SUDESH KUMAR
Role of B and T Lymphocytes in Immune Control of *Trypanosoma cruzi*
(Under the Direction of Dr. RICK L. TARLETON)

The study was undertaken to investigate the contribution of B cells, Th1/ Th2 subsets of $CD4^+$ and Tc1/Tc2 subsets of $CD8^+$ T cells to control of *Trypanosoma cruzi* infection in mice. B cell deficient mice exhibited a delay in parasitemia and an extended time to death relative to mice lacking $CD8^+$ T cells. But, both B cell- and $CD8^+$ T cell-deficient mice succumbed to the infection, suggesting the requirement of an antibody and a $CD8^+$ T cell response for control of *T. cruzi*. To investigate the role of Th1 and Th2 subsets of $CD4^+$ T cells in determining the outcome of *T. cruzi* infection in mice, we developed *T. cruzi* clones that express ovalbumin (OVA) and used OVA-specific Th1 and Th2 cells. Mice receiving OVA-specific Th1 cells and then challenged with OVA-expressing *T. cruzi* G-OVA.GPI showed significantly lower parasitemia and increased survival in comparison to mice that received no cells or Th2 cell recipients that developed higher parasitemias, exhibited higher tissue parasitism and inflammation and higher. Mice receiving a mixture of both Th1 and Th2 OVA-specific cells were also not protected from lethal challenge. OVA-specific Tc1 and Tc2 cells were also assayed for their ability to protect mice during infection with OVA-expressing *T. cruzi*. Mice receiving OVA-specific Tc1 or Tc2 cells developed lower parasitemia and tissue parasitism and survived a normally lethal infection with OVA-expressing *T. cruzi* GOVA.GPI. In contrast, mice receiving no cells, Tc1 cells or Tc2 cells but infected with wild-type *T. cruzi* developed significantly higher blood and tissue parasite burdens and succumbed to the infection. Tc1 cells continued to make primarily IFN-γ but the Tc2 population failed to retain a type 2 cytokine production pattern and instead exhibited predominantly a type 1 pattern of

cytokine production *in vivo*. Tc1 or Tc2 populations incapable of producing IFN-γ were unable to provide protection to infection with OVA-expressing *T. cruzi* suggesting that Tc1 cell-mediated protection in mice against lethal *T. cruzi* GOVA.GPI was dependent on the production of IFN-γ by these cells.

INDEX WORDS: Chagas Disease, Parasite Immunity, B Cells, CD4, CD8, *T. cruzi*

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# ROLE OF B AND T LYMPHOCYTES IN IMMUNE CONTROL OF *TRYPANOSOMA CRUZI*

by

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DEDICATION

To my wife Renu, my sons Nishant and Sungum

and

my family in India

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

*Trypanosoma cruzi* causing life threatening Chagas Disease in humans in South and Central America is a major public health problem. 16-18 million people are infected with the parasite and approximately 60 million people are at the risk of contracting the infection (1). *T. cruzi* is transmitted to the mammalian host as metacyclic trypomastigote by blood feeding reduviid bugs of the subfamily Triatominae. In the mammalian host, *T. cruzi* circulates in the blood as trypomastigote forms that can invade a wide variety of cells including cardiac and skeletal muscle cells, nerve cells and the cells of the reticuloendothelial system. Once inside the cells, the trypomastigotes convert to amastigotes which, following numerous rounds of replication, convert back to blood-form trypomastigotes that burst the host cell and re-enter the circulation.

*T. cruzi* infection in humans can be manifested in three stages (2) and these stages can be nicely replicated in infections involving various mice and parasite strain combinations. The acute stage of the infection in humans is characterized by clinical symptoms of pyrexia, hepatomegaly, splenomegaly and lymphadenopathy. In mice, intracellular nests of the amastigotes can be detected in several organs and tissues including skeletal, smooth and cardiac muscle and the nervous system (3). Most individuals infected with *T. cruzi*, progress from the acute stage to a clinically asymptomatic indeterminant phase (4, 5). This progression is brought about by the generation of a strong host immune response that reduces the parasite burden in the blood and tissues to near undetectable levels (6). As a consequence, tissue inflammation and necrosis is also thought to substantially subside during the indeterminant phase (4). Approximately 30-40% of *T. cruzi* infected

individuals after an extended asymptomatic period, develop chronic disease symptoms marked by progressive myocarditis characterized by inflammation and fibrosis of myocardial fibers (2). This condition may lead to several complications including myocardial infarction, thromboembolism and arteriosclerosis (7). Death may occur due to congestive heart failure and apical aneurisms (5). Throughout the course of infection however, *T. cruzi* remains a target of both humoral and cell-mediated immune responses.

## Immunity to *T. cruzi*

### Role of antibodies

*T. cruzi* in different mammalian hosts including humans is the target of a strong antibody response. Traditionally, antibody-mediated killing of the parasite *in vivo* has been suggested as the predominant effector mechanism against *T. cruzi*. Therefore, the majority of the early work focused on elucidation of different mechanisms by which antibodies cleared the parasite in vivo. Among the humoral immune responses, lytic (8), opsonic (9), ADCC (antibody-dependent cellular cytotoxicity) (10), complement activating (11, 12), and invasion blocking activities of antibodies (13) have been documented against *T. cruzi.*

"Lytic antibodies" are the antibodies from infected mice and humans that coat the surface of *T. cruzi* and render the parasite susceptible to lysis by the alternative complement-mediated pathway. In an attempt to elucidate the mechanism of lytic antibody-dependent activation of the alternative pathway by trypomastigotes, Kipnis et al. (1985) (14), demonstrated that Fab fragments derived from the IgG of chagasic patients could promote complement-mediated lysis of trypomastigotes. Therefore, the lysis-promoting activity of the antibodies was independent of the Fc region and was

instead related to the binding of Fabs to specific molecules on the parasite surface. On the contrary, Fab fragments of antibodies to *T. cruzi* failed to protect mice suggesting that other mechanisms (ADCC or phagocytosis) but not lysis may be involved in antibody-mediated clearance (9, 15). For example, some strains of *T. cruzi* are not sensitive to killing by lytic antibodies (16, 17) and not all strains of *T. cruzi* are capable of inducing lytic antibodies (18, 19). Other limitation of lytic antibodies is that these are not easy to elicit by immunization with dead parasites or with purified *T. cruzi* antigens (20), although they are readily detected in different mammalian hosts including humans infected with *T. cruzi* (21, 22).

Attempts have been made to identify targets of lytic antibodies and to determine how binding of lytic antibodies on these targets activates the alternative complement pathway. Among several molecules identified, the 160 kDa C regulatory protein (CRP) induces lytic antibodies necessary to activate the alternative C pathway on the surface membrane of the parasites for lysis to occur (23-25). T-DAF, a 87-93 kDa protein, accelerates the decay of C3 convertase and factor B binding, and the recombinant protein induces lytic antibodies and inhibits convertase activity (26-28). In addition, mucin glycans of infective trypomastigotes containing terminal α-galactosyl residues are epitopes recognized by lytic antibodies from chronic chagasic patients (29, 30). These anti-galactosyl antibodies have the ability to lyse trypomastigotes without assistance from classical or alternative complement pathway (31). Some of the targets for lytic antibodies have also been used to immunize mice and have been found to provide some degree of protection against subsequent *T. cruzi* infection (32-34).

A role for B cells in the control of *T. cruzi* infection has been shown in studies where protection induced by adoptive transfer of spleen cells from chronically infected mice into naive mice infected with *T. cruzi* was prevented by depletion of B cells (reviewed in (35). The importance of antibodies to the control of *T. cruzi* infection has also been shown in rats and mice which became highly susceptible to *T. cruzi* infection after treatment with anti-μ antibodies (36, 37). However, vaccination attempts designed to boost an antibody response alone have repeatedly failed to provide complete protection against *T. cruzi* infection (33, 38-41) making it clear that other immune responses, presumably, cell mediated immune responses, must be induced in order to control the infection. The availability of B cell deficient mice (42) provided us the opportunity to directly assess the role of B cells and to investigate if antibody production is the major mechanism of control as compared to T cell responses in *T. cruzi* infection. The ability of muMT (B cell deficient) mice to control infection with *T. cruzi* was tested by monitoring parasitemia, survival and tissue parasitism in comparison to mice lacking $CD8^+$ T cells. Immunization of muMT mice with a less virulent strain of *T. cruzi* followed by infection with a virulent strain helped us determine if a primed cell-mediated response in the absence of B cells and antibodies could confer protection in muMT mice.

**Role of $CD4^+$ T cells**

The requirement for $CD4^+$ T cells in immune control of *T. cruzi* was established by infection of mice in which genes encoding proteins required for generation of $CD4^+$ T cell were knocked out (37, 43, 44) and in mice given anti-CD4 antibodies (45). In the absence of $CD4^+$ T cells, mice succumb to infection early in the acute phase with high blood and tissue parasite load and the absence of inflammatory responses at the site of

infection. In $CD4^+$ T cell knockout mice, the levels of IFN-γ mRNA and nitric oxide production in response to *T. cruzi* infection are significantly lower as compared to wild-type mice (46). Altogether, these and other studies (47) suggest that $CD4^+$ T cells are required for protection against *T. cruzi* infection. The protective capacity of $CD4^+$ T cells in *T. cruzi* infection has also been analyzed by adoptively transferring *T. cruzi*-specific $CD4^+$ T cells in mice. Nickell *et al.* (1987) (48) showed that clones of $CD4^+$ T cells produced by stimulating splenocytes from immunized mice with whole trypomastigote stage antigens conferred significant protection in naive mice from challenge infection with *T. cruzi*. These cells also induced macrophage-mediated trypanocidal activity *in vitro.* Gruppi *et al.* (1995) (49) showed that adoptive transfer of lymph node cells from mice immunized with exoantigen Ea 4.5 of *T. cruzi* conferred some degree of protection in mice challenged with *T. cruzi*.

*Th1,Th2 cells and T. cruzi infection*

In the presence of antigen and appropriate co-satimulation plus cytokines, naïve CD4+ T cells proliferate and differentiate into effector cells with distinct cytokine production profiles. The development of Th1 cells is promoted by the presence of IL-12. This subpopulati8on of Th cells produces the signature cytokine IFN-γ and has a crucial role in macrophage activation and classical DTH responses. Th2 cells develop under the influence of IL-4 and the absence of IL-12. Th2 cells are identified by their production of the cytokines IL-4, IL-5 and IL-13 and participate in allergic responses, immune response to helminths and in the downregulation of Th1 responses. The existence of Th1 and Th2 subsets of $CD4^+$ T cells in mice was originally described by Mosmann et al. (50) and in humans by Romagnani and colleagues, (51, 52). The development of Th1 and Th2

responses determining different outcome of an infection was first demonstrated in different strains of mice infected with *Leishmania major* (53-55). Resistant C57BL/6 mice mounted a protective Th1 response against *L. major* while susceptible BALB/c mice generated a predominant Th2 response. Additional evidence has emerged in infection involving intracellular pathogens suggesting that a strong Th1 response is generally associated with protection while a Th2 response is linked to increased susceptibility (56-58). In *T. cruzi* infection, production of the type 1 cytokine, IFN-γ, in the acute phase of infection is associated with resistance (59, 60). Depletion of IFN-γ exacerbates parasitemia and results in increased mortality in *T. cruzi* infected mice (61, 62). Similarly, IL-10, a considered to be previously a type 2 cytokine, is associated with susceptibility to *T. cruzi* in several murine models (63) with an elevation of IL-10 production in susceptible mice strains as compared to resistant strains (64). The results of some studies suggest a requirement for a balanced type 1 and type 2 response to control infection with *T. cruzi* (61, 63, 65).

To investigate the contribution of Th2 cells in protection against *T. cruzi* infection in mice, Barbosa de Oleveira *et al.* (66) immunized mice with *T. cruzi* lysate and isolated *T. cruzi*-specific T cells secreting IL-4 and IL-10 from the lymph nodes of immunized mice. Mice receiving these Th2 cells became highly susceptible to infection with *T. cruzi* (Y strain). *In vitro* restimulation of splenocytes and lymph nodes cells from mice (that received Th2 cells) with Tc-Ag showed increased production of IL-4 and IL-10 and decreased secretion of IFN-γ. Altogether, these results suggested that transfer of type 2 cytokine producing cells in mice shifted the balance of cytokine production towards a Th2 type response, resulting in increased susceptibility to *T. cruzi.* Direct evidence for

the role of a Th1 vs. Th2 cells in controlling *T. cruzi* infection comes from infection of Stat-6 and Stat-4-deficient mice (67). Stat-6-deficient mice capable of making only a Th1 response survived infection with *T. cruzi* while Stat-4-deficient mice able to make only a Th2 response succumbed to the infection. These results demonstrate the requirement of a protective Th1 response for survival against *T. cruzi*. With the limited information available, a linkage between Th1 responses and resistance has not been so firmly established with respect to *T. cruzi* infection and a comprehensive analysis of the role of antigen-specific Th1 and Th2 cell in controlling T. cruzi infection remains to be done.

To determining the relative contribution of Th1 and Th2 cells in the control of *T. cruzi* infection in mice, we developed a Th1, Th2 cell transfer system. We wanted to ask if transfer of Th1 or Th2 cells could protect mice from a lethal infection with *T. cruzi*. This system could also allow us to monitor the persistence and expansion of these Th1 and Th2 cells *in vivo* as the infection progressed. However, in the absence of transgenic mice specific for a *T. cruzi* antigen, we did not have a ready source of *T. cruzi*-specific T cells (to generate Th1 and Th2 cells). Fortunately, with the availability of transgenic mice making T cells with single antigen specificity and the availability of monoclonal antibodies that recognize these cells, it is now possible to adoptively transfer the antigen specific cells into wild type mice and study their immune effector function, migration and expansion (68-71).

We studied the contribution of Th1 and Th2 cells in controlling *T. cruzi* infection by using OVA-specific transgenic T cells. Briefly, $CD4^+$ T cells from DO.11 OVA transgenic mice were used to generate clonal populations of Th1 and Th2 cells with

specificity to OVA peptide. A *Trypanosoma cruzi* strain expressing OVA protein was generated and served as a target for OVA-specific Th1 and Th2 cells. We determined if adoptive transfer of *T. cruzi*-specific Th1 or Th2 cells in naïve mice resulted in protection versus exacerbation of infection and /or disease after lethal challenge with *T. cruzi*. As stated above, this system enabled us to directly monitor the homing and expansion of OVA-specific Th1 and Th2 cells in lymphoid organs and at the site of infection in the recipient mice.

**Role of $CD8^+$ T cells**

Relatively recently, class I MHC restricted $CD8^+$ T cells have been identified as critical effectors in immunity to *T. cruzi* (72). Support for the importance of $CD8^+$ T cells in immune control of *T. cruzi* comes from studies reporting that depletion of $CD8^+$ T cells by antibody treatment or targeted disruption of genes necessary for the generation of mature $CD8^+$ T cells resulted in high susceptibility to *T. cruzi* in murine models (44, 73-76). In the absence of $CD8^+$ T cells, mice are highly susceptible to infection and die with high tissue parasitism early in acute phase of infection and without significant inflammatory responses at the site of infection (45, 75).

Further evidence for the possible role of $CD8^+$ T cells in mediating an active anti-*T. cruzi* inflammatory response in mice comes from studies in which $CD8^+$ T cells are shown to be associated with the presence of *T. cruzi* as a predominant cell population in the tissues (77). In chronic chagasic myocarditis, higher numbers of $CD8^+$ T cells were correlated with the presence of parasites in the cardiac muscle (78). These data suggest that $CD8^+$ T cells may contribute to the initiation and maintenance of a protective inflammatory response against *T. cruzi in vivo*.

Recently, the understanding of the role of $CD8^+$ T cells in immunity to *T. cruzi* has been extended with the presentation of information on the protective capacity of parasite-specific CTLs and the identification of antigen targets for some of these anti- *T. cruzi* $CD8^+$ T cells. Three members of the trans-sialidase family of surface proteins have now been identified as targets of anti-*T. cruzi* CTL responses in both mice (79, 80) and humans (81) and short term $CD8^+$ T cell lines producing type 1 cytokines specific for one of these molecules, TSA-1, have been shown to transfer some protection to naive mice (82). These T cell lines were highly cytolytic for peptide-pulsed or *T. cruzi*-infected target cells and were potent producers of both IFN-γ and TNF. However, the relative contribution of the cytolytic activity or cytokine production by these protective $CD8^+$ T cells in immune clearance of *T. cruzi* is not well understood.

$CD8^+$ T cells kill and/or mediate immune control via at least three distinct mechanisms. The perforin/granzyme cytolytic pathway was the first described mechanism of cytolysis by $CD8^+$ CTL and is still considered the primary cytolytic mechanism in most systems (83). Perforin/granzyme mediated cytolysis involves the release of granules containing perforin, which polymerizes to form pores in the target cell membrane. Granzymes, also a component of the granule contents, may enter through these pores and induce apoptosis of the target cell (83, 84). The generation of knockout mice lacking perforin or granzyme B has allowed investigators to explore the role of this pathway in $CD8^+$ T cell response in a number of systems (85-91).

In a number of viral infections, particularly those due to cytopathic viruses, cytokine production rather than killing via the perforin/granzyme pathway is a major factor in protection mediated by CTL and (86). Also, early control of infection with either

*Toxoplasma gondii* (85) or *Mycobacterium tuberculosis* (89) and irradiated sporozoite-induced protection in *Plasmodium berghei* infection (90) are all perforin-independent and involve production of type 1 cytokines.

$CD8^+$ CTL may also kill their targets by Fas/Fas-L interactions (83, 84). The Fas/Fas-L pathway of cytolysis has generally been considered to be of primary importance as an immune regulatory pathway (84, 88, 92). However, a number of recent studies have documented a prominent role for Fas/Fas-L interactions in CTL-mediated killing of infected or tumor targets (88, 92-94). Infection of FasL-deficient mice with *T. cruzi* increased parasite burden and mortality as compared to wild-type mice (95). However, this heightened susceptibility of FasL deficient mice was also attributed to increased production of type 2 and lack of type 1 cytokines.

We were interested in determining the role of perforin/granzyme pathways of cytolysis by $CD8^+$ T cells in *T. cruzi* infection. Parasitemia and mortality in perforin and granzyme B-deficient mice in comparison to wild-type C57BL/6 mice infected with Brazil strain of *T. cruzi* was the first direct measure of the role of these pathways in *T. cruzi* infection. Lysis of target cells (stimulated with *T. cruzi* peptide) by effectors cells from chronically infected perforin and granzyme B-deficient mice was another indicator of the possible role of perforin and granzme pathway of cytolysis in *T. cruzi* infection.

*Tc1, Tc2 cells and T. cruzi infection*

Analogous to Th1 and Th2 subsets of $CD4^+$ T cells, subsets of $CD8^+$ T cells producing predominantly type 1 (Tc1) or type 2 (Tc2) cytokines have been identified (96-101). Eventhough, Tc1 and Tc2 cells produce distinct cytokines, they seem to induce equally potent inflammatory reactions against their target antigen(102). The identification of Tc1

and Tc2 subsets of $CD8^+$ T cells and the ability of antigen-specific $CD8^+$ T cells to secrete different cytokines suggests that these cells perform distinct effector functions that might in turn influence the outcome of immune responses against infections. For example, in pulmonary viral infection, adoptively transferred virus-specific Tc1 cells but not Tc2 cells reduced the virus titer earlier in the lungs (103). *In vitro* generated Tc2 cells were less efficient than Tc1 cells to protect mice against LCMV infection and the inability of Tc2 cells to confer protection against LCMV was attributed to defective homing of Tc2 cells in the tissues despite high cytolytic activity and IFN-γ production by these cells (104). There is evidence for induction of Tc1 cells mediating protective immunity to *Mycobacterium tuberculosis* (an intracellular pathogen) in mice following immunization with heat killed *Mycobacterium vaccae* (105). Adoptive transfer of $CD8^+$ T cell line producing type 1 cytokines IFN-γ and TNF in mice has been shown to confer significant protection against *T. cruzi* (82). In *T. cruzi* infection, a comprehensive analysis of the role of antigen specific Tc1 and Tc2 cells in immune control of the parasites however, has not been done.

Our strategy to study Tc1 and Tc2 mediated immunoprotection against *T. cruzi* was similar to that outlined above for Th1 and Th2 cells. Briefly, OVA specific Tc1 and Tc2 cells were generated from CD8 OVA (OT-1) transgenic mice (106) and adoptively transferred into naïve mice that were challenged with *T. cruzi* expressing OVA. Survival of Tc1 and Tc2 cell recipient mice and their ability to control parasitemia after lethal challenge with OVA expressing *T. cruzi* was monitored as an indicator of Tc1 or Tc2 mediated immune protection. Adoptive transfer of Tc1 and Tc2 cells lacking the ability

to make IFN-γ into mice allowed us to ask if cytolytic activity in the absence of IFN-γ production could protect mice from challenge with *T. cruzi*.

In summary, several studies have documented the critical role of both $CD4^+$ and $CD8^+$ T cells in controlling *T. cruzi* infection in murine models. Lack of either of these T cell populations renders the mice highly susceptible to *T. cruzi* infection. A comprehensive elucidation of the role of B cells, Th1/Th2 and Tc1/Tc2 subsets of CD4 as well as $CD8^+$ T cells will help us to identify potential immune intervention strategies against *T. cruzi*.

**References**


1. 1991. Control of Chagas disease. Report of a WHO Expert Committee. *World Health Organ.Tech.Rep.Ser. 811:1*.
2. Santos-Buch, C. A. a. A. M. A. 1985. Pathology of Chagas Disease. In *Immunology and Pathogenesis of Trypanosomiasis*. T. I., ed. CRC Press, p. p. 152.
3. Postan, M., J. A. Dvorak, and J. P. McDaniel. 1983. Studies of Trypanosoma cruzi clones in inbred mice. I. A comparison of the course of infection of C3H/HEN- mice with two clones isolated from a common source. *Am J Trop Med Hyg 32:497*.
4. Andrade, Z. A. a. S. A. 1971. American Trypanosomiasis. In *Pathology of Protozoan and Helminthic Diseases*. R. A. Marcial-Rojas, ed. Williams and Wilkins Baltimore, p. p. 69.
5. Mott, K. E. 1976. Chagas' disease and left-axis deviation [letter]. *Lancet 2:801*.
6. Hoff, R., K. E. Mott, J. F. Silva, V. Menezes, J. N. Hoff, T. V. Barrett, and I. Sherlock. 1979. Prevalence of parasitemia and seroreactivity to Trypanosoma cruzi in a rural population of Northeast Brazil. *Am J Trop Med Hyg 28:461*.
7. Rossi, M. A. 1990. Myocardial damage in Trypanosoma cruzi myocarditis: a role for macrophages. *Can.J.Cardiol. 6:293*.
8. Lages-Silva, E., L. E. Ramirez, A. U. Krettli, and Z. Brener. 1987. Effect of protective and non-protective antibodies in the phagocytosis rate of Trypanosoma cruzi blood forms by mouse peritoneal macrophages. *Parasite Immunol. 9:21*.

9. Scott, M. T., and L. Moyes. 1982. 75Se-methionine labelled Trypanosoma cruzi blood trypomastigotes: opsonization by chronic infection serum facilitates killing in spleen and liver. *Clin.Exp.Immunol. 48:754*.

10. Lima-Martins, M. V., G. A. Sanchez, A. U. Krettli, and Z. Brener. 1985. Antibody-dependent cell cytotoxicity against Trypanosoma cruzi is only mediated by protective antibodies. *Parasite Immunol. 7:367*.

11. Brodskyn, C. I., A. M. Silva, H. A. Takehara, and I. Mota. 1989. IgG subclasses responsible for immune clearance in mice infected with Trypanosoma cruzi. *Immunol.Cell Biol. 67:343*.

12. Spinella, S., P. Liegeard, and M. Hontebeyrie-Joskowicz. 1992. Trypanosoma cruzi: predominance of IgG2a in nonspecific humoral response during experimental Chagas' disease. *Exp.Parasitol. 74:46*.

13. Almeida, I. C., S. R. Milani, P. A. Gorin, and L. R. Travassos. 1991. Complement-mediated lysis of Trypanosoma cruzi trypomastigotes by human anti-alpha-galactosyl antibodies. *J.Immunol. 146:2394*.

14. Kipnis, T. L., J. R. David, C. A. Alper, A. Sher, and W. D. da Silva. 1981. Enzymatic treatment transforms trypomastigotes of Trypanosoma cruzi into activators of alternative complement pathway and potentiates their uptake by macrophages. *Proc Natl Acad Sci U S A 78:602*.

15. Umekita, L. F., H. A. Takehara, and I. Mota. 1988. Role of the antibody Fc in the immune clearance of Trypanosoma cruzi. *Immunol.Lett. 17:85*.

16. Krettli, A. U., and Z. Brener. 1976. Protective effects of specific antibodies in Trypanosoma cruzi infections. *J Immunol 116:755*.

17. Murfin, D. J., L. Choromanski, and R. E. Kuhn. 1989. Relationship of route of infection to susceptibility and immune response of inbred mice to Y strain Trypanosoma cruzi. *Am J Trop Med Hyg 41:41*.

18. Muller, L. A., N. Anasco, and S. M. Gonzalez Cappa. 1986. Trypanosoma cruzi: isolate dependence in the induction of lytic antibodies in the mouse and rabbit. *Exp Parasitol 61:284.*

19. Iida, K., and V. Ley. 1991. Isolation and characterization of a 92-KD surface molecule of Trypanosoma cruzi amastigotes recognized by a monoclonal antibody that induces complement-mediated killing. *Am.J.Trop.Med.Hyg. 45:619.*

20. Krettli, A. U., and Z. Brener. 1982. Resistance against Trypanosoma cruzi associated to anti-living trypomastigote antibodies. *J Immunol 128:2009*.

21. Krettli, A. U., P. Weisz-Carrington, and R. S. Nussenzweig. 1979. Membrane-bound antibodies to bloodstream Trypanosoma cruzi in mice: strain differences in susceptibility to complement-mediated lysis. *Clin Exp Immunol 37:416*.

22. Krettli, A. U., and L. C. Pontes de Carvalho. 1985. Binding of C3 fragments to the Trypanosoma cruzi surface in the absence of specific antibodies and without activation of the complement cascade. *Clin Exp Immunol 62:270*.

23. Norris, K. A. 1998. Stable transfection of Trypanosoma cruzi epimastigotes with the trypomastigote-specific complement regulatory protein cDNA confers complement resistance. *Infect Immun 66:2460*.

24. Norris, K. A., B. Bradt, N. R. Cooper, and M. So. 1991. Characterization of a Trypanosoma cruzi C3 binding protein with functional and genetic similarities to

the human complement regulatory protein, decay-accelerating factor. *J Immunol 147:2240*.

25. Norris, K. A., G. Harth, and M. So. 1989. Purification of a Trypanosoma cruzi membrane glycoprotein which elicits lytic antibodies. *Infect Immun 57:2372*.

26. Tambourgi, D. V., T. L. Kipnis, W. D. da Silva, K. A. Joiner, A. Sher, S. Heath, B. F. Hall, and G. B. Ogden. 1993. A partial cDNA clone of trypomastigote decay-accelerating factor (T-DAF), a developmentally regulated complement inhibitor of Trypanosoma cruzi, has genetic and functional similarities to the human complement inhibitor DAF. *Infect Immun 61:3656*.

27. Rimoldi, M. T., A. Sher, S. Heiny, A. Lituchy, C. H. Hammer, and K. Joiner. 1988. Developmentally regulated expression by Trypanosoma cruzi of molecules that accelerate the decay of complement C3 convertases. *Proc Natl Acad Sci U S A 85:193*.

28. Joiner, K. A., W. D. daSilva, M. T. Rimoldi, C. H. Hammer, A. Sher, and T. L. Kipnis. 1988. Biochemical characterization of a factor produced by trypomastigotes of Trypanosoma cruzi that accelerates the decay of complement C3 convertases. *J Biol Chem 263:11327*.

29. Almeida, I. C., G. M. Krautz, A. U. Krettli, and L. R. Travassos. 1993. Glycoconjugates of Trypanosoma cruzi: a 74 kD antigen of trypomastigotes specifically reacts with lytic anti-alpha- galactosyl antibodies from patients with chronic Chagas disease. *J.Clin.Lab.Anal. 7:307*.

30. Almeida, I. C., M. A. Ferguson, S. Schenkman, and L. R. Travassos. 1994. Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas' disease

recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of Trypanosoma cruzi. *Biochem.J. 304:793.*

31. Gazzinelli, R. T., M. E. Pereira, A. Romanha, G. Gazzinelli, and Z. Brener. 1991. Direct lysis of Trypanosoma cruzi: a novel effector mechanism of protection mediated by human anti-gal antibodies. *Parasite Immunol. 13:345*.

32. Gonzalez, J., M. F. Araguth, and N. Yoshida. 1991. Resistance to acute Trypanosoma cruzi infection resulting from immunization of mice with a 90-kilodalton antigen from metacyclic trypomastigotes. *Infect Immun 59:863*.

33. Ruiz, A. M., M. Esteva, E. Subias, M. Moreno, d. C. Rosenstein, E. Velazquez, and E. L. Segura. 1990. Monoclonal antibodies against the flagellar fraction of epimastigotes of Trypanosoma cruzi: immunoprotection against metacyclic trypomastigotes obtained by immunization of mice with an affinity- purified antigen. *Mol.Biochem.Parasitol. 39:117*.

34. Segura, E. L., J. Bua, A. Rosenstein de Campanini, E. Subias, M. Esteva, M. Moreno, and A. M. Ruiz. 1986. Monoclonal antibodies against the flagellar fraction of epimastigotes of Trypanosoma cruzi: complement-mediated lytic activity against trypomastigotes and passive immunoprotection in mice. *Immunol Lett 13:165*.

35. Umekita, L. F., and I. Mota. 2000. How are antibodies involved in the protective mechanism of susceptible mice infected with T. cruzi? *Braz J Med Biol Res 33:253*.

36. Rodriguez, A. M., F. Santoro, D. Afchain, H. Bazin, and A. Capron. 1981. Trypanosoma cruzi infection in B-cell-deficient rats. *Infect.Immun. 31:524.*

37. Trischmann, T. M. 1984. Role of cellular immunity in protection against Trypanosoma cruzi in mice. *Parasite Immunol. 6:561*.

38. Basombrio, M. A. 1990. Trypanosoma cruzi: partial prevention of the natural infection of guinea pigs with a killed parasite vaccine. *Exp.Parasitol. 71:1*.

39. Basombrio, M. A., M. A. Segura, M. C. Mora, and L. Gomez. 1993. Field trial of vaccination against American trypanosomiasis (Chagas' disease) in dogs. *Am.J.Trop.Med.Hyg. 49:143*.

40. Basombrio, M. A., and S. Besuschio. 1982. Trypanosoma cruzi culture used as vaccine to prevent chronic Chagas' disease in mice. *Infect.Immun. 36:351*.

41. Scott, M. T., R. A. Neal, and N. C. Woods. 1985. Immunization of marmosets with Trypanosoma cruzi cell surface glycoprotein (GP90). *Trans.R.Soc.Trop.Med.Hyg. 79:451*.

42. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature 350:423*.

43. Rottenberg, M. E., D. A. Rodriguez, and A. Orn. 1992. Control of Trypanosoma cruzi infection in mice deprived of T- cell help. *Scand.J.Immunol. 36:261*.

44. Rottenberg, M. E., A. Riarte, L. Sporrong, J. Altcheh, P. Petray, A. M. Ruiz, H. Wigzell, and A. Orn. 1995. Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol.Lett. 45:53*.

45. Tarleton, R. L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect.Immun. 62:1820*.

46. Rottenberg, M. E., L. Sporrong, I. Persson, H. Wigzell, and A. Orn. 1995. Cytokine gene expression during infection of mice lacking CD4 and/or CD8 with Trypanosoma cruzi. *Scand.J.Immunol. 41:164*.

47. Brener, Z., and R. T. Gazzinelli. 1997. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. *Int.Arch.Allergy Immunol. 114:103*.

48. Nickell, S. P., A. Gebremichael, R. Hoff, and M. H. Boyer. 1987. Isolation and functional characterization of murine T cell lines and clones specific for the protozoan parasite Trypanosoma cruzi. *J.Immunol. 138:914*.

49. Gruppi, A., F. Cerban, M. C. Pistoresi-Palencia, and E. Vottero-Cima. 1995. Trypanosoma cruzi: transfer of protection by lymph node cells obtained from mice immunized with exoantigens of pI 4.5. *Exp.Parasitol. 80:382*.

50. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J.Immunol. 136:2348*.

51. Del Prete, G. F., M. De Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani. 1991. Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest 88:346*.

52. Parronchi, P., R. Manetti, C. Simonelli, F. S. Rugiu, M. P. Piccinni, E. Maggi, and S. Romagnani. 1991. Cytokine production by allergen (Der pI)-specific CD4+ T cell clones derived from a patient with severe atopic disease. *Int J Clin Lab Res 21:186.*

53. Sadick, M. D., F. P. Heinzel, B. J. Holaday, R. T. Pu, R. S. Dawkins, and R. M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *J Exp Med 171:115*.

54. Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley. 1989. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J.Exp.Med. 169:59*.

55. Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley. 1991. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc Natl Acad Sci U S A 88:7011*.

56. Geginat, G., M. Lalic, M. Kretschmar, W. Goebel, H. Hof, D. Palm, and A. Bubert. 1998. Th1 cells specific for a secreted protein of Listeria monocytogenes are protective in vivo. *J Immunol 160:6046*.

57. Zhou, P., M. C. Sieve, J. Bennett, K. J. Kwon-Chung, R. P. Tewari, R. T. Gazzinelli, A. Sher, and R. A. Seder. 1995. IL-12 prevents mortality in mice infected with Histoplasma capsulatum through induction of IFN-gamma. *J Immunol 155:785*.

