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EXPERIMENTAL CHAGAS' DISEASE

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Cover photo: Parasite nest in inflamed muscle tissue

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ABSTRACT

The main focus of this thesis was to develop and utilise experimental models using the protozoan parasite *Trypanosoma cruzi*. We were especially interested in studying the activation of NK cells early after *T. cruzi* infection and what factors that predispose to a chronic inflammatory disease after *T. cruzi* infection.

NK cells have previously been demonstrated to play a protective role early during *T. cruzi* infection. Here we define a model system that allows further characterisation of the activation of NK cells by describing kinetics of the induction of increased NK cell-mediated cytotoxicity and the production of IFN-γ after *T. cruzi* infection. NK cell-mediated cytotoxicity was increased during the whole first week post-infection and this increase was initially dependent on IFN-α/β. NK cell dependent, IL-12/IL-23-independent IFN-γ was transiently produced at 24 hours post-infection. Later during the first week IFN-γ was once again produced, but now by $CD4^+$ T cells in an IL-12/IL-23-dependent/NK cell-independent manner. IL-12/IL-23 but not IFN-α/β was necessary for survival, suggesting that NK cell-dependent cytotoxicity does not play a protective role during *T. cruzi* infection.

In order to study what factors are involved in the induction of a chronic inflammatory disease after *T. cruzi* infection we continued the characterisation of an experimental model of Chagas' disease previously established in our lab. We found that CBA/J mice infected with the Tulahuen strain of *T. cruzi* developed polymyositis, thus enabling the study of idiotypic inflammatory myopathies, a spectrum of diseases almost completely lacking appropriate model systems. To improve the models we have at hand for studying Chagas' disease we then went on to study the relative contribution of host and parasite genotypes, respectively. By combining different host and parasite genotypes through experimental infections we demonstrated that host genotypes could determine whether the final outcome is inflammatory disease or healing whereas parasite genotype at least can determine the phenotype as well as the severity of the inflammatory disease.

Finally we investigated the role of Toll-like receptors in experimental Chagas' disease. Utilising a previously established model we demonstrated that signalling through the Toll-like receptor adaptor protein MyD88 is not needed for a protective immune response to *T. cruzi*. Mice deficient of MyD88 did exhibit a less severe chronic inflammatory disease in skeletal muscles, however, suggesting that signalling by Toll-like receptors or IL-1/IL-18 plays a role in experimental Chagas' disease.

REPORTS CONSTITUTING THE THESIS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. Une C, Andersson J, Eloranta M-L, Sunnemark D, Harris RA and Örn A. Differential activation of NK cell cytotoxicity and IFN-γ expression during experimental infection with *Trypanosoma cruzi*. Clinical and Experimental Immunology 2000, 121:499-505.

II. Une C, Andersson J and Örn A. Role of IFN-α/β and IL-12 in the activation of natural killer cells and interferon-γ production during experimental infection with *Trypanosoma cruzi*. Accepted by Clinical and Experimental Immunology

III. Andersson J, Englund P, Sunnemark D, Dahlstedt A, Westerblad H, Nennesmo I, Örn A and Lundberg IE. CBA/J mice infected with *Trypanosoma cruzi*: An experimental model for inflammatory myopathies. Muscle & Nerve 2003, 27: 442–448.

IV. Andersson J, Örn A and Sunnemark D. Chronic murine Chagas' disease: the impact of host and parasite genotypes. Immunology Letters 2003, 86:207-212.

V. Andersson J, Takeuchi O, Akira A and Örn A. Importance of Toll-like receptors in murine Chagas' disease: MyD88 deficient mice exhibit an ameliorated chronic disease course. Manuscript.

LIST OF ABBREVIATIONS

CD	cluster of differentiation
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EVI	endocardial, vascular and interstitial
Fc	fragment crystallizable
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylphosphatidyl inositol
IBM	inclusion body myositis
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
NK	natural killer
NO	nitric oxide
PM	polymyositis
RNA	ribonucleic acid
PRR	pattern recognition receptor
SAPA	shed acute-phase antigen
STAT	signal transducers and activators of transcription
Th	T helper
TNF	tumor necrosis factor

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INTRODUCTION

Parasitism is a very successful way of life in which an organism lives at the expense of another, both of which have evolved independently time and time again during evolution. Parasitic life forms are generally well adapted to their host(s) and are usually not associated with disease. Parasites do strive to achieve a balance between transmission and the burden inflicted on the host, however, and this continuous optimisation of virulence is occasionally reflected by disease in some host-parasite relationships.

One obvious reason to study parasitic diseases is to improve existing and develop new approaches for preventing, diagnosing, treating and controlling this group of diseases. Humans have suffered and still suffer greatly from parasitic diseases such as Malaria, Schistosomiasis, Leishmaniasis and Trypanosomiasis.[1] Unfortunately, pharmaceutical companies have been reluctant to develop suitable drugs for parasitic diseases, since they mainly affect poor regions of the world. An improved understanding of parasitic disease might therefore allow the use of drugs originally designed against other diseases as a treatment for parasitic diseases. Another option is to try to develop drugs that target several closely related parasitic diseases possibly not only affecting Man, but which are also of veterinary importance, thus making it profitable for pharmaceutical companies to invest in such drugs.

Parasites are also studied for their extreme biology. Since the biology of parasites does not differ fundamentally from the biology of free-living organisms most of these phenomena have later been applicable to higher eukaryotes and non-parasitic organisms. Thus parasites provide outstanding model systems in many areas of biology. Biological phenomena first discovered in the order Kinetoplastida, to which the parasite studied of this thesis, *Trypanosoma cruzi*, belongs, include:

- Antigenic variation of surface glycoproteins[2]
- GPI anchors of membrane proteins[3]
- Mitochondrial RNA editing[4]
- Eukaryotic polycistronic transcription[5]
- Trans-splicing of pre-mRNAs[6]
- Unique organelles (kinetoplast, glycosome (peroxisome) and acidocalcisome)[7]
- Lysosomal repair of plasma membranes[8]

This rationale is also employed within the field of immunology. The best example of this is perhaps the use of *Leishmania* to study the Th1 versus Th2 polarisation.[9] The following study is concerned with the establishment and utilisation of experimental models for Chagas' disease in order to study NK activation and further the understanding of infection-induced inflammatory diseases.

TRYPANOSOMA CRUZI

The protozoan parasite *T. cruzi* is the causative agent of Chagas' disease, a chronic inflammatory disease mainly affecting the heart and gastrointestinal tract. *T. cruzi* is mainly transmitted by bloodsucking reduviid species but it is also commonly transmitted through blood transfusions. The World Health Organisation estimates that up to 20 million people in south and Central America are currently infected with *T. cruzi*, of which more then 30% will eventually develop a chronic disease.

