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Studies on the mechanisms of immune evasion in *Trypanosoma carassii* infections of the goldfish (*Carassius auratus* L.)

by

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ABSTRACT

Parasites possess variety of mechanisms to modulate or evade host defence systems to maintain chronic infection and ensure their transmission. The protozoan parasite *Trypanosoma carassii* is infective to a number of freshwater fish species and can cause significant mortality in aquaculture. *T. carassii* shares similarities with both *T. brucei*, the causative agent of sleeping sickness and *T. cruzi*, which causes Chagas' disease. It is not known how *T. carassii* escapes host immune responses.

My doctoral research focused on the interactions between *T. carassii* and its fish host with emphasis on the strategies used by the parasite to evade fish immune host defense.. and establish long lasting infections. I identified at the molecular level the key antigens of *T. carassii* present in excretory/secretory (ES) products and surface protein fractions of the parasite. As a first step toward understanding the importance of select antigens of *T. carassii*, I produced the recombinant proteins and characterized their roles in immune evasion.

Like heat shock protein 70 of other pathogens, I found that *T. carassii* hsp70 was immunogenic and was present in both ES products and surface protein fraction of the parasite. Recombinant parasite hsp70 significantly increased expression of pro-inflammatory genes and enhanced inflammatory response of goldfish macrophages. In contrast, another parasite surface molecule, glycoprotein 63 (Gp63) down-regulated both pathogen and cytokine-induced inflammatory responses of goldfish monocytes and macrophages. Parasite gp63 was associated with macrophages and appeared to interfere with signalling mechanisms. Since

complement-mediated lysis is one of the main host defence responses against trypanosomes, I cloned and characterized parasite surface molecule called calreticulin. Recombinant *T. carassii* calreticulin bound to first component of complement, C1q, of not only goldfish but also humans. Further, recombinant *T. carassii* calreticulin inhibited C1q-dependent hemolysis.

T. carassii infection of goldfish induced increased expression of pro- and anti-inflammatory cytokines. Increased cytokine mRNA levels were observed during the acute phase of infection, and then they returned to normal levels or were down-regulated during the elimination phase of the infection. These findings demonstrate that parasite surface molecules and those found in ES fraction have the capacity to manipulate host inflammatory and antimicrobial responses, thereby ensuring persistence of *T. carassii* in its host.

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LIST OF ABBREVIATIONS

ANOVA-analysis of variance

BCIP-5-bromo-4-chloro-3-indolyl phosphate

BSA-bovine serum albumin

cDNA-complementary DNA

CRP-complement regulatory proteins

DIC -differential interference contrast

DMSO-dimethyl sulfoxide

DNA -deoxyribonucleic acid

ES-excretory/secretory

FACS-fluorescence activated cell sorter

FCA-Freund's complete adjuvant

FIA-Freund's incomplete adjuvant

FITC-fluorescein isothiocyanate

GPI-glycosylphosphatidylinositol

HSP-heat shock protein

IFN-interferon

IgG-immunoglobulin G

IgM-immunoglobulin M

IL-interleukin

iNOS-inducible nitric oxide synthase

IP-intraperitoneal

IPTG-isopropyl-beta-D-galactopyranoside

kDa-kiloDalton

LAL-Limulus amebocyte lysate

LB-Luria-Bertani

LPS-lipopolysacharride

MAC-membrane attack complex

MS-mass spectrometry

MBL-mannose binding lectin

NBT-nitro blue tetrazolium

NFkB-nuclear factor kappa B

NO-nitric oxide

OD-optical density

PAMP-pathogen associated molecular pattern

PBS-phosphate buffered saline

PCR-polymerase chain reaction

PEG-polyethyleneglycol

PMSF-phenylmethylsulfonylchloride

Q-PCR-quantitative-polymerase chain reaction

RACE-rapid amplification of cDNA ends

RNA-ribonucleic acid

ROI-reactive oxygen intermediates

RT-PCR-reverse transcriptase polymerase chain reaction

SDS-PAGE-sodium dodecyl sulphate polyacrilamide gel electrophoresis

SEM-standard error of the mean

SRA-serum resistance-associated gene

SSH-selective subtractive hybridization

TBS-tris buffered saline

TGF-transforming growth factor

Th1/Th2-T-helper cell

TLTF-trypanosome lymphocyte triggering factor

TNF-tumor necrosis factor

TTBS-TBS+Tween 20

VSG-variable surface glycoprotein

VSP-variant surface protein

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

1.0 General introduction

Protozoan parasites have evolved remarkable immune modulation and evasion strategies to subvert host defence systems. These strategies enable their survival and persistence in various hosts and ensure their transmission. Parasite immune evasion processes are in large part dependent on their ability to manipulate host molecular mechanisms that constitute and regulate the immune response or the normal functioning of host cells. The study of these immune evasion processes has furthered our understanding of the functioning of the immune system [15]. The different parasites use several mechanisms either simultaneously or sequentially at different stages of the infection. Also, parasites produce molecules deployed in different ways for the purpose of evading host responses.

Generally, parasites immune evasion mechanisms can be classified as passive evasion where parasites hide from immune system by adopting an intracellular existence or by invading immune privileged tissue [2]. Parasites can become invisible to the immune system for example, by shielding surface components following recognition [3], or change their surface identity during the course of infection as in the case of antigenic variation [17]. Also, parasites can become temporarily inactive (hypobiosis and/or encystation) so as to escape immune system. Active modulation and interference is a mechanism where parasite actively interfere with hosts immune responses especially the regulatory

network such as cytokines responsible for generation of potent immune defence strategies. Parasites can also interfere with host cell functions by producing molecules that modulate immune response and cellular functions. By using a combination of the strategies mentioned above parasites can evade each step of the immune host defense.

Trypanosomes are digenetic protozoa that infect humans, domestic and wild animals. These parasites cause important medical and veterinary diseases making them a major public health concern. There are many species of trypanosomes and they infect hosts belonging to all vertebrate genera. Parasites also cycle between insect or leech vectors and vertebrate host where they undergo biochemical and morphological changes [10, 11, 16]. Trypanosomes have received attention in the last two decades because of the diseases they cause and their remarkable immune evasion mechanisms. The completed genome sequences of mammalian trypanosomes have revealed the extensive diversity of molecules that contribute to immune evasion by these parasites. The different species interact uniquely with their vertebrate hosts using an array of evasion strategies. Some of the most fascinating immune evasion mechanisms such as antigenic variation were first described in the trypanosomes.

African trypanosomes such as *T. brucei* have evolved an array of immune evasion strategies. The most important of these is switching of the variant surface glycoproteins (VSG) molecules. The VSGs are highly immunogenic and highly abundant molecules and their controlled expression allows parasites to keep one step ahead of host immune responses [6]. The *T. brucei* genome contains about

1000 different VSG genes and up to 20 VSG expression sites. An allelic exclusion mechanism ensures that only one VSG gene is expressed at any one time allowing the parasite to generate antigenically distinct glycoproteins and forcing the host to generate successive waves of antibodies [4]. Although the controlled expression of VSGs contributes to resistance of African trypanosomes to complement mediated lysis (CML), it is also believed that a major surface protease (MSP or GP63) plays a major role in parasite protection from CML [6, 7]. Additionally, these parasites secrete/excrete molecules known as trypanokines (cysteine proteases), that modulate host cytokine networks for parasites benefit.

A significant portion of the surface of *T. cruzi* is composed of mucin glycoproteins encoded by approximately 850 genes [5]. The mucin genes contain variable regions and their controlled expression protects the parasite from host immune responses. *T. cruzi* also expresses complement regulatory proteins (CRPs) on its surface. These molecules protect parasites from CML by restricting formation of active C3 convertase [12], and preventing assembly of membrane attack complex that kills parasites. Additionally, excreted/secreted CRPs may bind host complement molecules away from parasites surface.

There is increasing interest in fish parasitoses and defence mechanisms against these infections, because of their economic importance in aquaculture and fisheries. *Trypanosoma carassii* is thought to be a non-pathogenic trypanosome of fish in natural population. These parasites live in the vascular system of many economically important fish species. It is thought that the immunocompetent state of the host is responsible for their ability to control *T. carassii* infection, although

the prevalence of infection can be as high as 100% in aquaculture setting [9, 13]. In immunocompromised state, which can be caused by stress imposed by high population density, healthy fish can succumb to infection [13]. Mortality caused by *T. carassii* can be significant although it may not be due to high parasitemia but rather parasite-induced immunosuppression.

The goldfish-*T. carassii* model system represents a natural host-parasite system to dissect trypanosome evasion mechanisms. The investigation of the host-parasite interactions of the more studied trypanosomes *T. brucei* and *T. cruzi* are mostly carried out in mice that are not the natural hosts for these parasites. In this case, comparison of the results is usually difficult because different strains of hosts and parasites are used. The advantages of goldfish-*T. carassii* model system are as follows: (a) it is a natural host-parasite association which allows the study of protective immune responses of primitive ectothermic vertebrates to protozoan infection; (b) goldfish are hardy, relatively inexpensive and can be maintained easily; (c) *T. carassii* can be grown *in vitro* [16, 20]; and (d) goldfish can be readily infected with *T. carassii* [8, 18, 19].

This thesis focuses on some of the mechanisms used by *T. carassii* to evade goldfish immune responses. There are 8 chapters in this thesis. Chapter 2 provides a detailed review on trypanosome biology, interaction with their hosts and immune evasion strategies with emphasis on mammalian trypanosomes *T. brucei* and *T. cruzi*, and fish trypanosome *T. carassii.* Chapter 3 contains materials and methods used for the research described in this thesis while Chapter 4 focuses on the description of the course of infection in goldfish infected with *T.*

carassii, and examination of changes in immune gene expression in *T. carassii*-infected fish. Cloning of *T. carassii* heat shock protein 70 (Tcahsp70), the assessment of the effects of Tcahsp70 on goldfish immune cells, proteomics analysis of *T. carassii* surface proteins and excretory/secretory antigens were reported in Chapter 5. In Chapter 6, cloning of *T. carassii* glycoprotein 63 (Tcagp63), the assessment of the effects of Tcagp63 on goldfish monocyte and macrophages, and role of Th1/Th2 balance in trypanosome-induced immunosuppression were examined. Chapter 7 describes cloning of *T. carassii* calreticulin (TcaCRT), the assessment of TcaCRT effects on the function of classical pathway of complement as well the role of complement system in controlling trypanosome infection. General discussion of the parasite evasion mechanisms, the summary and implications of the results obtained and recommendations for future research are presented in Chapter 8.

1.1 Objectives and Specific Aims of the Thesis

The overall goal of my thesis research was to investigate the interactions between *T. carassii* and its fish host. The central objective of my thesis was to identify at the molecular level the key antigens of *T. carassii* and characterize the importance of these antigens to immune evasion strategies used by the parasite. Specific aims of my thesis were:

1. To investigate goldfish-*T. carassii* interaction by examining changes in the expression of goldfish immune genes during the course of infection.

2. To determine key antigens of *T. carrassii* in excretory/secretory products and surface protein extract using genomic and proteomic approaches.
3. To determine and clone the genes encoding these antigens and produce the recombinant protein for further characterization.
4. To functionally characterize the key antigens of *T. carrassii* and determine their contribution to evasion or modulation of goldfish immune responses.
5. To define the molecular mechanisms used by parasite to persist in the host by subverting or modulating host defence during the course of the infection.

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CHAPTER 2

LITERATURE REVIEW[1]

2.0 Introduction

The order Kinetoplastida includes genus *Trypanosoma* spp. and *Leishmania* spp. that infect hundreds of millions of humans and wild and domestic animals. These protozoans are characterized by the presence of kinetoplast a unique single mictochondrion, mitochondrial RNA editing, multicistronic transcription of nuclear genes and transplicing and metabolic compartmentalization [46, 213]. Trypanosomes have unique and extensive immune evasion strategies, which can be linked to their biology and life cycle. Their life cycle involves development of parasites that are morphologically and biochemically distinct within the vertebrate hosts and in the vectors. The development of different morphological forms during cell differentiation is orchestrated by the parasite cytoskeleton and is essential for the passage of parasites from vector to the host and vice versa. During development, these protozoans alternate between proliferative and non-proliferative forms adapted to a new environment, change gene expression patterns, translate specific proteins such as the variant surface antigens and generate a number of complement regulatory proteins [129, 128, 144].

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Although many species of trypanosome cause important veterinary diseases, only two species (*T. brucei* and *T. cruzi*), cause significant human morbidity and mortality. Trypanosomes cause more than 20 million infections and 100,000 deaths per year [193].

Sleeping sickness is a re-emerging disease caused by African trypanosomes. The disease occurs as a chronic form caused by *T. brucei gambiense,* which is found in West and Central Africa, and an acute form caused by *T. brucei rhodesiense* found in Eastern and Southern Africa [220]. Sleeping sickness occurs in 36 sub-Saharan countries and is prevalent within the tsetse fly zone. It is believed that over 60 million people living in this region are at risk of contracting the disease with over 300,000 new cases every year [220, 221]. Chagas disease, also referred to as American trypanosomiasis is caused by *T. cruzi* and has been reported in all Latin America countries where it constitutes the most important parasitic infection based on socio economic impact and strain on public health systems. Chagas disease affects about 18 million people with about 100 million people living in high risk areas, and 300,000 new cases are reported every year including 21,000 deaths per year [219].

In mammals African trypanosomes, which include *T. brucei, T. congolense* and *T. vivax,* thrive extracellularly in the bloodstream and other tissue fluids. Some *T. brucei* subspecies cause sleeping sickness in humans whereas *T. congolense* and *T. vivax* are not infectious to immunocompetent humans but causes a disease called Nagana in cattle. The natural resistance of human to *T. brucei brucei*, which causes disease in cattle results from the presence of

trypanolytic factors in human serum causing the lysis of the parasites. On the other hand, humans are highly susceptible to *T. brucei gambiense* and *T. brucei rhodesiense* since these parasites are resistant to serum-mediated lysis [137, 188, 189]. Resistance of *T. brucei rhodesiense* has been linked to the presence of serum resistance associated gene (SRA) in this parasite [43, 224].

The infection with African trypanosomes is initiated by tsetse fly bite, resulting in the inoculation of non-dividing metacyclic trypanosomes into the host. Metacyclic trypanosomes differentiate into bloodstream parasites with heterogenous population of proliferative slender forms in the ascending phase, and non-proliferative stumpy forms during peak parasitemia. The stumpy trypanosome is pre-adapted for differentiation into procylic trypanosomes that proliferate in tsetse fly midgut [128]. The surface of the bloodstream form of the African trypanosome is covered with highly abundant variant surface glycoproteins (VSG), while the procyclic trypanosomes are covered with the invariant surface glycoprotein, procyclin [162, 167]. The VSGs are used to evade host immune responses by antigenic switching [50] and are discussed further below.

T. cruzi the causal agent of Chagas disease is primarily an intracellular parasite. Although this parasite causes disease in humans it can also infect many other mammalian species [2]. *T. cruzi* infection starts when the insect vector takes a bloodmeal from an infected host. Blood stage trypomastigotes are taken up and transform to epimastigotes in the insect digestive tract. Epimastigotes divide and transform into metacyclic promastigotes that are excreted in feces. These

metacyclic trypomastigotes enters mammalian host and eventually invade variety of cell types where they transform into dividing amastigotes. Amastigotes transform to bloodstream trypomastigotes, which are released by rupturing of the cells. These trypomastigotes thrive extracellulary in the bloodstream and can infect other host cells. They also possess complement regulatory proteins that enable parasites to avoid complement-mediated lysis [143, 144].

Non-mammalian trypanosomes have been relatively neglected and very little is known about these parasites. The fish trypanosome, *T. carassii*, is transmitted by blood sucking leeches from infected fish to naive hosts and is believed to be exclusively extracellular. The parasite infects freshwater fish including carp (*Cyprinus carpio*), eel (*Anguilla* spp.) and tench (*Tinca tinca*) in Europe and Asia [112]. Prevalence of infection and mortality caused by *T. carassii* can be as high as 100% in aquaculture setting [4]. Experimental infection with *T. carassii* has been demonstrated for a number of fish species including goldfish [113, 222]. We are using the goldfish model system to study host-parasite interaction with the aim of dissecting the evasion mechanisms of *T. carassii*, as well as understanding how fish respond to infection. Presently, not much is known about the life cycle and interactions of this parasite with the leech vector and the fish host. However, few authors [158, 164] have reported that the trypanosome undergo series of developmental changes within the leech. It is not known precisely how *T. carassii* avoids complement-mediated lysis in the fish host, although its surface coat is very similar to that of *T. cruzi* [4**].** This suggests that *T carassii* like *T. cruzi* may have complement binding proteins on its surface.

In support of this, we have shown that trypsin treatment of *T. carassii* increases their susceptibility to lysis by the alternative complement pathway of the goldfish [156].

This review explores evasion mechanisms used by trypanosomes to evade or modulate host defences. The focus is on mammalian trypanosomes *T. brucei* and *T. cruzi* as well as non-mammalian trypanosome *T. carassii*. *T. carassii* shares fascinating similarities with both *T. brucei* and *T. cruzi.* These non-mammalian trypanosomes thrive extracellulary in the vascular system of the host like *T. brucei*, and possess a surface architecture similar to that of intracellular *T. cruzi.* Since trypanosome immune evasion strategies are closely linked to their surface coat, it is hypothesized that *T. carassii* will interact with the host defences like *T. cruzi*.

2.1 African trypanosomes and tsetse fly

2.1.1 *Tsetse fly defense against trypanosomes*

Tsetse flies (Diptera: Glossinidae) are known vector of African trypanosomes and there is evidence that 31 different species of tsetse flies are capable of transmitting trypanosomes [8]. Human infective *T. brucei rodesiense* and *T. brucei gambiense*, as well as *T. brucei brucei*, *T. congolense*, *T. vivax* and *T. simiae*, that infect domesticated animals, are all transmitted by tsetse flies [165]. With respect to fly infection and parasite establishment, the different species of trypanosomes develop in different organs of the fly. *T. vivax* develops exclusively in the mouthparts while *T. brucei* and *T. congolense* first colonise the

fly midgut following infection, and later migrate to the mouthparts where maturation occurs. *T. brucei* then migrate to the salivary gland [165]. In the tsetse fly host, trypanosomes are faced with myriads of immune challenges and have evolved a sophisticated system to cope with the fly host defences. In the tsetse fly midgut trypanosomes must deal with physical barriers, lectins, anti-microbial peptides and proteases. Variety of antimicrobial peptides including defensins, diptericins, attacins have been reported in tsetse fly midgut [75, 76, 78]. For example, the levels of attacin and defensin transcripts were found to increase by day 3 in the fat body of trypanosome-infected flies [76]. Also, targeted knockdown of attacin transcript increased trypanosome infection in the fly, indicating involvement of anti-microbial peptides in tsetse fly-trypanosome interaction [78]. It has also been established that recombinant attacin inhibits procyclic trypanosome proliferation *in vitro* [79].

Tsetse flies are refractory to trypanosomes and susceptibility to infection appears to be maternally inherited as demonstrated by breeding experiments [131, 134]. This pattern of inheritance was the focus of research leading to identification of midgut lectins, which were previously implicated in development of *T. cruzi* in triatomine bugs [155]. Lectin activity has been detected in tsetse fly midgut, and lectin inhibitory sugars fed to flies along with infective feed resulted in 100% infection [130]. Stimulation of fly immunity by bacteria before trypanosome infection led to significant decrease in trypanosome infection levels [76], suggesting that the innate immunity may be an important regulator of trypanosome infection in the flies [165]. It has been shown that not all tsetse flies

in an area are capable of transmitting trypanosomes. Thus, the vector capacity of tsetse fly is governed by its ability to acquire infectious forms during a blood meal and to subsequently develop an infection and transmit the parasites to a new vertebrate host. Transmission of trypanosomes to vertebrate hosts also depends on the density of the tsetse fly population, their longevity, susceptibility of tsetse flies to infection, and human behaviour or activities that increase man-tsetse fly contacts [220].

2.1.2 *T. brucei development in the tsetse fly*

Tsetse flies are infected with a non-replicating short stumpy form taken up in blood meal from infected mammal. The stumpy forms survive the attack by proteases in the fly midgut and differentiate into a dividing procyclic forms [172]. It has been suggested that proteases in the fly posterior midgut could be one of the natural triggers of differentiation of stumpy trypanosome into procyclic forms [165], although additional signals have also been reported [194]. Proteases have been shown to induce differentiation of stumpy trypanosomes into procyclic forms in culture [172]. This *in vitro* differentiation can also be induced by addition of citrate or *cis*-aconitate to the culture medium [228]. In the tsetse fly midgut, the bloodstream parasites are exposed to proteases resulting in death of the slender proliferating bloodstream forms [172]. Trypanosomes that are transformed from stumpy into procyclic parasites then shed their VSG coat, they leave the midgut and migrate to the salivary gland [217]. Following differentiation into procyclic form, the bloodstream VSG is cleaved by a surface metalloprotease to allow synthesis of the procyclic stage-specific coat known as

procyclin within few hours [69, 71]. Procyclins are immunodominant surface glycoproteins rich in proline residues and are different from VSG [162]. Procyclins are encoded by multi gene family and may be EP procyclins containing Glu-Pro dipeptide repeats or GPEET procyclins with Gly-Pro-Glu-Glu-Thr repeats [167]. Both types of procyclins have several isoforms and have been extensively reviewed [166, 167]. All isoforms of procyclins are expressed at similar levels few hours following differentiation into procyclic parasites [211]. Procyclins are resistant to proteases and are thought to protect procyclic trypanosomes from destruction in the fly midgut [3, 107]. They are also required for establishment of the infection as demonstrated by procyclin gene deletion experiment [168].

Arrival of procyclic trypanosomes to the testse fly salivary gland is followed by differentiation into the metacyclic form pre-adapted for life in the mammalian host. Metacyclic trypanosomes are quiescent and have been reported to express metacyclic VSGs on their surface although their repertoire of VSG at this point in the life cycle is limited [38]. The metacyclic parasites are introduced into mammalian host during tsetse fly bite and express one of the 10-15 different VSGs for about 7 days before switching to expression of bloodstream VSGs [50]. It has been reported that metacyclic VSGs can occasionally be re-expressed as bloodstream VSGs late in the infection [50]. VSGs are very important to bloodstream form of African trypanosomes. Interference with GPI anchor synthesis [139] or down-regulation of the VSG mRNA by RNA interference [182] is lethal to trypanosomes, indicating intact VSG coat is essential for trypanosome

survival. Interestingly, loss of procyclin is tolerated by insect stage parasites as shown by deletion of all procyclin genes and procyclin is neither essential for transmission of the parasite, nor required for establishment of the infection [138, 212]. However, procyclin appears to contribute to the efficiency of transmission, since genetically manipulated parasites bearing only one intact procyclin gene (GPEET) showed decreased infectivity [168].

A role for surface sialic acids in the protection of parasites in the tsetse fly midgut has been described. *T. brucei* cannot synthesize sialic acid, however, expression of surface glycosylphosphatidylinositols (GPIs)-anchored trans-sialidase enzyme by procyclic parasites has been described [57, 135]. Trypanosomes transfer sialic acids that they are unable to synthesize from the host's glycoconjugates to the GPIs on their surface using the trans-sialidase enzyme [138]. This process is important for the survival of trypanosomes in tsetse fly since parasites lacking sialic acids due to defect in synthesis of trans-sialidase are unable to survive unless sialylated by soluble trans-sialidase [138]. Interestingly, transfer of sialic acid from host's glycoconjugates onto mucins on the parasites surface has been described for both *T. cruzi* and *T. carassii* [4, 31, 63]. These parasites also use trans-sialidase enzyme, anchored to the parasite surface to transfer sialic acids. It has also been shown that trans-sialidase shed into the bloodstream binds receptors on the cell surface and influences defense mechanisms of the host [35, 199, 200].

Additional research is needed to further understand the mechanisms used by parasites to evade or modulate potentially harmful insect factors. The

completion of trypanosome genomes has revealed extensive diversity of genes that encode molecules that are differentially expressed in the different developmental stages of the parasite and are believed to be important for parasite defense strategies [219]. Progress in determining the array of insect molecules involved in the interaction with the parasites has been slow because of the limited genome information available for the insect vectors that is further complicated by the many different species of the vectors capable of transmitting trypanosomes. To understand the vector-parasite interaction on a genomic scale, the full genome sequence of insect vectors is necessary. Several tissue specific expressed sequence tags (ESTs) libraries have now been generated for tsetse fly *Glossina morsitans morsitans*. Also, there is information on the structure and size of the genome for different species, which is being used to decipher complete genome of *Glossina* [7]. EST library constructed from *Glossina* salivary gland have been sequenced to identify salivary proteins. More than 250 proteins were identified that may play a role in blood feeding, while new polypeptides families unique to *Glossina* were also identifed [9], although only few *Glossina* genes and proteins have been functionally characterized to date.

2.2 *T. cruzi* and its insect vectors

2.2.1 *T. cruzi* interaction with triatomine vectors

T. cruzi is present in the fecal droppings of infected insect vectors of the subfamily Triatominae and family Reduviidae, which are hematophagus bugs capable of transmitting the parasite. Their life cycle is characterized by five

nymph stages before sexual maturity, which may take up to six months depending on the species. Partly because of the control program employed against the domestic vectors, the normal pattern of transmission of *T. cruzi* (fecal route) is being replaced by secondary transmission patterns [179]. Thus, transmission by congenital transfer and blood transfusion are becoming increasingly common [219]. Recent report also indicated acquisition of infection after ingestion of food contaminated with metacyclic forms [226].

There are 140 species of Triatominae, all of which are potential vector of Chagas disease, although only *Rhodnius prolixus* and *Triatoma infestans* have been well studied [96, 178]. Adult insects can ingest blood of the host, 6-12 times their body weight, and store the blood in the anterior part of the midgut. These insects have saliva composed of various compounds to counteract hemostatic response of the mammalian host and overcome coagulation and platelet aggregation [160]. The blood is concentrated and moved into small intestine in barely digested form [64]. During this movement, the blood is prevented from clotting by anti coagulants from the salivary glands and stomach [49, 105]. Digestion and absorption of the blood occurs, and the remains of digestion are stored in the rectum and later removed by urine secreted rapidly after blood ingestion [64].

Following digestion of the blood, insects begin a starvation phase that may last for several months depending on species and climate [174]. Starvation is accompanied by shortage of nutrients in the insect gut that consequently affect parasite ability to multiply. Starvation does not appear to adversely affect

spheromastigotes stage of *T. cruzi*, since an increase in proportion of this developmental stage during starvation has been reported [97]. This led to suggestion that the spheromastigote stage of the life cycle may be adapted to survive under adverse conditions [97]. About one to four weeks following infection of insect, parasite population increases rapidly in the small intestine, more than in the rectum. In the later stages of infection, parasite multiplication is more rapid in the rectum than the small intestine [173]. Preferential multiplication and habitation of rectum by *T. cruzi* has been suggested to result from ability to better attach to the rectum rather than the effects of digestive enzymes on the parasite in the small intestine [12, 66, 96]. There is evidence for attachment of the flagellated parasites to the lining of rectal cuticle with the rectal pads being the most preferred site of colonization [175].

2.2.2 *T. cruzi development in Triatomines*

T. cruzi life cycle is complex and involves many morphologically and physiologically distinct stages that occur along the digestive tract of the vector. The bloodstream trypomastigotes are ingested from infected mammalian host by the insect vector during a blood meal. Parasite then undergoes series of transformations in the lumen of the digestive tract of the insect vector. After few days, the bloodstream trypomastigotes develop into dividing and non-replicative amastigotes, spheromastigotes and dividing epimastigotes [13, 96]. The epimastigotes later differentiates into non-replicative and infective metacyclic trypomastigotes in the rectum. Metacyclic trypomastigotes are adapted for infecting vertebrate hosts and are passed out in feces and urine [13, 96]. It has

been reported that all stages of parasite development in the insect vector are passed out in feces and urine, however, only trypomastigotes are capable of infecting mammalian host [180]. Since *T. cruzi* cannot penetrate intact skin, parasites gain entry into the mammalian hosts through skin abrasions, punctures as well as mucous membranes [180].

In the insect gut, parasites are faced with digestive enzymes, lectins, proteases and other component of insect gut, as well as factors derived from digestion of blood meal, which may affect parasite establishment and multiplication [13, 94, 96]. It has been reported that parasite's ability to survive within the digestive tract of the vector depends on the nutritional state of the host, the strain of the parasite, trypanolytic compounds present, digestive enzymes, lectins, bacteria living in the gut and the endocrine system of the vector [65]. Some of the molecules in insect gut that interact with parasite and may interfere with parasite multiplication or survival have been identified. Trialysin, a pore-forming molecule contained in saliva of *T. infestans* when ingested together in the blood meal has been shown to lyse the parasite [127]. Cathepsin B, the major digestive enzyme in the small intestine of triatomines, appears not to have any effect on the population density of *T. cruzi* [65]. However, elevated activity of cathepsin D has been observed in the small intestine of infected insects, one to three days after ingestion of blood meal containing *T. cruzi* compared to non-infected insects [26]. The haemolytic factors in the crop responsible for lysis of erythrocyte membrane to release free hemoglobin for digestion, as well as lectins, have been implicated shown to modulate parasite multiplication and

transformation [64]. It has been demonstrated that differential agglutinin and lysin activities exist in *R. prolixus* gut against different strains of *T. cruzi* [133].

There are also changes in temperature, osmolality, pH and nutrients, that parasite must cope with in the insect gut once it has been transferred from the mammalian host. In addition, microorganisms living in the gut of insect vectors may contribute significantly to parasite survival due to modulation of vector immunity [13]. Symbionts in the insect gut might reduce concentrations of the components of the innate immunity in the triatomines, resulting in an environment that is conducive for parasite growth [115]. Also, gut microorganisms themselves can produce factors that negatively impact parasite population density. *Serratia marcescens*, a haemolytic bacterium found in the stomach of *R. prolixus* [14] is capable of producing prodigiosin pigment that kills epimastigotes of certain *T. cruzi* strains [13, 14].

The parasites have adapted to survive attack by various elements of insect immunity. Antimicrobial peptides such as defensins are produced in the fat body, intestine and midgut of triatomines [65, 115]. Defensins are known to lyse Gram-positive and Gram-negative bacteria, fungi and parasites [28, 32]. *T. cruzi* infection induces changes in defensin expression in *Rhodnius prolixus* with higher expression levels in the intestine and midgut of infected insects, although parasites appear to be protected from the high concentration of defensins produced [115]. Thus far, three isoforms of defensins have been identified in *R. prolixus* [115]. Furthermore, five cDNAs encoding lysozymes have been characterized in triatomines [11, 18, 95]. These genes are induced in response to

experimental introduction of *T. cruzi* into the hemocoel of *R. prolixus* [11, 18], although the parasite does not invade hemocoel naturally. A change in transcriptional profile of lysozyme gene has been observed in *R. prolixus* following ingestion of blood containing *T. cruzi* [203]. In *T. infestans*, cysteine proteases and a cDNA encoding for lysozyme have been identified in the insect gut, although their involvement in the vector-parasite interactions is unknown [94, 95]. Lysozymes and defensins are the only antimicrobial proteins well characterized in triatomines, but there is also evidence for presence of other antimicrobial proteins in the insect vector [115, 215].

Nitric oxide generation is a conserved mechanism of innate immunity in invertebrates. In *R. prolixus, T. cruzi* induced variable expression of NOS genes according to the stage of infection. NOS gene expression was up-regulated in stomach and hemocytes during early stage of infections, ranging from one to two days post-infection, and corresponded with the period when differentiating epimastigotes inhabited the stomach. *T. cruzi* infection also induced nitrite production in the small intestine of *R. prolixus* during early stages of infection while a significant increase in nitrite levels were observed in the rectum of the insect at all stages of infection [218].

2.3 *T. carassii* and the leech vector.

2.3.1 *T. carassii* development in the leech vector

Progress on deciphering the development of *T. carassii* within the leech vector as well as interaction with the leech has been very slow. At present nothing

is known about parasite factors that contribute to survival in the leech as well as leech factors that hinder parasite growth. Transmission of fish trypanosomes by leeches has been reported [reviewed in 158]. The leech *Hemiclepis marginata* sucks blood from the fish host ingesting trypanosomes in the process. Following feeding, the leech becomes sluggish and motionless with a contracted body. Trypanosomes undergo series of morphological alterations in the leech crop as early as 3 - 5 hours after ingestion including repositioning of the kinetoplast. Parasites later differentiate into diving stumpy form one day after ingestion, then into crithidial form 2 days after ingestion. By day three, at least three distinct populations of trypanosomes were observed. The crithidial forms, the stumpy forms and a somewhat longer crithidial forms with narrow posterior and tapering anterior ends. These forms then undergo numerous alterations as the developmental cycle progresses. By day 7, tapering trypanosomes dominate the parasite population and by day 8, they become very long, slender flagellates that represent the final stage of development in the leech. These are the infective metacyclic trypanosomes some of which migrate to the proboscis sheath of the leech, to be introduced during the next blood meal. All stages of parasite development appear to occur in the leech crop [158], which is contrary to what has been observed in other trypanosomes where parasite development occurs in different regions of the insect gut.

Parasitic invertebrates such as leeches have evolved mechanisms to block host blood coagulation processes at many points during coagulation cascade. Inhibitors of factor Xa, platelets aggregation, thrombin and proteases have been

identified in the leech [22, 37, 170]. *Hirudo medicinalis*, the medicinal leech, has been reported to produce many proteinase inhibitors including inhibitors of trypsin, plasmin, elastase, kallikrein, chymotrypsin, cathepsin, factor Xa, thrombin and complement component, C1s [22]. It is not known whether these anti-coagulants would affect *T. carassii* development in the leech.

2.4 Immune evasion strategies of trypanosomes in mammalian hosts

2.4.1 *Trypanosome-induced immunosuppression*

Trypanosomes are masters at elaborating variety of escape mechanisms to circumvent host immune responses, while inducing factors that promote their growth. Suppression of T and B cell responses to trypanosome-related and -unrelated antigens is considered a major mechanism of evasion of the host immune response. Following infection with African trypanosomes, there is an increase in immunoglobulin (Ig) levels especially IgM due to activation of B cells by cytokines. These include trypanosome-specific antibodies as well as antibodies raised against auto antigens resulting from non-specific polyclonal B cell activation [214]. Surprisingly, some specific B cell and T cell responses are suppressed and several factors have been implicated in this immunosuppression. Early study on immunosuppression in trypanosome-infected cattle implicates polyclonal B cell activation and generation of suppressor T cells and suppressor macrophages as the cause of immunosuppression [83]. Several studies have reported an initial absence of specific host response towards the parasites, which was then followed by a transient proliferative T cell response to trypanosome

antigens in the first few days of infection [67]. In mice infected with *T. brucei*, T cell proliferation increased significantly in the spleen in the first few days of infection, and then the proliferative response abruptly stopped [214].

Suppression of T and B cell responses has been attributed to both suppressor T cells and suppressor macrophages. Endogenously produced IL-10 and IFNγ have been shown to mediate the suppression of T cell responses in *T. congolense*-infected mice [204, 205]. Infection of highly susceptible BALB/c and relatively resistant C57BL/6 mice to *T. congolense*, showed that C57BL/6 mice controlled their parasitaemia and survived much longer, while BALB/c mice did not and succumbed to the infection within days. Susceptible BALB/c mice had higher plasma levels of IL-10, IL-4 and IFNγ than the resistant C57BL/6 mice. Conversely, plasma TNFα levels were elevated significantly in the resistant relative to the susceptible mice. Concanavalin A (Con A)-induced secretions of IL-10, IL-4 and IFNγ by splenocytes from infected BALB/c mice were significantly higher than those from C57BL/6 mice, indicating suppression of Con A-induced proliferation of splenocytes from infected BALB/c mice. Suppression of splenocyte proliferative response was due to production of IL-10 and IFNγ, since addition of neutralizing anti-IL-10 or anti-IFNγ antibodies to the Con-A-stimulated spleen cell cultures from infected BALB/c mice effectively reversed this suppression. Also, administration of anti-IFNγ antibody to BALB/c mice early during infection dramatically shifted the phenotype of these susceptible mice to a more resistant-like phenotype by prolonging the survival period of the

hosts [204, 205]. It was later reported that $CD4^+$ T cells mediate the immunosuppression in *T. congolense* infected-BALB/c mice [206].

Several other lines of experimental evidence exist for the induction of immunosuppression during trypanosome infection. Defects in antigen processing and inability of macrophages to present antigens to T cells have been suggested to lead to production of immunosuppressive factors such as nitric oxide and prostaglandins [214]. There is also an increase in production of TGFβ, an immunosuppressive cytokine, which inhibits production of IL-4, IL-5 and IL-6 [58]. Some studies have reported deficiency in IL-2 production and IL-2 receptor expression, although prostaglandins and IFNγ produced by macrophages were thought to contribute to the suppression of IL-2 receptor expression [40, 184]. Macrophages are centrally involved in suppression of T-cell proliferative responses during *T. b. brucei* infection. T-cell proliferation is controlled by NO released by macrophages and suppressor macrophages have been shown to be responsible for suppression of T-cell responses to trypanosome antigens [70].

These suppressive effects are exerted through mechanisms that vary with tissue and stage of infection. In *T. b. brucei*-infected mice, T-cell proliferative responses, but not T-cell effector cell maturation and cytokine synthesis were impaired by prostaglandins and nitric oxide during early stages of infection [176]. It has also been suggested that NO preferentially targeted pathways important for proliferation, but not pathways required for cytokine synthesis, to account for this preferential suppressive effects [176]. Both IFNγ and TNFα appear to contribute to up-regulation of prostaglandin and NO synthesis during early stage of

infection, whereas T-cell proliferation seem to occur independent of prostaglandin and NO during late stage infection, although IFNγ produced by $CD8^+$ T cells appears to be important [40]. These results support the observation that macrophages from animals infected with trypanosomes show characteristics of cellular activation probably due to contact with IFNγ, a potent activator of macrophages, produced during infection. These macrophages also exhibit suppressor cell activity [154].

The identity of parasite antigens responsible for induction of auto antibodies as well as the role of auto antibodies in trypanosome-induced immunosuppression deserves further investigation. Few investigators have reported on the specificity of auto antibodies produced during infection. Some of these antibodies were produced in response to nucleic acids [81, 93], red blood cells [93], as well as myelin [85]. These antibodies appear to be generated by a non-specific B cells stimulation or molecular mimicry [41, 136]. Identification of epitopes of parasite antigens responsible for such non-specific B cell stimulation or molecular mimicry should be the focus of future research.

2.5 Immune evasion mechanisms of *T. brucei*.

2.5.1 *The variant surface glycoprotein*

The bloodstream parasites are continuously exposed to many components of the host immune system and have multiple mechanisms to evade host immune responses. Antigenic variation of the VSG molecules is the most important immune evasion mechanisms of this parasite. These highly immunogenic and

highly abundant glycoproteins form a dense cell surface coat that protects the invariant surface antigens from immune recognition. The VSG also protect the parasite from complement-mediated lysis **[**59, 197] and can induce production of important cytokines such as interleukin 1 (IL-1) and TNFα by macrophage [122, 194, 213]. VSG molecules can be cleaved from the parasite surface by a GPI-phospholipase C (GPI-PLC) a virulence factor that contributes to inflammatory response through action of soluble VSG and remnant GPI [124, 216]. Given the importance of VSG to African trypanosomes, soluble VSGs may have further roles in immune evasion that remain to be fully elucidated.

The *T. brucei* genome contains a repertoire of at least 1250 different VSG genes and up to 20 VSG expression sites [21, 23, 82, 209]. An allelic exclusion mechanism ensures that only one VSG gene is expressed at any one time, allowing the parasite to generate antigenically distinct glycoproteins and forcing the host to mount successive wave of VSG-specific antibodies [27]. When antibody production against a particular VSG has taken place, trypanosomes bearing this coat are lysed but a small number of parasites begin to express an antigenically distinct VSG, and eventually populate the vascular system of the host [201]. It has been reported that elimination of trypanosomes was due to phagocytosis and complement-mediated lysis that was mediated by anti-VSG antibodies [44, 119, 153]. The first class of anti-typanosomal antibody produced in infected mammal is the IgM generated against the specific VSG and this IgM fixes complement and aids in the assembly of complement components on the parasite surface [153]. The complement has been shown to efficiently lyse

parasites *in vitro* [72]. Phagocytosis of IgM opsonized parasites occurs by engagement of complement receptor CR3 (CD11b/CD18), and is facilitated by iC3b produced as a byproduct of antibody-induced complement activation and from activation of alternative pathway of complement [153]. In addition to IgM, IgG has also been reported to mediate phagocytosis of African trypanosomes [196]. To prevent anti-VSG antibody mediated phagocytosis, African trypanosomes restrict accumulation of VSG antibodies on their surface by endocytosis that occurs through the flagellar pocket of the parasites [56].

2.5.2 *Modulation of host immunity*

Presence of African trypanosomes in the bloodstream leads to polyclonal B cell activation resulting in increased production of IgM [20]. *In vivo* experiments have demonstrated the role of antibodies in clearance of trypanosome infections. IgG is involved in clearing *T. congolense* and *T. brucei* infections in mice [121, 153, 192]. In contrast, control of experimental *T. evansi* infection in mice appears to be IgM dependent, suggesting different antibody classes are involved in elimination of different trypanosome species [19].

Macrophages and other innate immune cells play important roles in host defense against trypanosomes. Macrophages are highly phagocytic and produce several pro-inflammatory cytokines, reactive oxygen and nitrogen intermediates upon appropriate activation. Macrophage activation has been reported in trypanosome infections. Interferon gamma (IFN-γ) produced by T-helper 1 (Th1) cells activated by parasite antigens is believed to serve as strong activation signal for macrophages. Complement receptor 3 (CR3)-dependent phagocytosis of IgM

or IgG opsonised parasites by activated macrophages and Kupffer cells (liver macrophages) results in TNFα production and down-regulation of NO synthesis [77, 153, 183, 192]. Since NO is toxic to African trypanosomes [52], this provide parasite with means to evade destruction by reactive nitrogen intermediates, one of the major antimicrobial mechanism of macrophages. TNFα has been demonstrated to kill parasites [116, 123], although resistance of some parasites to TNFα has also been reported [92]. Other macrophage receptors involved in parasite recognition include members of the TLRs family that signal through MyD88-dependent pathway [51, 153].

2.5.3 *Modulation of host cytokine gene expression*

African trypanosomes are known for their ability to secrete molecules that modulate host immune responses. These molecules, which are collectively known as trypanokines, are trypanosome proteins that modulate host cytokines for parasite benefit. Secretion of trypanokines is an example mechanism that parasites have evolved to enable their persistence in the host [207]. Trypanosomes cysteine proteases and T lymphocyte triggering factors (TLTF) are examples of trypanokines [88, 131]. TLTF triggers $CD8^+$ cells to produce IFN-γ, which stimulates parasites growth and production of TGF-β [15, 16, 17, 149, 150, 151]. TLTF is located in the flagellar pocket and interacts directly with the CD8 molecule on the surface of IFNγ-secreting cells to induce cytokine production [149, 207]. This molecule is produced by trypanosomes early during experimental infection, since the host is capable of generating neutralising antibodies that prevent TLTF function during later stages of infection [74].

Trypanosomes can also modulate host cytokines to enhance their survival in the host. It is well documented that parasites or parasite-derived molecules are capable of triggering immune response not required for their elimination [110, 124, 147, 154, 161, 181]. This strategy favours parasite establishment and persistence while diverting host responses away from parasite elimination. During the early stages of infection of mice with African trypanosomes higher levels of pro-inflammatory cytokines IFNγ and TNFα were reported [39, 191]. Similarly, the infection of vervet monkeys with *T. brucei rhodesiense* induced higher levels of IFNγ, TNFα and soluble TNF receptor 1 in serum of the infected animals [125]. These findings suggest preferential parasite-orchestrated induction of a Th1-type immune response, which is known to be ineffective against extracellular parasites. The survival of the host is thought to be dependent on a shift from pro-inflammatory cytokines (IFNγ and TNFα), to the anti-inflammatory cytokines (TGFβ and IL-10) production, enabling increased antibody response and eventual elimination of the parasites [140].

2.5.4 *Evasion of complement-mediated lysis*

T. brucei activates the alternative complement pathway in human hosts but it escapes lysis by preventing the complement cascade from proceeding beyond the association of C3 convertase with the parasite surface [45]. It was demonstrated that parasite incompletely activates the alternative pathway of complement without generating the terminal complex of the cascade resulting in inhibition of lysis [45]. Parasite VSG has been implicated in protection from complement-mediated lysis [197].

Trypanosomatid gp63 proteases have been suggested to be involved in host-parasite interaction and share sequence as well as structural similarities to the gp63 of their mammalian host and insect vectors. *T. brucei* homologues of *Leishmania* glycoprotein 63 (gp63) have been identified and well characterised. *Leishmania* gp63 is important for entry of promastigote into host macrophages and survival inside macrophages [34, 109, 190]. The protease activity of gp63 also contributes to the resistance of promastigotes to complement-mediated lysis [29], while immunization with recombinant protein partially protects animals in experimental infections [1, 36, 148, 225]. *T. brucei* appears to possess multiple genes encoding gp63 that are differentially expressed in the bloodstream parasites [55, 104]. It has been reported that gp63 may protect bloodstream trypanosomes from complement-mediated lysis by impairing a protein processing functions [55, 69, 104]. Gp63 is also required for removal of VSGs from the surface of bloodstream trypanosomes [69, 104].

