T. cruzi parasite-specific humoral immunity versus polyclonal activation

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The etiologic agent of Chagas' disease is *Trypanosoma cruzi*. Patent parasitemia leads to parasite spread throughout the host during acute phase disease. Parasitemia concomitant with polyclonal lymphocyte activation has been reported and is thought to contribute to parasite evasion of host immunity and subsequent parasite persistence, which leads to chronic disease. In the present studies, polyclonal B cell activation was evaluated in relatively susceptible Balb/c versus resistant C57Bl/6 mouse models. Hypergammaglobulinemia and B cell activation in susceptible mice was associated with a large number of antibody secreting cells (ASC) without appreciable parasite-specific ASC. In contrast, in resistant mice, B cell activation and expansion was associated with generation of parasite-specific humoral immunity. These data indicate that the outcome of B cell activation during early *T. cruzi* experimental infection varies according to host susceptibility.

T. cruzi encodes several proteins with mitogenic capacities that are thought to contribute to dysfunctional polyclonal B cell activation in susceptible mice. One recently identified *T. cruzi* mitogen is a proline racemase (TcPRAC). Characterization of B cell activation by recombinant protein in this study demonstrates that TcPRAC induced polyclonal B cell activation, evident by proliferation, antibody secretion, IL-10 production, and B cell surface phenotype. MZ B cells were more responsive to T-cell independent TcPRAC stimulation than were follicular mature

(FM) B cells. These data provide the first comprehensive characterization of B cell activation by TcPRAC.

During experimental *T. cruzi* infection, TcPRAC-specific IgG remained undetectable. Conversely, intradermal genetic immunization via gene-gun (GG) induced antigen-specific immunogenic responses, generating TcPRAC-specific high-titer IgG, bone marrow plasma cells, and memory B cells. TcPRAC-specific IgG bound mitogenic rTcPRAC, decreasing subsequent B cell activation. GG immunization with *TcPRAC* DNA was non-mitogenic and did not effect generation of specific IgG to another *T. cruzi* antigen, complement regulatory protein (CRP). These data demonstrate the utility of genetic immunization for the conversion of a protein mitogen into an effective immunogen. Furthermore, co-immunization of TcPRAC with another *T. cruzi* antigen indicated the usefulness of this approach for multivalent vaccine development.

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PREFACE

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Contributors to this thesis work are indicated as co-authors in the publications (in press or submitted) listed at the beginning of each chapter.

1.0 INTRODUCTION

Trypanosoma cruzi, the etiologic agent of Chagas' disease, is a haemoflagellate protozoan parasite. Carlos Chagas first discovered this parasite in 1909 and went on to characterize its vector and life cycle, and describe parasite induced pathology, although this parasite has been causing human disease from as early as about 7,000 BC (Ferreira, Britto et al. 2000). Today, Chagas' disease is a major health concern in many parts of Latin America and has uncertain disease prevalence in the United States (Hotez 2008). The World Health Organization estimates that approximately 12 million people are infected with *T. cruzi* resulting in 3.0–3.3 million symptomatic cases and an annual incidence of 200,000 cases in 15 countries (Morel and Lazdins 2003). Epidemiologic studies estimate that 30 percent of those infected will develop chronic disease (CD) which is characterized by parasite persistent and chronic inflammation of nerve and muscle fibers that lead to disturbances in cardiac conductance and progressive congestive heart failure and/or enlargement of digestive organs such as the esophagus and colon.

1.1 PARASITE LIFE CYCLE

T. cruzi cycles between reduviid insect vectors such as *Triatoma infestans* and mammalian hosts (Figure 1) (Tyler and Engman 2001).

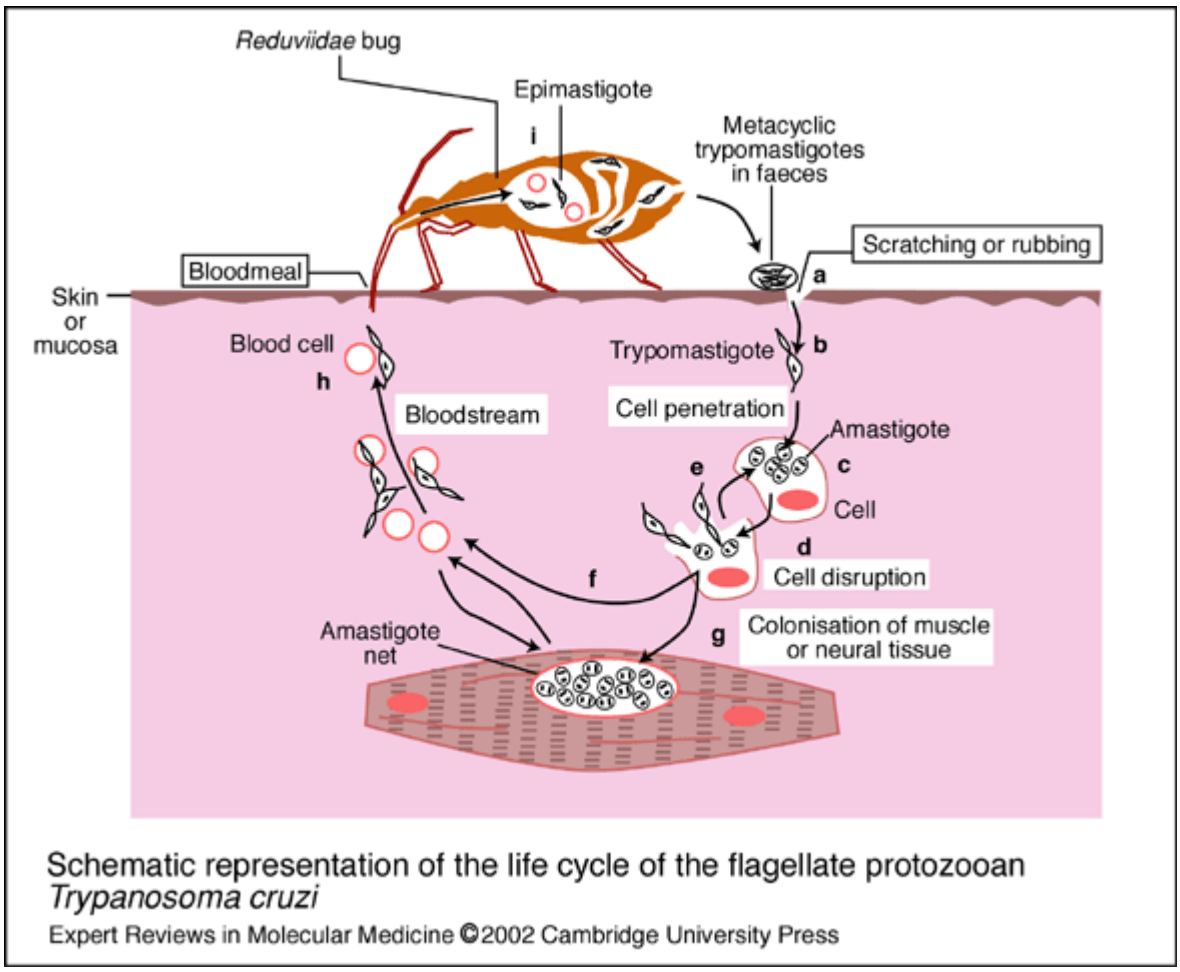

Figure 1: *T. cruzi* life cycle

Within the foregut of its insect host, *T. cruzi* undergoes reproduction via epimastigote stage parasites. As these parasites travel through the insect hindgut, they differentiate to the infective metacyclic trypomastigote form of the parasite. Parasites may then be transmitted to mammalian hosts by the insect vector after it takes a blood meal. The infectious parasite is passed through the insect feces and/or urine and can enter a mammalian host through the bite wound, a break in the skin, or mucosal surfaces, such as the eye. *T. cruzi* has wide host tropism, enabling it to carry out its infectious cycle in many vertebrate hosts. Upon entry into the host, trypomastigotes associate with the surface of mammalian cells and trigger recruitment and fusion of host cell lysosomes with the plasma membrane around the parasite, forming a vacuole. Parasites are

released into the cytoplasm following disruption of the vacuole and then differentiate into the amastigote forms (Burleigh and Woolsey 2002). In this form, parasites begin to divide by binary fission by 24 hours post infection and double every 12 hours (Tan and Andrews 2002). After four or five days, the first round of replication is complete, host cells have been filled with amastigote parasites, which then differentiate back into trypomastigotes. The host cell ruptures and releases parasites, which travel into the blood and are disseminated throughout the host. Extracellular amastigote stage parasite can also occur due to premature rupture of infected cells or differentiation of extracellular trypomastigotes. Amastigotes can be ingested by macrophages, where they can survive and complete the intracellular cycle (Burleigh and Andrews 1995). When taking a blood meal, the reduviid insect takes up circulating parasites and thereby continues the cycle.

1.2 CHAGAS' DISEASE

Chagas' disease causes the highest disease burden of the tropical diseases in Latin American: in 2002, the Chagas' disease mortality stratum was estimated as 2.7 times that of malaria, schistosomiasis, leishmaniasis, and leprosy combined (World_Health_Organization 2004). The World Health Organization estimates that approximately 10 million people are infected with *T. cruzi* resulting in 3.0–3.3 million symptomatic cases and an annual incidence of 217,000 cases in 15 countries; an estimated 667,000 disability-adjusted life years are attributed to Chagas' disease (Morel and Lazdins 2003; Mathers, Ezzati et al. 2007). While the most common route of infection is by the reduviid bug, congenital (mother to fetus) transmission is also a major route of transmission (Buekens, Almendares et al. 2008). Furthermore, *T. cruzi* can be transmitted

through blood or organ transplantation. Several instances of transplant or blood transfusion associated Chagas' disease in the United States have led to the development and FDA approval of an ELISA based screen of blood donations for parasite reactive antibodies (Leiby, Herron et al. 2002).

In many rural areas of Latin America, people live in housing made with mud walls and thatched housing in which the reduviid bugs can live (Figure 2).

Figure 2: Mud walls used in housing in rural Brazil (2005). Pictures used with permission from Wendell Meira.

This close association between infected insects and humans is associated with a high rate of *T. cruzi* infection in these areas (Gurtler, Kitron et al. 2007). To combat the domestic *Triatoma infestans* reduviid bug, which is a major source of *T. cruzi* in many of these areas, governments have formed the "southern cone initiative" to interrupt vector-borne transmission through insecticidal spraying of houses (Dias, Silveira et al. 2002). The necessity for repeat spraying poses the danger of insecticidal resistance, which has been reported in Argentina (Picollo,

Vassena et al. 2005). Vector control is further complicated by the many different vector species in some endemic countries (Tarleton, Reithinger et al. 2007). Potentially, improvements to housing could permanently reduce domestic vector transmission. But the cost, estimated at $200-2000 per home, are daunting in economically underdeveloped endemic areas (Tarleton, Reithinger et al. 2007). Yet, without these changes eradication of the domestic transmission of *T. cruzi* is unlikely (Gurtler, Kitron et al. 2007).

Acute phase Chagas' disease is characterized by mild symptoms including fever and malaise with occasional progression to symptomatic myocarditis and death. Drug treatment for Chagas is somewhat effective during acute phase disease, but lack of defining symptoms, cost, and poor tolerance, limit the use of these drugs (Viotti, Vigliano et al. 2009). Generally, acute phase symptoms resolve spontaneously, leading to an asymptomatic indeterminate phase with subpatent parasitemia and continued positive serology. Epidemiologic studies estimate up to thirty percent of those infected will progress from indeterminate phase to develop CD (Marin-Neto, Cunha-Neto et al. 2007).

Immune control resolves patent parasitemia during acute phase CD, but tissue infection persists for the life of the host and leads to chronic phase disease in as many as 30 percent of infected individuals (Tarleton 2001). Parasite persistence, measured by amplification of parasite DNA in cardiac lesions, is associated with the extent of inflammation, fibrosis, and progression of cardiac CD (Kirchhoff 2001; Schijman, Vigliano et al. 2004). Cardiac symptoms include diffuse myocardial damage and cardiac conduction defects (Voorhis 2000). In addition to cardiac manifestations, CD can also present as mega-esophagus or mega-colon, and some patients may

have both cardiac and gastro-intestinal (GI) symptoms (da Silveira, Lemos et al. 2007). In the GI, CD results in dilation and obstruction of the affected organs (colon, esophagus). Development of mega-colon after acute infection with *T cruzi* is associated with maintained invasion of enteric ganglia with cytotoxic T cells and loss of muscle innervations (da Silveira, Lemos et al. 2007). Drug treatment during chronic Chagas' disease (CD) is controversial due to relatively low efficacy at this stage and the absence of specific criteria for determining cure, since parasitemia is predominately undetectable and invasive procedures would be necessary to estimate parasite tissue burden (Viotti, Vigliano et al. 2006; Bern, Montgomery et al. 2007; Perez-Molina, Perez-Ayala et al. 2009). The lack of consensus regarding treatment for CD and the difficulty of assessing cure emphasize the need for the development of effective preventative measures to combat *T. cruzi* infection.

1.3 MOUSE MODELS OF CHAGAS' DISEASE

Due to the difficulties of human studies, the majority of research regarding immune control of parasite infection has been done in mouse models of disease, which develop detectable parasitemia during acute infection followed by chronic tissue parasitism that mimics human disease (Trischmann 1986; Russo, Starobinas et al. 1988; Sun and Tarleton 1993; Zhang and Tarleton 1999). Different strains of mice exhibit different kinetics and magnitude of acute disease symptoms (Trischmann, Tanowitz et al. 1978; Trischmann 1983; Haolla, Claser et al. 2009), which also vary depending on the strains of parasite used (Andrade, Barral-Netto et al. 1985). In general, susceptible mice exhibit higher parasitemia and increased mortality compared to resistant mice during acute infection and increased tissue parasitism during CD (Trischmann

1983; Russo, Starobinas et al. 1989; Powell and Wassom 1993; Tarleton, Grusby et al. 2000). Resistance versus susceptibility to *T. cruzi* in murine models has been linked to a variety of immune effector mechanisms (Minoprio 2002; Tarleton 2007; de Meis, Morrot et al. 2009).

1.4 CHAGAS' DISEASE IMMUNE EFFECTORS MECHANISMS

Control of *T. cruzi* infection depends on innate and acquired immunity, requiring the participation of macrophages, NK cells, T and B lymphocytes, and the production of cytokines, which play key roles in regulating both parasite replication and immune response (Zhang and Tarleton 1996). Mice lacking B cells, T cells, NK cells, IFN-γ, or IL-12 experience increased susceptibility to parasite infection compared to wild-type controls (Sardinha, Elias et al. 2006).

Survival of parasites within macrophages as well as non-phagocytic cells necessitates the induction of cell-mediated effector mechanisms to kill intracellular parasites (DosReis 1997). Induction of NK cells and helper T cells producing type I cytokines, particularly interferon gamma and interleukin 2 is critically important in immunity to *T. cruzi* in the mouse model (Hoft, Schnapp et al. 2000; Sardinha, Elias et al. 2006). NK cell activation has been detected in the spleen, peritoneal exudates, and liver early in infection (Lieke, Graefe et al. 2004; Duthie, Cetron et al. 2005; Newman and Riley 2007). Infection with *T. cruzi* activates cytokine secretion as well as lytic pathways of splenic NK cells and these cells can also develop contact-dependent effector mechanisms against free parasite during infection that leads to parasite lysis (Lieke, Graefe et al. 2004).

Humoral immunity has been shown to be important for control of parasite infection as mice lacking B cells (μMT mice) experience increased parasitemia and mice succumb to otherwise non-lethal infection (Kumar and Tarleton 1998). Adoptive transfer of antibodies in serum from chronic stage *T. cruzi* infected mice to acutely infected mice leads to rapid clearance of parasite from circulation (Brodskyn, Silva et al. 1989). Transfers of splenocytes from mice that have recovered from acute phase infection to naïve mice leads to protection against lethal *T. cruzi* infection, which is abolished by removal of B lymphocytes, but relatively insensitive to T cell or macrophage depletion (M.T. SCOTT 1981). These data indicate that effective *T. cruzi* humoral responses do develop and indicate that an early parasite-specific humoral response is capable of imparting resistance and clearance of blood stream parasite. Thus, developments of antibody mediated vaccines strategies are viable and desirable.

In general, T helper type 1 (Th1) responses increase protection and type 2 (Th2) exacerbate CD (Hoft, Schnapp et al. 2000; Tarleton, Grusby et al. 2000; Kumar and Tarleton 2001; Hoft and Eickhoff 2005). Direct evidence for this dichotomy was reported in Stat4 deficient mice, which show an increase in susceptibility and Sta6-deficient mice, which have increased resistance to *T. cruzi* infection (Tarleton, Grusby et al. 2000). Furthermore, *T. cruzi* antigen specific Th1 but not Th2 cells provide protection from lethal infection (Kumar and Tarleton 2001). Th1 IFN-γ responses early during infection are associated with resistance to infection and control of parasitemia, whereas late IFN-γ may participate in chronic inflammation and disease progression (Antunez and Cardoni 2000; Hoft, Schnapp et al. 2000). Th1 type T cells and their cytokines likely play important roles in macrophage activation and nitric oxide-mediated killing of *T. cruzi* in macrophages, as well as by providing help for antibody production

and CTL responses. CD8+ T cells have also been shown to be of prime importance since antibody-mediated depletion of and genetic knockout of CD8 causes mice to be exquisitely sensitive to *T. cruzi* infections and they are unable to survive low dose parasite challenge when lacking CD8 by either method (Padilla, Bustamante et al. 2009).

1.5 IMMUNE EVASION DURING ACUTE CHAGAS' DISEASE

Pathogens that cause persistent infections, such as *T. cruzi,* are adept at altering the host immune system to avoid efficient detection or clearance. The successful infection and persistence of *T. cruzi* in the mammalian host is due in part to the capacity of the parasite to evade multiple aspects of the innate and acquired host immune responses (reviewed in (Voorhis 2000)). Polyclonal lymphocyte activation and complement resistance are two mechanisms of parasite evasion that are thought to contribute to parasite immune evasion during blood stage infection. Both of these mechanisms may facilitate dissemination of the parasite throughout the host, as detailed in the next sections.

1.5.1 Polyclonal Lymphocyte Activation

Several studies have shown that immunosuppressive and mitogenic substances play a significant role in immune evasion strategies of pathogens (Montes, Acosta-Rodriguez et al. 2007). Polyclonal lymphoctye activation have been associated with many types of viral, bacterial, fungal and parasitic infections and B cell mitogenic activity is often associated with immunosuppressive activity (Coutinho, Gronowicz et al. 1974; Arala-Chaves, Ribeiro et al.

