA as well total parasite lysate on the basis that the host immune system is exposed to these Trypanosomal factors during the course of infection (Sternberg, 2004; Harris et al, 2006). Three treatment controls i.e. untreated, *E.coli* LPS and homologous mouse DNA were also incorporated to make a sum of six treatments which were applied to the macrophages for durations of 6hr and 24hrs.

The results showed that *E.coli* LPS, TcVSG and TcDNA listed in order of magnitude of potency, were able to significantly alter the secretion levels of the treated macrophages when compared against the untreated controls. The macrophages secretion levels of TNF-α, IL-1β, Il-6, IL12p40, CCL3, CXCL1 and CXCL10 all of which are type 1 cytokines and chemokines were significantly elevated by 6hrs of exposure to *E.coli* LPS, TcVSG and TcDNA. These cytokine and chemokine profiles were depictive of classically activated macrophages mediating an inflammatory response (Mosser, 2003). Macrophages of the trypanotolerant mouse model C57BL/6 generally secreted higher

levels of these proinflammatory mediators than macrophages derived from the trypanosusceptible model, A/J.

TcVSG compared positively with the classical endotoxin *E.coli* LPS in triggering an inflammation environment although there were clear variations in the levels of secretion by duration of exposure to treatment and host genotype. TcVSG triggered significantly elevated levels ($p<0.05$) of TNF-α, IL-1α, IL-6, IL-1β and IL12p40 as well as the counter-inflammatory cytokine, IL-10 by 6hrs. There was also parallel increase in secretion levels of CXCL1 and CXCL10. TcVSG therefore triggered an early inflammatory response that was controlled by 24hrs into the treatment as evidenced by the significantly diminished levels of both inflammatory and counter-inflammatory cytokines. These findings agree with previous reports that VSG moieties of *T.brucei* and *T.congolense* activate macrophages to an inflammatory phenotype (Tachado et al, 1997; Namangala et al, 2001; Magez et al, 2002). A strong association between increased systemic levels of TNF-α from caMΦ infection and *T.congolense*-induced immunopathologies has been advanced whereby TNF-α induction is said to be dependent upon early exposure of macrophages to IFN-γ (Magez et al, 2002). Based on the fact that TNF-α was significantly elevated in our set up where we utilized naive macrophages in an IFN-γ-free system, we demonstrate that TNF-α production by macrophages during T-*congolense* infection is not entirely IFN-γ-dependent and can be induced by TcVSG in the absence of IFN-γ.

Increasing levels of proinflammatory cytokines and chemokines in response to TcVSG were accompanied by a parallel increase in the counter-inflammatory cytokine, IL-10. IL-10 elicited a similar trend as the proinflammatory mediators with peak levels being achieved by 6hrs followed by significantly diminished levels by 24hrs of exposure to TcVSG. Elevation of IL-10 was also more pronounced in A/J than in C57BL/6 although the differences were not statistically significant. Increased secretion of IL-10 have previous been noted in both murine and bovine infections with *T.congolense* where trypanosusceptible Boran cattle and Balb/c mice elicited higher levels of the cytokine

than the trypanotolerant N'dama cattle and C57BL/6 mice (Kaushik et al, 1999; O'Gorman et al).

TcDNA stimulated the macrophages to secrete increased levels of the proinflammatory cytokines TNF-α, IL-1α, IL-10 and IL12p40 as well as the type I chemokines CCL3, CXCL1 and CXCL10. This cytokine and chemokine repertoire was indicative of classically activated macrophages. However, the secretion levels attained in response to TcDNA for these cytokines and chemokines were of a moderate nature in that they did not attain statistical significance ($p>0.05$) and were therefore not comparable those achieved in response to TcVSG and *E.coli* LPS. A distinct temporal pattern between macrophages of the two mouse models was also discerned. A/J mice showed an early response to TcDNA with peak proinflammatory cytokine and chemokine levels being attained by 6hrs after treatment, whereas, C57BL/6 displayed a delayed inflammatory response which peaked at 24hrs post-treatment.