58. Scharton-Kersten, T., L. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott. 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. *J Immunol 154:5320*.

59. Nabors, G. S., and R. L. Tarleton. 1991. Differential control of IFN-gamma and IL-2 production during Trypanosoma cruzi infection. *J.Immunol. 146:3591*.

60. Minoprio, P., C. M. el, E. Murphy, M. Hontebeyrie-Joskowicz, R. Coffman, A. Coutinho, and A. O'Garra. 1993. Xid-associated resistance to experimental Chagas' disease is IFN-gamma dependent. *J.Immunol. 151:4200*.

61. Petray, P. B., M. E. Rottenberg, G. Bertot, R. S. Corral, A. Diaz, A. Orn, and S. Grinstein. 1993. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of Trypanosoma cruzi. *Immunol.Lett. 35:77*.

62. Torrico, F., H. Heremans, M. T. Rivera, E. Van Marck, A. Billiau, and Y. Carlier. 1991. Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. *J.Immunol. 146:3626*.

63. Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed. 1992. Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection. *J.Exp.Med. 175:169*.

64. Reed, S. G., C. E. Brownell, D. M. Russo, J. S. Silva, K. H. Grabstein, and P. J. Morrissey. 1994. IL-10 mediates susceptibility to Trypanosoma cruzi infection. *J.Immunol. 153:3135*.

65. Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F. G. Araujo. 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J.Immunol. 158:3311*.

66. Barbosa de Oliveira, L. C., M. A. Curotto de Lafaille, G. M. Collet de Araujo Lima, and I. de Almeida Abrahamsohn. 1996. Antigen-specific Il-4- and IL-10-secreting CD4+ lymphocytes increase in vivo susceptibility to Trypanosoma cruzi infection. *Cell Immunol. 170:41*.

67. Tarleton, R. L., M. J. Grusby, and L. Zhang. 2000. Increased susceptibility of Stat4-deficient and enhanced resistance in Stat6-deficient mice to infection with Trypanosoma cruzi. *J Immunol 165:1520*.

68. Chen, Z. M., and M. K. Jenkins. 1998. Revealing the in vivo behavior of CD4+ T cells specific for an antigen expressed in Escherichia coli. *J.Immunol. 160:3462*.

69. Garside, P., E. Ingulli, R. R. Merica, J. G. Johnson, R. J. Noelle, and M. K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science 281:96*.

70. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen- specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J.Immunol. 155:1032*.

71. Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins. 1998. Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. *J.Immunol. 160:4719*.

72. Nickell, S. P., G. A. Stryker, and C. Arevalo. 1993. Isolation from Trypanosoma cruzi-infected mice of CD8+, MHC- restricted cytotoxic T cells that lyse parasite-infected target cells. *J.Immunol. 150:1446.*

73. Rottenberg, M. E., D. Sunnemark, T. Leandersson, and A. Orn. 1993. Organ-specific regulation of interferon-gamma, interleukin-2 and interleukin-2 receptor during murine infection with Trypanosoma cruzi. *Scand.J.Immunol. 37:559.*

74. Tarleton, R. L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine- induced immunity in mice infected with Trypanosoma cruzi. *J.Immunol. 144:717.*

75. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection [see comments]. *Nature 356:338.*

76. Tarleton, R. L., M. J. Grusby, M. Postan, and L. H. Glimcher. 1996. Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int.Immunol. 8:13.*

77. Sun, J., and R. L. Tarleton. 1993. Predominance of CD8+ T lymphocytes in the inflammatory lesions of mice with acute Trypanosoma cruzi infection. *Am.J.Trop.Med.Hyg. 48:161.*

78. Higuchi, M. D., M. M. Ries, V. D. Aiello, L. A. Benvenuti, P. S. Gutierrez, G. Bellotti, and F. Pileggi. 1997. Association of an increase in CD8+ T cells with the presence of Trypanosoma cruzi antigens in chronic, human, chagasic myocarditis. *Am.J.Trop.Med.Hyg. 56:485.*

79. Low, H. P., M. A. Santos, B. Wizel, and R. L. Tarleton. 1998. Amastigote surface proteins of Trypanosoma cruzi are targets for CD8+ CTL. *J.Immunol. 160:1817.*

80. Low, H. P., and R. L. Tarleton. 1997. Molecular cloning of the gene encoding the 83 kDa amastigote surface protein and its identification as a member of the Trypanosoma cruzi sialidase superfamily. *Mol.Biochem.Parasitol. 88:137.*

81. Wizel, B., M. Palmieri, C. Mendoza, B. Arana, J. Sidney, A. Sette, and R. Tarleton. 1998. Human infection with Trypanosoma cruzi induces parasite antigen- specific cytotoxic T lymphocyte responses. *J.Clin.Invest. 102:1062.*

82. Wizel, B., M. Nunes, and R. L. Tarleton. 1997. Identification of Trypanosoma cruzi trans-sialidase family members as targets of protective CD8+ TC1 responses. *J.Immunol. 159:6120.*

83. Berke, G. 1995. The CTL's kiss of death. *Cell 81:9.*

84. Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu.Rev.Immunol. Annu.Rev.Immunol. 14:207-32:207.*

85. Denkers, E. Y., G. Yap, T. Scharton-Kersten, H. Charest, B. A. Butcher, P. Caspar, S. Heiny, and A. Sher. 1997. Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. *J.Immunol. 159:1903.*

86. Guidotti, L. G., and F. V. Chisari. 1996. To kill or to cure: options in host defense against viral infection. *Curr.Opin.Immunol. 8:478.*

87. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell 76:977*.

88. Kagi, D., B. Ledermann, K. Burki, H. Hengartner, and R. M. Zinkernagel. 1994. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur.J.Immunol. Eur.J.Immunol. 24:3068*.

89. Laochumroonvorapong, P., J. Wang, C. C. Liu, W. Ye, A. L. Moreira, K. B. Elkon, V. H. Freedman, and G. Kaplan. 1997. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. *Infect.Immun. 65:127*.

90. Renggli, J., M. Hahne, H. Matile, B. Betschart, J. Tschopp, and G. Corradin. 1997. Elimination of P. berghei liver stages is independent of Fas (CD95/Apo- I) or perforin-mediated cytotoxicity. *Parasite Immunol. 19:145*.

91. Tang, Y., A. W. Hugin, N. A. Giese, L. Gabriele, S. K. Chattopadhyay, T. N. Fredrickson, D. Kagi, J. W. Hartley, and H. C. Morse. 1997. Control of immunodeficiency and lymphoproliferation in mouse AIDS: studies of mice deficient in CD8+ T cells or perforin. *J.Virol. 71:1808*.

92. Atkinson, E. A., and R. C. Bleackley. 1995. Mechanisms of lysis by cytotoxic T cells. *Crit.Rev.Immunol. 15:359*.

93. Garcia, S., M. Fevrier, G. Dadaglio, H. Lecoeur, Y. Riviere, and M. L. Gougeon. 1997. Potential deleterious effect of anti-viral cytotoxic lymphocyte through the CD95 (FAS/APO-1)-mediated pathway during chronic HIV infection. *Immunol.Lett. 57:53*.

94. Frost, P., C. P. Ng, A. Belldegrun, and B. Bonavida. 1997. Immunosensitization of prostate carcinoma cell lines for lymphocytes (CTL, TIL, LAK)-mediated apoptosis via the Fas-Fas-ligand pathway of cytotoxicity. *Cell Immunol. 180:70.*

95. Lopes, M. F., M. P. Nunes, A. Henriques-Pons, N. Giese, H. C. Morse, W. F. Davidson, T. C. Araujo-Jorge, and G. A. DosReis. 1999. Increased susceptibility of Fas ligand-deficient gld mice to Trypanosoma cruzi infection due to a Th2-biased host immune response. *Eur.J.Immunol. 29:81*.

96. Fitch, F. W., R. Stack, P. Fields, D. W. Lancki, and D. C. Cronin. 1995. Regulation of T lymphocyte subsets. *Ciba.Found.Symp. 195:68-80; discussion 80-5:68*.

97. Mosmann, T. R., S. Sad, L. Krishnan, T. G. Wegmann, L. J. Guilbert, and M. Belosevic. 1995. Differentiation of subsets of CD4+ and CD8+ T cells. *Ciba.Found.Symp. 195:42-50; discussion 50-4:42*.

98. Mosmann, T. R., L. Li, and S. Sad. 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin.Immunol. 9:87*.

99. Sad, S., R. Marcotte, and T. R. Mosmann. 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity. 2:271*.

100. Mosmann, T. R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol.Today 17:138.*

101. Carter, L. L., and R. W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr.Opin.Immunol. 8:336.*

102. Li, L., S. Sad, D. Kagi, and T. R. Mosmann. 1997. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J.Immunol. 158:4152*.

103. Cerwenka, A., T. M. Morgan, A. G. Harmsen, and R. W. Dutton. 1999. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J Exp Med 189:423*.

104. Wirth, S., M. van den Broek, C. P. Frossard, A. W. Hugin, I. Leblond, H. Pircher, and C. Hauser. 2000. CD8(+) T cells secreting type 2 lymphokines are defective in protection against viral infection. *Cell Immunol 202:13*.

105. Skinner, M. A., S. Yuan, R. Prestidge, D. Chuk, J. D. Watson, and P. L. Tan. 1997. Immunization with heat-killed Mycobacterium vaccae stimulates CD8+ cytotoxic T cells specific for macrophages infected with Mycobacterium tuberculosis. *Infect.Immun. 65:4525*.

106. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17*.

# CHAPTER 2

# RELATIVE CONTRIBUTION OF ANTIBODY PRODUCTION AND $CD8^+$ T CELL FUNCTION TO IMMUNE CONTROL OF *TRYPANOSOMA CRUZI*[1]

[1]Kumar Sudesh and Rick L. Tarleton. 1998. Parasite Immunology 200: 207-216.

**Abstract**

The life cycle of the protozoan parasite *Trypanosoma cruzi* in mammalian hosts includes both non-dividing trypomastigote forms which circulate in the blood and replicating intracellular amastigotes which reside within the cytoplasm of a variety of host cells. Many previous studies have focused on the ability of antibodies and/or activated macrophages to control *T. cruzi* infection by killing trypomastigotes. However, recent studies have documented the role of class I MHC-restricted T cells in immune control of *T. cruzi*, presumably at the level of the infected host cell. In this study we have used mice with induced mutations in genes responsible for either antibody production or cytolytic T lymphocyte (CTL) function to examine the relative contributions of these effector mechanisms to control of *T. cruzi*. Mice deficient in the production of antibodies exhibited a delay in the rise in acute phase parasitemia and an extended time to death relative to mice lacking $CD8^+$ T cells. Nevertheless, B cell deficient mice eventually succumbed to the infection. Prior infection with an avirulent strain of *T. cruzi* failed to protect either $CD8^+$ T cell-deficient mice or B cell deficient mice from challenge infection with virulent parasites. In contrast, mice with disruptions in the genes controlling perforin- or granzyme B-mediated cytolytic pathways had parasitemia and mortality rates similar to wild-type mice and were protected from secondary infection by prior exposure to avirulent parasites. Perforin and granzyme B knockout mice also retained significant cytolytic activity for target cells displaying *T. cruzi* peptides. These results suggest that although antibody production is secondary in importance to cellular responses mediated by both $CD4^+$ and $CD8^+$ T cells, elicitation of the humoral immune response is absolutely required for control of *T. cruzi* in primary and secondary

infections. Secondly, the mechanism of control of *T. cruzi* infection by $CD8^+$ T cells appears not to be dependent on perforin- or granzyme B-mediated pathways, and may be due to other lytic mechanisms or to cytokine production.

**Introduction**

*Trypanosoma cruzi* infects a wide variety of mammalian hosts and in humans can cause life threatening Chagas' disease. In mammals, *T. cruzi* cycles between intracellular amastigotes and extracellular trypomastigotes and evokes both humoral and cell mediated immune responses. Until recently, anti-*T. cruzi* antibodies and activated macrophages have been considered the major mechanisms of immune control of this parasite. The lytic (Lages-Silva et al., 1987), opsonic (Scott and Moyes, 1982) antibody dependent cellular cytotoxicity (ADCC) (Lima-Martins et al., 1985) complement activating (Mota and Umekita, 1989;Brodskyn et al., 1989;Spinella et al., 1992) and invasion blocking (Almeida et al., 1991) activities of antibodies have all been proposed to assist in immune control of *T. cruzi*. Likewise, activation of macrophage-mediated killing mechanisms by the production of Th1-type cytokines has also been shown to be critical to control of *T. cruzi* (Metz et al., 1993;Vespa et al., 1994;Munoz-Fernandez et al., 1992;Gazzinelli et al., 1992). Nevertheless, vaccination studies designed to activate primarily class II MHC-dependent T cell responses and antibody production have consistently failed to provide complete protection in experimental models (Rottenberg et al., 1988;Ruiz et al., 1986;Harth et al., 1994).

In recent years, class I MHC-restricted $CD8^+$ T cells have been added to the list of critical effectors in immunity to *T. cruzi* (Nickell et al., 1993). Support for the importance of $CD8^+$ T cells in immune control of *T. cruzi* comes from evidence that

depletion of $CD8^+$ T cells or targeted disruption of genes necessary for the generation of mature $CD8^+$ T cells results in high susceptibility to *T. cruzi* in murine models (Tarleton, 1990;Tarleton et al., 1992;Rottenberg et al., 1993;Rottenberg et al., 1995a;Tarleton et al., 1996). Further, transfer of $CD8^+$ T cells specific for TSA-1, a target of both murine and human anti-*T. cruzi* CTL responses, provides significant protection from *T. cruzi* infection (Wizel et al., 1997).

In this study we have utilized mice with targeted disruptions in genes controlling B cell development and $CD8^+$ T cell function to address the question of the relative contribution of these effector mechanisms in control of *T. cruzi* infection. As predicted from earlier studies, the failure to produce antibodies significantly hinders the generation of a protective response to *T. cruzi* in either a primary or challenge infection. Surprisingly, however, B cell-deficient mice are able to survive for significantly longer than do mice with defects in either $CD4^+$ or $CD8^+$ T cell function. With respect to the effector function of $CD8^+$ T cells in *T. cruzi* infection, the high susceptibility of mice with a deficiency in $CD8^+$ T cells is confirmed in studies using mice lacking TAP-1 gene function. However infections in mice with induced defects in the cytolytic function of $CD8^+$ T cells suggest that the control of *T. cruzi* infection by this population of T cells is not dependent on the granzyme/perforin cytolytic pathway.

**Materials and Methods**

**Mice**

Wild-type C57Bl/6J mice (common name B6), B cell deficient mice (C57BL/6-Igh-$6^{tm1Cgn}$; common name muMT; (Kitamura et al., 1991)), beta-2-

microglobulin deficient mice (C57BL/6J-B2m$^{tm1Unc}$; common name b2m$^{-/-}$; (Koller et al., 1990)), perforin deficient (C57BL/6-Pfp$^{tm1Sdz}$; common name perforin$^{-/-}$ (Kagi et al., 1994a) and granzyme B deficient (C57BL/6J-Gzmb$^{tm1Ley}$ ; common name granzyme B$^{-/-}$ (Heusel et al., 1994)) mice were either obtained from The Jackson Laboratory (Bar Harbor, ME) or were bred in our facility from stocks obtained from The Jackson Laboratory. TAP-1 deficient mice (common name TAP-1$^{-/-}$; (Van Kaer et al., 1992)) on the B6 background were bred from mating pairs obtained from Dr. Luc Van Kaer, Vanderbilt University).

**Parasites**

Blood-form trypomastigotes (BFT) of the *T. cruzi* Brazil strain were maintained in C3HHe/SnJ mice by biweekly passage and were injected at a dose of $10^3$/mouse by the intraperitoneal (i.p.) route. Trypomastigotes of the *T. cruzi* M/80 Miranda clone were obtained from culture in bovine embryonic smooth muscle cells and injected i.p. at a dose of $10^6$/mouse. Mice infected with this avirulent M/80 clone were subsequently challenge infected with 5 x $10^4$ BFT Brazil strain *T. cruzi* 294 days later. The parasitemias in infected mice were determined by weekly examination of tail blood in a hemacytometer and mortality was monitored daily.

**Measurements of anti-*T. cruzi* immune responses**

*T. cruzi*-specific IgM and IgG antibody levels were measured 25 and 35 days post-infection using a previously described procedure (Tarleton et al., 1996). Measurement of anti-*T. cruzi* TSA-1 peptide 77.2-specific cytolytic T cell activity was performed as previously described (Wizel et al., 1997). The percentage specific $^{51}$Cr-release from triplicate wells was calculated as {(average experimental cpm - average spontaneous

cpm)/(average maximum cpm - average spontaneous cpm)} X 100. Maximum and spontaneous $^{51}$Cr-release in counts per minute (cpm) were determined from triplicate wells containing labeled target cells only with no effectors in the presence or absence of 5% SDS, respectively.

**Histology**

Mice were killed by $CO_2$ inhalation at various times post-infection and tissues from heart, spleen, and skeletal muscle were collected in 10 percent buffered formalin. The five micron sections of paraffin embedded tissues were fixed, dehydrated and stained with hematoxylin and eosin and examined by light microscopy.

**Results**

Previous studies have utilized mice with targeted deletions in genes encoding proteins of immunological importance to determine the role of different immune effector mechanisms in immunity to *T. cruzi* (Tarleton et al., 1992;Rottenberg et al., 1993;Rottenberg et al., 1995a;Tarleton et al., 1996;Hunter et al., 1997;Santos Lima and Minoprio, 1996;Abrahamsohn and Coffman, 1996). To analyze further the mechanism of action of B cells and $CD8^+$ T cells in *T. cruzi* infection, we took advantage of mice with an induced mutation in the μ heavy chain immunoglobulin gene and mice with mutations in genes effecting the development and function of $CD8^+$ T cells. Our initial experiments compared the course of infection with the Brazil strain of *T. cruzi* in immunocompetent B6 mice, mice lacking *b2m* gene function, a defect previously shown to result in high susceptibility due to the absence of $CD8^+$ T cells (Tarleton et al., 1992), *TAP-1* deficient mice which also lack mature $CD8^+$ T cell function and muMT mice which are defective in immunoglobulin production. As expected, the course of *T. cruzi*

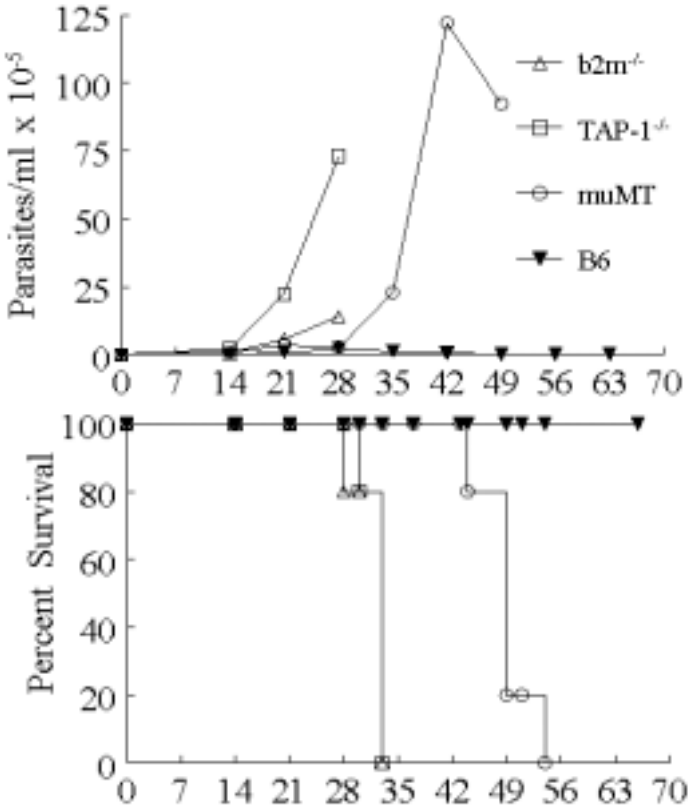



Figure 1. Parasitemia and mortality curves in immunoglobulin- and $CD8^+$ T cell-deficient mice infected with *T. cruzi*.

Legend: Mice were infected by intra-peritonially injecting 1000 BFT of virulent Brazil strain of *T. cruzi*. Blood parasite load was monitored at weekly intervals and mortality recorded daily.

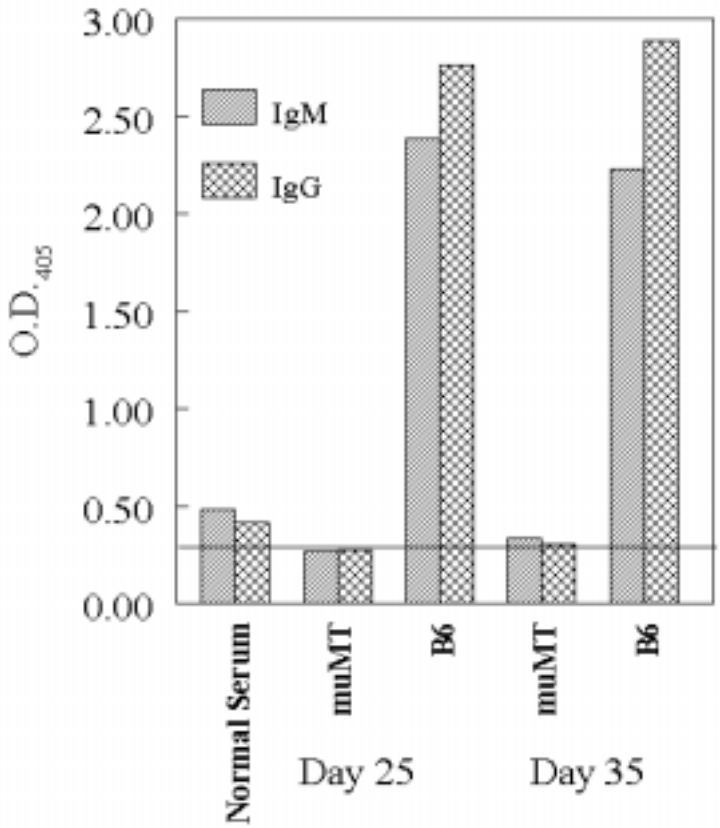



Figure 2. *Trypanosoma cruzi*-specific IgM and IgG levels in muMT and wild type B6 mice at day 25 and 35 post-infection.

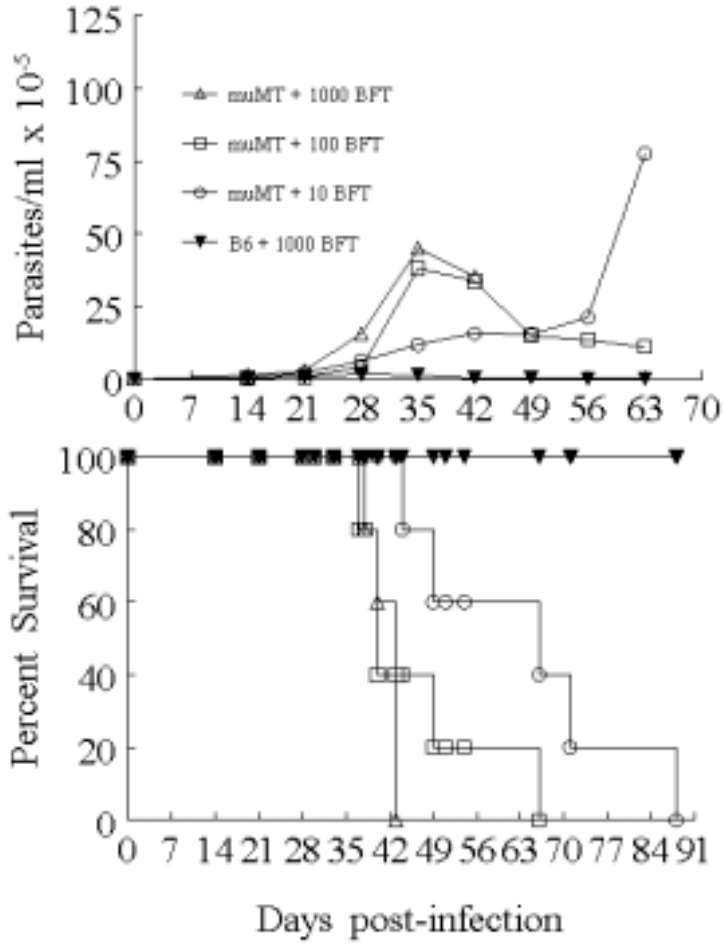



Figure 3. Parasitemia and mortality in muMT mice infected with varying doses of *T. cruzi* Brazil strain blood-form trypomastigotes (BFT).

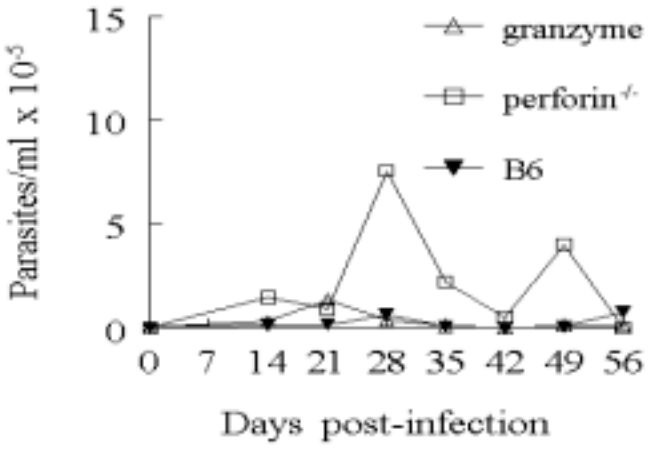



Figure 4. Parasitemia in mice with targeted deletions in the granzyme and perforin genes and infected with $10^3$ BFT of the Brazil strain of *T. cruzi*. Wild-type B6 mice serve as controls.

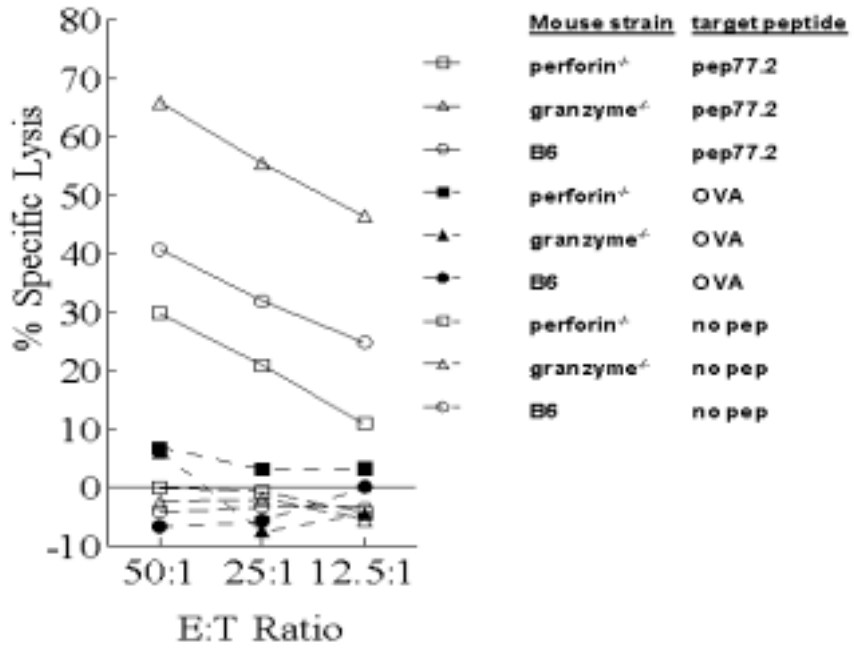
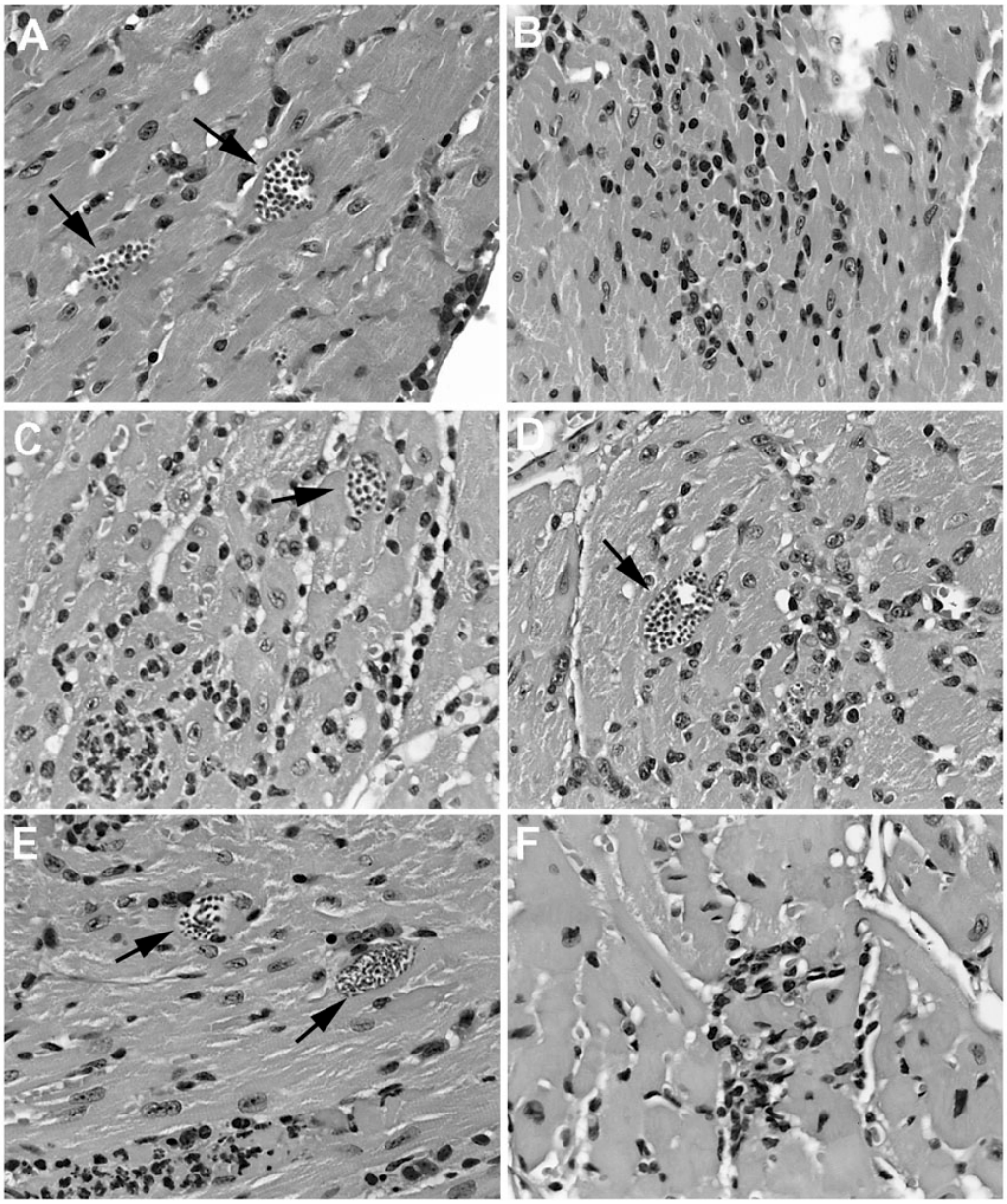



Figure 5. Percent specific lysis as an indicator of CTL activity in spleen cells obtained from wild-type (B6), perforin$^{-/-}$or granzyme B$^{-/-}$ mice on day 90 post-infection. Spleen cells were stimulated in vitro with *T. cruzi* TSA-1 derived pep77.2 for 6 days and tested for lytic activity ($^{51}$Cr release) on peptide-pulsed 5aKb target cells in a 6 hr assay as previously described (Wizel et al., 1997). 5aKb target cells were either pulsed with 1 uM pep77.2 (open symbols, solid lines) 1 uM ovalbumin peptide SIINFELK (Rotzschke et al., 1991) (closed symbols, dashed lines) or were incubated in the absence of peptide (open symbols, dashed lines).

Figure 6. Hematoxylin and eosin-stained tissue sections from hearts of mice infected with $10^3$ BFT of the Brazil strain of *T. cruzi*. A) TAP-1$^{-/-}$ day 23 post-infection, B) perforin$^{-/-}$ day 45 post-infection, C) muMT day 25 post-infection, D) B6 day 25 post-infection, E) muMT day 45 post-infection, and F) B6 day 45 post-infection. Note the absence of inflammation in the TAP-1$^{-/-}$ (A) mice in contrast to perforin$^{-/-}$ (B) muMT (C and E), and B6 (D and E) mice. Parasite-infected cells (arrows) are readily detected at 25 days post-infection in all strains. However parasites are not obvious at 45 days post-infection in perforin$^{-/-}$ and B6 mice but continue to increase in number in muMT mice until the eventual death of these animals. Tissues from granzyme B$^{-/-}$ mice (not shown) are similar in appearance to perforin$^{-/-}$ mice. Original magnification 400X.

Figure 6.