1986; Ferreira, Soares et al. 1988; Ferreira, Bras et al. 1997 Reina-San-Martin, 2000 #171). Polyclonal B cell activation of lymphocytes could inhibit effective humoral responses through increased production of non-pathogen specific antibodies that effectively dilute antigen specific responses, through increased activation of pathogen-specific clones, leading to exhaustion and anergy, and/or through production of cytokines which affect other immune effector mechanisms.

Indeed, polyclonal B cell activation that leads to hypergammaglobulinemia and delayed specific humoral immune response is generally accepted as a characteristic of acute phase Chagas' disease in humans and is reported in rodent experimental models of *T. cruzi* infection (Cunningham, Kuhn et al. 1978; Teixeira, Teixeira et al. 1978; Brener 1980; Reina-San-Martin, Cosson et al. 2000; Minoprio 2002). The acute phase polyclonal response to *T. cruzi* infection is associated with undetectable antigen specific responses in hybridomas made with splenic B cells after the first week of *T. cruzi* infection in mice (Reina-San-Martin, Cosson et al. 2000) and parasite specific antigen responses are often delayed several weeks post-infection in experimental models (Rowland, Mikhail et al. 1992; el Bouhdidi, Truyens et al. 1994). Studies indicate that B cell expansion in the spleen and lymph nodes during acute *T. cruzi* infection is associated with polyclonal, rather than specific responses (Minoprio, Eisen et al. 1986; Minoprio, Bandeira et al. 1989). Several parasite encoded mitogenic proteins (TcPRAC, *T. cruzi* trans-sialidase, *T. cruzi* malate dehydrogenase, Tc24) and mitogenic fractions (alkaline antigen) have been identified, but the relative contribution of these proteins to the observed polyclonal B cell activation during experimental *T. cruzi* infection has yet to be elucidated (Da Silva, Espinoza et al. 1998; Montes, Zuniga et al. 1999; Reina-San-Martin, Degrave et al. 2000; Gao, Wortis et al. 2002; Montes, Zuniga et al. 2002; Buschiazzo, Goytia et al. 2006). The analysis of relative

contribution of various mitogenic factors to immune evasion during *T. cruzi* infection has been hampered by the need to more fully understand how these factors interact with B cells and how those interactions may lead to immune evasion, as well as the difficulty of developing methods to test their effect in vivo. Generation of specific responses that neutralize mitogenic factors or genetic manipulations of the parasite to modulate the level of expression of these factors are technically challenging. In addition, several of the mitogenic factors have several functions during *T. cruzi* infection, so determining their effect on B cell activation is often inseparable from their other activities, which hampers analysis of experimental outcomes. Of the reported mitogenic factors from *T. cruzi*, the best characterized of them is a proline racemase (TcPRAC).

1.5.1.1 *T. cruzi* proline racemase (TcPRAC)

T. cruzi proline racemase (TcPRAC) was discovered through analysis of tissue culture trypomastigote supernatant for proteins that were able to stimulate proliferation in athymic and T-cell depleted splenocytes (Reina-San-Martin, Degrave et al. 2000; Buschiazzo, Goytia et al. 2006). TcPRAC is encoded by two independent homologous genes per haploid genome: *TcPRACA* and *TcPRACB* (Reina-San-Martin, Degrave et al. 2000). A small number of point mutations and the deletion of the amino terminal secretion signal distinguish TcPRACB from TcPRACA, although they contain the same active sites. TcPRACB has been found in the cytoplasm of non-infective epimastigotes. TcPRACA is secreted from the infectious trypomastigotes (Reina-San-Martin, Degrave et al. 2000).

TcPRACA proline racemase activity relies on dimerization; interconversion of L- and/or D-proline enantiomers by a two-base mechanism reaction uses one sulfhydryl group of the 330 Cys residue in the active site from each subunit (Buschiazzo, Goytia et al. 2006). Initial studies

suggested that mitogen activity of TcPRAC was dependent upon racemase functionality (Chamond, Gregoire et al. 2003), however Buschiazzo et al. have recently shown that these activities are separable (Buschiazzo, Goytia et al. 2006). Mutational analysis showed that racemase activity is dissociated from mitogenic activity and that mitogenic activity is likely dependent upon conformation-dependent epitopes of the native protein. Substitution of either catalytic residue abrogated racemase activity but did not hinder the mitogenic activity of the dimeric open configuration of the protein. In contrast, incubation with a competitive inhibitor of racemase activity, pyrrole-2-carboyxlic acid (PYC), causes the protein to assume the closed configuration and leads to the loss of mitogenic activity. Investigation of knock-down or over-expression of TcPRACA in parasites has provided evidence that expression of TcPRAC plays an essential role in parasite survival, infectivity and differentiation (Chamond, Goytia et al. 2005). Parasites with knock-down of TcPRACB were unable to survive, and parasites with knock-down of TcPRACA were less able to differentiate into the infective metacyclic form of the parasite. All together, these data suggest that TcPRAC is an important virulence factor during *T. cruzi* infection, with the capacity to induce or trigger polyclonal B cell activation during *T. cruzi* infection, as well as to mediate infectivity and parasite survival. Thus, this protein is an attractive vaccine target, as immunologic neutralization of this protein may benefit the host in multiple ways.

1.5.2 Evasion of Complement-Mediated Killing of *T. cruzi.*

As with other blood-dwelling pathogens, *T. cruzi* resistance to complement-mediated lysis is a developmentally regulated characteristic of the infectious stage. The insect forms (epimastigotes) are highly sensitive to lysis via the alternate complement pathway, whereas the

blood-form trypomastigotes are resistant (Nogueira, Bianco et al. 1975). Norris et al have characterized a parasite surface protein, which is able to confer complement resistance, *T. cruzi* complement regulatory protein (CRP) (Norris, Bradt et al. 1991). Complement-mediated lysis of trypomastigotes can occur via an antibody-dependent mechanism, yet lysis proceeds mainly via the alternate complement pathway (Krettli, Weisz-Carrington et al. 1979). $F(ab')_2$ and Fab fragments prepared from Chagasic patient antibodies were shown to be nearly as efficient as intact IgG in complement-mediated lysis assays under conditions where only the alternative pathway was active (> 90% cell lysis) (Kipnis, Krettli et al. 1985). Taken together, these results indicate that the in vitro lytic capacity of these anti-*T. cruzi* antibodies is related to their antigen binding sites rather than complement fixation via the Fc region of bound antibodies, suggesting that these antibodies may block the complement inhibitory activity of regulatory *T. cruzi* surface proteins, such as CRP. Indeed, antibodies that induce parasite lysis (lytic antibodies) have been shown to bind specifically to CRP and are associated with host protection (Norris, Harth et al. 1989). Furthermore, lytic antibodies are generated by immunization with CRP DNA (Sepulveda, Hontebeyrie et al. 2000). Thus, CRP is a marker of an effective parasite-specific humoral response and an attractive vaccine target, as strong humoral responses to CRP can generate lytic antibodies, which can block the complement regulatory function of this parasite surface protein, allowing for complement mediated lysis of blood-stream parasite.

1.5.2.1 *T. cruzi* complement regulatory protein (CRP)

Norris et al have shown that *T. cruzi* complement regulatory protein (CRP) inhibits the formation and stability of the C3 convertase (Norris, Bradt et al. 1991). CRP has a strong binding affinity for human complement components C3b and C4b, similar to the family of mammalian regulators of complement activation, such as DAF, Factor H and CR1 (Norris, Bradt et al. 1991). There are

14 full-length and 6 partial copies of CRP in the *T. cruzi* genome (Beucher and Norris 2008). It was determined that the predicted amino terminus of the protein was highly conserved among all family members whereas most of the diversity was found within the terminal 1/3 of the protein (Beucher and Norris 2008). Full-length genes from the most diverse and most similar groups were cloned and recombinant protein from each was shown to have complement regulatory activity through an ELISA based decay acceleration assay (Beucher and Norris 2008). While immunization studies demonstrate that genetic immunization with CRP generates lytic antibodies (Sepulveda, Hontebeyrie et al. 2000), future studies are necessary to determine the complement binding site of CRP as well as determining the cross reactivity of CRP-specific lytic antibodies with the diverse copies of CRP that are present in the *T. cruzi* genome.

1.6 CHAGAS' DISEASE VACCINE DEVELOPMENT

Despite some successful efforts to control the insect vectors in endemic areas, Chagas' disease remains an intractable, chronic infection afflicting approximately 12 million people in Latin America. Over 90 million people are considered at risk for infection and treatment regimens are of limited use, particularly once chronic phase symptoms have occurred (Morel and Lazdins 2003). As most individuals have sub-clinical symptoms during the early acute stage of disease, when drug treatment would be most effective, diagnoses are not generally made until after clinical symptoms of the chronic disease are apparent and irreversible damage is done. Historically, vaccine development against *T. cruzi* infections has not been vigorously pursued because of the possibility of an autoimmune component to the pathology, which arose due to the identification of host-parasite cross-reactive antigens (Kierszenbaum 2003). Convincing

evidence of an autoimmune etiology remains largely lacking (Voorhis 2000), although a recent study indicates a role for cross-reactive T cells isolated after chronic infection and then transferred to a naïve host, were able to imitate pathology in the absence of parasites (Girones, Carrasco-Marin et al. 2007). In contrast, Tarleton and coworkers demonstrated in a series of carefully controlled studies that persistence of parasites at the site of disease correlates with inflammation, leading to the parasite persistence theory, which hypothesizes that parasite persistence leads to chronic disease manifestations, such as heart and/or gut enlargement and dysfunction. In this scenario, immune-mediated clearance of the parasites at early stages of infection would ameliorate or prevent chronic disease (Zhang and Tarleton 1999; Tarleton 2001). The parasite persistence theory of disease development does not exclude the potential role of autoimmunity; rather, parasite persistence suggests that inflammation associated with chronic infection causes damage, one part of which may be mediated by autoimmune reactions (Zhang and Tarleton 1999; Tarleton 2001). In both cases, early clearance of the parasite is advantageous to diminish pathology due to CD.

In order to potentiate immune clearance of *T. cruzi,* vaccine design has followed research showing immune control of *T. cruzi* requires multiple effector mechanisms: activated macrophages, CD4+ and CD8+ T cells, and high levels neutralizing antibody production (Miyahira, Kobayashi et al. 1999; Sepulveda, Hontebeyrie et al. 2000; Wrightsman, Luhrs et al. 2002). As with many immunization strategies, the approach to *T. cruzi* immunization has been focused primarily on the introduction of immunodominant targets that would create a strong secondary response upon primary infection with parasite (Wizel, Nunes et al. 1997; Costa, Franchin et al. 1998; Wizel, Garg et al. 1998; Quanquin, Galaviz et al. 1999; Fujimura, Kinoshita

et al. 2001; Schnapp, Eickhoff et al. 2002; Wrightsman, Luhrs et al. 2002; Boscardin, Kinoshita et al. 2003; Fralish and Tarleton 2003).

Several different types of vaccines have been tested in murine models for protection from *T. cruzi* infection. Vaccines tested in mice include live nonvirulent strains, whole protein preparation, and recombinant subunit vaccines using protein, DNA, or viral vector delivery through several different routes of inoculation (Miyahira, Kobayashi et al. 1999; Quanquin, Galaviz et al. 1999; Garg and Tarleton 2002; Schnapp, Eickhoff et al. 2002). The most popular methods remain recombinant protein or DNA subunit vaccines through intramuscular/intradermal injection or gene-gun delivery as they allow for administration of single or multiple antigens. In recent years, several parasite encoded proteins have shown promise as vaccine targets, including *T. cruzi* CRP (Cazorla, Frank et al.).

1.6.1 CRP DNA immunization results in protection from lethal challenge

Modulation of complement sensitivity at different stages of the *T. cruzi* life cycle has been demonstrated. Complement resistance in blood form parasite as been linked to expression of CRP (Section 1.5.2) and parasite lytic antibodies have a high level of CRP reactivity; therefore, Norris et al set out to determine if an effective CRP-specific response could be generated through immunization. Evidence that CRP immunization could generate an effective humoral response was first demonstrated through raising polyclonal antibodies in response to *T. cruzi* derived CRP protein immunization. These antibodies were able to support high levels of in vitro complement-mediated lysis of trypomastigotes, as well as block the binding of CRP to C3b in vitro (Norris, Bradt et al. 1991). Isolation of CRP from the parasite is not a viable method for large scale

immunization, due to the technical difficulty and cost of this approach. In order to pursue further studies of CRP, Norris et al have generated recombinant CRP, which has complement regulatory activity (Norris 1998; Beucher, Meira et al. 2003). Intramuscular immunization of mice with plasmid DNA encoding recombinant CRP (rCRP) generated CRP-specific IgG response of mixed isotype. Splenocytes from rCRP-immune mice generate IFN-γ upon restimulation with rCRP protein. rCRP DNA immunization protected mice from challenge with an otherwise lethal dose of *T. cruzi* (Sepulveda, Hontebeyrie et al. 2000). In contrast, no protection was evident when rCRP purified from *E. coli* was used as an immunogen, suggesting that native folding and/or glycosylation is important for development of a protective humoral response. While significant protection from lethal challenge was achieved by vaccination with CRP, vaccination reduced but did not prevent parasitemia or chronic parasite persistence. Thus, it would be highly desirable to combine CRP with other vaccine targets to generate a final product capable of fully protecting the host from CD.

1.7 MITOGEN AS A VACCINE TARGET

Classical approaches to vaccine development focus on the induction of a robust secondary response to immunodominant microbial epitopes and the consequences of pathogen immune evasion strategies are not often considered. Despite effective immunization, protection from challenge infection may not be optimally achieved in cases where the pathogen induces a potent polyclonal B cell response that may delay secondary responses and dilute the existing immune effector mechanisms generated by vaccination (Reina-San-Martin, Cosson et al. 2000; Radwanska, Guirnalda et al. 2008). Yet, immunization studies with mitogens from several

pathogens have suggested an antigen-specific response to these mitogens may also be generated (Coutinho, Gronowicz et al. 1974; Lima, Bandeira et al. 1992; Tavares, Ferreira et al. 1995; Tavares, Ferreira et al. 2003). Effective in vivo immune responses to these proteins, which have demonstrated mitogenic activity in vitro, can decrease immune evasion and may improve host immunity to other antigens (Tavares, Salvador et al. 1993; Tavares, Ferreira et al. 1995; Minoprio 2001; Tavares, Ferreira et al. 2003; Dinis, Tavares et al. 2004). There is generally an inverse correlation between the dose of mitogen and polyclonal activation. Injection of low doses of mitogen induces an antigenic response to the mitogen, whereas higher doses of the mitogen induce polyclonal activation and non-specific responses (Coutinho, Gronowicz et al. 1974). This principle further demonstrated by Tarvares et al., in experiments showing that intradermal inoculations with low doses of a *Candida albicans* mitogen, p43, induce specific antibody response, whereas high dose inoculation induce a mitogenic response (Tavares, Ferreira et al. 2003). Similar results were also reported with *Streptococcus* B cell mitogens (Dinis, Tavares et al. 2004). A similar approach may be useful in generating a specific response to *T. cruzi* proteins with mitogenic activity, such as TcPRAC.

1.8 DNA IMMUNIZATION

DNA vaccines introduce plasmid DNA with the genes of interest under a eukaryotic promoter, the most effective of which is the CMV immediate early enhancer/promoter (Nagata, Aoshi et al. 2004). Genetic immunization is an appealing route for antigen delivery because of the ease of generation, stability of DNA plasmids, and the ability to generate robust cellular and humoral responses. Furthermore, DNA vaccines are advantageous over other types of vaccines due to

their ability to induce cytotoxic T lymphocytes and Th1 lymphocytes, without utilizing live organisms (Nagata, Aoshi et al. 2004). Intramuscular (i.m.) DNA immunization is associated with myocyte transfection and uptake of DNA by APC (Coelho-Castelo, Santos Junior et al. 2003; Liu 2003; Nagata, Aoshi et al. 2004). Intradermal (i.d.) injection and gene-gun bombardment immunizations involve delivery of DNA to epidermal Langerhans cells and keratinocytes (Raz, Carson et al. 1994). After intradermal delivery of DNA, keratinocytes are able to produce IL-1 and TNF-α, which can activate lymphocytes, macrophages, and dendritic cells (Raz, Carson et al. 1994). Langerhans cells move from the site of DNA uptake to the draining lymph nodes, where they present antigen to naïve T and B cells (Robinson and Torres 1997). Recently, particle-mediated injection of DNA has been shown to yield high titer antibody responses to many antigens with less DNA and fewer injections than with i.m. or i.d. needle inoculation (Yoshida, Nagata et al. 2000).

1.9 MURINE B CELL SUBSETS

Marginal zone (MZ) and follicular (FO) B cells constitute two functionally and anatomically distinct B cell subsets within the spleen (Oliver, Martin et al. 1997; Suarez, Lortholary et al. 2006; Allman and Pillai 2008; Radwanska, Guirnalda et al. 2008; Malkiel, Kuhlow et al. 2009).

MZ B cells ($CD19^{+}CD23^{-/low}CD21^{high}CD24^{high}CD9^{+}IgM^{high}IgD^{low}$) are primarily located at the marginal sinus of the spleen, although they also shuttle back and forth to the follicles (Martin and Kearney 2002; Cinamon, Zachariah et al. 2008). As peripheral blood enters the spleen through the marginal sinus, MZ B cells are first line responders to pathogens in the blood.

MZ B cells are more responsive to TI-antigens, respond quickly with natural antibody responses, and generate short-term plasma cells (Won and Kearney 2002). Short-term plasma cells generate large quantities of Ig quickly, are not typically thought to generate long term immunity or memory responses. Upon migration to the follicles MZ are also able to participate in germinal center T cell dependent (TD) reactions that may lead to long-term plasma cell formation or generation of specific memory B cells (Song and Cerny 2003). During this process, MZ B cells are also able to transport captured Ag and induce CD4 T cell expansion, stimulating CD4 T cells to produce high levels of polarizing cytokines (Attanavanich and Kearney 2004).