This is the first description to our knowledge of macrophage activation by *T.congolense* DNA and is in agreement with previous studies that have depicted the involvement of protozoan DNA in macrophage activation (Shoda et al, 2001). According to this report *T.brucei* and *T.cruzi* DNA was shown to activate macrophages to a classical phenotype in a similar fashion as bacterial DNA leading to increased secretion of IL-12, TNF-α and NO. It was proposed that trypanosome DNA contained unmethylated CpG motifs similar to bacterial CpG islands and that this feature conferred trypanosome DNA the capacity to induce CpG-dependent inflammation pathways (Harris et al, 2006). However, the unmethylated CpG content of *T.congolense* DNA remains currently uncharacterized.

Variations in cytokine profiles between macrophages of the two host genotypes (A/J and C57BL/6) were analyzed with an aim of providing clues to genetic mechanisms underlying tolerance to disease. Inherent differences in the cytokine profiles were detected between macrophages from trypanotolerant C57BL/6 and trypanosusceptible A/J mouse models. To this effect, IL12p40, TGF-β and IL-6 were significantly differentially secreted in response to treatment ($p<0.05$). C57BL/6 macrophages secreted

significantly higher levels of IL12p40 and IL-6 than A/J macrophages whereas A/J macrophages secreted higher levels of TGF-β than C57BL/6. A/J macrophages also secreted higher levels of IL-10 also not to a significant level ($p>0.05$).

IL-12 has been associated with trypanotolerance in bovine Trypanosomiasis where it induces INF-γ-dependent inflammation via macrophage and T-cell activation that mediate parasite clearance (Mertens et al, 1999; Silva et al, 2003). Uzonna et al showed that IL-12 mediated synthesis of antibody responses to trypanosome VSG antibodies (Uzonna et al, 1999). Based on these reports, our findings that tolerant C57BL/6 mice secrete significantly higher levels of IL-12 may allude to the fact that trypanotolerance in C57BL/6 is linked to comparably more efficient inflammatory and antibody responses and that lower levels of IL-12 in A/J may result in a delayed interferon-response.

IL-6 was secreted by C57BL/6 macrophages in significantly higher amounts that A/J from our findings. This pleiotropic cytokine is known to have capacity to exert both proinflammatory and anti-inflammatory activities. IL-6 is critical in development of pyrexia and cachexia in acute-phase responses and a role for its proinflammatory activity in nervous damage during *T.brucei* infection has also been shown (Oldenburg, 1993; de Vries et al, 2006). On the flip side IL-6 controls local and systemic acute inflammation by exerting anti-inflammatory effects on TNF-α, IL-1α and type I chemokines as CCL4 (Xing et al, 1998). This IL-6-specific anti-inflammatory activity cannot be compensated by other anti-inflammatory cytokines as IL-10. Secretion of higher levels of IL-6 by C57BL/6 macrophages may therefore point to the inherent capacity of the tolerant mouse model to efficiently mount an initial inflammatory environment to control *T.congolense* invasion which is effectively controlled in an autocrine fashion through sustained production of IL-6 thereby allaying excessive inflammation.

A/J macrophages secreted higher levels of the anti-inflammatory cytokines TGF-β and IL-10. IL-10 and TGF-β have been shown to modulate immunosuppression, downregulate inflammatory processes and production of reactive oxygen species all of which are crucial parasite clearance mechanisms executed by caMΦ (Namangala et al,

2000; Namangala et al, 2007). Silva et al demonstrated that high levels of TGF-β early in infection resulted in prolonged suppression of spleen cell immune responsiveness and decreased resistance to *T.cruzi* in tolerant C57BL/6 mice culminating in uncontrolled parasitemia and accelerated death due to inhibition of macrophage activation (Silva et al, 1991).IL-10 has been shown to uncouple chemokine receptors, thereby leading to reduced chemokine secretion levels which were also clear in our experimental findings (D'Amico et al, 2000). A/J mice are less efficient at clearing the first peak of parasitemia than C57BL/6 mice and this trait compromises their survival since long-term survival (Ogunremi and Tabel, 1995). Therefore higher levels of TGF-β and IL-10 and their inhibitory roles on macrophage activation during trypanosome infection may play a role in suppression of macrophage activation and diminished secretion of trypanotoxic proinflammatory mediators i.e. cytokines and nitric oxide thereby diminishing their capacity to control parasitemia and to survive infection.