2.6 Immune evasion mechanisms of *T. cruzi*

2.6.1 *Role of mucin glycoproteins*

T. cruzi amastigotes escape host humoral immune responses by adopting an intracellular existence. The parasite surface is covered in mucin glycoproteins that are essential for protection from recognition by host immune system.These mucins are acceptors of sialic acid residues, which are transferred from host glycoconjugates by the enzyme trans-sialidase. That *T. cruzi* genome contains ~ 850 mucin-encoding genes, which constitute ~ 1% of the genome and 6% of

predicted genes, highlights the significance of these proteins [31]. The mucin glycoproteins are also found on the surface of *T. carassii* [108] and *Cryptosporidium parvum* [198]. These molecules act at the interface between the parasites and the mammalian and non-mammalian hosts and protect the parasites against their immune responses [31].

The mucin glycoproteins are differentially expressed during *T. cruzi* life cycle. Both the amastigotes and blood trypomastigotes express variable mucin belonging to *T. cruzi* mucins (TcMUC) family, while the epimastigotes express the invariant mucins of T. *cruzi*, small mucin-like gene (TcSMUG) family. It is unclear which types of mucins are expressed by metacyclic trypomastigotes, however, it has been proposed that these parasites express the invariant mucins of the TcSMUG family similar to epimastigotes. TcMUC is characterized by an internal tandem repeats of Thr8-Lys-Pro2 sequence flanked by an N-terminal signal peptide and a GPI-anchor signal. The TcSMUG have a shorter central region with similar flanking regions, as observed in TcMUC [31]. Gene duplication, point mutations, deletions and insertions contribute to extensive diversity of the mucin genes [47, 48].

2.6.2 *Complement regulatory proteins*

The major evasion strategy of *T. cruzi* is the possession of several complement regulatory molecules, which allow the parasite to evade complement-mediated lysis (CML) [143, 210]. The bloodstream trypomastigotes possess the ability to resist CML using several surface glycoproteins that prevent complement activation [62, 86, 144]. The most studied of the complement binding molecules is

the 160kDa complement regulatory protein (160kDa CRP), which is expressed on the parasite surface and restricts activation of both the alternative and classical pathway of complement. This molecule binds complement components C3b and C4b, thereby interfering with the assembly of proteolytically active C3 convertase formation [144]. Transfection of epimastigotes with cDNA encoding trypomastigote 160 kDa CRP results in expression of the CRP on the epimastigote surface leading to transformation of complement sensitive epimatigotes to complement resistant parasites [142]. As shown in Figure 2.1, *T. cruzi* CRPs prevent assembly of C3 convertase enzymes of both the classical and alternative pathways of complement. Lysis of *T .cruzi* by complement is dependent on lytic antibodies [30, 101, 144], however, the alternative complement pathway is primarily responsible for the demise of the parasites. The classical pathway is inefficient in parasite lysis but enhances lysis by the alternative pathway of complement since sera from individuals that are genetically deficient in classical pathway component protein C2 lysed 50% of IgG-coated parasites, and the addition of purified C2 to the deficient sera significantly enhanced lysis of the parasites lysis [99].

The inability of the classical pathway of complement to efficiently destroy the parasites may be partly due to calreticulin (CRT), a parasite molecule (TcCRT) known to inhibit classical pathway-mediated lysis [6, 61, 146, 208]. TcCRT binds human C1q and inhibit C1q-dependent lysis [61], indicating that the presence of TcCRT on parasite surface could inhibit C1q-mediated activation of classical complement pathway at the earliest stage of complement activation.

Figure 2.1 indicates where trypanosome calreticulin acts to inhibit the classical pathway of complement. Additionally, secreted CRT molecules may perform other functions such as binding of host molecules away from the parasites, non-specific stimulation of immune responses or immunosuppression [146]. Parasite CRTs have multiple C1q-binding motifs, which are thought to enhance specific interaction with host C1q molecules [141]. Thus, multiple C1q binding motifs in parasite CRTs may represent an evolutionary strategy to compensate for multipoint interaction between antibody and C1q, to ensure complete inactivation of the classical complement pathway [141]. CRTs bind both collagenous tail and globular domains of C1q [90, 91, 141] and can compete with antibody for binding to C1q, indicating CRTs and antibodies share C1q binding sites [141]. TcCRT prevents human classical complement pathway activation by competitively inhibiting binding of $(C1r\text{-}C1s)_2$ tetrameric complex to collagenous tails of C1q and functionally interfering with C1s activities without releasing the serine protease from the C1q recognition complex [208]. Since *T. cruzi* infected individuals produce variable levels of specific antibodies against TcCRT in their serum [126], these antibodies may inhibit TcCRT-human C1q interaction that may benefit the hosts. Antibody raised against TcCRT inhibits calreticulin interaction with human C1q *in vitro* [5].

Several lines of evidence support the notion that *T. cruzi* elicits severe autoimmune responses in the host during infection and this contributes significantly to pathogenesis of Chagas' disease. This infection-induced autoimmune disease is believed to cause severe cardiac pathology observed in

about 30% of patients with chronic infection. TcCRT has high degree of homology (more than 50% identity) to human CRT (HuCRT), and is immunogenic in humans and mice. Anti-TcCRT antibodies generated in mice immunized with recombinant TcCRT (rTcCRT) reacted with rHuCRT suggesting that TcCRT may be involved in induction of autoimmunity in Chagas disease [159]. The anti-angiogenic and anti-tumor activities of TcCRT have also been described. TcCRT inhibits capillary morphogenesis, migration and proliferation of human endothelial cells, and growth of murine mammary tumor cell line. These observations, together with the ability to inhibit classical pathway of complement, suggest that TcCRT is an anti-inflammatory molecule that impairs anti-parasite immune responses [114].

2.6.3 *Gp63 proteases*

Annotation of *T. cruzi* genome revealed duplication of gp63 gene resulting in the division of the genes into four novel groups based on sequence features [118]. Complete genome and proteome analyses of *T. cruzi* have revealed at least 174 copies of *T. cruzi* gp63 (Tcgp63) that encode at least 29 different proteins. Many Tcgp63 genes are expressed in multiple life cycle stages, while others are stage specific. Antibodies raised against a Tcgp63 inhibit infection of cells by trypomastigotes as shown by neutralization assays [102]. These results suggest involvement of Tcgp63 in host-parasite interaction and evasion of phagocytic killing. Gp63 proteases of *T. cruzi* were extensively duplicated and contain several novel domains [118], suggesting Tcgp63 could play additional roles in immune evasion that are yet to be identified.

2.6.4 *Interference with cytokines and phagocytic cells*

Microbial pathogens have evolved strategies to benefit from immune regulatory mechanisms provided by cytokines [42]. *T. cruzi* induces the production of both Th1 and Th2 cytokines in infected individuals. Elevated expression of Th1 cytokines IFNγ and IL2 as well as Th2 cytokines IL-4 and IL-10 were reported [171]. Level of IL-10 mRNA was consistently elevated in *T. cruzi*-infected individuals leading to suggestion that the elevated IL-10 level may contribute to parasite persistence, since IL-10 is known to inhibit the protective Th1 immune responses [2, 186, 187]. Induction of IL-10 may be an important strategy for parasite survival, since elimination of parasites is dependent on Th1 mechanism that result in production IL-12, IFNγ, TNFα and the induction of nitric oxide response by activated macrophages [33, 80, 185]. IFNγ produced by NK cells during *T. cruzi* infection activates phagocytic cells to destroy internalised parasites through production of reactive nitrogen intermediates [10, 33, 68]. However induction of cytokines that leads to both cell mediated and humoral response has been suggested to be important for development of protective immunity [227].

2.7 Immune evasion mechanisms of *T. carassii*

2.7.1 *T. carassii infection of goldfish*

Although the prevalence of infection in aquaculture setting can reach 100%, *T. carassii* is considered to be non-pathogenic under natural conditions [111, 152]. The dose of parasites determines the severity of infection and

mortality is higher when fish are injected with high number of parasites [84]. In experimental infection of goldfish, symptoms such as anaemia, anorexia and hsitopathological changes in hematopoietic organs have been reported [53, 84]. It is unclear how *T. carassii* is able to maintain long-term infections in the fish host. As discussed earlier, *T. brucei* employ antigenic variation of their surface glycoproteins,to escape host immune attack. *T. cruzi* has mucin glycoproteins as well as complement regulatory proteins to survive host immune insults. Although *T. carassii* have mucin glycoproteins on their surface, these proteins have not been experimentally demonstrated to aid in parasite persistence.

2.7.2 *Evasion of humoral immunity*

T. carassii is believed to be exclusively extracellular since there is currently no evidence for intracellular stage of this parasite, suggesting predisposition to direct contact with components of both cellular and humoral immunity. In experimental infection of goldfish with *T. carassii* recovered host become immune to re-infection, suggesting the presence of immunological memory. Injection of serum or purified IgM from recovered fish conferred protection to naïve hosts in passive transfer studies [111, 152, 223]. Immunization of goldfish with parasite excretory/secretory product induces protective antibodies [24], however it is unclear how these antibodies function to eliminate parasites. Antibody-dependent complement-mediated lysis may be one way by which these antibodies mediate parasite clearance. Although the parasite antigens that induce protective antibodies in the host are yet to be fully elucidated, it is tempting to speculate that *T. carassii* possess surface molecules including complement

regulatory proteins similar to those of *T. cruzi*. Also, it is unclear whether *T. carassii* is able to endocytose surface bound antibodies for destruction, as observed for VSG-bound antibody in African trypanosomes.

2.7.3 *The search for complement regulatory proteins in T. carassii*

It is not known how *T. carassii* evades complement-mediated lysis in the fish host. The similarity in surface coat between *T. cruzi* and *T. carassii* has led to suggestion that *T. carassii* may also possess complement regulatory proteins on their surface for protection against complement lysis. This suggestion is supported by the observation that trypsin treated parasites were more susceptible to lysis by the alternative complement pathway of goldfish [156]. Parasites regained resistance to lysis 6-24 hrs after cultivation in the absence of trypsin and the resistance to lysis was abrogated after treatment of the parasites with protein synthesis inhibitor puromycin [156]. These results strongly suggest the existence of surface molecules that protect trypanosomes from lysis by fish complement cascades. Only few parasite molecules have been identified to date and none of these molecules is a major protective antigen. Parasite tubulin and cysteine proteinase have been reported as potential molecules that may participate in protection from complement-mediated lysis, but immunization of fish with the recombinant proteins only induced partial protection against a challenge infection [24, 87, 163]. These parasite molecules, although immunogenic, do not stimulate long lasting protection against re-infection. Immunization of mice with tubulin-rich preparation from *T. brucei,* and recombinant beta-tubulin from *T. evansi* offers partial protection against experimental challenge with these parasites [117,

106]. Alternatively, immunization with recombinant proteins may fail to induce effective lytic or protective antibodies as observed for *T. cruzi* CRPs where DNA but not protein immunization was found to be protective [177].

Recently, I [146] reported on the identification of *T. carassii* calreticulin (TcaCRT) as a secreted or excreted (ES) molecule that is abundantly expressed on the parasite surface. TcaCRT binds goldfish C1q, the first component of the classical pathway of fish complement and inhibits classical complement pathway-mediated lysis [146]. The ability of trypanosome calreticulin to inhibit complement lysis at the earliest stage of complement activation (Fig. 2.1) was first observed for *T. cruzi* calreticulin [61]. Since complement lyses trypsinized *T. carassii*, TcaCRT may contribute to protection of *T. carassii* from complement-mediated lysis. These results suggest TcaCRT inhibition of classical pathway may be partly responsible for the ineffective nature of goldfish classical complement pathway to cause parasite lysis [146, 156]. The presence of multiple C1q-binding motifs on TcaCRT may allow this molecule to competitively inhibit antibody binding or induce conformational changes that effectively abrogate the activation of classical complement pathway of goldfish [146].

2.7.4 *Modulation of host immunity*

We previously reported that *T. carassii* ES molecules are immunogenic and identified parasite tubilin, hsp70 and calreticulin in this protein fraction [24, 146, 147]. Antibodies raised against parasite tubulin inhibit parasite growth *in vitro*, although immunization of fish with the recombinant molecule only partially reduced parasitemia [24, 87]. Because tubulins are highly abundant and

immunogenic, they are targets of host immune response and antibody production during infection. These antibodies may benefit the host by eliminating the parasites. However, tubulins are also shed during *in vitro* cultivation of the parasites, indicating that they may be released from parasites *in vivo* and may direct immune responses away from the parasite surface. It is possible that parasite tubulin play other roles in immune evasion that are yet to be elucidated.

The ability of *T. carassii* hsp70 (Tchsp70) to enhance pro-inflammatory response of goldfish macrophages suggests the importance of this molecule in possible modulation of host immunity [147]. Tcahsp70 was identified in ES fraction as well as in membrane protein fraction of *in vitro* cultured parasites separated by 2-dimensional gel electrophoresis [147]. These results indicate a novel function of trypanosome hsp70 as a possible modulator of fish immune response. Recombinant Tcahsp70 (rTcahsp70) enhanced expression of pro-inflammatory cytokines and chemokines of goldfish macrophages. These findings suggest surface expression of Tcahsp70, and provide parasites with an excellent immune evasion strategy by preferentially inducing a Th1 type immune response to evade Th2 type response, which has been shown to play key role in parasite elimination [84, 87, 152, 223, 147]. The secreted hsp70 and calreticulin may have other roles in immune modulation. It is not clear at this point whether *T. carassii* suppresses host immunity or induces autoimmunity. Because of the similarity of *T. carassii* and *T. cruzi* surface coats, and the high degree of homology of calreticulins from both parasites [146], I speculate that extracellular TcaCRT may be involved in induction of autoimmunity, angiogenesis or immunosuppression.

I recently observed an increased expression of both pro-inflammatory and anti-inflammatory cytokines in *T. carassii* infected fish during the course of the infection [145]. I observed induction of INFγ and IL-10 during the acute phase of infection, suggesting a mixed Th1/Th2-type immune response [145]. An overlapping Th1/Th2 cytokine response can lead to generalized immunosuppression, which is a hallmark of African trypanosome infection [20, 204, 205, 206] although an overlapping Th1/Th2 cytokine patterns have also been observed in *T. cruzi* infected individuals [171]. Since increased expression of pro-inflammatory genes during the course of the infection paralleled the up-regulation of anti-inflammatory cytokine genes, these results suggest Th1/Th2-like responses may be important in the control of *T. carassii* infection in the goldfish. Furthermore, these findings indicate that IFNγ and IL-10 may play significant role in the control of fish trypanosomiasis.

2.7.5 *Possible role of proteases*

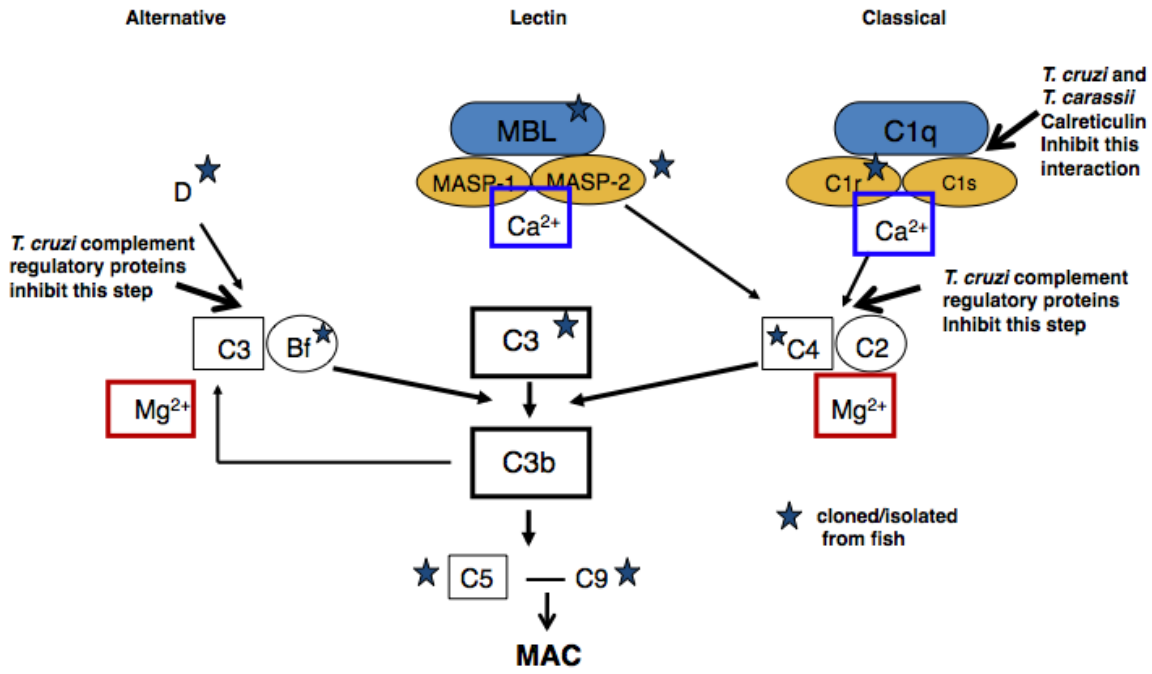
A cathepsin L-like proteinase (cysteine proteinase) of *T. carassii* has been cloned and characterised. This cysteine proteinase exhibits a high level of conservation within the Trypanosomatida in terms of structure and activity [163]. Immunization of carp with the recombinant proteinase induced an increase in proteinase-specific antibodies and slightly lowered parasitemia in experimentally challenged animals [163] suggesting that *T. carassii* cysteine proteinase is not a major protective antigen in carp. I have recently cloned *T. carassii* homologue of *Leishmania* gp63 and functionally characterized this protein. *T. carassii* gp63 shares sequence similarity with those of other kinetoplastids and it appears there

are multiple genes encoding gp63 in this parasite. Trypanosome gp63 warrant further study to understand the significance of these molecules in host-parasite interactions, as well as in parasite elimination, since these molecules elicit different effects.

2.8 Summary

In this review, I have provided an exploratory view of the current knowledge on immune evasion or modulation strategies employed by trypanosomes in both endothermic and ectothermic hosts. Various aspects of trypanosome biology that contribute to their remarkable immune evasion strategies were discussed so as to better understand these essential survival mechanisms of these highly successful protozoa. To emphasise the significant role played by the vectors of trypanosomes, I have included detailed discussion of parasite interactions with the insect or leech vectors. These parasites are fascinating with respect to their biology. They cycle between vertebrate and invertebrate hosts and must survive a plethora of antimicrobial immune responses in both hosts. Additionally, parasites have evolved ways to cope with change in temperature and nutrients as they cycle between hosts. Many of the molecules used by mammalian trypanosomes in host-parasite interaction and immune evasion have been described. More progress in this area is expected with the deluge of genome information available as investigators move from gene discovery to protein characterization and function.

The goldfish-*T. carassii* model system provides an excellent opportunity to study host-pathogen interaction that occur at the natural host-parasite interface. This is an obvious advantage over *T. brucei* and *T. cruzi*, where investigation of host-parasite interactions has been largely carried out in mice that are not the natural hosts for these parasites.



Modified from Nonaka and Smith, 2000.

Figure 2.1. The complement system. The alternative, lectin and classical pathways of complement are activated differently. Trypanosomes have developed strategies to inhibit complement-mediated lysis. *T. cruzi* complement regulatory proteins prevent formation of C3 convertase enzymes of both alternative and classical pathways. Both *T. cruzi* and *T. carassii* calreticulin can inhibit the classical pathway at the earliest level of complement activationComplement molecules that have been isolated or cloned in fish are indicated.

2.9 References

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CHAPTER 3

MATERIALS AND METHODS

3.0 General procedures

3.1 Fish and parasite culture conditions

3.1.1 Fish

Goldfish (*Carassius auratus* L.) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) or Aquatic Imports (Calgary, Alberta, Canada) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta, according Canadian Council for Animal Care (CCAC) guidelines. The fish were kept at 20^{o}C in a flow-through water system on a simulated natural photoperiod, and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments. All of the fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes were used in experiments. Fish were anaesthetized by immersion in tricaine methane sulfonate solution before bleeding, clipping and infection.

3.1.2 Fish blood and serum preparation

To obtain goldfish serum, blood was collected through caudal vein of fish anaesthetized with tricane methanosulfonate (TMS, Syndel Laboratories) using a 23-guage needle fitted to a syringe. Blood was allowed to clot for overnight at 4°C and serum was isolated by centrifugation at 3000 x *g* for 30 minutes. Serum used for culturing trypanosomes was heat-inactivated at 56°C for 30 minutes, filter sterilized (Millipore 0.22µm), and stored at -20°C until used.

Immune serum was obtained from individual goldfish infected with $6.25x10^6$ *in vitro* grown parasites. Sera were collected 7, 14, 21, 28, and 56 days post-infection, pooled and stored at -20^oC until used.

Serum for cultivation of goldfish monocytes and macrophages was obtained from common carp (*Cyprinus carpio*). Fish used for obtaining blood and serum preparations were maintained strictly for this purpose and were bled every 6-8 weeks. Carp serum was prepared as described for goldfish serum.

3.1.3 Parasites

The parasite used in this study was bloodstream form *T. carassii* strain TrCa (=*T danilewskyi* Laveran and Mesnil 1904). The parasite was isolated by Dr. J. Lom in 1977 from a crucian carp. Isolation and cultivation of this trypanosome strain has been previously described [4, 16]. Parasites cultures were maintained *in vitro* at 20°C in TDL-15 medium [2], supplemented with 10% heat inactivated goldfish serum. Components of TDL-15 medium are shown in Table 3.1. Parasites were harvested from 5-7day old cultures for use in the experiments.

3.1.4 Purification of parasites from blood

Blood from infected fish was mixed with tri-sodium citrate (100 mM tri-sodium citrate, 40 mM glucose, pH 7.3), to prevent clotting. Samples were centrifuged at 750 x g, and the supernatant was removed. The trypanosomes located in the layer on top of the red blood cell pellet were removed and gently re-suspended in separation buffer (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 4 mM KCl, 5 mM glucose, pH 8). Parasites were re-suspended twice to ensure

most of the blood cells were removed. Following the last centrifugation step, the vial was tilted for 5 minutes at a 20-degree angle, to allow parasites to separate from the pelleted cells and settle on the side- wall of the vial. The separated parasites were removed and used immediately in the experiments.

3.1.5 Infection of fish

Prior to use in the experiments all fish were examined for the presence of natural hemoflagellate infections. Blood samples were collected from individual fish and examined for the presence of hemoflagellates using the haematocrit centrifugation technique, as described previously [2, 10, 18]. All fish used in experiments were free of natural hemoflagellates.

Fish were infected intraperitoneally (ip) with $6.25x10^6$ *in vitro* cultured parasites suspended in 100 µL of TDL-15 medium (Table 3.1) using a 25-guage needle and a tuberculin syringe. This dose was selected based on previously published results from our laboratory, which showed that all fish inoculated with this number of parasites became infected [16].

3.1.6 Assessment of infection

In order to determine the number of circulating parasites during the course of infection, 100 µL of blood samples were collected from the caudal vein of the fish. Four microliters of blood was collected and mixed with 96 µL of tri-sodium citrate anticoagulant solution, and the number of trypanosomes was determined by counting the parasites using a microscope (400x), and an improved Neubauer hemocytometer fitted with a glass cover slip (22 x 22 mm).

Rarely, fish had very low number of circulating parasites that could not be detected using a hemocytometer. In this case, blood samples were collected in heparinized capillary tube and examined for parasites using hematocrit centrifugation technique [18]. When the number of parasites in the heparinized capillary tube was difficult to count accurately, the blood was placed in a separate capillary tube, centrifuged, and re-examined. The detection limit for assessment of parasites in the blood of fish was 10^3 trypanosomes per mL.

3.1.7 Preparation of parasite antigens

3.1.7.1 Excretory/secretory (ES) products

In vitro cultured trypanosomes were washed twice at 400 x *g* for 10 minutes in serum free culture medium and re-suspended to a density of 4.28 x 10^8/mL. Parasites were incubated at 20°C for 1 hour and the cultures were then centrifuged at 12,000 x *g* for 10 minutes. Supernatants containing ES products were removed. To ensure removal of any remaining parasites the supernatant was re-centrifuged again at 12,000 x *g* for 10 minutes, filtered (0.22 µm), concentrated 10 fold and stored at -20°C until used.

3.1.7. 2 Whole cell lysates

Trypanosome cell lysates were prepared by washing *in vitro* cultured parasites twice in serum free culture medium at 400 x *g* for 10 minutes. Parasites were re-suspended in culture medium containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride-PMSF, 1 µg/mL leupeptin, 1 µg/mL antipain, 5 µg/mL aprotinin) and subjected to 3 cycles of freeze-thaw.

3.1.8 Detergent solubilization of membrane proteins

Membrane proteins were prepared from 10^{10} bloodstream parasites using Triton X-114 (Sigma) [5]. Parasite suspensions were centrifuged at 400 x *g* for 10 minutes and washed 2 twice in serum free medium at 400 x *g* for 10 minutes. Parasites were re-suspended in 1 mL of PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 1 μg/mL antipain, 5 μg/mL aprotinin) on ice. One mL of Triton X-114 containing protease inhibitors was added and the suspension incubated on ice for 1 hour with intermittent mixing. Samples were centrifuged at 100,000 x *g* for 1 hour at 4°C using ultracentrifuge (Beckman Instruments). The supernatants containing solubilized membrane proteins were collected and used immediately for two-dimensional electrophoresis (2-DE). Protein concentrations were estimated using Micro BCA protein assay (Pierce).

3.1.9 Anti-carp IgM

Hybridoma cell supernatants containing monoclonal antibodies directed towards an epitope of the heavy chain of carp Ig (designated WCI 12) were obtained from previously grown cultures [3]. The specificity of monoclonal antibody for goldfish immunoglobulin was assessed using SDS-PAGE and immunoblotting. Immunoblotting revealed a single band of approximately 80 kDa further supporting the previous findings that indicate the size of the heavy chain to be approximately 79 kDa [17]

3.2 Goldfish kidney-derived monocyte/macrophage cultures

3.2.1 Culture medium

Goldfish leukocytes were cultured in MGFL-15 medium [15]. The components of the medium are shown in Table 3.2. Complete medium (C-MGFL-15) comprised of MGFL containing 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco), 100 µg/mL gentamicin (Gibco), 10% newborn calf serum (Hyclone) and 5% carp serum. Cultures were maintained at 20°C.

3.2.2 Isolation of goldfish kidney leukocytes

Leukocytes were isolated from goldfish kidneys as described previously [12, 15]. Briefly, fish were anaesthetized using TMS (approximately 40 mg/mL) and the kidneys were removed aseptically. The kidneys were placed in homogenizing solution (MGFL-15, Pen/Strep and heparin) and gently passed through sterile stainless steel screens using homogenizing solution. The resulting cell suspension was layered over 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 minutes at 4°C. Cells at the medium-Percoll interface were transferred into clean tubes and washed twice with MGFL-15 by centrifugation at 200 x *g* for 10 minutes at 4°C. The resulting cell pellets were re-suspended in complete medium (CMGFL-15) and cultured at 20°C. Goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (1.8-2 x 10^6 cells per flask from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of CMGFL-15 and 5 mL of cell-conditioned medium (CCM) from previous cultures. These primary kidney macrophage (PKM) cultures contain heterogenous

populations of cells including progenitors, monocytes and mature macrophages as determined by flow cytometry, morphology, cytochemistry and function [12]. Leukocytes that were cultured for 2-4 for days without CCM were predominantly monocytic cells, while macrophages were obtained from 6-8 days cultures, before use in assays.

3.2.3 Preparation of cell-conditioned medium (CCM)

Cultures of primary kidney macrophages maintained at 20°C for 6-8 days were centrifuged at 200 x *g* for 10 minutes at 4° and the resulting CCM (supernatants) from individual cultures were pooled, filter-sterilized (0.22 µm) and stored at 4°C until use.

3.3 Molecular procedures

3.3.1 Isolation of total RNA

Total RNA was extracted from parasite grown for 5-7 days *in vitro* using TRIZOL reagent (Invitrogen) according to manufacturer's instructions and RNA samples were stored at -80°C until used. The purity of RNA was confirmed by calculating the ratio of the absorbance at 260 nm and 280 nm. In all cases, this ratio was approximately 2.0, which is the generally acceptable value for pure RNA. The extracted RNA was reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) according to manufacturer's recommendation. The cDNA synthesized was used in polymerase chain reactions (PCR).

3.3.2 Cloning of *T. carassii* genes

3.3.2.1 *T. carassii* hsp70 (Tcahsp70)

Hsp70 fragment was amplified using primers designed to *T. carassii* spliced leader sequence and hsp70 peptides (IFDLGGGTFDVT LL and VVLVGGSTRIPKVMQ) identified by mass spectrometry. Table 3.3 shows the list of primers used. The rest of the sequence was amplified using 3' RACE, according to manufacturer's protocols (Clontech) and the full ORF was obtained. The sequence for Tcahsp70 has been submitted to GenBank (FJ970030).

3.3.2.2 *T. carassii* calreticulin (TcaCRT)

TcaCRT was originally identified in membrane protein fraction separated by 2-dimensional gel electrophoresis (2-DE) and identified by mass spectrometry. TcaCRT peptides identified in mass spectrometry analysis are (FYADAEK , CGGGYIK, YWLMFGPDR, EAPMIPNPK, QIPNPAYK). These peptides matched *T. cruzi* calreticulin (TcCRT) with accession number (XM_799098). TcaCRT fragment was amplified using primers (Table 3.3) designed to *T. carassii* spliced leader sequence and peptides identified by mass spectrometry. The rest of the sequence was amplified using 3' RACE, according to manufacturer's protocols (Clontech) and full ORF was obtained. The full sequence for TcaCRT has been submitted to GenBank (GQ406204).

3.3.2.3 *T. carassii* glycoprotein 63 (Tcagp63)

Tcagp63 fragment was amplified using primers (Table 3.3) designed to *T.*

carassii spliced leader sequence and a highly homologous region of trypanosome gp63 sequences. The rest of the sequence was amplified using 3' RACE, according to manufacturer's protocols (Clontech) and the full ORF was obtained. The full sequence for Tcagp63 has been submitted to GenBank (JN660149).

3.3.2.4 *In silico* analysis of *T. carasssii* genes

Protein sequence alignments were performed with Clustal W software. Phylogenetic analysis of Tcagp63 was conducted using Clustal X software neighbor joining method for relationship calculation and bootstrapped 10,000 times, with values expressed as percentages. Phylogenetic analysis was visualized using the njplot program. As indicated previously, all nucleotide and protein sequences used in this thesis have been submitted to GenBank. Protein sequences used for phylogenetic analysis of Tcagp63 and their accession numbers are: *T. cruzi* (XP_817187), *T. brucei* (XP_846998), *T. congolense* (CCC95446), *T. vivax* (CCC53361), *L. major* (XP_001684335), *L. mexicana* (CAA45733), *L. donovani donovani* (ACT31401), *L. infantum* (XP_003392667), *L. braziliensis* (XP_001562828), *T. carsssii* Cathepsin L (ABQ23397), *T. cruzi* cruzipain (AAF75547), *T. brucei* cysteine peptidase (XP_845218). *T. carsssii* Cathepsin L, *T. cruzi* cysteine protease and *T. brucei* cysteine peptidase were used as out-groups.

3.4 Using prokaryotic expression system for production of *T. carassii* recombinant proteins

3.4.1 Cloning of *T. carassii* genes into pET SUMO expression vectors

The production of recombinant parasite proteins characterized in this thesis has been described previously [13, 14]. *T. carassii* hsp70, calreticulin and gp63 were amplified from parasite cDNA using primers that allow cloning into the pET SUMO expression vector (Invitrogen). The PCR products were gel purified (QIAquick gel extraction kit) and ligated into the pET SUMO vector (Invitrogen). The recombinant molecules were used to transform competent *Escherichia coli* (One Shot chemically competent cell), plated onto LB plates containing kanamycin (50 μg/mL) and incubated overnight at 37°C. Recombinant clones were identified by colony PCR and cultured in LB broth containing kanamycin (50 μg/mL). Plasmid DNA was purified from recombinant clones using a QIAquickSpin miniprepkit (Qiagen) and sequenced using the vector specific primers (Table 3.3) to verify that insert was in correct orientation and in frame. The pET SUMO vector encodes an N-terminal 6XHis tags for purification and detection of the recombinant molecule.

3.4.2 Pilot expression studies of recombinant Tcahsp70, TcaCRT, Tcagp63

Recombinant plasmids containing Tcahsp70, TcaCRT or Tcagp63 insert were transformed into BL21 Star One Shot *E. coli* cells (Invitrogen) and the whole transformation mixture cultured overnight in 5 mL LB containing kanamycin (50 μg/mL). In this pilot run, 500 μL of the overnight culture was used

to inoculate 10 mL LB containing kanamycin (50 μg/mL), grown to mid-log phase (OD_{600} of 0.5-0.8) and split into two 5 mL cultures. IPTG (1 mM) was added to one of the cultures to induce expression of the recombinant protein. Samples were taken every 2 hours to determine the optimal induction times for each respective protein. Following sampling, bacteria lysate supernatant and pelleted bacteria fractions were resolved by SDS-PAGE and visualized by western blotting using mouse monoclonal anti-polyhistidine antibody (1:5000) (invitrogen) against the N-terminal 6X His tag on the recombinant proteins. From these pilot studies, I determined that the recombinant hsp70 and CRT were present in both supernatant and pellet fraction, gp63 was present in the pellet fraction, and the optimal induction time for all proteins to be 2 hours.

3.4.3 Scaled up expression of recombinant Tcahsp70 and TcaCRT in *E. coli*

Bacterial culture expressing the desired recombinant proteins were scaled up and incubated for the 2 hours optimal induction time. The pilot expression showed these proteins were present in both supernatant and pellet fraction although the majority of the proteins were present in the pellet fraction. The induced cells in the scaled up cultures were centrifuged at 10,000 x *g* for 10 minutes, the supernatants were removed and the cell pellets frozen at -20^{o}C until needed. Cells were suspended in lysis buffer (5 mL of 10X FastBreak cell lysis reagent (Promega) in 45 mL of denaturing wash buffer (100 mM Hepes, 10 mM imidazole, 7 M urea, 0.1 M PMSF; pH 7.5) and incubated with Magne-His Ni-particles (Promega) as recommended by the manufacturer. Ni-particles bound to

the recombinant proteins were retained by a PolyATtrack system 1000 magnet (Promega), were extensively washed with the wash buffer described above. The supernatants were discarded and the beads washed 3 times under denaturing condition using the denaturing wash buffer described above. The recombinant protein was eluted from the beads using 500 mM imidazole. Western blot using monoclonal anti-His antibody was performed to confirm the presence of the recombinant protein. The protein was renatured in 10 volumes of renaturation buffer (4 mM reduced glutathione, 2 mM oxidized glutathione, 50 mM sodium borate, 5 mM EDTA, pH 8.5) overnight and dialyzed against 4 L of 1X PBS overnight using 3 kDa MWCO Snakeskin dialysis tubing (Pierce), Protein concentration was done in dialysis tubing in polyethylene glycol flakes for 8 hours and protein was further dialyzed overnight against 1X PBS to remove traces of imidazole and urea. The recombinant protein was passed through an EndoTrap Red endotoxin removal column (Cambrex) as recommended by the manufacturer to remove bacteria LPS. Recombinant protein was analyzed using the *Limulus* amebocyte lysate kit (Cambrex) and found to be endotoxin-free (< 0.1 EU/mL). Protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce) and the presence of the recombinant protein verified by Western blot. The identity of the protein was confirmed by mass spectrometry. A vector control prepared by growing *E. coli* cells expressing an empty pET SUMO vector and following exact procedure described above was used as control in functional assays. The vector control had no protein contents as measured by Micro BCA Protein Assay (Pierce). The concentrations of rTcahsp70 used in functional assays

were based on previous studies [1]. Also, the concentrations of rTcaCRT used in hemolysis assay were based on previous studies [6].

3.4.4 Scaled up expression of recombinant Tcagp63 in *E. coli*

The pilot studies showed substantial degradation of the recombinant gp63 produced by vector expressing full ORF and ORF lacking the signal peptide. Stable gp63 protein was obtained from vector expressing fragment lacking N-terminal portion of the protein containing both the signal peptide and the catalytic domain. Bacteria culture expressing the desired recombinant protein was scaled up and incubated for the 2 hours optimal induction time. In the pilot expression study, recombinant gp63 was present in the pellet fraction. Therefore, the induced cells in the scaled up cultures were centrifuged at 10,000 x *g* for 10 minutes, the supernatants were removed and the cell pellets was frozen at -20^{o}C until needed. Cells were suspended in lysis buffer [5 mL of 10X FastBreak cell lysis reagent (Promega) in 45 mL of denaturing wash buffer (100 mM Hepes, 10 mM imidazole, 7 M urea, pH 7.5] and incubated with Magne His Ni-particles (Promega) as recommended by the manufacturer. The supernatants were discarded and the beads washed 3 times under denaturing condition using the denaturing wash buffer described above. The recombinant protein was eluted from the beads using 500 mM imidazole. Western blot using monoclonal anti-His antibody was performed to confirm the presence of the recombinant protein. The protein was re-natured in 10 volumes of renaturation buffer (40 mM Tris, pH 8) overnight and dialyzed against 4 L of 1X PBS using 3 kDa MWCO Snakeskin

dialysis tubing (Pierce). Protease inhibitor cocktail (AEBSF 1 mM, aprotinin 800 nM, bestatin 50 µM, E64 15 µM, leupeptin 20 µM, pepstatin A 10 µM) were added to all buffers to prevent protein degradation. Protein concentration was done in dialysis tubing in polyethylene glycol flakes for 8 hours and protein was further dialyzed overnight against 1X PBS to remove traces of imidazole and urea. The recombinant protein was passed through an EndoTrap Red endotoxin removal column (Cambrex) as recommended by the manufacturer to remove bacteria LPS. Recombinant protein was analyzed using the *Limulus* amebocyte lysate kit (Cambrex) and found to be endotoxin-free free (< 0.1 EU/mL). Protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce) and the presence of the recombinant protein verified by Western blot. The identity of the protein was confirmed by mass spectrometry. A vector control prepared by growing *E. coli* cells expressing an empty pET SUMO vector and following exact procedure described above was used as control in functional assays. The vector control had no protein contents as measured by Micro BCA Protein Assay (Pierce) however, volumes corresponding to recombinant proteins were used in functional assays. The concentrations of rTcagp63 used in functional assays were based on previous studies [9].

3.5 Quantitative expression analysis (Q-PCR) of goldfish immune genes

3.5.1 Q-PCR of monocytes and macrophages genes

The Q-PCR primer sequences used in this thesis are shown in Table 3.4. Expression primers design, validation Q-PCR conditions have been described

previously [7]. Briefly, primers were designed using Primer Express software (Applied Biosystems). Primers were validated by measuring relative quantification efficiency using a serially diluted goldfish cDNA. Primers were deemed acceptable for use if the experimentally derived R^2 values from the cDNA dilution curves were greater than 0.980. In most instances, subsequent to primer validation experiments, Q-PCR products were resolved by agarose gel electophoresis and the resulting products sequenced to confirm primer specificities.

Total RNA was extracted from *in vitro* cultured goldfish monocytes (2-4 days) and macrophages (6-8 days) using TRIzol reagent (Invitrogen) according to manufacturer's instructions and RNA samples were stored at -80°C until used. The extracted RNA was reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) according to manufacturer's recommendation. The cDNA synthesized was used in quantitative real-time PCR reactions. In all cases, equal amounts of RNA and cDNAs were used in the Q-PCR reaction. Thermocycling parameters were as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Analyses of gene expression data were performed using 7500 Fast software (Applied Biosystems).

3.5.1.1 Pro-inflammatory cytokine expression in goldfish macrophages treated with recombinant *T. carassii* hsp70

Macrophage cultures obtained from four individual fish (n = 4) were cultured for 8 days and seeded into individual wells of 96-well plates at a density

of $1x10^6$ cells per well. Cells were treated with medium alone or 5,000 ng/mL of *T. carasii* hsp70 (rhsp70) for 3 and 6 hours. Each treatment group consisted of $1x10^6$ cells in a final volume of 400 µL of complete medium. Total RNA was isolated from the cells at indicated times and RNA was reverse transcribed into cDNA as indicated previously. Changes in expression pattern of cytokines IFNγ, TNFα-1, TNFα-2, IL-1β-1, IL-1β-2; IL-12-p35; chemokines CXCL-8, CCL-1; and anti-microbial genes iNOS isoforms A and B were examined. Relative expression was determined in relation to elongation factor 1 alpha (EF-1α). All expression data were normalized using the baseline expression at 0 hour time point.

To confirm that hsp70 induced cytokine expression was due to cell surface signaling, macrophages were pre-treated with either culture medium only, or 10mM pronase for 30 minutes [1]. Cells were washed twice with incomplete medium and re-suspended in complete medium containing 5,000 ng/mL of rhsp70. Total RNA was isolated at 0, 3 and 6 hours post treatment and expression data normalized to baseline expression at 0 hour time point.

3.5.1.2 Immune gene expression in macrophages exposed to rTcagp63 and *A. salmoncida*

Goldfish macrophage cultures obtained from four individual fish (n = 4) were cultured for 8 days and seeded into individual wells of 96-well plates at a density of $1x10^6$ cells per well. Goldfish monocytes cultures obtained from four-day-old cultures and seeded into individual wells of 96-well plates at a density of

$1x10^6$ cells per well. Cells were treated with medium alone or 10 µg/mL of recombinant *T.* carassii gp63 (rTcagp63) at 20°C for 2 hours,, washed twice and then stimulated with heat-killed *A. salmonicida* at 20°C for 6 hours. Each treatment group consisted of $1x10^6$ cells in a final volume of 400 µL of complete medium. Total RNA was isolated from the cells and RNA was reverse transcribed into cDNA as indicated previously. Changes in expression pattern of select immune genes (iNOS, TNFα1 and 2, IL10 and NADPH oxidase component genes) were examined. Relative expression was determined in relation to elongation factor 1 alpha (EF-1α). All expression data were normalized using the baseline expression of cells incubated in medium alone.

3.5.1.3 Immune gene expression in macrophages treated with rTcagp63

Goldfish macrophage cultures obtained from four individual fish (n = 4) were cultured for 8 days and seeded into individual wells of 96-well plates at a density of $1x10^6$ cells per well. Cells were treated with 10 µg/mL of rTcagp63 for 0, 3 and 6 hours at 20°C. Each treatment group consisted of $1x10^6$ cells in a final volume of 400 µL of complete medium. Total RNA was isolated from the cells at the indicated times and RNA was reverse transcribed into cDNA as described previously. Changes in expression pattern of select immune genes (iNOS A and B, TNFα1 and 2, IL10) were examined. Relative expression was determined in relation to elongation factor 1 alpha (EF-1α). All expression data were normalized using the baseline expression of cells incubated in medium alone.

3.5.2 Quantitative expression analysis of immune genes in goldfish tissues

Spleen, liver and kidney tissues were removed from infected (n = 4) and non-infected (n=4) fish on days 7, 14, 21, 28 and 56 pi, and were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. Total RNA was extracted from goldfish tissues and RNA samples from different tissues were reverse transcribed into cDNA as described previously. In all cases, the purity of RNA was confirmed. Changes in the expression patterns of a panel of pro-inflammatory genes encoding IFN-γ, TNFα1 and TNFα2, IL-1β-1 and IL-1β-2, IL-12-p35 and IL-12-p40, CCL1, CXCL8, anti-inflammatory genes encoding IL-10 and TGFβ, and antimicrobial genes encoding iNOS A and iNOS B were examined. Relative expression was determined in relation to elongation factor-1 alpha (EF-1α). All expression data were normalized using the baseline expression in non-infected control fish for each gene. Results were provided as fold change in expression relative to non-infected control for each gene.

3.6 Measurement of the production of reactive oxygen and nitrogen intermediates: nitric oxide and respiratory burst assays

Individual cultures of primary macrophages were obtained from kidney of goldfish and the cells were cultured for 6 to 8 days (n = 7). Cells were harvested from cultures and seeded into individual wells of 96-well plates at a density of 3 x 10^5 cells per well. Cells were treated with culture medium alone (control) or with either heat-killed *A. salmonicida* or different concentrations of *T. carassii* and incubated at 20°C for 48 hours. Parasites were washed twice in serum-free culture

medium before use. Nitrite production was determined using the Griess reaction [8]. Briefly 1% sulphanilamide and 0.1% N-naphthyl-ethylenediamine were added to cultures and absorbance was measured at 540 nm. The amount of nitrite in culture supernatants was determined using nitrite standard curve.

For respiratory burst assay, individual cultures of goldfish monocytes were obtained from kidney of goldfish and cells were cultured for 3 to 4 days. Cells from 6 individual fish (n = 6) were seeded into 96-well plates at a density of 3×10^5 cells per well and incubated with medium alone (control), or with either macrophage activating factor (MAF) or different concentrations of *T. carassii*. ROI assays were incubated at 20°C for 48 hours. NBT (2 mg/mL, Sigma) and PMA (final conc. 100 ng/mL, Sigma) in PBS were added to the cultures and incubate at room temperature for an additional 30 minutes. The plates were than centrifuged at 400 × *g* for 10 minutes, the supernatants aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Absorbance from cells alone (no PMA) was subtracted from treatment values to factor in background NBT reduction.