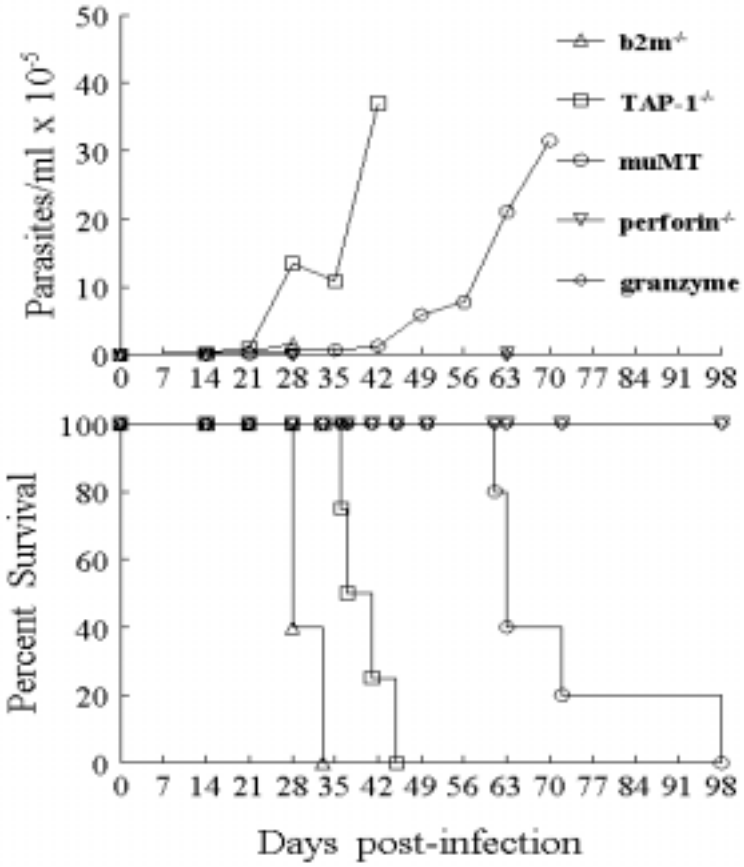



Figure 7. Parasitemia and mortality in immunodeficient and wild type B6 mice immunized with avirulent M80 Miranda clone parasites and challenged with virulent Brazil strain of *T. cruzi.*

Mice were infected with $10^6$ cultured derived trypomastigotes of *T. cruzi* M80 Miranda clone. After 294 days the mice were challenged with 5 x $10^4$ BFT of the Brazil strain of *T. cruzi*.

infection in TAP-1$^{-/-}$ mice closely resembles that of b2m$^{-/-}$ mice with high parasitemias and death between days 28-33 post-infection (Figure 1). In comparison muMT mice infected with same dose of *T. cruzi* displayed wild-type levels of parasitemia early in the infection (up to day 28 post infection). However, by 42 days post infection, parasitemias in the muMT mice had risen significantly above that of wild-type mice and all muMT mice were dead by day 55 post-infection. The absence of antibody production in the muMT mice was confirmed by analysis of serum from infected mice for the presence of anti-*T. cruzi* IgM and IgG (Figure 2). Thus in the absence of an antibody response, mice infected with *T. cruzi* exhibit evidence of an initial control of the infection but eventually fail to contain parasite replication and succumb to the infection.

We next asked if the survival of B cell deficient mice could be extended further by initiation of the infection with lower doses of parasites. muMT mice infected with 10 or 100 BFT of the Brazil strain exhibited control of the infection and survival for as long as 89 days post-infection (Figure 3). However all of these animals did eventually develop high parasiemia levels and die. Therefore immunoglobulin production is absolutely required for control of an even low initial level infection with a virulent strain of *T. cruzi*, although defects in other effector populations, in particular CD8$^{+}$ Figure 1 and (Tarleton et al., 1992) and/or CD4$^{+}$ T cells (Tarleton et al., 1996;Rottenberg et al., 1995b), leads to an even earlier time to death. Apart from antibody production, B cells also serve as antigen presenting cells and produce cytokines to stimulate T cells. Therefore, B cell deficient mice are also compromised in the presentation of *T. cruz*i antigens to T cells. The high susceptibility of the TAP-1$^{-/-}$ mice provides further confirmation of the requirement for the induction of parasite-specific CD8$^{+}$ T cells in immunity to *T. cruzi*.

Both cytolytic T lymphocyte (CTL) activity and cytokine production has been documented in anti-*T. cruzi* $CD8^+$ T cells (Wizel et al., 1997) but the requirement for either or both of these activities in immune control of *T. cruzi* has not been studied. To begin to address this question, mice with targeted defects in the pathways considered to be the most important in cytolytic killing by CTL were infected with *T. cruzi*. Perforin- and granzyme B-deficient mice infected with 1000 BFT of the Brazil strain of *T. cruzi* exhibited parasitemia levels (Figure 4) and survival rates (100% in all groups in this experiment) comparable to that of wild-type mice. Perforin$^{-/-}$ mice exhibited slightly higher peak blood parasite levels than either B6 or granzyme $B^{-/-}$ mice but parasitemia levels in all groups were undetectable by day 56 PI and remained so until greater than 100 days post-infection when the experiment was terminated.

Despite the absence of either granzyme B or perforin, spleen cells obtained from these deficient mice at day 90 post-infection displayed significant cytolytic T cell activity versus cells pulsed with peptide 77.2 (Figure 5), a *T. cruzi* TSA-1-derived peptide which had been previously shown to be a target of anti-*T. cruzi* CTL (Wizel et al., 1997). No similar activity was detected against target cells pulsed with an irrelevant peptide (OVA) or incubated without peptide. Both the ability of mice with defects in perforin or granzyme to control *T. cruzi* infection and the retention of cytolytic activity in splenocytes from these same mice suggest that at least part of the control function of $CD8^+$ T cells in *T. cruzi* infection is independent of a granzyme/perforin cytolytic pathway.

The results of histopathological analysis of heart tissues from immunodeficient mice confirm the evidence from parasitemia and longevity studies indicative of a heightened

susceptibility of the TAP-1$^{-/-}$ and muMT mice and of a relatively unaltered infection in the granzyme B$^{-/-}$ and perforin$^{-/-}$ mice (Figure 6). b2m$^{-/-}$ (Tarleton et al., 1992;Tarleton et al., 1996) and TAP-1$^{-/-}$ (Figure 6A) mice showed high tissue parasitism both in skeletal muscles (not shown) and heart with negligible inflammation in the acute phase of the infection (day 23). However, muMT mice displayed low levels of tissue parasites and moderate inflammation in heart and skeletal muscles at a comparable time point in the infection (day 25; Figure 6c), a response very similar to that of the wild-type B6 mice (Figure 6d). However, the tissue parasite burden and inflammation increased in heart muscle of muMT mice and was significantly more intense than that in B6 mice at 45 days post infection (Figures 6e and 6f, respectively). Tissue parasitism and inflammation in perforin$^{-/-}$ (Figure 6b) and granzyme B$^{-/-}$ (not shown) mice at day 45 post-infection was comparable to wild type B6 mice.

The ability of B cell deficient mice to survive for a significant period of time following infection with a virulent strain of *T. cruzi* suggests that the antibody response is relatively less important, in comparison to the responses mediated by CD4$^{+}$ or CD8$^{+}$ T cells, in control of the acute infection. This result prompted the question whether or not protection from infection could be induced by vaccination of animals which lack the ability to generate an antibody response. To address this question, wild-type and immunodeficient mice were infected with a low virulence strain of *T. cruzi* which had previously been shown to allow for survival in immunodeficient mouse strains (Tarleton et al., 1996). At approximately 300 days after this primary immunizing infection, the mice were challenged with virulent Brazil strain parasites and the parasitemia and longevity monitored (Figure 7). The challenge infection was lethal in all b2m$^{-/-}$ and

TAP-1$^{-/-}$ mice by 32 and 45 days post infection, respectively. B cell deficient mice again exhibited a delay in the rise of parasitemia and a significant increase in longevity relative to the CD8$^{+}$ T cell deficient mice. However these mice eventually died between 60 and 100 days post infection. Granzyme B$^{-/-}$ and perforin$^{-/-}$ mice were protected by the prior infection with avirulent parasites and had no detectable parasites in the blood at any point post-infection with the virulent Brazil strain.

**Discussion**

Immune control of *T. cruzi* infection involves multiple immune effector mechanisms (reviewed in (Tarleton, 1997)). Previous studies using cell depletion protocols and/or mice with induced mutations in genes controlling either CD4$^{+}$ (Araujo, 1989;Rottenberg et al., 1993;Rottenberg et al., 1995a;Tarleton et al., 1996) or CD8$^{+}$ (Tarleton, 1990;Tarleton et al., 1992;Rottenberg et al., 1993;Rottenberg et al., 1995a;Tarleton et al., 1996) T cell function have documented the absolute requirement for the activation of both of these T cell subpopulations for survival of mice during the acute phase of *T. cruzi* infection. Mice with deficiencies in both T cell subsets achieve higher parasite loads and die earlier in the infection than mice with single defects (Tarleton et al., 1996), indicating a non-overlapping mechanism of action for CD4$^{+}$ and CD8$^{+}$ T cells in control of *T. cruzi* infection. In addition, previous studies also provided strong evidence for an important role for antibodies in regulating *T. cruzi* in the infected host: anti-u suppressed rats (Rodriguez et al., 1981) and mice (Trischmann, 1983;Trischmann, 1984) are more susceptible to infection than are immunologically intact hosts.

The present study extends these findings by directly comparing the course of *T. cruzi* infection in mice lacking either CD8$^{+}$ T cells or antibody producing B cells. Mice unable

to make antibodies to *T. cruzi* live for substantially longer than mice lacking either $CD4^+$ (Tarleton et al., 1996) or $CD8^+$ T cell function, suggesting a more critical role for these T cell populations than for antibody production in the initial control of the infection. Nevertheless, even mice infected with very small numbers of virulent parasites or with an avirulent strain prior to challenge with virulent parasites are unable to control parasite growth and succumb during the acute parasitemic phase of the infection. Thus, in agreement with previous studies (Trischmann, 1983;Trischmann, 1984) T cells appear to be required for the initial control of the infection, but for long-term survival an effective antibody response is also critical. These results are also consistent with the failure to achieve vaccine-induced immunity with protocols based solely or primarily on induction of antibody responses (Brener, 1986). Elicitation of antibody production alone is insufficient to control the infection and effective vaccines are likely to additionally require activation of both the $CD4^+$ and $CD8^+$ T cell compartments.

The critical effector functions necessary for parasite control by anti-*T. cruzi* $CD4^+$ and $CD8^+$ T cells are not yet known. $CD4^+$ T cells likely have multiple roles, including providing help to B cells and $CD8^+$ T cells and as activators of effector cells such as macrophages. $CD8^+$ T cells have been shown to be able to lyse *T. cruzi*-infected target cells (Nickell et al., 1993;Wizel et al., 1997) and this cytolytic function has been presumed to be the major mechanism of action of these cells. Recently the understanding of role of $CD8^+$ T cells in immunity to *T. cruzi* has been extended with the presentation of information on the protective capacity of parasite-specific CTLs and the identification of antigen targets for some of these anti-*T. cruzi* $CD8^+$ T cells. Three members of the trans-sialidase family of surface proteins have now been identified as targets of anti-*T.*

*cruzi* CTL responses in both mice (Wizel et al., 1997;Low et al., 1997) and humans (Wizel and Tarleton, in preparation) and short-term $CD8^+$ T cell lines specific for one of these molecules, TSA-1, have been shown to transfer protection to naive mice (Wizel et al., 1997). These T cell lines where highly cytolytic for peptide-pulsed or *T. cruzi*-infected target cells and were potent producers of both IFN-gamma and TNF (Wizel et al., 1997). The relative contribution of the cytolytic activity or cytokine production of these protective $CD8^+$ T cells is not known.

$CD8^+$ T cells kill and/or mediate immune control via at least three distinct mechanisms. The perforin/granzyme cytolytic pathway was the first described mechanism of cytolysis by $CD8^+$ CTL and is still considered the primary cytolytic mechanism in most systems (Atkinson and Bleackley, 1995). Perforin/granzyme mediated cytolysis involves the release of granules containing perforin, which polymerizes to form pores in the target cell membrane, and granzymes which may enter through these pores and induce apoptosis of the target cell (Berke, 1995;Kagi et al., 1996b). The generation of knockout mice lacking perforin or granzyme B has allowed investigators to explore the role of this pathway in $CD8^+$ T cell response in a number of systems (Kagi et al., 1994a;Kagi et al., 1994b;Denkers et al., 1997;Guidotti and Chisari, 1996;Tang et al., 1997;Laochumroonvorapong et al., 1997;Renggli et al., 1997;Heusel et al., 1994). In the present work we show that in *T. cruzi* infection, perforin/granzyme-mediated cytolysis plays a rather minor role in the protective capacity of $CD8^+$ T cells. While mice deficient in $CD8^+$ T cells were highly susceptible to virulent strains of *T. cruzi* and uniformly die in the acute stage of the infection, mice deficient in either perforin or granzyme B function have little to no increase in susceptibility to primary infection, can

be protected by prior infection with an avirulent strain of *T. cruzi* and continue to exhibit cytolytic activity for host cells displaying *T. cruzi* peptides. *T. cruzi* infection is not unique with respect to the relative lack of importance of the perforin/granzyme pathway in $CD8^+$ CTL-mediated killing. In a number of viral systems, particularly cytopathic viruses, killing via the perforin/granzyme pathway is also a minor factor in protection mediated by CTL (Kagi et al., 1996b;Kagi and Hensgartner, 1996a;Guidotti et al., 1996). Also early control of infection with either *Toxoplasma gondii* (Denkers et al., 1997) or *Mycobacteria tuberculosis* (Laochumroonvorapong et al., 1997) and irradiated sporozoite-induced protection in *Plasmodium berghei* infection (Renggli et al., 1997) are all perforin-independent.

$CD8^+$ CTL may also kill by Fas/Fas-L interactions or via the surface display or secretion of cytokines such as TNF and IFN-gamma (Berke, 1995;Kagi et al., 1996b). The Fas/Fas-L pathway of cytolysis has generally been considered to be of primary importance as an immune regulatory pathway (Kagi et al., 1994b;Kagi et al., 1996b;Griffith et al., 1995;Atkinson et al., 1995). However a number of recent studies have documented a prominent role for Fas/Fas-L interactions in CTL-mediated killing of infected or tumor targets (Kagi et al., 1994b;Lowin et al., 1994;Clark et al., 1995;Stenger et al., 1997;Frost et al., 1997;Garcia et al., 1997b). Additionally, CTL activity and/or immune control mediated by $CD8^+$ T cells has been shown in some cases to be independent of both the perforin/granzyme and Fas/Fas-L pathways and has in most of these cases been ascribed to cytokine production, usually TNF and/or IFN-gamma (Kagi et al., 1996a;Guidotti et al., 1996;Elkon et al., 1997;Sutton et al., 1997);

Laochumroonvorapong, 1997 #3104]. The contribution of these other cytolytic pathways to control of *T. cruzi* infection are the focus of on-going studies in our laboratory.

The retention in perforin and granzyme B deficient mice of cytolytic activity specific for class I MHC-presented *T. cruzi* peptides suggests that the Fas/Fas-L or cytokine dependent cytolytic mechanisms are involved in the killer activity of *T. cruzi*-specific CTL. It is also possible that other activities of cytokines, in addition to or rather than cytolysis of target cells, are critical to the protective activity of $CD8^+$ T cells in *T. cruzi* infection. The virus inhibitory activity of IFN-gamma and possibly other cytokines are thought to be of greater importance than cytolysis in immunity to cytopathic viruses (Kagi et al., 1996a;Guidotti et al., 1996;Elkon et al., 1997;Ando et al., 1997). Studies in hepatitis B virus transgenic mice (Nakamoto et al., 1997) suggest that in vivo, both lytic (including perforin/granzyme, Fas/Fas-L and TNF dependent) and non-lytic (involving IFN-gamma and TNF production) mechanisms contribute to $CD8^+$ CTL-mediated target cell destruction. In this latter model it is the nature of the target cells which is proposed to be the primary determinant of the most efficient killing mechanism (Nakamoto et al., 1997).

Preliminary studies in our lab confirm that cytokines produced by *T. cruzi*-specific CTL can induce nitric oxide-dependent regulation of the growth of parasites in non-macrophage cell lines (Rosario and Tarleton, unpublished). In conjunction with the studies reported in the present study, these finding suggest the following model for immune control in *T. cruzi* infection. $CD4^+$ and $CD8^+$ cells have a primary role in regulation of the infection. $CD4^+$ T cells likely act through a number of mechanisms, primarily via providing the appropriate helper function for both antibody-producing B

cells and $CD8^+$ CTL. $CD4^+$ T cells may also serve an important role in the activation of macrophages for killing of intracellular parasites. However, in this latter function, the importance of $CD4^+$ T cells is probably secondary to that of $CD8^+$ T cells since the majority of the cells infected by *T. cruzi* in vivo are likely to be non-macrophage, non-MHC class II bearing cells. As reviewed above, the $CD8^+$ T cells have a variety of mechanisms by which to regulate parasite growth. The cytokines produced by $CD8^+$ T cells can significantly limit intracellular replication of *T. cruzi* but appear to be insufficient to completely control the infection. Likewise, cytolysis of infected cells by CTL would be expected to result in the premature release, but not necessarily the destruction, of intracellular amastigotes. The role of antibody production as a secondary mechanism of immune control can be explained by the requirement for these antibodies to mediate or potentiate the clearance of released amastigotes. Antibodies may also be important in the killing of trypomastigotes, although a considerable body of literature documents the multiple mechanisms by which trypomastigotes of *T. cruzi* may evade such antibody-mediated destruction (Garcia et al., 1997a;Rimoldi et al., 1989;Joiner et al., 1988;Sher et al., 1986;De Miranda-Santos and Compos-Neto, 1981;Hall and Joiner, 1993;Krettli and Brener, 1982;Murfin and Kuhn, 1989;Norris et al., 1989;Tomlinson et al., 1994;Schenkman et al., 1986). If our model is correct, then efforts toward the production of anti-*T. cruzi* vaccines should focus on protocols which elicit strong type 1 biased T helper and CTL responses and antibody responses to amastigote-derived molecules.

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**References**

Abrahamsohn, I. A. and Coffman, R. L. (1996) Trypanosoma cruzi: IL-10, TNF, IFN-gamma, and IL-12 regulate innate and acquired immunity to infection. *Exp Parasitol,* **84,** 231-244

Almeida, I. C., Milani, S. R., Gorin, P. A. and Travassos, L. R. (1991) Complement-mediated lysis of Trypanosoma cruzi trypomastigotes by human anti-alpha-galactosyl antibodies. *J Immunol,* **146,** 2394-2400

Ando, K., Hiroishi, K., Kaneko, T., Moriyama, T., Muto, Y., Kayagaki, N., Yagita, H., Okumura, K. and Imawari, M. (1997) Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J Immunol,* **158,** 5283-5291

Araujo, F. (1989) Development of resistance to Trypanosoma cruzi in mice depends on a viable population of L3T4+ (CD4+) T lymphocytes. *Infect. Immun.,* **57,** 2246-2248

Atkinson, E. A. and Bleackley, R. C. (1995) Mechanisms of lysis by cytotoxic T cells. *Crit. Rev. Immunol.,* **15,** 359-384

Berke, G. (1995) The CTL's kiss of death. *Cell,* **81,** 9-12

Brener, Z. (1986) Why vaccines do not work in Chagas' disease. *Parasitol. Today,* **2,** 196-197

Brodskyn, C. I., Silva, A. M. M., Takehara, H. A. and Mota, I. (1989) IgG subclasses responsible for immune clearance in mice infected with Trypanosoma cruzi. *Immunol. Cell. Bio.,* **67,** 343-348

Clark, W. R., Walsh, C. M., Glass, A. A., Huang, M. T., Ahmed, R. and Matloubian, M. (1995) Cell-mediated cytotoxicity in perforin-less mice. *Int Rev Immunol,* **13,** 1-14

De Miranda-Santos, I. K. and Compos-Neto, A. (1981) Receptor for immunoglobulin Fc on pathogenic but not on nonpathogenic protozoa of the Trypanosomatidae. *J Exp Med,* **154,** 1732-1742

Denkers, E. Y., Yap, G., Scharton-Kersten, T., Charest, H., Butcher, B. A., Caspar, P., Heiny, S. and Sher, A. (1997) Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. *J Immunol,* **159,** 1903-1908

Elkon, K. B., Liu, C. C., Gall, J. G., Trevejo, J., Marino, M. W., Abrahamsen, K. A., Song, X., Zhou, J. L., Old, L. J., Crystal, R. G. and Falck-Pedersen, E. (1997) Tumor necrosis factor alpha plays a central role in immune-mediated clearance of adenoviral vectors. *Proc Natl Acad Sci U S A,* **94,** 9814-9819

Frost, P., Ng, C. P., Belldegrun, A. and Bonavida, B. (1997) Immunosensitization of prostate carcinoma cell lines for lymphocytes (CTL, TIL, LAK)-mediated apoptosis via the Fas-Fas-ligand pathway of cytotoxicity. *Cell Immunol,* **180,** 70-83

Garcia, I. E., Lima, M. R., Marinho, C. R., Kipnis, T. L., Furtado, G. C. and Alvarez, J. M. (1997a) Role of membrane-bound IgM in Trypanosoma cruzi evasion from immune clearance. *J Parasitol,* **83,** 230-233

Garcia, S., Fevrier, M., Dadaglio, G., Lecoeur, H., Riviere, Y. and Gougeon, M. L. (1997b) Potential deleterious effect of anti-viral cytotoxic lymphocyte through the

CD95 (FAS/APO-1)-mediated pathway during chronic HIV infection. *Immunol Lett,* **57,** 53-58

Gazzinelli, R. T., Oswald, I. P., Hieny, S., James, S. L. and Sher, A. (1992) The microbicidal activity of interferon-gamma-treated macrophages against Trypanosoma cruzi involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. *Eur J Immunol,* **22,** 2501-2506

Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R. and Ferguson, T. A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science,* **270,** 1189-1192

Guidotti, L. G. and Chisari, F. V. (1996) To kill or to cure: options in host defense against viral infection. *Curr. Opin. Immunol.,* **8,** 478-483

Hall, B. F. and Joiner, K. A. (1993) Developmentally-regulated virulence factors of Trypanosoma cruzi and their relationship to evasion of host defences. *J Eukaryot Microbiol,* **40,** 207-213

Harth, G., Mills, A. A., Lin, T. and Araujo, F. G. (1994) Trypanosoma cruzi glycoprotein of M(r) 56,000 characterization and assessment of its potential to protect against fatal parasite infections. *Mol Microbiol,* **11,** 261-271

Heusel, J. W., Wesselschmidt, R. L., Shresta, S., Russell, J. H. and Ley, T. J. (1994) Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell,* **76,** 977-987

Hunter, C. A., Ellis-Neyes, L. A., Slifer, T., Kanaly, S., Grunig, G., Fort, M., Rennick, D. and Araujo, F. G. (1997) IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J Immunol,* **158,** 3311-3316

Joiner, K. A., daSilva, W. D., Rimoldi, M. T., Hammer, C. H., Sher, A. and Kipnis, T. L. (1988) Biochemical characterization of a factor produced by trypomastigotes of Trypanosoma cruzi that accelerates the decay of complement C3 convertases. *J Biol Chem,* **263,** 11327-11335

Kagi, D. and Hensgartner, H. (1996a) Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. *Curr. Opin. Immunol.,* **8,** 472-477

Kagi, D., Ledermann, B., Buerki, K., Hengartner, H. and Zinkernagel, R. M. (1994a) CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol,* **24,** 3068-3072

Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R. M. and Hengartner, H. (1996b) Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu. Rev. Immunol.,* **14,** 207-232

Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H. and Golstein, P. (1994b) Fas and perforin pathways as major mechanisms of T-cell mediated cytotoxicity. *Science,* **265,** 528-530

Kitamura, D., Roes, J., Kuhn, R. and Rajewsky, K. (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature,* **350,** 423-426

Koller, B. H., Marrack, P., Kappler, J. W. and Smithies, O. (1990) Normal development of mice deficient in beta-2-microglobulin, MHC class I proteins, and CD8+ T cells. *Science,* **248,** 1227-1230

Krettli, A. U. and Brener, Z. (1982) Resistance against Trypanosoma cruzi associated to anti-living trypomastigote antibodies. *J. Immunol.,* **128,** 2009-2012

Lages-Silva, E., Ramirez, L. E., Krettli, A. U. and Brener, Z. (1987) Effect of protective and non-protective antibodies in the phagocytosis rate of Trypanosoma cruzi blood forms by mouse peritoneal macrophages. *Parasite Immunol,* **9,** 21-30

Laochumroonvorapong, P., Wang, J., Liu, C. C., Ye, W., Moreira, A. L., Elkon, K. B., Freedman, V. H. and Kaplan, G. (1997) Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. *Infect Immun,* **65,** 127-132

Lima-Martins, M. V. C., Sanchez, G. A., Krettli, A. U. and Brener, Z. (1985) Antibody-dependent cell cytotoxicity against Trypanosoma cruzi is only mediated by protective antibodies. *Parasite Immunol,* **7,** 367-376

Low, H. P., Santos, M. A. M., Wizel, B. and Tarleton, R. L. (1997) Amastigote surface proteins of Trypanosoma cruzi are targets for CD8+ cytotoxic T lymphocytes. *J. Immunol.,* **in press**

Lowin, B., Hahne, M., Mattmann, C. and Tschopp, J. (1994) Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature,* **370,** 650-652

Metz, G., Carlier, Y. and Vray, B. (1993) Trypanosoma cruzi upregulates nitric oxide release by IFN-gamma-preactivated macrophages, limiting cell infection independently of the respiratory burst. *Parasite Immunol,* **15,** 693-699

Mota, I. and Umekita, L. F. (1989) The effect of C3 depletion on the clearance of Trypanosoma cruzi induced by IgG antibodies. *Immunol. Lett.,* **21,** 223-226

Munoz-Fernandez, M. A., Fernandez, M. A. and Fresno, M. (1992) Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. *Eur J Immunol,* **22,** 301-307

Murfin, D. J. and Kuhn, R. E. (1989) Relative resistance of Brazil strain trypomastigote forms of Trypanosoma cruzi to in vitro antibody-dependent complement-mediated lysis. *J. Parasitol.,* **74,** 1046-1050

Nakamoto, Y., Guidotti, L. G., Pasquetto, V., Schreiber, R. D. and Chisari, F. V. (1997) Differential target cell sensitivity to CTL-activated death pathways in hepatitis B virus transgenic mice. *J Immunol,* **158,** 5692-5697

Nickell, S. P., Stryker, G. A. and Arevalo, C. (1993) Isolation from Trypanosoma cruzi-infected mice of CD8+, MHC-restricted cytotoxic T cells that lyse parasite-infected target cells. *J Immunol,* **150,** 1446-1457

Norris, K., Harth, G. and So, M. (1989) Purification of a Trypanosoma cruzi membrane glycoprotein which elicits lytic antibodies. *Infect. Immun.,* **57,** 2372-2379

Renggli, J., Hahne, M., Matile, H., Betschart, B., Tschopp, J. and Corradin, G. (1997) Elimination of P. berghei liver stages is independent of Fas (CD95/Apo- I) or perforin-mediated cytotoxicity. *Parasite Immunol,* **19,** 145-148

Rimoldi, M. T., Tartanian, A. and Joiner, K. A. (1989) Lytic rabbit IgG for tissue culture trypomastigotes of Trypanosoma cruzi alters the extent and form of complement deposition. *Exp Parasitol,* **68,** 160-167

Rodriguez, A. M., Santoro, F., Afchain, D., Bazin, H. and Capron, H. (1981) Trypanosoma cruzi infection in B-cell deficient rats. *Parasite Immunol,* **31,** 524-529

Rottenberg, M. E., Bakhiet, M., Olsson, T., Kristensson, K., Mak, T., Wigzell, H. and Orn, A. (1993) Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with Trypanosoma cruzi or Trypanosoma brucei. *Infect Immun,* **61,** 5129-5133

Rottenberg, M. E., Cardoni, R. L., Titto, E. H. d., Moreno, M. and Segura, E. L. (1988) *Trypanosoma cruzi*: Immune response in mice immunized with parasite antigens. *Exp. Parasitol.,* **65,** 101-108

Rottenberg, M. E., Riarte, A., Sporrong, L., Altcheh, J., Petray, P., Ruiz, A. M., Wigzell, H. and Orn, A. (1995a) Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol Lett,* **45,** 53-60

Rottenberg, M. E., Sporrong, L., Persson, I., Wigzell, H. and Orn, A. (1995b) Cytokine gene expression during infection of mice lacking CD4 and/or CD8 with Trypanosoma cruzi. *Scand J Immunol,* **41,** 164-170

Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P. and Rammensee, H. G. (1991) Exact prediction of a natural T cell epitope. *Eur J Immunol,* **21,** 2891-2894

Ruiz, A. M., Esteva, M., Riarte, A., Subias, E. and Segura, E. L. (1986) Immunoprotection of mice against *Trypanosoma cruzi* with a lyophilized flagellar fraction of the parasite plus adjuvant. *Immunol. Letts.,* **12,** 1-4

Santos Lima, E. C. and Minoprio, P. (1996) Chagas' disease is attenuated in mice lacking gamma delta T cells. *Infect Immun,* **64,** 215-221

Schenkman, S., Guther, M. L. and Yoshida, N. (1986) Mechanism of resistance to lysis by the alternative complement pathway in Trypanosoma cruzi trypomastigotes: effect of specific monoclonal antibody. *J Immunol,* **137,** 1623-1628

Scott, M. T. and Moyes, L. (1982) 75Se-methionine labelled Trypanosoma cruzi blood trypomastigotes: opsonization by chronic infection serum facilitates killing in spleen and liver. *Clin. exp. Immunol.,* **48,** 754-757

Sher, A., Hieny, S. and Joiner, K. (1986) Evasion of the alternative complement pathway by metacyclic trypomastigotes of Trypanosoma cruzi: dependence on the developmentally regulated synthesis of surface protein and N-linked carbohydrate. *J Immunol,* **137,** 2961-2967

Spinella, S., Liegeard, P. and Hontebeyrie, J. M. (1992) Trypanosoma cruzi: predominance of IgG2a in nonspecific humoral response during experimental Chagas' disease. *Exp Parasitol,* **74,** 46-56

Stenger, S., Mazzaccaro, R. J., Uyemura, K., Cho, S., Barnes, P. F., Rosat, J. P., Sette, A., Brenner, M. B., Porcelli, S. A., Bloom, B. R. and Modlin, R. L. (1997) Differential effects of cytolytic T cell subsets on intracellular infection. *Science,* **276,** 1684-1687

Sutton, V. R., Vaux, D. L. and Trapani, J. A. (1997) Bcl-2 prevents apoptosis induced by perforin and granzyme B, but not that mediated by whole cytotoxic lymphocytes. *J Immunol,* **158,** 5783-5790

Tang, Y., Hugin, A. W., Giese, N. A., Gabriele, L., Chattopadhyay, S. K., Fredrickson, T. N., Kagi, D., Hartley, J. W. and Morse, H. C., 3rd (1997) Control of

immunodeficiency and lymphoproliferation in mouse AIDS: studies of mice deficient in CD8+ T cells or perforin. *J Virol,* **71,** 1808-1813

Tarleton, R. L. (1990) Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. *J. Immunol.,* **144,** 717-724

Tarleton, R. L. (1997) Immunity to *Trypanosoma cruzi*. In *Host Response to Intracellular Pathogens*(Ed, Kaufmann, S. H. E.) R.G. Landes Company, Austin, TX, pp. 227-247.

Tarleton, R. L., Grusby, M. J., Postan, M. and Glimcher, L. H. (1996) Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int. Immunol.,* **8,** 13-22

Tarleton, R. L., Koller, B. H., Latour, A. and Postan, M. (1992) Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection. *Nature,* **356,** 338-340

Tomlinson, S., Pontes de Carvalho, L. C., Vandekerckhove, F. and Nussenzweig, V. (1994) Role of sialic acid in the resistance of Trypanosoma cruzi trypomastigotes to complement. *J Immunol,* **153,** 3141-3147

Trischmann, T. M. (1983) Non-antibody mediated control of parasitemia in acute experimental Chagas' disease. *J. Immunol.,* **130,** 1953-1957

Trischmann, T. M. (1984) Role of cellular immunity in protection against Trypanosoma cruzi in mice. *Parasite Immunol,* **6,** 561-570

Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L. and Tonegawa, S. (1992) TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell,* **71,** 1205-1214

Vespa, G. N., Cunha, F. Q. and Silva, J. S. (1994) Nitric oxide is involved in control of Trypanosoma cruzi-induced parasitemia and directly kills the parasite in vitro. *Infect Immun,* **62,** 5177-5182

Wizel, B., Nunes, M. and Tarleton, R. L. (1997) Identification of a Trypanosoma cruzi trans-sialidase family member as a target of protective CD8+ Tc1 responses. *J. Immunol.,* **159,** 6120-6130

# CHAPTER 3

# ANTIGEN-SPECIFIC TH1 BUT NOT TH2 CELLS PROVIDE PROTECTION TO LETHAL *TRYPANOSOMA CRUZI* INFECTION IN MICE[1]

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**Abstract**

Infection with *Trypanosoma cruzi* results in the development of both type 1 and type 2 patterns of cytokine responses during acute and chronic stages of infection. To investigate the role of Th1 and Th2 subsets of $CD4^+$ T cells in determining the outcome of *T. cruzi* infection in mice, we have developed *T. cruzi* clones that express ovalbumin ($OVA^2$) and have used OVA-specific TCR-transgenic T cells to generate OVA-specific Th1 and Th2 cells. BALB/c mice receiving $10^7$ OVA-specific Th1 cells and then challenged with OVA-expressing *T. cruzi* G-OVA.GPI showed significantly lower parasitemia and increased survival in comparison to mice that received no cells. In contrast, recipients of OVA-specific Th2 cells developed higher parasitemias, exhibited higher tissue parasitism and inflammation and higher mortality than recipients of Th1 cells following infection with *T. cruzi* G-OVA.GPI. Mice receiving a mixture of both Th1 and Th2 OVA-specific cells were also not protected from lethal challenge. The protective effect of the OVA-specific Th1 cells was OVA-dependent as shown by the fact that transfer of OVA-specific Th1 or Th2 cells failed to alter the course of infection or disease in mice challenged with wild-type *T. cruzi*. Immunohistochemical analysis of OVA-specific Th1 and Th2 cells at 4, 15 and 30 days PI revealed the persistence and expansion of these cells in mice challenged with *T. cruzi* G-OVA.GPI but not in mice infected with wild-type *T. cruzi*. We conclude that transfer of antigen-specific Th1 cells but not Th2 cells protect mice from a lethal infection with *T. cruzi.*

**Introduction**

The protozoan parasite *Trypanosoma cruzi* causes Chagas disease in humans and is a major public health problem in Latin America. It is estimated that 18 million people are infected with the parasite and 90 million people are at risk of infection (1). Although vector control programs and the application of chemotherapeutics are partial solutions to control of the infection and disease, these approaches alone are unlikely to be long-term solutions to combating Chagas disease. Vaccination also has potential for reducing the severity of *T. cruzi* infection Chagas disease (2-4). The design of appropriate vaccination strategies against *T. cruzi* requires the elucidation of the mechanisms for immune protection. Experimental *T. cruzi* infection in murine models has provided the means for the identification of these protective immune mechanisms operating against the parasite.