4.5 CONCLUSION AND RECOMMENDATIONS

Macrophages are a key component of immune response to *T.congolense* infection through active secretion of cytokines and chemokines. This study demonstrates that macrophages are activated by TcVSG and TcDNA to a classical phenotype similar to macrophage activation by bacterial LPS which was characterized by increased secretion levels of proinflammatory cytokines and chemokines. This is the first study to explore involvement of TcDNA in macrophage activation, where TcDNA at a concentration of 100ng/µl induced a modest inflammatory signal in comparison to that induced by the same concentration for TcVSG. It is possible that the threshold required for macrophages activation by these two factors is different and perhaps a higher concentration of TcDNA may elicit a more pronounced effect. Nevertheless, we postulate that TcDNA is indeed be a potent stimulator of *T.congolense*-induced inflammation and acting in synergy with TcVSG. Further investigations are required to quantify CpG content of *T.congolense* DNA. Transcriptional profiling experiments would also be required to characterize the gene networks, signalling cascades involved in macrophage stimulation with *T.congolense* DNA and VSG.

This study also demonstrates differential secretion profiles of TGF-β, IL-10, IL12p40 and IL-6 by macrophages of the experimental murine trypanosusceptible and trypanotolerant models. This finding validates and harnesses fundamental knowledge on the genetic mechanisms underlying tolerance and susceptibility to *T.congolense* infections.

5 GENERAL DISCUSSION AND FUTURE PRESPECTIVES

African Bovine Trypanosomiasis continues to be a limiting factor for livestock production and diversity as well as a major threat to the livelihoods of small-scale livestock farmers in Sub-Saharan Africa. The disease mainly affects cattle, causing an initial acute phase associated with aggressive immune response and deaths. Individuals that do not recover or die of acute disease develop chronic disease which markedly diminishes productivity. No vaccine exists for Bovine Trypanosomiasis to date and billions of dollars are expended annually on relatively outdated but expensive drugs which aside from being highly toxic to livestock also have diminished effectiveness due development of drug resistance by the trypanosomes.

The minimal research efforts and resources being directed at this disease have seen it fall in the list of neglected diseases. This is indeed a paradox in context of the severity of the disease. This situation has precipitated the current paucity of information on the biological basis of the interactions between the major bovine trypanosome i.e. *T.congolense* and infected hosts which is crucial in the effective control of the disease. Although the existence of natural tolerance to the disease in specific indigenous cattle and availability of appropriate mouse models has generated knowledge on the genetic regions controlling host response to infection, a comprehensive perspective of the transcriptional responses of the host to *T.congolense* infection has been lacking (Kemp et al, 1997).

This thesis describes efforts to characterize host-pathogen interactions in acute infection with *T.congolense* in a bid to understand the bearing of these interactions in the development of pathology and pathogenesis of the disease.

The results validate inflammation as the key host response to infection during this stage. This *T.congolense*-induced inflammatory response triggers a gene-expression signature and a signaling cascade akin to that elicited by the classical endotoxin, LPS. The results, for the first time clearly characterize this inflammatory response by demonstrating host macrophages, TcVSG and TcDNA as the players involved in acute *T.congolense*-

induced inflammatory responses. In a standard *in vitro*model of mouse bone macrophages, this work for the first time potential of TcVSG and to a lesser extent TcDNA in activation of host macrophages to a classical phenotype that mediate the inflammatory response. These findings are discussed in this chapter in the context of development of pathogenesis and interventions of acute-phase *T.congolense* infections. Recommendations for follow up studies are also presented.