FO B cells ($CD19^{+}CD23^{high}CD21^{int}CD24^{neg}IgM^{low}IgD^{high}$) circulate through the lymph and are found in B cell follicles of the spleen. FO B cells respond to TD antigen and can become long-term plasma cells or memory B cells (Fairfax, Kallies et al. 2008). Due to their differential location and function, MZ and FO B cell subsets can have distinct roles in the development of long or short term pathogen-specific versus polyclonal B cell responses to pathogens (Gatto, Ruedl et al. 2004; Suarez, Lortholary et al. 2006; Radwanska, Guirnalda et al. 2008; Malkiel, Kuhlow et al. 2009). Analysis of B cell subsets and discussion of their potential roles in parasite-specific versus polyclonal B cell activation due to parasite derived mitogens or due to experimental infection have not been previously reported in the *T. cruzi* literature. In contrast, differential activation of MZ versus FO B cell subsets by LPS, a TLR4 dependent B cell mitogen, have indicated that these cells have intrinsic differences in their response to mitogenic stimuli (Meyer-Bahlburg, Bandaranayake et al. 2009) and display altered population profiles in response to different *Borrelia* species(Belperron, Dailey et al. 2007; Malkiel, Kuhlow et al. 2009). We hypothesize that these B cell subsets may have differential responsiveness to *T. cruzi*

mitogens, such as TcPRAC, and differentially expand in different experimental models of infection. The characterization of these differences may help us to better understand how to effectively immunize animals to generate the most effective humoral immune response against *T. cruzi*.

1.10 CONCLUSION

Chagas' disease, caused by *Trypanosoma cruzi*, remains endemic in many parts of Latin American, despite efforts by several governments to eradicate the insect vector. Treatment options for Chagas' disease are limited to acute phase disease and are poorly tolerated and expensive. It is now well established that disease progression is related to chronic inflammation due to parasite persistence within host tissues. Thus, many researchers are focusing on understanding effective versus adverse immune responses associated with Chagas' disease. In this study, we investigate parasite-specific humoral immunity versus polyclonal B cell activation during experimental *T. cruzi* infection in mouse models and in response to a parasite encoded mitogen. The ultimate purpose of these studies is to contribute to the rational design of immunization protocols that will prevent CD through immune effector mechanisms that target both the intracellular and extracellular, blood stage, parasite.

1.11 STATEMENT OF PURPOSE

As described above, polyclonal B cell activation that leads to hypergammaglobulinemia and delayed specific humoral immune response is generally accepted as a characteristic of acute phase Chagas' disease in humans and is reported in rodent experimental models of *T. cruzi* infection (Cunningham, Kuhn et al. 1978; Teixeira, Teixeira et al. 1978; Brener 1980; Reina-San-Martin, Cosson et al. 2000; Minoprio 2002). Preliminary data from our laboratory suggests that parasite-specific humoral responses to *T. cruzi* have differential kinetics and magnitude in relatively susceptible versus resistant mice, indicating a more complex host-pathogen reaction than has been generally acknowledged in the recent literature regarding *T. cruzi* humoral immunity. *Thus, we hypothesize that inoculation with doses of parasite that lead to similar timing and magnitude of initial parasitemia, susceptible and resistant mice will have measurable differences in parasite-specific and polyclonal humoral response*. TcPRAC has been shown to be a B cell polyclonal activator and is expressed by blood stream *T. cruzi* upon infection of a host, indicating it may contribute to B cell mitogenicity (Reina-San-Martin, Degrave et al. 2000; Chamond, Gregoire et al. 2003; Chamond, Goytia et al. 2005). Therefore, *we propose to further investigate in vitro B cell activation due to rTcPRACA (surface expressed/secreted protein) to better understand how it may contribute to the observed B cell mitogenic response during T. cruzi infection.* As a B cell mitogen, TcPRAC is an important virulence factor associated with infectivity and immune evasion. Immunologic neutralization of *T. cruzi* encoded B cell mitogen(s) presents an attractive proposition as a mitogen-specific host response could reduce polyclonal B cell activation during early *T. cruzi* infection (Reina-San-Martin, Cosson et al. 2000). Therefore, *this study will test the hypothesis that genetic immunization with rTcPRACA DNA can deliver TcPRAC as an antigen rather than a mitogen, resulting in the, generation of*

specific antibodies that bind to mitogenic protein. These hypotheses will each be tested in three separate specific aims.

Specific Aim 1: Evaluation of humoral responses to *T. cruzi* infection in relatively resistant C57Bl/6 and susceptible Balb/c mice.

To address Specific Aim 1, Balb/c and C57Bl/6 mice were infected with isolates of Y-strain parasite that correspond to an approximate 0.25 x LD50 in each mouse strain. Humoral responses in these mice were analyzed in serum and splenocyte populations. To further understand these responses, mice were analyzed for circulating cytokine levels compared to controls. The results of aim 1 are described in Chapter 2.

Specific Aim 2: Characterize the mitogenic capacity of rTcPRAC in vitro.

To address Specific Aim 2, Non-secreted *TcPRACA* was PCR amplified from tissue culture trypomastigote DNA and inserted into an expression vector to generate a recombinant His tagged protein (rTcPRAC). Polyclonal B cell activation was determined by in vitro stimulation assays to analyze B cell proliferation, antibody secretion, and cytokine production in response to rTcPRAC. Purified splenic B cells populations were evaluated to determine responsiveness to rTcPRAC. The results of aim 2 are described in Chapter 3.

Specific Aim 3: Test the hypothesis that *rTcPRACA* DNA immunization will deliver rTcPRACA as an immunogen without causing a B cell mitogenic response.

To address Specific Aim 3, *rTcPRACA* was cloned into a eukaryotic expression vector that was then delivered to the dermis of naïve mice via gene-gun (GG). Humoral responses were

evaluated after immunization to evaluate TcPRAC-specific immunity. To further determine the utility of GG immunization with *rTcPRACA* DNA in a multivalent vaccine approach, co-immunization with another *T. cruzi* antigen, *CRP*, was evaluated. The results of aim 3 are described in Chapter 4.

The appendix of this thesis contains additional observations made in the course of this study that fell outside the scope of the specific aims. These sections describe A) intradermal CRP DNA immunization via GG B) outcomes after parasite challenge of TcPRAC, CRP, or combined GG immunization regimens.

2.0 DECREASED POLYCLONAL B CELL ACTIVATION ASSOCIATED WITH IMPROVED SPECIFIC HUMORAL IMMUNITY TO *TRYPANOSOMA CRUZI* IN RESISTANT TH1 SKEWED MICE

Portions of this chapter are under review at PLoS Neglected Tropical Diseases. The authors of this submission are Marianne A. Bryan, Siobhan E. Guyach, and Karen A. Norris.

2.1 ABSTRACT

The etiologic agent of Chagas' disease is *Trypanosoma cruzi*. Acute infection results in patent parasitemia and polyclonal lymphocyte activation. Polyclonal B cell activation associated with hypergammaglobulinemia and delayed specific humoral immunity has been reported during *T. cruzi* infection in experimental mouse models. Based on preliminary data from our laboratory we hypothesized that variances in susceptibility to *T. cruzi* infections in murine strains is related to differences in the ability to mount parasite-specific humoral responses rather than polyclonal B cell activation during acute infection.

Relatively susceptible Balb/c and resistant C57Bl/6 mice were inoculated with doses of parasite that led to similar timing and magnitude of initial parasitemia. Longitudinal analysis of parasite-specific and total circulating antibody levels during acute infection demonstrated that

C57Bl/6 mice developed parasite-specific antibody responses by 2 weeks post-infection with little evidence of polyclonal B cell activation. The humoral response in C57Bl/6 mice was associated with differential activation of B cells and expansion of splenic $CD21^{high}CD23^{low}$ marginal zone like (MZ) B cells that coincided with parasite-specific antibody secreting cell (ASC) development in the spleen. In contrast, susceptible Balb/c mice demonstrated early activation of B cells and early expansion of MZ B cells that preceded high levels of ASC without apparent parasite-specific ASC formation. Cytokine analysis demonstrated that the specific humoral response in the resistant C57Bl/6 mice was associated with early T-cell helper type 1 (Th1) cytokine response, whereas polyclonal B cell activation in the susceptible Balb/c mice was associated with sustained Th2 responses and delayed Th1 cytokine production. The effect of Th cell bias was further demonstrated by differential total and parasite-specific antibody isotype responses in susceptible versus resistant mice. T cell activation and expansion were associated with parasite-specific humoral responses in the resistant C57Bl/6 mice.

The results of this study indicate that resistant C57Bl/6 mice had improved parasite-specific humoral responses that were associated with decreased polyclonal B cell activation. In general, Th2 cytokine responses are associated with improved antibody response. But in the context of parasite infection, this study shows that Th2 cytokine responses were associated with amplified polyclonal B cell activation and diminished specific humoral immunity. These results demonstrate that polyclonal B cell activation during acute experimental Chagas' disease is not a generalized response and suggest that the nature of humoral immunity during *T. cruzi* infection contributes to host susceptibility.

2.2 INTRODUCTION

The protozoan parasite, *Trypanosoma cruzi* is the etiologic agent of Chagas' disease. Chagas' disease is a chronic and debilitating syndrome that affects millions of people in Latin America. Infection with *T. cruzi* leads to patent parasitemia and systemic spread of the parasite throughout the host during acute phase disease. Immune control resolves patent parasitemia, but tissue infection persists for the life of the host and leads to chronic phase disease in as many as 30 percent of infected individuals (Tarleton 2001). Due to the difficulties of human studies, the majority of research regarding immune control of parasite infection has been done in experimental murine models, which develop detectable parasitemia during acute infection followed by chronic tissue parasitism that mimics human disease.

Control of *T. cruzi* infection depends on clearance of blood stream parasite through both innate and acquired immune mechanisms. Macrophages, NK cells, T and B lymphocytes, and the production of cytokines, which play key roles in regulating both parasite replication and immune response (Zhang and Tarleton 1996), are required to control parasitemia, as the depletion or absence of any given innate or adaptive effector mechanism leads to increased parasitemia and susceptibility to disease (Budzko, Pizzimenti et al. 1975; Kierszenbaum, Gottlieb et al. 1983; Tarleton 1990; Rottenberg, Rodriguez et al. 1992; Silva, Morrissey et al. 1992; Kumar and Tarleton 1998; Sardinha, Elias et al. 2006).

Humoral immunity is important for control of parasite infection as B cell depletion leads to increased parasitemia and mice succumb to otherwise non-lethal infection (Kumar and Tarleton 1998). Adoptive transfer of antibodies from late stage *T. cruzi* infected mice to naïve

mice leads to rapid clearance of parasite from circulation (Brodskyn, Silva et al. 1989). Transfers of splenocytes from mice that have recovered from acute phase infection to naïve mice confers protection against lethal *T. cruzi* infection, which is abolished by removal of B lymphocytes, but relatively insensitive to T cell or macrophage depletion (M.T. SCOTT 1981). Yet, evidence indicates that the majority of B cells are not parasite-specific during early *T. cruzi* infection (Minoprio, Burlen et al. 1988).

Polyclonal B cell activation that leads to hypergammaglobulinemia and delayed specific humoral immune response is generally accepted as a characteristic of acute phase Chagas' disease in humans and is reported in rodent experimental models of *T. cruzi* infection (Cunningham, Kuhn et al. 1978; Teixeira, Teixeira et al. 1978; Brener 1980; Reina-San-Martin, Cosson et al. 2000; Minoprio 2002). The acute phase polyclonal response to *T. cruzi* infection is associated with delayed specific responses (Reina-San-Martin, Cosson et al. 2000). Different IgG isotypes have been implicated in polyclonal B cell activation and parasite-specific antibody responses (d'Imperio Lima, Eisen et al. 1986; Minoprio, Eisen et al. 1987; Rowland, Mikhail et al. 1992; el Bouhdidi, Truyens et al. 1994). Several parasite encoded mitogenic proteins have been identified, but the role of each has yet to be elucidated (Da Silva, Espinoza et al. 1998; Montes, Zuniga et al. 1999; Reina-San-Martin, Degrave et al. 2000; Gao, Wortis et al. 2002; Montes, Zuniga et al. 2002; Buschiazzo, Goytia et al. 2006). B cell expansion in the spleen and lymph nodes during acute *T. cruzi* infection is associated with polyclonal, rather than specific responses (Minoprio, Eisen et al. 1986; Minoprio, Bandeira et al. 1989). Fas/FasL mediated apoptosis of parasite specific B cells and immature B cells in the bone marrow (BM) has also been reported in Balb/c mice (Zuniga, Motran et al. 2000; Zuniga, Motran et al. 2002; Zuniga,

Acosta-Rodriguez et al. 2005). Studies in Balb/c XID mice showed that the depletion of B cell subsets in this model led to an increased resistance to disease that was associated with improved IFN-γ responses, decreased hypergammaglobulinemia, and a skewed natural antibody repertoire (Minoprio, Coutinho et al. 1991; Minoprio, el Cheikh et al. 1993; Santos-Lima, Vasconcellos et al. 2001). Limited studies of B cell dynamics during *T. cruzi* infection in resistant versus susceptible mice have been reported (d'Imperio Lima, Eisen et al. 1986; Minoprio, Eisen et al. 1986).

Marginal zone (MZ) and follicular (FO) B cells constitute two functionally and anatomically distinct B cell subsets within the spleen (Oliver, Martin et al. 1997; Suarez, Lortholary et al. 2006; Allman and Pillai 2008; Radwanska, Guirnalda et al. 2008; Malkiel, Kuhlow et al. 2009). MZ B cells are located at the marginal sinus of the spleen, making these cells first line responders to pathogens in the blood. MZ B cells are more responsive to TI-antigens, respond quickly with natural antibody responses, and generate short-term plasma cells (Won and Kearney 2002). MZ are also able to migrate to the follicles and participate in germinal center T cell dependent (TD) reactions (Song and Cerny 2003). FO B cells circulate through the lymph and are found in B cell follicles of the spleen. FO B cells respond to TD antigen and can become long-term plasma cells or memory B cells (Fairfax, Kallies et al. 2008). Due to their differential location and function, these two B cell subsets can have distinct roles in the development of specific versus polyclonal B cell responses to pathogens (Gatto, Ruedl et al. 2004; Suarez, Lortholary et al. 2006; Radwanska, Guirnalda et al. 2008; Malkiel, Kuhlow et al. 2009).

While mouse models have been informative for analysis of immune responses to *T. cruzi* infection, inbred mouse strains experience variable disease progression and severity (Trischmann 1983; Haolla, Claser et al. 2009). Disease progression in these models also differ depending upon the strains of parasite used (Andrade, Barral-Netto et al. 1985). In general, Balb/c mice are more susceptible to infection compared to C57Bl/6 mice in terms of increased parasitemia and mortality given a similar parasite challenge (Hoft, Lynch et al. 1993). Kinetic analysis of cytokine production by lymphocytes provides evidence that resistance in C57Bl/6 mice is associated with increased early production of IFN-γ (Hoft, Lynch et al. 1993). Further studies show that immunization protocols capable of inducing polarized Th1 but not Th2 responses are able to protect Balb/c mice against *T. cruzi* challenge, but transfer of CD4 T cell alone was not enough to confer protection to naïve mice (Hoft, Schnapp et al. 2000). While these studies indicate that Th1 versus Th2 responses are correlated with protection versus resistance, the complete profile of effector mechanisms leading to increased resistance in Th1 skewed mice has yet to be elucidated.

In the present study, we analyzed the humoral response to *T. cruzi* experimental infection of susceptible Balb/c versus resistant C57Bl/6 mice. We infected Balb/c and C57Bl/6 mice with isolates from Y-strain parasite that generated similar timing and magnitude of initial patent parasitemia. The kinetics, magnitude, and isotype of the parasite-specific and total circulating antibody responses during acute *T. cruzi* infection were examined. We further evaluated humoral responses in the spleen and the association of parasite-specific antibody secreting cells (ASC) with activation of B cells and expansion of B cell subsets, as well as the expansion and activation of splenic T cells. The combined results of this study demonstrate that resistant C57Bl/6 mice

generated parasite-specific humoral responses that were associated with decreased hypergammaglobulinemia, differential kinetics of splenic B cell activation and B cell subset expansion, improved T cell help, and early IFN-γ production compared to more susceptible Balb/c mice.

2.3 MATERIALS AND METHODS

2.3.1 Parasites and Mice.

Balb/c and C57/Bl/6 mice were obtained from Jackson Laboratories and maintained in specific pathogen free housing. Mice husbandry and procedure protocols were reviewed and performed in accordance with the University of Pittsburgh IACUC. Y-strain parasites were grown in NIH 3T3 fibroblast cells and harvested by standard technique (Sepulveda, Hontebeyrie et al. 2000). Briefly, 3T3 fibroblasts were infected with trypomastigote parasites and cultured for 2 days at 37˚C (5% CO_2) in DMEM (Gibco) supplemented with 10% FBS, 10 mM HEPES, 0.2 mM sodium pyruvate, and 50 μg/mL gentamicin, to allow infection of the growing cells. On day 3, the media was exchanged and the culture was moved to a 34˚C incubator and maintained under anaerobic conditions until harvest of the tissue derived trypomastigotes (TCT) from the culture supernatant. To establish models of infection for analysis of kinetics and magnitude of parasite-specific humoral immunity versus polyclonal B cell activation in resistant C57Bl/6 and susceptible Balb/c mice, we evaluated experimental infection using two variants of *T. cruzi* Y-strain (Y-US and Y-Br). The LD_{50} was established for each model, using intraperitoneal injection of TCT in PBS plus 1% glucose. Survival curves for these infections indicated that the

Balb/c mice were much more susceptible to the Y-Br variant, with an approximately 1000 fold higher LD_{50}, but succumbed to infection with different kinetics than did C57Bl/6 mice (Supplementary Figure 1*A*). At similar LD_{50}, parasitemia was delayed in Y-Br-infected Balb/c compared to Y-Br-infected C57Bl/6 (Figure 26*A* in Appendix C). In contrast, using the Y-US variant, Balb/c mice experienced initial peak parasitemia comparable with C57Bl/6 mice given a similar LD_{50} dose of the Y-Br variant (Figure 26*B* in Appendix C). In both mouse strains, a dose of parasite equal to approximately 0.25 x LD_{50} was used to allow direct comparison of humoral responses in a sub-lethal infection of relatively resistant versus susceptible mice. The approximate 0.25 x LD_{50} dose corresponded to $2.5x10^5$ Y-US in Balb/c mice and $6.25x10^3$ Y-Br in C57Bl/6 mice. Control mice were inoculated with the same dose of parasite after it had been heat-inactivated at 56˚C for 25 minutes. Parasitemia was monitored by applying blood diluted 1/4 in RBC lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 115uM EDTA) to a hemocytometer and counting at 400x. Blood was collected at multiple time-points post infection for analysis by ELISA.