In infected hosts, *T. cruzi* circulates in the blood as non-replicating trypomastigote forms that invade a wide variety of cells and subsequently multiply intracellularly as amastigotes. Both amastigotes and trypomastigotes elicit a complex pattern of immune responses including substantial antibody production and cellular responses mediated by $CD4^+$ and $CD8^+$ T cells (5). In the absence of B cells, $CD4^+$ T cells or $CD8^+$ T cells, mice infected with *T. cruzi* develop high tissue parasite burden and die early in infection (6-12). Similar to infections with other intracellular pathogens (*Leishmania* (13), *Mycobacterium* (14) and *Listeria* (15)), where a strong Th1 response protects while a Th2 response increases susceptibility to infection (16, 17), there is some evidence for a protective role of Th1 cells (18) and an exacerbative role for Th2 cells (19) in *T. cruzi* infection. Production of the type 1 cytokine, IFN-γ, in the acute phase of *T. cruzi* infection is associated with resistance (20-22) and depletion of IFN-γ exacerbates

parasitemia and results in increased mortality in *T. cruzi* infected mice (23, 24). Similarly, IL-12, an inducer of the type 1 cytokine response, promotes resistance to *T. cruzi* in murine models (25). In contrast, IL-10, a cytokine suggested to induce type 2 response has been linked to susceptibility to *T. cruzi* in several murine models (26) with an elevation of IL-10 production in susceptible mice strains as compared to resistant strains (27).

In order to investigate further the role of antigen-specific Th1/Th2 cells in *T. cruzi* infection, we have developed a system by which we can generate and transfer "parasite-specific" Th1 and Th2 cell populations and determine their ability to protect naive mice from lethal *T. cruzi* infection. This system utilizes *T. cruzi* lines expressing chicken ovalbumin (OVA) as a source of infective parasites and OVA-specific Th1 and Th2 cells from DO11.10 TCR transgenic mice as the source of "parasite"-specific T cells (28). The adoptive transfer of OVA-specific Th1 cells protected mice while transfer of Th2 cells reversed the protective effect of Th1 cells transfer in mice infected with a lethal dose of OVA-expressing *T. cruzi*. Immunohistochemical analysis of spleens, lymph nodes and skeletal muscle of recipient mice showed that OVA-specific Th1 and Th2 cells persisted and expanded *in vivo* in response to OVA-expressing *T. cruzi* and not in response to wild-type parasites. These results suggest that a primed Th1 response and the absence of a Th2 response provides optimal control of *T. cruzi* infection.

**Materials and Methods**

*Mice and Parasites*

Mice transgenic for the DO11.10 TCR (I-$A^d$ restricted and OVA-specific) were obtained from Dr D. Loh (Washington University School of Medicine, St. Louis, MO) and wild

type BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Epimastigotes of *T. cruzi* (Brazil strain) were cultured at 28$^{o}$C in liver infusion tryptose (LIT) broth supplemented with 5% heat-inactivated fetal bovine serum (Hyclone Logan, UT). Infective metacyclic trypomastigotes were obtained from 4-5 week-old stationary-phase cultures of epimastigotes. Vero cells (African green monkey kidney fibroblasts, American Type Culture Collection, Rockville, MD) were infected with the infective metacyclic trypomastigotes to obtain fibroblast-derived trypomastigotes. Blood-form trypomastigotes (BFT) were maintained by biweekly passages in C3HHe/SnJ mice and were used for infection of mice. Trypomastigotes were converted to amastigotes extracellularly in LIT broth for flow cytometric analysis (29).

*Plasmid*

Plasmid pHD421β containing the *Trypanosoma brucei* β tubulin gene (a gift from Dr. Elizabeth Wirtz, Rockefeller University, NY) was modified by replacing the *T. brucei* β tubulin gene with approximately 0.7 kb of the β tubulin gene of *T. cruzi* (Brazil strain). A forward primer (5' GGTACCTGTATTGAAATGAAGCCCTGT 3') was designed at position 601 of the *T. cruzi* β tubulin gene (Gen Bank accession # M97956) to add a KpnI site and a reverse primer (5' CTCGACCTTCCTCCTCAATGGTGGCGGTC 3') was designed at position 1300 to add a XhoI site. These primers were used to amplify the β tubulin gene from *T. cruzi* (Brazil strain) genomic DNA and the amplified product was cloned in pHD421β at KpnI and XhoI sites. The Luciferase gene from the resulting plasmid was then removed by restriction digestion with HindIII and BamHI enzymes and replaced by a G-OVA.GPI construct (30), encoding the N-terminal signal sequence of *T. cruzi* glycoprotein gp-72 (aa 1-47); aa 139 to 357 of chicken ovalbumin (OVA) followed

by 45 amino acids of amastigote surface protein 1 (ASP-1) providing a C-terminal GPI cleavage/attachment site to yield pHD421β G-OVA.GPI.

*Transfection of T. cruzi and generation of T. cruzi G-OVA.GPI*

Mid-log phase *T. cruzi* epimastigotes were transfected with 25μg of the pHD421 β G-OVA.GPI plasmid linearized at a unique Not1 site in the *T. cruzi* β tubulin gene to allow for homologous recombination in one of the *T. cruzi* β tubulin genes loci. Hygromycin was added to a final concentration of 1.0 mg/ml after 48 h of incubation at $28^{O}$C for selection of transfectants. Clones of *T. cruzi* G-OVA.GPI were selected that continued to grow in the presence of hygromycin (1.0 mg/ml) at rates similar to those of wild-type *T. cruzi* growing in drug free media. After four weeks of drug selection, the parasites were grown in drug-free media.

*Flow cytometric analysis of T. cruzi G-OVA.GPI*

Amastigotes of *T. cruzi* were washed in PBS containing 0.1% sodium azide and 0.1% casein (PAC). In some experiments, parasites were also treated with $1 \times 10^{-2}$ U of *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PIPLC Boehringer Mannheim, Indianapolis, IN) in 100 μl of PIPLC buffer (30). For flow cytometric analysis, $1 \times 10^{6}$ parasites were suspended in 50 μl of PAC containing rabbit anti-OVA Ab (1/200) (Sigma Chemical Co., St Louis, MO) for 30 min at $4^{O}$C. After washing with 1 ml of PAC containing 0.01% Tween-20, the parasites were incubated with FITC-labeled goat $F(ab')_2$ anti-rabbit IgG (Southern Biotechnology, Birmingham, AL) (1/50 dilution in PAC) at $4^{O}$C for 30 min in the dark. Cells were then washed once with PAC containing Tween-20, resuspended in 250 μl of PAC and analyzed by flow cytometry on an EPICS Elite Analyzer (Coulter Corporation, Hialeah, FL).

*Generation of OVA-specific Th1 and Th2 cells*

Splenocytes from DO11.10 transgenic mice were depleted of RBCs by hypotonic lysis and were cultured at 5 x $10^6$ cells/well in 2 ml complete RPMI (Mediatech, Herndon, VA) containing 10% FBS (Hyclone, Logan, UT) in 24 wells plates. IL-2 (20 U/ml; Cetus Corporation, Emeryville, CA), IL-12 (10 ηg/ml; Genetics Institute, Cambridge, MA) and anti-IL-4 MAb 11B11 (10μg/ml) were added to cultures to generate Th1 cells, and IL-4 (100 U/ml; DNAX, Palo Alto, CA) and anti-IFN-γ MAb R4-6AB (75μg/ml) were added to generate Th2 cells. All wells also received OVA peptide (0.3 μM) containing aa 323-339 (SQAVHAAHAEINEAGRE) of chicken ovalbumin protein (31). After four days of stimulation, the frequency of cells expressing OVA TCR was determined by flow cytometric analysis using OVA TCR-specific MAb KJ1-26 (32); obtained from Dr. John Kappler; University of Colorado Health Science Center, Denver, CO). Th1 and Th2 cells (2.5 x $10^5$) were re-stimulated in 2 ml cultures with 4.5 x $10^6$ of RBC-depleted irradiated (2600 rads) H-$2^d$ BALB/c splenocytes, and OVA peptide. Supernatants from these cultures were collected after 48 hours and cytokine levels assayed by ELISA for IL-4 and IFN-γ. IFN-γ in the supernatant fluids was measured by ELISA as previously described (21) and IL-4 levels were determined using a commercial kit (Pharmingen, San Diego, CA) following the manufacturers instructions.

*Adoptive transfer of OVA-specific Th1 and Th2 cells*

OVA-specific Th1 and Th2 cells were purified over lymphocyte separation medium (LSM; ICN Biochemicals, Aurora, OH), and $10^7$ cells resuspended in 0.5 ml of DMEM (Life Technologies, Grand Island, NY) were transferred in naive BALB/c mice by injection into the tail vein. Control animals received DMEM alone. Mice (8 in each

group) were infected with 5 x $10^4$ or $10^5$ blood-form trypomastigotes (BFT) of *T. cruzi* by i.p. injection 12 hours after the injection of T cells. Parasitemias were monitored at weekly intervals by hemacytometer counts of parasites in tail blood and mortality was recorded daily.

*Flow cytometric analysis of cells for intracellular cytokines*

Flow cytometric analysis of splenocytes from recipients of Th1 or Th2 cells was accomplished using biotinylated KJ1-26 Mab plus streptavidin-cy7 (Molecular Probes, Eugene, OR) to identify OVA-specific $CD4^+$ T cells. Detection of intracellular IFN-γ and IL-4 was done using PE labeled- anti-IFN-γ MAb R46A-2 and anti-IL-4 MAb 11B11, respectively (Pharmingen), in single cell suspension of splenocytes in PAC buffer using Cytoperm/Cytofix (with GolgiPlug) kit (Pharmingen) as per the manufacturer's instructions.

*Histology and Immunohistochemistry*

Cardiac and skeletal muscle tissues were collected at 15 and 30 days post-infection in PBS and fixed in 10% buffered formalin. Sections (5 μm) from paraffin-embedded tissues were stained with hematoxylin and eosin for histopathological analysis. To detect OVA-specific Th1 and Th2 cells post-transfer, lymph nodes, spleens and skeletal muscle tissues from recipient mice were frozen in liquid nitrogen and 5-10 μm thick sections were analyzed as previously described with some modifications (33). Briefly, acetone fixed tissue sections were quenched with PBS containing 0.3% $H_2O_2$ and 0.1 % sodium azide and incubated with biotinylated KJ1-26 Ab in PBS at $4^0$C overnight. Enhanced color development was achieved using horseredishperoxidase and biotinyl tyramide (TSA-Indirect; NEN Biological Products, Boston MA) following manufacturer

instructions. Color was developed using diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO).

*Quantitation of T. cruzi DNA in the tissues*

DNA was isolated from proteinase K-treated (0.3 mg/ml in proteinase K buffer) skeletal muscle tissue by phenol:chloroform:isoamyl alcohol (Sigma, St. Louis, MO) extraction and ethanol precipitation as described previously (34). DNA in the tissues was quantitated by a real-time PCR protocol described by Cumming and Tarleton (manuscript in preparation) using a LightCycler™ (Roche Diagnostic Corporation, Indianapolis, IN). Data acquisition and analysis was performed using LightCycler™ Version 3.0 software. Standards of serially diluted *T. cruzi* DNA mixed with skeletal muscle DNA were used for quantification of samples. Standard curves generated were then used to determine parasite equivalents per 50 ηg of tissue DNA.

**Results**

In the absence of a clonal population of $CD4^+$ T cells that can recognize specific antigens expressed by *T. cruzi*, it is very difficult to directly analyze the role of antigen-specific Th1 and Th2 cells in susceptibility or exacerbation of *T. cruzi* infection and Chagas disease. Therefore, we developed a system which made use of DO11.10 OVA TCR transgenic mice as donors of T helper cells and *T. cruzi* expressing the OVA protein as targets of OVA-specific Th1 and Th2 cells.

*Generation of OVA-expressing T. cruzi G-OVA.GPI*

We have previously reported the generation of *T. cruzi* expressing both GPI-anchored (surface expressed) and secreted forms of ovalbumin (30). In addition, OVA secreted by intracellular parasites was processed and presented in association with class I MHC on

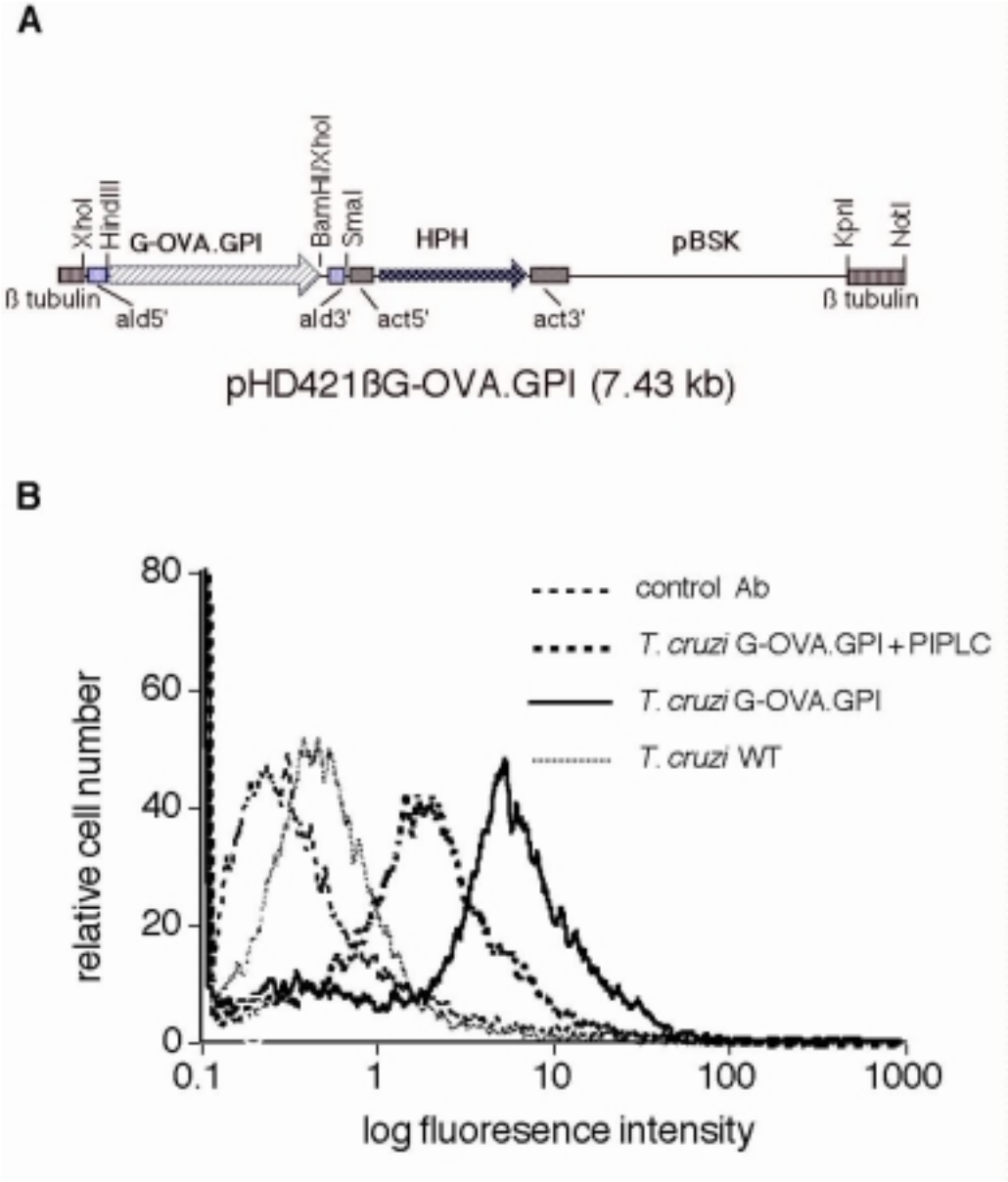
the surface of infected host cells (30). However, these OVA-expressing *T. cruzi* were not deemed useful for *in vivo* infection since continuous OVA expression depended upon continuous drug pressure on the parasites. To generate stable OVA-expressing *T. cruzi*, a plasmid pHD421βG-OVA.GPI containing the *T. cruzi* (Brazil strain) β-tubulin gene and G-OVA.GPI was constructed and electroporated into epimastigotes of *T. cruzi* for stable integration into one of the β-tubulin gene loci (Fig. 1 *A*). Trypomastigotes of *T. cruzi* G-OVA.GPI were generated by infection of VERO cells by metacyclic phase parasites and were converted into amastigotes by overnight incubation in LIT medium. Surface expression of OVA by the amastigotes of *T. cruzi* G-OVA.GPI was confirmed by FACS analysis using a polyclonal rabbit anti-chicken ovalbumin antibody (Fig. 1 *B*). The attachment of OVA via a GPI anchor was indicated by the fact that treatment of *T. cruzi* G-OVA.GPI with PIPLC resulted in loss of surface expression of OVA (Fig. 1 *B*). Stable expression of OVA was also documented by FACS analysis of parasites after passage of *T. cruzi* G-OVA.GPI through mice, after 5 months of culture in drug-free medium, and by the presence of anti-OVA antibodies in mice infected with *T. cruzi* G-OVA.GPI (data not shown). These results established that OVA was being expressed by *T. cruzi* G-OVA.GPI and thus could potentially be presented to OVA-specific Th1 and Th2 cells *in vivo* during infection.

*Generation of OVA specific Th1 and Th2 cells*

$CD4^+$ T cells in DO11.10 TCR transgenic mice express a clonotypic TCR that recognizes peptide fragment 323-339 from chicken ovalbumin protein in association with class II MHC molecules ($H\text{-}2^d$). Th1 and Th2 subpopulations of OVA-specific $CD4^+$ T cells were generated by *in vitro* stimulation of DO11.10 splenocytes with OVA peptide plus

**Figure 1.** Generation of OVA-expressing *T. cruzi*. *A*) The plasmid pHD421β G-OVA.GPI containing G-OVA.GPI insert (flanked by 5' and 3' UTR of *T. brucei* aldolase) and the hygromycin resistance gene (HPH) (flanked by 5' and 3' UTR of *T. brucei* actin) was linearized at a unique NotI site in β tubulin sequence and electroporated into epimastigotes of *T. cruzi* (Brazil strain) to allow for stable integration. *B*) Amastigotes of wild-type (WT) *T. cruzi* or OVA-expressing (G-OVA.GPI) *T. cruzi* were stained with a rabbit anti-OVA antibody followed by FITC-labeled goat anti-rabbit IgG and analyzed by FACS. FITC-labeled goat anti-rabbit IgG was used as control antibody. Some parasites were treated with PIPLC to cleave the GPI anchor, thus releasing OVA from the parasite surface.

Figure 1.


A
XhoI
HindIII
G-OVA.GPI
BamHI/XhoI
SmaI
HPH
pBSK
KpnI
NotI
ß tubulin
ald5'
ald3'
act5'
act3'
ß tubulin
pHD421ßG-OVA.GPI (7.43 kb)
B
control Ab
T. cruzi G-OVA.GPI + PIPLC
T. cruzi G-OVA.GPI
T. cruzi WT
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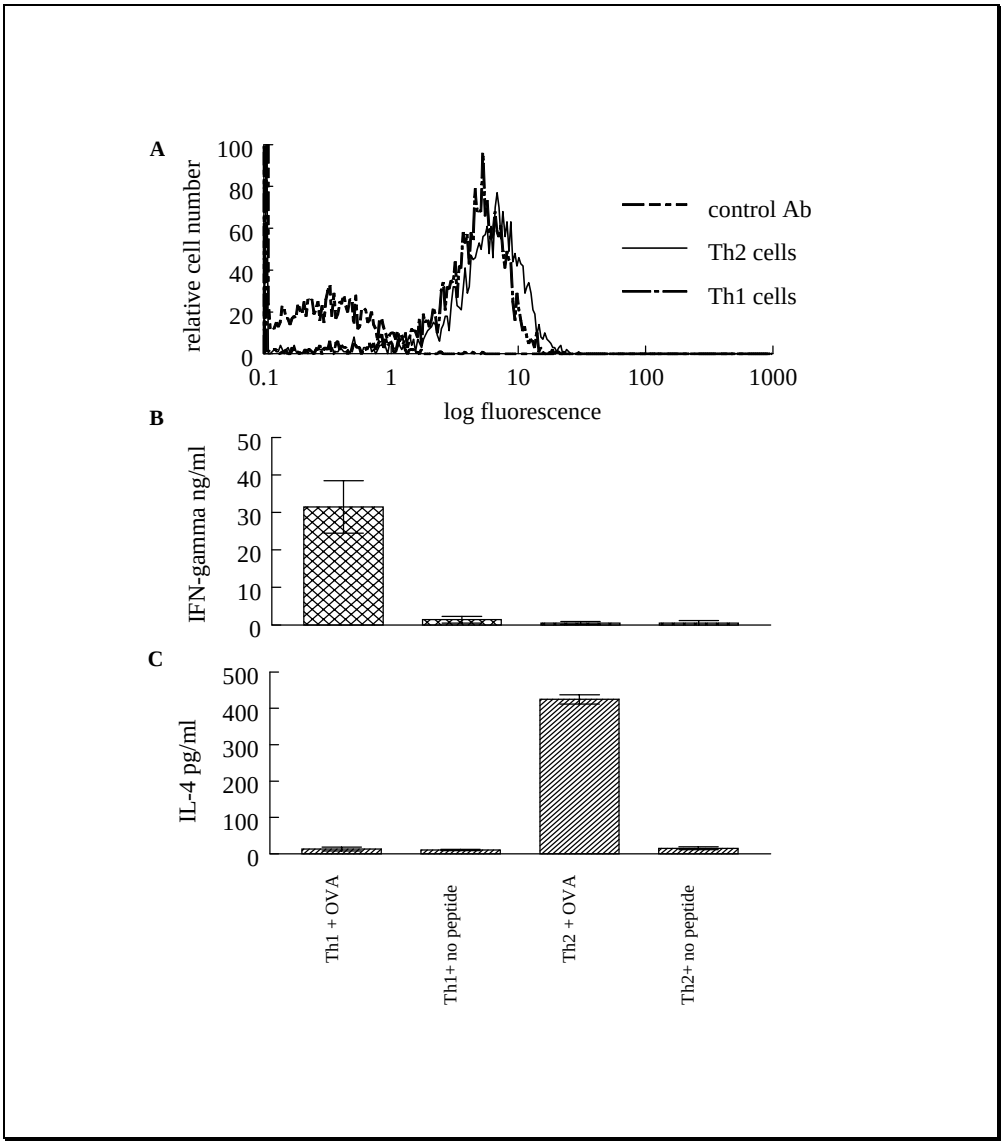



**Figure 2.** Generation and phenotypic analysis of OVA-specific Th1 and Th2 cells. DO11.10 splenocytes were stimulated with IL-2 plus IL-12 and anti-IL-4 Ab 11B11 to generate Th1 cells or IL-4 and anti IFN-γ Ab R4-6A2 to generate Th2 cells. OVA peptide was added to all cultures. *A*) After 4 days of culture, more than 90% of the cells expressed the OVA-specific clonotypic TCR receptor. *B* and *C*) Th1 and Th2 cells incubated with irradiated splenocytes plus OVA peptide secreted IFN-γ or IL-4, respectively (means +/- S.E.).

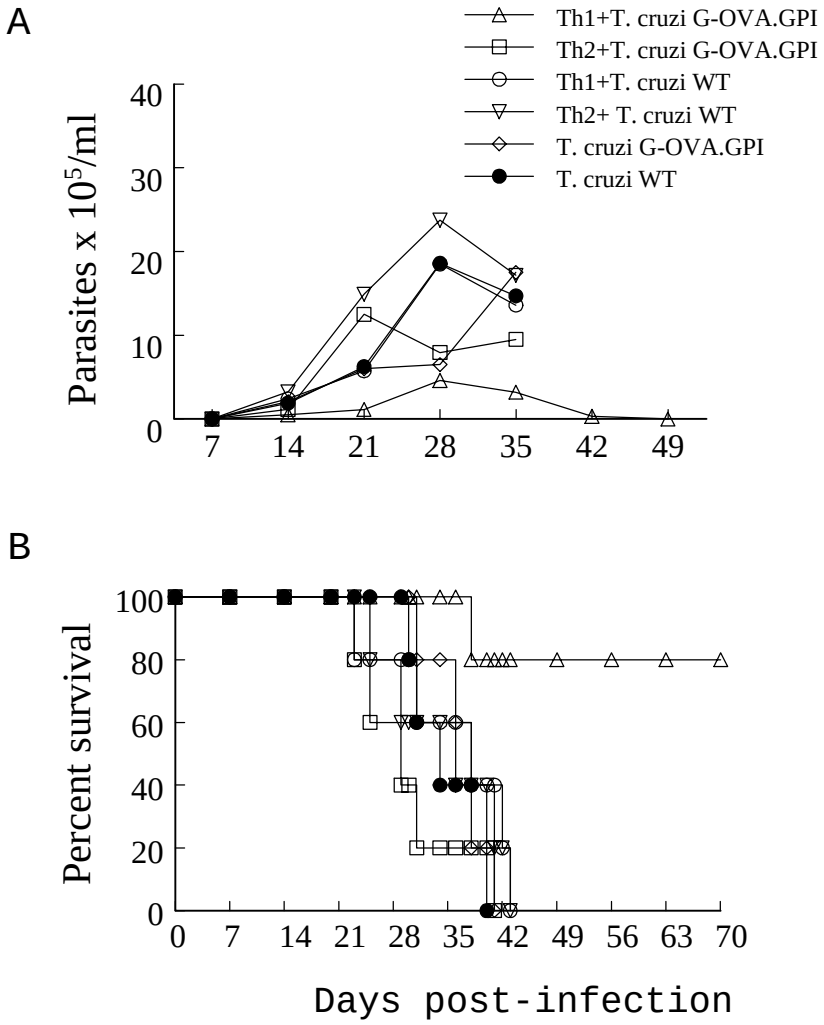



**Figure 3.** Protection conferred by adoptive transfer of OVA-specific Th1 or Th2 cells in groups of 5 mice each followed 12 hrs later by infection with 5 x $10^4$ BFT of *T. cruzi* G-OVA.GPI or WT *T. cruzi*. Parasites in the blood were counted weekly (*A*) and mortality recorded daily (*B*).

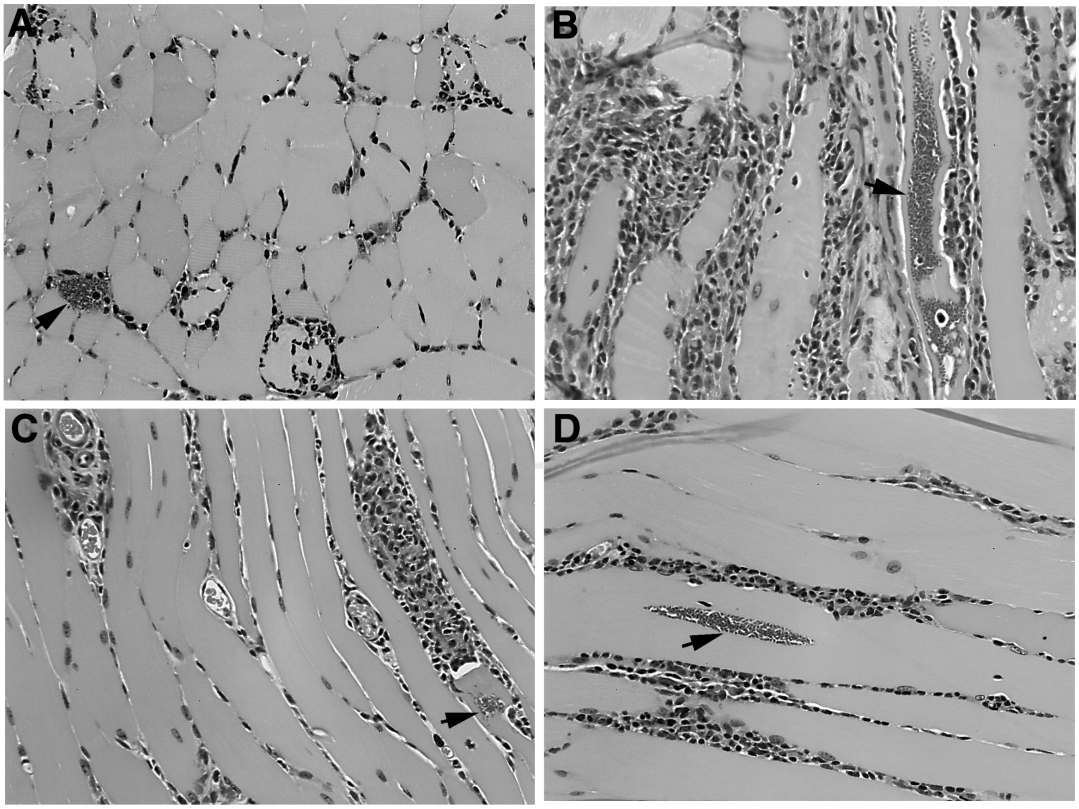



**Figure 4.** Histological analysis of skeletal muscle tissue 30 days post-infection in mice receiving Th1 or Th2 cells. Hematoxylin and eosin stained tissues from Th1 (*A*) or Th2 (*B*) cell recipient mice infected with *T. cruzi* G-OVA.GPI. Tissues from Th1 (*C*) or Th2 (*D*) cell recipient mice infected with wild-type *T. cruzi*. Arrows in B, C and D indicate "pseudocysts" of intracellular amastigotes.

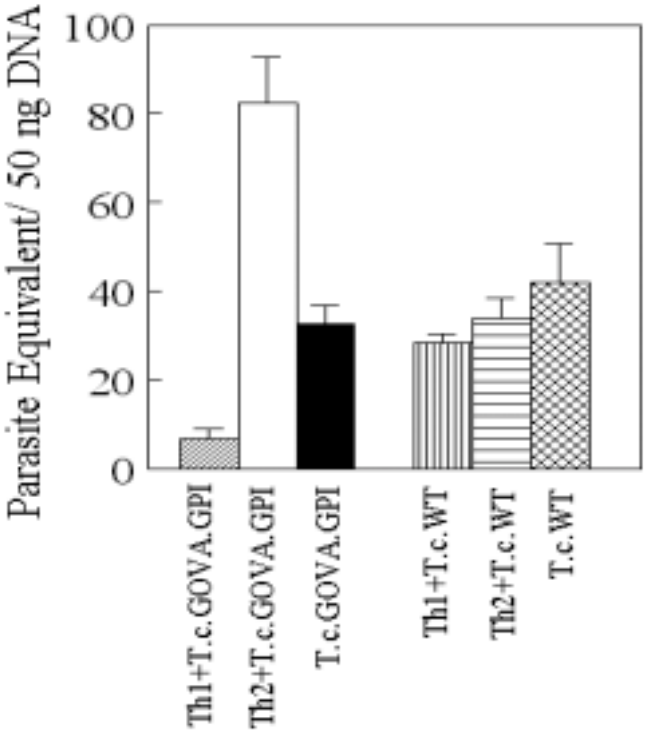
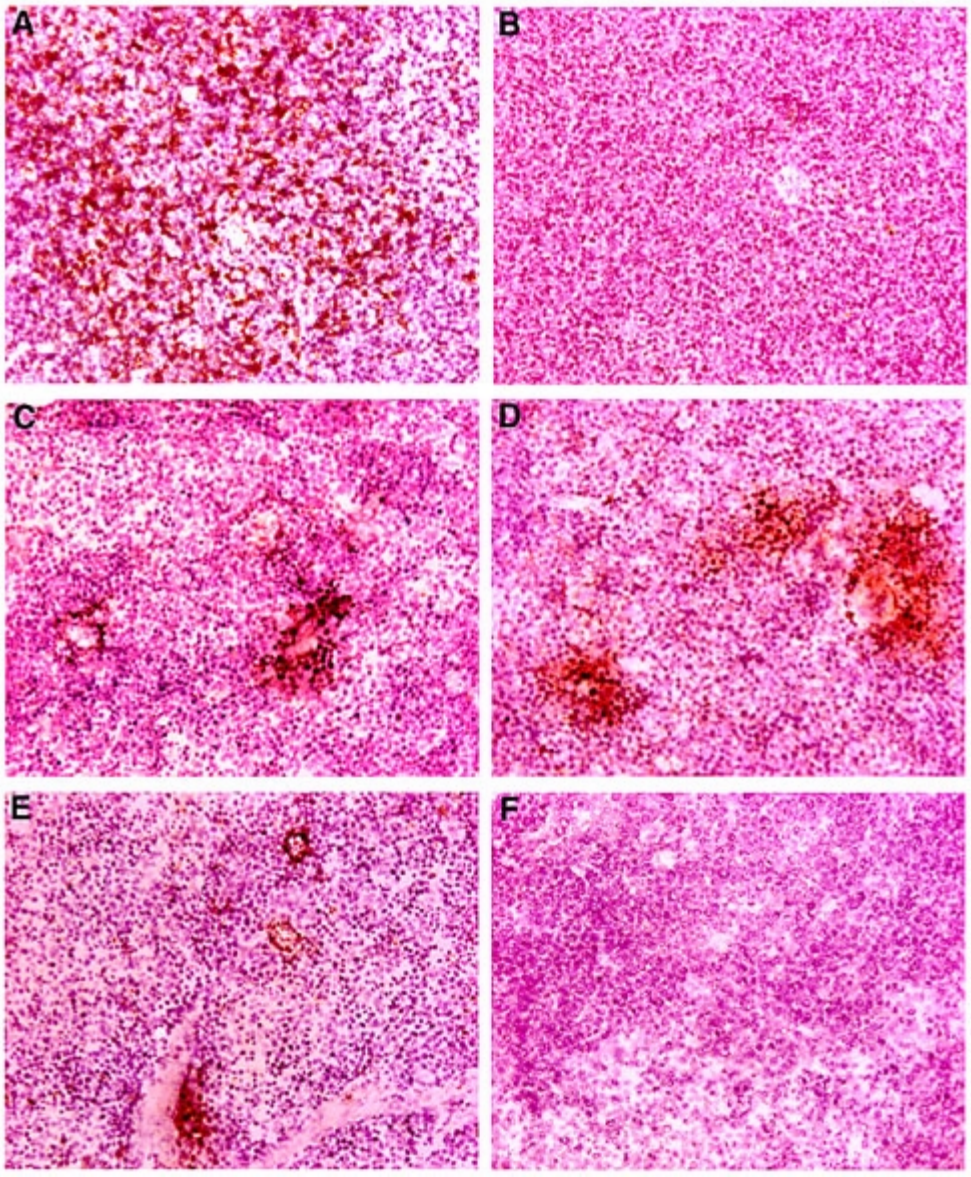



**Figure 5.** Th1 cell recipient mice have lower amounts of *T. cruzi* DNA than Th2 cell recipients infected with *T. cruzi* GOVA.GPI. At 30 days post-infection, *T. cruzi* kDNA was quantitated by real-time PCR from skeletal muscle tissue of mice receiving Th1 cells, Th2 cells, no cells and infected with *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. (means +/- S.E.).