3.7 Functional analysis of the effects of *T. carassii* proteins on antimicrobial responses of goldfish monocytes and macrophages

3.7.1 Assessment of the ability of rTcahsp70 to induce nitric oxide response of goldfish macrophages

Individual cultures of primary macrophages were obtained from kidney of goldfish and the cells were cultured for 6 to 8 days (n = 7). Cells were harvested from cultures and seeded into individual wells of 96-well plates at a density of 3 x 10^5 cells per well. Cells were treated with culture medium alone (control) or with either heat-killed *A. salmonicida* or rhsp70 (500 ng/mL, 5,000 ng/mL or 10,000 ng/mL) and incubated at 20°C for 72 hours. Nitrite production was determined using the Griess reaction as described previously [8]. To ascertain whether the results obtained were not due to other contaminating proteins, *E. coli* bacterial lysates were prepared and subjected to the same purification protocol as rhsp70-containing lysates. Following purification regimen, these lysates did not contain protein as indicated by micro BCA protein assay. Furthermore, separate goldfish macrophage cultures obtained from 4 fish (n = 4) were treated with either medium only (negative control), heat-killed *A. salmonicida* (positive control), rhsp70 (5,000 ng/mL, 10,000 ng/mL) or bacterial lysates (17 µL, 33 µL, equivalent volumes of rhsp70 lysates that corresponded to 5,000 and 10,000 ng/mL of rhsp70) and assayed for nitric oxide response using Griess reaction, as described previously [8].

3.7.2 Assessment of the effects of rTcagp63 on *A. salmonicida*-induced nitric oxide response of goldfish macrophages.

Individual cultures of primary macrophages were obtained from kidney of goldfish and the cells were cultured for 7 to 8 days. Cells from 6 individual fish (n = 6) were harvested from cultures and seeded into individual wells of 96-well plates at a density of 3×10^5 cells per well. Cells were incubated with culture medium alone (negative control), heat-killed *A. salmonicida* (positive control), rTcagp63 (1 µg/mL, 5 µg/mL or 10 µg/mL) plus heat-killed *A. salmonicida* at 20°C for 48 hours. Nitrite production was determined using the Griess reaction as described previously [8]. To ascertain that the results obtained were not due to other contaminating proteins, *E. coli* bacterial lysates were prepared and subjected to the same purification protocol as rTcagp63-containing lysates. Following purification regimen, these lysates did not contain protein as indicated by micro BCA protein assay. Furthermore, separate goldfish macrophage cultures obtained from 6 fish (n = 6) were treated with medium alone (negative control), heat-killed *A. salmonicida* (positive control), rTcagp63 (10 µg/mL) or bacterial lysates (4.5 µL, equivalent volumes of rTcagp63 lysates that corresponded to 10 µg/mL of rTcagp63) plus heat-killed *A. salmonicida* and assayed for nitric oxide response using Griess reaction. Also, recombinant goldfish TGFβ, purified using the same procedure as described for Tcagp63 were used in the NO assay at 10 µg/mL.

3.7.3 Assessment of the effects of rTcagp63 on *A. salmonicida*-induced respiratory burst response of goldfish monocytes.

Individual cultures of goldfish monocytes were obtained from kidney of goldfish and cells were cultured for 3 to 4 days. Cells from 6 individual fish (n = 6) were incubated with medium alone (control), *A. salmonicida* alone or *A. salmonicida* plus different concentration of rTcagp63. In another experiment cells were treated with culture medium alone (control), rTcagp63 (5 µg/mL or 10 µg/mL) for 2 hours and washed 3 times with incomplete medium before used to set up ROI assay. Cells were seeded into 96-well plates at a density of 3×10^5 cells per well. ROI assay was performed with cells incubated in medium alone or with rTcagp63 using heat-killed *A. salmonicida* and recombinant goldfish TNFα2 (rgTNFα2 100 ng/mL) to stimulate production of reactive oxygen species. ROI was determined as described in Section 3.6.

3.8 SDS-PAGE and western blot

3.8.1 SDS-PAGE

Proteins were separated and visualized by reducing SDS-PAGE according to the method described originally by Laemmli [11]. Briefly, samples were dissolved in an equal volume of Laemmli sample buffer (BioRad) and heated at 95 °C for 5 minutes. Samples were electrophoresed through polyacrylamide gels (4% stacking, 12% separating) at 100 V for 15 minutes followed by 185 V for 45 minutes. The polyacrylamide gels were stained with Coomassie brilliant blue (BioRad) to visualize protein bands in some experiments. Coomassie staining was

performed by soaking the gels in a solution of Coomassie brilliant blue (0.1% Coomassie brilliant blue, 10% acetic acid, 40% methanol) for 30 minutes at room temperature). Gels were destained using 10% acetic acid and 40% methanol.

3.8.2 Western blot

Following gel electrophoresis, proteins were transferred to 0.2µm nitrocellulose membranes (BioRad) at 100V for 1 hour in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes containing transferred proteins were immunoblotted in the following manner. The nitrocellulose were blocked with 0.5% BSA in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5; TBS) for 30 minutes at room temperature. Membranes were then incubated in a solution containing primary antibody in blocking buffer and incubated overnight at 4°C. Membranes were washed 3 times in TTBS and 3 times in TBS for 5 minutes each before incubation with secondary antibody diluted in blocking solution for 1 hour at room temperature. Blots were washed 3 times in TTBS and 3 times in TBS for 5 minutes each. Protein bands were visualized using chromogenic BCIP/NBT development solution according manufacturer's instructions (BioRad).

3.8.2.1 Analysis of excretory/secretory proteins using SDS-PAGE and western blot

Excretory/secretory proteins were separated by SDS-PAGE under reducing conditions. Briefly, 20 µg of proteins were mixed with equal volume of

Laemmli sample buffer (BioRad), heated at 95°C for 5 minutes and loaded onto the gel. Electrophoresis was performed using 1 mm gel containing 5% stacked and 12% separating SDS polyacrylamide at 100 V for 15minutes then 180 V for 45 minutes. Proteins were electrophoretically transferred to nitrocellulose membrane (BioRad) at 100V for 1 hour in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) for immunoblotting. Membrane strips containing ES proteins were blocked with 0.5% BSA in Tris-buffered saline/Tween 20 (TTBS; 0.1% Tween 20 in 100 mM Tris-HCl, 0.9% NaCl, pH 7.5; TBS) for 30 minutes at room temperature. Membranes were then incubated with either serum from recovered fish 56 days post infection or serum from non-infected fish (diluted 1: 25 in blocking buffer) overnight at 4°C. Membranes were washed 3 times in TTBS and 3 times in TBS for 5 minutes each before incubation with secondary antibody (hybridoma supernatant containing anti-carp IgM antibody) diluted 1:5 in blocking solution for 1 hour at room temperature. Blots were washed 3 times in TTBS and 3 times in TBS for 5 minutes and then incubated with alkaline phosphatase-labelled goat-anti-mouse IgG diluted 1:1500 in blocking buffer. Protein bands were visualized using chromogenic BCIP/NBT development solution according manufacturer's instructions (BioRad). Protein band corresponding to 68-70 KDa antigen detected on immunoblot was excised from a Coomassie blue stained SDS gel, analyzed by mass spectrometry and identified as *T. carassii* hsp70. Complete list of ES proteins identified using this method is shown in Table 3.5.

3.8.2.2 SDS-PAGE and western blot analysis of rTcahsp70, rTcaCRT and rTcagp63

Proteins were separated and visualized by reducing SDS-PAGE. Briefly, samples were dissolved in an equal volume of Laemmli sample buffer (BioRad), heated at 95 °C for 5 minutes and electrophoresed through polyacrylamide gels (4% stacking, 12% separating) at 100 V for 15 minutes followed by 185 V for 45 minutes. Proteins were transferred to 0.2 μm nitrocellulose membranes (BioRad) at 100 V for 1 hour in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes containing the transferred proteins were treated in the following manner. The nitrocellulose was first incubated in blocking solution consisting of 5% skimmed milk in PBS for 30 minutes at room temperature. The membranes were then incubated with primary antibody (mouse monoclonal anti-polyhistidine antibody 1: 5000 diluted in PBS and incubated overnight at 4 °C. Membranes were washed 3 times in TTBS and 3 times in TBS for 5 minutes each, prior to incubation with secondary antibody (alkaline phosphatase-labelled goat anti-mouse IgG 1:1500 or goat anti-rabbit IgG 1:3000) diluted in PBS for 1 hour at room temperature. Following incubation with the secondary antibody, the blots were washed 3 times in TTBS and 3 times in TBS for 5 minutes each. Protein bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturer's instructions (BioRad).

Polyclonal IgG antibodies raised against the recombinant proteins (1:2000) diluted in PBS were also used as primary antibody while alkaline phosphatase-labelled goat anti-rabbit IgG (1:3000) diluted in PBS were also used to visualize

the recombinant proteins. Only rabbit anti-rTcahsp70 and rabbit anti-rTcaCRT polyclonal IgG raised against the recombinant proteins react with the native proteins in whole cell lysate. Anti-rTcagp63 failed to react with native gp63 in *T. carassii* lysate in Western blot analysis.

Preparation of ES proteins and parasite cell lysate has been described previously [14]. For detection of native Tcahsp70 and TcaCRT in ES proteins and in whole cell lysate, ten μg of ES products or cell lysate were separated by SDS PAGE. Following gel electrophoresis, proteins were transferred to nitrocellulose membranes and immunoblotted. Membrane strips containing ES products or whole cell lysate proteins were blocked with 5% skimmed milk in PBS for 30 minutes at room temperature. Membranes were then incubated with either polyclonal rabbit anti-rTcahsp70 or rTcaCRT affinity purified IgG antibodies diluted in PBS (1:1500) overnight at 4°C. Membranes were washed 3 times in TTBS and 3 times in TBS for 5 minutes each before incubation with alkaline phosphatase-labelled goat anti-rabbit IgG antibody diluted in PBS (1:3000) for 1 hour at room temperature. Blots were washed 3 times in TTBS and 3 times in TBS for 5 minutes at room temperature. Protein bands were visualized using chromogenic BCIP/NBT development solution according manufacturer's instructions (BioRad).

3.8.2.3 SDS-PAGE and western blot analysis of carp and goldfish first complement component C1q

C1q-enriched fraction was isolated from fresh goldfish serum as

described previously [13]. Briefly, 2.5 mL of 30% (w/v) PEG4000 in 20 mM Tris-buffered saline (pH 7.5) was added to 10 mL of freshly collected carp or goldfish serum drop at a time with continuous mixing. The mixture was kept on ice for 15 minutes and centrifuged at 400 x *g* for 10 minutes. The supernatant was removed and pellet was re-suspended in 2 mL of TBS and protein concentration determined using Micro BCA Protein Assay Kit (Pierce). The fraction was separated on SDS-gel and transferred to nitrocellulose membrane for western blotting. C1q were detected with anti-carp C1q-A antiserum (a kind gift from Dr Miki Nakao, Kyushu University, Japan) diluted 1:1500 in PBS.

For detection of carp and goldfish C1q, the membranes were incubated with primary antibody rabbit anti-carp C1q-A antiserum 1:1500 diluted in PBS and incubated overnight at 4 °C. Membranes were washed 3 times in TTBS and 3 times in TBS for 5 minutes each, prior to incubation with secondary antibody (goat anti-rabbit IgG 1:3000) diluted in PBS for 1 hour at room temperature. Following incubation with the secondary antibody, the blots were washed 3times in TTBS and 3 times in TBS for 5 minutes each. Protein bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturer's instructions (BioRad).

3.8.2.4 Far western blot

Far western blotting was performed using a native gel. Native gels were prepared in the absence of SDS by lowering acrylamide concentration to 8% in separating and 3% in stacking gels. Protein samples were dissolved in native

sample buffer (0.125 M Tris pH 6.5, 10% glycerol, 0.005% bromophenol blue) and proteins were electrophoresed using native running buffer (25 mM Tris-HCl pH 8.8, 250 mM glycine). Following electrophoresis, proteins were were transferred to nitrocellulose membrane as described above. Nitrocellulose strips were incubated with PBS alone or with 200 μg of recombinant TcaCRT (rTcaCRT) diluted in PBS for 3 hours at room temperature. Blots were washed and then incubated with rabbit anti-rTcaCRT antibody (1:1500) for 3 hours at room temperature. Following washes with TTBS and TBS, blots were incubated with alkaline phosphatase-labelled goat anti-rabbit IgG (1:3000) for 1 h at room temperature. Protein bands were visualized with BCIP/NBT development kit.

3.9 Investigation of the effects of rTcagp63 on goldfish macrophages

3.9.1 The interaction between rTcagp63 and goldfish macrophages

Goldfish macrophage cultures obtained from three individual fish (n = 3), were cultured for 8 days. 10^7 cells were washed twice and incubated with rTcagp63 (10 μg/mL) for 0, 1, 2, 3 hours at 20°C. Cells were washed 3 times with PBS and lysed with 30 μL of RIPA buffer. 30 μL of Laemmli sample buffer was added and the mixture loaded into SDS PAGE for western blotting. Detection of rTcagp63 was done with rabbit anti-rTcagp63 antibody (1:2000) followed by HRP-conjugated goat anti-rabbit IgG (1:3000). Stripping of western blot membrane for re-probing was done with mild stripping buffer (15 g glycine, 1 g SDS, 10 mL Tween 20, pH 2.2 in 1I L of ultrapure water). PVDF membranes were incubated twice for 10 minutes at room temperature with stripping buffer

followed by incubation with PBS twice for 10 minutes. Blots were then incubated in TTBS twice for 5 minutes and checked to ensure signal was gone before blocking. Rabbit anti-actin-beta (NT) zebrafish polyclonal IgG (AnaSpec Inc.) (1:1000) was used for re-probing to detect goldfish actin which served as protein loading control.

3.9.2 Assessment of the effects of rTcagp63 on phospho-tyrosine protein pattern of goldfish macrophages

Goldfish macrophage cultures obtained from three individual fish (n = 3), were cultured for 8 days. 10^7 cells were washed twice and incubated with medium alone (control) or with rTcagp63 (10 µg/mL) for 6 hours. Cells were stimulated with *A. salmonicida* at 20°C for 10, 15 and 20 minutes before lysed with 30 µL of radio-immunoprecipitation assay (RIPA) buffer [150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 0.5% sodium deoxycholate, 1% Nonidet P-40 (NP-40), 10 mM 2-mercaptoethanol (pH 7.5)] containing 1 mM sodium vanadate, 1 mM p-nitrophenyl-phosphate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 50 µg/mL of aprotinin A, 25 µg/mL of leupeptin, and 25 µg/mL of pepstatin). 30 µL of Laemmli sample buffer was added and loaded in SDS PAGE for western blotting. Phospho-tyrosine protein patterns were detected with anti-phospho-tyrosine antibody (1: 4000) and HRP-conjugated goat anti-mouse monoclonal antibody (1: 3000). Membranes were developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

3.10 Production and purification of polyclonal rabbit anti-recombinant Tcahsp70, TcaCRT and Tcagp63 antibodies

3.10.1 Production of polyclonal rabbit anti-recombinant Tcahsp70, TcaCRT and Tcagp63 antibodies

Purified recombinant proteins were used to immunize rabbits for production of polyclonal antibodies. Rabbits were injected with 250 μg of purified recombinant protein in 750 μL mixed with 750 μL of Freund's complete adjuvant (FCA). Booster injections were performed every 4 weeks for 12 weeks using the same quantity of antigen in conjunction with Freund's incomplete adjuvant (FIA). The specificity of the antibody for the immunizing antigens and native Tcahsp70, TcaCRT and Tcagp63 present in *T. carassii* whole cell lysates was determined using Western blot as described above.

3.10.2 Affinity purification of anti-recombinant Tcahsp70, TcaCRT and Tcagp63 IgG from rabbit serum

The polyclonal antibodies were purified from rabbit serum using a HiTrap Protein A HP affinity column (GE Healthcare) according to the manufacturer's protocol. Briefly, 5 mL of polyclonal rabbit anti-recombinant Tcahsp70, TcaCRT or Tcagp63 was diluted in binding buffer 20 mM sodium phosphate (pH 7) and applied to a 1 mL HiTrap Protein A HP column that was pre-equilibrated with the binding buffer. The column was washed with binding buffer. The bound proteins were eluted from the column with a decreasing pH gradient (0.1 M citric acid pH 3–6). The pH of the eluted fractions was neutralized by the addition of 1 M Tris–

HCl, pH 9. Each fraction was tested for the presence and purity of IgG using SDS-PAGE and immunoblotting. Fractions containing IgG were pooled and the protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce). The purified IgG was dialyzed against PBS in 3 kDa MWCO Snakeskin dialysis tubing (Pierce), filter-sterilized (0.22 μm filter; Millipore) and stored at 4°C until used.

3.11 Functional assessment of TcaCRT and investigation of its interaction with goldfish complement component C1q

3.11.1 Assessment of interaction of goldfish complement component C1q with immobilized rTcaCRT using affinity chromatography

To study interaction between goldfish C1q and parasite CRT, 5 mg of purified rTcaCRT was coupled to CNBr-activated Sepharose (GE Healthcare) according to instructions provided by the manufacturer. Three mg of C1q containing serum fraction was loaded onto CRT-Sepharose column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$. The mixture was incubated at room temperature for 1 hour with gentle rocking to allow protein-protein interaction. Following incubation, the column was washed 4 times with 2 mL PBS. The bound proteins were subsequently eluted by increasing NaCl concentration (150 mM, 250 mM, 500 mM) and analyzed by SDS-gel electrophoresis. Proteins were transferred to nitrocellulose membrane and C1q was detected with anti-carp C1q-A antiserum (1:1500) using Western blot.

3.11.2 Assessment of interaction of goldfish complement component C1q and rTcaCRT using co-immunoprecipitation

To further show that TcaCRT interacts with goldfish C1q, I used co-immunoprecipitation assay. Fifty μg of anti-rTcaCRT or rTcahsp70 was coupled to agarose resin according to manufacturer's instruction (Pierce). Fifty μg of rTcaCRT was incubated with 50 μg of C1q-containing fraction eluted from CNBr-activated sepharose column with 500 mM NaCl in the presence of 20 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ for 6 hours at 4°C. The proteins were then incubated with the prepared agarose resin for 1 hour at room temperature. The columns were washed and bound proteins were eluted and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and eluted proteins were detected by Western blot.

3.11.3 Assessment of binding of human complement component C1q to immobilized rTcaCRT

Varying concentrations of rTcaCRT diluted in coating buffer (15 mM of Na_2CO_3, 35 mM of $NaHCO_3$, pH 9.6) were coated onto the wells of a microtitre plate (Corning) 100 μL per well for 6 hours at room temperature (22^oC to 25°C). Control wells received buffer alone or buffer with 3% w/v BSA. After each step, wells were washed 3 times with 0.05% Tween in PBS. Nonspecific binding sites were blocked with 3% w/v BSA in PBS for 1 hour at room temperature. Following blocking, 500 ng of pure human C1q (Sigma-Aldrich) in 100 μL in PBS/1% w/v BSA/0.05% Tween 20 was added to each well and the plate was

incubated at 4°C overnight. To detect bound C1q, affinity-purified rabbit anti-human C1q antiserum (DAKO) 1:1500 dilution was added in 100 µL final volume and the plates incubated at room temperature for 2 hours. Wells were washed with PBS-Tween followed by the addition of 100 µL affinity-purified goat anti-rabbit IgG-peroxidase conjugate (DAKO) 1:3000 dilution. Plate was kept at room temperature for 2 hours followed by washings with PBS-Tween. The bound peroxidase activity was measured by adding OPD (Sigma-Aldrich) as a substrate. The color produced was read at 492 nm in a microplate reader according to manufacturer's instruction.

3.11.4 Assessment of the effects of rTcaCRT on classical complement pathway-mediated haemolysis

To assess the effect of parasite CRT on activation of classical pathway of complement of goldfish, I used sheep red blood cell lysis assay. Briefly, washed sheep erythrocytes (SRBC) re-suspended in 0.9% NaCl were sensitized by adding an equal volume of 1:250 diluted de-complemented rabbit anti-sheep erythrocyte antiserum (Cappel) in PBS containing 1 mM $CaCl_2$. Cells were washed thrice with 0.9% NaCl by centrifugation at 400 x *g* for 10 minutes. A total 10^7 sensitized erythrocytes in 0.9% NaCl containing 1 mM $CaCl_2$ was added to wells of a microtitre plate containing varying concentrations of TcaCRT or BSA mixed with fresh normal rabbit serum (1/100 dilution) in a total assay volume of 200 µL. The plate was incubated at 37°C for 45 minutes and then kept at 4°C for 3 hours. Haemoglobin in the supernatant was measured by adding 50 µL of supernatant to

new wells containing 50 μL 0.9% NaCl and absorbance was measured at 415 nm. Total haemolysis was measured by lysing 10^7 sensitized erythrocytes with water and haemolytic activity was expressed as per cent of the total haemolysis.

3.11.5 Assessment of surface localization of TcaCRT using immunofluorescence and confocal microscopy

To determine whether TcaCRT is expressed on parasite surface, *in vitro* grown parasites were washed twice in serum-free medium and re-suspended to a concentration of 1×10^6/mL. 100 μL of parasites were incubated with 160 μg of rabbit anti-rTcaCRT IgG or rabbit anti-goldfish CSF-1 antibody (isotype control) for 24 hours at 20°C. Parasites were then spun onto poly-L-lysine coated glass slides using a Cytospin 2 at 55 x *g*, for 7 minutes (Shandon). The parasites were fixed to the slides in methanol at −20°C for 30 minutes and permeabilized using 1% Triton X-100 for 30 minutes at room temperature. Following permeabilization, the slides were washed 3 times for 20 minutes in Milli-Q water. The slides were then blocked with 0.5% BSA in PBS for 30 minutes at room temperature. The slides were then washed 3 times for 20 minutes each in Milli-Q water prior to incubation with FITC-conjugated goat anti-rabbit IgG 1:100 dilution (Sigma-Aldrich) for 2 hours at room temperature. To assess the level of TcaCRT expression on parasite surface, cells were treated the same way except permeabilization step was omitted. The cells were subsequently washed 3 times for 20 minutes each in Milli-Q water and observed using DIC imaging with a Leica confocal microscope. Parasite staining was observed using differential

interference contrast (DIC) imaging with a Leica confocal microspcope.

3.12 Proteomic analysis of *T. carassii* surface proteins

3.12.1 Two-dimensional gel electrophoresis (2-DE) of *T. carassii* surface protein fraction.

Triton X-114 was used to solubilize parasite membrane proteins in the presence of protease inhibitors and the solubilized proteins were analyzed by 2-DE. Protein concentration was determined by Micro BCA protein assay (Pierce) and samples were precipitated with acetone. Five hundred μg of proteins from *in vitro* cultured parasites were rehydrated overnight on 24 cm immobiline dry strips at pH 4-10 and separated by two-dimensional gel electrophoresis. In first dimension, samples were run on 24 cm, pH 4-10, Immobiline Drystrips (Amersham) on an IPGphor II IEF system (Amersham). Equilibration of strips were done in equilibration buffer (75 mM Tris-HCl pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) containing 100 mg/mL DTT for 15minutes and then in equilibration buffer containing 250 mg/mL iodoacetamide for 15 minutes. Strips were sealed to 12% acrylamide gels using 0.5% agarose in standard Tris-glycine electrophoresis buffer. Second dimension was run using Ettan DALT electrophoresis unit (Amersham) at 40 mA/gel and 16°C until tracking dye reached the end of the gel. Proteins were visualised by Sypro® Ruby fluorescence (Invitrogen). Gels were fixed overnight in 50% methanol, 7% acetic acid and stained overnight. Gels were destained in 10% methanol, 7% acetic acid for 30 minutes and stored in 7.5% acetic acid prior to imaging. Imaging was done with FLA-5000 laser scanner (Fujifilm) using 473 nm excitation and 450 nm

emission filters. Protein spots were detected using ImageMaster 2D platinum software. Spots were detected based on the following parameters: minimum area of 6, smoothness of 4 and saliency of 2500. Gel plugs containing the proteins were excised using Ettan spot picker (Amersham) and identified by mass spectrometry.

3.12.2 Mass spectrometry analysis

Excreted/secreted antigens and membrane proteins were separated by SDS-PAGE and 2-DE, respectively. Protein band excised from SDS gel were analyzed at the Institute for Biomolecular Design, University of Alberta. Protein spots from 2-D gels were analyzed at the Department of Chemistry, University of Alberta. The visualized bands or spots excised from gels were destained, reduced with DTT (Roche), alkylated with iodoacetamide (Sigma) and digested with trypsin (Promega Sequencing Grade Modified) overnight at 37°C. The extracted peptides were analyzed via LC-MS/MS on a nanoAcquity UPLC (Waters, MA) coupled with Q-ToF-Premier mass spectrometer (Micromass, UK/Waters, MA). Trypsin digested peptide fragments were separated using a linear water/acetonitrile gradient (0.1% Formic acid) on a nanoAcquity column (3 micron Atlantis dC18, 100 A pore size, 75 µm ID x 15 cm) (Waters, MA) as a loading /desalting column. Protein identification using the generated MS/MS spectra was performed by searching the NCBI non-redundant database using MASCOT or PEAKS studio 4.5 with consideration for carbamidomethylation of cysteine and the oxidation of methionine.

Analysis of LC-MS/MS was done with Mascot (Matrix science Ltd, London, UK) or PEAKS studio 4.5 software by comparing with the data in the National Centre for Biotechnology (NCBI) non-redundant database. Parameters used for analysis of MS results for both ES and membrane proteins included probabilistic score-based confidence interval, appearance of kinetoplastid proteins as top candidate, number of peptides matched and extent of sequence coverage. List of proteins identified in this study are shown in Table 3.5 for *T. carassii* ES fraction and Table 3.6 for surface proteins

3.13 Identification of *T. carassii* genes up-regulated *in vivo* using suppression subtraction hybridisation (SSH).

To identify differentially expressed transcripts between mRNAs isolated from *in vivo* and *in vitro* grown parasites, suppression subtraction hybridisation strategy was employed. This allows comparison of two mRNA populations to identify transcripts that are differentially expressed between two RNA samples. SSH was performed using cDNAs synthesised from *in vivo* cultured parasite as tester and *in vitro* cultured parasite as driver. For *in vivo* RNA, goldfish were infected with approximately $6.25x10^6$ parasites and allowed to live for 21 days to achieve peak parasitemia. Fish were checked for parasitemia a week after infection. Fish were exsanguinated and parasites were immediately purified from blood. In all cases, excessive washing of parasites was avoided to prevent possibility of mRNA degradation. Total RNA was extracted using TRIzol reagent (Invitrogen) and RNA samples stored at -80°C. Total RNA was also isolated from

in vitro cultured parasites grown for 5 days to exclude possibility of bloodstream parasite differentiating into stumpy form. mRNAs were isolated from total RNAs using μMACS mRNA Isolation Kit (Miltenyi Biotec). cDNA synthesis and SSH were performed with 2 μg of mRNAs using BD PCR-Select cDNA Subtraction kit (clonetech). Differentially expressed cDNAs were directly cloned into TOPO TA vector and used to transform Top10 Competent cells. Transformants were selected on Luria bertani (LB) medium containing ampicillin (100 μg/mL). Xgal and IPTG were added to LB plates to allow selection of white colonies, which are transformants containing inserts. About 180 colonies were selected and cultured in 200 μL of LB broth containing ampicillin in a 96 well culture plates. Cultures were used directly as templates in PCR reactions to further screen for transformants containing inserts. Clones containing inserts from gel electrophoresis of PCR products were used to inoculate 2 mL LB broth containing ampicillin. Plasmids were purified (Qiagen) and sequenced directly using vector-specific primers. Sequences were compared to databases at GeneDB or NCBI to identify the gene fragments. List of genes identified in the SSH study is shown in Table 3.7.

3.13.1 *In silico* analysis of differentially expressed gene fragments identified in suppression subtraction hybridisation

Gene fragments obtained by sequencing were used to identify the respective genes. A 50% identity cut off was used for gene identification. In some cases, protein sequences of gene fragments were used in database searches. Where

sequence fragments show no identity to databases sequences, domains and signatures present in the fragments were identified and used for gene identification. In most cases, gene fragments show identity to more than one parasite genes suggesting that the differentially expressed transcript could be any of those genes. Of the 120 sequenced clones, about 20 clones show no significant homology to any database sequence suggesting they could be *T. carassii* specific genes. About 40 differentially expressed transcripts show identity to uncharacterised parasites genes and are excluded from the list. Some identified genes were found in more than one clone. Since the cDNAs were digested with Rsa1 during SSH procedure, genes such as surface antigen, mucins, histones, and hsp40 which were identified in many clones could be different fragments of the same *T. carassii* gene. This indicates the protein product of these genes may be important for parasite immune escape strategies. The result of *in silico* analysis is hampered by low sequence homology between the identified sequences and the database sequences. This made gene identification difficult.

3.14 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's test. Probability level of $P < 0.05$ was considered significant. In all figures, error bars represent standard errors calculated from standard deviation and sample size. For gene expression analysis, four individual fish ($n = 4$) were used because less variability in gene expression was observed in previous studies conducted in our laboratory. For functional assays, between four

to seven individual fish were used since these assays are subject to much higher levels of variability as shown by previous studies in our laboratory.

Table 3.1

Components of *Trypanosoma carassii* TDL-15 Medium[a]

Reagents/Solutions	Amount/L	Specifications
Milli-Q water	300 mL	Endotoxin-free
Hank's Solution	40 mL	10X, no Ca^{2+} or Mg^{2+}
MEM amino acid solution[b]	12.5 mL	50X
MEM non-essential amino acid solution[b]	12.5 mL	100X
$NaHCO_3$	1.26 g	
NaOH	30 mL	1 N
Sodium pyruvate solution[b]	12.5 mL	10 mM
MEM vitamin solution[b]	10 mL	100X
Nucleic Acid Precursor Solution[c]	10 mL	2.5 mM
L-glutamine Solution	10 mL	200 mM
Gentamicin solution[b]	1 mL	50 mg/mL
2- Mercaptoethanol solution	1 mL	50 mM (fresh)
HEPES	2 g	
Insulin	0.005 g	
GFL-15 Medium[d]	500 mL	

[a] Developed by Wang and Belosevic [16]

[b] Supplied by Gibco/Invitrogen

[c] 2.5 mM each of : adenosine, cytidine, hypoxanthine, thymidine, uridine.

[d] 50% Leibovitz-15 and 50% Dulbecco's Modified Eagles Medium

Table 3.2

Components of MGFL-15 Medium

Reagents/Solutions	Amount/2L
Hepes	7 g
KH_2PO_4	0.688 g
K_2HPO_4	0.570 g
NaOH	0.750 g
$NaHCO_3$	0.340 g
L-glutamine	0.5844 g
Insulin	0.01 g
10X Hank's balanced salt solution	80 mL
MEM amino acid solution[a]	25 mL
MEM non-essential amino acid solution[a]	25 mL
Sodium pyruvate solution[a]	25 mL
MEM vitamin solution[a]	20 mL
Nucleic Acid Precursor Solution[b]	20 mL
2- Mercaptoethanol solution	7 µL
GFL-15 Medium[c]	1 L

[a] Supplied by Gibco/Invitrogen

[b] 0.067 g adenosine, 0.061 g cytidine, 0.031 g hypoxanthine, 0.061 g thymidine, 0.061 g uridine in 100 mL.

[c] GFL-15 medium: 1 package of dry powder each of Leibovitz-15 and Dulbecco's Modified Eagles Medium in 2 L.

Table 3.3

Primes used for molecular cloning of *Trypanosoma carassii* genes

Primers	**Sequences (3'- 5')**
Primers for amplification of Tcahsp70 fragments:	
Hsp70 forward	GATACAGTTTCTGTACTATATTG (Spliced leader)
Hsp70 reverse	TGCCGCCCACCAGCACCACATCAT (MS peptide)
Hsp70 3' RACE	GCTGACGATCGATGGGCGGCATCTTCGAG
Primers for cloning Tcahsp70 for expression in prokaryotic system	
SUMOHsp70 forward	ATGGCCTACGAAGGTGCGATTG
SUMOHsp70 reverse	TCAGTCAACTTCCTCCACCTTC
Vector-specific primers:	
M13 forward	GTA AAA CGA CGG CCA G
M13 reverse	ACA GCT ATG ACC ATG ATT AC
SUMO forward	AGA TTC TTG TAC GAC GGT ATT AG
T7 reverse	TAG TTA TTG CTC AGC GGT GG
Primers for amplification of TcaCRT gene fragments:	
CRT forward	(same as hsp70 forward)
CRT Reverse	TCGGGTTCGGAATCATCGGCGCTTC (MS peptides)
Primers for 3' RACE	GACTGGCCGATTGTTCAGCCCAAGG
Primers for cloning TcaCRT for expression in prokaryotic system:	
SUMOCRTforward	ATGCGTGCATCACTTCTCCTTATTG
SUMOCRTreverse	TTAGAGATCACCCTCCTCTTCCGCA
Primers for amplification of Tcagp63 fragments:	
Gp63 forward	(same as hsp70 forward)
3' RACE primer	CACCGATGACGAGGGAAAGGGCCCAACAGC

Primers for cloning the full coding sequence of Tcagp63 for expression:

Gp63 forward	ATGCGCTCTACATTCTCTAC
Gp63 reverse	TCAAAGAAAAACAAAGAAAACAATG

Primers for cloning Tcagp63 fragment lacking signal peptide (SP)

Gp63 forward	GCGCCCATTCGAATCAAGGTATTC
Gp63 reverse	TCAAAGAAAAACAAAGAAAACAATG

Primers for cloning Tcagp63 fragment lacking (SP) and zinc binding domain:

Gp63 forward	ATGACGAGGGAAAGGGCCCAACAG
Gp63 reverse	TCAAAGAAAAACAAAGAAAACAATG

Table 3.4

Quantitative real time primer sequences

Primer	Sequence (5'-3')
CCL-1 forward	AAG GTC ACC GAA CCC ATC AG
CCL-1 reverse	TCG TCA CAT GAT GGC CTT CA
CXCL-8 forward	CTG AGA GTC GAC GCA TTG GAA
CXCL-8 reverse	TGG TGT CTT TAC AGT GTG AGT TTG G
EF-1α forward	CCG TTG AGA TGC ACC ATG AGT
EF-1α reverse	TTG ACA GAC ACG TTC TTC ACG TT
gp91 phox forward	CCC ATC ACC TGT TCA TCG TCT T
gp91phox reverse	TAG TCT GGC CTC GCA CGA TA
IFNγ forward	GAA ACC CTA TGG GCG ATC AA
IFNγ reverse	GTA GAC ACG CTT CAG CTC AAA CA
IL-1β-1 forward	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 reverse	GTG ACA CAT TAA GCG GCT TCA C
IL-1β-2 forward	GAT GCG CTG CTC AGC TTC T
IL-1β-2 reverse	AGT GGG TGC TAC ATT AAC CAT ACG
IL-10 forward	CAAGGAGCTCCGTTCTGCAT
IL-10 reverse	TCGAGTAATGGTGCCAAGTCATCA
IL-12-p35 forward	TGT TTT ACG TGC ATT CCT TTG G
IL-12-p35 reverse	GGC GCC TGA AAA AAA TAC GA
IL-12-p40 forward	CTT CAG AAG CAG CTT TGT TGT TG
IL-12-p40 reverse	CAG TTT TTG AGA GCT CAC CGA TAT C
iNOS isoform A forward	TTG GTA CAT GGG CAC TGA GAT T
iNOS isoform A reverse	CCA ACC CGC TCA AGA ACA TT
iNOS isoform B forward	CAT CTT CCA TCC GAC CCT AGT G
iNOS isoform B reverse	AAA GCT ACG GAA GGG AGC AAT
p22 phox forward	TGG ACC CCT GAC CAG AAA CT
p22 phox reverse	AAC ATG AAC CCC CCT GGA A

p40 phox forward	TCC AAG AGC GGG AAT CAT G
p40 phox reverse	GTC GAT GCC CTC TGG CTG TA
p47 phox forward	CCA GGA ATG GGA CAC GAT CT
p47 phox reverse	GAG GAG AGC CTG AGT TTG CAA
p67 phox forward	TGC CTG GCA ACA TTG TCT TC
p67 phox reverse	CCC GCT TCT CAT TGA AAA CAA
TGFβ forward	GTA CAC TAC GGC GGA GGA TTG
TGFβ reverse	CGC TTC GAT TCG CTT TCT CT
TNFα-1 forward	CAT TCC TAC GGA TGG CAT TTA CTT
TNFα-1 reverse	CCT CAG GAA TGT CAG TCT TGC AT
TNFα-2 forward	TCA TTC CTT ACG ACG GCA TTT
TNFα-2 reverse	CAG TCA CGT CAG CCT TGC AG

Table 3.5

***Trypanosoma carassii* antigens identified in ES products by MS.**

Molecular Weight (KDa)	Protein Identity
36	Activated Kinase C receptor
52	Elongation factor 1α
55	Alpha and beta tubulin
55	Caleticulin
72	Hsp70
81	Hsp 83
95	Elongation factor
100	Pyruvate dikinase

Table 3.6

***Trypanosoma carassii* proteins identified in surface protein fraction by MS.**

Spot Number	Molecular Weight (KDa)	Protein Identity
1.	55	Tubulin alpha
2.	55	Tubulin alpha
3.	55	Tubulin beta
4.	58	Hsp60
5.	60	Hsp60
6.	78	Glucose regulated protein 78
7.	70	Hsp 70
8.	70	Hsp 70
9.	50	Calreticulin
10.	45	Hsp70
11.	45	Hsp 70
12.	40	Elongation factor 2
13.	36	Tubulin beta chain
14.	50	Enolase
15.	20	Tryparedoxin peroxidase
16.	54	Enolase
17.	17	Cyclophilin A
18.	40	MDR Efflux
19.	50	Enolase
20.	50	Actin
21.	40	Elongation factor 2
22.	40	Elongation factor 2
23.	36	Fructose-bisphosphate aldolase
24.	36	Enolase

Table 3.7

Differentially expressed *Trypanosoma carassii* genes identified by SSH.

Sequences	Possible Genes
1.	Surface antigen, Cysteine peptidase
2.	Histone H1, Cysteine peptidase, Integral membrane protein
3.	Surface antigen protein 2, Transialidase
4.	DnaJ protein i.e HSP40
5.	Mucin associated surface protein
6.	Cytochrome C oxidase Cox1 homologue
7.	Mucin associated surface protein, Interspersed repeat antigen
8.	Cysteine protease, Serine/threonine protein kinase
9.	Peptidase
10.	Mucin associated surface protein, Interspersed repeat antigen,
11.	Histone
12.	Surface antigen, Mucin TcSMUGS,
13.	Surface antigen, Mucin TcMUC1
14.	Ribosomal protein L15

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CHAPTER 4

THE COURSE OF INFECTION AND EXPRESSION ANALYSIS OF INFLAMMATORY AND ANTIMICROBIAL GENES IN THE GOLDFISH (*Carassius auratus* L.) INFECTED WITH *Trypanosoma carassii*[1].

4.0 Introduction

T. carasssii can naturally infect a number of fresh water fish including common carp (*Cyprinus carpio*), tench (*Tinca tinca*) and eel (*Anguilla* species) [23]. Experimentally, the parasite was shown to be infective to other fish species such as tin foil barb (*Barbus schwanenfeldi*), brown bullhead (*Ictalurus nebulosus*), and goldfish (*Carassius auratus*) [22, 60]. *T. carassii* is transmitted by blood sucking leeches of the genera *Hemiclepis* and *Pisciola* [47, 60]. In experimental studies, parasites isolated from fish can be used to establish infection in a new host. Also, parasite cultured *in vitro* can be used to establish new infection [9, 57, 60].

T. carassii is an extracellular protozoan parasite and morbidity and mortality caused by this parasite can be significant especially in aquaculture setting, where prevalence of infection can approach 100% [21, 40]. Goldfish are highly susceptible to trypanosome infection and while 60–80% mortality has been

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documented and fish that survive the infection become immune to homologous challenge [59, 60]. The nature of the protective immune responses induced by *T. carassii*, as well as parasite molecules that drive such responses, are yet to be fully elucidated. In addition, the host factors that contribute to induction of protective immunity and those that govern outcome of infection remain to be fully elucidated.

The role of cytokines in the control of trypanosome infections has been an area of intense research. For example, *T. brucei brucei* produces a trypanosome lymphocyte triggering factor (TLTF), which triggers $CD8^+$ cells to produce IFN-γ and TGF-β [5, 6, 39]. The trypanocidal effect of TNF-α has been reported [24], while IL-4 has been shown to promote antibody production important during the elimination phase of the infection [7]. IFN-γ produced by NK cells during *T. cruzi* infection activates phagocytic cells to destroy internalised parasites through production of reactive nitrogen intermediates [3, 11, 15]. Recently, it was reported that *T. carassii* infection of carp caused an induction of IFN-γ2 and IL-23, suggesting a role for a Th17-type immune response [49].

Parasites modulate host cytokine network to enhance their survival in the host. It is well documented that parasites or parasite-derived molecules are capable of inducing inflammatory response [20, 27, 38, 42, 49, 54]. Several studies using parasite model systems have shown an association between disease progression and inflammatory responses. For example, a decreased Th2 cytokine production and parallel increase in the Th1 cytokine levels were shown to correlate with resistance to *Leishmania* spp. infections [8, 34, 48, 53]. In

Schistosoma masoni infected mice, a down-regulation of Th1 cytokine production is accompanied by induction of Th2 response [43]. Mice infected with *T. cruzi* have decreased ability to produce interleukin-2 (IL-2) and exhibit reduction in the expression of IL-2 receptor [36, 50]. In addition, *T. cruzi* infected mice were shown to produce higher amounts of the pro-inflammatory cytokine IFN-γ during the second week of infection [36, 55]. IFN-γ is one of the central cytokines that promotes antimicrobial effector responses of macrophages resulting in the inhibition of parasite replication [15]. Similar results have been reported for IL-10 knockout mice infected with *T. cruzi*. These mice exhibit lower parasitemia in blood and tissues and higher levels of IFN-γ and nitric oxide production by spleen cells compared to wild type mice [1].

During the early stages of infection of mice with African trypanosomes, higher levels of pro-inflammatory cytokines IFN-γ and TNF-α have been reported [12, 56]. Similarly, the infection of vervet monkeys with *T. brucei rhodesiense* induced higher levels of IFN-γ, TNF-α and soluble TNF receptor 1 in serum but not in the cerebrospinal fluid of the animals [28]. It has also been postulated that in Aftican trypanosomiasis, the survival of the host was dependent on a shift in the production of pro-inflammatory cytokines (IFNγ and TNFα), to the production of anti-inflammatory cytokines (TGFβ and IL-10) [37].

The expression profiles of cytokines induced in the host during the acute and elimination phases of infection of goldfish with *T. carassii* and their roles in host defense have not been fully elucidated. My working hypothesis was that *T. carassii* induce Th1 type immune response to evade Th2 type response, which is

required for their elimination, or mixed Th1/Th2 responses to suppress specific or general immune response. In this study, I examined the expression patterns of a panel of pro-inflammatory genes encoding IFN-γ, TNFα1 and TNFα2, IL-1β-1 and IL-1β-2, IL-12-p35 and IL-12-p40, CCL1, CXCL8, anti-inflammatory genes encoding IL-10 and TGFβ and antimicrobial genes encoding iNOS A and iNOS B and those encoding the NADPH oxidase components.

4. 1 Experimental Design

4.1.1 Assessment of *T. carassii* growth *in vitro*

Trypanosomes were cultured in 25 cm^2 tissue culture flasks. Briefly, $1x10^5$ trypanosomes/mL of medium were added to 25 cm^2 tissue culture flasks, $20^oC \pm 2^oC$. The number of parasites in each culture (n = 4) was determined every two days for 8 days by performing hemocytometer counts on diluted samples. To determine the generation time (g) of the parasites, the following formula was used:

$$g = t_2\text{-}t_1/\log e^{n2/n1}$$

Where t_1 = time of inoculation; t_2 = end time point; n1 = number of parasites in the inoculum; n2 = number of parasites at the end point [14].

4.1.2 Course of infection in goldfish infected with *T. carassii*

Before experimental infections of fish with *T. carassii*, blood sample was taken from each fish and examined for the possible presence of natural hemoflagellate infections. The goldfish used in all studies in this thesis, were not

naturally infected with hemoflagellates. The cultured trypanosomes ($6.25x10^6$/fish) were used to inoculate individual fish intraperitoneally (n = 10). We have previously shown that all fish inoculated with this dose of parasites became infected. Non-infected control fish received an equal volume of serum-free culture medium. On days 3, 7, 14, 21, 28, and 56 pi, blood samples were collected and number of parasites/mL of blood was determined using a hemocytometer or hematocrit centrifugation technique, when parasites numbers were below detection limit of the hemocytometer [58]. In our system, the detection limit of hemocytometer is 10^3 parasites.