2.3.2 rCRP Cloning.

A full-length cDNA encoding the *T. cruzi CRP* was isolated by reverse transcription-PCR as previously described (Norris, Schrimpf et al. 1997). The *T. cruzi CRP* cDNA encoding the mature protein (starting at nucleotide 303) was subcloned into the pTrcHis expression vector (Invitrogen). *E. coli* strain SURE (Stratagene) were transformed with pTrcHis-CRP DNA for recombinant protein production with a histidine tag, as previously described (Sepulveda, Hontebeyrie et al. 2000). For eukaryotic expression, CRP was cloned into pcDNA3 with the

glycosylphosphatidylinositol (GPI) anchor signal sequence from human decay accelerating factor (daf), (Beucher, Meira et al. 2003).

2.3.3 Gene-gun immunization

pcDNA3_*CRP* DNA was purified from *E. coli* using endotoxin -free Mega prep kits (Qiagen). The DNA was coated on 1.0 μm gold particles (Bio-Rad) and loaded into Tefzel tubing (Bio-Rad) (Wang, Zhang et al. 2008). DNA (8 μg) was administered by Helios Gene-gun (Bio-Rad) at 400 psi, in two shots per mouse on shaved abdomen (Yoshida, Nagata et al. 2000). Four inoculations were performed at monthly intervals. Immunized mice were bled after boosting and the blood processed to obtain sera.

2.3.4 Protein Purification.

Expression of recombinant protein was induced by isopropyl-β-D-thiogalactoside (IPTG) (IBI Scientific, Peosta, IA) in transformed *E. coli* and the cells harvested by centrifugation (6,000 RCF, 10 min., 4˚C). The resulting cleared lysate was prepared under denaturing conditions and bound to cobalt metal affinity resin according to the Talon instruction manual with slight modifications (Clontech, Mountain View, CA). During binding of lysate to resin, 5-10mM imidazole (Sigma) was added into the binding buffer (50mM Tris, 300mM NaCl, pH 7.2). Protein was bound to the resin in batch for 2 hours. The bound protein was further washed and packed into a disposable column. Imidazole (150mM) was used to elute bound protein. Protein concentration in eluted fractions was determined by Bradford assay.

2.3.5 Quantitative IgM and IgG ELISA.

4 HBX Immulon ELISA plates (Thermo Scientific) were coated with 100ng of goat-anti-mouse Ig antibody (SouthernBiotech) overnight at 4°C, washed, and blocked with 1% milk in T-PBS (0.05% Tween-20, 1xPBS), washed and stored at -20°C until use. Serum samples were stored at less than -20°C then thawed and maintained at 4˚C during ELISA analysis. Serum was diluted in milk-T-PBS and applied to coated plates. A standard curve was generated with mouse IgM or IgG (SouthernBiotech, Birmingham, Alabama). Goat anti-mouse IgM or IgG conjugated with HRP was used as the secondary antibody (SouthernBiotech). After incubation with secondary antibody, plates were washed and developed with OPTEIA (BD) and analyzed for color change (OD_{450}). Standard curve fit and calculation of unknowns was performed using Prism software (GraphPad).

2.3.6 rCRP and whole parasite specific ELISA.

For rCRP analysis, 4 HBX Immulon ELISA plates (Thermo Scientific) were coated with 100 ng of purified protein and incubated overnight at 4°C. For whole parasite ELISA, plates were coated with $2x10^5$ heat-inactivated TCT per well and incubated overnight at 4°C. Plates were washed and blocked and stored at -20°C until used. Mouse serum was diluted in block and applied to ELISA plates overnight at 4°C. Plates were washed and developed with the appropriate secondary antibody. The estimated reciprocal endpoint titer (RET) was determined graphically based on the OD_{450} values from equivalent dilution of pooled mouse pre-immunization serum samples. RET was defined as the first dilution with a value below the pre-immune OD_{450} plus

SD (two to three replicates). Alternatively, equivalent units, determined by dividing the OD450 of test serum by the OD450 of pre-immune serum, were reported at a single dilution of sera.

2.3.7 Antibody-secreting-cell (ASC) ELISPOT.

Spleens were processed for single cells, by gentle mashing in a 40μM cell strainer, treated with RBC lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 115uM EDTA), washed with 1 x PBS, and suspended in cRPMI. Multiscreen HTS 96-well ELISPOT plates (BD Biosciences) were coated with 2.5 μg/mL of rCRP or 5 μg/mL of goat anti-mouse Ig (SouthernBiotech) and incubated overnight. ELISPOT plates were washed with T-PBS and blocked with cRPMI for 2 hrs. Blocking media was removed and cells were plated into the ELISPOT plates (5 wells per sample) at several dilutions. After 5-6 hrs, the cells were washed off with PBS (3x) followed by T-PBS (3x). Secreted antibodies were detected by incubating with anti-mouse IgG conjugated to biotin (16 hrs, 4˚C), washing with T-PBS (3-4x), incubation with avidin-peroxidase complex (30 min, RT)(Vector Laboratories, Burlingame, CA), washing with T-PBS (3x) and PBS (3x), followed by incubation with AEC ELISPOT substrate (8 min, RT)(BD Biosciences). The reaction was stopped by washing with PBS. Spots were analyzed using ImmunoSpot image acquisition 4.5 and ImmunoSpot 5.0 Professional DC software (ImmunoSpot). The frequency of ASC per 10^6 splenocytes was determined (Slifka and Ahmed 1996; Slifka, Antia et al. 1998; Crotty, Kersh et al. 2003).

2.3.8 Flow cytometry.

Splenocytes were isolated, counted, and plated at $5x10^5$ cells per well in 96 well plates. Cells were collected by centrifugation (500 x g, 5 min, 4˚C) and washed with FACS staining buffer (1 x PBS with 2.5% FCS, 1% goat serum, and 1% human AB serum). 10^7 cells per mL were incubated with fluorescently labeled abs, diluted in FACS buffer, for 20 min on ice or 5 min at 4˚C. Abs used for staining included anti-CD19 (MB19-1), CD3 antibodies (17A2), CD69 (H1.2F3), CD86 (GL1), CD21 (eBio4E3), CD23 (B3B4), CD4 (RM4-5, L3T4), CD8a (53-6.7), PanNK (DX5), CD95 (15A7), CD95L (MFL3). For determining population gating on cells with multiple stains, florescence minus one controls were used. For analysis of surface activation, B and T cells were counter stained and doublets were excluded to ensure only the reported lymphocyte population was analyzed. Antibodies were purchased from BD Biosciences or eBioscience. Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star). The data were analyzed using bi-exponential transformation for complete data visualization.

2.3.9 Statistical analysis

2-way ANOVA was used for comparing the two mouse models over time. Bonferroni post-test analysis, Student's *t* test, or Mann-Whitney tests were used for comparison of individual doses or time-points, either between infected and control mice or between models.

2.4 RESULTS

2.4.1 Balb/c and C57Bl/6 models of *T. cruzi* infection

Balb/c and C57BL/6 mice were infected with a sub-lethal parasite dose (0.25 LD_{50}) of TCT. The magnitude and timing of the initial peak parasitemia was similar in both models, although Balb/c mice experienced a second wave of parasitemia at day 15 post-infection that was not evident in the C57Bl/6 mice (Fig 3*A*). Furthermore, Balb/c mice displayed significant weight loss by day 15 post-infection ($p = 0.005$, Student's *t*-test) that did not rebound until after acute infection (> 30 days post-infection), whereas C57Bl/6 mice maintained their weight over the course of infection ($p = 0.009$, 2-way ANOVA) (Fig 3*B*). These data show that given the same relative dose with similar early parasitemia kinetics, Balb/c mice remained more susceptible to the adverse effects of *T. cruzi* infection than were C57Bl/6 mice.

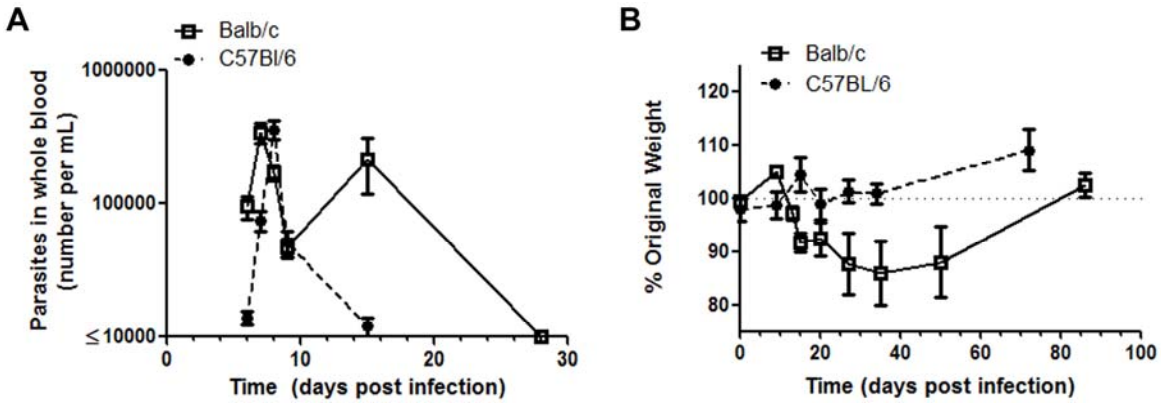

Figure 3: Disease severity and parasitemia in Balb/c and C57Bl/6 mice

Mice were injected with tissue culture trypomastigotes (i.p.). *A*, Parasitemia levels were analyzed in mouse blood at days 6-9, 15, and 28 post-infection ($n \geq 5$ mice per group). *B*, Mouse weights were collected at multiple time-points post-infection and the change from baseline determined for each mouse ($n = 5$ mice per group).

2.4.2 Th1 skewed mice have decreased hypergammaglobulinemia and improved parasite specific antibody response

To investigate systemic antibody responses during infection, serial pooled serum samples were collected and analyzed for levels of circulating IgG and IgM as well as antibodies specific to a *T. cruzi* surface antigen, *T. cruzi* complement regulatory protein (CRP), a member of the transialidase superfamily (Sepulveda, Hontebeyrie et al. 2000; Meira, Galvao et al. 2002; Meira, Galvao et al. 2004; Beucher and Norris 2008). The profiles of total IgM and IgG response in Balb/c and C57Bl/6 mice were significantly different during early infection (Fig 4*A*). C57Bl/6 mice had more circulating IgM after infection than did Balb/c mice. In contrast, Balb/c mice had significantly greater hypergammaglobulinemia post-infection than did C57Bl/6 mice (Fig 4*B*).

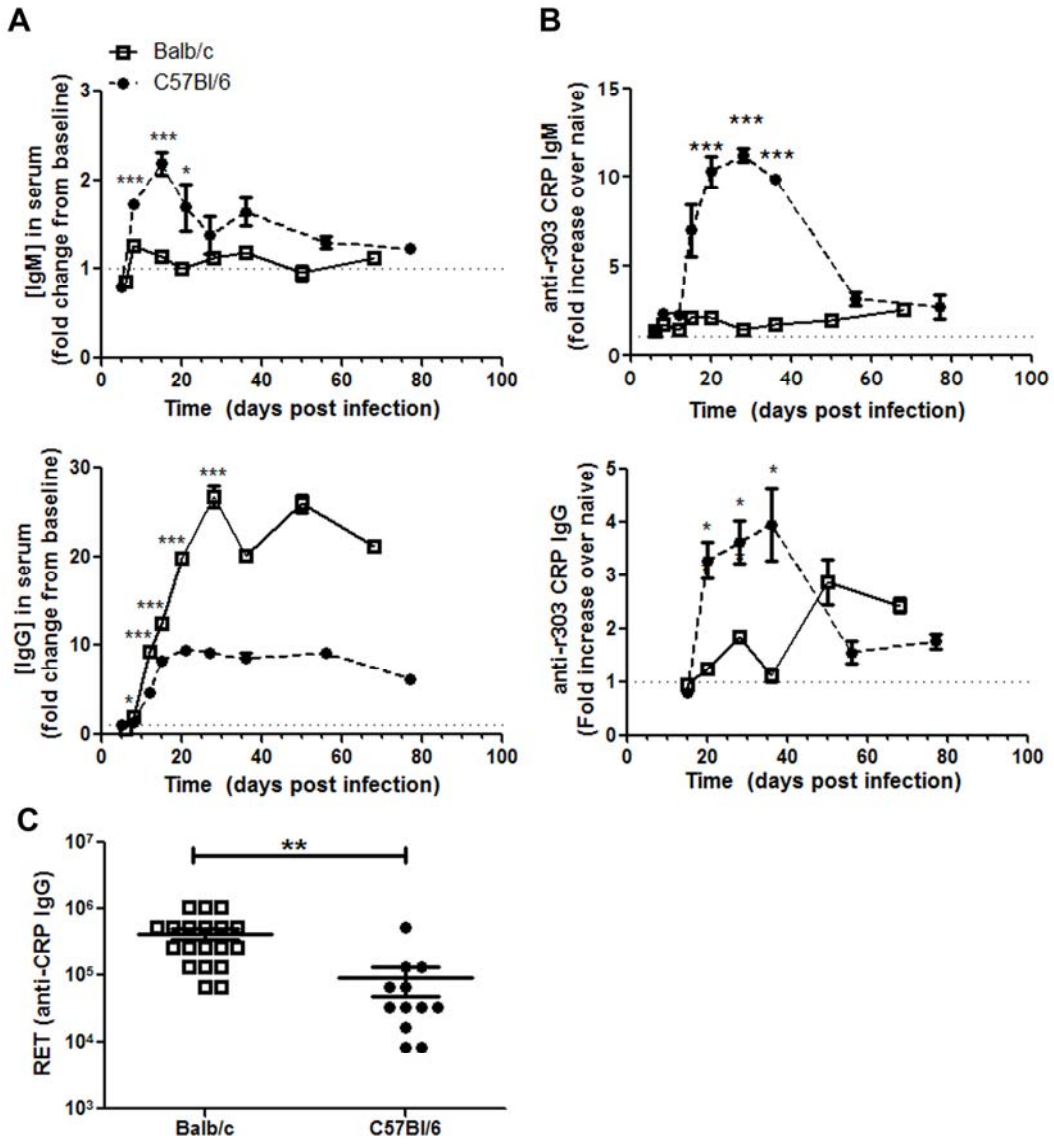

Figure 4: Circulating Total and CRP-specific IgM and IgG after *T. cruzi* infection.

A, Infected mice (n = 5 per group) were bled at multiple time-points post-infection and their pooled sera analyzed for total IgM (top) and total IgG (bottom), with triplicate repeats for each time-point. *B*, Pooled sera were analyzed for CRP-specific IgM and CRP-specific IgG by ELISA in duplicate. *C*, Balb/c generate a higher titer CRP-specific IgG than do C57Bl/6 mice in response to genetic immunization ($p = 0.002$). Each data point represents one mouse, data pooled from several individual experiments. * $p < 0.05$, *** $p < 0.001$ by Student's *t* test comparing Balb/c and C57Bl/6 models.

Parasite-specific antibody responses were evaluated by measurement of *T. cruzi* CRP-specific IgM and IgG following infection of Balb/c and C57Bl/6 mice. C57Bl/6 mice developed CRP-specific antibodies by day 20 post-infection (3.3±0.5 fold increase from baseline) (Fig 4*B*), whereas Balb/c mice had minimal CRP-specific responses until late in acute phase (Fig 4*B*). Delayed parasite-specific IgG response was also evident when Balb/c mice were inoculated with the low doses of the Y-BR variant (data not shown). In contrast, to the relative delay in CRP specific antibody responses in Balb/c versus C57Bl/6 mice during experimental infection, genetic immunization with CRP resulted in an improved CRP-specific IgG response in Balb/c mice compared to C57Bl/6 mice (Fig 4*C*). Taken together, these data suggest that the delayed CRP-specific antibody response in Balb/c mice compared to C57Bl/6 mice was not due to an inherent inability of Balb/c mice to respond to CRP antigen, but rather a host-pathogen interaction leading to diminished generation of parasite-specific antibody responses.

2.4.3 Earlier IFN-γ / Th1 skewed response in resistant mice versus delayed IFN-γ / increased Th2 cytokines in susceptible mice.

Cytokines influence the generation of cellular and humoral responses to infection and can be differentially produced in resistant versus susceptible mice during *T. cruzi* infection (Hoft, Lynch et al. 1993; Hoft, Schnapp et al. 2000). Cytokine production from T helper (Th) cells fall broadly into classification as T helper type 1 (Th1), T helper type 2 (Th2), or T helper 17 (Th17) categories: Th1 are defined by production of pro-inflammatory cytokines, particularly IFN-γ and TNF, Th2 by IL-4, IL-5, and IL-10, and Th17 cells by IL-17 (Forsthuber and Ji 2007). To evaluate levels of circulating cytokines post-infection, multi-plex cytokine analysis was performed on serum samples from day 8, 15, and 28 post-inoculation, and compared to control

mice inoculated with heat-inactivated parasites. The timing of IFN-γ, IL-10, IL-5, and IL-6 responses were significantly different in Balb/c mice compared to C57Bl/6 (Fig 5). In C57Bl/6 mice, an early significant IFN-γ response was evident along with increased IL-6 and IL-10, which then decreased to near control levels by day 15 post-infection. In comparison to C57Bl/6 mice, Balb/c had delayed IFN-γ and IL-6 response, an early IL-10 that persisted to day 15 and increased by day 28 post-infection, and IL-5 levels that were significantly elevated by day 15 and remained so at day 28 post-infection. In addition, Balb/c mice had IL-4 by day 28 post-infection, whereas Balb/c controls and C57Bl/6 mice (infected and control) did not produce detectable IL-4 in their serum (data not shown). These data indicate that C57Bl/6 responded to infection with an early Th1 biased cytokine response, whereas Balb/c mice responded to infection with a more Th2 skewed response.