**Figure 6.** OVA-specific Th1 cells expand *in vivo* in response to infection with *T. cruzi* G-OVA.GPI. Spleens from Th1 cell recipient mice at 4 and 30 days post-transfer and infection were stained for OVA-specific Th1 cells using anti-OVA TCR Ab KJ1-26. Spleens from OVA TCR transgenic DO11.10 mice stained with KJ1-26 MAb (*A*) or with mouse anti-rat IgG negative control antibody (*B*), were used as positive and negative staining controls, respectively. KJ1-26 positive cells were apparent in the spleens of Th1 recipient mice infected with either *T. cruzi* G-OVA.GPI (*C*) or wild-type *T. cruzi* (*E*) at 4 days post-infection. Further expansion of the KJ1-26 positive cells was obvious 30 days post-infection in the spleens of Th1 cell recipients infected with *T. cruzi* G-OVA (*D*) but no KJ1-26 positive cells were detectable in spleens of Th1 recipient infected with wild-type *T. cruzi* (*F*).

Figure 6.



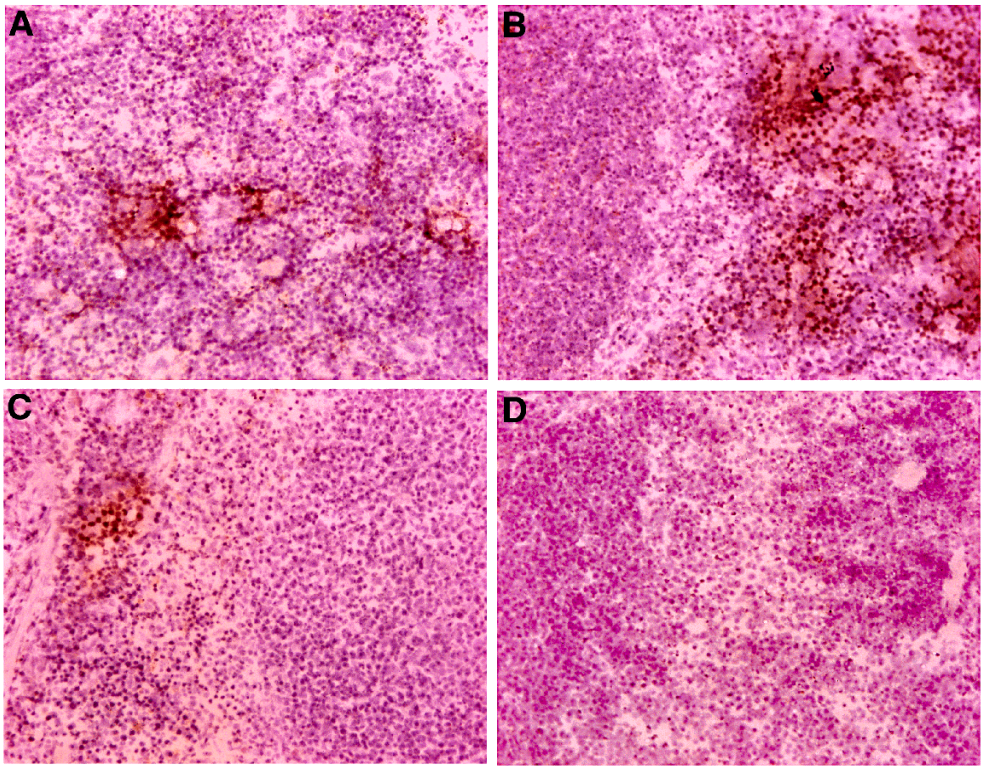
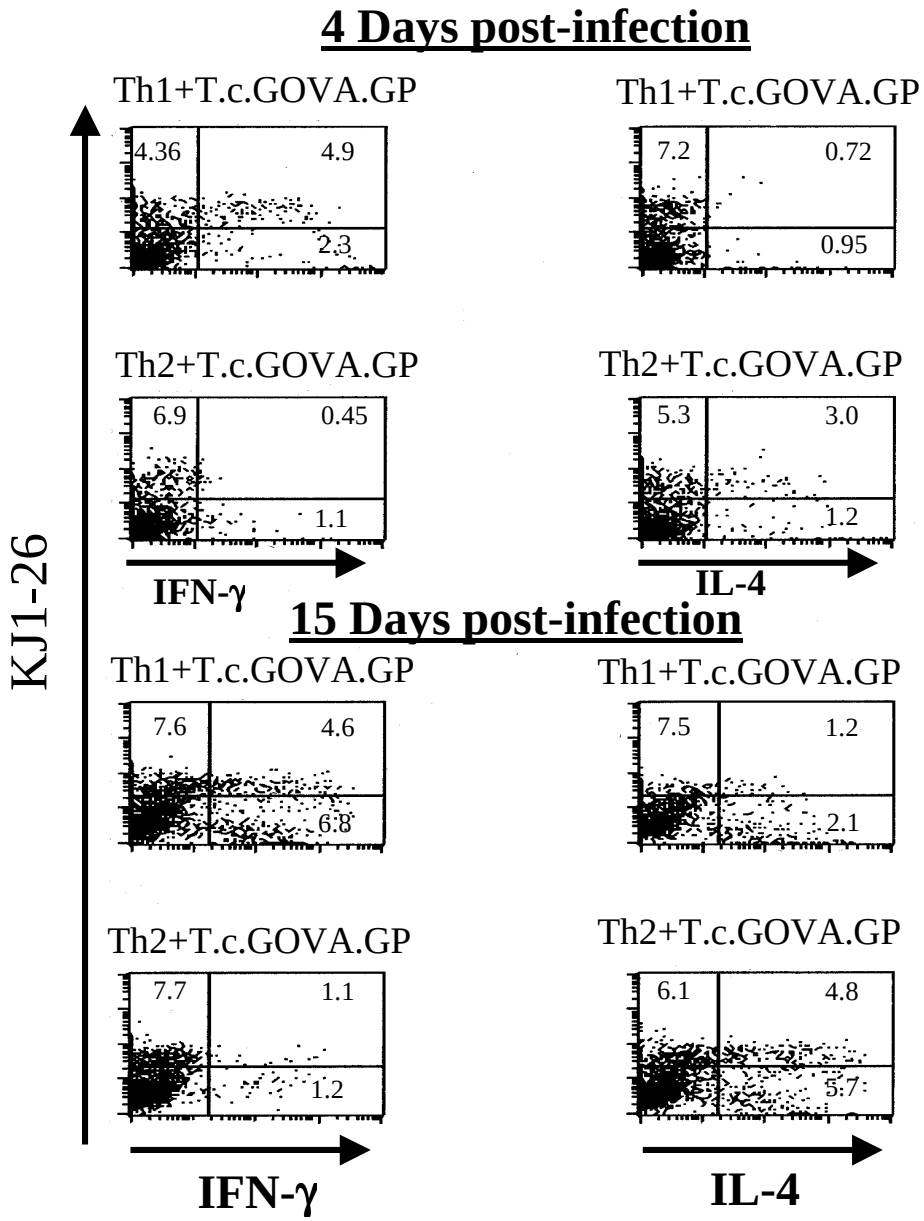



**Figure 7.** Th2 cells persist and expand in the spleen selectively in response to OVA-expressing parasites. Similar to Figure 5, at 4 days PI, Th2 cells were detected in the spleens of mice infected with *T. cruzi* G-OVA.GPI (*A*) and wild-type *T. cruzi* (*C*). But, at 30 days PI, expansion of these cells could only be detected in the spleens of *T. cruzi* G-OVA.GPI infected mice (*B*) and not in the spleens of mice infected with wild-type *T. cruzi* (*D*).

**Figure 8.** OVA-specific Th1 and Th2 cells continue to make IFN-γ and IL-4 cytokines, respectively, *in vivo*. Splenocytes collected from Th1 and Th2 cell recipient mice infected with *T. cruzi* GOVA.GPI at 4 and 15 days post-infection were stimulated with 5μM OVA peptide for 15 hours including addition of GolgiPlug during last 10 hours of stimulation. Two-color analysis for KJ1-26 positive and IFN-γ or IL-4 positive cells was done on $10^5$ events collected. Data shown is representative of two mice in each group.

Figure 8.


4 Days post-infection
Th1+T.c.GOVA.GP
4.36
4.9
2.3
Th1+T.c.GOVA.GP
7.2
0.72
0.95
Th2+T.c.GOVA.GP
6.9
0.45
1.1
Th2+T.c.GOVA.GP
5.3
3.0
1.2
IFN-γ
IL-4
KJ1-26
15 Days post-infection
Th1+T.c.GOVA.GP
7.6
4.6
6.8
Th1+T.c.GOVA.GP
7.5
1.2
2.1
Th2+T.c.GOVA.GP
7.7
1.1
1.2
Th2+T.c.GOVA.GP
6.1
4.8
5.7
IFN-γ
IL-4

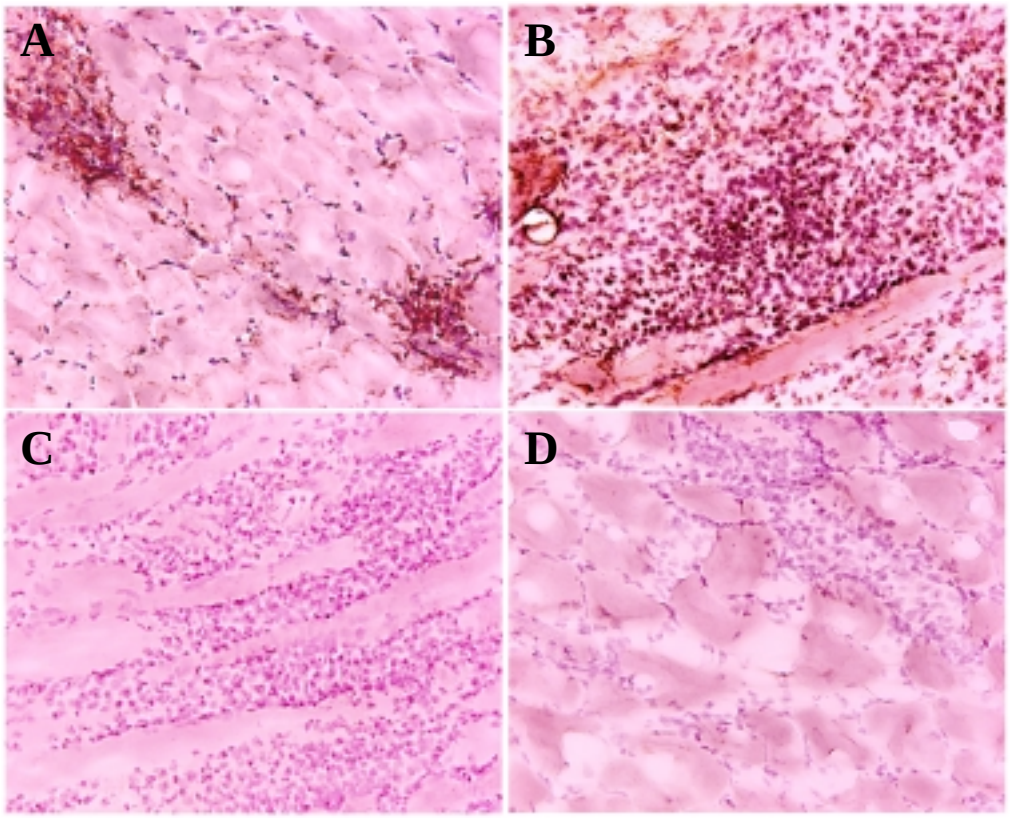



**Figure 9.** OVA-specific Th1 and Th2 cells are present in the skeletal muscle lesions in *T. cruzi* G-OVA.GPI infected mice. At 30 days PI, KJ1-26 positive cells are detected in the skeletal muscles of Th1 (*A*) and Th2 (*B*) cell recipient mice infected with *T. cruzi* G-OVA.GPI but not in mice receiving Th1 (*C*) or Th2 (*D*) cells and infected with wild-type *T. cruzi*.

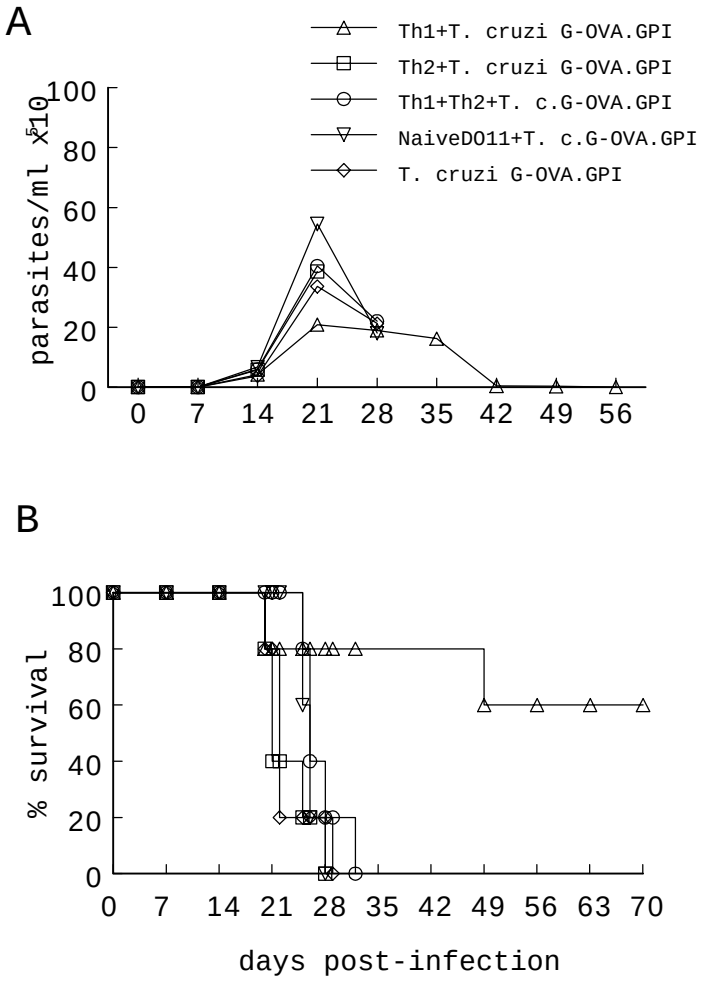



**Figure 10.** Adoptive transfer of Th2 cells abrogates the protective effect of Th1 cells in mice from *T. cruzi* G-OVA.GPI infection. Groups of 8 mice received 5 x $10^6$ Th1 and 5 x $10^6$ Th2 cells each, $10^7$ naive DO11.10 T cells, $10^7$ Th1 or $10^7$ Th2 cells. The mice were then challenged with $10^5$ BFT of *T. cruzi* G-OVA.GPI and parasitemia (*A*) and mortality (*B*) were recorded as described in the legend to Figure 3. We observed similar parasitemia and mortality in mice receiving 5 x $10^6$ Th1 or Th2 cells compared to mice receiving $10^7$ Th1 or Th2 cells and challenged with *T. cruzi* GOVA.GPI (data not shown).

IL-2, IL-12 and anti-IL-4 Ab (for Th1) or IL-4 and anti-IFN-γ Ab (for Th2). FACS analysis of cells after four days of culture using the TCR-specific KJ1-26 monoclonal antibody showed that more than 90% of the cells expressed the clonotypic TCR (Fig. 2 *A*). Phenotypic analysis of Th1 and Th2 cells was accomplished by measuring production of IFN-γ and IL-4 cytokines, after incubation with irradiated APCs. OVA peptide-stimulated Th1 cells produced predominantly IFN-γ but no IL-4 while Th2 cells produced IL-4 but not IFN-γ (Fig. 2 *B* and *C*). No IFN-γ and IL-4 was produced by Th1 and Th2 cells in the absence of OVA peptide stimulation (Fig. 2 *B* and *C*).

*Adoptive transfer of OVA-specific Th1 cells but not Th2 cells protects naive mice from infection with T. cruzi G-OVA.GPI*

The ability of antigen-specific Th1 and Th2 cells to modulate the outcome of a lethal *T. cruzi* infection was investigated by adoptive transfer of Th1 and Th2 cells to naive BALB/c mice and then challenge of these mice with a lethal dose of *T. cruzi* G-OVA.GPI. Mice infected with 5 x $10^4$ BFT of either *T. cruzi* G-OVA.GPI or wild-type *T. cruzi* developed similar parasitemia levels and died by day 42 post-infection suggesting that the OVA-transgenic parasites were equally as virulent as wild-type parasites (Fig. 3 *A* and *B*). Transfer of OVA-specific Th1 cells protected mice from lethal infection with *T. cruzi* G-OVA.GPI but not from infection with wild-type *T. cruzi*. Parasitemias in these protected animals were below the level of detection by 49 days after infection and 80% of the mice survived the infection until 200 days post-infection when the experiment was terminated. In contrast, mice receiving Th2 cells developed high parasitemias and died between days 23 and 43 following infection with either *T. cruzi* G-OVA.GPI or wild-type *T. cruzi* (Fig. 3 *A* and *B*). Thus, transfer of OVA-specific Th1 cells conferred resistance

in susceptible BALB/c mice to lethal *T. cruzi* infection. This protection occurred in an antigen-specific manner as mice receiving Th1 cells and infected with *T. cruzi* G-OVA.GPI were protected while Th1 cells recipients infected with wild-type *T. cruzi* were not.

*Histology*

Histopathological analysis of tissues at 30 days post-infection revealed higher tissue parasitism and more severe pathology in skeletal muscles than in the hearts of mice in all groups (data not shown). Skeletal muscle of Th1 cell recipient mice showed mild to moderate inflammation with a predominant lymphocytic infiltration and relatively lower tissue parasitism (Fig. 4 *A*). In contrast, the skeletal muscles of Th2 cell recipient mice were heavily parasitized with severe inflammation consisting predominantly of polymorphonuclear cells and significant tissue destruction and necrosis (Fig. 4 *B*). Mice that received Th1 or Th2 cells and were then infected with wild-type *T. cruzi* showed high tissue parasitism and moderate to severe inflammation in the skeletal muscles (Fig. 4 *C* and *D*). The differential control of tissue parasite load in Th1- and Th2-recipient mice infected with *T. cruzi* GOVA.GPI was confirmed by quantitation of parasite DNA by real-time PCR. Recipients of Th2 cells had significantly higher amounts of *T. cruzi* DNA in the skeletal muscle tissue as compared to Th1 cell recipients following infection with *T. cruzi* GOVA.GPI (Fig. 5). Thus, even though the Th2 cells recipient mice mounted an intense inflammatory response, this response was clearly less effective in controlling parasites in the tissues of these mice. We conclude from these results that antigen-specific Th2 cells were incapable of mediating clearance of infection with *T. cruzi* from the tissues in spite of eliciting a strong inflammatory response.

*OVA-specific Th1 and Th2 cells persist and expand in response to T. cruzi G-OVA.GPI infection in vivo*

The OVA system provided a tool to monitor the distribution and expansion of "parasite-specific" T cells in infected mice. For this purpose, the OVA-TCR specific monoclonal antibody KJ1-26 was used to detect OVA-specific Th1 and Th2 cells in the spleen and lymph nodes of mice infected either with *T. cruzi* G-OVA.GPI or wild-type *T. cruzi*. KJ1-26 positive cells were present in small clusters around the arterioles at day 4 (Fig. 6 *C* and *E*) and day 15 post-infection (data not shown) in spleens of mice receiving Th1 cells and infected with either OVA-expressing or wild-type *T. cruzi*. At 30 days post-infection, there was an increase in the frequency of KJ1-26 positive cells in spleens of mice receiving Th1 cells and infected with *T. cruzi* G-OVA.GPI and these cells were localized in T cell-rich areas around the lymphoid follicles (Fig. 6 *D*). However, KJ1-26 positive cells could not be detected in the spleens of Th1 cell recipients infected with wild-type *T. cruzi* at 30 days post-infection (Fig. 6 *F*). Similar persistence and expansion of OVA-specific cells was observed in the spleens of recipients of Th2 cells challenged with *T. cruzi* G-OVA.GPI (Fig. 7 *A* and *B*) but not in mice receiving Th2 cells and challenged with wild-type *T. cruzi* (Fig. 7 *C* and *D*).

To determine whether or not the transferred Th1 or Th2 cells continued to maintain their respective cytokine production phenotype following transfer, flow cytometric analysis was used to monitor intracellular IFN-γ and IL-4 production in KJ1-$26^+$ cells from spleens (Fig. 8). At 4 days post-infection, the majority of KJ1-$26^+$ Th1 or Th2 cells produced the respective type-specific cytokine IFN-γ or IL-4 following *in vitro* stimulation with OVA peptide. This was also the case at day 15 post-infection.

However, at this time point, an increasing proportion of KJ1-26$^+$ cells from Th1 or Th2 recipients produced IL-4 or IFN-γ, respectively. It is also noteworthy that in the Th2 recipients, a lower percentage of KJ1-26 negative cells produced IFN-γ when compared to Th1 recipients. One interpretation of these data is that the transferred Th1 and Th2 cells influence the cytokine production pattern of cells responding to parasite antigens other than OVA.

To determine if OVA-specific Th1 and Th2 cells migrated to the sites of parasite multiplication in muscle tissue, skeletal muscle from mice at 30 days post-infection were stained for the presence of OVA-specific T cells (Figure 9). Tissues from mice infected with *T. cruzi* G-OVA.GPI (Fig. 9 *A* and *B*) but not those from mice infected with wild-type *T. cruzi* (Fig. 9 *C* and *D*) revealed the presence of OVA-specific Th1 or Th2 cells. These results demonstrate the antigen-driven proliferation of OVA-specific Th1 and Th2 cell in the lymphoid organs, the maintenance of type1 and type 2 phenotype and the homing of these cells to sites of active infection.

*Adoptive transfer of OVA specific Th2 cells abrogates the protective effect of Th1 cells against T. cruzi G-OVA.GPI infection*

The data above demonstrate that Th1 but not Th2 cells help mediate protection in *T. cruzi*-infected mice. The T helper cell response to *T. cruzi* in both susceptible and resistant strains of mice normally exhibits a mixed Th1/Th2 cytokine production profile (33). Thus it was of interest to determine if the adoptive transfer of a combination of Th1 and Th2 cells or of naive DO.11.10 T cells (which could differentiate into Th1 and Th2 cells *in vivo*) could confer protection to *T. cruzi* infection equivalent to that of Th1 cells alone. Adoptive transfer of OVA-specific Th1+Th2 cells (5 x $10^6$ each) or $10^7$ naive

DO.11.10 cells failed to provide protection in mice challenged with $10^5$ BFT of *T. cruzi* G-OVA.GPI (Fig. 10 *A* and *B*). The higher challenge dose used in this experiment as compared to the results shown in Figure 3 ($10^5$ vs. 5 x $10^4$), resulted in higher parasitemia levels and earlier time to death in all groups except the group receiving $10^7$ Th1 cells. It is noteworthy that we have compared transfer of $10^7$ Th1 or Th2 cells with only 5 x $10^6$ Th1+Th2 cells in this experiment. Therefore, we transferred 5 x $10^6$ Th1 or Th2 cells and the same number of Th1+Th2 cells into mice that were infected with $10^5$ BFT of either *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. Mice receiving 5 x $10^6$ Th1 or Th2 cells developed similar levels of parasitemia and mortality as mice receiving $10^7$ Th1 or Th2 cells while mice receiving Th1+Th2 cells again died of infection (data not shown). These results indicate that adoptive transfer of parasite-specific Th1 cells provides significant protection from lethal *T. cruzi* infection in naive mice, but co-transfer of Th2 cells abrogated this protective ability of Th1 cells. The protection provided by Th1 cells was again antigen-specific and antigen-dependent as demonstrated by the fact that mice receiving OVA-specific Th1 cells and challenged with wild-type *T. cruzi* were not protected.

**Discussion**

The requirement for $CD4^+$ T cells as critical components of a protective immune response against *T. cruzi* has been established by studies in mice deficient in genes encoding class II MHC or CD4 molecules and thereby lacking $CD4^+$ T cells (7, 8, 11, 35). Mice deficient in $CD4^+$ T cells become highly susceptible to infection with *T. cruzi*, develop high tissue parasite burden and die early in the acute phase of the infection with negligible inflammation (11, 36). Resistance to infection with other intracellular

pathogens including *Leishmania major* (37-43), *Mycobacterium tuberculosis* (44-46) and *Listeria monocytogenes* (15), has been associated with a strong and polarized Th1 response while a Th2 response has been shown to associate with susceptibility (16, 17). In contrast, a linkage between Th1 responses and resistance has not been so firmly established with respect to *T. cruzi* infection. The results of some studies suggest a requirement for a balanced type 1 and type 2 response to control infection with *T. cruzi* (23, 26, 47), while other studies attribute protection to the production of type 1 but not type 2 cytokines *in vivo* in different mouse and parasite strain combinations (18-20, 24, 48, 49).

To investigate directly the role of primed antigen-specific Th1 and Th2 cells in modulating the host immune response to *T. cruzi*, we developed a system by which Th1 or Th2 cells specific for a parasite-expressed antigen could be generated and tested for their ability to protect naive mice from a lethal infection with *T. cruzi*. We were constrained by the lack of a source for a clonal population of Th1 and Th2 cells specific for a bona fide *T. cruzi*-derived class II MHC epitope, and therefore, we generated *T. cruzi* that expressed the model antigen chicken ovalbumin and used DO.11 TCR transgenic mice (28) as source of OVA-specific $CD4^+$ T cells. $CD4^+$ T cells in these mice express a clonotypic T cell receptor that recognizes OVA peptide aa 323-339 in the context of the class II molecules ($H\text{-}2^d$). Using the DO.11.10 TCR-specific monoclonal Ab KJ1-26 (32), we were also able to follow the transferred cells *in vivo* and monitored persistence and expansion of these cells in response to infection with *T. cruzi* G-OVA.GPI.

Mice receiving OVA-specific Th1 cells controlled the infection with *T. cruzi* G-OVA.GPI and showed reduced pathology in the skeletal muscles. In contrast, recipients of Th2 cells remained highly susceptible to infection with *T. cruzi* G-OVA.GPI and developed much higher blood and tissue parasitism than Th1 recipients. The susceptibility of the Th2 cell recipient mice was not due to the absence of a potent response to the parasite, either systemically or in infected tissues. Th2 recipient mice exhibited expansion of OVA-specific cells in the lymphoid tissues, the homing of these cells to sites of infection in peripheral tissues, and vigorous inflammatory responses with predominantly polymorphonuclear cells at sites of active infection. However, this response appeared to be relatively unproductive and not sufficient to control parasite replication in the tissues. As a consequence, all Th2 cell recipients died in the acute phase of infection, while the majority of Th1 cell recipients controlled the infection and became aparasitimic. We demonstrate that this protection occurred in an antigen-specific manner as only the mice that received OVA-specific Th1 cells and challenged with OVA-expressing *T. cruzi* were protected while the mice challenged with wild-type *T. cruzi* were not.

In naive animals, antigenic stimulation causes an increase in the frequency of clonal populations of T cells specific for their cognate antigens. However, due to the broad repertoire of antigen-specific T cells that are stimulated in a protozoal infection, it is difficult to monitor the change in frequency of individual clones of T cells (50). Hence, the system of adoptive transfer of a clonal population of antigen-specific cells obtained from TCR transgenic mice and Abs specific for the clonotypic TCR are valuable tools to analyze persistence, activation and expansion of clonotypic T cells *in vivo* (51-56). Using

KJ1-26 MAb, we showed persistence and expansion of OVA-specific Th1 and Th2 cells in the spleens, lymph nodes and skeletal muscle of recipient mice.

A number of studies have suggested that a "polyclonal", antigen non-specific expansion of the T and B cell compartments occurs in *T. cruzi* infection in mice (57-60). These conclusions are based on the normal representation of V beta TCR in proliferating $CD4^+$ T cells (60) and on the dramatic expansion of B cells and T cells which does not appear to be specific for *T. cruzi* antigens (57-59). The results of the present study suggest that OVA-specific Th1 and Th2 cells did not undergo polyclonal activation but expanded *in vivo* only in the presence of OVA in an antigen-specific manner. Thus, with respect to primed OVA-specific $CD4^+$ T cells, *T. cruzi* does not appear to induce a true polyclonal immune response.

The adoptive transfer system described herein provides the means to also follow the fate of naive OVA-specific T cells after infection with WT or OVA-expressing *T. cruzi* to (1) further explore the phenomenon of polyclonal activation and (2) to determine if these cells differentiate into Th1 or Th2 cells during infection. Understanding the development of Th1 and Th2 cells and their relatively ability to control *T. cruzi* infection and the development of Chagas disease is crucial for designing appropriate immune intervention strategies for this infection and disease. Using the Th1/Th2 cell transfer system, we demonstrate that initial priming of a Th1 response is required for control of *T. cruzi* infection. This conclusion is supported by the recent results of Hoft et al. demonstrating that immunization of mice under conditions that promote a Th1 response results in protection from challenge infection with *T. cruzi* (47). In the future, the Th1/Th2 cell

transfer system will also allow us to identify ways in which to modulate the course of the immune response to *T. cruzi* so as to achieve a strong type-1 biased cytokine response.

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**Footnotes**

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2. Abbreviations: OVA, ovalbumin, BFT, blood form trypomastigotes, GPI, glycosylphosphatidylinositol

**References**

1. 1991. Control of Chagas disease. Report of a WHO Expert Committee. *World Health Organ.Tech.Rep.Ser. 811:1*.

2. Rodrigues, M. M., M. Ribeirao, V. Pereira-Chioccola, L. Renia, and F. Costa. 1999. Predominance of CD4 Th1 and CD8 Tc1 cells revealed by characterization of the cellular immune response generated by immunization with a DNA vaccine containing a Trypanosoma cruzi gene. *Infect Immun 67:3855*.

3. Wizel, B., N. Garg, and R. L. Tarleton. 1998. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal Trypanosoma cruzi infection. *Infect Immun 66:5073*.

4. Costa, F., G. Franchin, V. L. Pereira-Chioccola, M. Ribeirao, S. Schenkman, and M. M. Rodrigues. 1998. Immunization with a plasmid DNA containing the gene of trans-sialidase reduces Trypanosoma cruzi infection in mice. *Vaccine 16:768.*

5. Tarleton, R. L. 1991. Regulation of immunity in Trypanosoma cruzi infection. *Exp.Parasitol. 73:106.*

6. Kumar, S., and R. L. Tarleton. 1998. The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite Immunol. 20:207*.

7. Rottenberg, M. E., D. A. Rodriguez, and A. Orn. 1992. Control of Trypanosoma cruzi infection in mice deprived of T- cell help. *Scand.J.Immunol. 36:261*.

8. Rottenberg, M. E., A. Riarte, L. Sporrong, J. Altcheh, P. Petray, A. M. Ruiz, H. Wigzell, and A. Orn. 1995. Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol.Lett. 45:53*.