4.1.3 Assessment of the effects of *T. carassii* infection on expression of pro-inflammatory and antimicrobial genes of goldfish.

Spleen, liver and kidney tissues were removed from infected (n = 4) and non-infected (n = 4) fish on days 7, 14, 21, 28 and 56 pi, and were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. Total RNA was extracted from goldfish tissues and RNA from the different tissues was reverse transcribed into cDNA. The purity of RNA was confirmed by calculating the ratio of the absorbance at 260 nm and 280 nm. Also, equal amounts of RNA and cDNA were used. Relative expression was determined in relation to elongation factor-1 alpha (EF-1α). All expression data were normalized using the baseline expression in non-infected control fish for each gene. Results were reported as fold change in expression relative to non-infected control for each gene.

4. 2 Results

4.2.1 The course of *T. carassii* infection in the goldfish

Image of *in vitro* grown parasite is shown in Fig 4.1. All fish infected with *in vitro* cultured *T. carassii* and their blood was positive for parasites on day 3 pi. In the early stages of infection, I observed a continuous rise in parasitemia from day 3 (pi) with peak parasitemia between days 14 and 21 pi (Table. 4. 1). After day 21 pi, the mean number of parasites gradually declined (days 28 to 56 pi) (Table. 4.1) and all fish remained infected at day 56 pi. The first phase of infection (days 3-21) is subsequently referred to as the acute phase while the second phase (days 28-56) is referred to as elimination phase. No mortality was observed in both non-infected control fish and infected fish.

Assessment of parasite growth *in vitro* showed that parasite proliferated in TDL-15 medium supplemented with 10% heat-inactivated goldfish serum (Fig. 4.2). The generation time of parasites was found to be 47.1 hours based on the above formula and the average number of parasites from the cultures on day 8. This represents a greater than 8-fold increase in parasite concentration over the course of growth (Fig. 4.2).

4.2.2 *T. carassii* induces differential cytokine gene expression during the acute and elimination phases of the infection in the goldfish

To understand the immune response elicited during *T. carassii* infection of goldfish, I measured the expression of genes encoding pro-inflammatory cytokines CCL1, CXCL8, IFN-γ, TNFα1 and TNFα2, IL-1β-1 and IL-1β-2, IL-

12-p35 and IL-12-p40, and anti-inflammatory cytokines TGFβ, and IL-10 using quantitative PCR. I compared immune gene expression in the kidney, spleen and liver in infected and non-infected goldfish. All of the fish that were experimentally infected with *T. carassii* had parasites in their blood. In the kidney, I observed significant up-regulation of IL-1β-2, IFN-γ and IL-10 by day 7 pi (Fig. 4.3). By day 14 pi, IL-1β-2 mRNA level returned to normal while IFN-γ and IL-10 mRNA levels were significantly higher in infected compared to non-infected fish (Fig. 4.3). With the exception of IL-1β-2 and CXCL8 (day 28 pi), all cytokines were significantly up-regulated by day 21 and 28 pi (Fig. 4.3). All cytokines were either down-regulated or returned to normal levels by day 56 pi (Fig. 4.3).

In the spleen, I observed up-regulation of IFN-γ, IL-1β-1 and IL-10 during the acute phase of infection, days 7 and 14 pi (Fig. 4.4). There were also significant increases in the mRNA levels of CCL1, IL-12-p35, IL-12-p40 and TNFα2 on day 14 pi. By day 21 pi, cytokine mRNAs returned to normal levels with the exception of IL-10, which was still significantly up-regulated (Fig. 4.4). A significant down-regulation of mRNA levels of majority of cytokine genes was observed on day 28 pi, and by day 56 pi the mRNA levels of all cytokines examined, except for IL-10, were similar in infected and non-infected fish (Fig. 4.4).

In the liver, I observed a significant up-regulation of IL-1β-1 by day 7 pi, IL-1β-1 mRNA level returned to the level observed in non-infected fish by day 14 pi (Fig. 4.5). In addition, the mRNA levels of CCL1, IFN-γ and IL-10 were

significantly higher in infected compared to non-infected fish on day 14 pi. A significant up-regulation of all cytokines was observed (with the exception of IL-1β-1) on day 21 pi, whereas only CXCL8 and IL-10 mRNA levels were significantly higher in infected fish on day 28 pi. The cytokines mRNA levels returned to levels in non-infected fish by day 56 pi with the exception of IL-10 (Fig. 4.5).

4.2.3 Infection of goldfish with *T. carassii* induces delayed expression of iNOS genes

Nitric oxide production regulated by iNOS genes is up-regulated during trypanosome infection in mammals [16]. For this reason, I examined the expression of iNOS genes in *T. carassii* infected and non-infected fish. I observed a delayed (day 14 or 21 pi) but a significant up-regulation of iNOS genes (both isoforms A and B) in the kidney and liver of infected fish (Fig. 4.6). I also observed a lesser but significantly different increase in expression of iNOS genes in the spleen on days 14 and 21 pi, compared to non-infected animals (Fig. 4.6). These results indicate that *T. carassii* infection induced enhanced iNOS gene expression that was delayed during the critical period of establishment of parasites in the host (first week pi).

Finally, I examined the effects of *T. carassii* infection of goldfish on expression of NADPH oxidase gene components. Both phox40 and phox67 showed non-significant increases in expression in the kidney of infected goldfish on day 21 pi (Fig. 4.7)

4.3 Discussion

The course of infection reported in this thesis is consistent with previously observed course of infection in *T. carassii*-infected goldfish [10, 46]. Also, the generation time determined is consistent with previously reported observation for parasite grown under the same condition [14, 46]. The number of parasite in the inoculum has been suggested to be the major determinant of mortality in *T. carassii*-infected fish [57, 59]. Although a high dose of parasites was used in this study, no mortality was observed. The *in vitro* cultured parasites were highly infective since all inoculated fish developed trypanosome infections as early as day 3 pi.

The inability of *in vitro* grown parasites to cause mortality may be due to many factors among which is the source of the infecting parasites since parasites can lose virulence after prolonged *in vitro* cultivation. However, *T. carassii* grown in culture supplemented with 5% carp serum are indistinguishable from parasites isolated from infected fish with respect to morphology, surface structure and reactivity with monoclonal antibody [40]. It is unlikely that *in vitro* cultured parasites have decreased infectivity since the course of infection reported in this thesis is consistent with previous observations [10, 46]. Another reason for decreased virulence of *T. carassii* may be due to the adaptation of the developmental form of the parasite used to infect fish. For example, African trypanosomes have different developmental forms adapted to live in different hosts [29, 30]. Different developmental forms of *T. carassii* have also been observed [47], but it is not clear how these different forms are adapted to live in

fish and leech hosts. There is evidence that *in vitro* grown bloodstream forms of *T. carassii* decrease from 80% to 60%, after one week of cultivation as shown by morphometric studies [10]. These observations suggest that a lower number of virulent parasites may be present in the inoculums since I used parasites obtained from 5-7 day-old cultures to infect fish.

The ability to grow *T. carassii in vitro* and the ability of these parasites to cause an infection in the goldfish provides an excellent experimental model system to study the immune evasion mechanism in this host-parasite association.

Cytokines produced by exposure to parasites or parasite-derived molecules determine whether a Th1, Th2 or Th17 type response is elicited. The levels of IL-12 or IL-4 early during infection determine whether Th1 or Th2 type immune response predominates, while Th17 type response is regulated by the production of IL-23 [14, 33, 35, 44]. The elaboration of Th1 and Th2 T-cell subsets, eventually results in efficient host defence against intracellular and extracellular parasites, respectively. Since *T. carassii* is an extracellular parasite, the induction of IFN-γ (a Th1 cytokine) may cause reduction in Th2-type cytokine production and affect host's humoral immune responses, an immune evasion mechanism similar to that shown for *S. mansoni* [43]. It is well established that anti-parasite antibody responses are essential for *T. carassii* clearance and maintenance of long lasting resistance to re-infection [17, 18, 41, 59].

From the literature, it is clear that exclusively extracellular trypanosomes, such as *T. brucei*, have adapted to exclusively live in the blood of their hosts. The key to that adaptation is that these parasites express on their

surface Variable Surface Glycoproteins (VSGs), and that they are able to evade host immune responses in the blood (primarily anti-parasite antibody responses of the host) by expressing different VSGs on their surface (there are many as 1200 genes that encode VSGs). In contrast, intracellular trypanosomes, such as *T. cruzi*, do not express VSGs on their surface, instead their surface coat is very rich in sialic acid residues. From an evolutionary perspective, although *T. carassii* is considered to be an extracellular parasite at present, it does not express VSGs on its surface (like *T. brucei*), but it does have a surface coat that is very rich in sialic acid residues like *T.cruzi* [2, 41].

I observed that *T. carassii* induced different cytokine profiles in the three tissues of the goldfish during the trypanosome infection. Interestingly, *T. carassii* induced higher expression of both pro-inflammatory and anti-inflammatory cytokines during the acute phase of the infection, while the mRNA levels of these cytokine genes either returned to normal levels or were down-regulated during the elimination phase of infection, with notable exception of the IL-10. In addition, the expression of majority of the cytokine genes appeared to be sustained longer in the kidney of the infected fish (until day 28 pi), compared to the cytokine mRNA levels in the spleen (day 21 pi). An up-regulation of IL-1β observed during the acute phase of infection was expected since IL-1 is an important mediator of inflammatory response and tissue damage [4]. Furthermore, I observed that IFN-γ and IL-10 were significantly up-regulated in the spleen of infected fish by day 7 pi. These results support the observation of previous studies

using mammalian model systems [11, 36, 55] and suggest a role for both IFN-γ and IL-10 in regulation of *T. carassii* infection in goldfish.

I found a delayed (day 14 pi) and significant up-regulation of the antimicrobial genes encoding iNOS isoforms, especially on day 21 pi in all tissues examined. It has been reported that *T. carassii* does not induce significant NO response in the carp [51], while parasite antigen (hsp70) has been shown to induce NO response of goldfish macrophages *in vitro* [38]. *T. carassii* is also susceptible to NO [51], suggesting that a mechanism may exist *in vivo*, to dampen a significant NO response particularly during the establishment of *T. carassii* infection in the goldfish. It has been established that while *T. brucei*-infected mice exhibit pro-inflammatory cytokine responses and NO response [52], the infected humans have only a marginal increase in plasma nitrite levels [26]. Interestingly, humans infected with African trypanosomes, also have significantly elevated levels of Th1 cytokine, IFNγ [26].

The increased expression of pro-inflammatory genes during the course of the infection paralleled the up-regulation of anti-inflammatory cytokine genes, such as IL-10, suggesting Th1/Th2 cross-regulation may be important in the outcome of *T. carassii* infection in the goldfish. The regulatory T-cells (Tregs), which produce IL-10 and TGF-β are induced by bacterial, viral and parasite antigens. The Tregs can suppress protective Th1 responses thereby encouraging parasite survival [13, 19, 25, 31, 32]. In support of this are reports that demonstrated that antigens of *Plasmodium falciparum* [45] and *Onchocerca*

volvulus [13] induce IL-10-producing T cells that caused a down-regulation of T-cell proliferation.

I reported for the first time a comprehensive analysis of expression of genes that encode pro- and anti-inflammatory cytokines during both acute and elimination phases of a protozoan infection in fish. My findings indicate that IFN-γ and IL-10 may play significant role in the progression of host immune responses in fish trypanosomiasis.

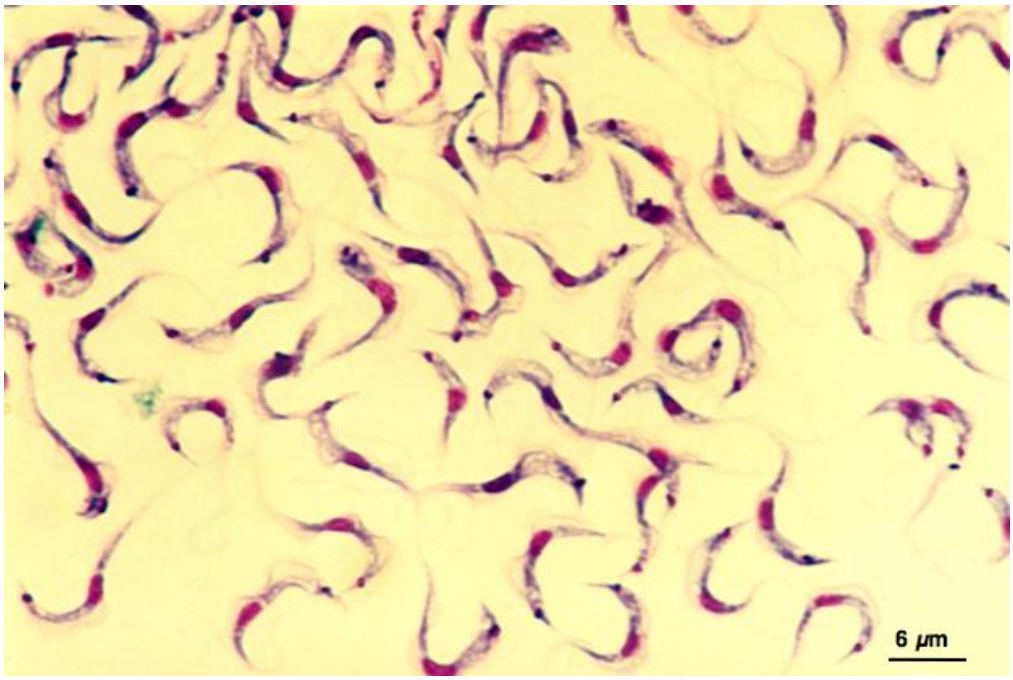

Figure 4.1. Image of *in vitro* cultivated *T. carassii*. Wright's-stained smear of *T. carassii* grown for 6 days in culture medium supplemented with 10% goldfish serum. Adapted from [10].

Days post-infection	Number of Trypanosomes/mL (log_{10}) (Mean ± SEM)
3	4.32 ± 0.82
7	5.18 ± 0.76
14	6.02 ± 1.01
21	5.92 ± 1.17
28	5.07 ± 1.13
56	4.12 ± 0.90

Table 4.1. **Parasitemia in goldfish infected with *T. carassii***. Fish (n = 6) were inoculated with 6.25 x 10^6 *in vitro* grown *T. carassii*. Blood samples were collected from individual fish at the indicated time period and the number of parasites was enumerated. The results are mean number of parasites/mL of blood ± SEM.

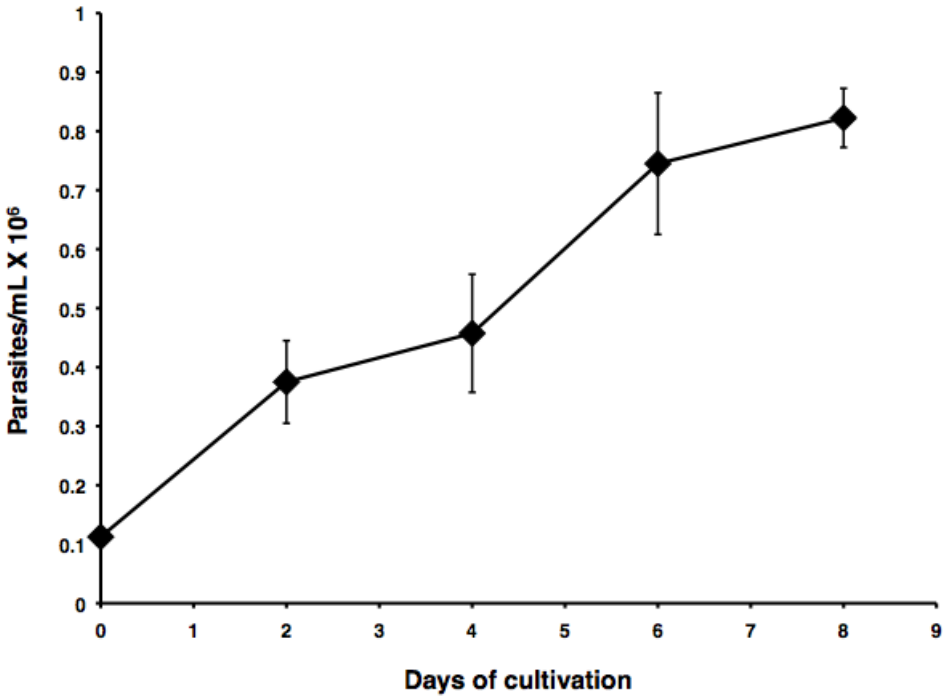

Figure 4.2. Growth of *T. carassii in vitro* in medium supplemented with 10% heat-inactivated goldfish serum. Trypanosomes were inoculated at 1 x 10^5 cells/mL into 25 cm^2 culture flasks (n = 4). Number of parasites was enumerated at the indicated time period. Each dot represents mean number parasites/mL of medium ± SEM.

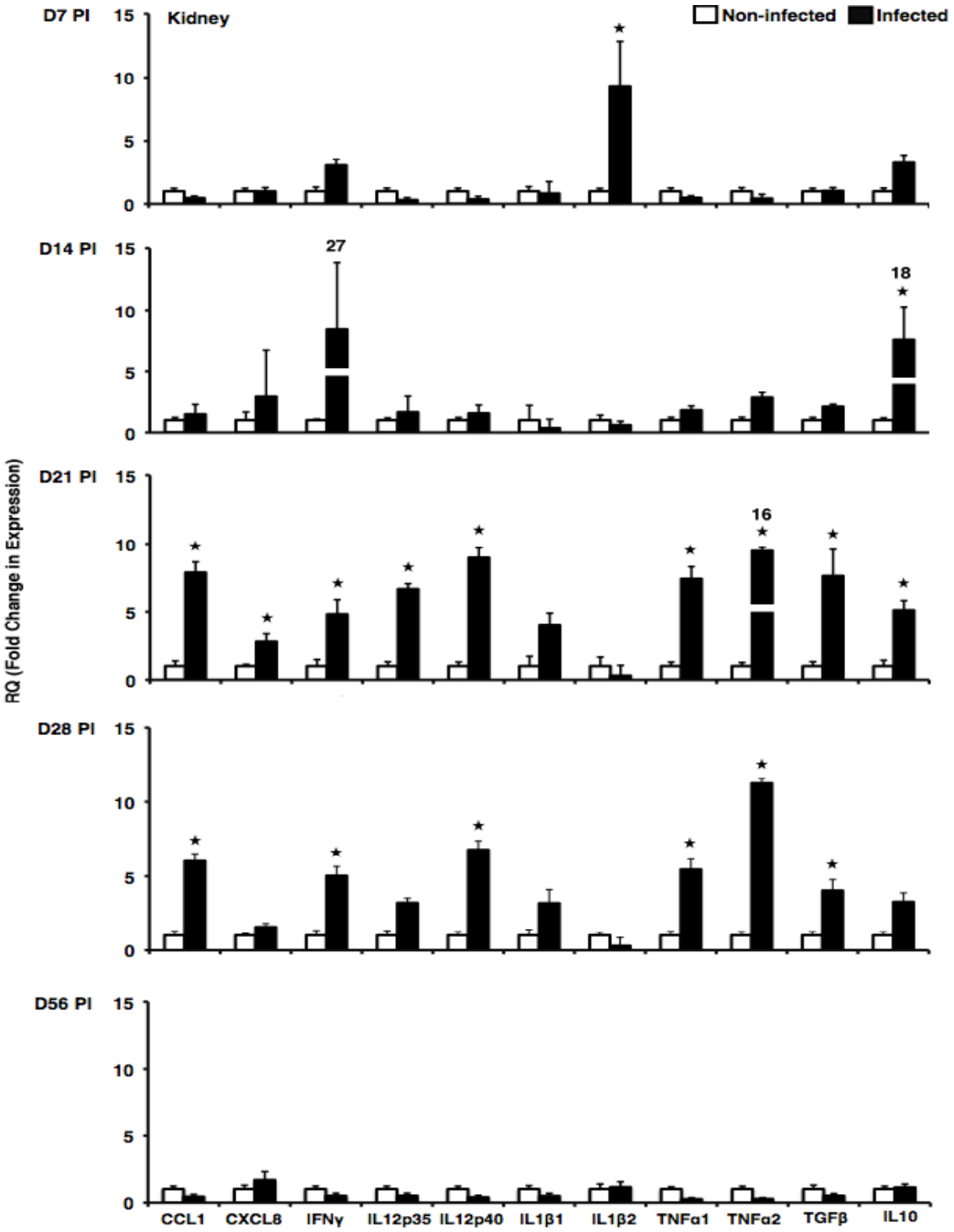

Figure 4.3. **Quantitative expression analysis of goldfish immune genes in the kidney of *T. carassii*-infected and non-infected fish.** The reported expression was relative to EF-1α. The expressions were normalized against those observed in non-infected control fish respectively for each gene. Statistical analysis was performed using ANOVA (n = 4 for each experimental group/observation period).

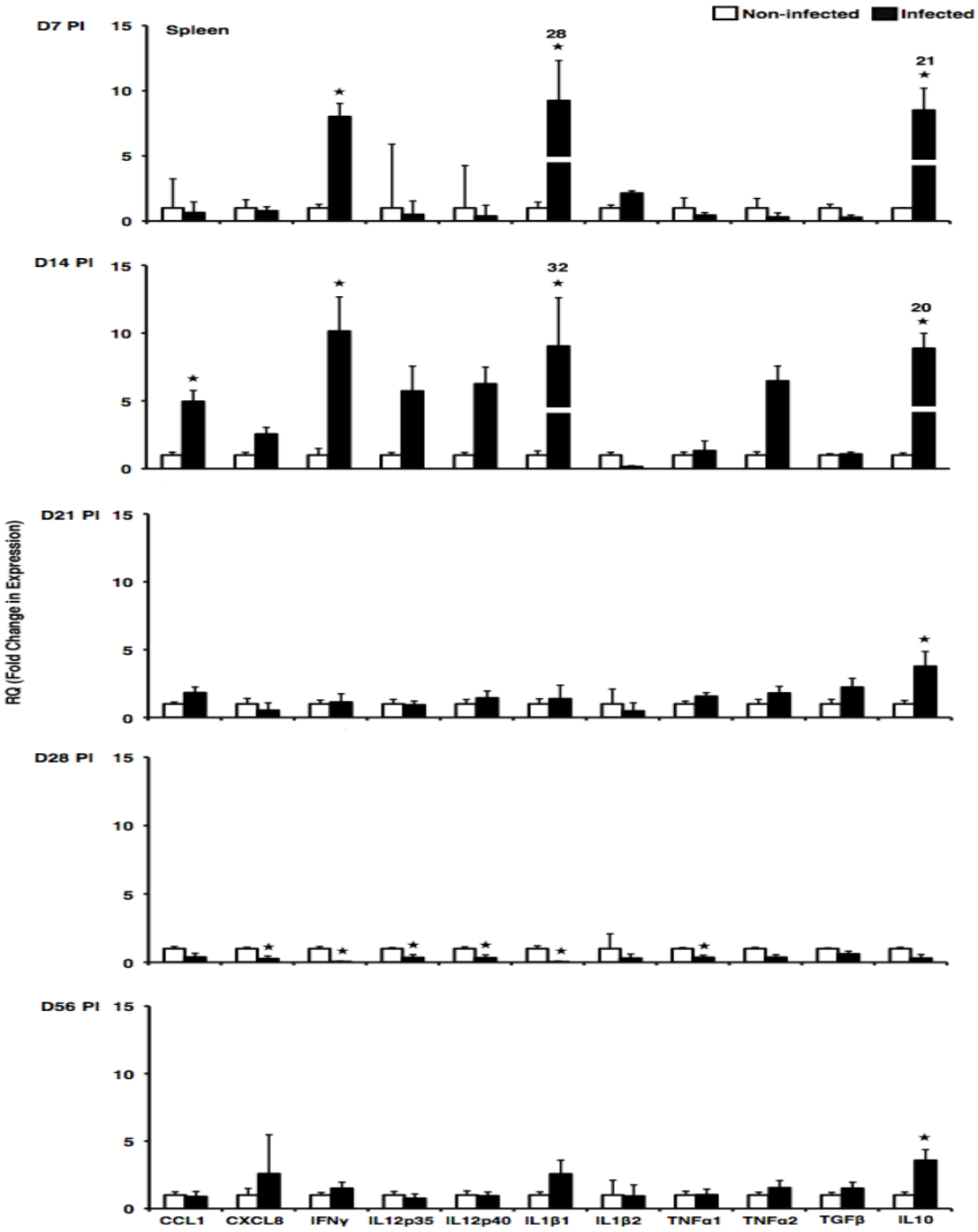

Figure 4.4. **Quantitative expression analysis of goldfish immune genes in the spleen of *T. carassii*-infected and non-infected fish.** The reported expression was relative to EF-1α. The expressions were normalized against those observed in non-infected control fish respectively for each gene. Statistical analysis was performed using ANOVA (n = 4 for each experimental group/observation period).

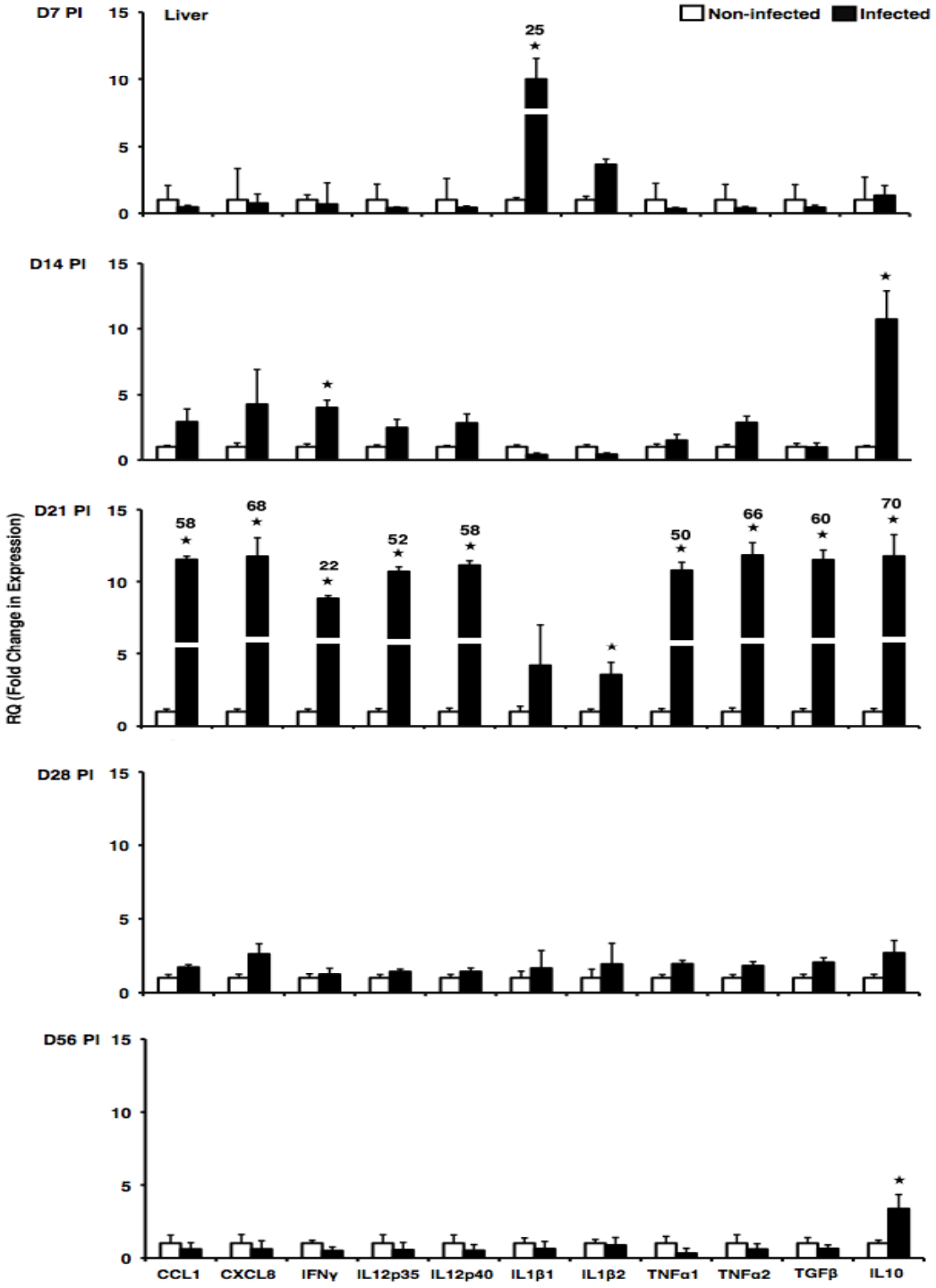

Figure 4.5. Quantitative expression analysis of goldfish immune genes in the liver of *T. carassii*-infected and non-infected fish. The reported expression was relative to EF-1α. The expressions were normalized against those observed in non-infected control fish respectively for each gene. Statistical analysis was performed using ANOVA (n = 4 for each experimental group/observation period).

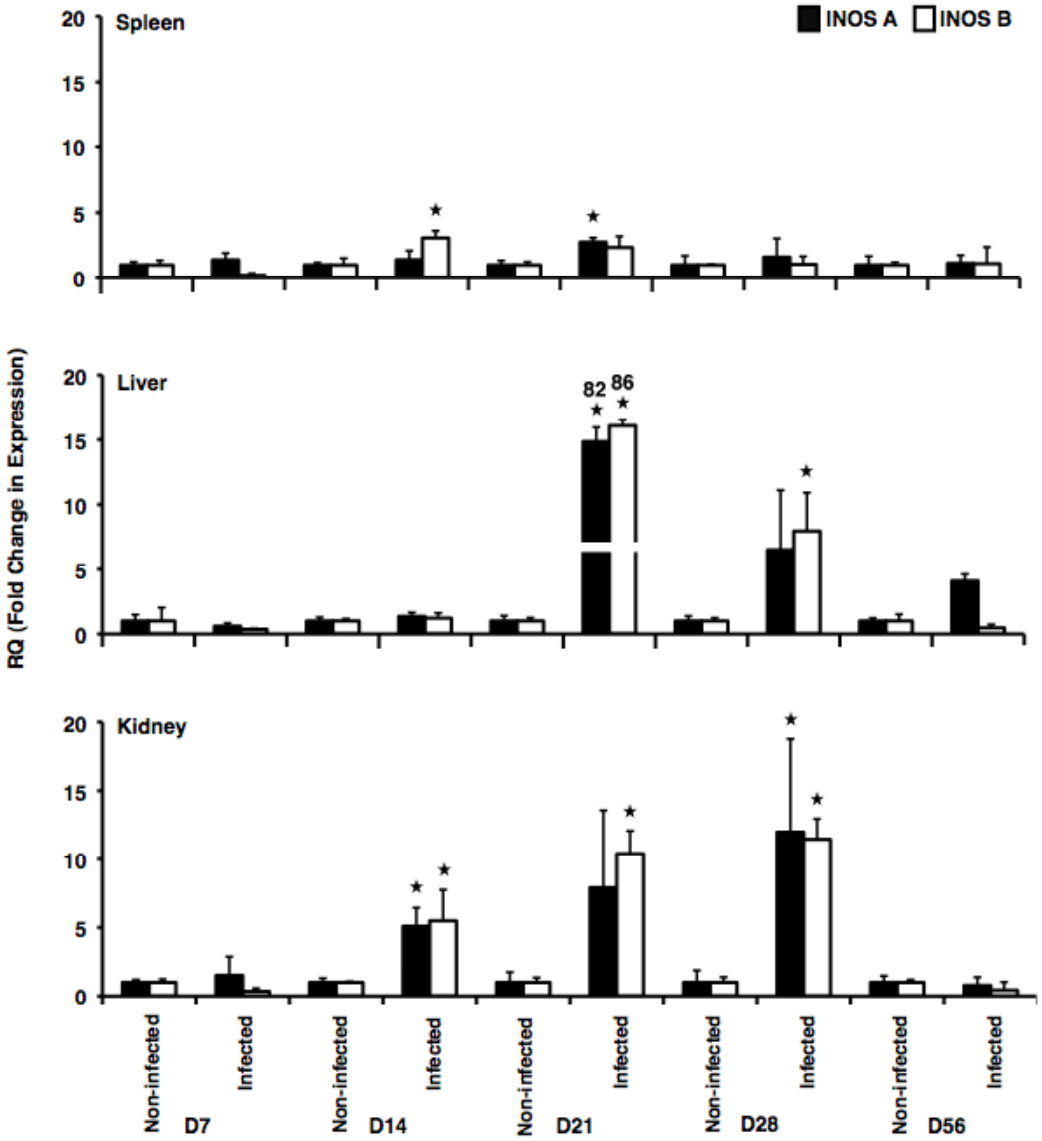

Figure 4.6. Quantitative expression analysis of goldfish iNOS genes in *T. carassii*-infected and non-infected fish. The reported expression was relative to EF-1α. The expression data were normalized against those observed in non-infected control fish respectively for each gene in the different tissues. Statistical analysis was performed using ANOVA (n = 4) for both experimental groups/observation period) and the results were deemed to be significant at $p < 0.05$. (*) denotes significantly different ($p < 0.05$) from the respective controls.

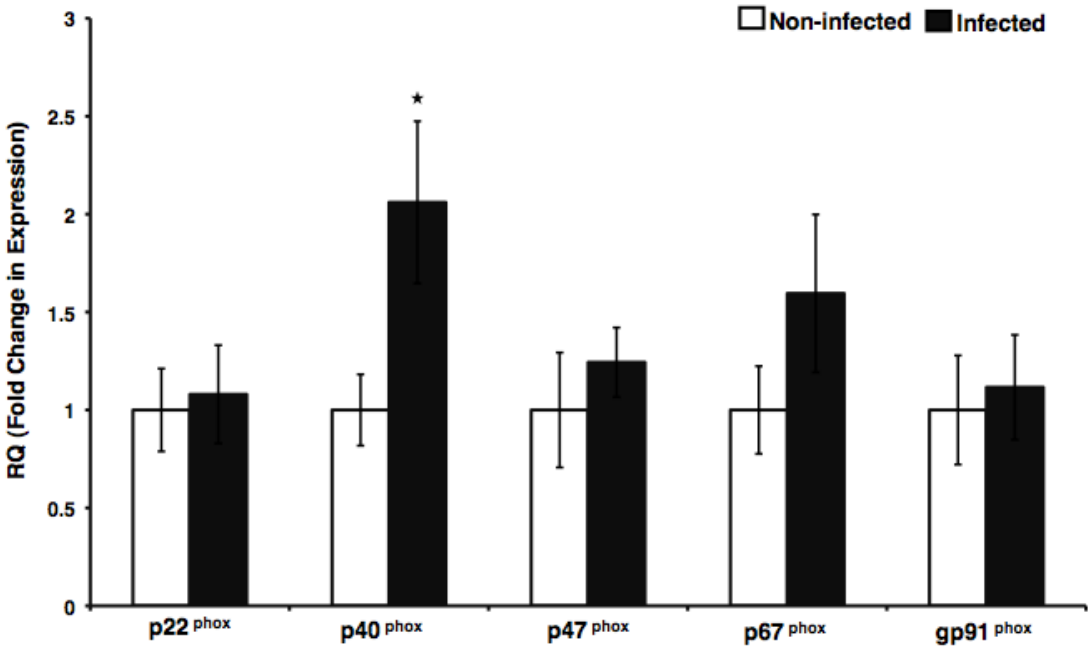

Figure 4.7. Quantitative expression analysis of goldfish NADPH oxidase component genes in kidney of *T. carassii*-infected (day 21 pi) and non-infected fish. The reported expression was relative to EF-1α. The expression data were normalized against those observed in non-infected control fish respectively for each gene in the different tissues. Statistical analysis was performed using ANOVA (n = 4 for both experimental groups/observation period) and the results were deemed to be significant at $p < 0.05$. (*) denotes significantly different ($p < 0.05$) from the respective controls.

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CHAPTER 5

IDENTIFICATION OF HEAT SHOCK PROTEIN 70 (hsp70) AND OTHER ANTIGENS IN EXCRETORY/SECRETORY PRODUCTS AND SURFACE PROTEIN FRACTION FROM *Trypanosoma carassii*[1].

5.0 Introduction

Excreted/secreted (ES) and surface molecules of protozoan parasites are important for establishing infection, protecting parasites from early attack of host immune system, and acting as invasive as well as evasive agents [10, 41, 19]. These molecules can increase parasite survival and help maintain long-lasting infection by interfering with macrophage antimicrobial functions, cytokine production, antigen presentation, and effector cell activation [19, 36]. These effects are achieved by variety of mechanisms including down-regulation of gene expression, protein degradation or post-translational modification, interference with host cell signaling, and activation of suppressive pathways and molecules [11, 17, 19, 36]. Many of the parasite proteins that contribute to immune evasion or modulation have been identified in both ES products and on parasites surface [8, 30, 34, 49].

The heat shock proteins (hsps) are highly conserved molecules and are present in subcellular compartments in eukaryotes and prokaryotes. Under normal

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physiological conditions, hsps are expressed at low levels but during conditions of stress a vast array of physiological, environmental and pathological stimuli can induce significant up-regulation of hsp mRNAs [29].

The heat shock response is a general homeostatic mechanism that protects cells or organisms from damaging effects of stressful stimuli. Hsps have been termed molecular chaperones because they play crucial roles in protein folding and unfolding, protein translocation, assembly and disassembly of protein complexes. However, there is increasing evidence for the involvement of hsps in antigen processing and presentation and in other immunological processes. For example, the formation of multimeric molecules such as T-cell receptors (TCR), immunoglobulins (Ig) and major histocompatibility complex (MHC) requires functional hsps [52].

During infection, pathogens are faced with an array of stressful conditions such as changes in temperature, pH and immune responses of the host. To survive inside the host, pathogens must activate several immune evasion strategies including synthesis of hsps [52]. The importance of hsps during infection has been reported [9]. For example, mutation in *Salmonella typhimurium* HtrA gene, which encodes a chaperone results in increased susceptibility to killing by activated macrophages and produces a non-virulent bacteria *in vivo* [26]. Although a high degree of similarity exists between parasite and host hsps, parasite hsps have been reported to be immunogenic probably because of selective immune responses to the non-homologous regions in the C-terminus of hsps sequences [14, 32, 47]. There is about 73% amino acid sequence similarity between human and

Trypanosoma cruzi hsp70, yet *T. cruzi* hsp70 induces significant antibody response during human infection [14]. Surprisingly, sera from *Leishmania donovani* infected patients recognize *L. donovani* hsp70 and hsp90 but not *T. cruzi* hsps despite more than 80% amino acid identity between these proteins [12]. This suggests that hsps may be exploited for serodiagnostic purposes for mixed infections in endemic countries.

The role of hsps in induction of proliferative responses in immune cells, as well as their immunostimulatory activities, is currently generating interests. Hsps have been reported to directly activate macrophages to secrete cytokines, stimulate proliferation of NK cells and induce maturation of dendritic cells in mammals [3, 16, 28, 33, 40]. These effects are believed to be due to interaction of hsps with specific receptors on target cells [4, 5, 48]. Trypanosomes and related parasites are known to possess all major families of hsps with hsp40, hsp60 and hsp70 family members present in large numbers indicating they play important roles in parasite biology [15]. Unfortunately, the precise role of these parasite hsps in modulation of host immune responses by the parasite is yet to be elucidated. Hsp70 from *Plasmodium falciparum* has been reported to be a major immunogen in protein fraction shown to experimentally protect *Saimiri sciureus* monkeys [6].

The extracellular existence of *T. carassii* predisposes it to direct contact with components of both the cellular and humoral branches of the immune system. Goldfish are highly susceptible to trypanosome infection and fish that survive the infection become immune to homologous challenge [50, 51]. Also,

passive transfer studies have shown that immunity to re-infection can be transferred to naïve goldfish using plasma or purified IgM from a recovered host [27, 31, 37]. Resistance to re-infection was long lasting and was believed to be due to the sequestration of parasites in the internal organs resulting in constant priming of the immune system [25, 50, 51]. Protective antibodies have been induced in naïve goldfish after immunization with parasite excretory/secretory products [7] or parasite beta tubulin [27]. These results provides strong evidence for antibody-mediated control of *T. carassii* infection in goldfish, and molecules in ES products as well as surface protein fractions may be crucial for vaccine development to combat this infection.

In this chapter, I report on identification of an immunogenic hsp70 from *T. carassii*. My working hypothesis was that *T. carassii* hsp70 modulates host immune defense by enhancing cytokine gene expression and antimicrobial responses of goldfish macrophages. *T. carassii* hsp70 was recognized by serum from infected fish among other excreted/secreted (ES) molecules and was present in solubilized membrane protein fraction. Furthermoree, recombinant parasite hsp70 enhanced expression of pro-inflammatory cytokines and induced nitric oxide response of goldfish macrophages.

5.1 Experimental Design

5.1.1 Proteomic analysis of ES products and surface protein fraction.

ES products and membrane protein fractions were prepared from *in vitro* cultured bloodstream parasites. ES proteins separated by SDS-PAGE were

immunoblotted with immune sera collected from infected fish. ES were also separated with SDS-PAGE and stained with Coomasie blue. Proteins that reacted with immune sera were excised from SDS-PAGE gels and identified by MS. *T. carassii* surface protein fractions were separated by two-dimensional electrophoresis (2-DE) and selected proteins were identified by mass spectrometry.

5.1.2 Molecular cloning and expression of *T. carassii* hsp70

Protein band corresponding to 68-70KDa antigen detected on immunoblot of ES product with sera from infected fish was identified by MS as *T. carassii* hsp70. Hsp70 fragment was amplified using primers designed to *T. carassii* spliced leader sequence and peptides identified in MS studies **(**IFDLGGGTFDVTLL and VVLVGGSTRIPKVMQ). The full sequence of Tcahsp70 was amplified, cloned and expressed in prokaryotic system. Purified rTcahsp70 was used to immunize rabbits for production of polyclonal antiserum.

5.1.3 Assessment of effects of recombinant *T. carassii* hsp70 on cytokine gene expression in goldfish macrophages

Macrophage cultures prepared from four individual fish (n = 4) were seeded into at a density of $1x10^6$ cells per well into individual wells of 96-well plates. Cells were treated with medium alone or 5000 ng/mL of *T. carasii* hsp70 (rhsp70) for 3 hours and 6 hours. Total RNA was isolated from cells and reverse transcribed into cDNA. cDNAs were used for Q-PCR and relative expression was

determined in relation to EF-1α. All expression data were normalized using the baseline expression at 0 hour time point.

To show that rTcahsp70 effects were due to cell surface signaling, macrophages pre-treated with either culture medium only or 10 mM pronase for 30 min [3] were washed twice with incomplete medium and incubated with 5000 ng/mL rTcahsp70. Total RNAs isolated at 0, 3 and 6 hours post treatment were used in Q-PCR.

5.1.4 Assessment of effects of recombinant *T. carassii* hsp70 on anti-microbial response of goldfish macrophages

Individual cultures of primary macrophages obtained from kidney of goldfish were seeded into individual wells of 96-well plates at a density of 3×10^5 cells per well. Cells were then treated with culture medium alone (control) or with either heat-killed *Aeromonas salmonicida* or different concentrations of rTcahsp70 (500 ng/mL, 5000 ng/mL or 10000 ng/mL) and incubated at 20°C for 72 h. Nitrite production was determined using the Griess reaction. To ascertain that the results were due to rTcahsp70 effects, *E. coli* bacterial lysates were prepared and subjected to the same purification protocol as rTcahsp70-containing lysates. Volumes of purified *E. coli* lysate equivalent to volumes of rTcahsp70 were used in NO assay were assayed.

5.2 Results

5.2.1 Identification of *T. carassii* antigens in ES products

Previously, it was reported that sera from infected fish recognized several high molecular weight proteins, 102-104, 68-72 and 50-59 KDa present in ES supernatants of *in vitro* grown *T. carassii* [7]. The 50-59 KDa proteins were identified as *T. carassii* alpha and beta tubulins [39]. I repeated these experiments and my analysis of MS results of ES antigens led to identification of the 68-72 KDa antigen as parasite hsp70. *T. carassii* antigens identified in ES products so far are shown in Table 3.5.

5.2.2 Proteomic analysis of *T. carassii* surface protein fraction

I used Triton X-114 to solubilize parasite membrane proteins and analyzed the solubilized proteins by 2-DE. The reproducibility of my sample preparation, IEF, gel electrophoresis and gel staining was tested by running 4 replicate gels with proteins prepared from different batches of parasites cultured on different days. In all cases, approximately 300 protein spots were detected using same detection parameters. This suggests that the majority of the proteins in solubilized membrane fraction could be resolved with a high degree of reproducibility (Fig. 5.1). I selected 30 protein spots for mass spectrometry analysis based on the results presented in Table 3.5 that indicated that goldfish immune serum recognized primarily higher molecular weight proteins (55-97KDa) in immunoblotting of ES products. The full list of protein identified in surface protein fraction is shown in Table 3.6. Proteins such as tubulin, hsps and

enolase were detected in more than one spot. This probably reflects the highly abundant nature of these proteins and their degradation products. Also, several isoforms of these proteins may have been produced. Their abundant nature suggests they may play important roles in parasite interaction with the host.