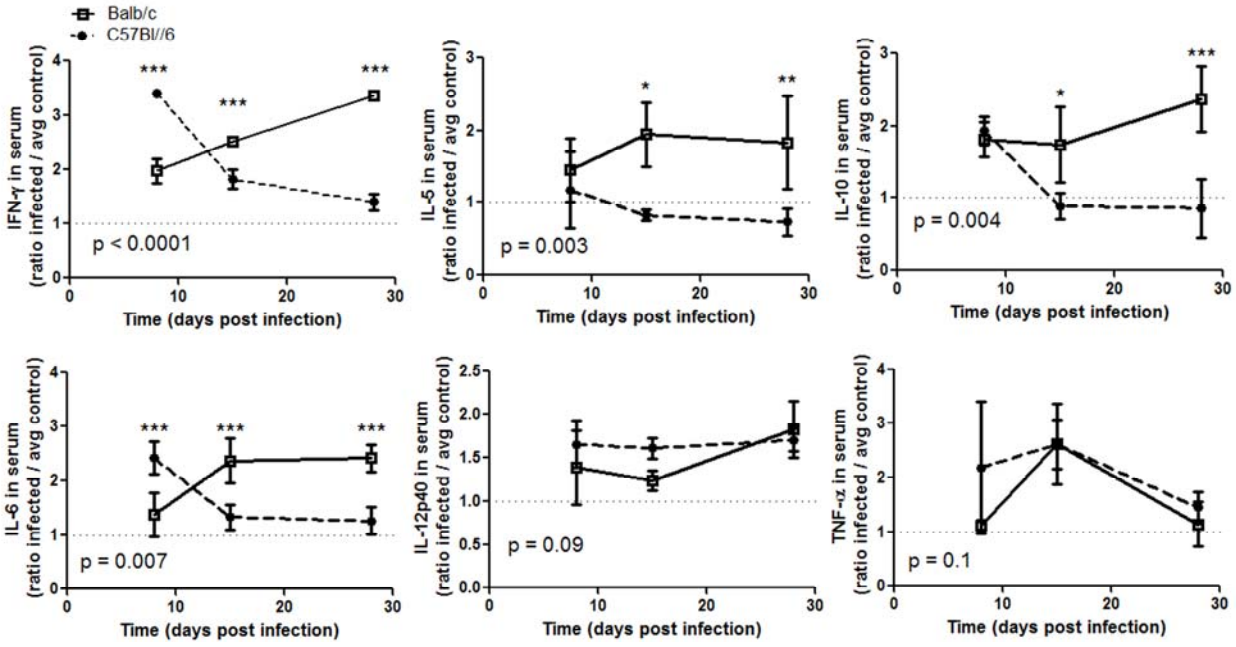

Figure 5: Cytokine profile after *T. cruzi* infection.

Luminex analysis of cytokine levels in mouse serum samples at days 8, 15, and 28 post-infection. Values are reported as the concentration in infected mice relative to control mice receiving heat-inactivate parasite. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Bonferroni post-test after 2-way ANOVA comparing Balb/c and C57Bl/6 models (p values reported on graphs) and/or Student's *t* test.

2.4.4 IgG1 and IgG2a predominate in total and specific response of susceptible versus a more mixed isotype response in resistant mice.

Different IgG isotypes have been implicated in effective immunity and polyclonal B cell responses to *T. cruzi* (d'Imperio Lima, Eisen et al. 1986; Minoprio, Eisen et al. 1987; Rowland, Mikhail et al. 1992; el Bouhdidi, Truyens et al. 1994). To compare the level of IgG isotype switching in Balb/c versus C57Bl/6 mice, serum samples were analyzed over the course of infection for concentration of IgG1, IgG2a (Balb/c) or IgG2c (C57Bl/6), IgG2b, and IgG3 (Martin, Brady et al. 1998). The serum levels for each isotype were significantly different in Balb/c versus C57Bl/6 mice over the time-points tested (day 8 through 125, post-infection) (Fig 6*A*). IgG1 and IgG2a were increased in Balb/c mice compared to C57Bl/6 mice ($p < 0.0001$ for each, 2-way ANOVA). IgG2b was increased in C57Bl/6 mice compared to Balb/c mice ($p < 0.0001$, 2-way ANOVA). The profile of IgG3 showed an overall difference that was significant at days 15 and 125 post-infection ($p < 0.01$ and $p < 0.001$, respectively, by Bonferroni post-test; $p = 0.0007$ by 2-way ANOVA). Serum samples from infected mice were analyzed for CRP-specific IgG isotype responses at day 28 post-infection, revealing a significant difference in the isotype profile of parasite specific IgG ($p = 0.0006$, 2-way ANOVA), with predominantly IgG1 and IgG2a produced in Balb/c, with a mixed response including IgG2b and IgG3 in C57Bl/6 mice (Fig 6*B*).

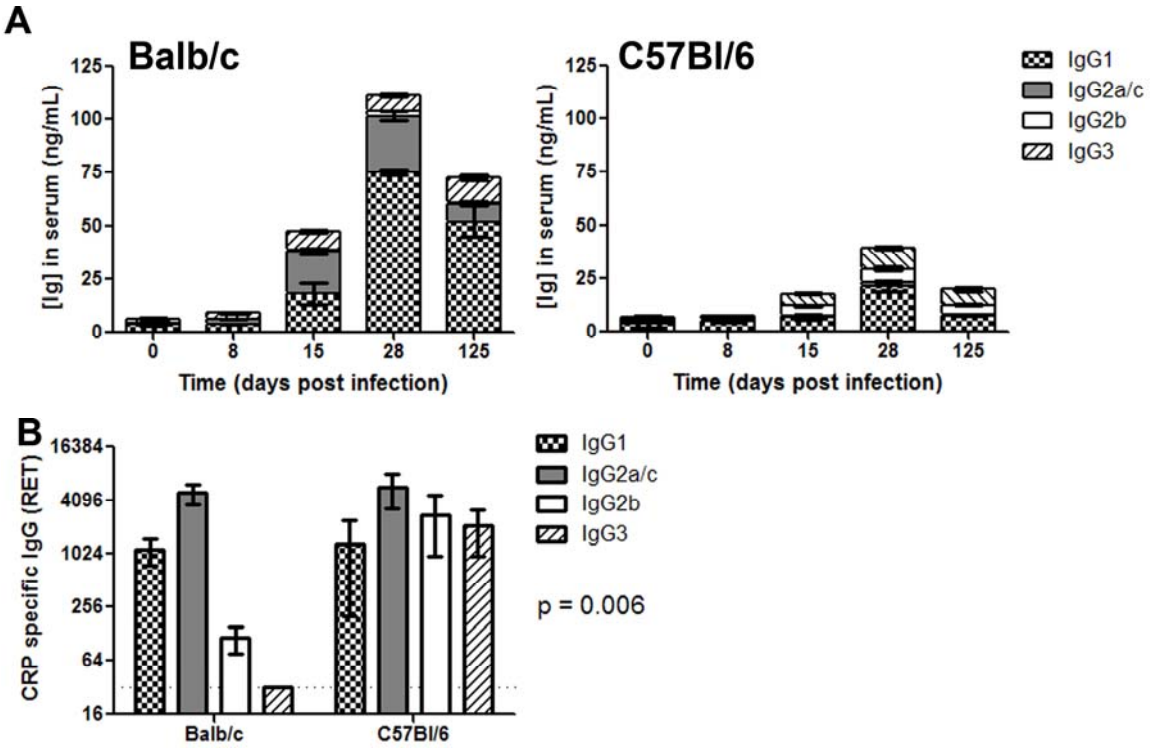

Figure 6: Antibody isotype and cytokine profile after *T. cruzi* infection.

A, Serum samples from infected mice were analyzed by ELISA to determine the isotype of the total Ig at days 8, 15, 28, and 125 post-infection. For each isotype the profile was significantly different in Balb/c versus C57Bl/6 mice. IgG1 and IgG2a were increased in Balb/c mice compared to levels of IgG1 and IgG2a in C57Bl/6 mice, respectively ($p < 0.0001$, 2-way ANOVA). IgG2b was increased in C57Bl/6 mice compared to Balb/c mice ($p < 0.0001$, 2-way ANOVA). The profile of IgG3 showed an overall difference that was significant at days 15 and 125 post-infection ($p < 0.01$ and $p < 0.001$, respectively, by Bonferroni post-test; $p = 0.0007$ by 2-way ANOVA). Five mice were used per group per time-point. *B*, Serum samples from infected mice were analyzed for CRP-specific IgG isotype responses at day 28 post-infection by determining reciprocal endpoint titers (RET) using pre-infection sera. $p = 0.006$ by 2-way ANOVA comparing the CRP-specific isotype profile of Balb/c versus C57Bl/6 mice. Five mice were used per group.

2.4.5 Resistant mice generate parasite specific IgG ASC; susceptible mice generate non-specific IgG ASC and have increased numbers of B cell blasts.

Acute *T. cruzi* infection has been reported to induce expansion of B and T cell subsets in the spleen without generation of parasite specific responses (Kierszenbaum 1981; Minoprio, Eisen et al. 1986). To determine whether resistant and susceptible mice display altered humoral responses in the spleen, splenocytes were analyzed for total B cells and large B cell blasts, as well as total and parasite-specific IgG antibody secreting cells (ASC) after *T. cruzi* infection (Fig 7) (For representative plots, see Appendix C). *T. cruzi* infection led to expansion of splenic B cells in both Balb/c and C57Bl/6 mice compared to controls by day 8 post infection (1.92±0.60 fold increase $p < 0.01$ and 1.57±0.54 fold increase $p = 0.04$, respectively) (Fig 7A). B cell blasts, defined by their increased size and granularity, were increased to a greater extent in Balb/c mice compared to C57Bl/6 mice post-infection (Fig 7B). Balb/c mice had B cell blasts levels ≥2.4 fold higher than controls at day 8 and 15 post infection ($p = 0.002$ for both time-points), with levels falling to 1.7±0.9 by day 28 post-infection ($p = 0.03$). In contrast, C57Bl/6 mice first experienced a significant increase in total B cell blasts compared to controls (2.0±0.9, $p = 0.02$) at day 15 post-infection. Elevated levels of total IgG ASC were seen in both models but to a greater extent in Balb/c mice (Fig 7*C*). Furthermore, the increase in IgG ASC in Balb/c mice was not associated with an increase in parasite-specific ASC measured by CRP-specific ASC (Fig 7*D*). In contrast, C57Bl/6 show a parasite-specific response in the spleen at day 15 post-infection (Fig 7*D*). These data indicate that splenic B cells in Balb/c mice were more susceptible to *T. cruzi* induced polyclonal activation, but were less capable of generating a specific response to *T. cruzi* antigen. C57BL/6 mice were able to generate specific IgG ASC against a parasite specific protein, indicating an improved humoral response in the spleens of these mice.

Furthermore, these data demonstrate that while there were similar changes in total B cell numbers in these two mouse models during *T. cruzi* infection, these changes were associated with different outcomes and that these differences in B cell effector function were also linked to phenotypic differences, such as blast formation.

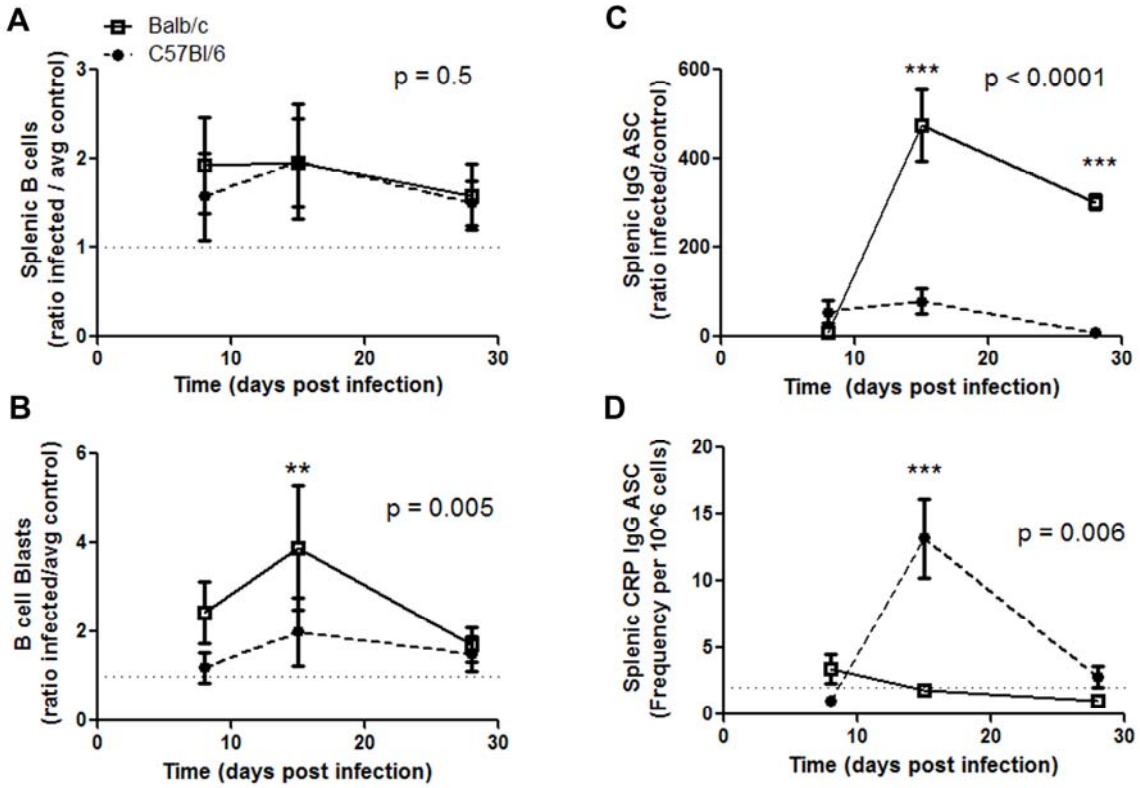

Figure 7: Splenic B cell expansion with or without parasite specific ASC.

A, Splenocytes were stained and analyzed by flow cytometry for enumeration of B cells. *B*, B cells were analyzed by forward and side scatter for blast formation. These plots show the ratio of total splenic B cells in infected mice as a ratio to the total number of splenic B cells in control mice of the same genotype. *C*, B cell ELISPOT analysis was completed to enumerate total IgG ASC from spleens of infected and control mice. The results are presented as the ratio of infected to average control of the same genotype. D, B cell ELISPOT analysis was used to enumerate the frequency of CRP-specific IgG ASC in the spleen of infected mice. ** $p < 0.01$, *** $p < 0.001$ by Bonferroni post-test after 2-way ANOVA comparing Balb/c and C57Bl/6 models (p values reported on graphs).

2.4.6 Analysis of B cell activation by surface expression of CD69, CD86, Fas, and FasL

To analyze activation status of splenic B cells post-infection, flow cytometry was performed to measure surface expression of CD69, CD86, CD95 (Fas) and CD95L (FasL) at days 8, 15, and 28 post-inoculation, comparing expression on B cells in infected mice to control mice receiving heat-inactivated parasite (Fig 8). CD69 was increased post-infection in both models, but with different profiles ($p = 0.0002$, 2-way ANOVA). C57Bl/6 mice initially had higher CD69 on the surface of B cells (4.6±2.2 fold higher than controls at day 8 $p = 0.002$), which then decreased over the course of infection. Balb/c mice maintained approximately a 2 fold elevation in CD69 expression (2.3±0.2 fold higher than controls at day 8 $p < 0.0001$). CD86 was increased post-infection in both models, but with different profiles ($p = 0.0001$, 2-way ANOVA). CD86 was initially elevated on B cells in both strains, then decreased in C57Bl/6 mice and increased in Balb/c mice at d28 post-infection. In both models, Fas and FasL positive B cells were increased post-infection, but with different profiles ($p < 0.0001$ and $P = 0.03$, respectively, 2-way ANOVA). Fas and FasL on B cells in infected C57Bl/6 mice increased between d8 and d15, then declined between d15 and d28. In contrast, Fas and FasL on B cells in infected Balb/c mice increased between d15 and d28 post-infection. Together, these data indicate that B cells were differentially activated in susceptible versus resistant mice.

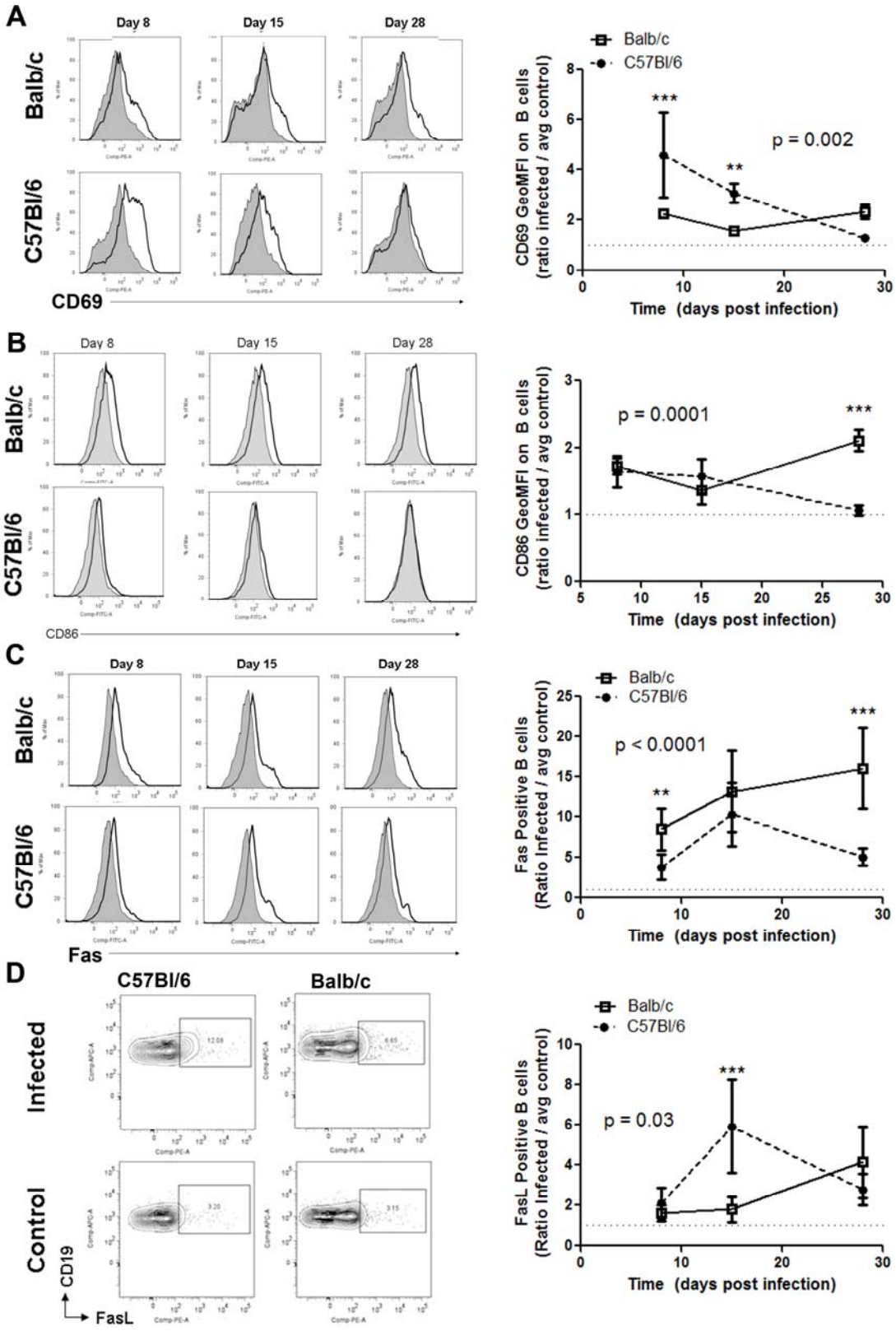

Figure 8: Splenic B cell activation.