Evidence for an early LPS-like inflammatory response was revealed on analysis of transcriptional profiles of *T.congolense*-infected mice as presented in chapter 3 of this thesis. This response corroborated previous findings that highlighted an increase in circulating bacterial endotoxins during trypanosomiasis (Alafiatayo et al, 1993; Nyakundi et al, 2002). However, as we found no evidence for bacterial and endotoxin contamination in *T.congolense*-infected mice, it became apparent that specific substances of *T.congolense* origin were responsible for the inflammation. Further characterization in an *in vitro*model as shown in chapter 4 of this report revealed TcVSG triggered an LPS-like inflammatory response macrophages obtained from both trypanosusceptible and tolerant mouse models. Indeed the resukts show that 100ng/µl TcVSG triggered a comparable inflammatory response to that elicited by 10ng/µl of E.coli LPS. This finding echoed those of Tizard and Magez et al, who reported the potential of trypanosome VSG as the causative of systemic inflammation in human trypanosomiasis (Tizard et al, 1978; Magez et al, 1998).

Proof in recent studies attributes the inflammatory activity of trypanosome VSG to the GPI anchor. Following cleavage of VSG by PLC and release of soluble VSG trypanosome GPI anchors become exposed to the host immune system. Two distinct moieties of the GPI anchor of *T.brucei* (Magez et al, 2002) and *T.cruzi* (Almeida and Gazzinelli, 2001) stimulate macrophages to varying degrees. Additionally, protozoan GPI molecules as Plasmodium and Leishmania have been deemed as determinants of pathogenicity and virulence (Tachado et al, 1997). The role of the GPI anchor of TcVSG in stimulation of inflammatory response is currently speculative and is recommended as a subject of future research.

Prior studies have shown that vaccination with monoclonal antibodies raised against trypanosome GPI blocked the GPI-induced inflammatory response in *T.cruzi* (Tachado et al, 1999). Efforts are underway to develop GPI-based vaccines for malaria and leishmaniasis (Schofield et al, 2002). Evidence shows that synthetic GPI molecules not only reduce inflammatory responses in infected animals, but also elicit production of protective antibodies, implying that these preparations may be applied as immune-therapies and vaccines. Growing interest is being garnered around carbohydrate-based vaccines presumed to be more efficacious than those based on peptide antigens (Astronomo et al, 2010). Taken together, these efforts may highlight the need to revisit vaccine development against Trypanosomiasis and to explore GPI-based formulations as potential therapies.

Signalling pathwayss involved in *T.congolense* inflammatory responses have been largely unknown. However, findings in this study indicate the *T.congolense* induced inflammation is signaled via toll-like receptors. TLR1, TLR2 and TLR4 genes were significantly perturbed as described in chapter 3 of this thesis. These findings are in contrast to those of Drennan et al who reported no evidence of TLR, TLR2 and TLR4 in induction of type 1 innate response in *T.brucei*-infected mice (Drennan et al, 2005). Evidence shows that TLR2 is crucial in recognition of VSG-GPI anchors of *T.brucei*, *T.cruzi* and P.falciparum (Schofield et al, 1993; Campo et al, 2001; Coller et al, 2003). Indeed, TLR2 has been confirmed to have a functional receptor for GPI (Almeida and Gazzinelli, 2001). TLR1 has also been demonstrated to recognize the lipid moieties of parasite GPI anchors and lipoproteins (Takeuchi et al, 2002). A case for the involvement of TLR4 in inflammatory responsiveness to *T.cruzi* GPI anchor has also been made (Oliveira et al, 2004). Indeed, a review of investigations on mutant mice for different toll-like receptors indicates a strong association between exacerbated disease and defective TLR signaling (Gazzinelli et al, 2004). In combination, these observations add to the knowledge that toll-like receptors play vital roles in recognizing pathogen molecular patterns, initiating early immune responses.