5.2.3 *T. carassii* hsp70 (Tcahsp70) is immunogenic

The first attempt at identifying *T. carassii* ES proteins came from the observation that sera from infected fish recognized several high molecular weight proteins in ES supernatants of *in vitro* grown *T. carassii* [7]. By using a more concentrated ES preparation, I showed that additional proteins were recognized by immune serum (Table 3.5). Protein band corresponding to the 68-78KDa antigen excised from a Coomassie stained SDS-PAGE gel was identified as parasite hsp70 using mass spectrometry (Fig. 5.2A). Tcahsp70 was cloned and its full sequence was determined. Alignment of Tcahsp70 with hsp70 from other trypanosomatids as well as sequences from mammalian and non-mammalian vertebrates is shown in Fig.5 3. Fig. 5.4 shows the protein structure of *T. carassii* hsp70 and *T. cruzi* hsp70. Recombinant parasite hsp70 was produced in prokaryotic system and purified using the N-terminal His-tag. Figure 5.2C shows detection of rTcahsp70 using anti-His antibody. Interestingly, the *T. carassii* recombinant hsp70 was also recognized by fish immune serum as shown in Figure 5.2D, suggesting proper folding of the recombinant molecule.

5.2.4 *T. carassii* hsp70 is present in surface protein fraction

Parasite hsp70 have been reported in the soluble antigens and in the secretome of *Leishmania donovani* [24, 46]. Because *T. carassii* hsp70 was found in the ES preparations, I examined whether *T. carassii* hsp70 was present in solubilized surface protein fraction of the parasite. Parasite hsp70 were detected in more than one spot (Fig. 5.5) in 2-D gel of solubilized surface proteins suggesting degradation products or different forms of this molecule.

5.2.5 Recombinant parasite hsp70 induces expression of pro-inflammatory genes in goldfish macrophages

The ability of recombinant hsp70 (rhsp70) to directly activate macrophages to secrete cytokines, stimulate proliferation of NK cells and induce maturation of dendritic cells has been reported for mammalian and bacterial hsp70 [3, 16, 40]. For this reason, I examined whether rTcahsp70 can modulate pro-inflammatory gene expression of goldfish macrophages. Macrophage pro-inflammatory genes examined include cytokines (IFNγ, TNFα-1, TNFα-2; IL-1β-1, IL-1β-2; IL-12-p35, IL-12-p40), chemokines (CXCL-8, CCL-1), and iNOS isoforms A and B at different times after treatment with rhsp70. Treatment of macrophages with rTcahsp70 results in up-regulated expression of IFNγ, TNFα1 and TNFα2 (Fig. 5.6 A and B) and the expression of IL-1β-1, IL-1β-2 and IL-12-p35 (Fig. 5.6 C and D). I also quantified the expression of chemokines CCL-1 and CXCL-8 (IL-8) in macrophages activated with rTcahsp70. rTcahsp70 up-regulated expression of both chemokines (Fig. 5.7)

That hsp70 effects were induced following interaction of hsp70 with receptor(s) on the surface of activated goldfish macrophages was supported by the observation that pre-treatment of macrophages with pronase abrogated the expression of select immune genes (Fig. 5.8).

5.2.6 Recombinant parasite hsp70 induces nitric oxide response of goldfish macrophages

Because *T. carassii* hsp70 may be secreted during infection or expressed on the parasite surface, I examined whether the rTcahsp70 induced nitric oxide response in activated goldfish macrophages. Primary macrophages obtained from goldfish kidney were used to establish cultures from individual fish and treated with culture medium (control), either heat-killed *A. salmonicida* or rTcahsp70. Macrophages were treated with 5000 ng/mL rhsp70 because this concentration has been shown to be effective in a previous report [40]. As shown in Figure 5.9, *T. carassii* rhsp70 induced increased expression of both iNOS isoforms as well as a strong nitrite production by activated goldfish macrophages (Fig. 5.10). The bacterial lysates subjected to the same purification regimen as those containing rTcahsp70 were also tested for their ability to induce nitric oxide response in goldfish macrophages (Fig 5.11). One way analysis of variance indicated that rTcahsp70 treatment induced significant ($P < 0.05$) nitric oxide response of goldfish macrophages when compared to either medium control or bacterial lysate groups.

5.3 Discussion

The identity of proteins secreted/excreted by *T. carassii* as well as those expressed on the parasite surface is of particular interest to us because of the possible roles these molecules might play in host-parasite interactions. It has been previously shown that immunization of fish with ES products from *in vitro* cultured parasites increased fish resistance to challenge infection [7]. Parasite alpha and beta tubulins were identified in the ES product and immunization studies showed that recombinant beta tubulin partially protected fish against a challenge infection [27, 39]. I [35] reported that *T. carassii* hsp70 is also one of the major proteins in ES supernatants and that it was identified in the solubilized parasite membrane fraction. Although it is not clear how *T. carassii* may export hsp70 to its surface, my observations are consistent with reports from other studies that identified parasite hsp70 in soluble antigen preparations and secretome of *L. donovani* [24, 46]. Absence of a clear N-terminal signal sequence in *T carassii* hsp70, may suggest the existence of a non-classical secretory pathway for this protein. The fact that *T. carassii* hsp70 was secreted and expressed on the parasite surface, suggests that it may play a role in the host-parasite interactions. Indeed hsp70 has been reported to be an important antigen in several infectious diseases [52].

During infection, fish macrophages respond by up-regulating the expression and secretion of cytokines including TNF-α, IL-1 and enhanced nitric oxide response [13, 42, 43, 44, 45]. Activation of macrophages by parasite antigens is common in trypanosome infection [22, 23, 38]. My results further

support these earlier studies and suggest interaction between *T. carassii* hsp70 and goldfish macrophages potentially leading to development of Th1-like immune response. Recombinant hsp70 from *T. carassii* activated goldfish macrophages and up-regulated expression of cytokines IFNγ, TNFα-1, TNFα-2, IL-1β-1, IL-1β-2; IL-12-p35 and chemokines CXCL-8, CCL-1. The expressions of iNOS isoforms A and B were also up-regulated but at different levels after treatment with rTcahsp70. I did not see a significant increase in expression for IL-12-p40 at the time points examined. It is possible that this cytokine is expressed at later time points as shown previously in goldfish macrophages stimulated with recombinant goldfish interferon gamma [18] Previous reports have shown possible links between cytokine expression and protozoan infection in fish. *Trypanoplasma borreli* induced differential expression of TNF-α with a higher expression of TNFα-2 in carp head kidney phagocytes *in vitro* and up-regulated IL-1β mRNA *in vivo* [32-34]. *T. borreli* also stimulates nitrite production by carp phagocytes *in vitro* [45]. In contrast, these authors reported that *T. carassii* organisms when added to macrophages did not induce nitric oxide response *in vitro* and that there was no change in serum nitrate level in fish infected with this parasite [44]. I have also observed that *T. carassii* induces ROI response of goldfish monocytes but failed to induce NO response of goldfish macrophages. In this study, I showed that *T carassii* recombinant hsp70 induced a strong nitric oxide response in goldfish macrophages and was demonstrated to be present in ES supernatants generated *in vitro.* My observations are consistent with those reported for *T. cruzi*, which showed that *T. cruzi* surface proteins (GPIs) are able induce a potent

nitric oxide response of mouse macrophages [2]. Interestingly, *T. carassii* and *T. cruzi* have very similar surface architecture [1, 30] that may account for differential induction of nitric oxide response of macrophages by *T. borreli* and *T. carassii*.

My results indicate a novel function of trypanosome hsp70 in ectothermic vertebrates, as a modulator of host immune response. *T. carassii* recombinant hsp70 induced increased expression of pro-inflammatory cytokines and chemokines in goldfish macrophages. The recombinant hsp70-induced cytokine expression was abrogated by pronase treatment of macrophages suggesting that rTcahsp70 acted through receptor(s) on macrophage surface. This recognition and signal transduction has been reported to occur partly through CD14 pathway [3]. It is also possible that hsp70 may use different receptors on different immune cells, since hsp70 activation of NK-cell mediated killing was reported to be dependent on the interaction of hsp70 with C-type lectin receptor CD94 [20, 21]. The effects of *T. carassii* hsp70 reported here may result in non-specific stimulation of immune responses that may contribute to general immunosuppression, which is one of the hallmarks of trypanosome infections in mammals. Given that *T. carassii* is an extracellular parasite, stimulation of pro-inflammatory immune response by parasite antigens may down-regulate host humoral immune responses thereby ensuring their establishment and survival in the host. We [27], and others [25, 51] have shown that humoral immune responses play a major role in the elimination and the maintenance of long-lasting resistance to reinfection against *T. carassii*.

Considering that hsps are highly conserved molecules, it is somewhat surprising that *T. carassii* hsp70 activated goldfish macrophages. Although *T. carassii* and goldfish hsp70 sequences are similar (68% similarity), there are notable differences between the host and parasite hsp70 sequences at the C-terminal end of the proteins. *T. carassii* hsp70 possess five GMPG repeats while goldfish hsp70 contains only one repeat. Similarly, *T. cruzi* hsp70 contains at least ten GMPG repeats and human hsp70 has no GMPG repeats at the C-terminal end of the protein. It is possible that the GMPG repeats may be major antigenic determinants of parasite hsp70. A further investigation of this repeat sequence is warranted to understand its importance in host-parasite interactions.

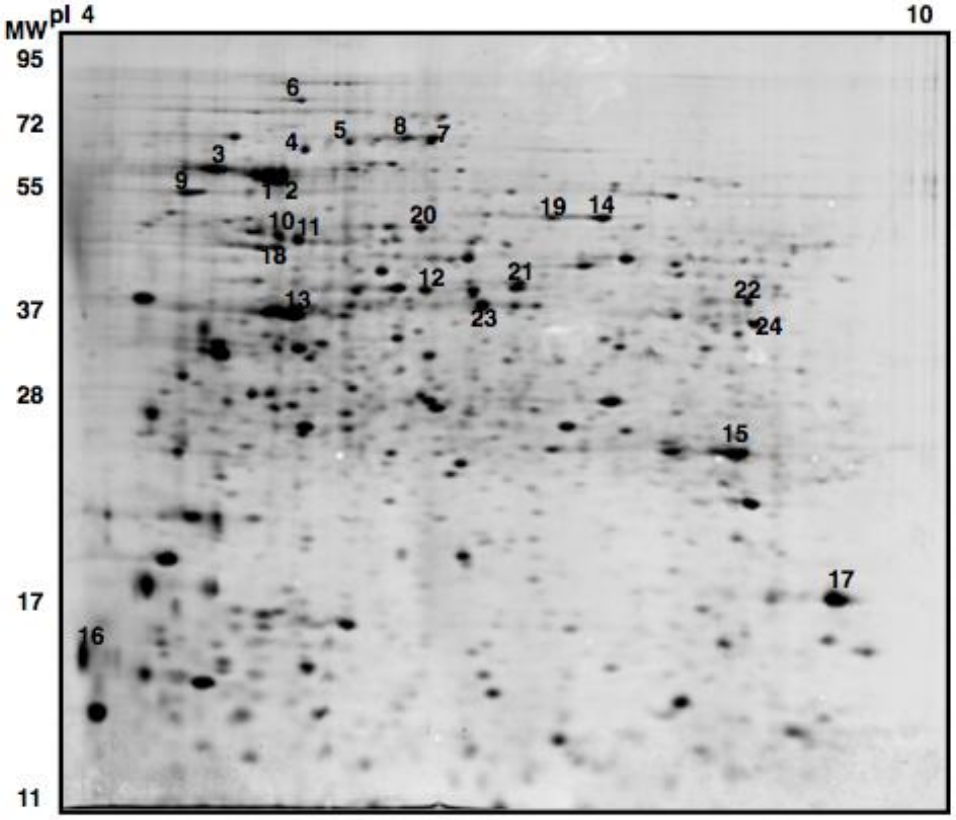

Figure 5.1. Two-dimensional gel electrophoresis of *T. carassii* surface proteins. Five hundred µg of Triton X-114 membrane solubilised proteins were separated in the first and second dimensions. Proteins were visualised using Sypro® ruby fluorescent stain. The labelled spots were selected for mass spectrometry based on their abundance and molecular weight. Some of the labelled proteins and their identity are (1) alpha tubulin (2) alpha tubulin (3) beta tubulin (4) Hsp60 (5) Hsp60 (6) Grp78 (7) Hsp70 (8) Hsp70 (9) Calreticulin (10) Hsp70. The full list of identified proteins is reported in Table 3.6.

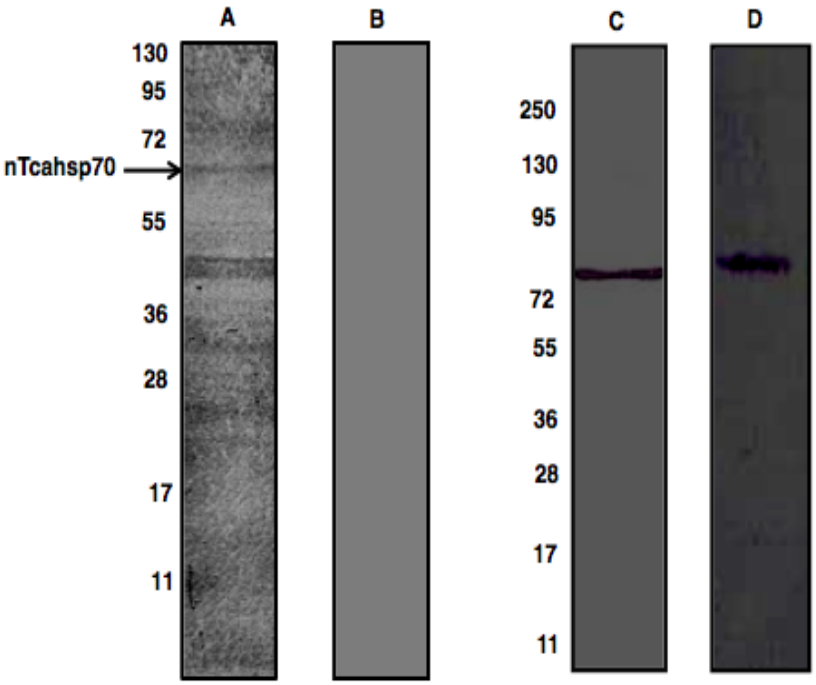

Figure 5.2. Immunogenicity of *T. carassii* hsp70

Filtered ES product was separated by SDS-PAGE and transferred to nitrocellulose (A) Immunoblotting ES products performed with serum from recovered fish diluted 1:25 in blocking buffer (56 days post infection). The 68-70kDa band (arrow) was identified as *T carassii* hsp70. (B) Immunoblot of ES products using serum from non-infected fish diluted 1:25 in blocking buffer. (C) Immunoblot of recombinant *T. carassii* hsp70 recognized by anti-His antibody. (D) Immunoblot of recombinant goldfish hsp70 recognized by immune goldfish serum (56 days post infection).

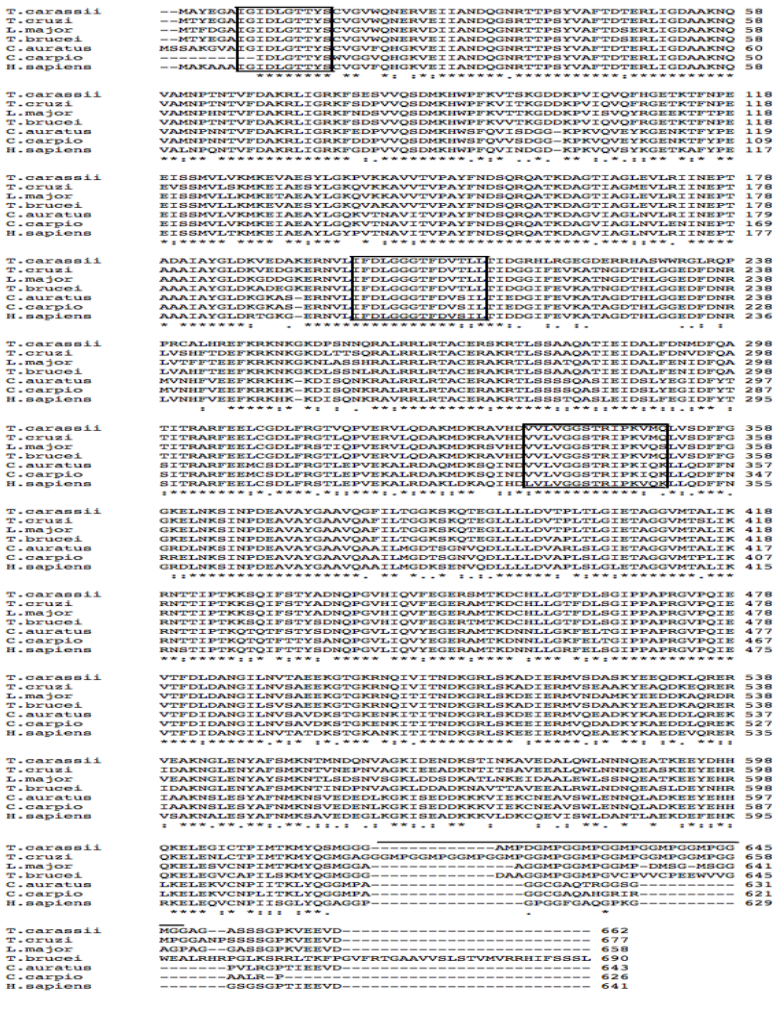

Figure 5.3. **Amino acid alignment of *T. carassii* hsp70 sequence and those from other trypanosomatids (*T. cruzi*, *T. brucei* and *L. major*) as well as sequences from mammalian (*Homo sapiens*) and non-mammalian (*Carassius auratus* and *Cyprinus carpio*) vertebrates**. Sequences were obtained from GenBank with accession numbers *T. cruzi* (XM_812645), *L. major* (XM_001684512), *T. brucei* (XM_824105), *C. auratus* (BAC67184), *C. carpio* (AAM81603), *H. sapiens* (NP_005336). (*) indicates identical residues, (:) indicates conserved residues while (.) indicates semi-conserved residues. (-) are gaps introduced to maximize the alignment. The boxed residues represent the three signature patterns of the hsp70 family predicted by PROSITE server. The region containing the GMPG repeats were shown with a bar at the C-terminal end of the sequences to highlight the presence of this repeat in parasites but not in the host molecules.

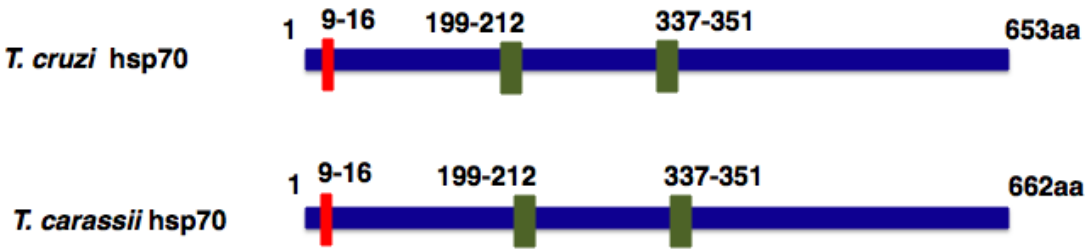

Figure 5.4. Schematic of protein structure of *T. carassii* hsp70. *T. carassii* hsp70 was compared with *T. cruzi* hsp70. The coloured boxes represent hsp70 family signatures. The approximate locations of the hsp70 family signatures are shown.

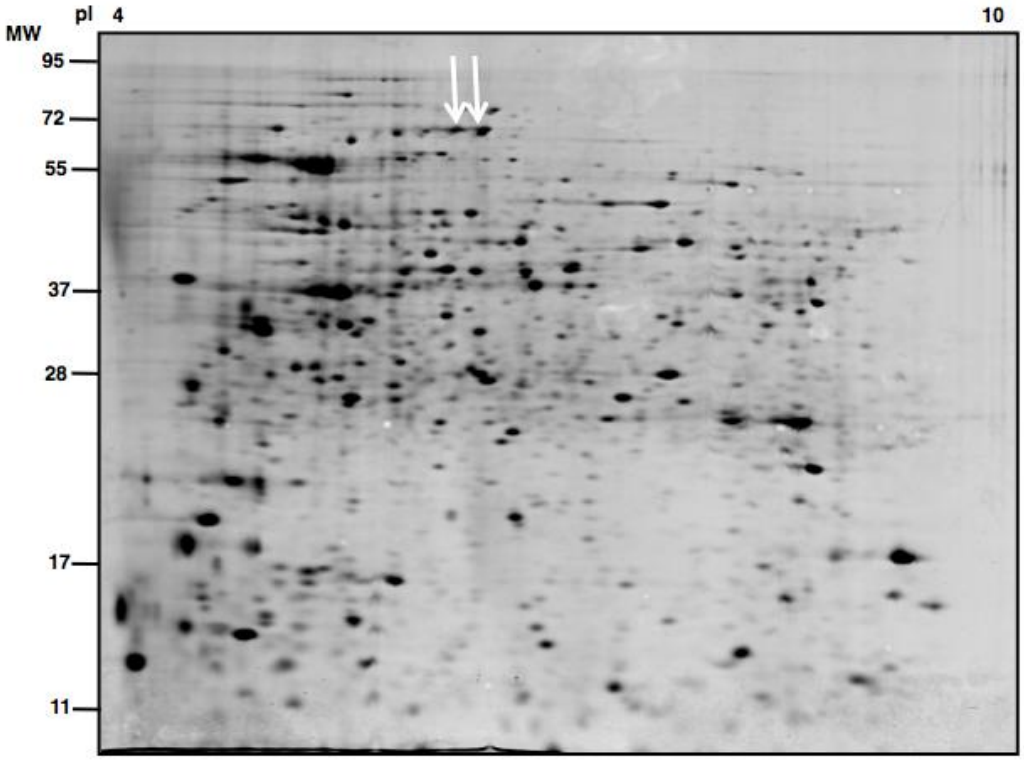

Figure 5.5. *T. carassii* hsp70 is present in membrane surface protein fraction. Five hundred μg of Triton X-114 membrane solubilised proteins were separated in the first and second dimensions as described. Proteins were visualised using Sypro® ruby fluorescent stain. Arrows indicates *T. carassii* hsp70.

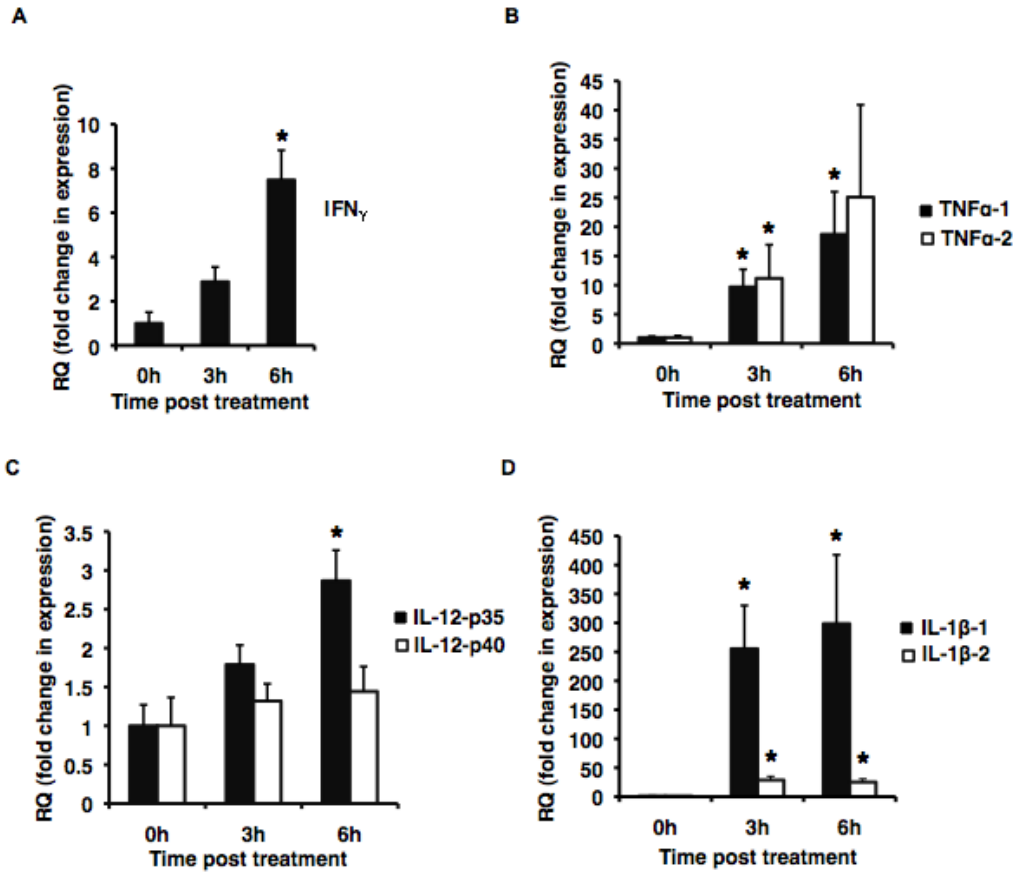

Figure 5.6. Analysis of pro-inflammatory cytokine expression of goldfish macrophages treated with recombinant *T. carassii* hsp70 (rTcahsp70). Quantitative expression of cytokines IFNγ, TNFα-1, TNFα-2, IL-12-p35, IL-12-p40, IL-1β-1, IL-1β-2 were reported relative to endogenous control EF-1α. The expression data were normalized against 0 hour time point expression level for each gene. The results are mean ± SEM of macrophage cultures established from from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. Expression levels were considered to be significant at $P < 0.05$. (*) denotes significantly different from the 0 hour time point.

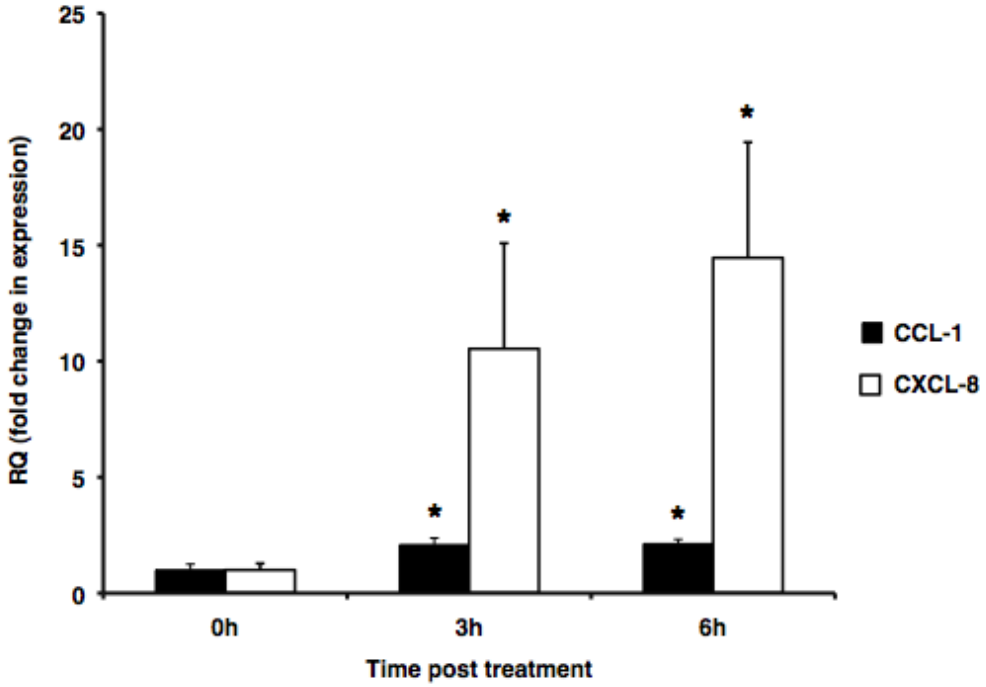

Figure 5.7. Analysis of chemokine gene expression in goldfish macrophages treated with rTcahsp70. Quantitative expression of chemokines CCL-1 and CXCL-8 relative to endogenous control EF-1α. The expression data were normalized against 0 hour time point expression level for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. Expression levels were considered to be significant at $P < 0.05$. (*) denotes significantly different from the 0 hour time point.

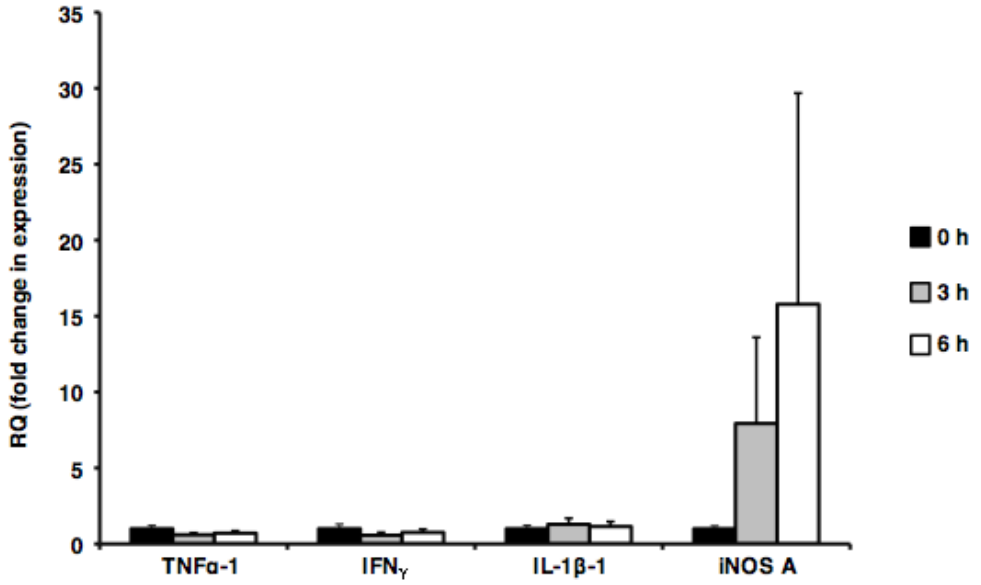

Figure 5.8. Analysis of immune gene expression in goldfish macrophages pre-treated with pronase before incubation with rTcahsp70. Quantitative expression of select genes of goldfish macrophages pre-treated with pronase before incubation with rTcahsp70. Expression of TNFα-2, IFNγ, IL-1β-1 and iNOS A was relative to endogenous control EF-1α. The expression data were normalized against 0 hour time point expression level for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. None of the expression levels were significant at $P < 0.05$ (*) from the 0 hour time point for each gene. Although the expression of iNOS A gene is slightly up-regulated, this expression level is about 30-fold less than expression level in macrophages incubated with rTcahsp70 without pronase pre-treatment.

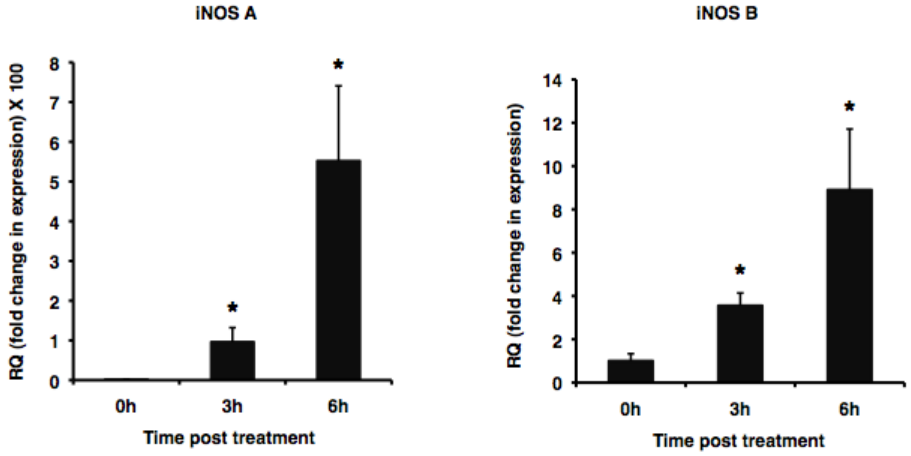

Figure 5.9. *T. carassii* hsp70 up-regulates iNOS gene expression of goldfish macrophages. Quantitative expression of iNOS isoforms A and B relative to endogenous control EF-1α in goldfish macrophages following treatment with rTcahsp70. The expression data were normalized against 0 hour time point expression level for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. Expression levels were considered to be significant at $P < 0.05$. (*) denotes significantly different from the 0 hour time point.

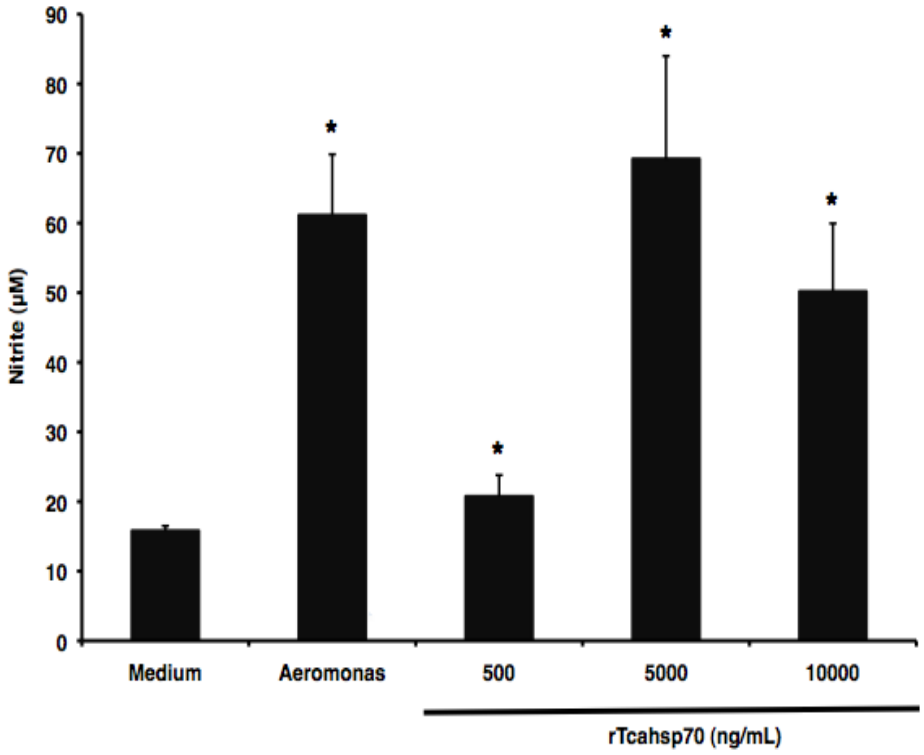

Figure 5.10. *T. carassii* hsp70 enhances nitric oxide response of goldfish macrophages. Nitric oxide production by goldfish macrophages was determined using the Griess reaction and nitrite concentration determined using a nitrite standard curve. The mean ± SEM nitrite production determined for macrophage cultures established from seven individual fish (n = 7). NO assay was performed twice with same results. At concentration greater than 5000 ng/mL, rTcahsp70 effect is unchanged probably because the system is saturated. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control.

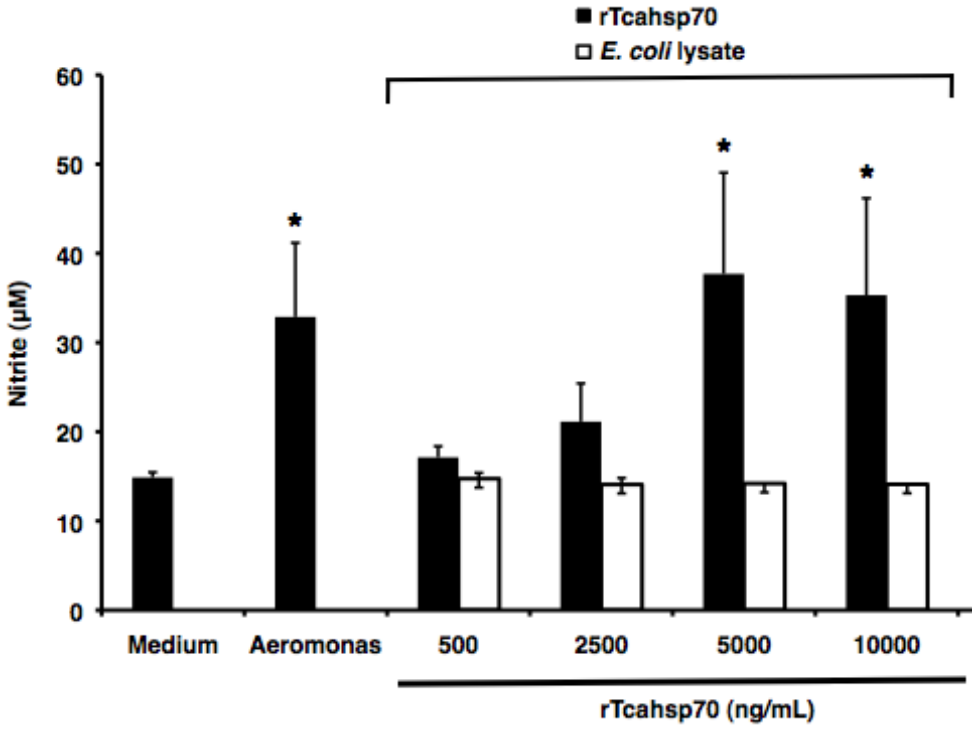

Figure 5.11. Nitric oxide response of goldfish macrophages is due to rTcahsp70. Nitric oxide production by goldfish macrophages was determined using the Griess reaction and nitrite concentration determined using a nitrite standard curve. The mean ± SEM nitrite production determined for macrophage cultures established from seven individual fish (n = 7). *E. coli* lysate failed to enhance NO response of goldfish macrophages. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control.

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CHAPTER 6

GLYCOPROTEIN 63 (gp63) OF *Trypanosoma carassii* SUPPRESSES ANTIMICROBIAL RESPONSES OF GOLDFISH (*Carassius auratus* L.) MACROPHAGES[1]

6.0 Introduction

Glycoprotein 63 (gp63) is a metalloprotease belonging to the metizincin class and is characterized by a conserved motif HEXXH, an N-terminal pro-peptide that maintains the protein in an inactive form that is lost upon activation [44]. The pro-peptide is cleaved from the N-terminal end of nascent gp63 by autocatalytic mechanism that unblocks the active site through cysteine switch mechanism [10, 62]. Mature protease consists of N-terminal domain comprising the catalytic component of the zinc protease, the central domain and C-terminal domain containing the glycosylphosphatidylinositol (GPI) anchor addition site [43, 66]. The protein is synthesized as an N-glycosylated pro-enzyme [41, 44].

Gp63 is also known as major surface protease (MSP) or leishmanolysin and is a major membrane glycoprotein of *Leishmania* spp. [8, 20]. This molecule plays many roles in host-parasite interactions and has been shown to be important for entry of promastigote stage of the parasite into host macrophages and survival inside macrophages [13, 38, 58]. Gp63 is involved in the attachment of the

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promastigote to host cell surface receptors, such as the fibronectin receptor [13, 58]. The protease activity of gp63 contributes to the resistance of promastigotes to complement-mediated lysis [9] while immunization with recombinant protein partially protects animals after challenge infection [1, 14, 53, 65]. The findings of a recent report indicate that gp63 is also capable of generating a partial Th1 type protective response in *Leishmania donovani*-infected mice [33]. The immunized animals had high levels of IFN-γ and IL-2 and low levels of IL-4 and IL-10 indicating generation of a protective Th1 response and suppression of Th2 type immune response [33]. The *Leishmania* parasites interfere with host immunity using sophisticated mechanisms involving impairment of host cell signal transduction such as inhibition of JAK/STAT signalling and inactivation of transcription factors AP-1, NF-$_k$B, and CREB. These activities result in subversion of macrophage anti-microbial functions, including cytokine-induced functions and nitric oxide (NO) production [26, 52]. Gp63 has been demonstrated to be centrally crucial to the processes that lead to impairment of host cell functions. Gp63 is involved in cleavage of NF-$_k$B, AP-1, protein tyrosine phosphatases (PTP), and actin cytoskeleton regulators [15, 22, 25, 29]

Trypanosome homologues of leishmania gp63 have been identified although the contribution of trypanosome gp63 to host-parasite interactions and immune modulation is yet to be fully elucidated. In *T. brucei,* genes encoding gp63 have been reported and these gene show differential expression in the bloodstream form parasites [18, 36]. This differential expression has led to suggestion that gp63 may protect bloodstream trypanosomes from complement

lysis by impairing a protein processing functions [18, 23, 36]. Trypanosome gp63 is also required for removal of variable surface glycoproteins (VSGs) from the surface of bloodstream trypanosomes [23, 36]. *T. cruzi* gp63 genes have been divided into four novel groups based on sequence features [40]. Some *T. cruzi* gp63 (Tcgp63) were differentially expressed in the different life cycle stages and antibodies raised against a Tcgp63 inhibited the entry of trypomastigotes into cells as shown in neutralization assays [16, 35]. These results suggest involvement of Tcgp63 in host-parasite interaction and evasion of phagocytic killing.

T. carassii shares extracellular existence with African trypanosomes although its surface architecture closely resembles *T. cruzi* [37]. Transmission of *T. carassii* from infected fish to naive hosts by leech vectors occurs through blood sucking, another characteristic shared with *T. brucei.* A number of economically important fish can be infected and 100% prevalence of *T. carassii* infection have been reported [39, 55]. However, fish that survive the infection become immune to homologous challenge [63, 64], suggesting induction of protective immunity and absence of antigenic variation. Although we have identified some parasite molecules that may play role in evasion of goldfish immunity [50, 51), those involved in induction of protective immune responses are yet to be identified. In this study, I cloned and expressed *T. carassii* gp63 (Tcagp63) to further understand how *T. carassii* interacts with the fish host and modulates host immunity. My working hypothesis was that Tcagp63 is used by parasite to down-regulate inflammatory, antimicrobial and cytokine-induced responses thereby providing *T carassii* with a strategy to modulate the balance of pro- and anti-

inflammatory responses to ensure maintenance of long lasting infections and their transmission.

6.1 Experimental design

6.1.1 Molecular cloning and expression of Tcagp63

Total RNA extracted from *in vitro* grown parasites was reverse transcribed into cDNA. The cDNA synthesized was used in polymerase chain reactions (PCR) to amplify Tcagp63 fragment using primers designed to *T. carassii* spliced leader sequence and a highly homologous region of trypanosome gp63 sequences. The full sequence of Tcagp63 was amplified and the recombinant protein was produced using a prokaryotic expression system. Purified rTcagp63 was used to immunize rabbits for production of polyclonal antiserum.

6.1.2 Assessment of the effect of Tcagp63 on nitric oxide response of goldfish macrophages

Primary macrophages cultures obtained from kidney of goldfish (n = 6) were seeded into individual wells of 96-well plates at a density of 3×10^5 cells per well. Cells were incubated with culture medium alone (negative control), heat-killed *A. salmonicida* (positive control), rTcagp63 (1 µg/mL, 5 µg/mL or 10 µg/mL) plus heat-killed *A. salmonicida* at 20°C for 48 hours. Nitrite production was determined using the Griess reaction as described previously [24]. To ascertain that the results obtained were not due to other contaminating proteins, *E. coli* bacterial lysates were prepared and subjected to the same purification

protocol as rTcagp63-containing lysates. Separate goldfish macrophage cultures obtained from 6 fish (n = 6) were treated with medium alone (negative control), heat-killed *A. salmonicida* (positive control), rTcagp63 (10 µg/mL) or bacterial lysates (4.5 µL, equivalent volumes of rTcagp63 lysates that corresponded to 10 µg/mL of rTcagp63) plus heat-killed *A. salmonicida* and assayed for nitric oxide response using Griess reaction. Also, recombinant goldfish TGFβ, purified using the same procedure as described for Tcagp63 were used in the NO assay at 10 µg/mL.

6.1.3 Assessment of the effect of rTcagp63 on respiratory burst response of goldfish monocytes

Cultures of goldfish monocytes obtained from kidney of goldfish (n = 6) were incubated with medium alone (control), *A. salmonicida* alone or *A. salmonicida* plus different concentration of rTcagp63. In another experiment aimed at understanding how rTcagp63 exerts its effects cells were treated with culture medium alone (control), rTcagp63 (5 µg/mL or 10 µg/mL) for 2 hours and washed 3 times with incomplete medium before used to set up ROI assay. Cells were seeded into 96-well plates at a density of 3×10^5 cells per well. ROI assay was performed with cells incubated in medium alone or with rTcagp63 using heat-killed *A. salmonicida* and recombinant goldfish TNFα2 (rgTNFα2 100 ng/mL) to stimulate production of reactive oxygen species. ROI assays were incubated at 20°C for 48 h. NBT (2 mg/mL, Sigma) and PMA (final conc. 100 ng/mL, Sigma) in PBS were added to the cultures and incubate at room

temperature for an additional 30 minutes. The plates were than centrifuged at 400 x *g* for 10 minutes, the supernatants aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Absorbances from cells alone (no PMA) were subtracted from treatment values to factor in background NBT reduction.

6.1.4 Assessment of effect of rTcagp63 on immune gene expression of goldfish macrophages activated with *A. salmoncida*

Goldfish monocyte or macrophage cultures (n = 4) were seeded into individual wells of 96-well plates at a density of $1x10^6$ cells per well. Cells were treated with medium alone or 10 µg/mL of rTcagp63 at 20°C for 2 hours, washed twice and then stimulated with heat-killed *A. salmonicida* at 20°C for 6 hours. Total RNA was isolated from cells and reverse transcribed into cDNA. Changes in expression pattern of select immune genes (iNOS, TNFα1 and 2, IL10 and NADPH oxidase component genes) were examined. Relative expression was determined in relation to elongation factor 1 alpha (EF-1α) and all expression data were normalized using the baseline expression of cells incubated in medium alone.