Splenocytes were harvested and stained for surface protein analysis by flow cytometry at multiple time-points post-infection with *T. cruzi* in Balb/c and C57BL/6 mice. In each section, representative histograms (A-C) or contour plots (D) are shown to the left of the complete analysis. ** $p < 0.01$, *** $p < 0.001$ by Bonferroni post-test after 2-way ANOVA comparing Balb/c and C57Bl/6 models (ANOVA p values reported on graphs).

2.4.7 Differential B cell subset profiles in resistant versus susceptible mice

FO and MZ B cells are functionally and phenotypically distinct B cell populations within the spleen occupying distinct locations in the spleen. MZ B cells are poised at the marginal sinus of the spleen, and these cells are a source of natural antibody and T-independent (TI) responses (Lopes-Carvalho and Kearney 2005; Pillai, Cariappa et al. 2005). In contrast, FO B cells are located in spleen follicles and respond to antigen in a T cell dependent (TD) manner (Allman and Pillai 2008). To analyze changes in these B cell subsets, B cell gates were determined based on controls and naïve mice, using published population statistics as a guide (Allman and Pillai 2008). B cell subsets were defined as $CD19^{+}CD21^{high}CD23^{low/-}$ (MZ B cells) population, $CD19^{+}CD21^{int}CD23^{+}$ (FO B cells) and $CD19^{+}CD21^{low}CD23^{low/-}$ (transitional or newly formed B cells or cells that may have lost CD21 and CD23 expression due to activation). This gating strategy has been used to demonstrate changes in phenotypically defined MZ and FO B cell splenic subsets due to infection with other microbes (Achtman, Khan et al. 2003; Belperron, Dailey et al. 2005; Radwanska, Guirnalda et al. 2008; Guay, Mishra et al. 2009; Malkiel, Kuhlow et al. 2009). While CD21 and CD23 levels have been shown to be modulated by virus (Cordier-Bussat, Billaud et al. 1993), in particular in B cells infected with virus (Collins, Boss et al. 2009), the extent that other microbes modulate expression of these markers has not been determined. Analysis of CD21 and CD23 levels on B cells from *T. cruzi* infected mice indicates

that the expression of both markers decreased overall on B cells by day 8 post-infection in resistant C57Bl/6 mice, after which expression increased over the course of infection; CD21 remained relatively unchanged in susceptible Balb/c mice at day 8 and 15, then slightly increased at day 28 post-infection, while CD23 levels were decreased at every time-point analyzed (Fig 9*A*). The changes in expression of these markers on total B cells were then reflected in apparent changes in these phenotypically defined B cell subsets post-infection with *T. cruzi* in both mouse models. Representative plots of B cell subsets for C57Bl/6 and Balb/c mice at d8 post-infection are shown in Figure 9*B*.

In Balb/c mice, the percentage of $CD19^{+}CD21^{int}CD23^{+}$ (phenotypically defined as FO B cells) within the B cell gate decreased to 36±17 percent of controls ($p < 0.0001$) and in C57Bl/6 decreased to 74±41 percent of controls ($p = 0.02$), which was a significant retention of FO B cells in C57Bl/6 mice compared to Balb/c mice (Fig 9*C*), although absolute numbers of FO B cells were not significantly different from controls in either model. Analysis of FO B cells within the total B cell blast population indicates that FO B cell blasts represent a higher proportion of the total B cell blasts at day 8 and 15 post-infection in resistant C57Bl/6 mice than in susceptible Balb/c mice ($p < 0.01$ for both time-points)(Fig 9*C*).

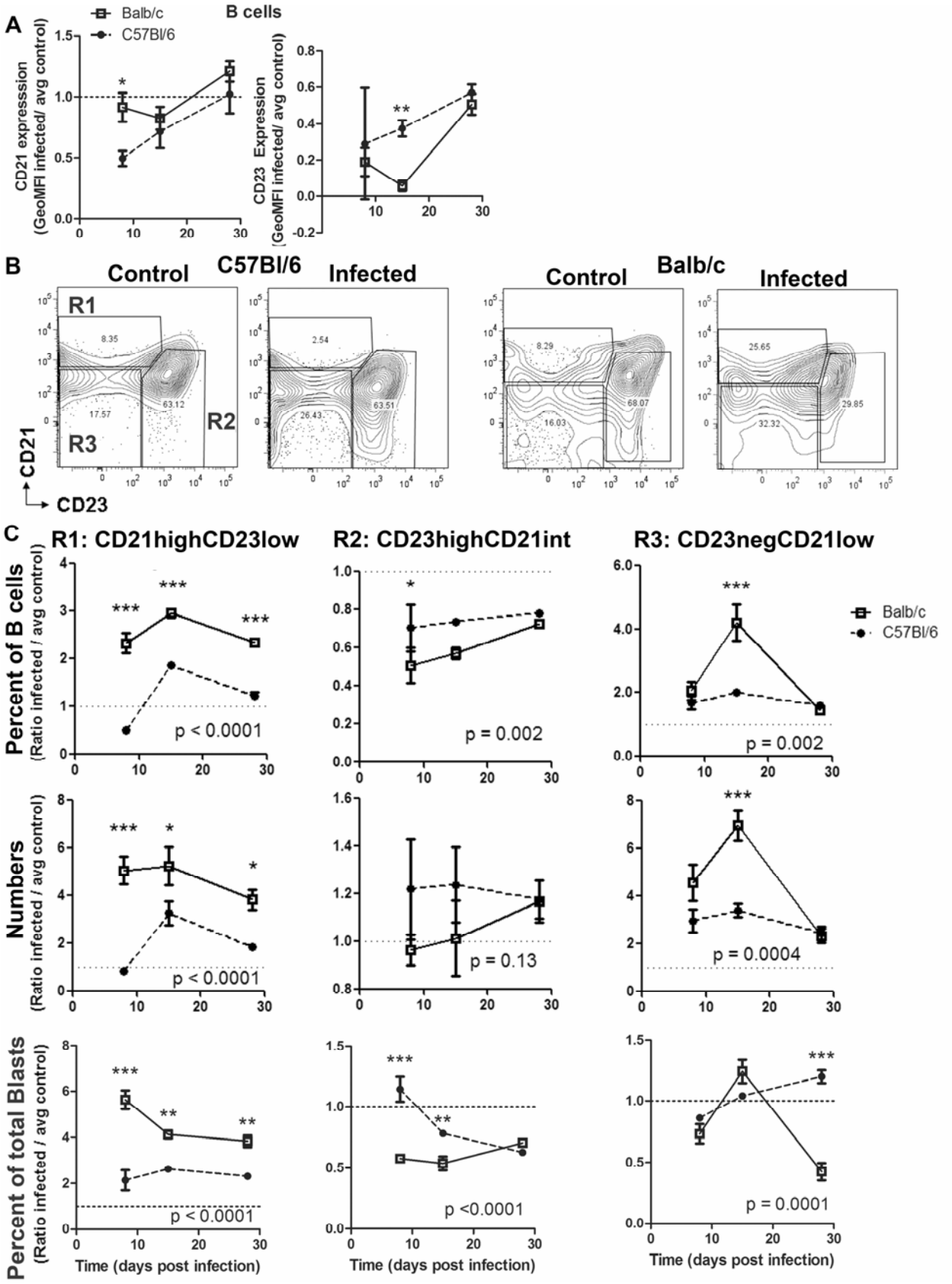
A
Balb/c
C57Bl/6
B cells
CD21 expresssion (GeoMFI infected/ avg control)
CD23 Expression (GeoMFI infected/ avg control)
B
Control
C57Bl/6
Infected
Control
Balb/c
Infected
R1
R2
R3
CD21
CD23
C
R1: CD21highCD23low
R2: CD23highCD21int
R3: CD23negCD21low
Percent of B cells
(Ratio infected / avg control)
Numbers
(Ratio infected / avg control)
Percent of total Blasts
(Ratio infected / avg control)
p < 0.0001
p = 0.002
p = 0.002
p < 0.0001
p = 0.13
p = 0.0004
p < 0.0001
p <0.0001
p = 0.0001
Time (days post infection)
Time (days post infection)
Time (days post infection)

Figure 9: Splenic B cell subsets population dynamics.

Splenoctyes were harvested from infected and control mice at days 8, 15, or 28 post infection and analyzed by surface markers for B cell populations. A, Analysis of CD21 and CD23 expression levels over the course of infection. B, Representative contour plots from day 8 post-infection show gates used for subset determination. C, These plots show the relative change in splenic B cell phenotype between infected and control mice. The first row graphs show percent of B cells accounted for by each of the subsets, the second row graphs show the absolute numbers for each population, the third row graphs shows the percent of B cell blasts accounted for by each of the subsets. In these sets of graphs, the y-axis shows the ratio of infected to average control (n = 5 mice per group per time-point). Graphs for a given population are underneath that title. Balb/c are represented by open squares, solid line; C57Bl/6 mice by closed circles, dashed line. Reported p values indicate the result of 2-way ANOVA analysis between Balb/c and C57Bl/6 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Bonferroni post-test after 2-Way ANOVA comparing Balb/c and C57Bl/6 models (p values reported on graphs) and/or Student's t test.

$CD19^{+}CD21^{high}CD23^{low/-}$ splenic B cells (defined as MZ by phenotype) responses also differed in Balb/c mice and C57Bl/6 mice. The percentage of B cells with the MZ phenotype was significantly different in Balb/c versus C57Bl/6 mice ($p < 0.0001$, 2-way ANOVA). While in Balb/c mice, MZ B cells accounted for an increased percentage of the total B cells at d8, in C57Bl/6 mice, the percentage of MZ B cells were decreased (Fig 9*C*). The percentage of B cells accounted for by MZ remained elevated at all three time-points for Balb/c mice. C57Bl/6 experienced an increased percentage of MZ B cells at d15 post-infection, which then declined by d28 post-infection. Absolute numbers of MZ B cells were significantly different in Balb/c versus C57Bl/6 mice over the course of infection ($p < 0.0001$) (Fig 9*C*). By d8 post-infection, MZ B cell numbers were increased 4.5±2.3 fold above controls in Balb/c mice ($p = 0.01$). In contrast, MZ B cell numbers were similar to controls in C57Bl/6 mice (0.83±0.29, $p = 0.4$). MZ B cell numbers were increased at d15 post-infection in C57Bl/6 and then declined, but remained elevated above controls at d28 post-infection. Analysis of $CD19^{+}CD21^{high}CD23^{low/-}IgM^{high}$ B

cells showed these same trends of altered B cell numbers (data not shown). The percent of total B cell blasts that were represented by the MZ phenotype followed this same pattern of increased representation in the B cells from susceptible Balb/c mice, significantly more than in the resistant C57Bl/6 mice ($p < 0.0001$).

$CD19^{+}CD21^{low/-}CD23^{low/-}$ B cells numbers and percentages within the B cell gate were expanded in both models by day 8, Balb/c mice experienced further increased numbers and levels measured at d15 post-infection, whereas C57Bl/6 mice did not ($p < 0.0001$, $p = 0.002$, numbers and percentage of B cells, respectively)(Fig 9*C*). These cells represented similar amounts of the total B cell blasts for both models until day 28 post-infection, when the numbers of blast cells represented by this subset decreased in Balb/c mice compared to C57Bl/6 ($p < 0.001$)

2.4.8 T cell activation and expansion in resistant versus susceptible mice

To determine the expansion and activation of splenic T cells, the number of total T cells and T cell blasts were assessed at days 8, 15, and 28 post-inoculation, comparing T cells in infected mice to control mice receiving heat-inactivated parasite (Fig 10*A,B)*. C57Bl/6 mice had increased T cells at d15 that decreased but remained elevated by d28 (Fig 10*A*). This coincided with high levels of T cell blasts at d15 (Fig 10*B*). Balb/c mice did not have increased T cells, although T cell blasts were increased at d15. Fas and FasL profiles were significantly different on T cells from Balb/c versus C57Bl/6 mice (Fig 10*C*), especially at d15. CD4 and NKT cells levels were significantly higher in C57Bl/6 mice compared to Balb/c mice at d15 post-infection (Fig 10*D*). C57Bl/6 mice maintained or slightly increased splenic CD4 numbers at d15 pi,

whereas Balb/c mice had decreased CD4 numbers post-infection, suggesting a lack of T cell help in the Balb/c mice compared to the C57Bl/6 mice (Fig 10*D*).

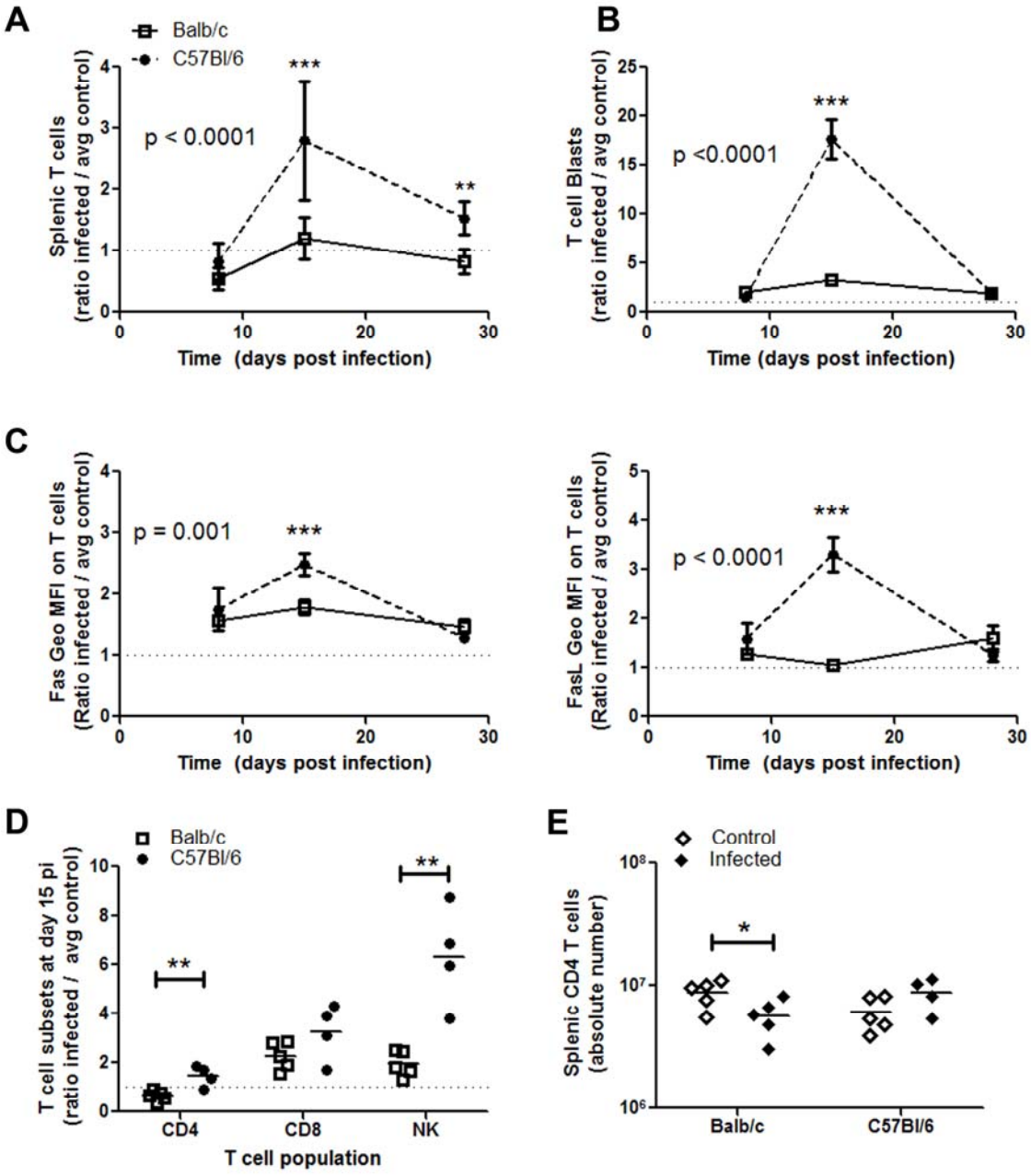

Figure 10: T cell expansion and activation in the spleen.

Splenoctyes were harvested from Infected and control mice at days 8, 15, or 28 post infection and analyzed by surface markers for T cell populations. Populations are reported the ratio of absolute cell numbers or geometric mean florescent intensity (GeoMFI) on T cells in infected mice to the average absolute numbers for control mice.

Data represent four-five infected mice per time-point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Bonferroni post-test after 2-way ANOVA comparing Balb/c and C57Bl/6 models (p values reported on graphs) and/or Student's *t* test.

The results presented in this study demonstrate that increased polyclonal B cell antibody responses were associated with decreased parasite-specific humoral immunity and increased disease susceptibility during *T. cruzi* infection (Figure 11).