The role of *T.congolense* DNA had previously not been explored although evidence drawn from prior studies had demonstrated immunostimulatory capacity of DNA from trypanosomes and other protozoans (Shoda et al, 2001; Harris et al, 2007). The results of this study demonstrate, for the first time the immunogenicity of *T.congolense* DNA thereby validating existing evidence. As presented in chapter 4 of this thesis, *T.congolense* DNA was able to activate macrophages to a classical phenotype and stimulate the secretion of pro-inflammatory cytokines and chemokines. The results show that at equal concentrations TcDNA was less immunogenic to macrophages in comparison to TcVSG, implying that while TcDNA may play a role, TcVSG was the fundamental trigger in T-*congolense* -induced inflammatory responses.

Previous studies have attributed the immunogenic capacity of trypanosome DNA to presence of unmethylated CpG motifs. This fact could not be deduced from this study and the CpG content of *T.congolense* DNA is recommended as a subject of further investigations. In other reports, protozoan DNA is reported be recognized by TLR9 signaling inflammatory responses through MyD88-dependent pathways (Gazzinelli and Denkers, 2006). Transcriptional profiles of *T.congolense*-infected mice described in chapter 3 of this thesis did not demonstrate perturbation of TLR9. This result is not however conclusive given the mild immunogenic capacity of TcDNA. Transcriptional profiling of macrophages exposed to TcDNA is therefore recommended to accurately delineate the role of TcDNA in triggering toll-like receptor signaling pathways.

Macrophages facilitate host early responses to pathogens and act as central effectors of innate immune responses. The nature of macrophage responses is reflected by their activation status. Consequently, macrophages switch between classical and alternative phenotypes which mediate inflammatory and anti-inflammatory responses respectively. This work presents for the first time a comprehensive investigation of macrophage activation in response to TcVSG and TcDNA based on a panel of sixteen cytokines and chemokines. Activation of macrophages by both TcVSG and TcDNA to a classical phenotype was confirmative of the role of type I macrophages in early inflammatory responses to *T.congolense* infection. These results corroborated similar investigations in

T.brucei where classically–activated macrophages developed in early stage infection (Namangala et al, 2001).

Additionally, inflammation was more marked in tolerant C57BL/6 macrophages that in susceptible A/J macrophages. IL-6 and IL12-p40 were particularly highly secreted by tolerant mouse macrophages in agreement with previous findings (Tabel et al, 2000). These observations were credible as classically activated macrophages have been associated with capacity to control trypanosome infection due to their inflammatory, cytotoxic, and antiproliferative functions. However, due to the increased pathologies associated with inflammation, a timely switch to alternatively-activated macrophages has been proposed to favour host survival and recovery. IL-10 and TGF-β which are shown to exert anti-inflammatory activities and favour the development of alternatively macrophages were elevated in the findings of this study.

Macrophage activation has been proposed to be dependent of initial priming by IFN-γ and previous *in vitro* studies on macrophage responses to trypanosomes have entailed addition exogenous of IFN-γ. This study demonstrates macrophage activation by TcVSG and TcDNA in the absence of IFN-γ, and therefore contravenes previous investigations that have reported macrophage activation to be compromised by absence of IFN-γ (Hertz and Mansfield, 1999). These observations suggest that either macrophage activation due to TcVSG and TcDNA is effected by IFN-γ-independent pathways or point to the existence of inherent mechanisms of macrophage priming.

6 FINAL CONCLUSION

This study validates inflammation as the key host response to early response to infection in acute *T.congolense* infection. Findings of this study also show that *T.congolense* inflammation is comparable to bacterial sepsis as it triggers gene expression signatures, signaling cascades and cytokine/chemokine repertoire akin to those elicited by bacterial endotoxin. These findings provide grounds for testing of established chemotherapies and immunotherapies for bacterial sepsis for control of acute *T.congolense* infections. Such explorations may offer insights to novel preventive measures against development of pathologies associated with persistent inflammation.