6.1.5 Assessment of effect of rTcagp63 on immune gene expression of goldfish macrophages

Goldfish macrophage cultures (n = 4) were seeded into individual wells of 96-well plates at a density of $1x10^6$ cells per well. Cells were treated with medium alone or 10 µg/mL of rTcagp63 for 0, 3 and 6 hours at 20°C. Total RNA was isolated from the cells and reverse transcribed into cDNA. Changes in expression pattern of select immune genes (iNOS A and B, TNFα1 and 2, IL10) were examined. Relative expression was determined in relation to elongation factor 1 alpha (EF-1α). All expression data were normalized using the baseline expression of cells incubated in medium alone.

6.1.6 Assessment of interaction of rTcagp63 with goldfish macrophages

Goldfish macrophage cultures (n = 3) were established and 10^5 cells were washed twice and incubated with rTcagp63 (10 µg/mL) for 30 minutes at room temperature. Cells were washed twice in PBS and incubated with affinity purified rabbit anti-rTcagp63 (80 µg) or affinity purified rabbit IgG (80 µg) diluted in PBS for 1 hour. This is followed by washing twice in PBS before incubation with FITC-labelled goat anti-rabbit IgG (1:100) diluted in PBS for 1 hour at room temperature. Cells were washed twice in PBS and rTcagp63 binding to macrophages was analyzed using FACS Calibur flow cytometer. A total of at least 10,000 cells were analyzed in each sample.

To assess whether rTcagp63 enter and accumulates in goldfish macrophage, 10^7 cells were washed twice and incubated with rTcagp63 (10 µg/mL) for 0, 1, 2,

3 hours at 20°C. Cells were washed 3 times with PBS and lysed with 30 µL of RIPA buffer. 30 µL of Laemmli sample buffer was added and the mixture loaded into SDS PAGE for western blotting. Accumulated rTcagp63 was detected with rabbit anti-rTcagp63 antibody (1:2000) followed by HRP-conjugated goat anti-rabbit IgG (1:3000). Blots were stripped and re-probed rabbit anti-actin-beta (NT) zebrafish polyclonal IgG (AnaSpec Inc.) (1:1000) for detection of goldfish actin, which served as protein loading control.

To assess the effect of rTcagp63 on phospho-tyrosine protein pattern of goldfish macrophages, macrophage cultures (n = 3) were established and 10^7 cells were washed twice and incubated with medium alone (control) or with rTcagp63 (10 µg/mL) for 6 hours. Cells were stimulated with *A. salmonicida* at 20°C for 10, 15 and 20 minutes before lysed with 30 µL of RIPA buffer. 30 µL of Laemmli sample buffer was added and loaded in SDS PAGE for western blotting. Phospho-tyrosine protein patterns were detected with anti-phospho-tyrosine antibody (1:4000) and HRP-conjugated goat anti-mouse monoclonal antibody (1:3000).

6.2 Results

6.2.1 Tcagp63 is similar to gp63 of other trypanosomes.

Sequence alignment (Fig. 6.1) shows Tcagp63 shares some sequence similarities with gp63 of *T. cruzi* (Tcgp63) and *T. brucei* (Tbgp63). The highest identity is seen with Tcgp63 46%. Interestingly, the catalytic site of Tcagp63 is different from that of *T. cruzi* and *T. brucei* as well as Leishmania gp63 (Lmg63). The protein structure of Tcagp63 compared with Tcgp63 and Lmgp63 is shown in

Fig. 6.2. Metalloprotease catalytic site denoted by HEXXH is different compared to Tcagp63 catalytic site (HELRTH) which has an insertion of a polar threonine residue with neutral side chains. The implications of this are unclear at this point although introduction of a larger polar residue with neutral side chains may destabilize the structure of this protein at least at the catalytic site. Destabilization of structure may affect the overall functions of the protein or results in a non-functional catalytic site. The glutamic acid residue has been shown to be important for the catalytic activity as demonstrated in mutagenesis experiment [44], while substitution of either histidine residue leads to intracellular degradation of the mutant protein [44]. Phylogenetic analysis of Tcagp63 predicted protein sequence grouped it with trypanosome gp63 sequences and showed the most similarity to *T. cruzi* gp63 (Fig 6.3).

6.2.2 rTcagp63 is unstable and undergoes rapid degradation

To assess the functions of Tcagp63, I expressed the full coding sequence in the prokaryotic system. The protein products of the full coding sequence and protein produced from Tcagp63 fragment that lacks the N-terminal signal peptide were rapidly degraded following purification. I was able to produce relatively stable recombinant Tcagp63 from fragment lacking both the signal peptide and the catalytic site (Fig 6.4 A). The protein product of this fragment was therefore used in antibody production and in subsequent functional assays. Antibody raised against rTcagp63 recognized the recombinant protein (Fig. 6.4 B) but not native gp63 from *T. carassii* lysate. It has been reported that gp63 is post-

translationally modified [10, 43, 44, 62, 66]. Post-translational modification by N-glycosylation has also been described [13] and was thought to stabilize the protein and its enzymatic activity [34]. Three potential N-glycosylation sites have been reported in gp63 proteins and analysis of these sites by site-directed substitution indicated they were important for intracellular stability of the protein [44]. However, functional gp63 proteins have also been produced in prokaryotic system [11, 57]

6.2.3 rTcagp63 abrogated *A. salmonicida*-induced nitric oxide response of goldfish macrophages.

Leishmania spp. parasites use sophisticated mechanisms to subvert macrophage anti-microbial functions including inhibition of nitric oxide (NO) production and macrophage cytokine-inducible functions [52]. These effects have been largely attributed to *Leishmania* spp. gp63 [15, 22]. For this reason, I tested the ability of rTcagp63 to inhibit *A. salmonicida*-inducible macrophage antimicrobial responses. As shown in Figure 6.5, rTcagp63 abrogated *A. salmonicida*-induced NO response of goldfish macrophages. This effect was specific to rTcagp63 since recombinant goldfish TGFβ (rgTGFβ) and bacteria lysate purified using the same procedure as rTcagp63 had no effect on *A. salmonicida*-induced NO response of macrophages (Fig. 6.6). Cells were viable after addition of recombinant molecules as shown by microscopy, RNA isolation and measurement of metabolic rates by MTT assay.

6.2.4 rTcagp63 abrogated *A. salmonicida*-induced respiratory burst response of goldfish monocytes

To further characterize the effects of Tcagp63 on antimicrobial responses of goldfish, I examined whether incubation of goldfish monocytes with rTcagp63 would affect their ROI response. As shown in Figure 6.7, rTcagp63 down-regulated *A. salmonicida*-induced ROI response of goldfish monocytes. The ability of rTcagp63 to inhibit ROI response was not limited to *A. salmonicida*-activated monocytes. The recombinant protein also inhibited cytokine-inducible effects. As shown in Figure 6.8, rTcagp63 also inhibited rgTNFα2-indced ROI response of activated goldfish monocytes.

6.2.5 rTcagp63 exerts its effects partly by association with immune cells.

It has been suggested that gp63 could function by cleaving surface receptors on immune cells or by entering cells and activating protein tyrosine phosphatases leading to alteration of signalling and attenuation of antimicrobial responses [22, 30]. I investigated the mechanisms by which rTcagp63 exert its effects. Goldfish monocytes were incubated with rTcagp63 for 2 hours, washed three times and used to set up ROI assay for 48 hours. As shown in Figure 6.9, pre-incubation of cells with rTcagp63 inhibited both *A. salmonicida*- and rgTNFα2-induced ROI response of the monocytes. These findings suggest that rTcagp63 may use mechanism other than cleavage of surface receptors to exert its effects. However, I cannot exclude the possibility that a combination of receptor

cleavage and alteration or attenuation of intracellular signalling occurred after rTcagp63 treatment of monocytes.

6.2.6 Pre-incubation of cells with rTcagp63 altered immune gene expression

Since incubation of cells with rTcagp63 abrogates *A. salmonicida*- and cytokine-induced production of reactive nitrogen and oxygen intermediates, I examined whether pre-incubation of cells with rTcagp63 before incubation with *A. salmonicida* had an effect on the expression of several immune genes. Pre-treatment of cells with rTcagp63 for 2 hours prior to incubation with *A. salmonicida* for 6 hours caused a significant decrease in expression of iNOS A, TNFα1 and TNFα2, compared to that in cells incubated with *A. salmonicida* alone (Fig. 6.10). Although I observed a decrease in the expressions of IL-10 and $p47^{phox}$, they were not statistically significant (Fig. 6.11). These results are consistent with previous findings that gp63 interfered with host cell signalling [22, 25, 29]. I then examine whether incubation of cells with rTcagp63 alone had any effect on immune gene expression. As shown in Fig 6.12, only slight increases in expressions of immune genes were observed when cells were incubated with rTcagp63 alone for 6 hours. These results suggest rTcagp63 have no significant effect on immune gene expression at least at the time points investigated in this study.

6.2.7 rTcagp63 associates with goldfish macrophages and altered phospho-tyrosine protein patterns of macrophages.

I examined the ability of rTcagp63 to bind to goldfish macrophages by incubating cells with rTcagp63 (10 µg/mL) for 30 minutes. Cells were washed twice in PBS and incubated with rabbit anti-rTcagp63 (80 µg) or rabbit IgG (80 µg) isotype control for 1 hour. Following incubation, cells were washed twice in PBS and incubated with FITC-labelled goat anti-rabbit IgG (1:100) for 1 hour. Cells were washed twice in PBS and analyzed by flow cytometry. As shown in Figure 6.13, cells incubated with rTcagp63 followed by anti-rTcagp63 showed increased fluorescence intensity indicated by a shift in surface staining compared to cells incubated with isotype control antibody (anti-rabbit IgG). The non-specific binding of isotype control antibody to macrophages observed (Fig. 6.13) may result from effects of rTcagp63 on the cells. Cleavage of surface molecules by rTcagp63 may expose epitopes recognized by rabbit IgG. Nonetheless, a significant shift in staining suggests binding of rTcagp63 to macrophages.

To further investigate how rTcgp63 interacted with macrophages, I incubated cells with the recombinant protein for 0, 1, 2, 3 hours before washing three times with PBS. Cells were lysed with RIPA buffer and the lysates were subjected to Western blot analysis. rTcagp63 was detected using the anti-rTcagp63 antibody as early as 1 hour after incubation (Fig. 6.14). The degree of rTcagp63 association with macrophages was time dependent (Fig. 6.14). I used rabbit anti-zebrafish actin to detect goldfish actin that served as loading control to ensure presence of protein in all lanes.

To investigate whether rTcagp63 affected the phospho-tryrosine protein patterns of macrophages, cells incubated with either medium alone or with rTcagp63 for 6 hours were washed twice and incubated with *A. salmonicida* for the indicated time. Cells were lysed with RIPA buffer and the lysates were subjected to western blot analysis. I observed changes in phospho-tyrosine protein patterns as early as 10 minutes in cells pre-treated with rTcagp63 (Fig 6.15). I used cells incubated with medium alone as control to determine basal phospho-tyrosine protein levels (Fig 6.15).

6.3 Discussion

A number of trypanosome proteins that participate in immune evasion are abundantly expressed on the parasite surface and some have been identified in ES fraction [8, 18, 37, 47, 48, 50, 51, 60]. There is evidence that gp63 a GPI-anchored surface protein and can be released from parasites [17, 32, 43, 67], suggesting that it can participate in immune evasion or modulation extracellulary. In my proteomic studies of the excretory/secretory products and surface proteins of *in vitro* grown *T. carassii*, I did not identify gp63 as a constituent of these fractions [50, 51] probably due to very low levels of protein expressed on the surface of *in vitro* grown trypomastigotes. Alternatively, the low level of sequence similarity among parasite gp63 molecules especially at amino acid levels might make detection of Tcagp63 by MS difficult. Moreover, rabbit IgG antibody produced using rTcagp63 failed to detect native protein in Western blot analysis,

which may be due to either very low protein levels or post-translational modification of Tcagp63.

Among parasite surface and secreted molecules, the roles of *T. brucei* VSG [59, 60], *T. cruzi* complement regulatory proteins (CRP) [47, 48], Leishmania gp63 and lipohosphoglycan (LPG) [15, 22, 25, 29, 52, 56] are well documented. The rhomboid proteases of *Entamoeba histolytica* cleave surface lectins and have been implicated in immune evasion [5]. *E. histolytica* is an extracellular parasite and must evade host immunity for successful colonization of the host. The parasite uses surface receptor capping whereby surface receptors that have been recognized by the host are shed from the parasite surface [19]. *Plasmodium* rhomboid proteases aid in host cell invasion since they cleave parasite adhesins that engage host cell receptors [3]. Successful invasion of host cells requires disengagement of adhesin-receptor interactions. These results in release of adhesins, and rhomboid proteases that are responsible for this activity have been identified in all invasive stages of the malaria life cycle [3].

Crithidia fasciculata a trypanosomatid that does not seem to have mammalian host and complete its life cycle in mosquito has been reported to possess a homologue of *Leishmania* gp63 [31]. *C. fasciculata* appears to have two developmental forms in the insect but the biological role of gp63 in this organism remains to be determined [31, 42]. It is thought that *C. fasciculata* gp63 might protect the organism from insect immune response [31]. This suggests evolutionary conservation of gp63 metalloprotease [18].

Replacement of the bloodstream stage VSG with procyclic stage procyclin, is a hallmark of differentiation in *T. brucei*. VSG is released during differentiation by at least two mechanisms, endoproteolytic cleavage by gp63 and GPI hydrolysis by endogenous bloodstream trypanosome glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) which hydrolyzes GPI anchor present on VSG [27, 28]. Electrophoretic mobility of the VSG released in culture medium can be used to distinguish between the two forms. Full-length soluble VSG results from GPI hydrolysis while truncated VSG is due to proteolysis [27]. Several genes encoding gp63 have been identified in trypanosomes, but the contribution(s) of trypanosome gp63 to immune modulation is yet to be fully investigated. *T. brucei* gp63 molecules show differential expression in the bloodstream form of the parasites, and are required for removing VSG from the surface of bloodstream trypanosomes [18, 23, 27, 28, 36]. It has been suggested that gp63 is up-regulated during differentiation from bloodstream parasites to procyclic forms to release VSG and is maintained on the surface of procyclic trypanosomes following differentiation [28]. *T. cruzi* gp63 molecules also show differential expression in the different life cycle stages and have been reported to be involved in infection of host cells [16, 35]. Although *T. carassii* is an extracellular parasite of ectothermic vertebrates, it has a surface coat that is rich in sialic acid residues like *T. cruzi* that is primarily an intracellular parasite [2]. It is clear that Tcagp63 is not involved in infection of host cells or the cleavage of VSG molecules, since *T. carassii* does not have VSGs on its surface [2, 54].

I showed that rTcagp63 associates with goldfish macrophages and that cells treated with the recombinant protein exhibited altered phospho-tyrosine protein patterns. These findings are similar to those that showed that the incubation of macrophages with *L. major* or recombinant gp63 from *L. major* lead to internalisation of native and recombinant proteins within 1 hour of incubation [22]. Internalised gp63 interacted with and activated protein tyrosine phosphatases causing down-regulation of JAK/STAT and MAPK signalling and inhibition of macrophage functions [7, 45, 21].

rTcagp63 abrogated *A. salmonicida*- and rgTNFα2-induced production of reactive oxygen and nitrogen intermediates of goldfish monocytes and macrophages, respectively. These effects may result from alteration in signalling events. Attenuation of signalling or alteration in signalling events will affect activity of transcription factors and consequently gene transcription. I observed significant decrease in the expression of iNOS isoform A that encodes the enzyme (iNOS) responsible for the production of reactive nitrogen intermediates. In addition, the mRNA levels of TNFα1 and TNFα2 in macrophages pre-incubated with rTcagp63, and then activated by *A. salmonicida* were significantly reduced. These observations are consistent with previous finding that *Leishmania* gp63 altered host cell signalling through cleavage of protein tyrosine phosphatase [22] and that it mediated the inactivation of p38 mitogen-activated protein kinase [29] and transcription factors [15]. Since incubation of macrophages with the recombinant protein followed by washing to remove the protease before used for ROI assay failed to reverse the effects of rTcagp63, rTcagp63 likely mediated

these effects by associating with macrophages and interfering with signal transduction. This undoubtedly leads to attenuation or alteration of signalling and inhibition of the activity of transcription factors and eventual impairment of downstream macrophage antimicrobial functions.

The recombinant Tcagp63 used in this study lacks the metalloprotease catalytic site. However, Tcagp63 catalytic site denoted by HELRTH is different from the putative metalloprotease catalytic site denoted by HEXXH. Although Tcagp63 catalytic site contains the important residues required for catalytic activity and stabilization of the protein (Section 6.2.1), insertion of a larger polar threonine residue with neutral side chains may destabilize the structure of Tcagp63 catalytic site. It is possible that Tcagp63 catalytic site is inactive or that this protein retained other functions that are independent of the catalytic site.

This is the first report showing that a trypanosome gp63 down-regulates pro-inflammatory responses of macrophages. I showed previously that a balanced Th1/Th2 response is important for the outcome of *T. carassii* infection in the goldfish [49], and that one of the immune evasion strategies of the parasite is the secretion of hsp70 that causes up-regulation of macrophage antimicrobial responses [51]. The results reported here indicate *T. carassii* gp63 may be one of the molecules responsible for dampening pro-inflammatory responses induced by Tcahp70 and related molecules. This hypothesis is supported by observations that both Th1 and Th2-type cytokine genes were up-regulated in *T. carassii*-infected goldfish compared to non-infected controls [49]. Taken together, my findings suggest that *T. carassii* has the ability to regulate and balance pro- and anti-

inflammatory responses of the host during infection enabling it to evade immune responses and persist in its ectothermic host. However, these activities are likely to result in inability of the fish host to mount a strong humoral response to eliminate the parasites thereby leading to generalised immunosuppression. These findings suggest that induction of immunosuppression may be an important immune evasion and modulation strategy of *T. carassii*. Immunosuppression is common in mammalian trypanosome infections [4, 61] and untimely death of host may occur due to secondary infections.

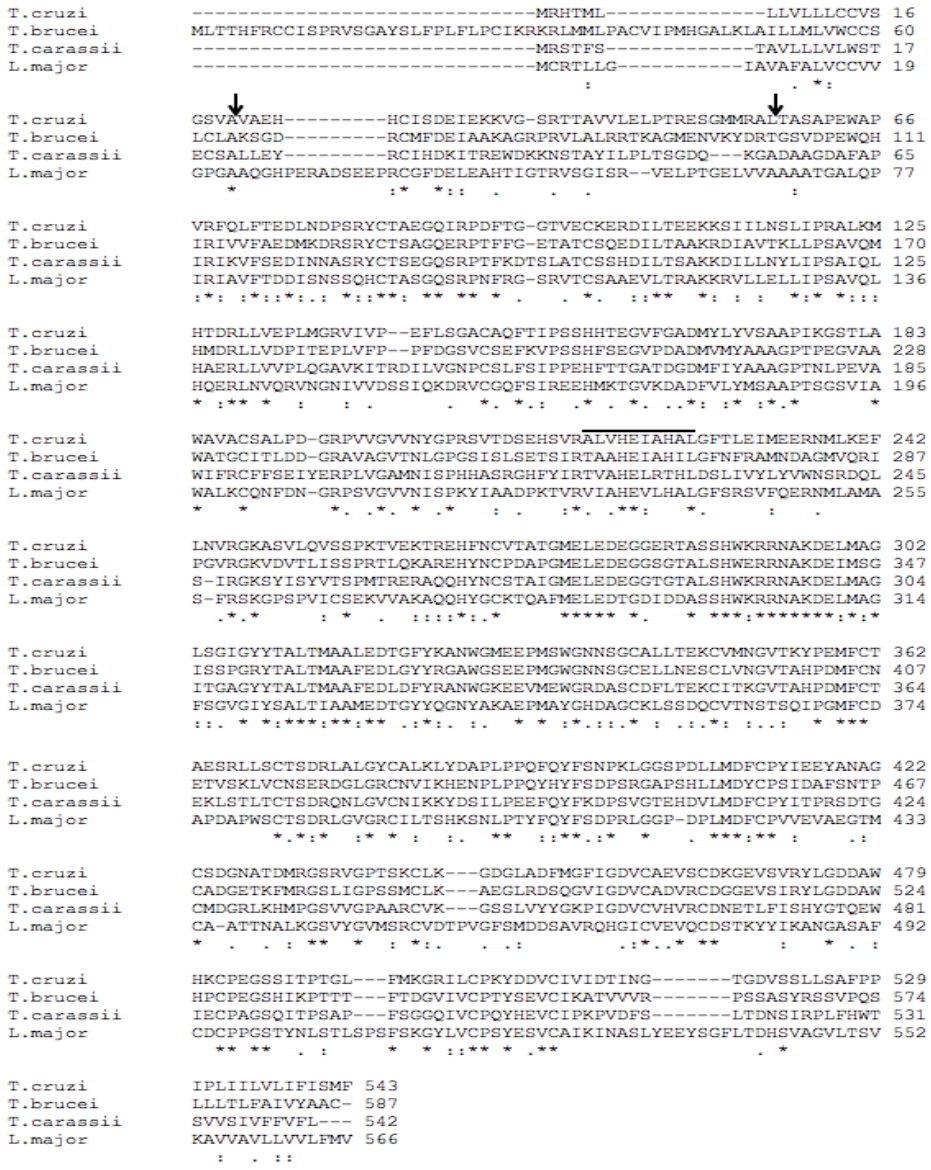

Figure 6.1. Amino acid alignment of *T. carassii* gp63 (Tcagp63) sequence and those from *T. cruzi*, *T. brucei* and *Leishmania major*. Sequences were obtained from GenBank accession numbers *T. cruzi* (XP_817187), *T. brucei* (XP_846998), *L. major* (XP_001684335). (*) indicates identical residues, (:) indicates conserved residues while (.) indicates semi-conserved residues. (-) are gaps introduced to maximize the alignment. The predicted cleavage site for Tcagp63 signal peptide and pro-region respectively are indicated with arrow. A bar denotes the putative metalloprotease zinc binding site.

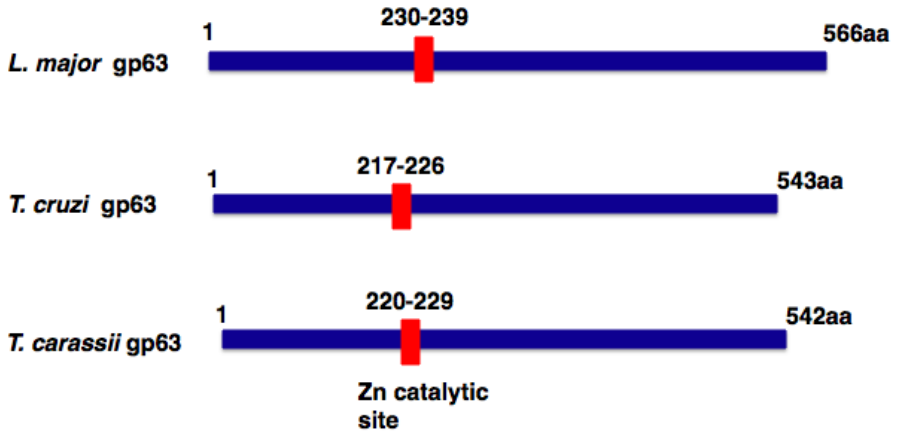

Figure 6.2. Schematic of protein structure of *T. carassii* gp63. *T. carassii* gp63 was compared with *T. cruzi* and *L. major* CRT sequences. The coloured boxes represent the putative gp63 zinc metalloprotease catalytic site. The approximate locations of the catalytic sites are shown.

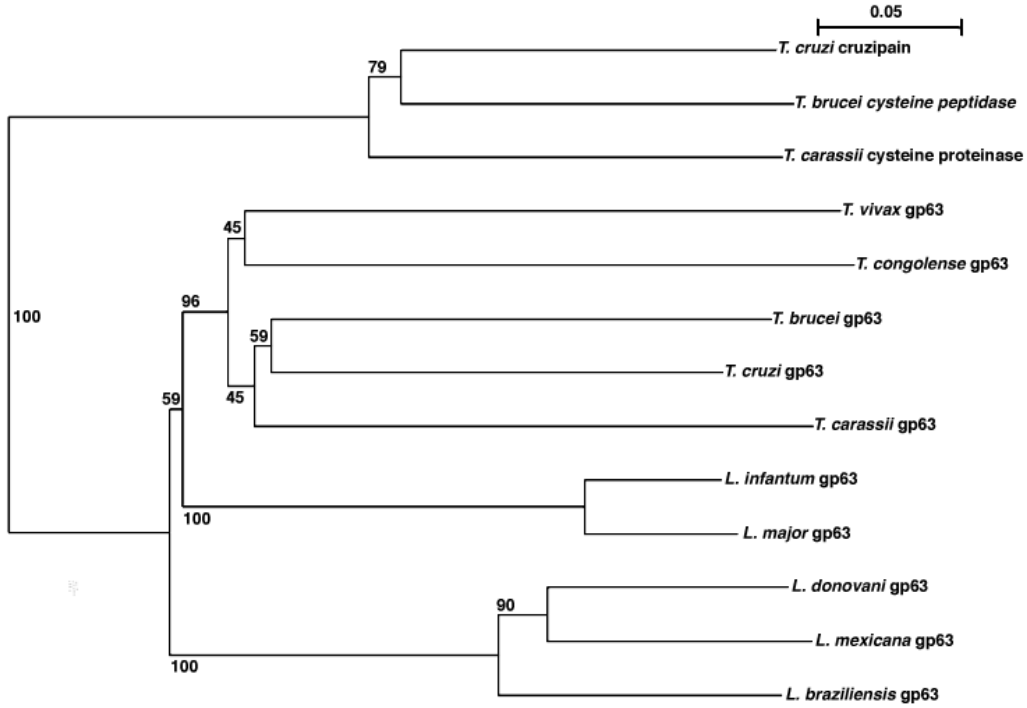

Figure 6.3. Phylogenetic analysis of the *T. carassii* gp63 demonstrated closest relationship to *T. cruzi* and *T. brucei* gp63 sequences. Protein sequences used and their accession numbers are: *T. cruzi* (XP_817187), *T. brucei* (XP_846998), *T. congolense* (CCC95446), *T. vivax* (CCC53361), *L. major* (XP_001684335), *L. mexicana* (CAA45733), *L. donovani donovani* (ACT31401), *L. infantum* (XP_003392667), *L. braziliensis* (XP_001562828), *T. carsssii* Cathepsin L (ABQ23397), *T. cruzi* cruzipain (AAF75547), *T. brucei* cysteine peptidase (XP_845218). *T. carsssii* Cathepsin L, *T. cruzi* cysteine protease and *T. brucei* cysteine peptidase were used as out-groups.

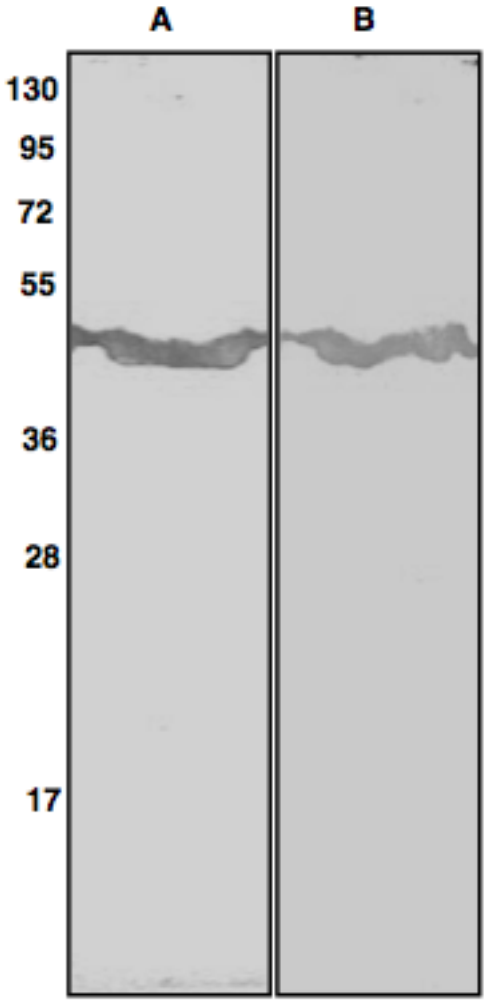

Figure 6.4. Western blot analysis of rTcagp63. Western blot analysis of rTcagp63. rTcagp63 was expressed in prokaryotic system and purified as described. rTcagp63 is recognized by Mouse anti-histidine monoclonal antibody (1:5000) (A) and Rabbit anti-rTcagp63 affinity purified IgG polyclonal antibody (1:2000) raised against rTcagp63 (B).

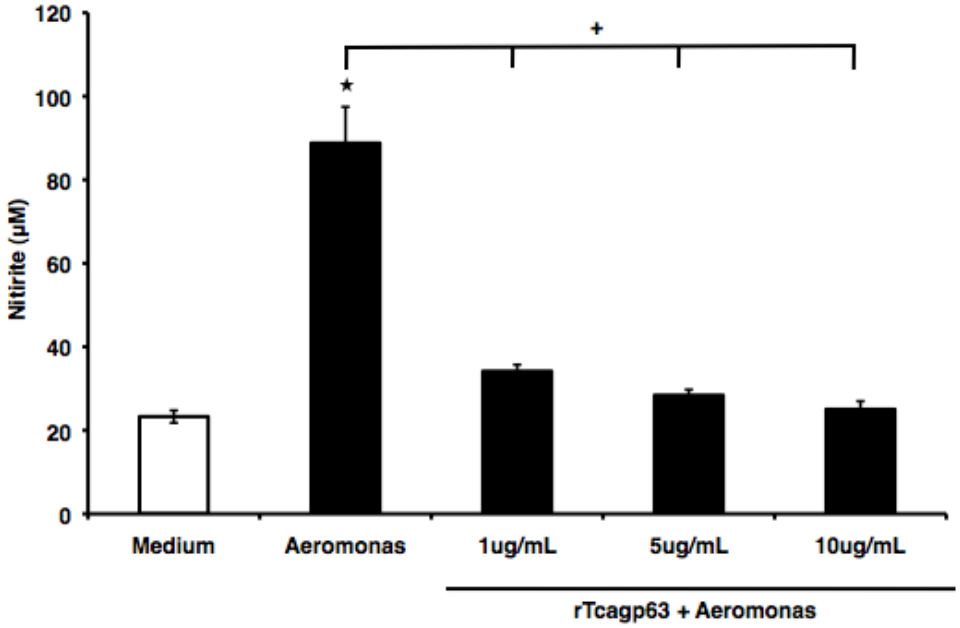

Figure 6.5. Recombinant *T. carassii* gp63 abrogated *A. salmonicida*-induced NO response of goldfish macrophages. NO production by goldfish macrophages was determined using the Griess reaction and nitrite concentration determined using a nitrite standard curve. The mean ± SEM nitrite production was determined for macrophage cultures established from six individual fish (n = 6). NO assay was performed twice with same results. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

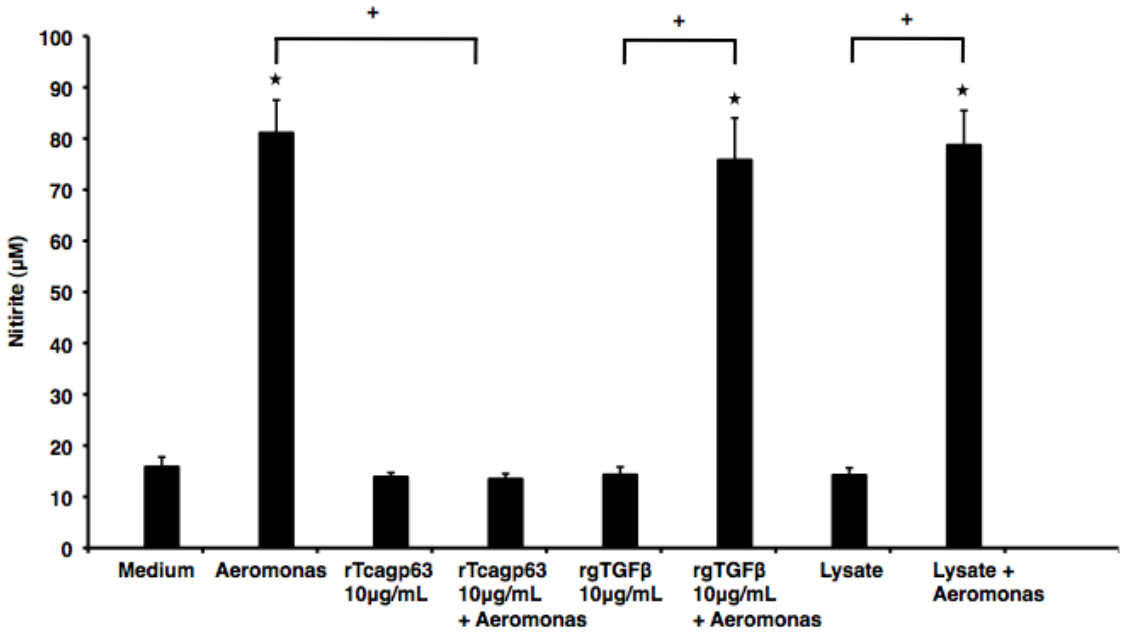

Figure 6.6. Abrogation of *A. salmonicida*-induced NO response of goldfish macrophages is due to rTcagp63. NO production by goldfish macrophages was determined using the Griess reaction and nitrite concentration determined using a nitrite standard curve. The mean ± SEM nitrite production was determined for macrophage cultures established from six individual fish (n = 6). NO assay was performed twice with same results. *E. coli* lysate and rgTGFβ purified using the same procedure as rTcagp63 have no effect on *A. salmonicida*-induced NO response of macrophages. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

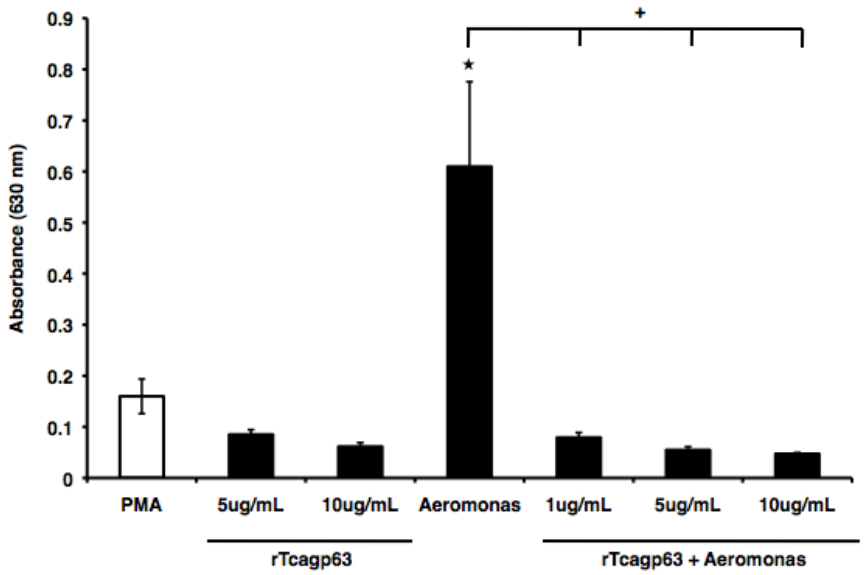

Figure 6.7. Recombinant *T. carassii* gp63 abrogated *A. salmonicida*-induced ROI response of goldfish monocytes. ROI (mean ± SEM) assay was performed with cells from 6 individual fish (n = 6) incubated in medium alone or with rTcagp63 using heat-killed *Aeromonas salmonicida* to stimulate production of reactive oxygen species. ROI assays were incubated at 20°C for 72 hours. NBT (2 mg/mL, Sigma) and PMA (final conc. 100 ng/mL, Sigma) in PBS was added to the cultures and incubated at room temp for an additional 30 minutes. The supernatants were aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

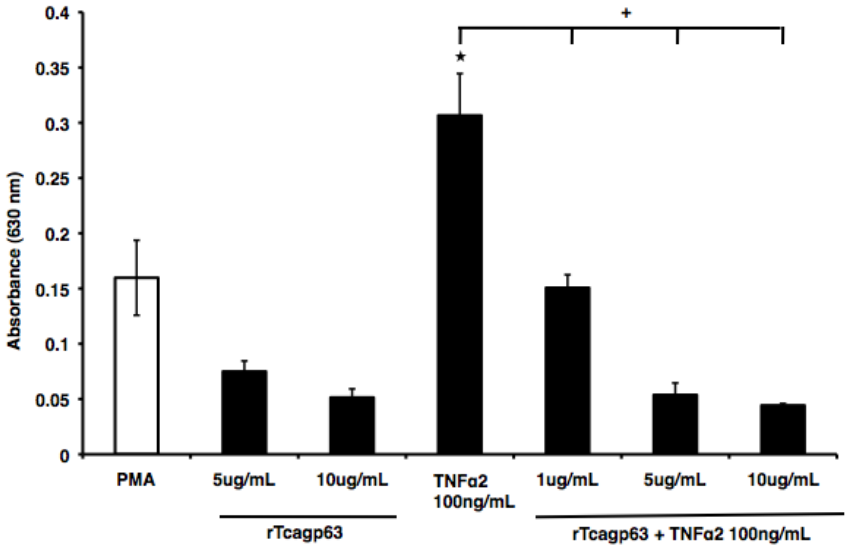

Figure 6.8. Recombinant *T. carassii* gp63 abrogated rgTNFα2-induced ROI response of goldfish monocytes. ROI (mean± SEM) assay was performed with cells from 6 individual fish (n = 6) incubated in medium alone or with rTcagp63 using rgTNFα2 (100 ng/mL) to stimulate production of reactive oxygen species. ROI assays were incubated at 20°C for 72 hours. NBT (2 mg/mL, Sigma) and PMA (final conc. 100 ng/mL, Sigma) in PBS was added to the cultures and incubated at room temp for an additional 30 minutes. The supernatants were aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

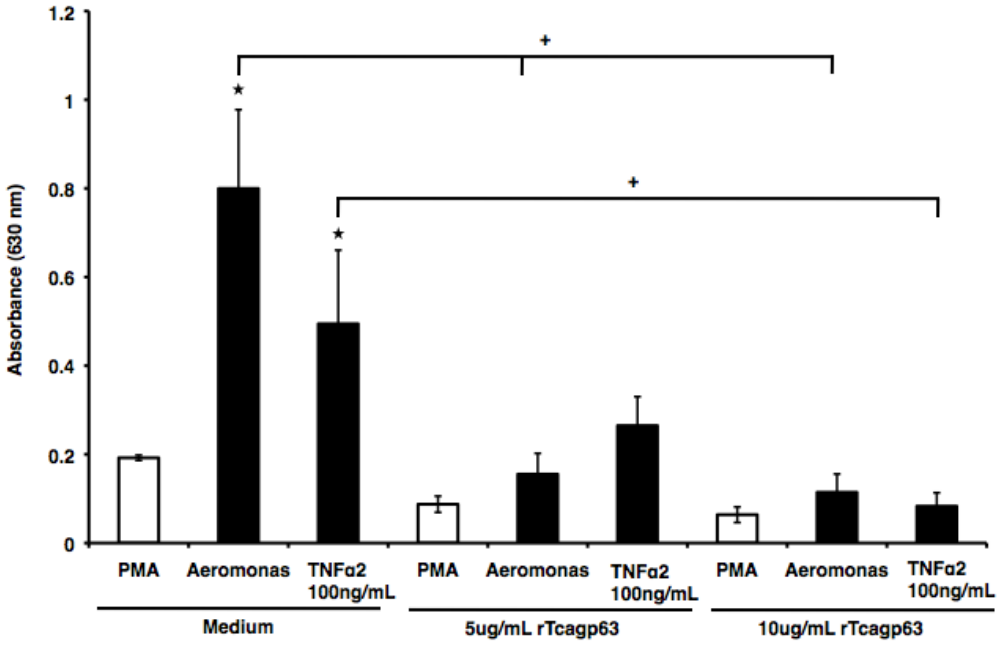

Figure 6.9. Pre-incubation of cells with rTcagp63 and subsequent washing failed to reverse *A. salmonicida* and TNFα2-induced ROI response of goldfish monocytes. ROI (mean± SEM) assay was set up with cells from 6 individual fish (n = 6) incubated in medium alone or with rTcagp63 (5 µg/mL and 10 µg/mL) for 2 hours. Cells were washed to remove the protease and used to set up ROI assay. Cells were stimulated with heat-killed *Aeromonas salmonicida* or rgTNFα2 (100 ng/mL) for production of reactive oxygen species. ROI assays were incubated at 20°C for 72 hours. NBT (2 mg/mL, Sigma) and PMA (final conc. 100 ng/mL, Sigma) in PBS was added to the cultures and incubated at room temp for an additional 30 minutes. The supernatants were aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

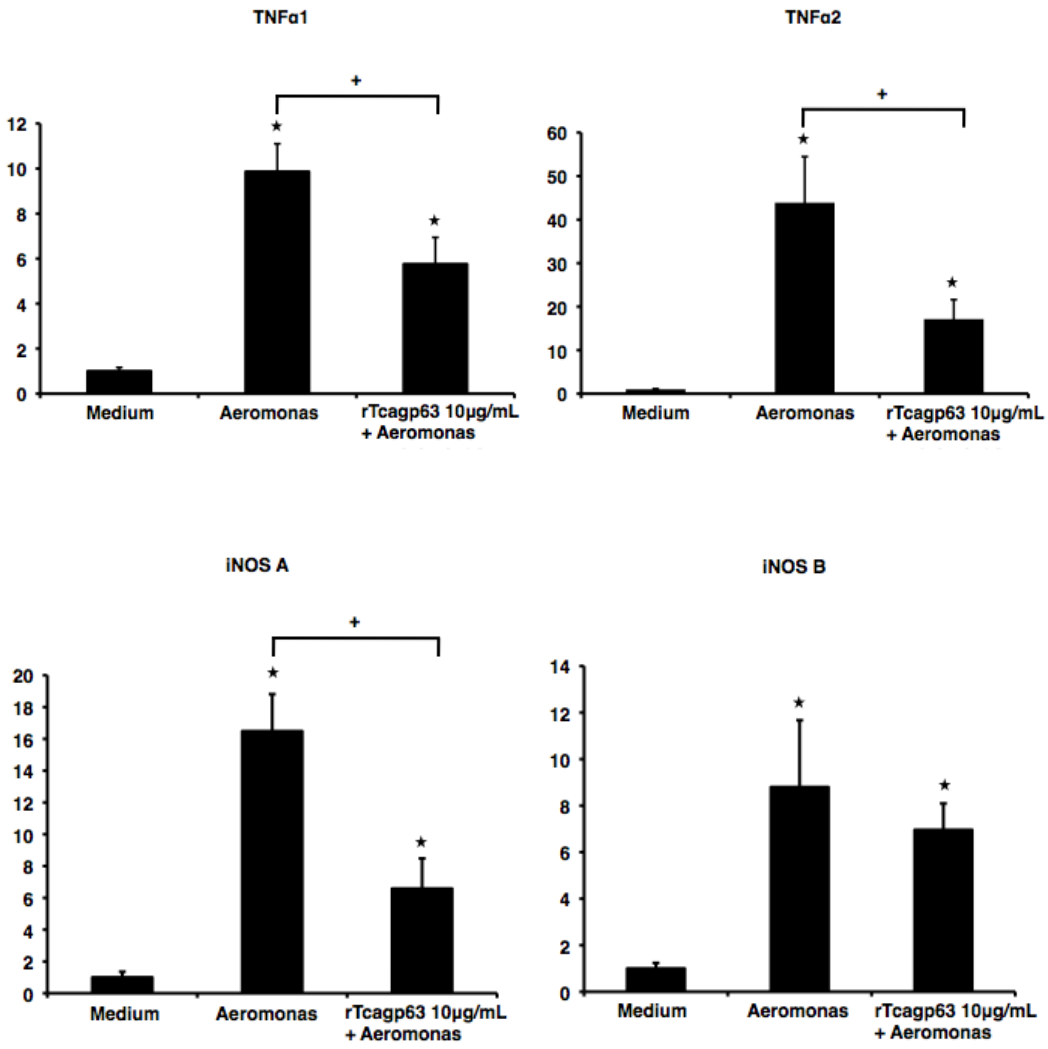

Figure 6.10. Analysis of immune gene expression of goldfish macrophages pre-treated with rTcagp63 before stimulation with *A. salmonicida*.
Quantitative expression of cytokines TNFα-1 and TNFα-2, iNOS A and B were reported relative to endogenous control EF-1α. The expression data were normalized against medium control for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. Expression levels were considered to be significant at $P < 0.05$. (*) denotes significantly different from the medium control. (+) denotes statistical difference ($P < 0.05$) between experimental groups.