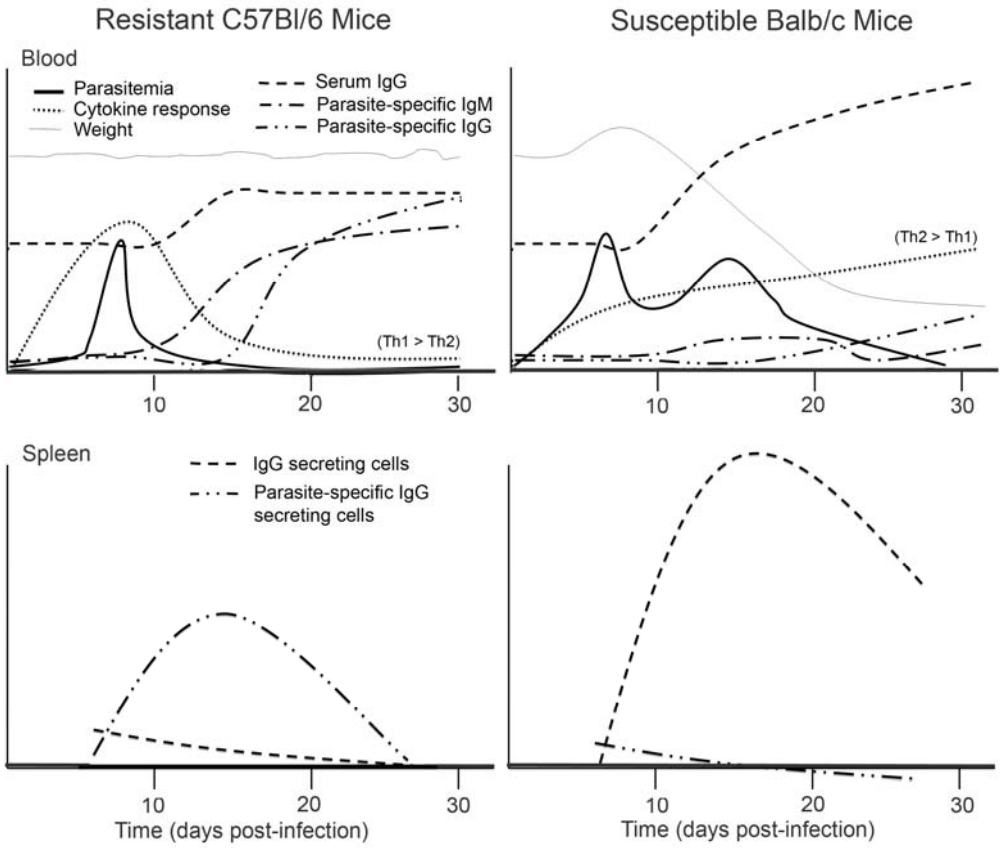

Figure 11 Differential antibody and cytokine responses associated with resistance to *T. cruzi*.

These plots model responses in T. cruzi infected C57Bl/6 and Balb/c mice compared to control mice inoculated with heat-inactivated parasite (original data in Fig 1-5) and show the association of parasite-specific antibody responses in the blood and spleen with decreased polyclonal antibody responses and differential cytokine expression (kinetics and quality summarized here). Serum cytokine levels (see Fig 5 for details) in C57Bl/6 compared to controls were IFN-γ > TNF-α = IL-6 > IL-10 with significantly elevated IL-12p40 and undetectable IL-5. The overall cytokine profile of Balb/c mice was IL-10 ≥ IL-5, IL-6, and TNF-α, with increased IFN-γ and IL-12p40 later during infection

(Fig. 5). The isotype of serum IgGs for C57Bl/6 mice were IgG1 > IgG3 > IgG2b > IgG2c, whereas Balb/c mice were IgG1 > IgG2a > IgG3 > IgG2b (Fig 4). Weight; the y-intercept is 100% and the origin is approximately 80% of the baseline. Parasitemia; the origin is the limit of detection (104 parasites/mL) and peak parasitemia approximately 5x105 parasites/mL. For cytokine responses, serum IgG, and parasite-specific IgM/G in both the blood and the spleen, the y-axis is the relative change in infected versus control mice and is to scale for each measure (i.e. directly compare IgG between C57Bl/6 and Balb/c mice). The plots for serum IgG are offset above the others for clarity.

Hypergammaglobulinemia was more pronounced in susceptible Balb/c mice and was associated with delayed generation of parasite-specific antibodies. The parasite-specific humoral immunity in the resistant C57Bl/6 mice was concomitant with a Th1-skewed cytokine burst. In comparison, delayed and then maintained Th2-skewed cytokine production in Balb/c mice was associated with polyclonal B cell activation. B cell activation in resistant mice was associated with low levels of total ASC and appreciable parasite-specific ASC in the spleen. Total B cell expansion and activation in the spleen of susceptible mice was associated with the development of high levels of antibody secreting cells (ASC) without detection of parasite-specific response. Figure 11 provides a model for the association of polyclonal versus parasite-specific antibody with disease severity and cytokine responses in resistant and susceptible mice. Furthermore, analysis of B cell surface markers (CD69, CD86, Fas, FasL, CD21, CD23) indicated differential profiles in the context of polyclonal versus parasite-specific activation in resistant C57Bl/6 versus susceptible Balb/c mice. B cells with the marginal zone (MZ) phenotype were differentially expanded in the spleen of resistant C57Bl/6 mice in association with parasite-specific responses. MZ-like B cells were expanded early and remained elevated in susceptible Balb/c mice. Changes in T cell surface marker expression (Fas, FasL, CD69) and subsets (CD4,

CD8, NK) were observed in resistant versus susceptible mice and associated with differential humoral responses.

2.5 DISCUSSION

As in many experimental models of infection, there are significant differences in susceptibility to pathogens among mouse strains; however the host-pathogen interactions that account for these differences are not completely clear. In the case of *T cruzi* experimental infection, protective cytotoxic CD8 responses and Th1 responses have been well characterized (Martin and Tarleton 2004; Hoft and Eickhoff 2005; Tarleton 2007; Padilla, Bustamante et al. 2009). The extent of B cell activation during *T. cruzi* infection and its association with effective parasite-specific immunity versus adverse polyclonal responses remains unclear.

It has been previously been shown that *T. cruzi* induces polyclonal B cell activation during acute infection and that B cell mitogens are expressed by infectious trypomastigotes (Minoprio, Eisen et al. 1986; Minoprio, Andrade et al. 1989; Hontebeyrie-Joskowicz and Minoprio 1991; Reina-San-Martin, Degrave et al. 2000). It has been postulated that this aberrant B cell response may contribute to a delayed parasite-specific humoral response, thus promoting infection (Minoprio, Burlen et al. 1988; Hontebeyrie-Joskowicz and Minoprio 1991; Minoprio, Coutinho et al. 1991; Reina-San-Martin, Cosson et al. 2000). In this study, we examined the humoral responses of two murine strains with differing susceptibility to *T. cruzi* infection to 1) determine whether induction of polyclonal B cell activation was associated with increased

disease susceptibility and 2) determine whether changes in circulating cytokines, splenic B cell function and phenotype, and splenic T cell activation and subset expansion were associated with polyclonal versus parasite-specific humoral responses.

Analysis of total circulating IgG and IgM indicated that resistant C57Bl/6 mice demonstrated increased total IgM followed by an IgM response to a parasite specific antigen (CRP) (Fig 2). The IgM responses in C57Bl/6 mice developed as parasites were cleared from circulation and were followed by a rise in total IgG and then parasite-specific IgG. In contrast, Balb/c mice had very little change in total IgM in the blood post-infection and minimal parasite-specific IgM responses. In contrast to the lack of IgM response, Balb/c mice had an increased hypergammaglobulinemia response compared to C57Bl/6 mice. The increased hypergammaglobulinemia evident in Balb/c mice was associated with a further delay and diminished parasite-specific IgG response compared to C57Bl/6. This lack of a robust IgG response to a parasite specific antigen (CRP) in Balb/c mice during experimental infection with *T. cruzi* was not due to an inherent inability to generate a response to this *T. cruzi* antigen, as evident by the magnitude of the IgG response to CRP after genetic immunization in Balb/c versus C57Bl/6 mice. Rather, these data suggest underlying host-parasite interactions that determine the balance of hypergammaglobulinemia versus the development of parasite specific responses.

As Th responses play a role in the generation of specific immune responses, as well as polyclonal B cell activation (Minoprio, Eisen et al. 1987), we hypothesized that the differential humoral immune response to *T. cruzi* infection may be associated with differential production of

cytokines in these two models. Analysis of circulating cytokines post-infection confirmed the skewing of Balb/c mice toward Th2 responses and the C57BL/6 mice toward Th1 responses (Fig 3). These data are in agreement with previous reports that early Th1 IFN-γ responses are associated with resistance to infection (Antunez and Cardoni 2000; Hoft, Schnapp et al. 2000) and that Th2 cytokines, especially IL-4 and IL-10 are associated with susceptibility to infection (Minoprio, el Cheikh et al. 1993; Barbosa de Oliveira, Curotto de Lafaille et al. 1996; Hiyama, Hamano et al. 2001; Kumar and Tarleton 2001; Muller, Kohler et al. 2001; Acosta-Rodriguez, Montes et al. 2004; Vogt, Alba Soto et al. 2008). The results of the present study show that early Th1-skewed cytokine burst was coincident with control of parasitemia and preceded the generation of parasite-specific humoral immunity in resistant C57Bl/6 mice (Fig. 9). Susceptible Balb/c mice had a delayed cytokine response, which was Th-2 skewed including IL-5 and IL-4 production, and was associated with delayed parasite-specific responses and exacerbated hypergammaglobulinemia (Fig. 9). The apparent delay in cytokine responses and the lack of detectable IL-4 until day 28 post-infection in susceptible Balb/c mice may have been due to rapid consumption of these cytokines, especially IL-4, rather then lack of their production. Overall, these data suggest that the early Th-1 cytokine burst in susceptible mice may have dampened parasite-mediated polyclonal B cell activation, allowing for improved parasite-specific humoral responses, whereas sustained Th-2 cytokine production in susceptible mice may exacerbate parasite-induced polyclonal B cell activation. As B cells have been shown to produce cytokines in response to parasite-derived mitogenic factors (Bryan and Norris), it is possible that B cell activation may account for some of the observed increases in cytokine levels post-infection with *T. cruzi*.

Cytokines can drive antibody production and influence isotype switch (Acosta-Rodriguez, Merino et al. 2007).The differential production of cytokines in susceptible Balb/c versus resistant C57Bl/6 mice was associated with differential total and parasite-specific IgG isotype response over the course of acute infection (Fig 2, 3, and 9). IgG1 and IgG2a made up the majority of the hypergammaglobunemia in Balb/c mice, whereas C57Bl/6 mice had a much lower IgG1 and IgG2a responses and increased total IgG2b (Fig 4). While IgG1 was the most elevated isotype in both mouse models, IgG2a showed a much greater increase from baseline in Balb/c and IgG2b in C57Bl/6 mice. C57Bl/6 mice experienced isotype switching of parasite-specific antibodies to IgG1, IgG2c, IgGb, and IgG3. In contrast, the Balb/c mice had limited isotype switching; the parasite-specific response remained predominately IgG1 and IgG2a out to day 28 post-infection. These data confirm the previously reported trend towards differential IgG isotype responses in resistant versus susceptible mice (d'Imperio Lima, Eisen et al. 1986; el Bouhdidi, Truyens et al. 1994) and further support the association of parasite-specific IgG2, particularly IgG2b, with increased resistance to *T. cruzi* (Takehara, Perini et al. 1981; Brodskyn, Silva et al. 1989; Powell and Wassom 1993).

Previous studies have reported B cell expansion in the spleen during *T. cruzi* infection (d'Imperio Lima, Eisen et al. 1986; Minoprio, Eisen et al. 1986; Minoprio, Eisen et al. 1987; Minoprio, Burlen et al. 1988; Zuniga, Motran et al. 2000 ; Marinho, Bastos et al. 2004). This expansion is increased when intact CD4 T-cell responses are present (Minoprio, Eisen et al. 1987) and evidence shows that it is dissociated from parasite specific responses (Minoprio, Burlen et al. 1988), although some studies report both total and specific B cell responses. In the present study, we performed a detailed analysis of the B cell response to infection and found that

although total numbers of B cells were expanded to similar extents in susceptible and resistant mice, the outcome of these expansions were different in terms of production of total and specific IgG ASC, activation status by CD69, CD86, Fas/FasL expression, and B cell subset expansion (Fig 5-7).

Splenic B cell expansion in susceptible Balb/c mice was associated with increased numbers of B cell blasts, as well as with increased numbers of IgG ASC, without appreciable expansion of parasite specific IgG ASC. In contrast, B cell expansion in resistant C57Bl/6 mice led to moderately increased B cell blast formation, moderate to low levels of IgG ASC, and the formation of parasite-specific IgG ASC (Fig 5). CD69, a marker of lymphocyte activation (Powell and Wassom 1993), was moderately increased on Balb/c splenocytes throughout infection, whereas in C57Bl/6 mice, CD69 was differentially expressed over the course of acute infection, with an early peak expression that was two fold higher on C57Bl/6 B cells compared to Balb/c B cells. The early rise in CD69 on C57Bl/6 B cells preceded parasite-specific ASC formation, after which CD69 levels decreased (Fig. 6). These data demonstrated that transient high level CD69 expression preceded parasite-specific B cell activation, while moderate sustained CD69 expression was associated with polyclonal B cell activation. Previous studies have indicated a role for increased CD86 on B cells during *T. cruzi* infection in leading to increased immunoglobulin production through interaction with NK cells (De Arruda Hinds, Alexandre-Moreira et al. 2001). In this study, CD86 expression on B cell was not significantly different between these two models early in infection, but diverged later with increased levels of Balb/c B cells at day 28 post-infection concomitant with peak hypergammaglobulinemia in these mice.

Recent studies have indicated that *T. cruzi* causes parasite specific B cells to undergo Fas-FasL mediated fratricide (Zuniga, Motran et al. 2002). Fas is also expressed on B cells during germinal center reactions, without leading to apoptosis (reviewed in (van Eijk, Defrance et al. 2001)). Our data shows that Fas and FasL were differentially expressed on the surface of B cells in C57Bl/6 versus Balb/c mice during *T. cruzi* infection (Fig 6). In resistant C57Bl/6 mice, increased Fas and FasL positive B cell numbers were associated with CRP-specific IgG ASC. Fas and FasL were also increased on T cells in conjunction with this CRP-specific IgG response in the spleen. Together, these data suggest that the Fas/FasL expression on B cells in C57Bl/6 mice was associated with a productive, germinal-center like, reaction in the spleen. In contrast, elevated Fas/FasL positive B cell numbers in susceptible Balb/c mice were not associated with a parasite-specific response. Rather, the sustained elevated expression of these death ligands may have limited the non-specific expansion of B cells in these mice through B-cell fratricide, as increased activation and blast formation did not lead to overwhelming expansion of B cells during acute infection.

To better understand which B cells account for the observed increase in splenic B cells during *T. cruzi* infection, we evaluated B cell subsets based on CD21 and CD23 expression of CD19+ B cells (Fig 7). These markers have previously been used to define splenic B cell subsets ex vivo during microbial infection (Achtman, Khan et al. 2003; Belperron, Dailey et al. 2005; Radwanska, Guirnalda et al. 2008; Guay, Mishra et al. 2009; Malkiel, Kuhlow et al. 2009). As the expression of these markers may be modulated by activation induced by infection, the definition of these subsets is not definitive. Future studies are necessary to define the affect of *T.*

cruzi infection on these B cell subsets in vivo. For the purposes of this study, B cell subset populations were defined as $CD19^{+}CD21^{high}CD23^{low/-}$ (MZ B cells), $CD19^{+}CD21^{int}CD23^{+}$ (FO B cells) and $CD19^{+}CD21^{low}CD23^{low/-}$ cells. In Balb/c mice, B cells retained CD21 expression, but decreased CD23 expression, the low affinity IgE receptor. Susceptible mice had an apparent increase in B cells with the MZ phenotype within total B cells and B cell blasts that was associated with polyclonal rather than parasite-specific humoral responses (Fig. 5). Two possible explanations for the expansion of B cells with the MZ phenotype are that MZ B cells proliferated or parasite activation induced B cells differentiation toward a MZ B cell phenotype. MZ B cells were not expanded early in infection in C57Bl/6 mice. Rather, transient expansion in MZ phenotype within B cells and B cells blasts coincided with the development of parasite-specific IgG ASC in resistant C57Bl/6 mice. The percentages of B cells with a FO phenotype decreased in both models, but were retained to a greater extend in the C57Bl/6 mice, although the variability between mice was quite high, leading to a lack of significant difference in absolute numbers of FO in C57Bl/6 versus Balb/c mice. The relative amount of B cells with the FO phenotype within the B cell blast population was higher during early infection of C57Bl/6 mice compared to Balb/c mice, suggesting that in the context of parasite-specific response B cell blasts retain the FO phenotype to a greater extend than in the context of polyclonal B cell activation. Analysis of $CD19^{+}CD21^{low}CD23^{low/-}$ cells indicated that Balb/c mice experienced increased expansion of these cells compared to C57Bl/6 mice. The expansion of transitional B cells without parasite-specific ASC in susceptible mice suggests that this expansion results from polyclonal B cell activation rather than parasite-specific humoral immunity. The relatively low representation of this population in the total number of B cell blasts in susceptible mice suggests that perhaps decreased CD23 expression, rather than proliferation led to the relative expansion of

this subset compared to controls. All together, these data indicate differential changes in B cell subset phenotype in resistant versus susceptible mice. Further studies are needed to fully define the functional consequence of these apparent B cell subset changes and their contribution to humoral response during *T. cruzi* infection.

To evaluate whether these differences in humoral response were associated with differences in T cell dynamics in resistant versus susceptible mice, bulk splenic T cells were analyzed for expansion, blast formation, and expression of Fas and FasL (Fig 8). T cell expansion was coincident with the generation of parasite specific IgG responses at day fifteen post-infection in resistant mice, but remained near control levels in during the polyclonal B cell activation in susceptible mice at this same time-point. CD69 expression on total T cells was similar early during infection for both models. Later in infection, T cells from infected Balb/c mice experienced a second wave of CD69 activation, which was coincident with clearance of parasite from circulation and the first detection of parasite-specific IgG in circulation, although these levels did increase much until later on during infection (after day 36). Low Fas and FasL expression in Balb/c mice suggests that the contribution of T cells to control of B cell numbers via apoptosis was minimal in these mice. The expression of Fas and FasL on T cells coincided with parasite-specific B cell responses in C57Bl/6 mice, suggesting they may have formed a productive association. Further analysis of T cells at this time-point indicated that resistant mice had significantly higher levels of CD4 and NK T cells than susceptible mice, both of which can provide B cell help (Lang 2009). While previous studies show that lack of CD4 T cells led to decreased polyclonal B cell activation (Minoprio, Eisen et al. 1987), these results suggest that maintenance of CD4 and increased NK T cells may also be important for directing the efficacy

of the specific humoral response to parasite antigen. Activation of NK T cells has previously been linked to increased resistance to *T. cruzi*, but depends upon the presence of CD8 and CD4 T cells, as well as IFN-γ production (Duthie and Kahn 2002; Duthie and Kahn 2006). The increased numbers of NK T cells in resistant C57Bl/6 mice is particularly intriguing as they may provide B cell help through direct interaction (CD40L) or rapid cytokine responses, especially IFN-γ, which was produced to a much greater extent in these resistant mice (Leadbetter, Brigl et al. 2008). These data provide rationale for further studies to fully define the contribution of CD4 and NK T cells to polyclonal versus parasite-specific humoral immunity during *T. cruzi* infection of susceptible versus resistant mice.