T. cruzi is characterised by a flagellum and a single mitochondrion in which is situated the kinetoplast, a specialized DNA-containing organelle. Kinetoplast DNA constitutes 20-25% of the parasites total DNA.[7] It is organised in a network of mini and maxi circles, which contain essential genetic information as parasites lacking a kinetoplast cannot undergo a complete lifecycle. *T. cruzi* exists in several distinct morphological states during its life cycle (Fig. 1). These forms are identified by the relative position of the kinetoplast in relation to the cell nucleus and the flagellum.[7]

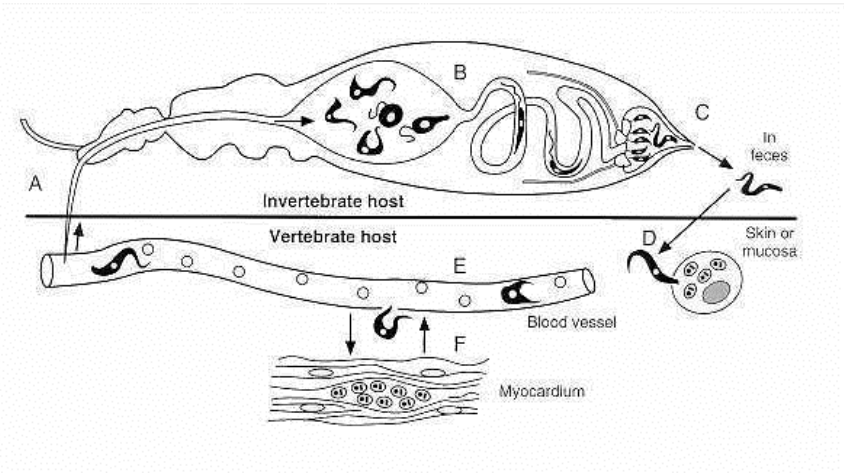

Figure 1. Life cycle of *T cruzi*. A Reduviid bug feeds on an infected host thus allowing *T. cruzi* trypomastigotes to enter the insect (A). Trypomastigotes then transform in the stomach and midgut to epimastigotes, possibly through a transient spheroid/amastigote stage (B).[10] Epimastigotes attach to the walls of the hindgut and produce infective metacyclic trypomastigotes. When the bug takes a blood meal it defecates and the parasites enter through the bite wound (C). Metacyclic trypomastigotes now enter host cells and differentiate into amastigotes that undergo several rounds of replication (D). After replication amastigotes transform into trypomastigotes, possibly through a transient epimastigote form,[11] which invade other tissues (E and F). (adapted from: http://gsbs.utmb.edu /microbook/ch082.htm)

In the trypomastigote stage the kinetoplast is at the posterior end of the parasite (and nucleus) and the flagellum emerges from the flagellar pocket, which is located near to the kinetoplast. In epimastigote stages the kinetoplast and the flagellar pocket are found in a position anterior to the nucleus. The replicative amastigote stages are rounded stages displaying a short inconspicuous flagellum.

The non-replicative trypomastigote's ability to invade almost all mammalian nucleated cells is a key feature in the parasites ability to propagate and hide from the immune system. Cell invasion is initiated when the trypomastigote attaches to mammalian host cell. Diverse families of parasite receptors[12-15] mediate this interaction through sialic acid containing and heparin sulphate host molecules.[14, 16-18] The trypomastigote will then recruit lysosomes to the attachment site in order to exploit the ubiquitous cell membrane wound repair mechanism to enter the cell.[19, 20] Lysosomal recruitment is mediated by synaptotagmin VII and is dependent on parasite-induced Ca^{2+} signalling in the host cell.[21] The molecular mechanisms behind such Ca^{2+} signalling remain controversial, as three exclusive mechanisms have been presented.[22] Finally after the trypomastigotes have entered the lysosome -derived parasitophorous vacuole, the acidic environment will activate a pore-forming molecule, TcTox,[23, 24] and the vacuole will be disrupted. Moreover the acidic environment in the lysosome-derived vacuole is thought to be an important trigger for trypomastigote to amastigote differentiation.[25]

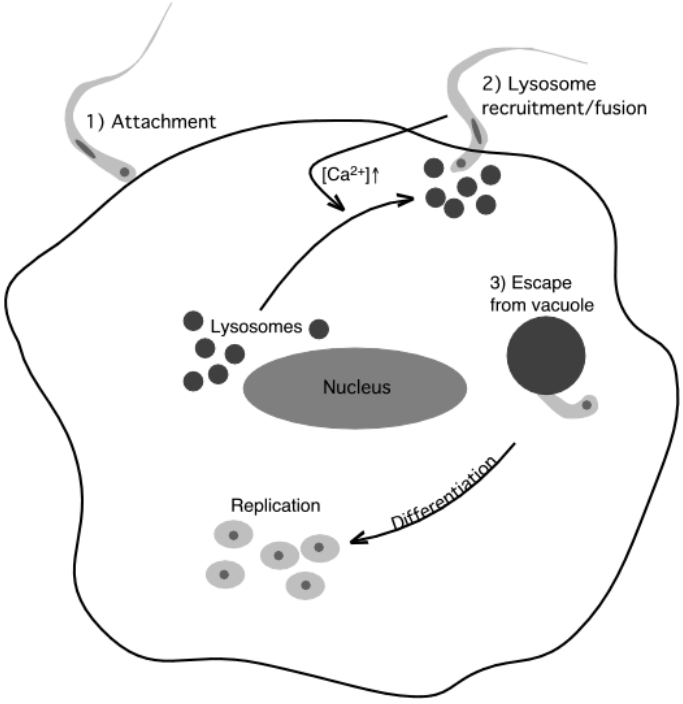

Figure 2. *T. cruzi* cell invasion. Trypomastigotes attach to the surface of the mammalian cell (1). The parasite then initiates host-signalling leading to increased [Ca^{2+}] dependent on gp82 or oligopeptidase B or cruzipain (2). Lysosomes are then recruited to the attachment site followed by fusion with the membrane and generation of a vacuole. A pore forming *T. cruzi* molecule facilitates escape from the vacuole (3). Parasites released into the cytoplasm will differentiate into replicative amastigotes.

HOST DEFENSE

Upon entry into the host *T. cruzi* will invade cells and start multiplying intracellularly. The first line of defence, the innate immune response, is then activated in order to limit parasite proliferation and dissemination. The key event in this early stage of infection is the activation of macrophages. A non-activated macrophage has a very limited ability to kill parasites and instead functions as an important site for further parasite replication[26]. The most well studied and apparently most potent macrophage activator during *T. cruzi* infection is IFN-γ.[27-29] Other cytokines that act as potent macrophage activators include GM-CSF[30] and TNF-α.[31] A relatively recent discovery is that conserved parasite antigens also have the ability to induce macrophage activation *in vitro* through Toll-like receptor 2.[32, 33] The trypanocidal activity of macrophages is associated with the production of nitric oxide rather than production of respiratory burst metabolites.[34, 35] The protective effect of NO is apparent in mice deficient of iNOS[36] or treated with NO inhibitors,[37, 38] which are highly susceptible to *T. cruzi* infection. In contrast, scavengers of respiratory burst metabolites do not inhibit the *in vitro* trypanocidal activity of activated macrophages.[34]

Macrophage activation is not sufficient to mount a protective immune response to *T. cruzi* infection, however. Depletion of defined cell populations prior to infection has demonstrated a protective role of both neutrophils and NK cells. The protective effect of neutrophils was limited to some mouse strains and was correlated with an enhanced Th1 response.[39] NK cell depletion also leads to an exacerbated disease after *T. cruzi* infection,[40, 41] but exactly how NK cells exert their protective effect is not well understood.