9. Russo, M., P. Minoprio, A. Coutinho, and M. Hontebeyrie-Joskowicz. 1988. Depletion of L3T4+ T lymphocytes during acute Trypanosoma cruzi infection abolish macrophage and B lymphocyte activation but not tissue inflammatory reaction. *Mem.Inst.Oswaldo.Cruz. 83 Suppl 1:527.*

10. Tarleton, R. L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine- induced immunity in mice infected with Trypanosoma cruzi. *J.Immunol. 144:717.*

11. Tarleton, R. L., M. J. Grusby, M. Postan, and L. H. Glimcher. 1996. Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int.Immunol. 8:13.*

12. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection [see comments]. *Nature 356:338.*

13. Locksley, R. M., F. P. Heinzel, M. D. Sadick, B. J. Holaday, and K. D. Gardner, Jr. 1987. Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. *Ann Inst Pasteur Immunol 138:744.*

14. Zhu, X., N. Venkataprasad, H. S. Thangaraj, M. Hill, M. Singh, J. Ivanyi, and H. M. Vordermeier. 1997. Functions and specificity of T cells following nucleic acid vaccination of mice against Mycobacterium tuberculosis infection. *J.Immunol. 158:5921.*

15. Geginat, G., M. Lalic, M. Kretschmar, W. Goebel, H. Hof, D. Palm, and A. Bubert. 1998. Th1 cells specific for a secreted protein of Listeria monocytogenes are protective in vivo. *J.Immunol. 160:6046.*

16. Romagnani, S. 1996. Understanding the role of Th1/Th2 cells in infection. *Trends.Microbiol. 4:470*.

17. Sher, A., and R. L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu.Rev.Immunol. 10:385-409:385*.

18. Nickell, S. P., M. Keane, and M. So. 1993. Further characterization of protective Trypanosoma cruzi- specific CD4+ T-cell clones: T helper type 1-like phenotype and reactivity with shed trypomastigote antigens. *Infect.Immun. 61:3250*.

19. Barbosa de Oliveira, L. C., M. A. Curotto de Lafaille, G. M. Collet de Araujo Lima, and I. de Almeida Abrahamsohn. 1996. Antigen-specific Il-4- and IL-10-secreting CD4+ lymphocytes increase in vivo susceptibility to Trypanosoma cruzi infection. *Cell Immunol. 170:41*.

20. Reed, S. G. 1988. In vivo administration of recombinant IFN-gamma induces macrophage activation, and prevents acute disease, immune suppression, and death in experimental Trypanosoma cruzi infections. *J.Immunol. 140:4342*.

21. Nabors, G. S., and R. L. Tarleton. 1991. Differential control of IFN-gamma and IL-2 production during Trypanosoma cruzi infection. *J.Immunol. 146:3591*.

22. Minoprio, P., C. M. el, E. Murphy, M. Hontebeyrie-Joskowicz, R. Coffman, A. Coutinho, and A. O'Garra. 1993. Xid-associated resistance to experimental Chagas' disease is IFN-gamma dependent. *J.Immunol. 151:4200*.

23. Petray, P. B., M. E. Rottenberg, G. Bertot, R. S. Corral, A. Diaz, A. Orn, and S. Grinstein. 1993. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of Trypanosoma cruzi. *Immunol.Lett. 35:77.*

24. Torrico, F., H. Heremans, M. T. Rivera, E. Van Marck, A. Billiau, and Y. Carlier. 1991. Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. *J.Immunol. 146:3626.*

25. Silva, J. S., J. C. Aliberti, G. A. Martins, M. A. Souza, J. T. Souto, and M. A. Padua. 1998. The role of IL-12 in experimental Trypanosoma cruzi infection. *Braz.J.Med.Biol.Res. 31:111*.

26. Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed. 1992. Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection. *J.Exp.Med. 175:169.*

27. Reed, S. G., C. E. Brownell, D. M. Russo, J. S. Silva, K. H. Grabstein, and P. J. Morrissey. 1994. IL-10 mediates susceptibility to Trypanosoma cruzi infection. *J.Immunol. 153:3135*.

28. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science 250:1720.*

29. Santos, M. A., N. Garg, and R. L. Tarleton. 1997. The identification and molecular characterization of Trypanosoma cruzi amastigote surface protein-1, a member of the trans- sialidase gene super-family. *Mol.Biochem.Parasitol. 86:1*.

30. Garg, N., M. P. Nunes, and R. L. Tarleton. 1997. Delivery by Trypanosoma cruzi of proteins into the MHC class I antigen processing and presentation pathway. *J.Immunol. 158:3293*.

31. Mosmann, T. R., L. Li, H. Hengartner, D. Kagi, W. Fu, and S. Sad. 1997. Differentiation and functions of T cell subsets. *Ciba.Found.Symp. 204:148-54; discussion 154-8:148*.

32. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J.Exp.Med. 157:1149*.

33. Zhang, L., and R. L. Tarleton. 1996. Characterization of cytokine production in murine Trypanosoma cruzi infection by in situ immunocytochemistry: lack of association between susceptibility and type 2 cytokine production. *Eur.J.Immunol. 26:102*.

34. Garg, N., M. Postan, K. Mensa-Wilmot, and R. L. Tarleton. 1997. Glycosylphosphatidylinositols are required for the development of Trypanosoma cruzi amastigotes. *Infect.Immun. 65:4055*.

35. Russo, M., N. Starobinas, P. Minoprio, A. Coutinho, and M. Hontebeyrie-Joskowicz. 1988. Parasitic load increases and myocardial inflammation decreases in Trypanosoma cruzi-infected mice after inactivation of helper T cells. *Ann.Inst.Pasteur Immunol. 139:225*.

36. Tarleton, R. L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect.Immun. 62:1820*.

37. Chatelain, R., S. Mauze, K. Varkila, and R. L. Coffman. 1999. Leishmania major infection in interleukin-4 and interferon-gamma depleted mice. *Parasite Immunol 21:423*.

38. Himmelrich, H., C. Parra-Lopez, F. Tacchini-Cottier, J. A. Louis, and P. Launois. 1998. The IL-4 rapidly produced in BALB/c mice after infection with Leishmania major down-regulates IL-12 receptor beta 2-chain expression on CD4+ T cells resulting in a state of unresponsiveness to IL-12. *J Immunol 161:6156*.

39. Kopf, M., F. Brombacher, G. Kohler, G. Kienzle, K. H. Widmann, K. Lefrang, C. Humborg, B. Ledermann, and W. Solbach. 1996. IL-4-deficient Balb/c mice resist infection with Leishmania major. *J Exp Med 184:1127*.

40. Louis, J. A., F. Conceicao-Silva, H. Himmelrich, F. Tacchini-Cottier, and P. Launois. 1998. Anti-leishmania effector functions of CD4+ Th1 cells and early events instructing Th2 cell development and susceptibility to Leishmania major in BALB/c mice. *Adv Exp Med Biol 452:53*.

41. Louis, J., H. Himmelrich, C. Parra-Lopez, F. Tacchini-Cottier, and P. Launois. 1998. Regulation of protective immunity against Leishmania major in mice. *Curr Opin Immunol 10:459*.

42. Mattner, F., K. Di Padova, and G. Alber. 1997. Interleukin-12 is indispensable for protective immunity against Leishmania major. *Infect Immun 65:4378*.

43. Sjolander, A., T. M. Baldwin, J. M. Curtis, and E. Handman. 1998. Induction of a Th1 immune response and simultaneous lack of activation of a Th2 response are required for generation of immunity to leishmaniasis. *J Immunol 160:3949*.

44. Castro, A. G., R. A. Silva, and R. Appelberg. 1995. Endogenously produced IL-12 is required for the induction of protective T cells during Mycobacterium avium infections in mice. *J Immunol 155:2013*.

45. Kobayashi, K., N. Nakata, M. Kai, T. Kasama, Y. Hanyuda, and Y. Hatano. 1997. Decreased expression of cytokines that induce type 1 helper T cell/interferon-gamma responses in genetically susceptible mice infected with Mycobacterium avium. *Clin Immunol Immunopathol 85:112*.

46. Kobayashi, K., M. Kai, M. Gidoh, N. Nakata, M. Endoh, R. P. Singh, T. Kasama, and H. Saito. 1998. The possible role of interleukin (IL)-12 and interferon-gamma-inducing factor/IL-18 in protection against experimental Mycobacterium leprae infection in mice. *Clin Immunol Immunopathol 88:226*.

47. Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F. G. Araujo. 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J.Immunol. 158:3311*.

48. Hoft, D. F., A. R. Schnapp, C. S. Eickhoff, and S. T. Roodman. 2000. Involvement of CD4(+) Th1 cells in systemic immunity protective against primary and secondary challenges with Trypanosoma cruzi. *Infect Immun 68:197*.

49. Humphrey, J. S., T. S. McCormick, and E. C. Rowland. 1997. Parasite antigen-induced IFN-gamma and IL-4 production by cells from pathopermissive and pathoresistant strains of mice infected with Trypanosoma cruzi. *J.Parasitol. 83:533*.

50. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen- specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J.Immunol. 155:1032*.

51. Healey, D., P. Ozegbe, S. Arden, P. Chandler, J. Hutton, and A. Cooke. 1995. In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. *J.Clin.Invest. 95:2979*.

52. Ohta, A., N. Sato, T. Yahata, Y. Ohmi, K. Santa, T. Sato, H. Tashiro, S. Habu, and T. Nishimura. 1997. Manipulation of Th1/Th2 balance in vivo by adoptive transfer of antigen- specific Th1 or Th2 cells. *J.Immunol.Methods 209:85*.

53. Powrie, F., R. L. Coffman, and R. Correa-Oliveira. 1994. Transfer of CD4+ T cells to C.B-17 SCID mice: a model to study Th1 and Th2 cell differentiation and regulation in vivo. *Res.Immunol. 145:347*.

54. Rizzo, L. V., R. H. DeKruyff, D. T. Umetsu, and R. R. Caspi. 1995. Regulation of the interaction between Th1 and Th2 T cell clones to provide help for antibody production in vivo. *Eur.J.Immunol. 25:708*.

55. Rogers, W. O., C. T. Weaver, L. A. Kraus, J. Li, L. Li, and R. P. Bucy. 1997. Visualization of antigen-specific T cell activation and cytokine expression in vivo. *J.Immunol. 158:649*.

56. Swain, S. L. 1994. Generation and in vivo persistence of polarized Th1 and Th2 memory cells. *Immunity. 1:543*.

57. Minoprio, P., A. Bandeira, P. Pereira, T. Mota Santos, and A. Coutinho. 1989. Preferential expansion of Ly-1 B and CD4- CD8- T cells in the polyclonal lymphocyte responses to murine T. cruzi infection. *Int Immunol 1:176.*

58. Montes, C. L., E. Zuniga, P. Minoprio, E. Vottero-Cima, and A. Gruppi. 1999. A Trypanosoma cruzi alkaline antigen induces polyclonal B-cell activation of normal murine spleen cells by T-cell-independent, BCR- directed stimulation. *Scand J Immunol 50:159.*

59. Pestel, J., J. P. Defoort, H. Gras-Masse, D. Afchain, A. Capron, A. Tartar, and A. Ouaissi. 1992. Polyclonal cell activity of a repeat peptide derived from the sequence of an 85-kilodalton surface protein of Trypanosoma cruzi trypomastigotes. *Infect Immun 60:715*.

60. Leite-de-Moraes, M. C., A. Coutinho, M. Hontebeyrie-Joskowicz, P. Minoprio, H. Eisen, and A. Bandeira. 1994. Skewed V beta TCR repertoire of CD8+ T cells in murine Trypanosoma cruzi infection. *Int.Immunol. 6:387.*

# CHAPTER 4

# IFN-γ PRODUCTION IS REQUIRED FOR PROTECTION MEDIATED BY TC1 CELLS IN *TRYPANOSOMA CRUZI* INFECTION[1]

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**Abstract**

In mammalian hosts, *Trypanosoma cruzi* is the target of multiple immune effector mechanisms, among these $CD8^+$ T cells, which are required for control of the infection. In order to investigate the relative contribution of Tc1 and Tc2 subsets of $CD8^+$ T cells to control of *T. cruzi* infection in mice, we utilized a cell transfer/infection system in which ($OVA^2$)-specific Tc1 and Tc2 cells were assayed for their ability to protect mice during infection with OVA-expressing *T. cruzi*. Mice receiving OVA-specific Tc1 or Tc2 cells developed lower parasitemia and tissue parasitism and survived a normally lethal infection with OVA-expressing *T. cruzi* GOVA.GPI. In contrast, mice receiving no cells or mice receiving Tc1 and Tc2 cells but infected with wild-type *T. cruzi* developed significantly higher blood and tissue parasite burdens and succumbed to the infection. Immunohistochemical analysis of lymphoid tissues revealed the persistence of OVA-specific Tc1 and Tc2 cells in mice infected with *T. cruzi* GOVA.GPI but not in mice infected with wild-type *T. cruzi*. Analysis of cytokines produced by the transferred cells revealed that Tc1 cells continued to make primarily IFN-γ but the Tc2 population failed to retain a type 2 cytokine production pattern and instead exhibited predominantly a type 1 pattern of cytokine production. Tc1 or Tc2 populations derived from OT-1 mice backcrossed to IFN-γ knockout mice (and thus incapable of producing IFN-γ), were unable to provide protection to infection with OVA-expressing *T. cruzi*. We conclude that Tc1 cell-mediated protection in mice against lethal *T. cruzi* GOVA.GPI was dependent on the production of IFN-γ by these cells.

**Introduction**

*Trypanosoma cruzi* is the cause of potentially fatal Chagas disease in humans and is a major health problem throughout Latin America. In the mammalian host, *T. cruzi* cycles between extracellular non-dividing trypomastigotes that invade a variety of cell types and cytoplasmically localized, replicative amastigotes. Both extracellular trypomastigotes and intracellular amastigotes are targets of multiple immune responses (1, 2). $CD8^+$ T cells are critical to control of *T. cruzi* as demonstrated by the absolute susceptibility of mice deficient in $CD8^+$ T cells (3, 4), mice depleted of $CD8^+$ T cells (5, 6) and mice lacking β2m (7).

Recently, it has been documented that upon antigenic stimulation and in the appropriate cytokine environment, $CD8^+$ T cells can differentiate into Tc1 or Tc2 cells with a cytokine production pattern similar to Th1 and Th2 subsets of $CD4^+$ T cells (8-13). The Tc1 $CD8^+$ T cells secrete IFN-γ and IL-2 and Tc2 cells secrete predominantly IL-4, IL-5 and IL-13 and little IFN-γ. Both Tc1 and Tc2 subsets exhibit cytolytic activity.

While there is considerable evidence for the natural development of Tc1-type cells during infections of various types (14-17), the documentation of Tc2 cells in infection is more limited. Nevertheless, evidence for the existence of $CD8^+$ T cells secreting type 2 cytokines *in vivo* comes from infections like HIV (18, 19). IL-5 producing $CD8^+$ T cells have been shown to be associated with airway eosinophilia in individuals infected with lymphocytic choriomeningitis virus (20).

In *T. cruzi* infected mice (21-23) and in individuals sero-positive for *T. cruzi* (24), $CD8^+$ T cells specific for *T. cruzi*-derived antigens have been detected. In addition, transfer of *T. cruzi*-specific $CD8^+$ T cells capable of cytolytic activity and of secreting

IFN-γ has also been shown to provide significant protection in mice from lethal infection with the parasite (22). These observations suggest a possible role for IFN-γ producing CTLs in controlling *T. cruzi* infection *in vivo* but do not discriminate between the contribution of cytolysis and IFN-γ production in this protection.

*T. cruzi* infection in mice is known to induce the differentiation of both Th1 and Th2 $CD4^+$ T cell populations. However, whether or not a similar expansion of $CD8^+$ T cells also occurs and what the relationship of such differentiation is to control of the infection is not known.

To investigate the relative contribution of Tc1 and Tc2 subsets of CD $8^+$ T cells to the control of *T. cruzi* infection in mice, we have developed a cell transfer system which uses *in vitro*-generated OVA-specific Tc1 or Tc2 cells and infection of mice with *T. cruzi* expressing chicken ovalbumin. Transfer of OVA-specific Tc1 or Tc2 cells protected mice from lethal infection with OVA-expressing *T. cruzi* but, in both cases, this protection was dependent on the ability of the Tc population to produce IFN-γ. These results document a critical role for the production of IFN-γ by $CD8^+$ T cells in controlling *T. cruzi* infection in mice.

**Materials and Methods**

*Mice and Parasites*

OT-1 TCR transgenic mice expressing class I MHC ($H\text{-}2^b$)-restricted Vα2 TCR specific for the SIINFEKL peptide from chicken ovalbumin produced by W. Heath and F. Carbone (25) and obtained from Dr. Michael Bevan (University of Washington Seattle, WA) were bred in our animal facility under specific pathogen-free conditions. Wild-type C57BL/6 mice and IFN-$\gamma^{-/-}$ mice (C57BL/6-$Ifng^{tm1Ts}$), 6-10 weeks of age, were

purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous IFN-γ knockout mice expressing OVA-specific TCR transgene were generating by backcrossing OT-1 mice with IFN-γ gene knockout mice. Blood-form trypomastigotes (BFT) of Brazil strain of OVA-expressing *T. cruzi* (*T.cruzi* GOVA.GPI)[1] and wild-type *T. cruzi* were obtained by infection of C3H(He)/SnJ mice with tissue culture-derived trypomastigotes.

*Generation of OVA-specific Tc1 and Tc2 cells*

Splenocytes from OT-1 mice were depleted of RBCs by hypotonic lysis and 5 x $10^6$ cells were cultured in 24 wells plates in 2 ml complete RPMI (Mediatech, Herndon, VA) containing 10% FBS (Hyclone, Logan, UT) plus 3μM OVA peptide (synthesized at Molecular Genetics and Instrumentation Facility of The University of Georgia). To generate Tc1 cells, IL-12 (2.5 ηg/ml; Genetics Institute, Cambridge, MA), IL-2 (20 U/ml; Cetus Corporation, Emeryville, CA) and anti-IL-4 MAb 11B11 (25 μg/ml) was added to cultures. Tc2 cells were generated by addition of IL-2 (20 U/ml), IL-4 (500 U/ml; DNAX, Palo Alto, CA) and anti-IFN-γ MAb R4-6AB (75 μg/ml) to cultures. Fresh media, cytokines, antibodies and peptides were added at day 4 of culture and the Tc1 and Tc2 lines were harvested following another 3 days of culture. For measurement of cytokines produced by the effector cells, Tc1 and Tc2 cells (2.5 x $10^5$) were re-stimulated in 2 ml cultures with 4.5 x $10^6$ RBC-depleted irradiated (2600 rads) H-$2^b$-expressing splenocytes and OVA peptide. Supernatants from these cultures were harvested 48 hrs later and cytokine levels were measured by ELISA as previously described for IFN-γ ( ) or using a commercial kit (Pharmingen, San Diego, CA) for IL-4.

*Assay for cytolytic activity of Tc1 and Tc2 cells*

Cytolytic activity of Tc1 and Tc2 cells was determined by a standard $^{51}Cr$-release assay described previously (22). Briefly, RMA-S cells ($10^6$/2ml/well of 24-well plate) were incubated with 5 uM OVA peptide and 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Life Sciences Inc., Arlington Heights, IL) overnight in complete RPMI with 10% FBS at $37^0$C in 5% $CO_2$. After washing three times with the media, various numbers of effector cells were incubated with 5 x $10^3$ target cells in a total 200 μl /well volume in 96 wells round-bottom plates (Corning, Corning, NY) at $37^0$C. Supernatants were harvested 5 hours later using a Skatron Multi Harvesting System (Skatron Instruments, Inc., Sterling, VA). $Cr^{51}$ release was determined on a Cobra II Auto gamma counter (Packard Instrument Co., Downers Grove, IL). The percent specific release of $^{51}Cr$ was determined using the formula: [(experimental counts per minute – spontaneous counts per minute)/(maximum counts per minute – spontaneous counts per minute)] X 100. To determine maximum and spontaneous release, $^{51}Cr$-labeled targets with no effector cells were incubated in the presence or the absence of 5% SDS, respectively.

*Flow Cytometric Analysis*

Flow cytometric analysis of Tc1 and Tc2 cells from cultures and splenocytes from Tc1 and Tc2 cell recipient mice was done as follows: Single cell suspension of cells were washed in staining buffer consisting of 1% FBS and 0.1% sodium azide in Phosphate Buffered Saline (PBS, pH 7.2). APC-labeled OVA tetramers consisting of biotin conjugated H-$2^b$/β2m molecules carrying OVA (SIINFEKL) peptide (synthesized at The Tetramer Core Facility, Emory University, GA) and PE anti-CD8 MAb (Pharmingen) were used to identify the OVA-specific $CD8^+$ T cells. Detection of intracellular IFN-γ and IL-4 was done using Cytoperm/Cytofix (with GolgiPlug) kit (Pharmingen).

Splenocytes (5 x $10^6$/2ml/well of 24-wells plate) were stimulated with 5 μM OVA peptide for 12-24 hours. GolgiPlug was added during the last 8 hours of stimulation. After three washes, $10^6$ cells were incubated with 1 μg Fc Block (anti-CD16/CD32; Pharmingen) in 100 μl of staining buffer for 15 minutes at $4^0$C. The cells were washed and then stained with 1:2000 dilution of APC OVA tetramers for 30 minutes at $4^0$C. The cells were washed twice again, permeabilized and fixed with Cytoperm/Cytofix solution for 10 minutes at $4^0$C. After two washes with 1 X Perm/Wash solution, cells were stained with PE-labeled rat IgG1 (isotype control), PE anti-IFN-γ or PE anti-IL-4 MAbs for 30 minutes at $4^0$C. The cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA) with CellQuest 3.0 software.

*Staining of Tc1 and Tc2 cells with CFSE*

Prior to adoptive transfer into mice, Tc1 and Tc2 cells were purified over lymphocyte separation medium (LSM; ICN Biochemicals, Aurora, OH) before staining with CFSE as previously described with minor modifications (26). Cells were suspended at $10^7$/ml in PBS and labeled with 5 μM CFSE dye (5-(and –6)-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR) at $37^0$C for 10 minutes by intermittent shaking. The excess dye was quenched by addition of an equal volume of 10% FBS and incubation at $37^0$C for 10 minutes. CFSE-labeled cells were washed three times in complete RPMI with 10% FBS and re-suspended at $10^7$ cells in 0.5 ml of DMEM (Life Technologies, Grand Island, NY).

*Adoptive transfer of OVA-specific Tc1 and Tc2 cells and infection with T. cruzi*

$10^7$ OVA-specific Tc1 and Tc2 cells were transferred in each C57BL/6 naïve mouse by injection into the tail vein. Control animals were injected with DMEM alone. 12 hours

after injection of cells, the animals were infected with $10^4$ or $10^5$ BFT of *T. cruzi* by i.p. injection. A total of 8 mice were included in each group. Parasitemia in infected mice was monitored at weekly intervals by examination of tail blood and counting parasites in a hemacytometer. Three mice from each group were randomly selected (one mouse each) at different time points post-infection for FACS analysis, histology and DNA isolation for real-time PCR. Mortality was recorded daily.

*Immunohistochemistry and histology*

To detect adoptively transferred Tc1 and Tc2 cells *in situ* by immunohistochemistry, spleens, lymph nodes and skeletal muscle tissues from recipient mice were frozen in liquid nitrogen. 10 μm thick tissue sections were cut and stained according to previously described protocol with minor modifications (27). Briefly, tissue sections fixed in acetone and quenched with PBS containing 0.3% $H_2O_2$ and 0.1% sodium azide were incubated with biotinylated anti-fluorescein Ab (Vector) in PBS at $4^0$C for 12 hours. Tyramide Signal Amplification kit (TSA-Indirect, NEN Biological Products, Boston, MA) was used to enhance color development. Color was developed using diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO). Cardiac and skeletal muscle tissues were collected at 30 days post-infection in PBS and fixed in 10% buffered formalin. Sections (5 μm thick) from paraffin-embedded tissues were stained with hematoxylin and eosin for standard histopathological analysis.

*Quantitation of T. cruzi DNA in the skeletal muscle by real-time PCR*

Skeletal muscle tissue from infected mice was treated with proteinase K (0.3 mg/ml in proteinase K buffer). DNA was isolated by extraction with phenol:chloroform:isoamyl alcohol (Sigma, St. Louis, MO) and by precipitation with ethanol as described previously

(28). The genomic DNA was reconstituted at 25 μg/ml in PCR-grade $H_2O$. *T. cruzi* DNA in the tissues was quantitated by a protocol described elsewhere[2] using a LightCycler™ (Roche Diagnostic Corporation, Indianapolis, IN). Briefly, *T. cruzi* DNA was amplified using *T. cruzi* minicircle specific primers S36 (5' GGGT TCGATTGGGGTTGGT-GT-3') and S35 (5' AAATAATGTACGGGKGAGATGC ATGA-3') in a 20 μl PCR reaction consisting of 2 μl DNA Master Sybr Green 1 (Roche), 3mM $MgCl_2$, 0.16 μl of TaqStart Antibody (0.22μg/ml stock; Sigma), 2 μl of template DNA and PCR-grade $H_2O$. Roche LightCycler™ capillaries loaded with PCR reaction mixes were capped, centrifuged and placed into the LightCycler™. Data acquisition and analysis was performed using LightCycler™ Version 3.0 software. Standards of serially diluted *T. cruzi* DNA mixed with skeletal muscle DNA were used for quantification of samples. Standard curves generated were then used to determine parasite equivalents per 50 ηg of tissue DNA.

**Results**

*Generation of OVA-specific Tc1 and Tc2 cells*

$CD8^+$ T cells from OT-1 transgenic mice were stimulated *in vitro* to generate Tc1 and Tc2 cells. After 6 days of stimulation, more than 95% of the Tc1 and Tc2 population were CD8 positive and expressed OVA-specific TCR as determined by staining with OVA tetramers (Fig. 1 *A*). Flow cytometric analysis to measure intracellular IFN-γ and IL-4 showed that cells in the Tc1 pool secreted IFN-γ but not IL-4. In contrast cells in the Tc2 pool secreted IL-4 but a small proportion of these cells made IFN-γ (Fig. 1 *B*). To measure the total levels of IFN-γ and IL-4 production in bulk cultures, Tc1 and Tc2 cells were incubated with OVA peptide-pulsed syngeneic APCs and supernatants were

**Figure 1.** Phenotypic and functional analysis of OVA-specific Tc1 and Tc2 cells. OVA-specific Tc1 and Tc2 cells were generated as described in the Materials and Methods and analyzed after 6 days of stimulation. *A*) Expression of OVA-specific TCR and *B*) intracellular IFN-γ and IL-4 by Tc1 and Tc2 cells. Tc1 and Tc2 cells incubated with irradiated syngeneic splenocytes plus OVA peptide secreted IFN-γ and IL-4, respectively. *D*) Tc1 and Tc2 cells were incubation with $Cr^{51}$ labeled, OVA peptide pulsed RMA-s cells to determine their cytolytic activity. As a negative control, RMA-s cells were pulsed with control peptide 77.2 (22). Radioactivity in the media was measured to calculate percent specific lysis.

Figure 1.

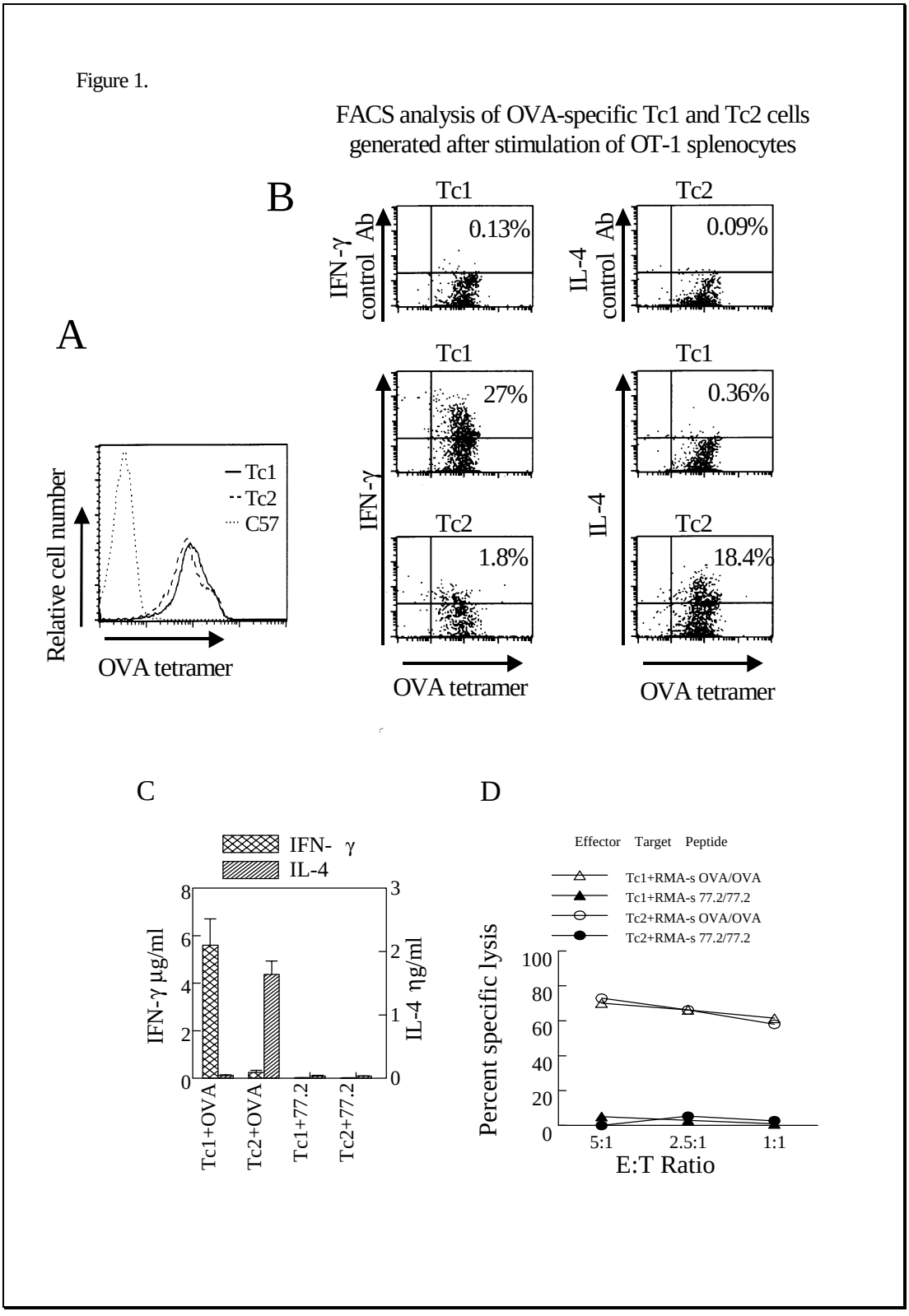


Figure 1.
FACS analysis of OVA-specific Tc1 and Tc2 cells generated after stimulation of OT-1 splenocytes
A
Relative cell number
Tc1
Tc2
C57
OVA tetramer
B
Tc1
Tc2
IFN-γ control Ab
IL-4 control Ab
0.13%
0.09%
27%
0.36%
1.8%
18.4%
IFN-γ
IL-4
OVA tetramer
C
IFN- γ
IL-4
IFN-γ μg/ml
IL-4 ηg/ml
Tc1+OVA
Tc2+OVA
Tc1+77.2
Tc2+77.2
D
Effector Target Peptide
Tc1+RMA-s OVA/OVA
Tc1+RMA-s 77.2/77.2
Tc2+RMA-s OVA/OVA
Tc2+RMA-s 77.2/77.2
Percent specific lysis
E:T Ratio
5:1
2.5:1
1:1

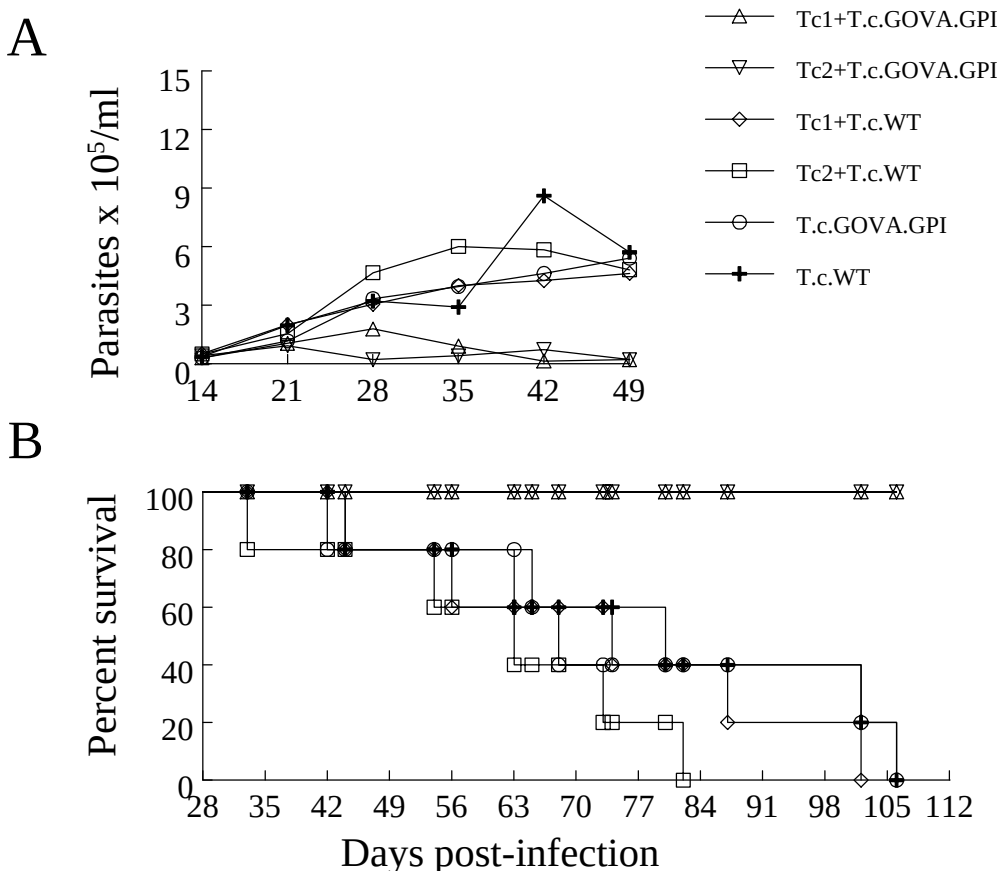



**Figure 2.** Parasitemia and survival in mice receiving OVA-specific Tc1 or Tc2 cells and challenged with 5 x $10^4$ BFT of *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. 5 mice in each group were infected with BFT of *T. cruzi*. Parasites in the blood were counted weekly (*A*) and mortality recorded daily (*B*).