T.congolense VSG is characterized in this study as the primary driver while classically-activated macrophages are the key effectors of this response. *T.congolense* DNA is demonstrated for the first time to play a contributory role to generation of inflammatory responses albeit to a lesser degree compared against VSG. Signaling of the *T.congolense*-induced inflammation occurred via toll-like receptor pathways notably TLR1 TLR2 and TLR4. The involvement of TLR1 and TLR2 strongly imply that the GPI anchor of *T.congolense* VSG might be responsible for *T.congolense*-induced inflammation. Based on the fact that GPI anchors are highly conserved and protective antibodies can be raised against them, there exist opportunities for exploration of a GPI-based vaccine for Africa Trypanosomiasis.

The findings of this work therefore highlight the pertinent role of VSG in *T.congolense* inflammation and pathology and affirm new opportunities in the search for novel immune interventions for the control of acute disease. Indeed, effective control and management of Bovine Trypanosomiasis through novel interventions will contribute positively in conservation of existing diversity of African cattle.

7 APPENDIX

7.1 Standard Operating Procedures (SOPs)

7.1.1 Expansion of *T.congolense* IL1180 parasites

Stabilates of *T.congolense* clone IL1180 which is a derivative of a *T.congolense* Savannah type isolate that was originally collected from a lion in the Serengeti in Tanzania (Geigy and Kaufmann, 1973) were obtained from the biological services unit in ILRI, Kenya. The stabilates were thawed in a 37°C water bath and initial expansion of the parasites was performed by injecting the parasites into a donor A/J mouse that had received gamma irradiation for immunosuppression. On attaining parasiteamia of $>10^7$ parasites/ml, the mouse was euthanized by CO_2 asphyxiation and the parasitized blood harvested aseptically by cardiac puncture. This parasitized blood was then diluted 10^6 parasites/200µl in PBS containing 1mg/ml glucose (PBSG). A further expansion of the parasites was then performed in immunosuppressed Sprague Dawley rats by injecting them with 10^6 parasites each. The rats were monitored regularly for parasitemia until they attained levels of $>10^8$ parasites/ml. They were then euthanized by CO_2 asphyxiation and parasitized blood collected aseptically by cardiac puncture. Blood was maintained on ice prior to isolation of the trypanosomes.

7.1.2 Isolation of *T.congolense* parasites from rat blood

This was done according to the method previously described (Lanham and Godfrey, 1970). Briefly, the parasitized blood was diluted 1:1 in ice-cold PBSG pH 8.0. The diluted blood was then passed over a diethylaminoethyl cellulose type 52 (DEAE 52) column equilibrated to pH 8.0 with PBSG. Under these conditions, the cellular components of the blood were bound to the column whereas the free trypanosomes swam through the column and were collected in the eluate. The trypanosomes were concentrated by centrifugation at 1500 x g at 4°C washed three times in ice cold PBSG, counted on a haemocytometer and maintained on ice.

7.1.3 Purification of *T.congolense* VSG

Parasite pellets were resuspended for hypotonic lysis in uncoated siliconized vacutainer tubes (BD, UK) at 10^8 parasites/ml in ice cold lysis buffer. The lysis buffer was composed of a cocktail of serine and cysteine protease inhibitors (Sigma-Aldrich, UK) in PBSG. The composition of protease inhibitors per ml of buffer was 20µM AEBSF, 10µM EDTA, 1.3µM bestatin, 140nM E64, 10nM leupeptin and 3nm aprotinin. The trypanosomes were incubated in lysis solution at 4°C overnight.

The tubes were shaken for 30mins on a rotatory shaker and spun at 3000 x g for 10mins at 4°C. The supernatant was collected and retained, while the pellet was resuspended in fresh lysis buffer for a further 30mins at 37°C. The tubes were cooled to 4°C and spun at 10,000 x g for 15mins. The supernatant from this and the previous step were pooled and applied onto a DEAE 52 cellulose column equilibrated to pH 8.0 with PBSG. VSG was eluted in the first peak, purified and concentrated using YM-30 centripreps (Amicon, UK). The VSG was then run on a 12% SDS-PAGE gel and quantified using the Nanodrop™ 1000 spectrophotometer (Thermo Scientific) at 280nm. The VSG preparation was stored at -80°C until further use.