Around two weeks after the initial infection the innate immune response is complemented with an adaptive immune response.[42] Utilising knockout mice it has become evident that both B and T cells are necessary to mount an efficient protective immune response.[43-45] Perhaps the most important component of the adaptive response is T helper cells that function to orchestrate an immune response suitable for the offending pathogen. An often applicable model used to describe T cell responses is to divide them into either a Th1 type of response which corresponds to a predominantly cellular response and is associated with IFN-γ and IL-2 production, and a Th2 type of response which corresponds to a predominantly humoral response and is associated with IL-4, IL-5 and IL-13 production.[46, 47] Infections of STAT-4 and STAT-6 deficient mice, which have impaired Th1 or Th2 polarisation, respectively, have demonstrated

that a strong cell-mediated response is necessary for surviving *T. cruzi* infection.[48] While $CD4^+$ T cells are absolutely necessary for mounting a successful response against *T. cruzi*, $CD8^+$ T cells play a somewhat less crucial role. In fact CD8 deficient mice have the ability to survive infections with low, but not high, virulent *T. cruzi* strains.[45] The distinguishing feature of $CD8^+$ T cells is their capability to kill aberrant cells and in this case infected cells.[49] The importance of such killing remains controversial as different experiments utilising Granzyme B and perforin deficient mice have resulted in different outcomes concerning their susceptibility to *T. cruzi* infection.[43, 50-52] The observation that $CD8^+$ T cells are capable of releasing IFN-γ and TNF-α has raised the possibility that CD8 cells mediate their effects in an analogous way to CD4 positive cells, namely through releasing cytokines.

B cells are also important for inducing a protective response as mice lacking B cells do not survive *T. cruzi* infection.[43] Early after infection B cells are polyclonally activated and the antibody response will therefore be relatively inefficient[53]. The parasite also protects some of its cell surface bound enzymes from antibodies by surrounding them with immunodominant domains.[54] When an antibody binds to the enzyme it will most often bind to the immunodominant domain rather then the active site thus not affecting the enzyme function.[54, 55] This protective effect (from the parasites point of view) is temporary and antibodies that impair enzymatic activities arise around the time when the level of parasites in the blood starts to decrease.

In order to persist, thereby increasing the possibility of further transmission, *T. cruzi* has evolved a multitude of different mechanisms to escape the host defence. One such mechanism is the parasites ability to invade all nucleated cells, thereby hiding from the immune system. Other strategies, include:

- Polyclonal activation of B cells and T cells[53, 56-58]
- Unresponsiveness to and impaired production of IL-2[59, 60]
- Impaired MHC class II presentation[61]
- Impaired DC maturation[62]
- Immunodominant non-essential enzyme epitopes protecting the active site[54, 55]
- Alternative activation of macrophages[63]
- Parasite Fc receptors[64]
- Complement inactivation[65, 66]
- Preferential persistence in tissues with poor regenerative ability[67]
- Induction of in CD4 T-cell anergy[68]

These mechanisms combined often allow parasites to persist for decades. While this lack of clearance is at least partially responsible for causing chronic Chagas' disease it should not necessarily be considered a failure but rather a compromise between limiting parasite growth and causing excessive damage to the host. A more forceful immune response may very well eradicate the parasite but at the same time cause irreversible damage to the host and lead to death. This notion is supported by the fact that mice deficient in the immunosuppressive cytokine IL-10 exhibit lower levels of parasitaemia but a higher rate of mortality.[69]

PATHOGENESIS OF CHAGAS' DISEASE

Upon vector borne transmission of *T. cruzi* a small sore develops at the bite wound where the parasite enters the body. The initial acute phase of Chagas' disease now ensues, which is characterised by a transient parasitaemia lasting for up to two months.[70] Fever, nausea and swollen lymph nodes accompany this phase of the disease. While the acute phase is most often mild or unsymptomatic it occasionally results in severe myocarditis and death, especially in young children.[70]

The chronic phase of Chagas' disease, which follows after the acute infection, is commonly divided into two different forms, the indeterminate form and the chronic form.[70] The indeterminate phase of Chagas' disease is defined as a period when the patient has parasitological and/or serological evidence of continuing *T. cruzi* infection and a normal electrocardiogram. It is poorly characterised as most experimental model systems lack an indeterminate phase and practical limitations concerning clinical studies of this form. The few studies that have been performed suggest that this phase is an equilibrium between the parasite and the host immune system.[71] 30-40% of all people infected with *T. cruzi* will eventually develop a chronic disease most commonly characterised by megacolon, megaesophagus and severe focal myocarditis.[70] Several hypotheses have been forwarded to explain the pathogenesis of Chagas' disease, as discussed below with emphasis on the cardiac form of the disease.

Parasite persistence in chronic Chagas' disease

Parasite mediated tissue damage is an undisputable cause of acute Chagas' disease that results in tissue destruction and inflammation. During the chronic phase of the disease it is significantly more difficult to detect parasites and this has led to the birth of several other hypotheses, which could also explain the excessive inflammation occuring in diseased hearts of Chagasic patients. During the last 10 years it has however become increasingly accepted that parasite persistence plays a role in Chagas' disease. The studies supporting this line of thought can be summarised as the following:

I. In 1993 Jones *et al.* reported that *T. cruzi* DNA was present in diseased hearts from Chagasic patients but not in hearts of cadavers seropositive for *T. cruzi* but lacking signs of cardiopathy.[72] This study has later been followed up by several other similar clinical and experimental studies, which together demonstrate an almost perfect association between the presence of *T. cruzi* DNA and diseased organs.[73-78]

II. The presence of *T. cruzi* DNA in diseased organs is indeed believed to reflect the persistence of viable intact parasites based on a study by Anez *et al.* that demonstrate presence of intact amastigotes in 22 of 26 myocardial biopsies.[73] This issue has been also addressed in a murine system utilising infections with parasites transgenic for β-galactosidase.[79] Histological sections collected during the chronic phase of the disease demonstrated that these parasites expressed β-galactosidase, as assessed with x-gal staining, thus obviously being alive at the time of organ harvest.

III. Parasite DNA or parasite antigens are found in the vicinity of inflammatory infiltrates.[77, 80] This could also explain the multifocal inflammation apparent in Chagas' disease, something that has been considered difficult to reconcile with an autoimmune genesis.[70]

IV. Regimens that lessen the parasite burden alleviate chronic Chagas' disease,[81-85] in contrast to immunosuppressive treatments/diseases that aggravate the inflammatory disease.[86-93]

V. In 1992 dos Santos *et al.* demonstrated that syngenic heterotopically transplanted hearts were rejected by chronically infected mice.[94] For a long time this was considered the strongest indication that Chagas' disease had an autoimmune component. In 1997 Tarleton *et al*. repeated the experiment with a completely different outcome where the heterotopically transplanted hearts survived for more than 1 year post the transplantation process.[95] The current interpretation of these two studies is that these differences are due to the differential invasiveness of the parasite strains used. Ribeiro-dos-Santos *et al.*, in contrast to Tarleton *et al.*, used a highly virulent *T. cruzi* strain that that could have colonised the transplanted heart, thereby resulting in rejection.