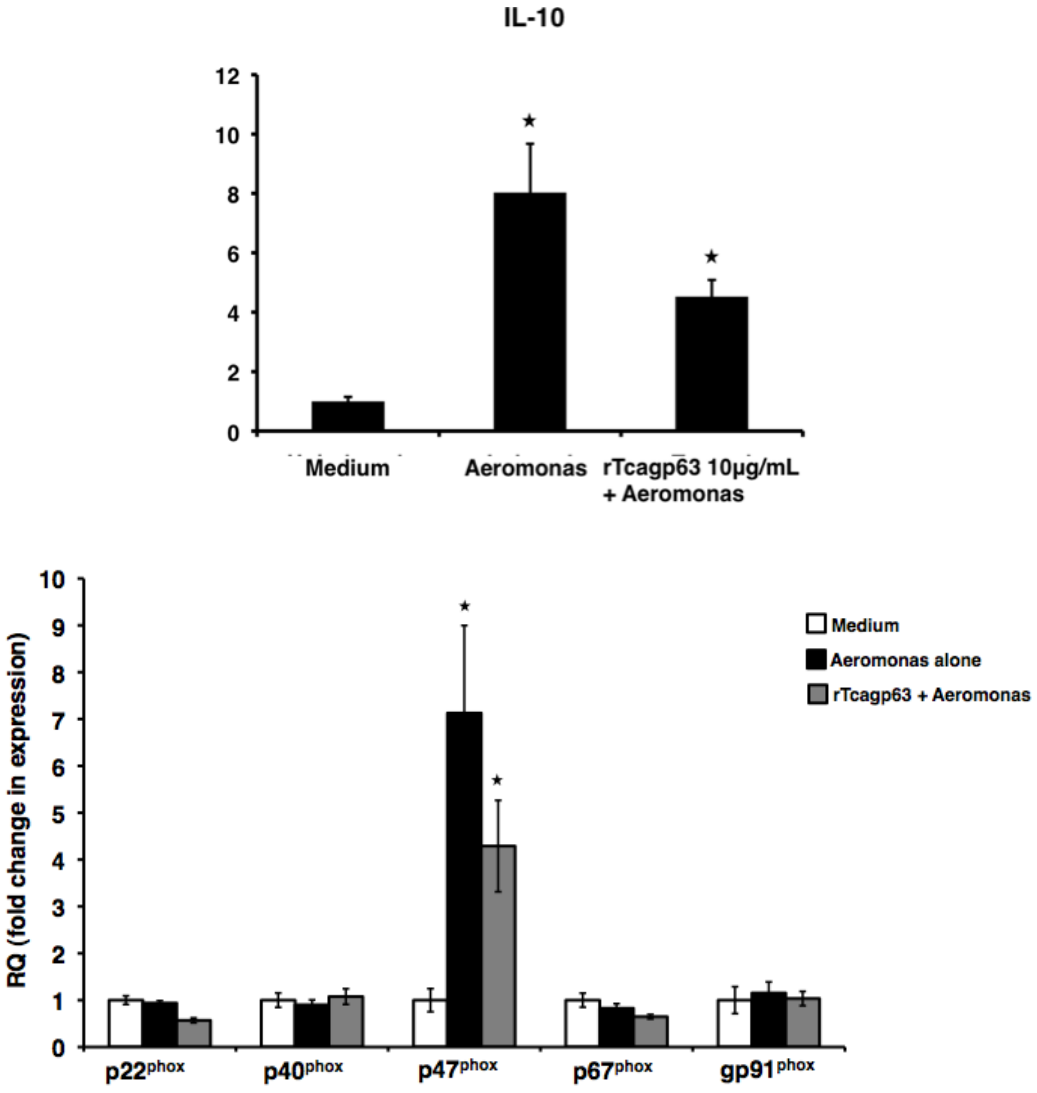

Figure 6.11. Analysis of IL-10 and NADPH oxidase components gene expression in goldfish macrophages and monocytes pre-treated with rTcagp63 before stimulation with *A. salmonicida*. Quantitative expression of IL-10 and NADPH oxidase component genes were reported relative to endogenous control EF-1α. The expression data were normalized against medium control for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. Expression levels were considered to be significant at $P < 0.05$. (*) denotes significantly different from the medium control.

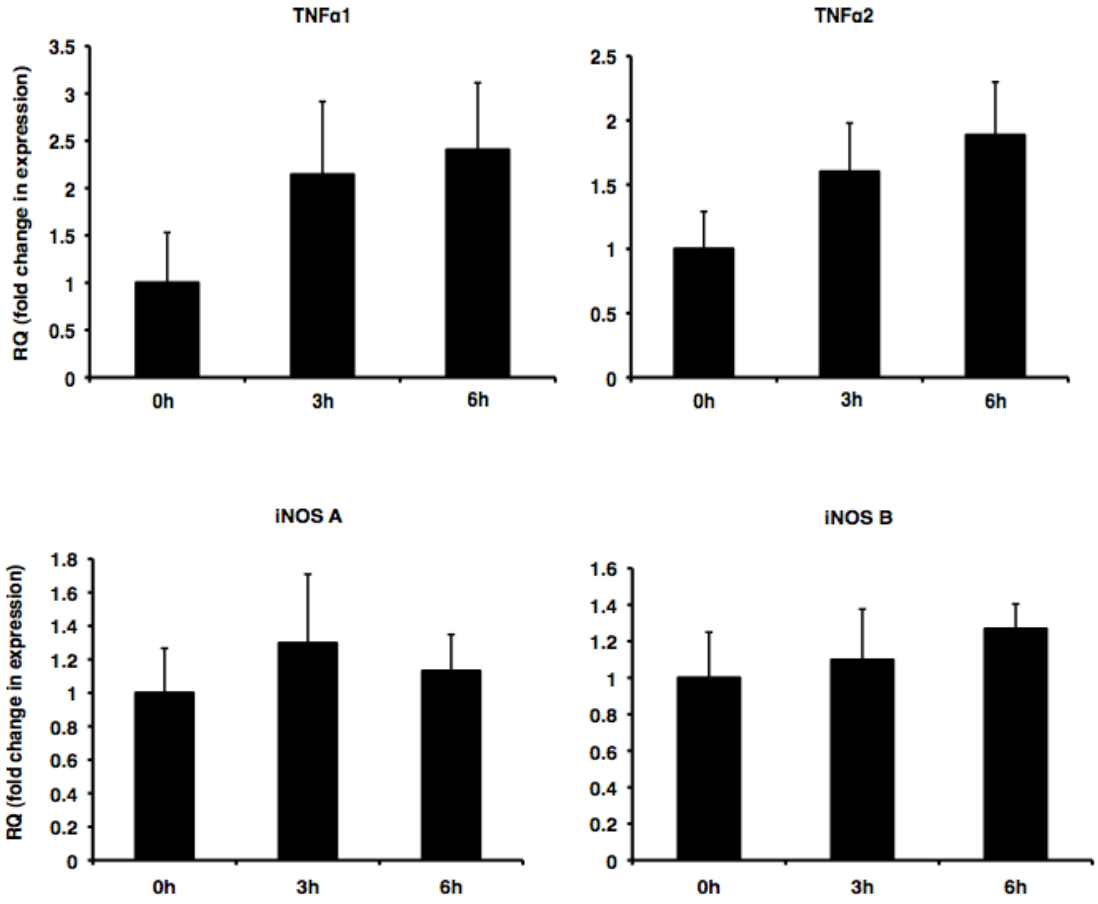

Figure 6.12. Analysis of immune gene expression of goldfish macrophages treated with rTcagp63. Quantitative expression of cytokines TNFα-1 and TNFα-2, iNOS A and B were reported relative to endogenous control EF-1α. The expression data were normalized against medium control for each gene. The results are mean ± SEM of macrophage cultures established from four individual fish (n = 4). Statistical analysis was done with one-way ANOVA. None of the expression levels were considered to be significant at $P < 0.05$.

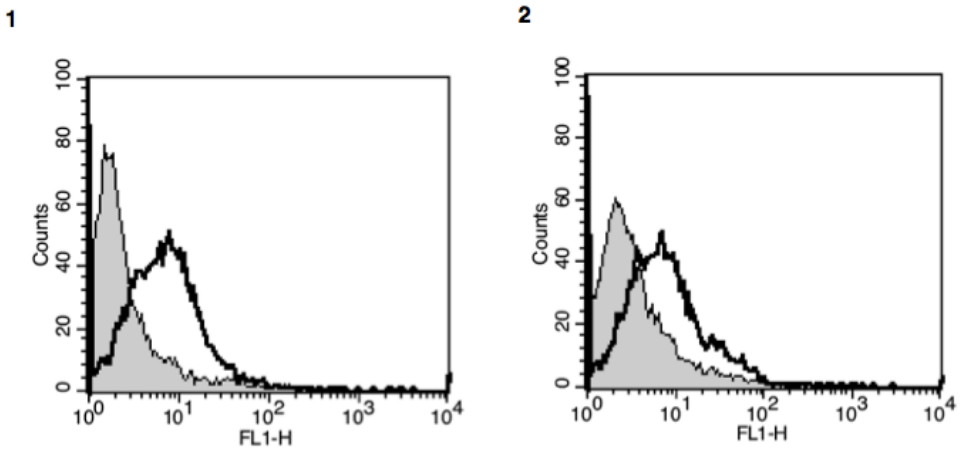

Figure 6.13. Recombinant *T. carassii* gp63 was bound to goldfish macrophages. Goldfish macrophage cultures obtained from two individual fish (n = 2) were used in all assays. 10^5 cells were washed twice and incubated with rTcagp63 (10 µg/mL) for 30 minutes. Cells were washed twice in PBS and incubated with affinity purified rabbit anti-rTcagp63 IgG (80 µg) or affinity purified rabbit IgG (80 µg) in PBS for 1 hour. Following incubation, cells were washed twice in PBS and incubated with FITC-labelled goat anti-rabbit IgG (1:100) for 1 hour. Cells were washed twice and analyzed by flow cytometry. A total of at least 10,000 cells were analyzed in each sample. 1 & 2 indicate results from separate experiments.

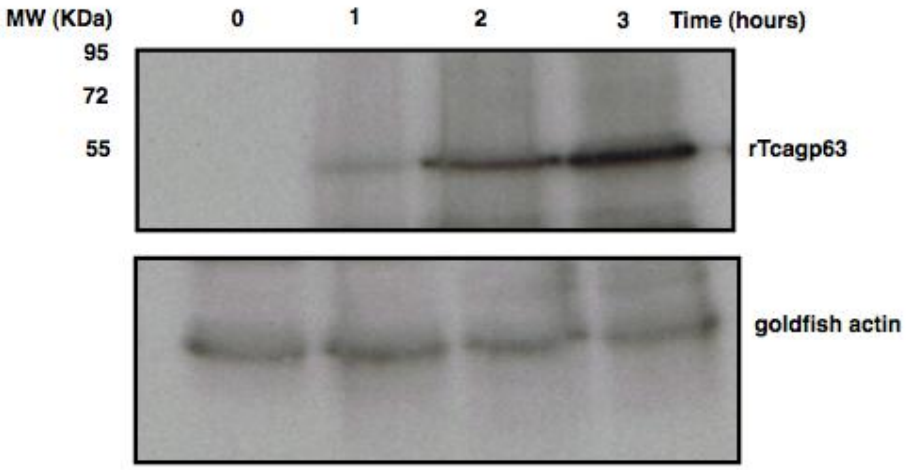

Figure 6.14. Recombinant *T. carassii* gp63 associates with goldfish macrophages. 10^7 cells were washed twice and incubated with rTcagp63 (10 µg/mL) for 0, 1, 2, 3 hours at 20°C. Cells were washed 3 times with PBS and lysed with 30 µL of RIPA buffer. rTcagp63 in lysate was detected with rabbit anti-rTcagp63 antibody (1:2000) and HRP-conjugated goat anti-rabbit IgG (1:3000). Blots were stripped as described and reprobed with rabbit anti-zebrafish actin polyclonal antibody (1:1000) and HRP-conjugated goat anti-rabbit IgG (1:3000) for detection of goldfish actin which was used as loading control.

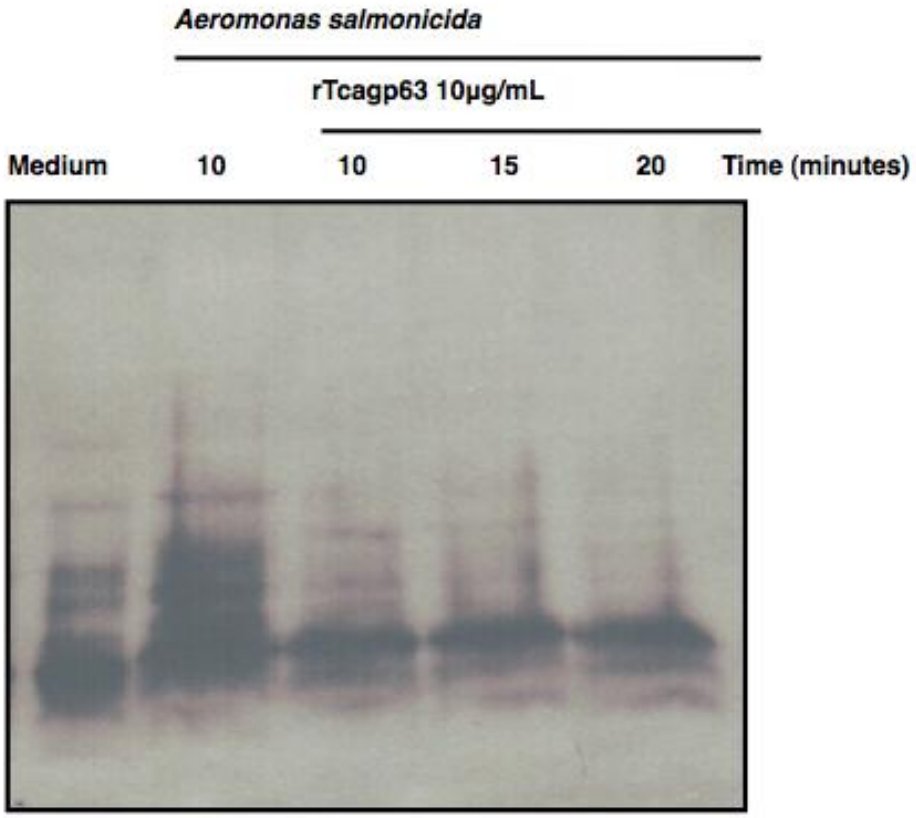

Figure 6.15. Recombinant *T. carassii* gp63 altered phospho-tyrosine protein pattern of goldfish macrophages. 10^7 cells were washed twice and incubated with medium alone (control) or with rTcagp63 (10 µg/mL) for 6 hours. Cells were stimulated with *Aeromonas salmonicida* at 20°C for 10, 15 and 20 minutes before lysed with RIPA buffer. Phospho-tyrosine protein patterns were analysed by western blotting with mouse anti-phosphotyrosine antibody (1:4000) and HRP-conjugated goat anti-mouse monoclonal antibody (1:3000). Results are representative of three individual fish.

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CHAPTER 7

Trypanosoma carassii CALRETICULIN BINDS HOST COMPLEMENT COMPONENT C1q AND INHIBITS CLASSICAL COMPLEMENT PATHWAY-MEDIATED LYSIS[1].

7.0 Introduction

Calreticulin (CRT) is a highly conserved multifunctional protein originally identified as a major calcium binding protein of the endo/sarcoplasmic reticulum [24]. CRT has since been reported in every cell of the higher organisms with exception of the erythrocytes. CRTs possess lectin-like chaperone activity, participate in lytic activity of perforins from T and natural killer cells, modulate gene expression, enhance phagocytosis of apoptotic cells, inhibit tumoral growth, mediate autoimmunity and inhibit C1q-dependent complement activation [13, 23]. All CRT proteins contain three structural domains: a globular N-terminal domain, a proline rich P domain and an acidic C-terminal domain. The N-terminal domain is involved in protein-protein interactions, RNA and autoantibody binding ([8, 9, 14]. The proline rich P domain binds Ca^{2+} with high affinity and low capacity while the C-terminal domain, which is the least conserved domain among CRTs, binds Ca^{2+} with low affinity [5, 24]. The CRT sequence starts with a signal peptide and ends with KDEL or related endoplasmic reticulum retention sequence [2, 14, 23].

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The role of CRT in host-parasite interactions has recently become an important area of research. CRT molecules from a number of human parasites have been cloned and these sequences display about 50% identity with human CRT [10]. Parasite CRTs bind host C1q and inhibit C1q-dependent complement activation [10, 15, 25, 34]. It has been reported that *Haemonchus contortus* CRT binds host C-reactive protein (CRP) and is present in the ES molecules of the adult worms [34, 35]. These studies indicate parasite CRT may inhibit activation of the classical pathway of complement by binding C1q or CRP. The binding of CRT to CRP, which has anti-parasite as well as procoagulant activities may prevent blood clotting and allow the parasite to feed longer on its host. The ectoparasite *Amblyomma americanum* secretes CRT during feeding [12] suggesting that CRT may use its anti-coagulant ability to prevent blood clotting and allow the parasite to feed longer on the host or divert host anti-parasite responses.

The presence of CRT in the penetration gland cells of schistosome cercariae suggests that this molecule may be important during penetration of host skin [16]. Parasite CRT is important in worm infection and has been reported to induce Th2 type immune response in mice infected with *Heligmosomoides polygyrus* [31]. Experimental vaccination with recombinant *Necator americanus* CRT in absence of adjuvant reduced worm burden by 43-49% in lungs of infected mice compared to control mice [38]. Among protozoan parasites, *T. cruzi* calreticulin (TcCRT) has been fairly characterized. TcCRT binds human C1q and specifically inhibits classical pathway of complement [2, 10]. TcCRT colocalized

with human C1q on parasite surface and antibody against TcCRT inhibits its interaction with human C1q [1, 10]. Taken together, these studies highlight the importance of parasite CRT during infection.

We have reported previously that *T. carassii* is resistant to complement-mediated lysis but removal of parasite surface proteins with trypsin increases their susceptibility to lysis in *in vitro* studies. Lysis occurred through the alternative complement pathway of goldfish since specific antibody was not required and magnesium but not calcium ion was required for lysis. Parasites regained resistance to lysis 6-24 hours following cultivation in the absence of trypsin and resistance to lysis was abrogated with a protein synthesis inhibitor, puromycin [30]. Although *T. carassii* shares similar surface architecture with *T. cruzi*, this fish parasite is also related to *T. brucei* by its extracellular existence. The extracellular nature of *T. carassii* suggests that host humoral responses may also play roles in parasite lysis *in vivo* through the classical pathway of complement. Thus, there is need to further investigate the importance of this pathway in control of infection and how parasite may evade it. This is the first report on the molecular characterization of CRT molecule of *T. carassii* (TcaCRT). My working hypothesis was that TcaCRT binds goldfish complement component C1q and inhibits the classical complement pathway. I showed that TcaCRT is located on parasite surface and is present in the supernatants of cultured parasites. Recombinant TcaCRT binds goldfish C1q *in vitro* and inhibits classical pathway-mediated lysis of sensitized sheep erythrocytes.

7.1 Experimental design

7.1.1 Molecular cloning of *T. carassii* calreticulin

TcaCRT was originally identified in surface protein fraction separated by 2-dimensional gel electrophoresis (2-DE) and analyzed by mass spectrometry (MS). TcaCRT peptides identified in MS analysis are (FYADAEK, CGGGYIK, YWLMFGPDR, EAPMIPNPK, QIPNPAYK). These peptides matched TcCRT with accession number (XM_799098). CRT fragment was amplified using primers designed to *T. carassii* spliced leader sequence and peptides identified in MS studies. The full sequence was amplified and the recombinant protein was produced in prokaryotic system. Purified rTcaCRT was used to immunize rabbits for production of polyclonal antiserum.

7.1.2 Assessment of TcaCRT localization using immunofluorescence and confocal microscopy.

The *in vitro* grown parasites were washed twice in serum-free medium and re-suspended to a concentration of 1×10^6/mL. 100 μL of parasites were incubated with 80 μg of rabbit anti-rTcaCRT IgG, rabbit anti-goldfish CSF IgG (isotype control) or with PBS for 24 hours at 20°C. Parasites were then spun onto poly-l-lysine coated glass slides using a Cytospin 2 at 55 x *g*, for 7 minutes. The parasites were fixed to the slides in methanol at −20 °C for 30 minutes and permeabilized using 1% Triton X-100 for 30 minutes at room temperature. Following permeabilization, the slides were washed 3 x for 20 minutes each in Milli-Q water. The slides were then blocked with 0.5% BSA in PBS for 30

minutes at room temperature. The slides were then washed 3 times for 20 minutes each in Milli-Q water prior to incubation with FITC-conjugated goat anti-rabbit IgG 1:100 dilution for 2 hours at room temperature. The cells were subsequently washed 3 times for 20 minutes each in Milli-Q water and observed using DIC imaging with a Leica confocal microscope.

7.1.3 Examination of interaction of goldfish complement component C1q with immobilized rTcaCRT.

To study the interaction between goldfish C1q and parasite CRT, 5 mg of purified rTcaCRT was coupled to CNBr-activated Sepharose (GE Healthcare) according to instructions provided by the manufacturer. Three mg of C1q containing serum fraction was loaded onto CRT-Sepharose column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$. The mixture was incubated at room temperature for 1 hour with gentle rocking to allow protein-protein interaction. Following incubation, the column was washed 4 times with 2 mL PBS. The bound proteins were subsequently eluted by increasing NaCl concentration (150 mM, 250 mM, 500 mM) and analyzed by SDS-gel electrophoresis. Proteins were transferred to nitrocellulose membrane and C1q was detected with anti-carp C1q-A antiserum using Western blot.

Binding of human C1q to immobilized rTcaCRT was examined to test whether CRT-C1q interaction is an evolutionarily conserved event. Varying concentrations of rTcaCRT diluted in coating buffer (15 mM of Na_2CO3, 35 mM of $NaHCO_3$, pH 9.6) were coated onto the wells of a microtitre plate (Corning)

100 μL per well for 6 hours at room temperature (22^{o}C to 25°C). Control wells received buffer alone or buffer with 3% w/v BSA. After each step, wells were washed 3 times with 0.05% Tween in PBS. Nonspecific binding sites were blocked with 3% w/v BSA in PBS for 1 h at room temperature. Following blocking, 500 ng of pure human C1q (Sigma-Aldrich) in 100uL in PBS/1% w/v BSA/0.05% Tween 20 was added to each well and the plate was incubated at 4°C overnight. To detect bound C1q, affinity-purified rabbit anti-human C1q antiserum (DAKO) 1:1500 dilution was added in 100 μL final volume and the plates incubated at room temperature for 2 hours. Wells were washed with PBS-Tween followed by the addition of 100μL affinity-purified goat anti-rabbit IgG-peroxidase conjugate (DAKO) 1:3000 dilution. Plate was kept at room temperature for 2 hours followed by washings with PBS-Tween. The bound peroxidase activity was measured by adding OPD (Sigma-Aldrich) as a substrate. The color produced was read at 492 nm in a microplate reader according to manufacturer's instruction.

Co-immunoprecipitation assay was performed to further show that TcaCRT interacts with goldfish C1q. Fifty μg of anti-rTcaCRT or rTcahsp70 was coupled to agarose resin according to manufacturer's instruction (Pierce). Fifty μg of rTcaCRT was incubated with 50 μg of C1q-containing fraction eluted from CNBr-activated sepharose column with 500 mM NaCl in the presence of 20 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ for 6 hours at 4°C. The proteins were then incubated with the prepared agarose resin for 1 hour at room temperature. The columns were washed and bound proteins were eluted and separated by SDS-

PAGE. Proteins were transferred to nitrocellulose membranes and eluted proteins were detected by Western blot.

7.1.4 Haemolytic assays

To assess the effect of parasite CRT on activation of classical pathway of complement, washed sheep erythrocytes (SRBC) re-suspended in 0.9% NaCl were sensitized by adding an equal volume of 1:250 diluted decomplemented rabbit anti-sheep erythrocyte antiserum in PBS containing 1 mM $CaCl_2$. Cells were washed thrice with 0.9% NaCl by centrifugation at 400 x *g* for 10 min. A total 10^7 sensitized erythrocytes in 0.9% NaCl containing 1 mM $CaCl_2$ was added to wells of a microtitre plate containing varying concentrations of TcaCRT or BSA mixed with fresh normal rabbit serum (1/100 dilution) in a total assay volume of 200 µL. The plate was incubated at 37°C for 45 minutes and then kept at 4°C for 3 hours. Haemoglobin in the supernatant was measured by adding 50 µL of supernatant to new wells containing 50 µL 0.9% NaCl and absorbance was measured at 415 nm. Total haemolysis was measured by lysing 10^7 sensitized erythrocytes with water and haemolytic activity was expressed as per cent of the total haemolysis.

7.2 Results

7.2.1 TcaCRT is present in membrane protein fraction and may be secreted

I [28] reported previously the identification of *T. carassii* hsp70 in ES products and surface protein fraction. TcaCRT was also identified in the surface

protein fraction (Fig. 7.1), suggesting surface expression of this molecule and a possibility that it may be secreted. I [27] cloned TcaCRT and expressed the recombinant protein in prokaryotic system. Alignment of TcaCRT with CRTs from other trypanosomatids is shown (Fig. 7.2). TcaCRT shares 62% similarity with TcCRT. The protein structure of TcaCRT compared with TcCRT is shown in Fig. 7.3. The recombinant protein, ES products and whole cell lysate were separated using SDS-PAGE and CRT was detected using Western blot with mouse anti-polyhistidine antibody (Fig. 7.4A), and rabbit anti-rTcaCRT antibody (Fig. 7.4B). Rabbit anti-rTcaCRT antibody reacted with TcaCRT in ES products (Fig. 7.4C) and native TcaCRT in whole cell lysate (Fig. 7.4D). Identification of TcaCRT in ES products was consistent with reports from other parasites that identified CRT in the ES products [11, 15, 32, 35]. Secreted *T. carassii* CRT has been suggested to play a role in immune evasion or immune modulation by parasites.

7.2.2 TcaCRT is expressed on parasite surface

That TcaCRT is identified in ES and surface protein fractions suggests surface expression of this molecule. To further show that TcaCRT is expressed on the parasite surface and may act as receptor for host C1q *in vivo*, I used indirect immunofluorescence. Parasites were incubated with rabbit anti-TcaCRT antibody, isotype control antibody or PBS. Bound antibodies were detected with FITC-conjugated goat anti-rabbit IgG (1:100). The result of confocal microscopy indicates TcaCRT is expressed on the surface of the parasites (Fig. 7.5C).

7.2.3 Anti-carp C1q-A antibody cross-reacts with goldfish C1q.

Interaction between parasite CRT and host C1q have been shown for *T. cruzi* and several heminths that infect humans [10, 15, 34, 35]. To assess possible interactions between goldfish C1q and TcaCRT, I examined whether carp anti-C1q-A antibody cross-reacted with goldfish C1q. C1q-containing serum fraction was prepared and protein fraction was separated by SDS-PAGE. Western blot of nitrocellulose containing serum proteins indicate anti-carp C1q-A antiserum recognized goldfish C1q (Fig. 7.6).

7.2.4 rTcaCRT binds goldfish C1q *in vitro*

I showed that rTcaCRT immobilized on activated sepharose retained structural conformation necessary for interaction with host C1q (Fig. 7.7A). Goldfish C1q bound immobilized CRT, as well as other serum proteins. Non-specifically bound proteins were removed by washing the column 4 times with PBS and with 150 mM NaCl. C1q was specifically eluted at higher salt concentration (500 mM) and recognized by anti-carp C1q-A antiserum (Fig 7.7B). C1q is a large molecule of about 460KDa composed of 18 polypeptide chains held together by disulfide bonds [4, 33]. The individual polypeptide chains are tightly held in the C1q complex and are difficult to separate from this complex. Since protein separation was performed on SDS-PAGE under denaturing and reducing condition, it is likely that the three prominent protein bands seen on the stained blot are different subunits of C1q. I used anti-carp-C1q-A antibody, which was raised against carp C1q-A for detection and this antibody may not recognize C1q-

B and C1q-C. Alternatively, these bands may be degradation products of C1q, which can no longer be recognized by anti-carp C1q-A antibody. Calreticulin also binds other serum proteins including members of the collectin family mannose binding protein (MBP), conglutinin and lung surfactant protein (SP-A). The binding of host CRP with a molecular weight of 24KDa and blood clotting protein Factor X with molecular weight of 55KDa to immobilized parasite CRT has been reported [34, 35].

The binding of other serum proteins to immobilized rTcaCRT may prevent rTcaCRT from binding most of the C1q in the serum fraction, which may explain the detection of C1q in the unbound fraction. To further demonstrate that CRT bound host C1q, I employed far Western blot technique. C1q-containing serum fractions were separated using native PAGE and transferred to nitrocellulose membranes. Blots were incubated with rTcaCRT or PBS as control. Following washes with TTBS and TBS, bound rTcaCRT was detected using rabbit anti-rTcaCRT IgG, while non bound rTcaCRT was detected on blots incubated with PBS alone (Fig. 7.8A and B).

Co-immunoprecipitation experiments were done to examine the interaction between rTcaCRT and goldfish C1q. Rabbit anti-rTcaCRT IgG was coupled to agarose resin and used to precipitate rTcaCRT and goldfish CIq. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with rabbit anti-carp C1q-A antiserum (1:1500), mouse monoclonal anti-polyhistidine antibody (1:5000) and rabbit anti-rTcaCRT antibody (1:1500). Blots were washed and proteins bands

detected with alkaline phosphatase conjugated goat anti-rabbit IgG (1:3000) or goat anti-mouse IgG (1:1500). C1q in the immunoprecipitate was detected using anti-carp C1q-A antiserum (Fig. 7.9, lane 1). TcaCRT in the immunoprecipitate was detected with anti-histidine antibody (Fig. 7.9, lane 2) and anti-rTcaCRT antibody (Fig. 7.9, lane 3). No proteins were detected in elution from the control resin (Fig. 7.9, lane 4) and elution from anti-rTcahsp70 column (Fig. 7.9, lane 5).

7.2.5 rTcaCRT binds human C1q

Heligmosomoides polygyrus is a parasitic nematode of mice whose CRT molecule has been reported to bind human C1q [31]. For this reason, I investigated whether TcaCRT can interact with human C1q using ELISA. Result of ELISA shown in Figure 7.10 indicates that like *H. polygyrus* CRT, the *T carassii* recombinant CRT bound human C1q, suggesting an evolutionarily conserved interaction irrespective of species.

7.2.6 rTcaCRT inhibits C1q-dependent complement lysis

The implications of CRT-C1q interaction was investigated using classical complement pathway-dependent haemolysis of sensitized sheep erythrocytes (Fig. 7.11). I used 1/100 dilution of normal rabbit serum as source of complement since majority of C1q is associated with C1r and C1s under normal physiological conditions. Under these conditions, we observed 80-100% haemolysis in distilled water. Lysis of sensitized sheep erythrocytes was significantly inhibited in the presence of rTcaCRT as shown by decreased haemolytic activity (15.33% to

13.62%) compared to BSA control that showed no significant decrease in haemolytic activity (94.33% to 79.29%) (Fig. 7.11). Addition of anti-rTcaCRT restored hemolysis to about 80% suggesting that inhibition of hemolysis is due to rTcaCRT.

7.3 Discussion

During host-parasite interaction, important roles of parasite molecules present in ES may include helping parasites to establish infection, protect parasite from host immune molecules by binding those molecules away from parasites, divert immune responses away from parasites by causing non-specific stimulation of immune response and tissue penetration [11, 28, 32]. *T. carassii* is an extracellular pathogen and molecules secreted by parasites may play roles in evasion or modulation of host immunity. It has been demonstrated in previous studies that *in vitro* shed ES antigens from *T. carassii* confer protection against challenge infection [6]. I [27, 28] originally identified parasite TcaCRT in 2-DE and mass spectrometry analysis of parasite surface protein fractions suggesting surface expression of this molecule. For this reason, I examined the ES products of parasites and identified secreted TcaCRT in the ES products. In *T. cruzi* cell surface expressed TcCRT has been shown to interact with host C1q [10], providing the parasites with a strategy to inhibit C1q-dependent activation of the classical pathway. The ability of CRT molecules of human helminth parasites to interact with host C1q the recognition molecule of the classical pathway of complement has been documented [15, 25]. In this study, I demonstrated that

CRT from the fish parasite *T. carassii* interacted with host C1q and inhibited C1q-dependent haemolytic activity *in vitro*. I [27] showed that TcaCRT was expressed on parasite surface and that it may provide the parasites with an elegant strategy to evade complement-mediated lysis. This is the first report of CRT-C1q interaction in ectothermic vertebrates.

CRT was also shown to interact with CRP [34], and since CRP may activate the classical complement pathway by binding to C1q, this may be another mechanism by which CRT may influence complement-mediated lysis. As an acute phase protein, CRP has been shown to exhibit antiparasite effects. For example, the incubation of adult *Hymenolepis diminuta* in serum containing CRP resulted in a loss of motility and damaged tegument, reminiscent of damage caused by complement [36]. CRP-depleted serum showed no antiparasitic activity while de-complemented serum was fully active suggesting that this effect was CRP-specific. These findings suggest CRT-CRP interactions may have important physiological role in parasite survival and we have initiated studies to further characterize CRP-CRT interactions in goldfish infected with *T. carassii*.

Although recombinant TcaCRT failed to bind calcium probably due to improper folding resulting from expression in prokaryotic system, it retained the structural architecture required for interaction with C1q, as indicated by the inhibition of C1q-mediated haemolysis, following addition of rTcaCRT. Also, functional forms of CRT have been produced in prokaryotic system [5].

I [27] showed that TcaCRT interacted with goldfish C1q and inhibited classical complement pathway-mediated haemolysis of sensitized sheep

erythrocytes. TcaCRT was secreted by the *in vitro*-grown trypanosomes and indirect immunofluorescence showed that this molecule is abundantly expressed on the parasite surface. The presence of TcaCRT on parasite surface may provide parasite with an elegant means to inhibit C1q-mediated activation of classical complement pathway. Additionally, secreted CRT molecules may perform other functions such as binding of host molecules away from the parasites, non-specific stimulation of immune responses or immunosuppression. That TcaCRT also bound human C1q suggests an evolutionarily conserved interaction between these two molecules. This observation was further supported by our haemolytic assay results, demonstrating that the interaction of rTcaCRT with C1q in normal rabbit serum inhibited classical complement pathway. Parasite CRTs have been reported to possess multiple C1q-binding motifs, which are thought to enhance specific interaction with host C1q molecules [25], and it was suggested that multiple C1q-binding motifs in parasite CRTs may be an evolutionary strategy to compensate for multipoint interaction between antibody and C1q to ensure complete inactivation of the classical complement pathway [25]. The presence of such multiple C1q motifs in parasite CRTs may allow CRTs to bind diverse C1q molecules.

It has been documented that complement-mediated lysis of *T. cruzi* was primarily due to the action of alternative compement pathway since serum depletion of factor B and Properdin abrogated lysis of IgG-coated trypanosomes [20]. The classical pathway appeared to be ineffective in parasite lysis, but its role was to amplify the lytic effects of the alternative complement pathway. Sera from

individuals that are genetically deficient in the classical pathway component C2 lysed 50% of IgG-coated parasites compared to controls treated with normal sera and addition of increasing amount of purified C2 to the deficient sera restored lysis to levels comparable to that of controls [20]. We have previously shown that trypsin treatment of *T. carassii* increases their susceptibility to lysis by the alternative complement pathway of goldfish, suggesting a similar mechanism to lysis of *T. cruzi.* Parasites regained resistance to lysis 6-24 hrs after cultivation in the absence of trypsin and resistance to lysis was abrogated with puromycin a protein synthesis inhibitor [30]. The expression of CRT on the parasite surface may be the reason why the classical compement pathway appears to play a minor role in control of *T. cruzi.* Similarly, it is probable that *T. carassii* CRT present on the surface of the trypanosomes may inhibit the classical complement pathway. This would provide the parasite with a strategy to interfere with host complement system at the earliest stage of complement activation. *T. carassii* is an extracellular parasite like the African trypanosomes but does not exhibit antigenic variation [29]. The similarity in surface architecture between *T. cruzi* and *T. carassii* suggest presence of major complement regulatory proteins in *T. carassii* that are similar to that of *T. cruzi* [22, 26]. The TcaCRT may be part of a cascade of complement binding molecules elaborated on the surface of *T. carassi* that may be used in evasion of host immune attack.

T. cruzi infected individuals produce variable levels of specific antibodies against TcCRT in their serum [3, 21] and these antibodies may inhibit TcCRT-human C1q interaction which may benefit the hosts. Indeed antibody raised

against TcCRT inhibits its interaction with human C1q *in vitro* [1]. In this study, I observed that that the addition of anti-rTcaCRT antibody restored haemolysis of sensitized erythrocytes *in vitro*. Since TcaCRT shows 62% similarity to TcCRT, it possible that TcaCRT will have same functional effects as TcCRT. Furthermore, during the course of chronic *T. cruzi* infection, vertebrate hosts produce antibodies that convert complement resistant trypomastigotes into complement sensitive parasites. These antibodies have been referred to as lytic antibodies [18] and are responsible for antibody-dependent complement lysis of parasites primarily via the alternative pathway [7, 20]. It has been demonstrated that Fab and Fab antibody fragments derived from the IgG of Chagasic patients promoted complement-mediated lysis of *T. cruzi* trypomastigites [17] indicating that the lysis-promoting activity of antibodies occur independently of Fc portion of antibodies .

At present, anti-TcCRT antibody is not classified as a lytic antibody perhaps because of the inefficient nature of the classical pathway-mediated lysis on trypanosomes. However, a number of *T. cruzi* molecules, which includes the major complement regulatory proteins that elicit production of lytic antibodies in the host, have been reported [18]. Characterization of parasite molecules that elicit lytic antibodies is hampered by the fact these antibodies are difficult to elicit by immunization with dead parasites or with purified parasite antigens [19]. However, lytic antibodies have been elicited by inoculation with attenuated *T. cruzi* trypomastigotes and with trypomastigotes ES antigens [37]. These observations suggest that molecules that elicit lytic antibodies are labile and shed

proteins are important and can sufficiently confer complement resistance.

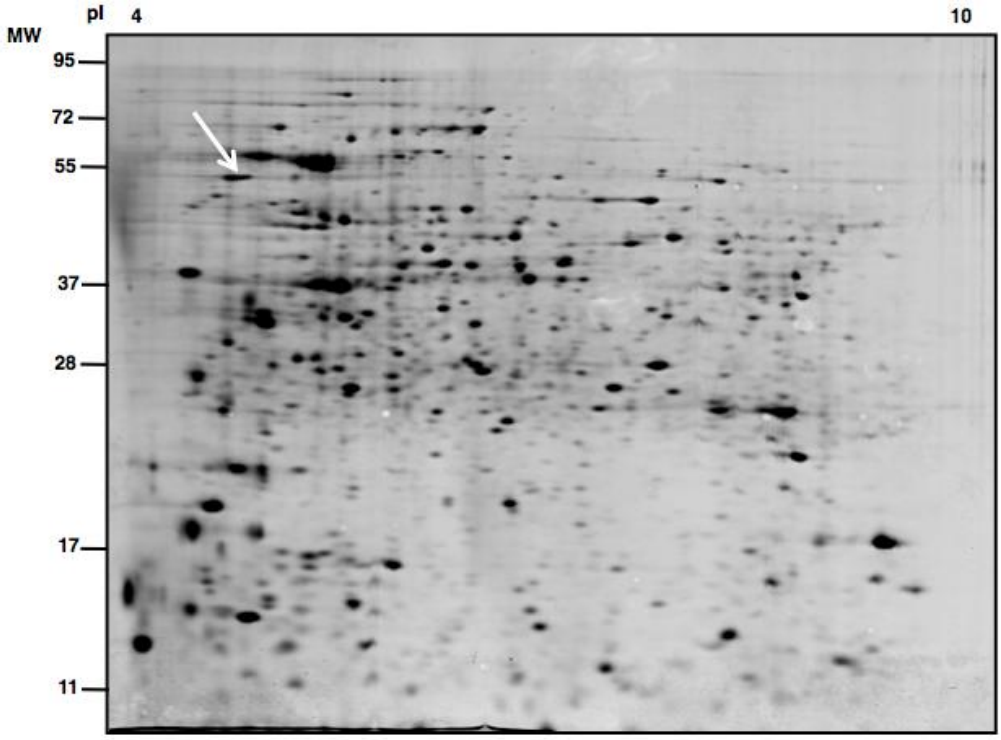

Figure 7.1. A representative gel of two-dimensional gel electrophoresis of *T. carassii* membrane proteins. Five hundred micrograms of Triton X-114 membrane solubilized proteins were separated in the first and second dimensions. Proteins were visualized using Sypro® ruby fluorescent stain and spots were selected for MS analysis. TcaCRT is shown with the arrow.

Figure 7.2. Amino acid alignment of *Trypanosoma carassii* calreticulin (TcaCRT) sequence and those from *T. cruzi* and *T. brucei*. Sequences were obtained from GenBank accession numbers *T. cruzi* (XM_799098), *T. brucei* (XM_842477). (*) indicates identical residues, (:) indicates conserved residues while (.) indicates semi-conserved residues. (-) are gaps introduced to maximize the alignment. The possible cleavage site for TcaCRT molecule is indicated with arrow. A bar denotes the endoplasmic reticulum retention signal sequences at the C-terminal end of the molecules.

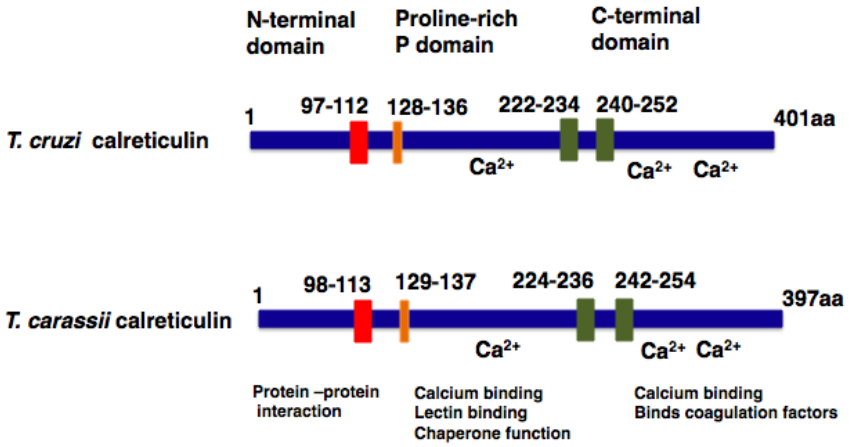

Figure 7.3. Schematic of protein structure of *T. carassii* CRT. *T. carassii* CRT was compared with *T. cruzi* CRT. The coloured boxes represent CRT family signatures. The approximate locations of the CRT family signatures are shown. The three domains of CRT molecules, their approximate positions and functions are indicated.

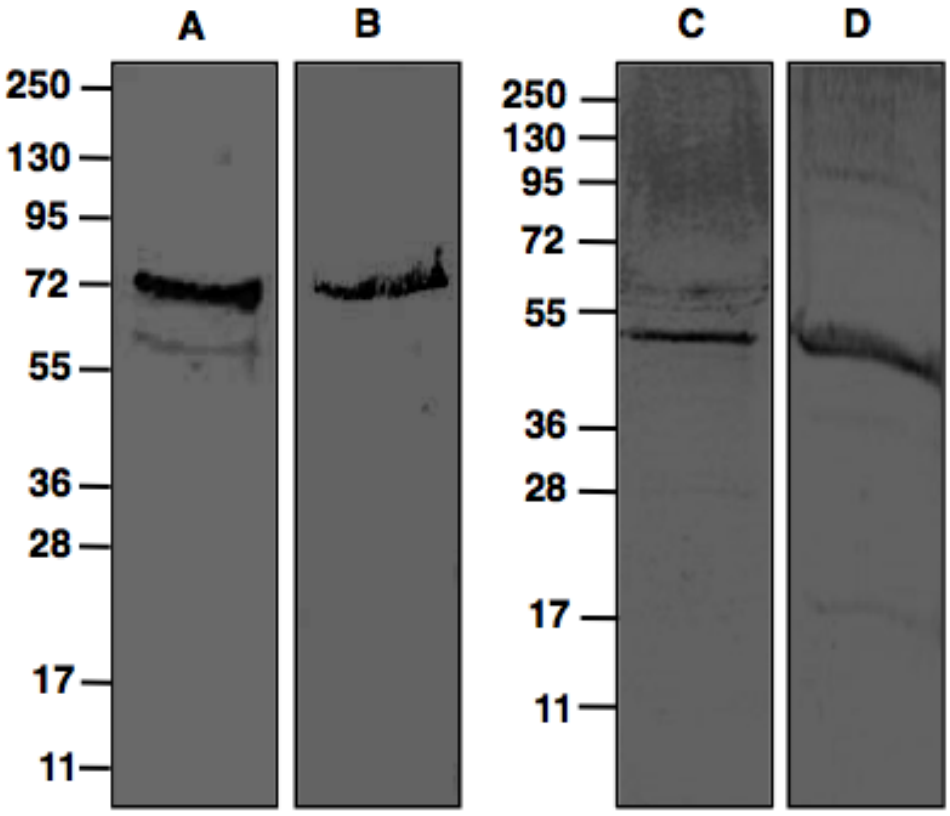

Figure 7.4. Rabbit anti-recombinant TcaCRT affinity purified rabbit IgG polyclonal antibody recognizes TcaCRT in ES products and whole cell lysate of *T. carassii*. rTcaCRT (A and B), concentrated *T. carassii* ES products (C) and whole cell lysates (D) were separated by SDS-PAGE and transferred to nitrocellulose. (A) Western blotting of rTcaCRT performed with mouse anti-polyhistidine antibody (1: 5000). (B) Western blot of rTcaCRT with rabbit anti-rTcaCRT IgG (1:1500). (C) Western blot analysis of *T. carassii* ES products using rabbit anti-rTcaCRT IgG (1: 1500). (D) Western blot of *T. carassii* whole cell lysate with rabbit anti-rTcaCRT IgG (1: 1500). The difference in size of the recombinant molecules compared to the native molecules is due to the additional vector sequence.