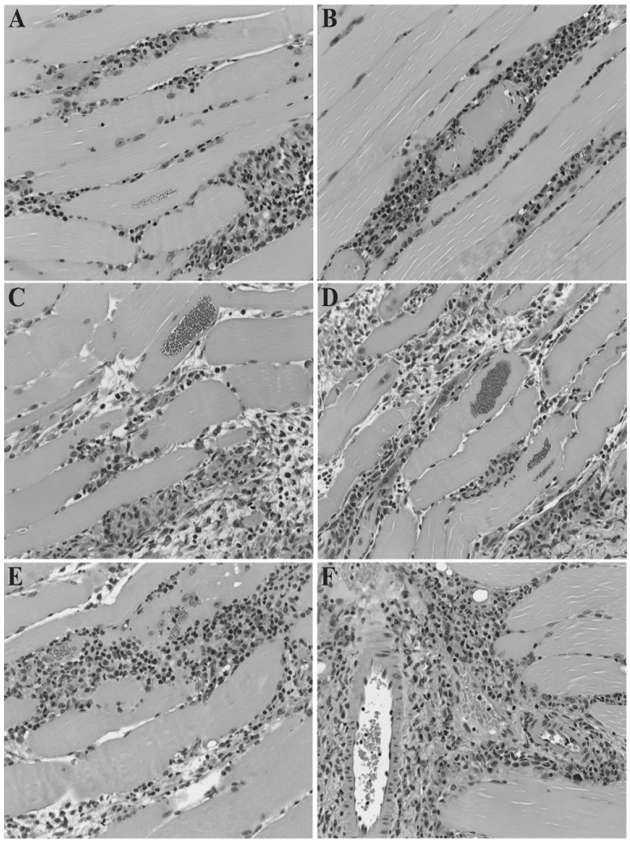



**Figure 3.** Histology of skeletal muscle tissue from mice receiving Tc1 or Tc2 cells. Hematoxylin and eosin stained skeletal muscle tissues 30 days post-infection from mice receiving Tc1 (*A*) or Tc2 (*B*) cells and infected with *T. cruzi* GOVA.GPI. Tissues from Tc1 (*C*) or Tc2 (*D*) cell recipient mice infected with wild-type *T. cruzi*. Tissues from mice infected with either *T. cruzi* GOVA.GPI (*E*) or wild-type *T. cruzi* (*F*).

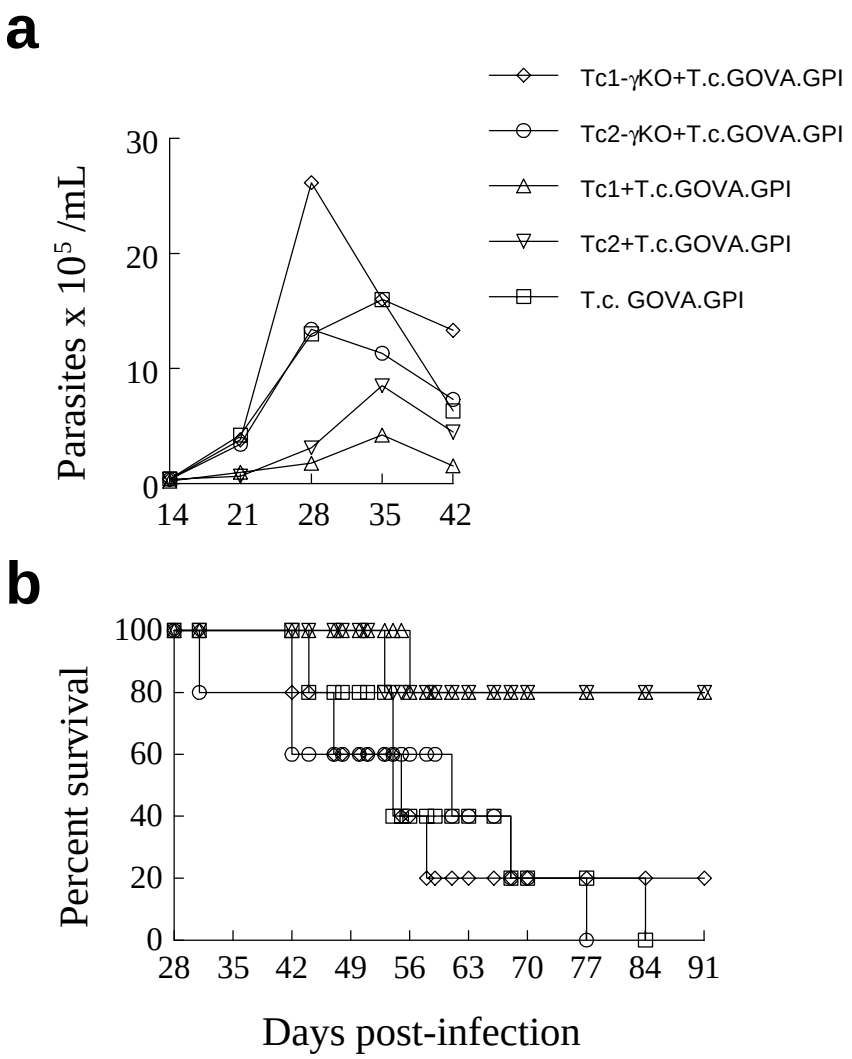



**Figure 4.** Infection of Tc1 and Tc2 cell recipient mice with a higher dose of *T. cruzi* GOVA.GPI. Five Tc1 or Tc2 cell recipient mice in each group were infected with 1 x $10^5$ BFT of *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. Parasitemia was observed daily (*A*) and mortality recorded weekly (*B*).

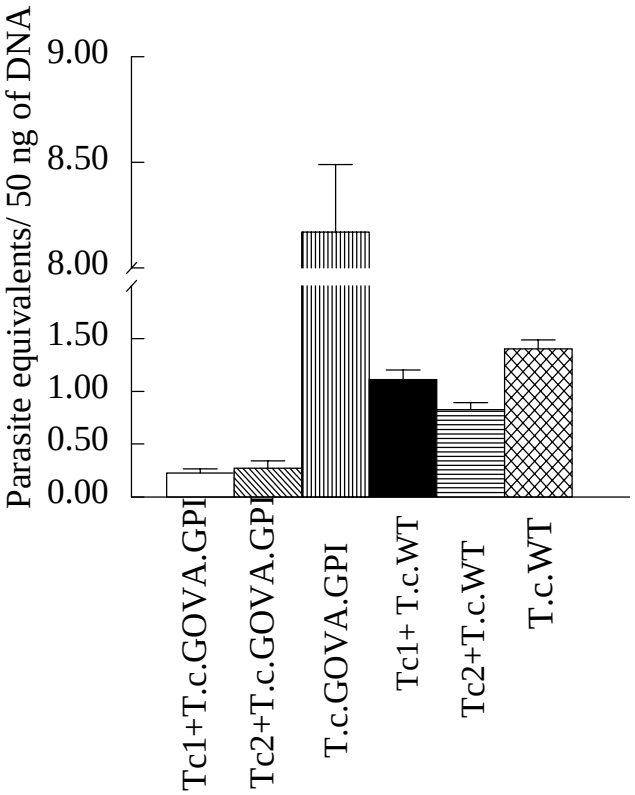
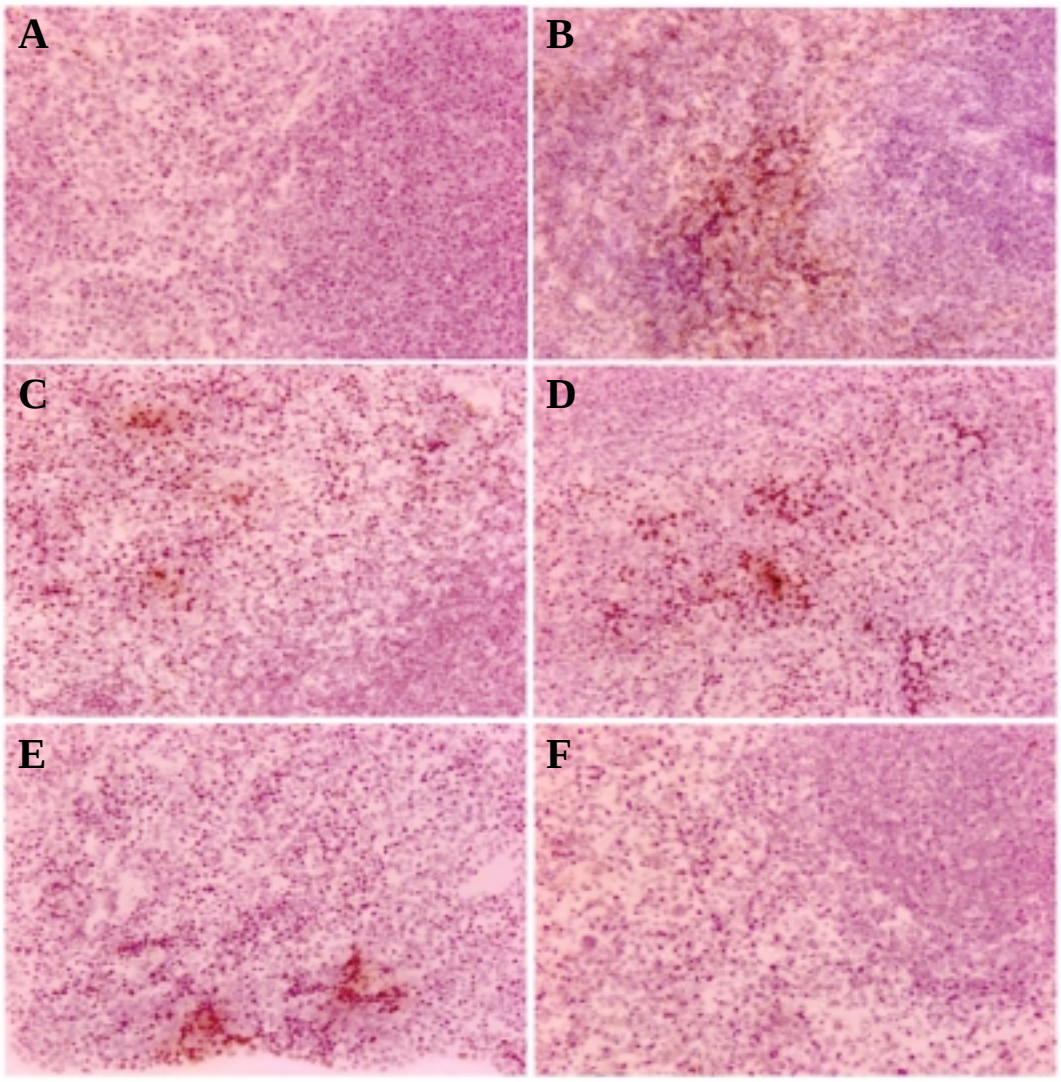



**Figure 5.** Real time PCR analysis of *T. cruzi* DNA in the skeletal muscle of mice in the acute phase of infection. *T. cruzi* DNA in the skeletal muscle of mice receiving Tc1 or Tc2 cells and infected with 1 x $10^5$ BFT of *T. cruzi* GOVA.GPI or wild-type *T. cruzi* was quantitated (mean +/- S.E.) by real time PCR.

**Figure 6.** OVA-specific Tc1 cells persist *in vivo* in mice infected with *T. cruzi* GOVA.GPI.

Spleens from mice at 4 and 30 days post-infection were stained with anti-fluorescein Ab to detect CFSE labeled Tc1 cells. Spleen from uninfected mouse not injected with (*A*) or injected with 50 μM CFSE in PBS (*B*) and stained with anti-fluorescein antibody served as negative and positive controls, respectively. CFSE positive cells were present in the spleens of mice infected with *T. cruzi* GOVA.GPI (*C*) or wild-type *T. cruzi* (*E*) at 4 days post-infection. However at 30 days post-infection, CFSE positive cells could only be detected in the spleens of mice infected with *T. cruzi* GOVA.GPI (*D*) and not in the spleens of mice infected with wild-type *T. cruzi* (*F*).

Figure 6.



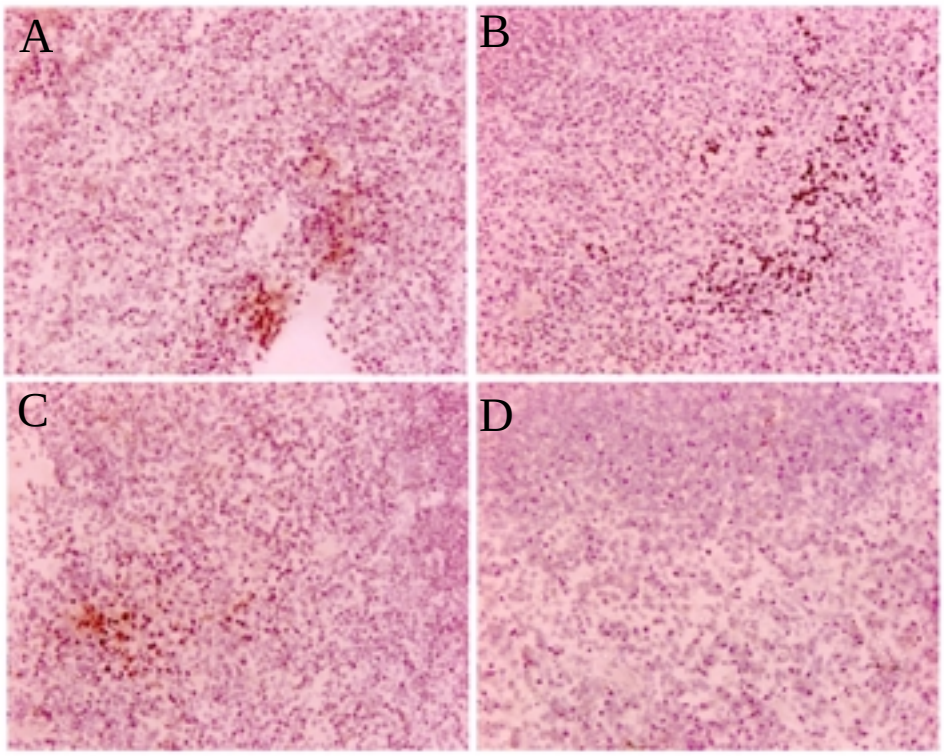



**Figure 7.** OVA-specific Tc2 cells only persist in the spleens of mice infected with *T. cruzi* GOVA.GPI.

Similar to figure 5, CFSE positive cells could be detected in the spleens of mice infected with either *T. cruzi* GOVA.GPI (*A*) or wild-type *T. cruzi* (*C*) at 4 days post-infection. But, at 30 days post-infection, CFSE positive cells were apparent only in the spleens of mice infected with *T. cruzi* GOVA.GPI (*B*) and not in the spleens of mice infected with wild-type *T. cruzi* (*D*).

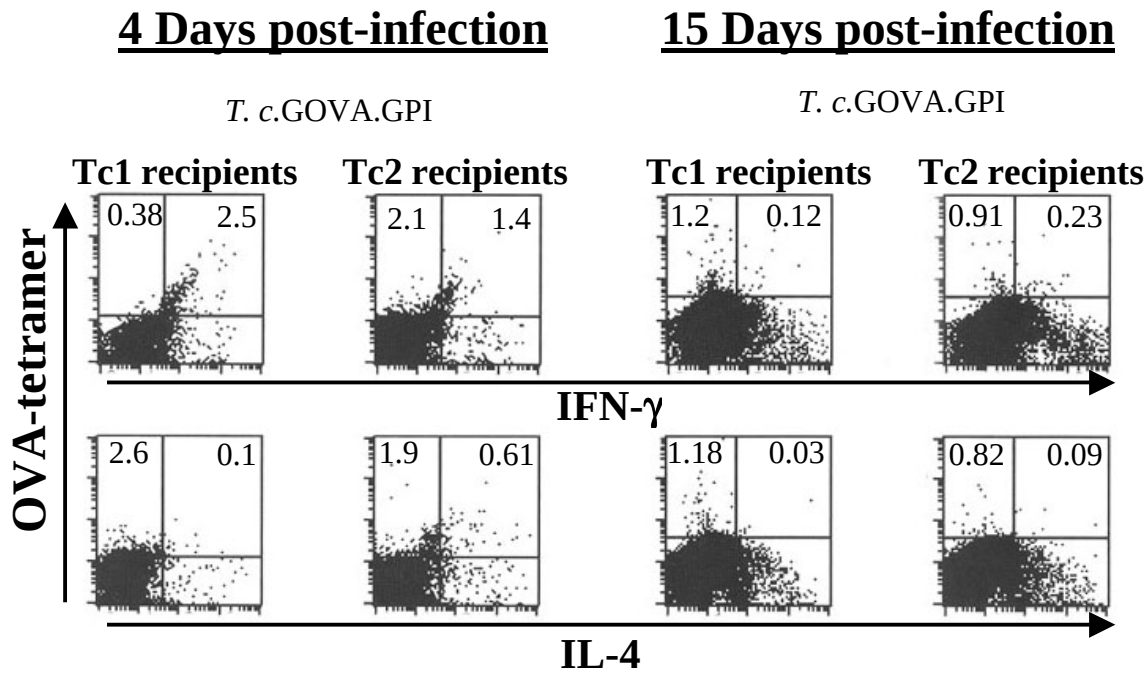
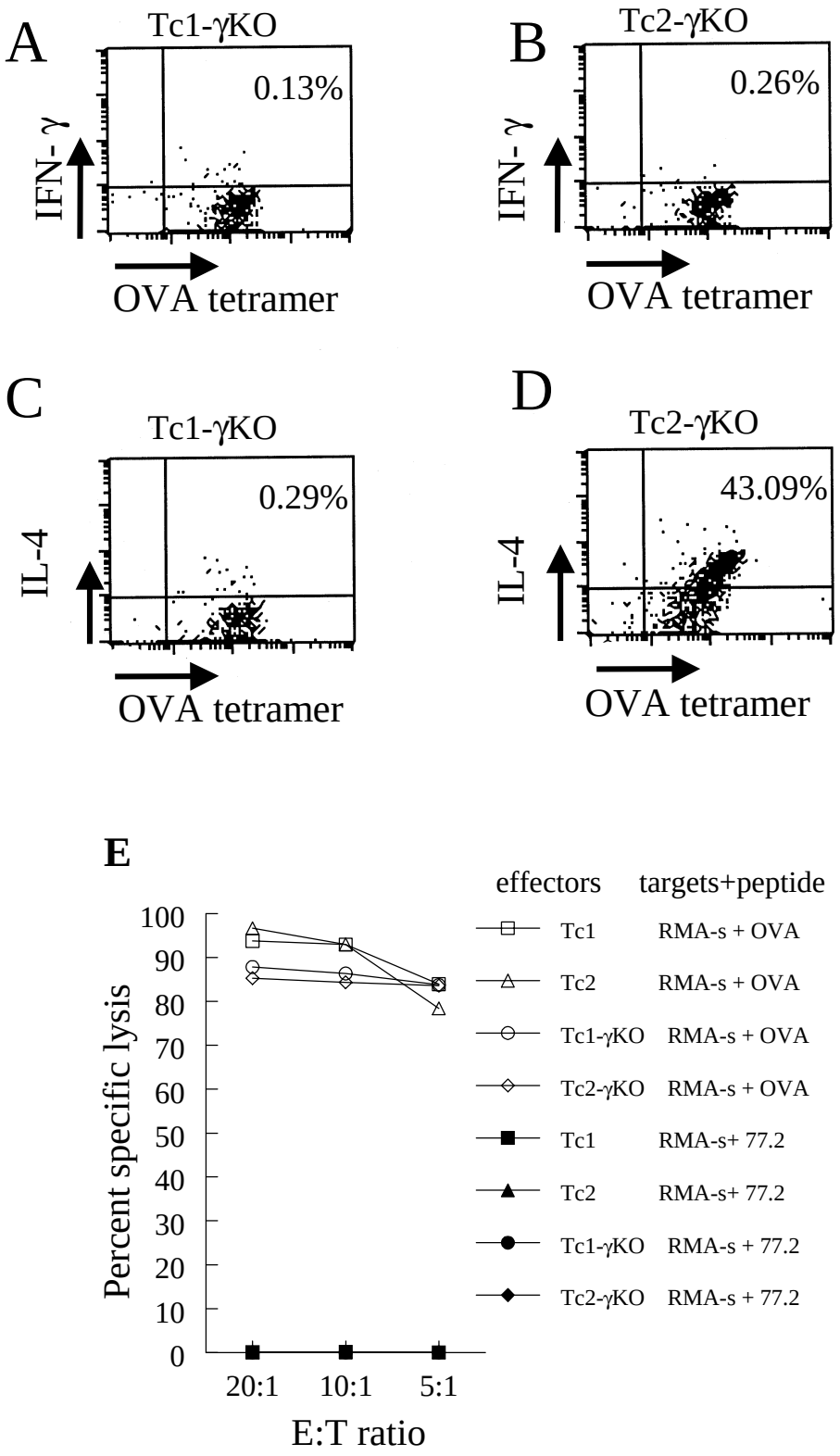



**Figure 8.** Cytokine production by adoptively transferred Tc1 and Tc2 cells. Spleens from Tc1 and Tc2 cell recipient mice were harvested at 4 and 15 days post-infection. Splenocytes were stimulated with OVA peptide and OVA tetramer positive cells were analyzed for the production of IFN-γ or IL-4 cytokines by intracellular cytokine staining.

**Figure 9.** Generation and phenotypic analysis of Tc1-KO and Tc2-KO cells. Splenocytes from IFN-γ deficient OT-1 transgenic mice were stimulated under Tc1 or Tc2 cells generating conditions. After 6 days of culture Tc1-γKO and Tc2-γKO cells were analyzed for the production of IFN-γ and IL-4 cytokines by intracellular cytokine staining. Tc1-γKO cells (*A*) and Tc2-γKO cells (*C*) stained with OVA tetramers plus anti-IFN-γ Ab. Tc1-γKO cells (B) and Tc2-γKO cells (D) stained with OVA-tetramer and anti-IL-4 Ab. *E*) Cytolytic activity of Tc1-γKO and Tc2-γKO cells was determined in standard $Cr^{51}$ release assay along with wild type Tc1 and Tc2 cells.

Figure 9.


A
Tc1-γKO
0.13%
IFN- γ
OVA tetramer
B
Tc2-γKO
0.26%
IFN- γ
OVA tetramer
C
Tc1-γKO
0.29%
IL-4
OVA tetramer
D
Tc2-γKO
43.09%
IL-4
OVA tetramer
E
effectors
targets+peptide
Tc1 RMA-s + OVA
Tc2 RMA-s + OVA
Tc1-γKO RMA-s + OVA
Tc2-γKO RMA-s + OVA
Tc1 RMA-s+ 77.2
Tc2 RMA-s+ 77.2
Tc1-γKO RMA-s + 77.2
Tc2-γKO RMA-s + 77.2
Percent specific lysis
100
90
80
70
60
50
40
30
20
10
0
20:1
10:1
5:1
E:T ratio

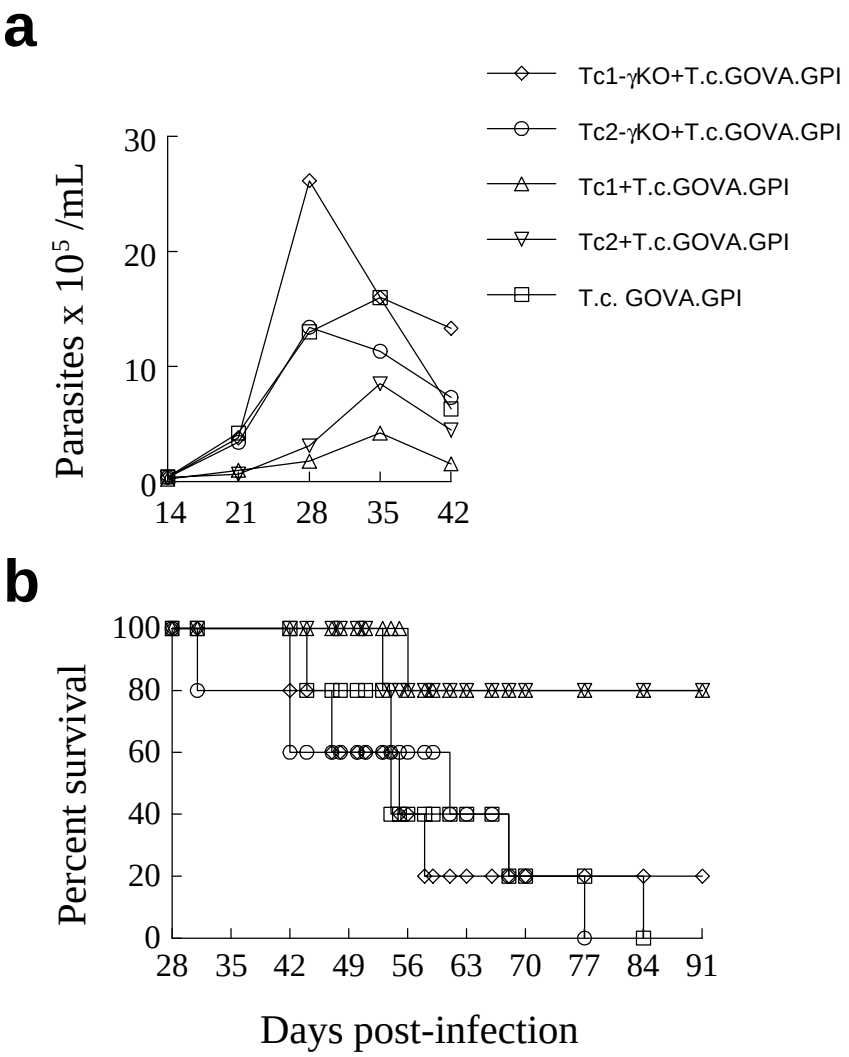



**Figure 10.** Adoptive transfer of Tc1-γKO or Tc2-γKO does not confer protection against lethal infection with *T. cruzi* GOVA.GPI. 1 x $10^7$ Tc1-γKO or Tc2-γKO cells were injected in groups of five mice each along with similar numbers of regular Tc1 and Tc2 cells followed by infection with 1 x $10^5$ BFT of *T. cruzi* GOVA.GPI. Parasitemia (*A*) and mortality were recorded (*B*).

analyzed. As shown in Figure 1 *C*, Tc1 cells secreted IFN-γ but not IL-4 and Tc2 cells secreted predominantly IL-4. Neither Tc1 nor Tc2 cells produced IFN-γ or IL-4 after stimulation with a control peptide 77.2 from *T. cruzi* protein TSA-1. Both Tc1 and Tc2 cells exhibited high cytolytic activity against RMA-s cells pulsed with OVA peptide (Fig. 1 *D*). However, neither Tc1 nor Tc2 cells lysed target cells presenting the control peptide 77.2 (Fig. 1 *D*). Thus, these culture conditions generate highly polarized OVA-specific Tc1 and Tc2 populations, both of which have potent cytolytic activity.

*Adoptive transfer of OVA-specific Tc1 or Tc2 cells protects naïve mice from lethal infection with T. cruzi GOVA.GPI*

OVA-specific Tc1 and Tc2 cells were adoptively transferred into naïve C57BL/6 mice to evaluate the ability of these effector cells to protect mice from lethal infection with OVA-expressing *T. cruzi* GOVA.GPI. Mice infected with $5 \times 10^4$ BFT of *T. cruzi* GOVA.GPI and receiving $10^7$ Tc1 or Tc2 cells developed similar parasitemias that became undetectable by day 49 post-infection. All of these mice survived the infection (Fig. 2 *A* and *B*). In comparison, mice receiving OVA-specific Tc1 or Tc2 cells and challenged with wild-type *T. cruzi* developed substantially higher blood parasite levels similar to those of mice that did not receive any cells and were infected with either *T. cruzi* GOVA.GPI or wild-type *T. cruzi* (Fig. 2 *A*). All mice receiving Tc1 or Tc2 cells and challenged with wild-type *T. cruzi* as well as mice not receiving any cells and infected with either *T. cruzi* GOVA.GPI or wild-type *T. cruzi* succumbed to the infection and died between 35 and 105 days after infection (Fig. 2 *B*).

To compare parasite burden and pathology in the tissues, skeletal muscle and heart tissues of representative mice from all groups were analyzed by histology at 30 days post-

infection. Cardiac muscle in mice from all the groups showed less severe pathology and tissue parasitism than the skeletal muscle (data not shown). Skeletal muscle tissue from Tc1 and Tc2 cell recipient mice infected with *T. cruzi* GOVA.GPI showed moderate inflammation consisting of predominantly lymphocytic cells with few to no parasites (Fig. 3 *A* and *B*). In contrast, skeletal muscles of Tc1 and Tc2 cell recipients infected with wild-type *T. cruzi* were more severely inflamed with greater necrosis and higher tissue parasite burden (Fig. 3 *C* and *D*). Similar high tissue parasitism and inflammation was observed in mice receiving no cells and infected with either wild-type *T. cruzi* or T.cruzi GOVA.GPI (Fig. 3 *E* and *F*).

Although parasitemia data and the screening of tissue sections did not suggest a difference in parasite load between Tc1 and Tc2 recipients, quantitative analysis by real-time PCR of tissues from surviving animals from each of these groups indicated a higher level of parasite kDNA in the Tc2 recipients as compared to the Tc1 recipients (data not shown). This result prompted us to ask if infection with a higher dose of *T. cruzi* might reveal a greater susceptibility of mice receiving Tc2 cells in comparison to mice receiving Tc1 cells. Therefore, OVA-specific Tc1 and Tc2 cells were transferred into mice that were subsequently challenged with $10^5$ BFT (a two-fold higher dose) of either *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. As in the experiment using $5 \times 10^4$ BFT as a challenge dose, recipients of OVA-specific Tc1 or Tc2 cells had similar parasitemia levels and mortality (1 of 5 mice in each group died by day 90 post-infection) after challenge with $10^5$ BFT of *T. cruzi* GOVA.GPI (Fig. 4 *A* and *B*). Real-time PCR analysis of skeletal muscle tissue from mice in all the groups at 30 days post-infection confirmed the parasitemia data. Mice receiving Tc1 or Tc2 cells and infected with *T. cruzi*

GOVA.GPI had lower levels of *T. cruzi* DNA in comparison to mice receiving Tc1 cells, Tc2 cells or no cells and infected with wild-type *T. cruzi* (Fig. 5). These results indicate that both Tc1 and Tc2 OVA-specific cells were able to confer similar levels of protection in mice infected with *T. cruzi* GOVA.GPI.

*OVA-specific Tc1 and Tc2 cells persist in vivo only in T. cruzi GOVA.GPI infected mice*

In the absence of a monoclonal antibody reactive with the OVA-specific TCR expressed by OT-1-derived Tc1 and Tc2 cells, these populations were labeled with CFSE before adoptive transfer into mice and their trafficking and persistence was monitored with an anti-flourescein MAb. CFSE-positive cells could be detected as early as 4 days post-infection in the spleens of mice receiving Tc1 cells and infected with either *T. cruzi* GOVA.GPI or wild-type *T. cruzi* (Fig. 6 *C* and *E*). However, at 30 days post-infection, CFSE-positive cells were detectable in the spleens of mice infected with *T. cruzi* GOVA.GPI but not in the spleens of wild-type *T. cruzi* infected mice (Fig. 6 *D* and *F*). Persistence of CFSE-positive cells in the spleens of Tc2 cell recipient mice infected with *T. cruzi* GOVA.GPI was evident at 4 as well as at 30 days-post-infection (Fig. 7 *A* and *B*) but could only be detected at 4 days and not at 30 days post-infection in the spleens of mice infected with wild-type *T. cruzi* (Fig. 7 *C* and *D*). Similar results on the persistence of OVA-specific Tc1 and Tc2 cells in the lymph nodes were obtained at 4 and 30 days post-infection (data not shown). We conclude from these results that OVA-specific Tc1 and Tc2 cells persist in the lymphoid organs of infected mice only in the presence of antigenic stimulation provided by infection with OVA-expressing *T. cruzi*.

*Cytokine production by OVA-specific Tc1 and Tc2 cells in vivo*

To examine whether adoptively transferred Tc1 and Tc2 effector cells were maintaining their type 1 and type 2 phenotype *in vivo*, we stimulated splenocytes from mice receiving either Tc1 or Tc2 cells with OVA peptide *in vitro* and analyzed intracellular cytokine production by FACS. As shown in Figure 8, a higher proportion of OVA tetramer positive Tc1 cells continued to make IFN-γ but little or no IL-4 at 4 and 15 days post-infection, respectively. Surprisingly, approximately 40 % cells from the Tc2 pool were producing IFN-γ while only 24 % cells continued to produce IL-4 at 4 days post-infection and nearly equal proportions of cells from the Tc2 pool were making IFN-γ and IL-4 at 15 days post-infection (Fig. 8). We conclude from these results that Tc2 cells were either switching towards the production of type 1 cytokine IFN-γ or there was a preferential expansion of Tc1 cells present among the Tc2 cells *in vivo.* Tc1 cells however, were stable in their production of this cytokine.