Autoimmunity in chronic Chagas' disease

The idea that chronic Chagas' disease has an autoimmune element was first forwarded in the 1940s. It is now known that a multitude of self-reactive T cells and antibodies of various specificities develop after *T. cruzi* infection in both animal models and humans.[96] The challenge concerning the autoimmune hypothesis has been to directly link this self-reactivity to the chronic inflammatory disease. Autoimmunity as an adaptive immune response against self is a normal feature of the immune system and appears to be necessary for appropriate immune regulation.[97] In some situations such self-reactivity can be deleterious and result in disease, i.e. autoimmune disease.[98] The dual role of self-reactivity in immune regulation and immunological disease makes it very difficult to directly prove that a disease has an autoimmune genesis. To acquire direct evidence that a human disease has an autoimmune component it is necessary to be able to transfer the disease from humans to either humans or animals.[99] Since there are no suitable cell transfer systems from humans to animals it is in practise only possible to directly assess the role of autoantibodies. Even here caution is required since only the antibody but not the antibody producing B cell is transferred. The potential role of B cells in peripheral tolerance was recently highlighted when it was demonstrated that self-reactive IL-10 producing B cells are responsible for the spontaneous remission seen in murine experimental autoimmune encephalomyelitis.[100]

Thus considering these practical limitations most investigators commonly try to obtain indirect or circumstantial evidence of autoimmunity in disease by using animal models. A few of the better characterised examples of self-reactivity in Chagas' disease include:

I. Antibodies reactive to endocardial, vascular and interstitial (EVI) antigens were initially forwarded as the first example of autoreactive antibodies in Chagas' disease.[101] These EVI reactive antibodies were detected in large numbers of Chagasic patients and could be removed by adsorption with *T. cruzi* epimastigote lysate. Thus EVI reactive antibodies were suggested to be crossreactive and responsible for tissue damage in Chagas' disease. This study was later retracted when the investigators found that reactivity to EVI antigens was confined to non-human tissues that had been used in the original study.[102] Even though this study was retracted it gave rise to the notion that cross-reactive antibodies could be of importance in the pathogenesis of Chagas' disease, an area that since then has received a lot of attention.

II. Antibodies to a *T. cruzi* flagellar surface protein, termed FL-160, have been shown to crossreact with a 48 kDa protein present in axonal and myenteric plexus cells.[103] Combined with the finding that around 50% of all patients with Chagas' disease have antibodies directed to FL-160 it was suggested that this might be a mechanism responsible for nerve tissue damage.[104] It is now known that the levels of a-FL-160 antibodies do not correlate with the clinical disease, a finding that has dampened the efforts of further studying this antigen.[104]

III. Girones *et al.* identified an autoantigen, named *Cha* that was recognised by more than 90% of all Chagasic sera tested.[105] *Cha* has two regions of homology to the *T. cruzi* proteins SAPA and TENU2845, respectively, suggesting a possible role of molecular mimicry resulting in Cha autorectivity.[106] *Cha* can be recognised by both T cells and antibodies in diseased patients and the recognition of the R3 epitope of *Cha* appears to be 100% specific for sera from Chagasic patients.[105] Transfer of *Cha* specific T cells in a murine model transferred disease, but it should be noted that these experiments were lacking appropriate controls to exclude the possibility that parasites were transferred together with the T cells.[106] The levels of *Cha*-specific antibodies correlate with the disease progression of Chagas' disease, but significantly drop after antiparasitic chemotheraphy, suggesting that they mainly recognise the parasite antigen.[105] While it is unclear what role, if any, *Cha*-specific immune responses have in Chagas' disease it appears to be an excellent marker that should be helpful in the diagnosis and monitoring of Chagas' disease.[105]

IV. A very interesting example of molecular mimicry in Chagas' disease is antibodies that recognise functional target molecules. Already in 1976 Sterin-Borda *et al*. demonstarted that IgG from chagasic patients could affect the contraction frequency of atrial preparations *in vitro* through β_1-adrenergic receptors.[107] Later studies have demonstrated that these β_1-adrenergic antoibodies also recognise the ribosomal P0 protein of *T. cruzi* (*TcP0)*.[108] Immunisation with *TcP0* resulted in arrhythmia and conduction abnormalities in 13/16 mice but no inflammation.[109] Our understanding of the role of β_1-adrenergic in the pathogenesis of Chagas' disease is complicated by the

fact that not only does α-β_1-adrenergic receptor antibodies appear in chagasic sera but also α-muscarinic acetylcholine receptor antibodies, which trigger the opposite effects on contractility.[110, 111]

Once again it should be emphasised that autoimmunity and parasite-driven disease are in no way mutually exclusive, but may very well help to facilitate eachother. One such possibility would be that persisting parasites could be recognised through TLRs and result in the production of IL-6, a cytokine found in high levels in inflamed hearts.[112] IL-6 in turn has the ability to override T suppressor cell function[113], which might be a prerequisite for developing autoimmune disease.[114] Several other hypotheses have also been forwarded in relation to the pathogenesis of Chagas' disease, including:

Neurogenic disease. Here it is proposed that cardiac dilatation and enlargement is due to the selective destruction of postganglionic neurons involved in the autonomic control of the heart.[115] The data supporting this hypothesis is controversial since different studies have yielded different results concerning the magnitude of heart denervation. Cardiac denervation is also in other heart diseases, which raises the question whether the denervation is a primary event in the disease progression or secondary to Chagas' disease.

Microvasular disease. That microvascular changes are associated with Chagas' disease has been realised almost since Carlos Chagas' first described the disease in 1909. In 1990 Rossi postulated that alterations in the heart microvasculature might lead to ischaemia, eventually followed by heart damage.[116] Several investigators have since tried to evaluate the role of microvascular changes in Chagas' disease. Unfortunately these experiments have utilised acutely infected animal models and it is therefore very difficult to assess how relevant they are in the context of the chronic disease. Thus it is still not really known to what extent microvascular changes contribute to the pathogenesis of Chagas' disease.

Continual granulocytic activation. According to this hypothesis granulocytes are recruited into the infected heart where they then degranulate, causing additional tissue damage which in turn leads to further recruitment of granulocytes. [117] *In vitro* studies have also demonstrated that eosinophils degranulate when exposed to *T. cruzi* amastigotes[118]. Such a circle could then possibly continue after the removal of the parasite. The experimental evidence supporting this model is the finding that the number of granulocytes (a minor component of Chagasic inflammatory infiltrates) correlates with disease severity.

CONTROL OF CHAGAS' DISEASE

There are several different ways in which infectious diseases can be controlled. In principle these intervention strategies either aim to interrupt transmission of the infectious agent, or to directly kill it, as with vaccines or anti-microbial agents, respectively.