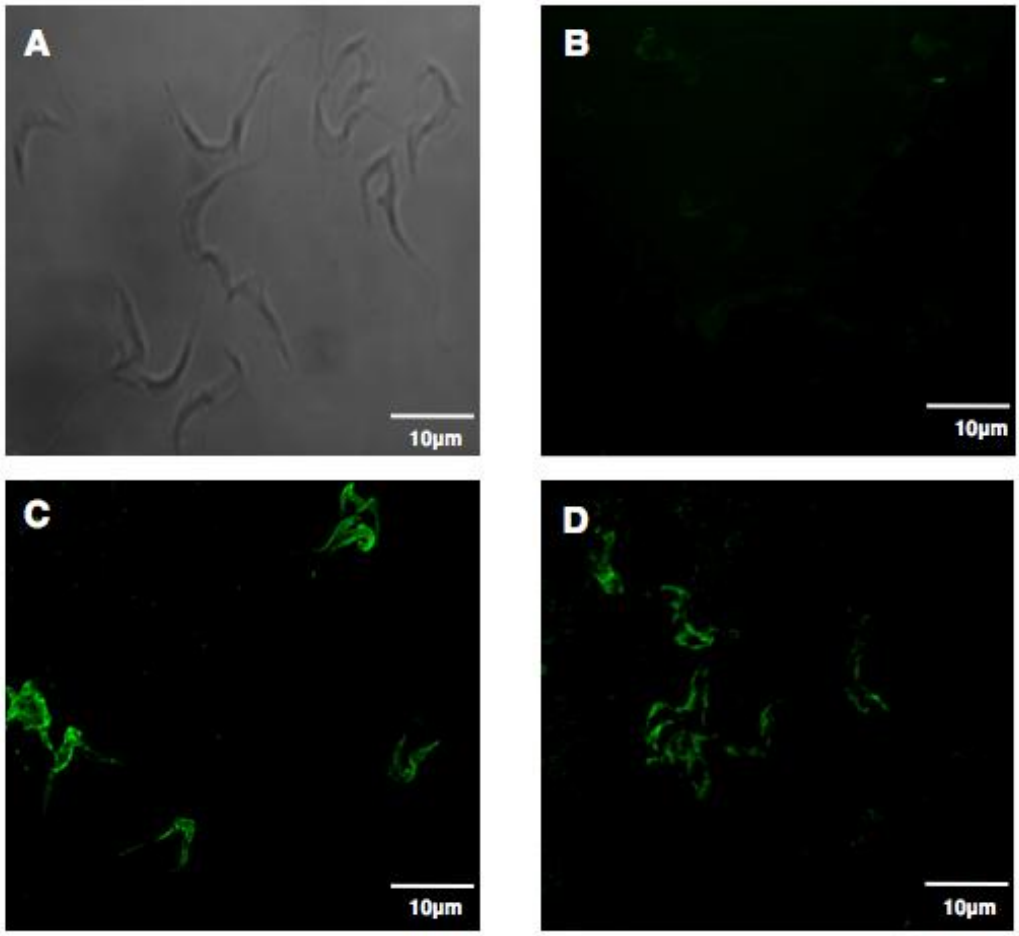

Figure 7.5. *T carassii* CRT is expressed on the parasite surface. Trypanosomes were fixed, blocked and incubated with PBS, rabbit anti-goldfish rCSF IgG or rabbit anti-rTcaCRT IgG as described. TcaCRT expression was detected by staining with FITC-conjugated goat anti-rabbit IgG (1:100). No fluorescence was observed when slides were incubated with the PBS control (A). Background staining was observed with cells incubated with anti-goldfish rCSF (B). Cells incubated with rTcaCRT IgG show surface staining indicating TcaCRT expression on parasite surface (D) and internal structures (C).

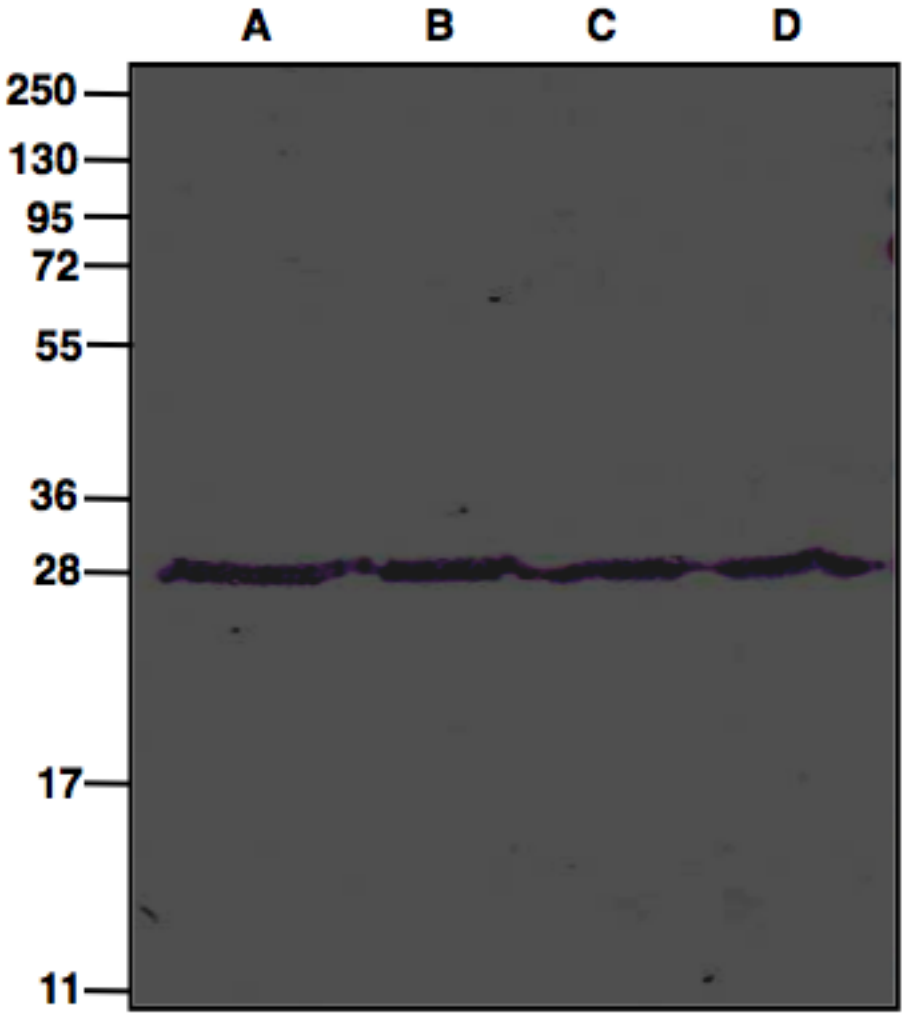

Figure 7.6. Rabbit anti-carp C1q-A antiserum recognizes goldfish C1q. Sera were collected form carp and goldfish blood and C1q fraction obtained by PEG precipitation. Two μL of goldfish or carp serum (lanes A and D) and 5 μg of goldfish or carp PEG 4000 precipitated C1q-containing fractions (lanes B and C) were separated by SDS-PAGE and transferred to nitrocellulose membrane. C1q was detected using rabbit anti-carp C1q-A antiserum (1:1500) and AP-conjugated goat anti-rabbit IgG (1:3000).

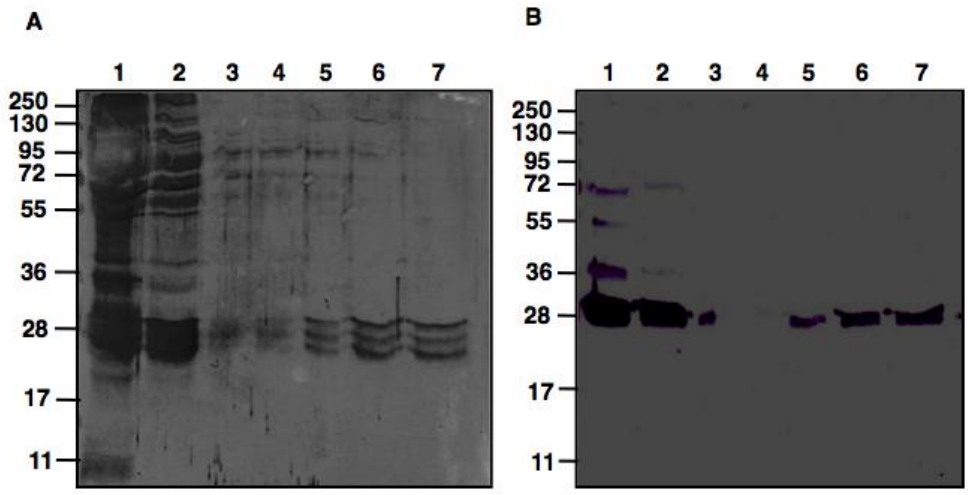

Figure 7.7. Goldfish C1q binds immobilized rTcaCRT. Following coupling of rTcaCRT to CNBr-Sepharose, C1q-containing serum fraction was added to the column. CRT-C1q column was incubated at room temperature to allow for protein interaction and column was washed with phosphate-buffered saline. C1q was eluted using 500 mM NaCl. Eluted fractions were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were stained with colloidal gold total protein stain (Bio-Rad) (A). Western blot using rabbit anti-carp C1q-A antiserum (B). Protein concentration was similar in all lanes of the gels. Lane 1: C1q-containing serum fraction before addition to the column. Lane 2: Unbound proteins eluted from column after rTcaCRT-C1q interaction. Lanes 3 and 4: Proteins eluted using 150 mM NaCl following washes with PBS. Lane 5: Proteins eluted using 250 mM NaCl. Lanes 6 and 7: Proteins eluted using 500 mM NaCl.

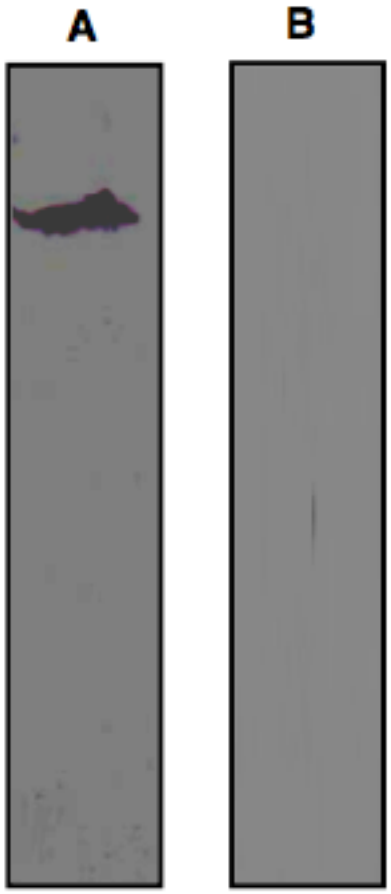

Figure 7.8. The far western blot showing that goldfish C1q interacted with rTcaCRT. Fifteen μg of C1q-containing fraction was separated by native gel electrophoresis and transferred to nitrocellulose membrane. Blots were incubated with 200 μg of rTcaCRT in PBS or PBS alone for 3 hours at room temperature. Following washing with TTBS and TBS, blots were incubated with rabbit anti-rTcaCRT IgG (1:1500) for 3 hours at room temperature. Bound rTcaCRT was detected using AP-conjugated goat anti-rabbit IgG (1:3000) (A), and no bound CRT was detected on blot incubated with PBS (B).

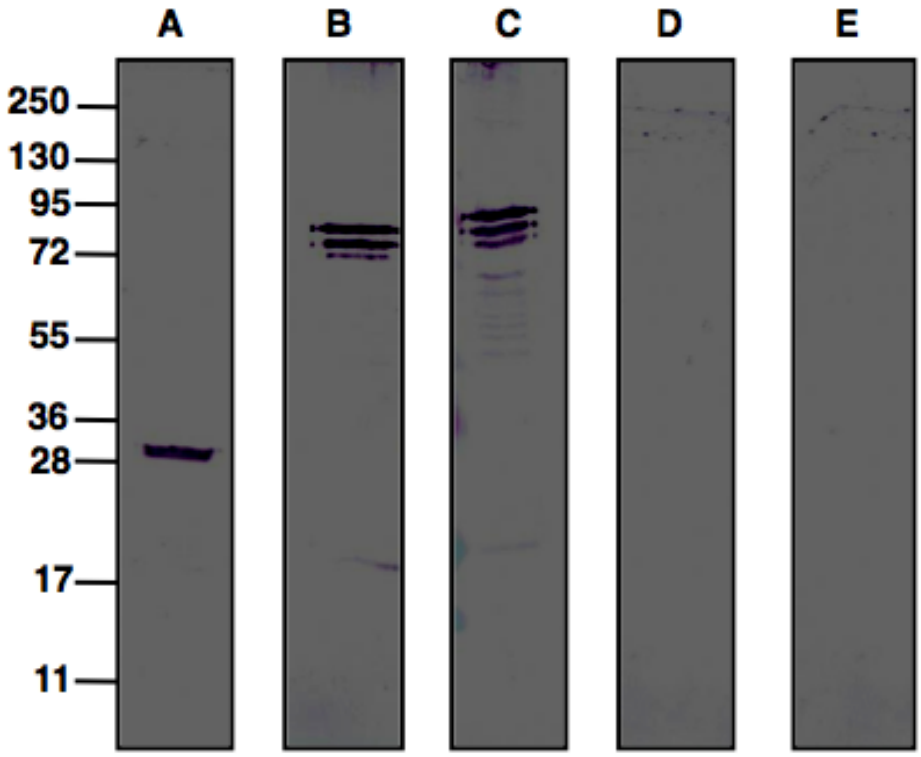

Figure 7.9. Goldfish C1q interacted with rTcaCRT. Recombinant TcaCRT and C1q were co-immunoprecipitated as described. Goldfish C1q was detected with anti-carp C1q-A antiserum (1:1500) (lane A), rTcaCRT was detected using anti-histidine antibody (1:5000) (lane B) and anti-rTcaCRT antibody (1:1500) (lane C). Characteristic degradation of recombinant CRTs was observed with TcaCRT. No C1q was detected in elution from control resin (lane D) and rTcahsp70-coupled resin (line E).

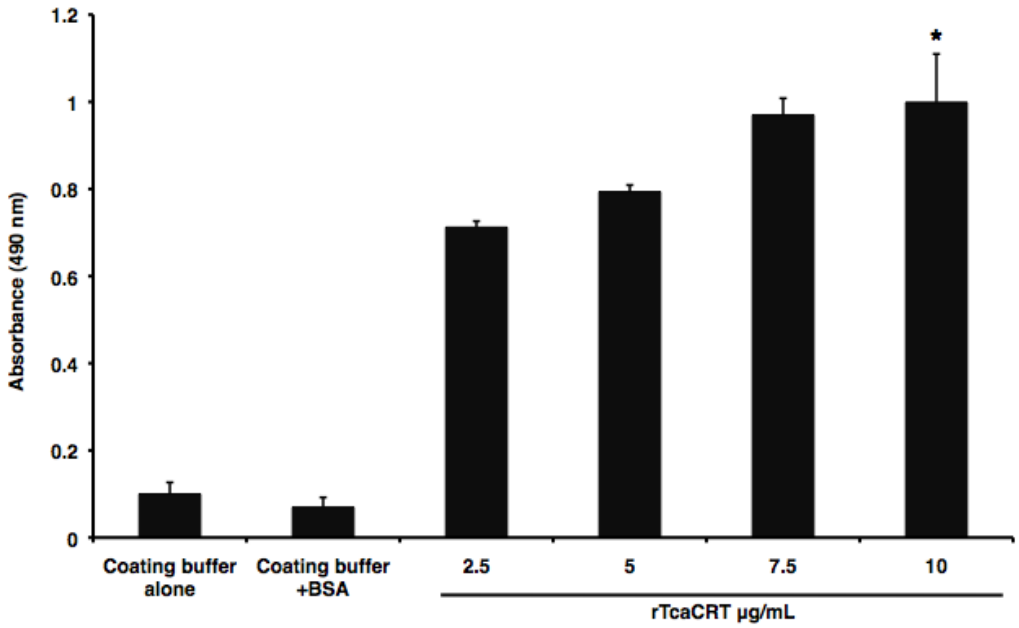

Figure 7.10. *T. carassii* CRT binds human C1q. Varying concentrations of rTcaCRT were coated onto wells of ELISA plate as described. Control wells were coated with buffer alone or coating buffer containing BSA. Wells were blocked with PBS/1% w/v BSA/0.05% Tween 20 before incubation with pure human C1q. Bound C1q was detected using anti-human C1q IgG (1:1500) and HRP-conjugated goat anti-rabbit IgG (1:3000). Statistical analysis was done using one-way ANOVA. (*) denotes significantly different ($P < 0.05$) from coating buffer alone or coating buffer + BSA. The experiment was performed twice with similar result.

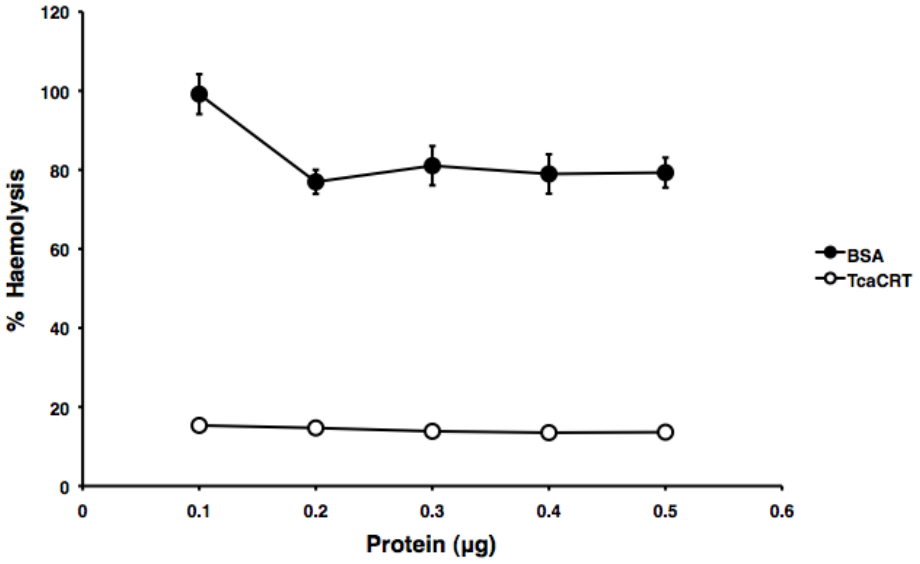

Figure 7.11. Recombinant *T. carassii* CRT inhibited classical complement pathway-mediated haemolysis. Different concentrations of the rTcaCRT or BSA controls were added to wells of a microtiter plate containing 50 µL of normal rabbit serum 1/100 dilution. 10^7 antibody-sensitized cells were then added to each well. The percentage of lysis was calculated with reference to 100% lysis of the cells in distilled water. Results are mean ± SEM of three independent experiments and the percent haemolysis for each CRT differed no more than 5% from the mean in each experiment. Haemolysis was restored to approximately 80% in the presence of anti-TcaCRT IgG at 1/100 dilution.

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CHAPTER 8

GENERAL DISCUSSION

8.0 Evasion of Host Immune Responses by Parasites

Parasites are eukaryotic pathogens and broadly consist of protozoa, fungi, helminths and arthropods. They complete part or all of their lifecycle within another organism, the host. As is the case with every other pathogenic organism, parasites have evolved ways to survive the highly potent immune response encountered in their various hosts. The immune evasion was described over 100 years ago by Paul Ehrlich (Nobel Lecture 1908). Although, a relatively faint idea at the time, Ehrlich reported on the 'disappearance of receptors' in African trypanosomes. This mechanism is now known as antigenic variation. Antigenic variation has since been studied in detail in parasites and bacteria [13, 26, 53, 98].

The immune evasion strategies employed by parasites are varied and include avoidance of immune recognition, immune suppression and diversion of host immune attack mechanisms. All the major parasite groups are known to deploy immune evasion mechanisms [11, 23, 85, 91].

Parasites use armamentarium of immune evasion strategies to interfere with, disrupt or manipulate host immune defences at the different stages of immune response. Hosts deploy their immune system to prevent infections [82, 88]. Ultimately, any immune response generated against the parasite must employ mechanisms that are able to control, contain or kill the parasite. The innate immune system is the first line of defense against pathogens and includes the pattern recognition receptors (PRRs) that recognise pathogen-associated

molecular patterns (PAMPs). Following detection, the host uses a number of innate immune components such as NK cells, phagocytes, complement and antimicrobial peptides as the eventual means by which the invading parasites are eliminated [59]. Innate immunity can inhibit parasite growth but it rarely eliminates successful parasites. Thus, lymphocytes of the adaptive immune system are activated by actions of innate immune cells leading to induction of T cell proliferation and antibody production by B cells [59]. These antigen-specific T and B cells are effector cells of the adaptive immunity and generate a potent reaction to eliminate infection. Evasion or modulation of adaptive immune response is therefore crucial for parasite survival and persistence [89].

Protozoan parasites that proliferate rapidly in the hosts possess strategies to subvert host innate and adaptive immune systems. These ensure survival of these parasites, persistence of infection and increase the transmission potential. The African trypanosomes use antigenic variation whereby expression of distinct surface molecules allows new variants to avoid early recognition and repopulate the vascular system of the host, and replace those parasites killed by adaptive immune system [13]. *T. cruzi* has adopted an intracellular existence to escape recognition by host immunity. The surface of *T. cruzi* is covered by mucin glycoproteins that further allow parasite to evade recognition by host immunity and aid in establishment of infection [15].

During the passage of *P. falciparum*-infected erythrocytes through the spleen, parasite-derived proteins are exported to the erythrocyte surface to avoid clearance from the blood. These proteins of which the best characterized is *P.*

falciparum erythrocyte membrane protein 1 (PfemP1), bind to specific receptors on venular endothelial cells and allow the parasite to be retained [6]. Host antibodies can recognize these proteins and prevent adherence of infected erythrocytes to different molecules on venular endothelium. However, a mechanism of clonal antigenic diversity of PfemP1 encoded by over 50 '*var'* genes per genome is required to maintain sequestration and increase transmission potential of the parasite [14]**.** It has been suggested that cytoadherence evolved as a mechanism of immune evasion to avoid splenic clearance but plays a secondary role in conferring pathogenicity [81]. This phenomenon, described as antigenic diversity, which involves expression of antigenically different alleles of a gene in the different *P. falciparum* is different from antigenic variation. Antigenic variation described above for the African trypanosome is a rapid, stochastic process by which a clonal parasite population switches its antigenic phenotype and has also been observed in different *Plasmodium* species [10, 42, 62, 83].

Antigenic variation has also been described as a major immune evasion strategy in *Giardia*, the flagellated protozoan parasites of small intestine that causes diarrhoea in humans and other animals. *Giardia* surface antigens known as variant-specific surface proteins (VSPs) play roles in parasite attachment and are continuously switched to evade host humoral response [63, 65]. The VSPs are highly immunogenic and cover the entire surface of the parasite acting as a dense interface between the parasite and the host [78]. At any one time, only 1 of approximately 200 VSP genes encoded by the genome is expressed on the surface of an individual parasite, although simultaneous expression of two different VSPs

can occur during VSP switching [66, 80]. It has been reported that trophozoites of *Giardia* undergo antigenic variation both within the host and in cell culture [66]. *Giardia*-derived products have also been reported to contribute to immune evasion and establishment of chronic infection [24].

Slow maturing multicellular helminths do not replicate in their human host and do not use antigenic variation or switching. They have larger genome size and have evolved several alternative immune evasion mechanisms. The large size of helminth parasites provides them with a large and varied number of antigens that must be recognized by the host immune system for development of an effective immune response. Some helminths possess ability to mask themselves from an effective immune response by acquiring antigens from their host [93]. Schistosomes establish chronic infections in their host by acquiring host molecules including immunoglobulins [48, 102], major histocompatibility complex [19, 92], complement components [87, 94], C3 decay- accelerating factor [43], β_2-microglobulin [96] and α_2-macroglobulin [27, 49]. These parasites express a surface form of invertebrate muscle protein paramyosin which is functionally and antigenically related to human CD59, a membrane bound complement regulatory protein. Paramyosin, like CD59 binds human C8 and C9, and inhibits assembly of membrane attack complex C5b-9 [28, 77]. Paramyosin has also been found in the tegument and on the surface of *Echinococcus granulosus* [54], *Taenia solium* [64] and *Fasciola hepatica* [16]**.**

Generally, the excretory/secretory products of worms may induce immunosuppression by impairing or modulating immune cell functions. ES

products from gastrointestinal nematode *Heligmosomoides polygyrus* were shown to inhibit cytokine and chemokine production, and co-stimulatory molecules expression induced by TLR ligation in dendritic cells. These effects led to impairment of dendritic cell functions and suppression of both Th1 and Th2 immune responses [90]. *Trichinella spiralis*, a zoonotic nematode and food borne parasite suppresses host immune response during infection. *T. spiralis* infection poses serious challenge to the host since parasite lifecycle is completed in one host resulting in exposure of the host to parasite and parasite-derived molecules from all stages of development. The ES products of *T. spiralis* modulate macrophage function by reducing the capacity of macrophages to express LPS-induced pro-inflammatory cytokines [1]. Treatment of macrophages with *T. spiralis* ES alone enhances anti-inflammatory cytokine expression and molecular studies showed that *T. spiralis* ES products significantly inhibited LPS-induced nuclear translocation of nuclear factor-*k*B and phosphorylation of extracellular signal-regulated protein kinase and p38 mitogen-activated protein kinase [1]. These results suggest worm ES products participate in immune evasion by suppressing macrophage functions and pro-inflammatory responses.

Since NO plays an important role in inducing intestinal physiology and inflammation during *T. spiralis* infection [57], abrogation of LPS-induced iNOS expression in macrophages by *T. spiralis* ES products reflects a potent immunomodulatory strategy. *T. spiralis* infection has been reported to down-regulate iNOS gene expression [9], suggesting parasite and parasite-derived molecules may modulate host immune responses to allow survival. Since parasite

ES products enhance anti-inflammarotory cytokine expression, ES products may modulate balance of pro- and anti-inflammatory responses thus providing parasite with a favourable environment for infection and growth [1]. *T. spiralis* ES products also induce Th2 type responses in rat bone-marrow derived dendritic cells *in vitro* and *in vivo*, while *in vivo* T-cell priming generated a mixed Th1/Th2 cytokine response [38]. Recently, it was reported that recombinant paramyosin from *T. spiralis* bound complement components C8 and C9, inhibited C9 polymerization and prevented formation of MAC [103]. Recombinant *T. spiralis* paramyosin also inhibited lysis of rabbit erythrocytes via alternative complement pathway [103].

Parasites have evolved a variety of ways to evade antimicrobial responses of macrophages and other immune cells. To understand how *T. carassii* interacts with fish macrophages, I examined the effects of *T. carassii* hsp70 on goldfish macrophage functions. I observed that Tcahsp70 activated goldfish macrophages and stimulated the production of pro-inflammatory cytokines [73]. Parasite hsp70 also up-regulated the expression of iNOS isoforms A and B, and induced a strong nitric oxide response of goldfish macrophages [73]. Trypanosomes are known to possess all major classes of Hsps [33] but the precise roles of trypanosome hsps in immune modulation are yet to be investigated. However, hsp70 of protozoan parasites have been shown to be immunogenic and induced protection in immunization studies [7, 46].

A functioning immune response depends on its signalling network. In response to an infection, host signalling molecules, such as cytokines and

chemokines, and antimicrobial molecules are produced by macrophages. Parasites interfere with immune cell signalling in many ways. For example, *Leishmania* modulate host cells by interfering with signalling mechanisms leading to impairment of immune cell functions [37, 75]. This effect is dependent on *Leishmania* gp63 [21, 35]. I showed that rTcagp63 down regulated *Aeromonas salmonicida* and recombinant goldfish TNFα2-induced production of reactive oxygen and nitrogen intermediates. Macrophages treated with rTcagp63 also exhibited significant reduction in the expression of cytokines TNFα-1 and TNFα-2, and iNOS-A genes. Recombinant Tcagp63 was internalized by goldfish macrophages and altered phospho-tyrosine protein pattern suggesting that Tcagp63 may act by altering the signalling events, which are important in downstream macrophage antimicrobial and other cytokine-induced functions. *T. brucei* gp63 is required for removing VSGs from the surface of bloodstream parasites [39, 55], while *T. cruzi* gp63 may participate in infection of host cells [25]. However, none of trypanosome gp63 molecule reported so far have been shown to modulate immune cell functions.

T. cruzi cruzain a cysteine protease was recently reported to mediate immune evasion by cleaving signalling molecule NF-kB thereby inhibiting host cell activation [29]. Cruzain also inhibits cytokine expression in *T. cruzi*-infected macrophages treated with LPS suggesting impairment of signalling mechanisms required for macrophage activation. Cruzain gene knockout parasites activate macrophages, induced ikB phosphorylation, NF-kB nuclear translocation, cytokine expression and were unable to survive within macrophages [29]. These

results further lend support to reports that parasite proteases mediate immune evasion.

It appears that *T. carassii* possess molecules that can induce pro- and anti-inflammatory responses presenting the parasite with an excellent strategy to manipulate host inflammatory responses to their benefit. This assertion is supported by results described in this thesis. For example, I observed significant and parallel increases in mRNA levels of genes encoding pro-inflammatory and anti-inflammatory cytokines in the different tissues of *T. carassii*-infected fish compared to non-infected control [71]. Expressions of majority of genes encoding pro- and anti-inflammatory cytokines were up-regulated during the acute phase of infection (days 7 to 21 post-infection). The mRNA levels of these cytokines returned to normal levels or were down-regulated during the elimination phase of infection (days 28 to 56). These results suggest Th1/Th2-like responses may be important for controlling *T. carassii* infection in the goldfish. Alternatively, parasite and parasite-derived molecules may induce pro- and anti-inflammatory responses to suppress specific or general immune responses. I favour the latter and propose that immunosuppression could be a major evasion strategy used by *T. carassii*.

In vertebrates, the complement system is an important part of both innate and adaptive immunity. In innate immunity, complement system recognizes the invading pathogen, communicate with and activate the adaptive immunity [67, 99, 100]. It involves cascades of circulating and cell surface-bound proteins which are activated sequentially through three pathways known as the classical, lectin and

alternative pathways. The classical and lectin complement pathways are initiated by C1q and MBL. The alternative pathway is activated after binding of complement component C3b to microbial surfaces. The end product of all complement activation pathways is the coating of the invading organism with C3 and assembly of the membrane attack complex leading to destruction of the pathogen [56]. Regulators of complement activation down-regulate complement action at several steps in the complement cascades and prevent deleterious effects of complement activation on host cells. Because of the significant evolutionary pressure exerted on parasites by host complement system, they have evolved mechanisms to escape the deleterious effects of complement. The investigation and characterization of complement evasion strategies used by pathogens is an expanding area of research. Generally, pathogens avoid complement lysis by evading recognition by complement activators, by expressing soluble and membrane-bound molecules that inhibit or degrade complement components or by recruiting complement regulators [23, 50, 77, 97, 103]. These mechanisms have led to a multitude of complement evasion strategies by different pathogens [8, 12, 22, 32, 44, 51, 104].

Trypanosomes are resistant to complement-mediated lysis (CML) and their complement evasion strategies have been fairly studied. In *T. brucei*, it has been reported that VSG and gp63 proteases play roles in protection of parasites from CML [30**,** 36, 55, 95]. *T. cruzi* possess variety of complement regulatory molecules, which allow the parasite to evade CML [68, 69]. Complement-mediated lysis of trypanosomes has been reported to occur primarily via the

alternative complement pathway [52, 79]. The classical pathway appeared to be ineffective in parasite lysis, although it is believed to amplify the lytic effects of the alternative complement pathway. Calreticulin, a secreted and cell surface-bound protein is thought to be partly responsible for the inability of the classical complement pathway to lyse trypanosomes [31, 72]. Despite the importance of calreticulin as an immune evasion molecule, only *T. cruzi* calreticulin (TcCRT) has been fairly characterized among protozoa parasites. TcCRT binds human C1q and specifically inhibits classical pathway of complement [31]. I reported that *T. carassii* calreticulin is identified in both the ES and surface protein fractions and is abundantly expressed on the parasite surface [72]. Recombinant *T. carassii* calreticulin bound first complement component, C1q of the goldfish and inhibited C1q-dependent lysis, suggesting that TcaCRT may be used by the parasite to inhibit host classical complement pathway *in vivo.*

8.1 Future direction

Further characterization of *T. carassii* antigens in ES and surface protein fractions is necessary to determine identity of these antigens and investigate their role in host-parasite interactions. Although immunoblot of surface proteins separated by 2D-electrophoresis and probed with immune sera presents technical challenges, such approach may reveal candidate antigens expressed on the parasite surface. Another approach that may accelerate identification of protective antigens of *T. carassii* would be to compare leech stage parasites with those in fish using proteomics and transcriptomics. Two-dimensional electrophoresis, cDNA library construction and suppression subtraction hybridization may be used

for such large-scale studies. Results of these studies can be validated using quantitative real time PCR, and western or northern blotting techniques. A European based collaborator will be required for these studies since cultivation of leech stage parasite is not allowed in Canada. (*T.carassii* is not indigenous in North America).

The expression analysis of pro- and anti-inflammatory cytokines presented in this thesis is the first step towards understanding goldfish-*T. carassii* interactions. It may be necessary to validate the results reported at protein levels to further understand the type of immune response generated in *T. carassii*-infected fish. *T. carassii* infection induced expression of IFNγ and TNFα in goldfish. TNFα has been reported to cause lysis of *T. brucei* and *T. cruzi in vitro,* while treatment of *T. brucei*-infected mice with anti-TNFα antibodies increased parasitemia in the blood and tissues [58, 74]. On the other hand, IFNγ acts as a growth factor and promotes the growth of African trypanosomes [2, 3, 4]. Like *T. brucei, T. carassii* is an extracellular parasite and it would be interesting to investigate the effects of goldfish TNFα on this parasite. If goldfish TNFα cause lysis of *T. carassii*, this observation will further strengthen the functional significance of Tcagp63 as parasite-derived molecule that mediates immune evasion by dampening pro-inflammatory responses. That both pro- and anti-inflammatory responses are produced in *T. carassii*-infected goldfish supports the idea that parasite may use immunosuppression as an evasion strategy.

Further investigation of innate immunity of goldfish to *T. carassii* infection should be undertaken because of heavy reliance of fish on non-specific immunity.

A more detailed examination of parasite interaction with goldfish immune cells *in vitro* should provide new insights into host-parasite interactions. I have observed that *T. carassii* induces respiratory burst response of monocytes but fails to induce nitric oxide response of goldfish macrophages *in vitro* studies. I reported that *T. carassii* induced iNOS gene expression *in vivo* [71], while it is susceptible to NO *in vitro* [86]. The reasons for these discrepancies are not clear at this point, however, it would be interesting to examine the effects of activated macrophages on parasites. From my observations, it is clear that *T. carassii* ES products contain at least one molecule that can induce significant pro-inflammatory responses and at least one molecule that can down-regulate such responses. Effects of the ES products on goldfish monocyte and macrophages functions need to be investigated. Although, I could not detect Tcagp63 in parasite ES products there is evidence for extracellular release of gp63 [45, 61]. ES fraction should be evaluated for protease activity by examining its proteolytic effects on immune molecules of the goldfish.

In mice infected with the worm *Heligmosomoides polygyrus*, worm calreticulin skews immune response toward a Th2 type phenotype [84], suggesting that parasite CRTs may possess ability to induce a Th2 type response. This hypothesis may be tested by examining cytokine gene expression in macrophages treated with rTcaCRT. Alternatively, goldfish can be immunized with rTcaCRT in the presence and absence of adjuvants, and antibody responses as well as cytokine gene expression in the different organs examined. The result of this study should provide further support for the observation that parasite

induced a parallel up-regulation of pro- and anti-inflammatory cytokines in the different tissues of infected goldfish.

The general biology and functions of parasite CRTs warrants further studies. It has been reported that CRT can inhibit perforin-mediated lysis at physiologically relevant concentration leading to proposition that CRT may stabilize cell membranes to prevent formation of polyperforin pore [34]. This indicates CRT can regulate lytic functions and it would be interesting to test whether parasite CRT can perform this function or use this as a strategy for immune modulation. Additionally, CRT binds azurocidin, a secretory protein in neutrophils that has been shown to induce cytokine production in monocytes. Azurocidin-induced cytokine production is inhibited by anti-CRT antibodies suggesting that interaction of cell surface CRT with azurocidin is required for monocyte activation [70]. It is possible that parasite CRTs generally use their chaperone activity to interact with other proteins and indirectly participate in immune cell activation.

Any potential protective antigen would need to be evaluated for its ability to protect experimentally challenged animals in immunization studies. A single antigen or combination of antigens may be evaluated for their protective efficacy. Immunization studies with recombinant antigens or DNA may be considered since DNA vaccination may induce better level of protection. Experimental vaccination with recombinant *Necator americanus* CRT in absence of adjuvant reduced worm burden by approximately 49% in the lungs of infected mice compared to control mice [101]. *Leishmania* parasite hsp70 was shown to be

protective in immunization studies [46]. Recently, a cocktail of *Leishmania* gp63 and hsp70 was shown to induce significant levels of protection in experimentally challenged mice [47]. The immunized animals generated high levels of IgG2a and expressed high levels of IFNγ and IL-2 but low levels of IL-4 and IL-10 [47]. These results suggest generation of a protective Th1 response and suppression of Th2 type immune response. I [73] reported that Tcahsp70 stimulated inflammatory responses of goldfish macrophages while Tcagp63 down-regulates inflammatory responses. It would be interesting to test these antigens separately and in combination in immunization studies. Also, TcaCRT another parasite chaperone may be tested together with Tcagp63 in immunization studies.

Although several hundred antimicrobial peptides have been described from diverse species, there is little information on antimicrobial peptides in fish and their effects on *T. carassii* are yet to be elucidated. These small molecules have characteristic cationic charge and amphipathic conformation, and play crucial roles in immunity [41]. Antimicrobial peptides have been shown to kill *T. brucei* and *T. cruzi* trypomastigotes *in vitro* [40, 60, 5]. To date, only a few fish antimicrobial peptides have been identified [17, 18, 20, 76]. I have attempted to clone goldfish cathelicidin using primers designed to homolgous regions of fish cethelicidin sequences with no success thus far. The effects of these antimicrobial peptides can be tested on *in vitro* grown *T. carassii*. The progress in genome sequencing should accelerate identification of more fish peptides that can be tested**.** Since these molecules exhibit sequence conservation, the effects of mammalian antimicrobial peptides may also be tested on *T. carassii*.

8.2 Proposed model of *T. carassii* evasion mechanisms

Based on the work described in this thesis, I propose two mechanisms of immune evasion by *T. carassii*. For the first mechanism, *T. carassii* may use hsp70 and other parasite molecules capable of inducing inflammatory responses to induce a Th1 type immune response (Fig 8.1) early during infection (from days 1-21 pi). This will results in down-regulation of Th2 cytokine secretion and antibody production, which is required for parasite elimination. This environment will favour parasite establishment and maintenance of infection. TcaCRT is also expected to function during early stages of infection to prevent classical complement pathway-mediated lysis. Later in infection (between days 21-28 dpi), parasites may produce Tcagp63 to dampen inflammatory responses and prevent inflammation-associated tissue damage to the host. However, this is to the detriment of parasites, as this would allow host to generate Th2 type responses and produce parasite specific antibodies that will eliminate the parasites.

In the second mechanism, both Tcahp70 and Tcagp63 may be produced during early stages of infection (days 1-21 pi) (Fig. 8.1) leading to mixed Th1/Th2 responses causing general immunosuppression. This will also enhance parasite establishment in the host. Both mechanisms will ensure that parasites maintain a chronic infection to ensure their transmission to a new host.

The roles of TcaCRT in Th1/Th2 responses deserve further investigation. Calreticulins have anti-complement effects resulting in decreased chomotaxis and activation of immune cells because of decrease in the generation of

anaphylatoxins, C3a and C5a. Thus, TcaCRT may play additional roles early during infection by contributing to suppression of inflammatory responses.
It is possible that parasites modulate concentration of these molecules within the host to regulate the balance of Th1/Th2 responses. The timing of production and dose of these antigens may be important in the cross-regulation of host Th1/Th2 responses.

8.3 Summary

The work presented in this thesis showed that *T. carassii* uses a variety of mechanisms to evade or modulate goldfish immune responses. *T. carassii*-infection induced a parallel expression of both pro- and anti-inflammatory cytokines in goldfish. The profile of cytokine induced in *T. carassii*-infected fish suggests that a balance of Th1 and Th2 responses may play roles in controlling trypanosome infection in fish.

T. carassii ES products contain several immunogenic molecules, among which are Tcahsp70, TcaCRT and Tcagp63. I showed that rTcahsp70 can stimulate inflammatory responses while rTcagp63 may down-regulate inflammatory responses. These observations lend further credibility to assertion that a balance Th1/Th2 responses are important in controlling *T. carassii* infection. Also, TcaCRT a surface expressed molecule may be partly responsible for inefficient lysis of parasite by classical complement pathway. Recombinant TcaCRT bound and inhibited C1q-dependent lysis. It is clear that *T. carassii* uses a variety of strategies to persist in the host and ensure continuous transmission.

This host-parasite model system presents some challenges such as lack of complete genome information to accelerate identification of parasite genes that may be involved in immune evasion as well as host factors that may play roles in induction of protective immunity. Thus, considerable time and effort was required to generate reagents such as recombinant proteins and antibodies. Additionally, inability to culture the leech stage parasites in Canada prevents the use of large-scale comparative proteomic and transcriptomic studies to identify parasite evasion molecules that are differentially regulated. Nevetheless, this model system presents an opportunity to study protective immune responses in ectothermic vertebrates and results obtained using this system will provide valuable information into an host-parasite interaction that occurs naturally. More research using this model system should be encouraged to advance our knowledge of immune evasion strategies used by this protozoan of ectothermic vertebrates.

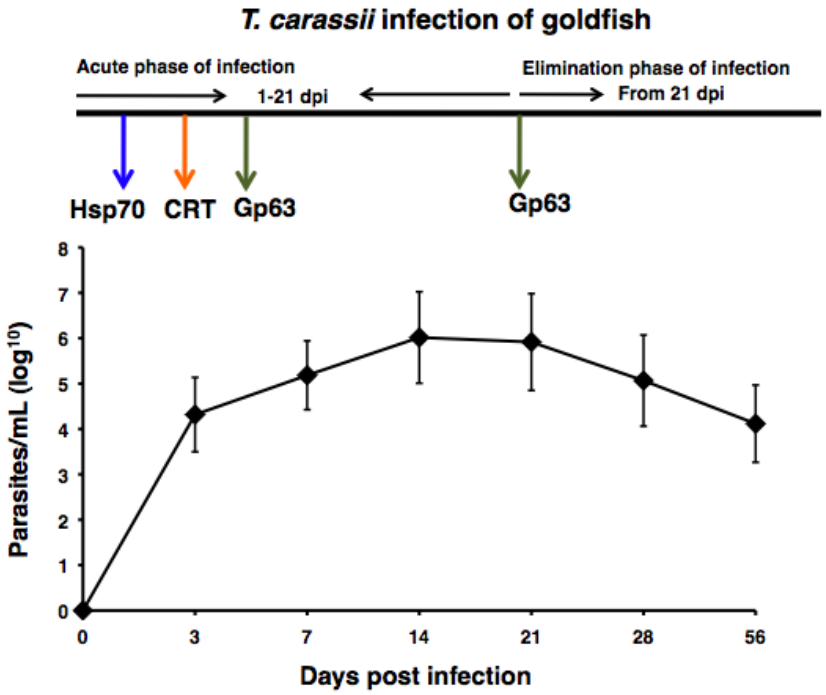

Figure 8.1. Proposed mechanisms of *T. carassii* immune evasion. Course of *T. carassii* infection in the goldfish is shown. During early stage of infection, Tcahsp70 may be produced to induce a Th1 type immune response leading to enhancement of parasite establishment. TcaCRT may be produced at the same time to prevent classical complement pathway-mediated lysis. TcaCRT can also act as anti-inflammatory molecule due to its anti-complement effects. Tcagp63 may be produced early during infection to generate mixed a Th1/Th2 responses and immunosupression. Also, Tcagp63 may be used at later stage (days 21-28 pi) to down-regulate hsp70-induced inflammatory responses. Down-regulation of inflammatory responses may allow host humoral response to eliminate the parasite but the prolonged infection would ensure parasite transmission to a new host.

8.4 References

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