7.1.4 Preparation *T.congolense* DNA

T.congolense parasites isolated from the DEAE 52 cellulose were washed in PBSG and pelleted by spinning at 1500 x g for 10mins. 466µl Tris EDTA NaCl (TE/NaCl) buffer pH 8.0, 30µl 10% SDS, 3µl proteinase K and 1µl 20µg/ml RNAse A was added to the parasite pellet and incubated for 2hrs at 37°C with gentle shaking. An equal volume of phenol chloroform isoamyl alcohol equilibrated to the ratio 24:24:1 was added and the tubes mixed by gentle inversion for 1 min, then spun at 10,000 x g for 10mins.

The upper aqueous phase was collected into a fresh tube and a tenth of the volume of 3M sodium acetate was added and mixed by gentle inversion. This was followed by

addition of two volumes of cold absolute ethanol and mixing by gentle inversion. The mixture was then incubated for 2hrs at -80°C and the DNA was precipitated by spinning at 10,000 x g for 10mins. The DNA precipitate was then washed twice with 75% ethanol and the pellet dried in a vacuum drier. The DNA was resuspended in Tris EDTA pH 8.0. Quality and quantity controls were performed by running the DNA on a 0.8% agarose gel and spectrophotometry analysis at 260nm (Nanodrop). The DNA preparation was stored at -80°C.

7.1.5 Preparation of homologous mouse DNA

Mouse DNA was separately prepared from BMDM cell cultures of both A/J and C57BL/6 mice and hence the term homologous mouse DNA. A similar procedure to the one described above for *T.congolense* DNA was used to prepare mouse DNA from the macrophage cells cultures.

7.1.6 Preparation of *T.congolense* total parasite lysate

Total parasite lysate was prepared according established protocols (Uzonna et al, 1998). *T.congolense* parasites isolated from the DEAE 52 cellulose were washed in PBSG and pelleted by spinning at 1500 x g for 10mins. The parasites in batches of 10^8/ml were resuspended in PBSG without protease inhibitors and subjected to three freeze-thaw cycles (-80°C freezing followed by 37°C thawing per cycle). Protease inhibitors were not added to lysate to prevent interference with downstream treatments on cell cultures. The lysate preparations were then placed on ice and sonicated for 10 cycles 30secs/cycle at 50% duty cycle with 30secs interval between cycles. Total parasite lysate was stored at -80°C until further use.

7.1.7 L929 cell cultures

L929 clone NCTC is an immortalized fibrosarcoma cell line originally established from mouse subcutaneous areolar and adipose tissue (Sanford et al, 1948). L929 cells secrete a factor identical to murine M-CSF which facilitates differentiation of peripheral blood mononuclear cells into macrophages and monocytes (Warren and Vogel, 1985). Consequently, L929 conditioned medium is recommended as a crude source of murine M-CSF (Genovesi et al, 1989; Chodakewitz et al, 1990; Moore et al, 1994).

Frozen L929 cell stabilates were resuscitated by heating in a 37°C water bath and excess pre-warmed medium was added to the cells to dilute out DMSO cryopreservative. The suspension was washed twice in fresh medium by spinning for 5mins at 1500 x g and discarding the supernatant. The washed cells were then cultured in fresh RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin and gentamycin at 37°C and 5% CO_2. On attaining confluence, conditioned medium was harvested and stored at -20°C. The cells were then trypsinized and subcultured for further harvests of conditioned medium. The conditioned medium obtained from L929 cell cultures was then utilized as a crude source of murine M-CSF and added at 30% concentration to bone marrow cell cultures (above) to facilitate maturation of macrophages.

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