In 1991 the *Southern Cone Initiative* was launched in an effort to interrupt vector-borne transmission by eradicating insect vectors living in close proximity to humans.[119] While the *Southern Cone Initiative* initially only comprised Argentina, Brazil, Chile, Paraguay and Uruguay, several other countries in South and Central America have joined since then. The strategy in use includes spraying of houses with residual insecticides. It has also been necessary to screen blood banks in order to avoid transmitting *T. cruzi* through infected blood. These combined efforts have resulted in a dramatic decrease of new cases of Chagas' disease. In fact, some areas of Latin America are now considered totally free from transmission of *T. cruzi*.[70] However, the large number of animal hosts makes it impossible to eradicate *T. cruzi*. Thus future efforts should be concerned with limiting reinfestation of sprayed houses and interrupting the sylvatic life cycle exhibited by some vector species.

To truly control Chagas' disease it will be necessary to develop vaccines as well as better chemotherapeutic agents. The development of vaccines was for as long time held back by the belief that Chagas' disease had an autoimmune genesis. Thus people feared that a vaccine might induce an inflammatory disease rather than being protective. It should be noted that this is no longer the predominant view of the research community, as voiced by the WHO expert committee: "Recent experimental, histological, and clinical observations tend to show that Chagas' disease should be regarded mainly as a parasitic infection rather than as an exclusively autoimmune disease. One of the consequences of this pathogenic interpretation is that it may be possible to achieve a favourable outcome in infected patients by the administration of specific parasiticidal treatment".[70] This change of opinion has led to an increase in research concerning vaccines against *T. cruzi*, but several obstacles still remain. While vaccines have been very successful in combating bacterial and viral diseases almost no progress has been made towards functional vaccines for any parasitic disease. This is possibly due to the fact that parasites need to persist in order to confer a protective immune response, a phenomenon known as concomitant immunity.

Today there exist two types of drugs that are used to treat *T. cruzi* infections, nitrofurans and nitroimidazoles.[120] Until recently they were only used to treat acute and congenital Chagas' disease but relatively recently it was shown that Benzidazole has a beneficial effect on chronic Chagas' disease.[84] The main drawback is that these drugs often produce severe side-effects when in use. Efforts are currently underway to develop inhibitors of sterol metabolism, protein prenylation, phospholipid signalling and proteases.[70] The majority of these drugs have only been used in experimental studies, however, so it remains to be seen if they will be used in clinical settings.

SCOPE OF THE THESIS

The main focus of this thesis was to develop and utilise experimental models using the protozoan parasite *T. cruzi*. We were especially interested in studying:

I. Mechanisms involved in NK cell activation during early *T. cruzi* infection.

II. Factors that predispose to a chronic inflammatory disease after *T. cruzi* infection.

RESULTS AND DISCUSSION

Activation of NK cells early during *T. cruzi* infection (Papers I and II)

NK cells are lymphocytes capable of secreting cytokines, chemokines and mediating cytotoxic activity.[121] These effector functions are essential components of protective immune responses against several infectious agents.[122, 123] The importance of NK cells in host defense against *T. cruzi* is highlighted by the increased mortality and parasitaemia exhibited by NK-depleted *T. cruzi* infected mice.[40, 41] Several aspects concerning the role of NK cells during *T. cruzi* infection remain poorly understood, including which mechanisms are involved in NK cell activation and how NK cells exert their protective effects. In **paper I** we define a model system that allowed further investigations of how NK cells are activated to produce IFN-γ and mediate cytotoxicity after *T. cruzi* infection. In **paper II** we utilised this model system to specifically determine the role of IFN-α/β and IL-12/IL-23 in NK cell activation and in protective immunity against *T. cruzi*.

In order dissect the mechanisms involved in NK cell activation we began by determining the kinetics of increased NK cytotoxicity and production of IFN-γ, IL-12, TNF-α and IFN-α after *T. cruzi* infection. C57BL/6 mice were infected with either a low (50) or a high dose (10^5 or $5x10^5$) of parasites from the Tulahuen strain of *T. cruzi.* Infection with the low dose of parasites induced small amounts of IFN-α but none of the other cytokines were upregulated and NK cell cytotoxicity was unaltered. Thus we choose to focus on the use of the higher dose of parasites in this and successive studies. Mice infected with the high dose of parasites exhibited a transient peak of IFN-α, IFN-γ, IL-12 and TNF-α at 24 hours post-infection (Fig. 3). Two days post-infection all cytokines were again expressed at baseline levels. IFN-α, IL-12 and TNF-α remained at baseline levels throughout the rest first week of infection whereas a second peak of IFN-γ appeared at 3-4 days post-infection (Fig. 3). Depletion of NK cells resulted in the disappearance of the first IFN-γ peak, demonstrating that this IFN-γ is dependent on NK cells. In contrast, it was shown that the second IFN-γ peak was mainly produced by $CD4^+$ T cells. NK cytotoxicity was increased from 24 h post-infection and remained increased during the whole first week (Fig. 3). The differential kinetics of increased NK cell cytotoxicity and IFN-γ production implies that these effector functions are regulated by different mechanisms.

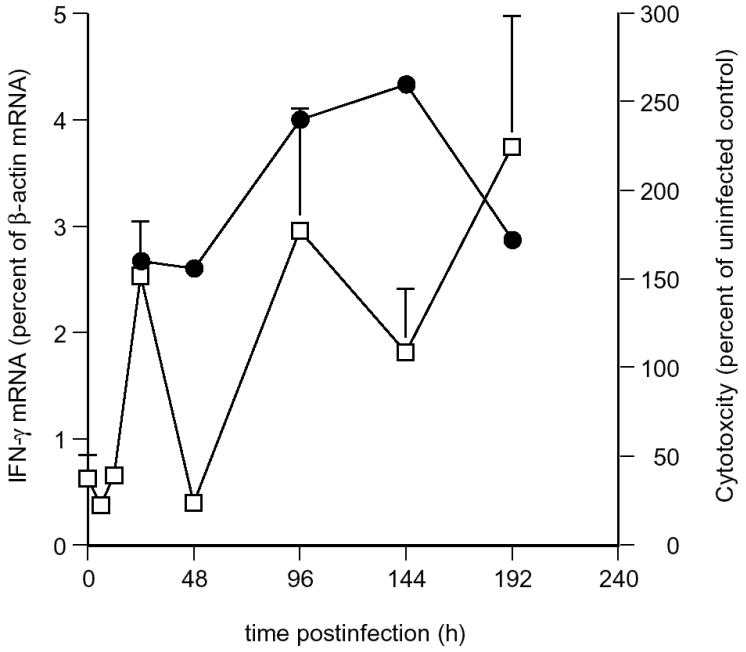

Figure 3. Kinetics of IFN-γ mRNA production and NK cell cytotoxicity after infection with 10^5-$5x10^5$ blood trypomastigotes. IFN-γ mRNA (□) is expressed as mean value ± s.e.m (n=4-5). NK cell cytotoxicity (●) is expressed as the relative ability of spleenocytes from infected versus uninfected animals to lyse Yac-1 cells (E:T ratio is 200:1). The absolute values of cytotoxicity varied between 5-20% for uninfected mice and between 20-50% for infected mice.