*OVA-specific Tc1 and Tc2 cells lacking the ability to make IFN-γ are unable to protect mice from lethal infection with T. cruzi GOVA.GPI*

Both Tc1 and Tc2 cells possess cytolytic activity and both populations produce IFN-γ when transferred to mice infected with *T. cruzi*. To investigate the contribution of IFN-γ to protection provided by Tc1 and Tc2 cells, we generated OVA-specific Tc1 and Tc2 cells from homozygous IFN-$\gamma^{-/-}$ OT-1 mice (termed Tc1-γKO and Tc2-γKO, respectively). FACS analysis of *in vitro* generated Tc1-γKO and Tc2-γKO subpopulations confirmed that Tc1-γKO and Tc2-γKO cells failed to produce IFN-γ after stimulation with OVA peptide (Fig. 9 *A* and *B*). However, approximately 43% of Tc2-γKO cells produced IL-4 while Tc1-γKO cells did not (Fig. 9 *C* and *D*). Tc1- and Tc2-

γKO cells were similar to wild-type Tc1 and Tc2 cells in their CTL activity against OVA peptide pulsed target cells (Fig. 9 *E*). These results demonstrate that we can generate Tc1 and Tc2 cells lacking the ability to make IFN-γ but retaining cytolytic activity at a level similar to that of wild-type Tc1 and Tc2 cells.

Tc1-γKO or Tc2-γKO cells were adoptively transferred into mice and these mice were challenged with $10^5$ BFT of T.cruzi GOVA.GPI. Recipients of Tc1-γKO and Tc2-γKO cells were susceptible to *T. cruzi* GOVA.GPI infection and all of the Tc2-γKO cell recipient mice and all except one Tc1-γKO recipient mouse died by day 90 post-infection (Fig. 10 *B*). Eighty percent of wild-type Tc1 and Tc2 cell recipients however, controlled parasitemia and survived the infection (Fig. 10 *A* and *B*). These data demonstrate that the inability to make IFN-γ compromises the ability of Tc1 and Tc2 cells to protect mice from *T. cruzi* infection and thus cytolytic activity alone in the absence of IFN-γ production was not sufficient to allow control of *T. cruzi* infection.

**Discussion**

A number of studies have shown that $CD8^+$ T cells are critical mediators of protective immunity against the protozoan parasite *T. cruzi* (reviewed in (1)). The requirement for a potent $CD8^+$ T cell response in mice infected with *T. cruzi* was initially demonstrated by depletion of $CD8^+$ T cells by treatment with anti-CD8 antibody (5). Depletion of $CD8^+$ T cells in chronically infected mice abrogated the resistance to *T. cruzi* and increased tissue parasite burden (6). Subsequent studies involving infection with *T. cruzi* of mice lacking the genes encoding CD8 (3, 4), β2m (7, 29) or TAP (30) provided additional evidence that $CD8^+$ T cells are absolutely required to control *T. cruzi* in infected mice. Further evidence for the role of $CD8^+$ T cells in mediating an active anti-*T. cruzi* inflammatory

response in mice comes from studies in which $CD8^+$ T cells are shown to be associated with *T. cruzi* and *T. cruzi*-infected cells in tissues (31). In persons with chronic chagasic myocarditis, higher numbers of $CD8^+$ T cells were correlated with the presence of parasites in the cardiac muscle (32). These data suggest that $CD8^+$ T cells may contribute to the initiation and maintenance of a persistent inflammatory response against *T. cruzi in vivo*. The presence of $CD8^+$ CTLs specific to several *T. cruzi* antigens in mice (21-23) and in humans (24) and the ability of *T. cruzi* antigen-specific $CD8^+$ T cell of Tc1 phenotype to confer significant protection in mice (22) suggest that these cells play an important role in immune control of *T. cruzi*.

$CD8^+$ T cells can control infection with intracellular pathogens either by direct cytolysis of infected target cells or via production of cytokines (33). Cytolysis of target cells by CTLs can either be mediated by perforin and granzyme release or Fas/Fas-L interaction. In infections with non-cytopathic LCMV and *Listeria monocytogenes*, perforin -mediated killing of infected cells is considered to be the predominant cytolytic mechanism (34, 35). The Fas/Fas-L pathway of cytolysis has generally been considered to be of primary importance as an immune regulatory pathway (35). However a number of recent studies have documented a prominent role for Fas/Fas-L interactions in CTL-mediated killing of viral infected or tumor cells(36-38). The ability of perforin or granzyme B-deficient mice to control *T. cruzi* infection similar to wild-type mice suggests that perforin- and granzyme-mediated cytolysis is not required for the control of *T. cruzi* infection (30).

Recently, it has been shown that naïve $CD8^+$ T cells upon antigen stimulation and the presence of cytokines, can differentiate into Tc1 or Tc2 subpopulations similar to Th1

and Th2 subsets of $CD4^+$ T cells (8-13, 39). The Tc1 cells secrete IFN-γ, TNF-α and IL-2 while Tc2 cells make IL-4, IL-5, IL-13 and IL-10 and little IFN-γ. However, both Tc1 and Tc2 subsets have direct cytolytic activity against their targets. The identification of Tc1 and Tc2 subsets of $CD8^+$ T cells and the ability of antigen-specific $CD8^+$ T cells to secrete different cytokines suggests that these cells perform distinct effector functions that might in turn influence the outcome of immune responses against infections. For example, in pulmonary viral infection, adoptively transferred virus-specific Tc1 cells but not the Tc2 cells reduced the virus titer earlier in the lungs (40). Virus-specific Tc1 cells entered the lungs earlier and localized near the infected epithelium as compared to Tc2 cells which showed delayed kinetics of entering the lungs and hence localized away from the inflamed epithelium. *In vitro* generated Tc2 cells were less efficient than Tc1 cells to protect mice against LCMV infection and the inability of Tc2 cells to confer protection against LCMV was attributed to defective homing of Tc2 cells in the tissues despite high cytolytic activity and IFN-γ production by these cells (41). There is much evidence for the existence of $CD8^+$ T cells of Tc1 phenotype (14-17) and some evidence for the presence of $CD8^+$ T cells secreting type 2 cytokines in different infections i*n vivo* (18). In this study, stability of Tc1 population to continue to make IFN-γ and relative dominance of IFN-γ producing cells among the Tc2 cell pool in mice infected with *T. cruzi* is noteworthy. One possible explanation for this observation could be that *T. cruzi* induces development of Tc1 cells but not of Tc2 cells in infected mice. Switching of Tc2 cells towards a Tc1 phenotype may also be due to the inherent instability of Tc2 cells relative to Tc1 cells.

In the present study, the relative contribution of adoptively transferred Tc1 and Tc2 subpopulations of $CD8^+$ T cells to the control of *T. cruzi* infection in mice was investigated. As we lacked a ready source of parasite specific Tc1/Tc2 cells, we expressed the model antigen OVA in *T. cruzi* and used *in vitro* generated OVA-specific Tc1 and Tc2 cells. Following transfer of OVA-specific Tc1 and Tc2 cells, the recipient mice were infected with a lethal dose of *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. Adoptive transfer of Tc1 as well as Tc2 cells protected mice from lethal infection with *T. cruzi* GOVA.GPI and these mice had lower blood and tissue parasite burden and less severe pathology in the skeletal muscle compared to wild-type *T. cruzi* infected mice as determined by routine histology and quantitative PCR. As a consequence, mice receiving Tc1 and Tc2 cells and infected with wild-type *T. cruzi* were unable to control the infection and died. Therefore, Tc1 and Tc2 cells promoted survival of the recipient mice against *T. cruzi* in an antigen-specific manner.

The persistence of OVA-specific Tc1 and Tc2 cells in the lymphoid organs occurred only in mice infected with OVA-expressing *T. cruzi* but not in mice infected with wild-type *T. cruzi*. Analysis of cytokine production by adoptively transferred Tc1 and Tc2 cells showed that a high proportion of Tc1 cells continued to make only IFN-γ but more Tc2 cells were making IFN-γ rather than IL-4. The observation that more Tc2 cells were producing IFN-γ after transfer into mice suggest that protection conferred by the transfer of Tc2 cells could be due to the production of IFN-γ by these cells.

Transferring OVA-specific Tc1-γKO and Tc2-γKO cells lacking the ability to produce IFN-γ into mice challenged with *T. cruzi* allowed us to ask the following questions: 1) Does IFN-γ production by wild-type Tc1 and Tc2 cells contribute to the protection of

mice from *T. cruzi*? and 2) Could cytolytic function in the absence of IFN-γ production confer such protection? The mice receiving Tc1-γKO or Tc2-γKO cells succumbed to infection with *T. cruzi* GOVA.GPI suggesting that IFN-γ production was critical in the protective ability of wild-type Tc1 and Tc2 cells and that cytolytic activity alone was insufficient to provide protection against the parasite.

Several studies have shown a critical role of IFN-γ in regulation of *T. cruzi* infection in mice (42-44). Production of IFN-γ during the acute phase conferred resistance, and depletion of IFN-γ increased mortality in mice to *T. cruzi* (45, 46). In *T. cruzi* infection, IFN-γ is also suggested to contribute to enhanced parasite clearance by activation of macrophages and production of NO. More recently, supernatants from $CD8^+$ Tc1 cell cultures stimulated muscle cells (the primary cells invaded by *T. cruzi*) to produce NO and thus increased the ability of muscle cells to control parasite growth *in vitro* (T. Rosario and R. L. Tarleton manuscript in preparation). The ability of $CD8^+$ T cells to promote the control of parasite growth by muscle cells could be important given the fact that $CD8^+$ T cells are the predominant cell population at the lesion site. Evidence for the role of IFN-γ in controlling *T. cruzi* also comes from experiments in which adoptively transferred antigen-specific Th1 cells but not Th2 cells provided protection against lethal *T. cruzi* infection in mice (S. Kumar and R. L. Tarleton, manuscript in preparation). Similarly, Stat6-deficient mice capable of making only a type 1-biased response were resistant to *T. cruzi* infection compared to Stat4- deficient mice that only make a type 2 response (47). Our data on protection of mice from *T. cruzi* by adoptively transferred antigen-specific IFN-γ producing Tc1 cells in conjunction with the results demonstrating significant protection conferred by $CD8^+$ Tc1 cells in mice infected with *T. cruzi* (22)

suggest that a strong type 1 response is crucial for the control of *T. cruzi*, and supports our attempts to elucidate mechanisms and vaccination strategies that would promote a strong type 1 response to *T. cruzi*.

**References**

1. Brener, Z., and R. T. Gazzinelli. 1997. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. *Int Arch Allergy Immunol 114:103*.

2. Tarleton, R. L. 1991. The role of T-cell subpopulations in experimental Chagas' disease. *Res Immunol 142:130*.

3. Rottenberg, M. E., M. Bakhiet, T. Olsson, K. Kristensson, T. Mak, H. Wigzell, and A. Orn. 1993. Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with Trypanosoma cruzi or Trypanosoma brucei. *Infect Immun 61:5129*.

4. Rottenberg, M. E., A. Riarte, L. Sporrong, J. Altcheh, P. Petray, A. M. Ruiz, H. Wigzell, and A. Orn. 1995. Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol Lett 45:53*.

5. Tarleton, R. L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. *J Immunol 144:717*.

6. Tarleton, R. L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. *Infect Immun 62:1820*.

7. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection [see comments]. *Nature 356:338*.

8. Croft, M., L. Carter, S. L. Swain, and R. W. Dutton. 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med 180:1715*.

9. Sad, S., R. Marcotte, and T. R. Mosmann. 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity 2:271*.

10. Halverson, D. C., G. N. Schwartz, C. Carter, R. E. Gress, and D. H. Fowler. 1997. In vitro generation of allospecific human CD8+ T cells of Tc1 and Tc2 phenotype. *Blood 90:2089*.

11. Li, L., S. Sad, D. Kagi, and T. R. Mosmann. 1997. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J Immunol 158:4152*.

12. Mosmann, T. R., L. Li, and S. Sad. 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Semin Immunol 9:87*.

13. Mosmann, T. R., L. Li, H. Hengartner, D. Kagi, W. Fu, and S. Sad. 1997. Differentiation and functions of T cell subsets. *Ciba Found Symp 204:148*.

14. Sarawar, S. R., and P. C. Doherty. 1994. Concurrent production of interleukin-2, interleukin-10, and gamma interferon in the regional lymph nodes of mice with influenza pneumonia. *J Virol 68:3112*.

15. Falchetti, R., G. Lanzilli, I. A. Casalinuovo, R. Gaziano, A. T. Palamara, P. Di Francesco, G. Ravagnan, and E. Garaci. 1996. Splenic CD4+ and CD8+ T cells

from influenza immune mice concurrently produce in vitro IL2, IL4, and IFN-gamma. *Cell Immunol 170:222*.

16. Serbina, N. V., and J. L. Flynn. 1999. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of Mycobacterium tuberculosis-infected mice. *Infect Immun 67:3980*.

17. Pathan, A. A., K. A. Wilkinson, R. J. Wilkinson, M. Latif, H. McShane, G. Pasvol, A. V. Hill, and A. Lalvani. 2000. High frequencies of circulating IFN-gamma-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted Mycobacterium tuberculosis epitope in M. tuberculosis-infected subjects without disease. *Eur J Immunol 30:2713*.

18. Maggi, E., M. G. Giudizi, R. Biagiotti, F. Annunziato, R. Manetti, M. P. Piccinni, P. Parronchi, S. Sampognaro, L. Giannarini, G. Zuccati, and et al. 1994. Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J Exp Med 180:489*.

19. Maggi, E., R. Manetti, F. Annunziato, and S. Romagnani. 1995. CD8+ T lymphocytes producing Th2-type cytokines (Tc2) in HIV-infected individuals. *J Biol Regul Homeost Agents 9:78*.

20. Coyle, A. J., F. Erard, C. Bertrand, S. Walti, H. Pircher, and G. Le Gros. 1995. Virus-specific CD8+ cells can switch to interleukin 5 production and induce airway eosinophilia. *J Exp Med 181:1229*.

21. Low, H. P., M. A. Santos, B. Wizel, and R. L. Tarleton. 1998. Amastigote surface proteins of Trypanosoma cruzi are targets for CD8+ CTL. *J Immunol 160:1817*.

22. Wizel, B., M. Nunes, and R. L. Tarleton. 1997. Identification of Trypanosoma cruzi trans-sialidase family members as targets of protective CD8+ TC1 responses. *J Immunol 159:6120*.

23. Nickell, S. P., G. A. Stryker, and C. Arevalo. 1993. Isolation from Trypanosoma cruzi-infected mice of CD8+, MHC-restricted cytotoxic T cells that lyse parasite-infected target cells. *J Immunol 150:1446*.

24. Wizel, B., M. Palmieri, C. Mendoza, B. Arana, J. Sidney, A. Sette, and R. Tarleton. 1998. Human infection with Trypanosoma cruzi induces parasite antigen-specific cytotoxic T lymphocyte responses. *J Clin Invest 102:1062*.

25. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17*.

26. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J Immunol Methods 171:131*.

27. Zhang, L., and R. L. Tarleton. 1996. Persistent production of inflammatory and anti-inflammatory cytokines and associated MHC and adhesion molecule expression at the site of infection and disease in experimental Trypanosoma cruzi infections. *Exp Parasitol 84:203*.

28. Garg, N., M. Postan, K. Mensa-Wilmot, and R. L. Tarleton. 1997. Glycosylphosphatidylinositols are required for the development of Trypanosoma cruzi amastigotes. *Infect Immun 65:4055*.

29. Tarleton, R. L., M. J. Grusby, M. Postan, and L. H. Glimcher. 1996. Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class

I- and class II-restricted T cells in immune resistance and disease. *Int Immunol 8:13.*

30. Kumar, S., and R. L. Tarleton. 1998. The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite Immunol 20:207*.

31. Sun, J., and R. L. Tarleton. 1993. Predominance of CD8+ T lymphocytes in the inflammatory lesions of mice with acute Trypanosoma cruzi infection. *Am J Trop Med Hyg 48:161*.

32. Higuchi, M. D., M. M. Ries, V. D. Aiello, L. A. Benvenuti, P. S. Gutierrez, G. Bellotti, and F. Pileggi. 1997. Association of an increase in CD8+ T cells with the presence of Trypanosoma cruzi antigens in chronic, human, chagasic myocarditis. *Am J Trop Med Hyg 56:485*.

33. Berke, G. 1995. The CTL's kiss of death. *Cell 81:9.*

34. Kagi, D., P. Seiler, J. Pavlovic, B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1995. The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. *Eur J Immunol 25:3256.*

35. Kagi, D., B. Ledermann, K. Burki, H. Hengartner, and R. M. Zinkernagel. 1994. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur J Immunol 24:3068.*

36. Conceicao-Silva, F., M. Hahne, M. Schroter, J. Louis, and J. Tschopp. 1998. The resolution of lesions induced by Leishmania major in mice requires a functional Fas (APO-1, CD95) pathway of cytotoxicity. *Eur J Immunol 28:237*.

37. Lopes, M. F., M. P. Nunes, A. Henriques-Pons, N. Giese, H. C. Morse, 3rd, W. F. Davidson, T. C. Araujo-Jorge, and G. A. DosReis. 1999. Increased susceptibility of Fas ligand-deficient gld mice to Trypanosoma cruzi infection due to a Th2-biased host immune response. *Eur J Immunol 29:81*.

38. Frost, P., C. P. Ng, A. Belldegrun, and B. Bonavida. 1997. Immunosensitization of prostate carcinoma cell lines for lymphocytes (CTL, TIL, LAK)-mediated apoptosis via the Fas-Fas-ligand pathway of cytotoxicity. *Cell Immunol 180:70*.

39. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science 254:279*.

40. Cerwenka, A., T. M. Morgan, A. G. Harmsen, and R. W. Dutton. 1999. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J Exp Med 189:423*.

41. Wirth, S., M. van den Broek, C. P. Frossard, A. W. Hugin, I. Leblond, H. Pircher, and C. Hauser. 2000. CD8(+) T cells secreting type 2 lymphokines are defective in protection against viral infection. *Cell Immunol 202:13*.

42. Minoprio, P., M. C. el Cheikh, E. Murphy, M. Hontebeyrie-Joskowicz, R. Coffman, A. Coutinho, and A. O'Garra. 1993. Xid-associated resistance to experimental Chagas' disease is IFN-gamma dependent. *J Immunol 151:4200*.

43. Reed, S. G. 1988. In vivo administration of recombinant IFN-gamma induces macrophage activation, and prevents acute disease, immune suppression, and death in experimental Trypanosoma cruzi infections. *J Immunol 140:4342*.

44. Nabors, G. S., and R. L. Tarleton. 1991. Differential control of IFN-gamma and IL-2 production during Trypanosoma cruzi infection. *J Immunol 146:3591*.

45. Torrico, F., H. Heremans, M. T. Rivera, E. Van Marck, A. Billiau, and Y. Carlier. 1991. Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. *J Immunol 146:3626*.

46. Petray, P. B., M. E. Rottenberg, G. Bertot, R. S. Corral, A. Diaz, A. Orn, and S. Grinstein. 1993. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of *Trypanosoma cruzi*. *Immunol Lett 35:77*.

47. Tarleton, R. L., M. J. Grusby, and L. Zhang. 2000. Increased susceptibility of Stat4-deficient and enhanced resistance in Stat6-deficient mice to infection with *Trypanosoma cruzi*. *J Immunol 165:1520*.

# CHAPTER 5

# CONCLUSIONS

Chagas disease remains a major health problem in South and Central America. Despite decades of efforts, there is no single effective treatment to cure *Trypanosoma cruzi* infection. The available drug, Benznidazole has to be continuously administered for 30-60 days and has harmful side effects. Vector control strategies, even though successful at times, have failed to prevent disease transmission completely. Efforts to develop vaccines against *T. cruzi* have been discouraged in part due to the popular belief in autoimmune etiology of Chagas disease. However, research done in recent years has clearly established that disease development in the host is linked to parasite persistence (1, 2). This has paved the way for efforts to develop effective immune interventions against *T. cruzi*. For these efforts to succeed, further elucidation of protective immune mechanisms against *T. cruzi* is necessary.

Earlier work on immunity to *T. cruzi* had focused primarily on antibody-mediated mechanisms of immune control of *T. cruzi* while the intracellular location of the amastigote stage of *T. cruzi* was generally considered sequestered away from the immune system. However, evidence of presentation of *T. cruzi* antigens via the class I MHC presentation pathway and the susceptibility of mice deficient in genes required for $CD4^+$ and $CD8^+$ T cell function has clearly established the requirement for a potent T cell response against *T. cruzi* (3-7).

The focus of this study was to analyze the contribution of B cells and type 1 and type 2 subsets of $CD4^+$ and $CD8^+$ T cells to the control of *T. cruzi* infection in mice. Infection

of B cell-deficient (muMT) mice (8) allowed us to analyze the contribution of B cells and antibody production to control *T. cruzi*. muMT mice infected with *T. cruzi* showed delay in the development of parasitemia and lived substantially longer than mice deficient in CD8+ T cells (β2m and TAP-1 deficient mice) but eventually developed high parasitemia and died. On the contrary, wild-type mice survived the same dose of *T. cruzi* infection with undetectable parasitemia. Infection with lower doses of *T. cruzi* prolonged survival of muMT mice but these mice eventually became highly parasitemic and died in chronic phase of infection. Prior infection with an avirulent strain of *T. cruzi* could not protect β2m and TAP-1-deficient mice from subsequent challenge with virulent parasites and all of these mice died again in the acute phase of infection. Even though muMT mice infected with avirulent parasites were able to delay development of parasitemia and onset of mortality after infection with virulent parasites, muMT mice were unable to control parasite burden and died during the chronic phase of infection. We conclude from these experiments that the $CD8^+$ T cell response appears more critical than an antibody response to control *T. cruzi* during the acute phase of infection. However, for long-term survival, an effective antibody response is also critical.

The requirement for $CD4^+$ T cells as important components of a protective immune response against *T. cruzi* has been established by studies in mice deficient in genes encoding class II MHC or CD4 molecules and thereby lacking $CD4^+$ T cells (3-7, 9). Even though in infection with other intracellular pathogens, a strong and polarized Th1 response has been associated with resistance while a Th2 response has been shown to associate with susceptibility (10), a linkage between Th1 response and resistance has been less clearly established with respect to *T. cruzi* infection. Some studies, both in

susceptible and resistant strains of mice, have suggested the requirement of a Th1 and Th2 response (11-13). To investigate directly the role of antigen specific Th1 and Th2 cells in modulating the host immune response *in vivo* and in determining the outcome of a lethal *T. cruzi* infection, we developed a system by which Th1 or Th2 cells specific for a parasite-expressed antigen were tested for their ability to protect naive mice from a lethal infection with *T. cruzi*. We were constrained by the lack of a source for clonal populations of Th1 and Th2 cells specific for a bona fide *T. cruzi*-derived class II MHC epitope. Therefore, we generated *T. cruzi* that expressed the model antigen chicken ovalbumin and used DO.11 TCR transgenic mice (14) as source of OVA-specific $CD4^+$ T cells. Using the DO.11.10 TCR-specific monoclonal Ab KJ1-26 (15), we were also able to follow the transferred cells *in vivo* and to monitor persistence and expansion of these cells in response to infection with *T. cruzi* G-OVA.GPI.

Mice receiving OVA-specific Th1 cells controlled infection with *T. cruzi* G-OVA.GPI and showed reduced pathology in the skeletal muscles. In contrast, recipients of Th2 cells remained highly susceptible to infection with *T. cruzi* G-OVA.GPI and developed much higher blood and tissue parasitism than Th1 recipients, as confirmed by histology and quantitation of parasite DNA by real-time PCR. The susceptibility of the Th2 cell recipient mice was not due to the absence of potent systemic and local responses to the parasite. Th2 recipient mice exhibited a vigorous inflammatory response with predominently polymorphonuclear cells at the site of infection but it seemed to be an unproductive response, insufficient to control parasite replication in the tissues. As a consequence, all Th2 cell recipients died in the acute phase of infection while the majority of Th1 cells-recipients controlled the infection and became aparasitemic. The

availability of DO.11.10 TCR-specific MAb KJ1-26 allowed us *in vivo* monitoring of persistence and expansion of OVA-specific Th1 and Th2 cells in the recipient mice. Transferred Th1/Th2 cells expanded in an antigen-specific manner as there was no polyclonal activation of OVA specific T cells in response to infection with wild-type parasites. All together, we showed that augmentation with a polarized Th1 response protected susceptible mice against a lethal *T. cruzi* infection. This protection occurred in an antigen-specific manner as only the mice that received OVA-specific Th1 cells and challenged with OVA expressing *T. cruzi* were protected while those challenged with wild-type *T. cruzi* were not. Using the Th1/Th2 cells transfer system, we demonstrated that a strong Th1 response is absolutely required while a Th2 response or a mixed Th1:Th2 response is an unproductive response for controlling *T. cruzi* infection.

We extended the cell transfer system mentioned above to study the relative contribution of adoptively transferred Tc1 and Tc2 subpopulations of $CD8^+$ T cells to the control of *T. cruzi* infection in mice. OT-1 transgenic mice (16) were used as a source of OVA-specific $CD8^+$ T cells. Tc1 and Tc2 subpopulations of OVA-specific $CD8^+$ T cells were generated by stimulation of OT-1 splenocytes *in vitro* under type 1 and type 2 phenotype promoting conditions. Following transfer of OVA-specific Tc1 and Tc2 cells, the recipient mice were infected with a lethal dose of *T. cruzi* GOVA.GPI or wild-type *T. cruzi*. Adoptive transfer of Tc1 as well as Tc2 cells protected mice from lethal infection with *T. cruzi* GOVA.GPI and these mice had lower blood and tissue parasite burden and less severe pathology in the skeletal muscle compared to wild-type *T. cruzi* infected mice as determined by routine histology and quantitative PCR. As a consequence, mice receiving Tc1 and Tc2 cells and infected with wild-type *T. cruzi* were unable to control

the infection and died. Therefore, Tc1 and Tc2 cells promoted survival of the recipient mice against *T. cruzi* in an antigen-specific manner.

Persistence of OVA-specific Tc1 and Tc2 cells in the lymphoid organs occurred only in mice infected with OVA-expressing *T. cruzi* but not in mice infected with wild-type *T. cruzi*. However, analysis of cytokine production by adoptively transferred Tc1 and Tc2 cells showed that a high proportion of Tc1 cells continued to make only IFN-γ but more Tc2 cells were making IFN-γ rather than IL-4. The observation that more Tc2 cells were producing IFN-γ after transfer into mice suggest that protection conferred by the transfer of Tc2 cells could be due to the production of IFN-γ by these cells.

The transfer of OVA-specific Tc1-γKO and Tc2-γKO cells lacking the ability to produce IFN-γ into mice challenged with *T. cruzi* allowed us to examine the role of IFN-γ production by Tc1 and Tc2 cells to the protection of mice from *T. cruzi*. It also allowed us to ask if cytolytic function in the absence of IFN-γ production could confer such protection. Mice receiving Tc1-γKO or Tc2-γKO cells succumbed to infection with *T. cruzi* GOVA.GPI suggesting that IFN-γ production was critical in the protective ability of Tc1 and Tc2 cells and that cytolytic activity alone was insufficient to provide protection against the parasite.

$CD8^+$ T cells kill their targets and/or mediate immune control via perforin/granzyme pathway, Fas/Fas-L induced apoptosis and cytokine production (17). In order to investigate the role of perforin and granzyme B-mediated lytic pathway in the control of *T. cruzi* infection, perforin (18) and granzyme B-deficient mice (19) were infected with *T. cruzi*. Perforin and granzyme B-deficient mice controlled infection with *T. cruzi* similarly to wild-type mice. Despite the absence of either perforin or granzyme B, spleen

cells obtained from these *T. cruzi* infected deficient mice displayed significant cytolytic T cell activity versus cells pulsed with peptide 77.2, a *T. cruzi* TSA-1-derived peptide which has been previously shown to be a target of anti-*T. cruzi* CTL (20). These results suggest that perforin- or granzyme B-mediated lytic pathway is not required for control of *T. cruzi* infection.

In summary, we have developed a cell transfer system that has allowed us to investigate the role of subsets of CD4- and $CD8^+$ T cells in the control of *T. cruzi* infection. Protection of mice receiving type 1 cytokine producing CD4- and $CD8^+$ T cells demonstrated that a strong type 1 cell-mediated response is required for control of *T. cruzi* infection. Infection of muMT mice suggests that antibodies are essentials in controlling *T. cruzi* in the post-acute phase of infection. Our results also suggest that cytokine production rather than cytolytic activity of $CD8^+$ T cells are important in the control of *T. cruzi*. The results of this study make it possible for us to design immunotherapy or immunoprophylaxis that would induce above described protective immune responses against *T. cruzi*.

This work also supports our attempts to generate transgenic mice in which the T cells express clonotypic TCR specific to a *T. cruzi*-derived antigen. These mice can serve as a source of a clonal population of T cells that recognize a true *T. cruzi* antigen. In the mean time, the OVA system will allow us to monitor activation of naive $CD8^+$ T cells in *T. cruzi* infection in the future. By transferring OVA-specific OT-1 $CD8^+$ T cells into mice challenged with *T. cruzi* GOVA.GPI, we can analyze activation, type 1 and/or type 2 cytokine production and development of memory phenotype by the transferred cells as

the disease progresses. We can also examine whether the transferred cells preferentially home to certain organs in response to infection.

Another possible application of the OVA system is to address the question of polyclonal activation and immunosuppression in *T. cruzi* infection. Several studies have suggested that *T. cruzi* causes immunosuppression in the host during the acute phase of infection (21-25). *T. cruzi*-infected mice show disturbances in the peripheral immune system such as polyclonal activation, and immunosuppression of T lymphocytes. The parasite has been shown to inhibit T and B lymphocyte functions *in vitro*. In spite of the evidence for suppressor activity of *T. cruzi* in the host, there is no clear evidence about the underlying mechanisms of immunosuppression and polyclonal activation of T cells. By adoptively transferring OVA specific $CD4^+$ and $CD8^+$ T cells in mice, and monitoring their activation and proliferation in response to infection, we could potentially address the question of polyclonal activation and immunosppression of T cells in *T. cruzi* infection.

**References**

1. Tarleton, R. L., and L. Zhang. 1999. Chagas disease etiology: autoimmunity or parasite persistence? *Parasitol Today 15:94*.

2. Zhang, L., and R. L. Tarleton. 1999. Parasite persistence correlates with disease severity and localization in chronic Chagas' disease. *J Infect Dis 180:480*.

3. Rottenberg, M. E., A. Riarte, L. Sporrong, J. Altcheh, P. Petray, A. M. Ruiz, H. Wigzell, and A. Orn. 1995. Outcome of infection with different strains of Trypanosoma cruzi in mice lacking CD4 and/or CD8. *Immunol.Lett. 45:53*.

4. Rottenberg, M. E., D. A. Rodriguez, and A. Orn. 1992. Control of Trypanosoma cruzi infection in mice deprived of T- cell help. *Scand.J.Immunol. 36:261*.

5. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection [see comments]. *Nature 356:338*.

6. Tarleton, R. L., M. J. Grusby, M. Postan, and L. H. Glimcher. 1996. Trypanosoma cruzi infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int.Immunol. 8:13*.

7. Tarleton, R. L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine- induced immunity in mice infected with Trypanosoma cruzi. *J.Immunol. 144:717*.

8. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature 350:423*.

9. Russo, M., P. Minoprio, A. Coutinho, and M. Hontebeyrie-Joskowicz. 1988. Depletion of L3T4+ T lymphocytes during acute Trypanosoma cruzi infection abolish macrophage and B lymphocyte activation but not tissue inflammatory reaction. *Mem.Inst.Oswaldo.Cruz. 83 Suppl 1:527.*

10. Romagnani, S. 1996. Understanding the role of Th1/Th2 cells in infection. *Trends.Microbiol. 4:470.*

11. Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F. G. Araujo. 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. *J.Immunol. 158:3311.*

12. Petray, P. B., M. E. Rottenberg, G. Bertot, R. S. Corral, A. Diaz, A. Orn, and S. Grinstein. 1993. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of Trypanosoma cruzi. *Immunol.Lett. 35:77.*

13. Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed. 1992. Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection. *J.Exp.Med. 175:169.*

14. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science 250:1720.*

15. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J.Exp.Med. 157:1149.*

16. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17*.

17. Kagi, D., B. Ledermann, K. Burki, R. M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu.Rev.Immunol. 14:207-32:207*.

18. Kagi, D., B. Ledermann, K. Burki, H. Hengartner, and R. M. Zinkernagel. 1994. CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. *Eur.J.Immunol. 24:3068*.

19. Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell, and T. J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell 76:977*.

20. Wizel, B., M. Nunes, and R. L. Tarleton. 1997. Identification of Trypanosoma cruzi trans-sialidase family members as targets of protective CD8+ TC1 responses. *J.Immunol. 159:6120*.

21. Beltz, L. A., F. Kierszenbaum, and M. B. Sztein. 1990. Trypanosoma cruzi-induced suppression of human peripheral blood lymphocytes activated via the alternative (CD2) pathway. *Infect.Immun. 58:1114*.

22. Lopez, H. M., M. K. Tanner, F. Kierszenbaum, and M. B. Sztein. 1993. Alterations induced by Trypanosoma cruzi in activated mouse lymphocytes. *Parasite Immunol. 15:273*.

23. Lopes, M. F., V. F. da Veiga, A. R. Santos, M. E. Fonseca, and G. A. DosReis. 1995. Activation-induced CD4+ T cell death by apoptosis in experimental Chagas' disease. *J.Immunol. 154:744*.

24. Majumder, S., and F. Kierszenbaum. 1996. Mechanisms of Trypanosoma cruzi-induced down-regulation of lymphocyte function. Inhibition of transcription and expression of IL-2 receptor gamma (p64IL-2R) and beta (p70IL-2R) chain molecules in activated normal human lymphocytes. *J.Immunol. 156:3866.*

25. Minoprio, P., A. Bandeira, P. Pereira, T. Mota Santos, and A. Coutinho. 1989. Preferential expansion of Ly-1 B and CD4- CD8- T cells in the polyclonal lymphocyte responses to murine T. cruzi infection. *Int Immunol 1:176*.