Our data is in agreement with previous studies that have demonstrated IFN-γ production early after infection. The study also supports the findings of Reed *et al*. and Cardillo *et al*.,[41, 124] that NK depletion will lead to less production of IFN-γ after *T. cruzi* infection. The mRNA levels of TNF-α were not affected by NK cell depletion. Thus even while TNF-α is a vital component of the protective immune response in *T. cruzi* infection it does not appear to be involved in the protective role exerted by NK cells. The levels of IL-12 mRNA were somewhat reduced in after NK cell depletion, implying that NK cells could contribute to but are not necessary for induction of IL-12. As the IFN-γ production at 8 days post-infection is not dramatically reduced it appears that NK cells are not important in the initial induction of a type 1 immune response.

In contrast to an earlier study performed by Hatcher *et al.*,[125] NK cell cytotoxicity remained elevated during the whole first week post-infection in our study. One difference between these studies is that we utilise a larger dose of parasites, which could potentially explain this disparity.

The induction of IFN-α and IL-12 at 24 hours post-infection combined with their involvement in NK cell activation in other experimental systems prompted us to investigate their role in induction of IFN-γ and increased NK cell cytotoxicity after *T. cruzi* infection (**paper II**). IFN-α/βR deficient and p40 deficient mice were infected with a high dose of parasites and NK cell cytotoxicity and IFN-γ production were analysed. Mice lacking p40 are deficient for both IL-12 and the related heterodimer IL-23, which also has been implicated in NK cells ability to produce IFN-γ.[126] Neither IFN-α, IFN-β, IL-12 nor IL-23 appeared to be involved in the regulation of NK cell-dependent IFN-γ since both types of knockout mice produced IFN-γ at 24 hours post-infection. IL-12/IL-23 is needed for production of IFN-γ at 8 days post-infection, however, since p40 deficient mice were not able to produce IFN−γ at this timepoint. IL-12/IL-23 deficient mice exhibit normal regulation of NK cell

cytotoxicity whereas IFN-α/βR deficient mice are unable to activate NK cell cytotoxicity at 24 hours post-infection (Fig. 4). Later during the infection other factors, possibly including IL-2, result in upregulation of NK cell cytotoxicity.

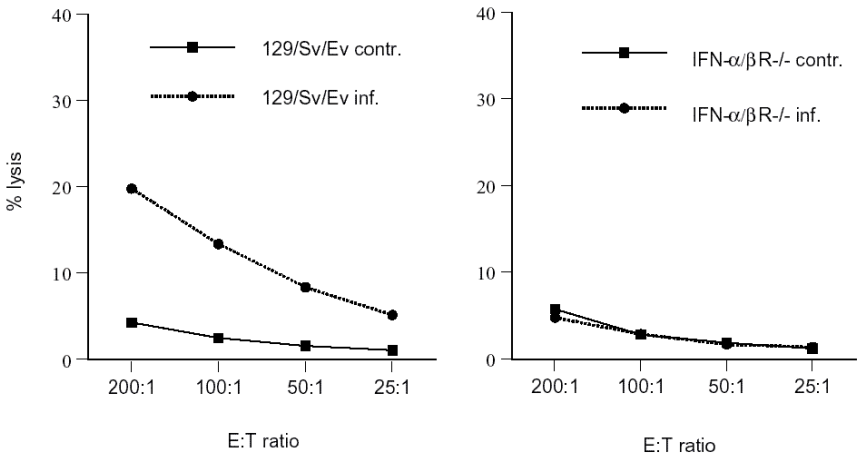

Figure 4. NK cell cytotoxicity at 24 hours postinfection after infection with 10^5 trypomastigotes. Values are expressed as mean (n=4). 100% = lysis of all target cells which was determined by lysis of target cells with a detergent.

Since IFN-α/βR deficient mice lack upregulation of NK cell cytotoxcicity during the first few days post-infection and at the same time exhibit comparable levels of mortality and parasitaemia as wildtype mice, it appears that NK cell cytotoxicity does not play a protective role during *T. cruzi* infection. In contrast, IL-12/IL-23 is absolutely necessary since mice deficient for p40 lack the ability to induce a protective Th1 response. While $CD4^+$ T cell-derived IFN-γ is dependent on IL-12/IL-23 it seems that IL-12/IL-23 is not necessary for the induction of NK cell-dependent IFN-γ. We have not been able to directly prove or disprove that NK cell-dependent IFN-γ is of importance in the protective response against *T. cruzi*. In theory there are several ways in how NK cell-derived IFN-γ could exert its protective effects. First, by activating the trypanocidal activity of macrophages. Second, by augmenting a protective Th1 response. Third, by nonhemopoietic cell-derived IFN-γ-dependent mechanisms.[127]

Our study suggests that neither IFN-α, IFN–β, IL-12 nor IL-23 are involved in the induction of NK cell-dependent IFN-γ. Thus it remains to be determined which factor(s) is responsible for induction of the initial peak of IFN–γ. In addition to IL-12 and IFN-α/β, other cytokines as well as NK cell receptors can induce IFN-γ. The following list contains some factors that might be involved in the production of the NK cell-dependent IFN-γ evident after early *T. cruzi* infection:

IL-18, originally known as interferon-γ inducing factor, which was originally described as a factor able to active NK cells and produce IFN-γ.[128] IL-18 has been implicated to participate in early production of IFN-γ and production of IL-12-independent $CD4^+$ T cell derived IFN-γ, after *T. cruzi* infection.[52, 129]

IL-12 and IFN-α/β related cytokines have been described and deserve attention considering the fundamental role of IL-12 and type I interferons in NK cell biology. Cytokines related to IL-12 does not only include IL-23 but also IL-27, whereas both IL-28 and IL-29 are related to type I interferons. IL-27 is a dimeric cytokine composed

of EBI3, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide.[130] IL-27 is known to enhance IFN-γ production by NK cells cultured with IL-2 and IL-12.[130] IL-28 and IL-29 are distantly related to type I interferons.[131] The signalling of all these cytokines are similar in the sense that they all result in components of the signalling cascade interacting with gene regulatory elements known as interferon stimulated response elements. The similarities between IL-28, IL-29 and type I interferons are further underscored by their antiviral activities.[131] No data are currently available on the role of these cytokines in Chagas' disease.

NK cell receptors, such as the human receptors KIR2DL4 and NKAR and the murine receptor NKR-P1 are able to produce IFN-γ upon ligation/crosslinking.[132-134] Another cell surface receptor that can induce IFN-γ production is the IgG receptor FcγRIII (CD16).[135] No data are currently available on the role of these receptors in Chagas' disease.

Establishment and characterisation of experimental models for polymyositis and Chagas' disease (Papers III and IV)

Animal models are excellent tools for studying biological phenomena *in vivo*. They have successfully been used to provide increased knowledge about the pathogenesis of many diseases. In **paper III** we continued the characterisation of a murine model previously established in our lab,[112, 136] in which we investigated the skeletal muscle involvement of CBA/J mice infected with the Tulahuen strain of *T. cruzi*. The rationale behind this experiment was firstly that increased knowledge about the model system would increase its usefulness, especially considering that skeletal muscle is often the primary target organ in murine Chagasic disease. Secondly, a careful characterisation of the skeletal muscle involvement might also allow us to use this model to study idiotypic inflammatory myopathies, which almost completely lack useful animal models.

Histological and immunohistochemical characterisation of skeletal muscles from CBA/J mice infected with the Tulahuen strain of *T. cruzi* revealed diffuse skeletal muscle inflammation during the acute phase of the disease. Later during the chronic phase, when the parasitaemia had been cleared, a more focal pattern of inflammation mainly located in the endomysium became apparent. The inflammation was mainly comprised of T-cells and macrophages throughout the disease. Using image analysis we demonstrated that $CD4^+$ T cells predominated around blood vessels, in contrast to $CD8^+$ T cells which congregated in the endomysium during chronic disease. Other characteristic features of the chronic inflammation included MHC class I expression on invaded non-necrotic muscle fibers, as well as some intact fibers. No signs of fibrosis or increased connective tissue formation were evident.

The differential distribution of $CD4^+$ and $CD8^+$ T cells is a feature shared between all types of human idiopathic inflammatory myopathies.[137] The predominant localization of infiltrates in the endomysium rather than in the perivascular areas is a characteristic feature of PM and IBM, but differs from that of DM in which perivascular infiltrates predominate.[137] The similarity of our model with Polymyositis (PM) and inclusion body myositis (IBM) is further accentuated by the frequent occurrence of $CD8^+$ T-cells invading non-necrotic muscle cells (Fig. 5), all of which express MHC class I.[137] Since

no vacuolated muscle fibers were ever noted, the model lacks the characteristic immunohistopathological features of IBM. Thus this model most closely resembles human PM. How useful this model will be in studies of human idiopathic inflammatory myopathies remains to be seen. The use of an infectious organism as the instigating agent in this model may limit its practical applicability. Another obvious concern is how well *T. cruzi* infection mimics the initiating events of human idiopathic inflammatory myopathies. This is a difficult question to answer, as the etiogenesis of most cases of inflammatory myopathies remains unknown. Infectious agents, including *T. cruzi*, have been implicated in some cases of inflammatory myopathies, but in the majority of cases it is not known whether infections play a role or not. However, it is interesting to note that all infectious agents associated with PM are intracellular. Thus hopefully this model could be a representative and valuable experimental tool for examining the nature and order of events that eventually lead to chronic muscle inflammation with T-cell infiltrates as well as their relationship to impairment of muscle function.

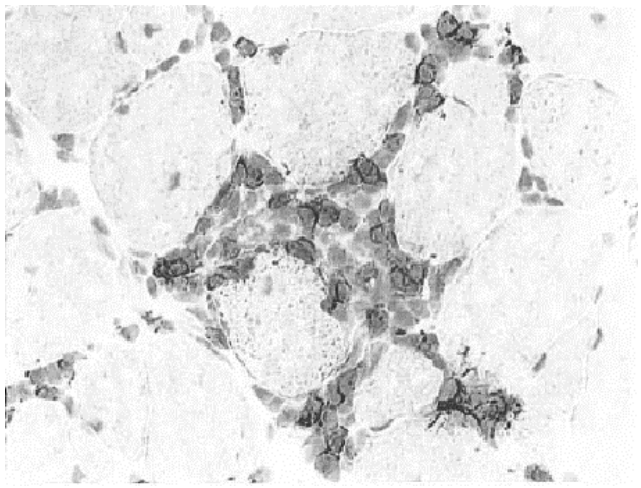

Figure 5. A nonnecrotic muscle fiber in the endomysium invaded by $CD8^+$ T-cells. The tissue section was collected at 4 months post-infection

In another attempt to improve the animal models at hand and to further the understanding of *T. cruzi* chronic inflammatory diseases, we investigated the role of the parasite and host genotypes. This was achived by combining different parasite strains/clones and mouse strains through experimental infections. Tulahuen infected mice exhibited a severe acute disease characterised by high levels of parasitaemia and substantial mortality. In contrast, all CA-1 infected mice survived the acute phase of the disease and had lower levels of parasitaemia. C57BL/6 and BALB/c mice infected with the CA-1 clone of *T. cruzi* both exhibit myocarditis. C57BL/6 mice also develop very severe polymyositis, again characterised by inflammatory infiltrates predominantly localised in the endomysium and degenerating muscle fibers. BALB/c mice infected with the CA-1 clone of *T. cruzi* only exhibit perivasculitis in skeletal muscle. BALB/c mice were resistant to a chronic disease after infection with the Tulahuen strain of *T. cruzi* whereas CBA/J mice developed PM and mild myocarditis.

Parasite	**CA-1**		**Tulahuen**	
Mouse	heart	muscle	heart	muscle
BALB/c	myocarditis	vascular disease	-	-
C57BL/6	myocarditis	PM	-	?
CBA/J	myocarditis	vascular disease	myocarditis	PM

Table 1. The genotype of both the parasite and the host determines the outcome of the *T. cruzi* infection. PM = polymyositis.

Our data thus demonstrates that the severity of the acute phase of Chagas' disease does not correspond to the severity of the chronic disease, contrary to an earlier report.[138] The study also suggests that it might be appropriate to take the parasite genotype into account in this type of clinical study. The most intriguing aspect of this study, however, is the differential outcome, chronic disease versus healing, exhibited by CBA/J or BALB/c mice, respectively, infected with the Tulahuen strain of *T. cruzi*. This difference will make it possible to define factors that may play a role in causing Chagas' disease by comparing the infection during the acute phase between these two strains. Thus it will be possible to investigate whether any correlations between cytokine profiles or autoimmunity and chronic disease exist.

Toll-like receptors (TLRs) in experimental Chagas' disease (Paper V)

Several lines of evidence have suggested a role for persisting parasites in Chagas' disease. It is not known how such persisting parasites are recognised by the immune system. One possibility is that pattern recognition receptors (PRRs) recognise conserved antigens from the parasite, thereby propagating the inflammatory response. Several different types of PRRs exist, including mannan-binding protein,[139] C-reactive protein,[140] the mannose receptor,[141] the scavenger receptor,[142] PKR[143] and TLRs.[143] In this study we chose to focus on the role of TLRs as they are known to recognise two structures from *T. cruzi*, GPI anchors and the protein Tc52.[32, 144] Even though both of these structures are recognised by TLR2 we chose to use mice deficient for an adaptor protein MyD88, which is involved in much of the signalling from all TLRs.[145] MyD88 deficient mice were selected as there might be other conserved *T. cruzi* structures that are recognised by other TLRs.

We first addressed the question of whether MyD88 dependent signalling is important in the protective immune response. Thus MyD88-deficient mice were infected with the CA-1 clone or Tulahuen strain of *T. cruzi*. As these infected MyD88-deficient mice exhibited a similar parasitaemia and cumulative mortality, it appears that MyD88-dependent signalling does not play an important role in inducing a protective immune response against *T. cruzi*. As MyD88 is not only required for signalling through TLRs but also for signalling through IL-1 and IL-18 receptors it seems that neither IL-1 nor IL-18 are absolutely required for the induction of a protective immune response to *T. cruzi*.[146] In this respect *T. cruzi* behaves like *Plasmodium berghei*, where MyD88-deficient and wildtype mice had similar parasitaemia and mortality.[147] In contrast, MyD88-deficient mice are more susceptible than wildtype mice to *Toxoplasma gondii* infections, with significantly increased mortality and parasite burden.[148] Why MyD88 is needed to combat some pathogens but not others remains unclear. It is tempting to speculate, however, that *T. cruzi* deliberately allows itself to be recognised by TLRs. This is based on the fact that the secreted protein Tc52 is recognised by TLRs. If this recognition would be deleterious for the parasite it would seem likely that this protein would have been modified during evolution to avoid such recognition. Tc52 ligation also results in differential macrophage activation compared to that induced by GPI anchors. The potentially voluntary recognition of Tc52 might be responsible for the extreme activation of the immune system seen after *T. cruzi* infection, which in turn might result in ill-advised allocation of the immune systems' resources.

One of the primary purposes of this study was to investigate if TLR signalling plays a role in the chronic inflammatory disease induced by *T. cruzi*. Histopathological examination of skeletal muscles collected at 7 months post-infection demonstrated that MyD88-deficient mice exhibited a significantly less severe chronic inflammatory disease. Hearts collected at the same timepoint exhibited a substantially less severe inflammatory disease than in skeletal muscles but there were no differences between infected MyD88-deficient and wildtype mice. Whether this differential influence of MyD88 has anything to do with the larger numbers of parasites in skeletal muscles than the heart remains to be seen.

	Inflammatory infiltrates in skeletal muscle (%)	Inflammatory infiltrates in heart muscle (%)
Infected wildtype	28±24	1.2±1,6
Infected MyD88$^{-/-}$	6±8	1.0±2
Uninfected wildtype	0	0
Uninfected MyD88$^{-/-}$	0	0

Table 2. Percentage of inflammatory infiltrates in skeletal muscle tissue and heart muscle tissue.

The ameliorated myositis apparent in MyD88-deficient mice could be could be dependent on several different mechanisms, including:

Firstly, TLRs could recognise *T. cruzi*-derived ligands from persisting parasites resulting in a continued proinflammatory response, which could fuel the chronic inflammation.

Secondly, TLRs could recognise endogenous TLR ligands, such as heat shock protein 60, fibronectin, fibrinogen and hyaluronan.[149-152] These ligands might be available to the immune system after tissue damage. Thus it is possible that this constitutes a vicious cycle in which tissue damage is followed by release of endogenous TLR ligands, resulting in a continued inflammation and more tissue destruction.

Thirdly, MyD88-deficient mice are not only deficient in TLR signalling but also in IL-1 and IL-18 signalling. Either of these two factors may thus be responsible for the decreased inflammation.[146]

Fourthly, MyD88-deficient mice have lower amounts of *T. cruzi*-specific antibodies at 7 months post-infection. The amounts of host/parasite-crossreactive antibodies should also be decreased. If such antibodies are important in the chronic inflammatory process evident in experimental Chagas' disease (which in itself is highly debatable), it could explain the decrease in inflammation.

The relevance of TLRs in human Chagas' disease is still an open question as the major target organ in Man, the heart, did not exhibit any decreased inflammation in this study. The possibility that TLRs are involved in the chronic human disease should not be discounted, however. The reason why it is important to address the role of TLRs in human Chagas' disease is the fact that TLRs are considered as drug targets for other diseases.[153] Thus if inhibitors to TLRs prove beneficial in chronic Chagas' disease it is far more probable that such inhibitors would be available from pharmaceutical companies rather than the possibility of these companies developing a parasite-specific drug.

CONCLUDING REMARKS

NK cells are needed for the production of a transient IL-12-independent peak of IFN-γ at 24 hours after infection with a high dose of *T. cruzi*. In contrast to the transient nature of IFN-γ production, NK cell cytotoxicity remains elevated during the whole first week after infection. The increase of NK cell cytotoxicity was initially dependent on IFN-α/βR. IFN-α/βR deficient mice are not more susceptible to *T. cruzi* infection, suggesting that early NK cell activation does not play a major role in defence against *T. cruzi*. IL-12-deficient were more susceptible to *T. cruzi* infection, however, probably due to their impaired ability to mount a Th1 response.

Mice infected with *T. cruzi* can develop both myocarditis, PM and perivascular inflammation. Thus it is possible to use murine *T. cruzi* infection not only as a model for Chagas' disease but also for idiopathic inflammatory myopathies. The chronic disease is dependent on both the host and parasites genotypes but not on the severity of the acute infection.

TLR/IL-1/IL-18 signalling appears to play no major protective role during acute *T. cruzi* infection as mice lacking MyD88 exhibit a normal acute phase in respect to cumulative mortality and parasitaemia. MyD88-dependent signalling does play a deleterious role during the chronic infection, however, as MyD88-deficient mice developed less severe PM.

FUTURE PERSPECTIVES

The studies included in this thesis have, as with most scientific studies, raised more questions than they have answered. They also span over a relatively wide area, and thus future studies should benefit from a more focused effort to in detail investigate one of these questions that have arised.

One such question that remains to be answered is what factors are responsible for the production of NK cell-dependent IFN-γ. Obvious candidates include IL-18, homologues of IFN-α/β and IL-12 as well as NK cell receptors. Understanding how NK cell-dependent IFN-γ is induced might also allow us to directly investigate whether it the NK cells' ability to produce IFN-γ early after infection that is important for its protective function.

The experimental models established in **papers III and IV** also require further characterisation in order to maximise their usefulness. The following issues should be clarified: First, do mice that develop PM also have a decreased muscle function *in vivo*? Second, do parasites persist in all chronically diseased organs? Third, will parasite chemotheraphy ameliorate the disease? While the last two questions have been addressed in other experimental models of Chagas' disease it would still be appropriate to repeat these experiments in our models.

Lastly, it will be very interesting to follow up on the role MyD88 in chronic inflammation induced by *T. cruzi*. An obvious experiment that should be done is to see how TLR2 deficient mice respond to *T. cruzi* infection. Such an experiment might allow us to distinguish between the role of TLR and IL-1/IL-18 signalling. To differ between the potential role of endogenous and exogenous TLR ligands will be more difficult. However, if it turns out that *T. cruzi* is only recognised by TLR2 it will be possible to compare severity of inflammation between TLR2-deficient mice and mice defective in multiple TLRs (including TLR2 and some which recognise endogenous ligands). Such a comparison would then allow us to determine the relative role of endogenous and exogenous TLR ligands in the inflammatory disease process.

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