While susceptibility and resistance of Balb/c and C57Bl/6 mice have been documented and explored in terms of cellular and cytokine responses to *T. cruzi* experimental infection (Starobinas, Russo et al. 1991; Hoft, Lynch et al. 1993; Leite de Moraes, Minoprio et al. 1994; Abrahamsohn and Coffman 1996; Zhang and Tarleton 1996; Antunez and Cardoni 2000; Tarleton, Grusby et al. 2000; Antunez and Cardoni 2001; Kumar and Tarleton 2001; Planelles, Thomas et al. 2003), humoral responses in these studies have been largely neglected. This is the first study to examine both polyclonal and specific humoral responses in these mice in the context of equivalent initial parasitemia. Taken together, the results in this study support the hypothesis that polyclonal B cell activation leading to hyper-IgG responses are associated with increased disease susceptibility and highlights the importance of host-parasite interactions in development of humoral responses to *T. cruzi.* By further characterizing associations between Th1 and Th2 responses, the development of polyclonal versus parasite-specific humoral responses, and the potential contribution of B cell subsets to these processes, this present study

provides a more detailed understanding of the development of effective versus detrimental humoral responses during *T. cruzi* infection. Furthermore, these results have implications for vaccine design in *T. cruzi,* as host genetic biases that lead to differential polyclonal B cell activation may have profound effects on the development of humoral immunity to *T. cruzi* target antigens (Reina-San-Martin, Cosson et al. 2000; Minoprio 2001; Montes, Acosta-Rodriguez et al. 2007).

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3.0 TcPRAC INDUCED POLYCLONAL B CELL ACTIVATION

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3.1 ABSTRACT

Trypanosoma cruzi is the etiologic agent in Chagas' disease. Acute *T. cruzi* infection results in polyclonal B cell activation and delayed specific humoral immunity. TcPRAC, a *T. cruzi* B cell mitogen, may contribute to this dysfunctional humoral response. Stimulation of murine splenocytes with recombinant protein (rTcPRAC) induced B cell proliferation, antibody secretion, IL-10 production, and up-regulation of CD69 and CD86 on B cells. Marginal zone (MZ) B cells are more responsive to T-cell independent rTcPRAC stimulation than are follicular mature (FM) B cells in terms of proliferation, antibody secretion, and IL-10 production.

3.2 INTRODUCTION

T. cruzi proline racemase (TcPRAC) has been identified as a T cell-independent (TI) B cell mitogen (Reina-San-Martin, Cosson et al. 2000; Reina-San-Martin, Degrave et al. 2000; Chamond, Gregoire et al. 2003). TcPRAC is a dimeric protein encoded by two paralogous genes per haploid genome: *TcPRACA* and *TcPRACB*. *TcPRACA* encodes a secreted or transmembrane anchored protein, although an alternative second initiation site can result in a cytoplasmic protein (Chamond, Goytia et al. 2005). TcPRACB has been found in the cytoplasm of insect stage epimastigotes and is distinguished from TcPRACA through several point mutations and the deletion of the amino terminal secretion signal (Chamond, Gregoire et al. 2003; Chamond, Goytia et al. 2005). In this study, we focused on TcPRACA, as the expression of this isotype by infectious and blood form trypomastigote parasites suggest its possible involvement in polyclonal B cell activation during *T. cruzi* infection. TcPRACA was first isolated from the supernatant of infectious trypomastigote parasites and was defined as a B cell mitogen due to TcPRACA-induced proliferation of athymic or T-cell depleted splenoctyes (Reina-San-Martin, Cosson et al. 2000), however the effect of TcPRACA on the activation and function of total splenic B cell or subsets thereof has not been directly shown.

Marginal zone (MZ) and follicular mature (FM) B cells constitute two functionally and anatomically distinct B cell subsets within the spleen (Allman and Pillai 2008). MZ B cells are located at the marginal sinus of the spleen. MZ B cells are considered first line responders to pathogens in the blood. MZ B cells are more responsive to TI-antigens and generate short-term plasma cells (Won and Kearney 2002). FM B cells circulate through the lymph and are found in B cell follicles of the spleen. FM B cells respond to T-dependent (TD) antigen and can become

long-term plasma cells or memory B cells (Fairfax, Kallies et al. 2008). The differential contribution of these two B cell populations to immunity during infectious disease is still under investigation (Oliver, Martin et al. 1997; Suarez, Lortholary et al. 2006; Allman and Pillai 2008; Radwanska, Guirnalda et al. 2008; Malkiel, Kuhlow et al. 2009). While differences in MZ and FM responsiveness to LPS and other TLR ligands has been reported (Gunn and Brewer 2006; Meyer-Bahlburg, Bandaranayake et al. 2009), the response of these B cell subsets to pathogen derived B cell mitogenic proteins has remained largely unexplored.

3.3 MATERIALS AND METHODS

3.3.1 Parasites and Mice.

Balb/c, C57/Bl/6, and C3H/HeJ mice were obtained from Jackson Laboratories and maintained in specific pathogen free housing. Mice husbandry and procedure protocols were reviewed and performed in accordance with the University of Pittsburgh IACUC. Y-strain parasites were grown in NIH 3T3 cells and harvested by standard technique (Sepulveda, Hontebeyrie et al. 2000). Two isolates of Y-strain parasite were used, a highly virulent Y-Br and a more attenuated isolate, Y-US. Balb/c mice were experimentally infected by intraperitoneal injection with one LD50 of Y-US (10^6). C57Bl/6 mice were experimentally infected by intraperitoneal injection with one LD50 ($2x10^4$) Y-Br parasites. Blood was collected at multiple time-points post infection for analysis by ELISA.

3.3.2 rTcPRAC and rCRP Cloning.

TcPRACA (without a secretion signal) was cloned from Y-strain *T. cruzi* genomic DNA using the same primers as previously used to generate rTcPRAC from CL strain parasite (Bg45 and Hi45) (Reina-San-Martin, Degrave et al. 2000). The amplified TcPRACA open reading frame (ORF) was cloned into pCRII-Blunt-TOPO vector (Invitrogen). The insert was then cut out with SacI and ligated in frame with a histidine tag in the pET28b (EMD, Novagen) vector. pET28B_TcPRACA was transformed into BL21(DE3) E. coli cells (EMD, Novagen). For eukaryotic expression, the amplified TcPRACA sequence was cloned into the pcDNA3.1/VF-his-TOPO vector (Invitrogen) and transformed into BL21(DE3) (EMD, Novagen). A full-length cDNA encoding the *T. cruzi* CRP was isolated by reverse transcription-PCR as previously described (Norris, Schrimpf et al. 1997). The *T. cruzi* CRP cDNA encoding the mature protein (starting at nucleotide 303) was subcloned into the pTrcHis expression vector (Invitrogen). E. coli strain SURE (Stratagene) were transformed with pTrcHis-CRP DNA for recombinant protein production with a histidine tag, as previously described (Sepulveda, Hontebeyrie et al. 2000). For eukaryotic expression, CRP was cloned into pCDNA3 with the glycosylphosphatidylinositol (GPI) anchor signal sequence from human DAF, the full description of this cloning was previously published (pcDNA3-CRP.daf) (Beucher, Meira et al. 2003).

3.3.3 Protein Purification.

Expression of recombinant protein was induced by isopropyl-β-D-thiogalactoside (IPTG) (IBI Scientific, Peosta, IA) in transformed E. coli and the cells harvested by centrifugation (6,000

RCF, 10 min., 4˚C). The resulting cleared lysate was prepared under native or denaturing conditions and bound to cobalt metal affinity resin according to the Talon instruction manual with slight modifications (Clontech, Mountain View, CA). During binding of lysate to resin, 5-10mM imidazole (Sigma) was added into the binding buffer (50mM Tris, 300mM NaCl, pH 7.2). Protein was bound to the resin in batch for 2 hours. The bound protein was further washed and packed into a disposable column. An imidazole step gradient (25-150mM) was used to elute bound protein. Protein concentration in eluted fractions was determined by Bradford assay. Elution fractions containing rTcPRAC protein were further selected for active dimers, concentrated, and buffer exchanged into 1xPBS with micro-concentrators with a molecular weight cut-off of 50 kDa (Microcon YM-50 Pierce). After purification, the concentration of rTcPRAC was calculated by subtracting the amount of protein present in the equivalent eluted negative control fractions from the protein amount in eluted rTcPRAC fractions.

3.3.4 In vitro stimulation of B cells.

Freshly isolated splenocytes from Balb/c mice or C3H/HeJ mice were stained with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma), washed with PBS, and cell number adjusted to 106 cells per mL in cRPMI (GIBCO RPMI+Glutamax, 10% FCS, 1xHEPES, Pen/Strep)(Invitrogen) for stimulation. Cells were stimulated with rTcPRAC; empty vector IMAC purified protein as a negative control; or 0.001-10 μg/mL LPS (Sigma) as a positive control. Cells were maintained at 37°C, 5% CO2 until harvest. Induced empty vector IMAC purified protein was chosen as the appropriate negative control for rTcPRAC stimulation as it preserves any co-purifying heat-labile and heat-stable stimulatory components. LPS was measured as less than 100 EU/mL (10 ng/mL) by LAL assay (Lonza, Basel Switzerland) in 25

μg/mL rTcPRAC. To negate any potential LPS contamination 5-10 μg/mL polymyxin B (PMB) was added to the culture containing rTcPRAC except where indicated (5 μg/mL PMB decreased B cell proliferation in cultured stimulated with 1 μg/mL LPS to background levels).

3.3.5 Cell sorting for MZ and FM B cells.

Total Balb/c splenocytes were isolated as described above. Cells were incubated with magnetic bead-conjugated anti-CD43 Abs according to manufacturer's instructions (Miltenyi Biotec). Immediately after anti-CD43 staining, cells were stained with specific Abs for sorting by FACSAria, followed by CD43 depletion using Automacs columns (Miltenyi Biotec) to collect the untouched CD43 negative cells. Pre and post-CD43 depletion fractions were analyzed for CD19 expression, with 95% CD19+ cells in the CD43 negative population and 33% CD19+ cells in the CD43+ population. The CD43 negative cells were further sorted by a FACSAria sorter with Diva software (BD Biosciences). FM B cells were sorted as CD21int, CD23+, CD24int; MZ B cells as CD21high, CD24high cells (Meyer-Bahlburg, Bandaranayake et al. 2009). B cell numbers were adjusted to approximately 10^6 cells per mL in cRPMI for stimulation.

3.3.6 Flow Cytometry

For analysis post-stimulation, cells were collected by centrifugation (500 x g, 5', 4˚C) and washed with FACS staining buffer (1 x PBS with 2.5% FCS, 1% Goat serum, and 1% human AB serum). 10^6 cells per mL were incubated with fluorescently labeled Abs, diluted in FACS buffer, for 20 min on ice or 5 min at 4˚C. Whole blood cells were directly stained with anti-CD19 and CD3 antibodies, treated with lysing solution (BD Biosciences), washed, and data collected by

flow cytometry. Abs used for staining included anti-CD19 (MB19-1), CD3 antibodies (17A2)(eBioscience), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), CD21 (eBio4E3), CD23 (B3B4), and CD24 (M1/69). All antibodies were purchased from BD Biosciences or eBioscience. Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star). The data were analyzed using bi-exponential transformation for complete data visualization.

3.3.7 Quantitative IgM, IgG, and IL-10 ELISA.

4 HBX Immulon ELISA plates (Thermo Scientific) were coated with 100ng of goat-anti-mouse Ig antibody (SouthernBiotech) overnight at 4°C, washed, and blocked with 1% milk in T-PBS (0.05% Tween-20, 1xPBS), washed and stored at -20°C until use. Upon harvest of cells, culture supernatent was collected and stored at less than -20°C. Culture supernatants were thawed, diluted, and applied to coated plates. A standard curve was generated with mouse IgM or IgG (SouthernBiotech, Birmingham, Alabama). Goat anti-mouse IgM or IgG conjugated with HRP was used as the secondary antibody (SouthernBiotech). IL-10 ELISA was performed according to manufacturer's instructions (BD Biosciences), briefly ELISA plates were coated overnight with IL-10 capture antibody, then washed (T-PBS) and blocked with 10% FCS in PBS. Culture supernatant or standards were applied to the plates and incubated for 2 hours at room temperature. Plates were then washed and incubated with anti-IL-10 HRP conjugated secondary antibody. After incubation with secondary antibody, plates were washed and developed with OPtEIA (BD) and analyzed for color change (OD_{450}). Standard curve fit and calculation of unknowns was performed using Prism software (GraphPad).

3.3.8 Statistical analysis.

2-way ANOVA was used for comparing two treatments over a range of doses or after passing of time. Bonferroni post-test analysis or Student's *t* tests were used for comparison of individual doses or time-points. For analysis of correlation between two treatments, the Pearson test was applied.

3.4 RESULTS

3.4.1 Y-strain derived rTcPRAC-induced B cell proliferation

Full length TcPRACA was cloned from *T. cruzi* Y-strain parasite and the predicted amino acid sequence was greater than 99% identical to the reported TcPRACA primary protein sequence from CL strain (9). TcPRACA (rTcPRAC) was subcloned into a prokaryotic expression vector and the purified rTcPRAC yielded the expected 45 kDA molecular weight monomeric protein with minimal background protein contamination (Fig 12*A*). To confirm that this protein retained the functional activity of the mitogenic dimeric form of rTcPRAC, Balb/c splenocytes were stimulated for 72 hr with rTcPRAC and analyzed for B cell proliferation by CFSE dilution. Purified rTcPRAC was compared to a negative control consisting of an equal volume addition of mock-induced empty vector IMAC elution fraction (Student's *t* test, $p < 0.0005$) (Fig 12*B*). To evaluate the kinetics of rTcPRAC-induced B cell proliferation, splenocytes were stimulated with 10 μg/mL of rTcPRAC. Proliferation was determined by CFSE dilution at 20, 44, 68, and 72 hours post-rTcPRAC stimulation (Fig 12*C*). rTcPRAC-induced B cell proliferation was detected

at 44 hours and was declining by 92 hours post-stimulation. These results confirm the mitogenic capacity of Y-strain derived rTcPRAC and indicate that three days was the optimal stimulation time for analysis of rTcPRAC-induced B cell proliferation, which was used throughout the rest of this study.

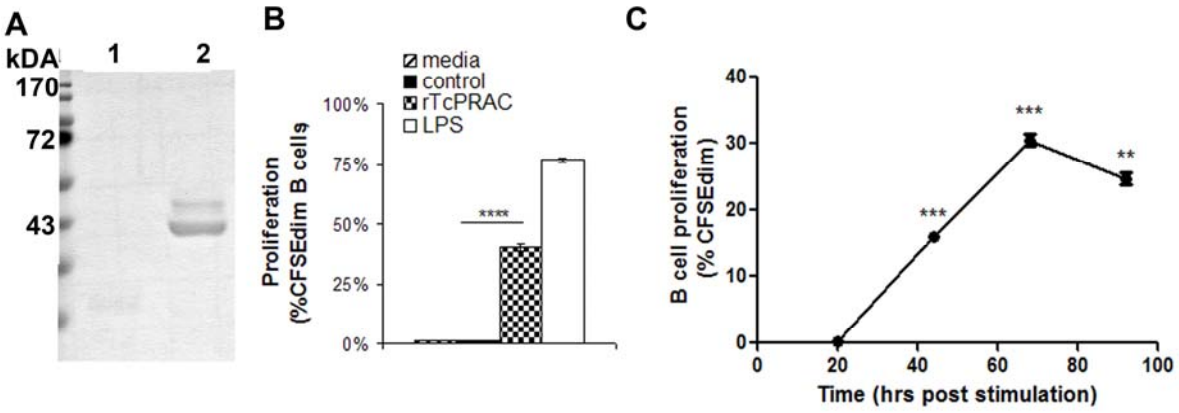

Figure 12 Y-strain derived rTcPRAC-induced B cell proliferation.

A, IMAC purified protein from empty vector IMAC purified protein (lane 1) and from IMAC purified rTcPRAC (lane 2). *B,* rTcPRAC-induced B cell proliferation compared with negative control in the absence of PMB treated media or 10 μg/mL LPS as a positive control. *C,* Time-course analysis of rTcPRAC-induced B cell proliferation, statistics compare each time-point with the previous one. Data represent the mean percentages of triplicate repeats ± the SD. **** $p < 0.00001$, *** $p < 0.0001$, ** $p < 0.001$, Student's *t* test.

3.4.2 Dose dependent rTcPRAC-induced B cell CD69 and CD86 expression and IgG secretion

Up-regulation of surface activation markers and co-stimulatory markers are hallmarks of lymphocyte activation and has been reported in the context of polyclonal B cell mitogenicity (Montes, Acosta-Rodriguez et al. 2007). Therefore, we assessed the correlation between rTcPRAC-induced B cell proliferation and the expression of activation and co-stimulatory

surface proteins. To assess whether rTcPRAC-induced B cells activation increased surface expression of the early activation marker CD69 and the co-stimulatory molecules CD80 and CD86, Balb/c splenocytes were stimulated with 1 to 20 μg/mL of rTcPRAC or equal volume of negative control. rTcPRAC stimulation resulted in B cell proliferation that was significant compared to negative control stimulation ($p < 0.0001$) and correlated with rTcPRAC dose ($p = 0.02$; $R^2 = 0.86$) (Fig 13*A*). rTcPRAC treatment increased the number of CD69 positive B cells and increased the geometric mean fluorescence intensity (GeoMFI) of CD86 on B cells compared to negative controls ($p < 0.0001$ for both) (Fig 13*B*). The expression of these proteins correlated with rTcPRAC dose ($p = 0.008$; $R^2 = 0.93$ and $p = 0.009$; $R^2 = 0.92$, respectively). CD80 expression on B cells was not altered after rTcPRAC stimulation (data not shown). The percentage of CD69 positive B cells and the GeoMFI of CD86 on B cells are plotted against rTcPRAC-induced B cell proliferation (Fig 13*C*). Both the level of CD69 positive B cells and the level of CD86 after rTcPRAC correlated with rTcPRAC-induced B cell proliferation ($p < 0.001$; $R^2 = 0.98$ and $p = 0.01$; $R^2 = 0.90$, respectively).

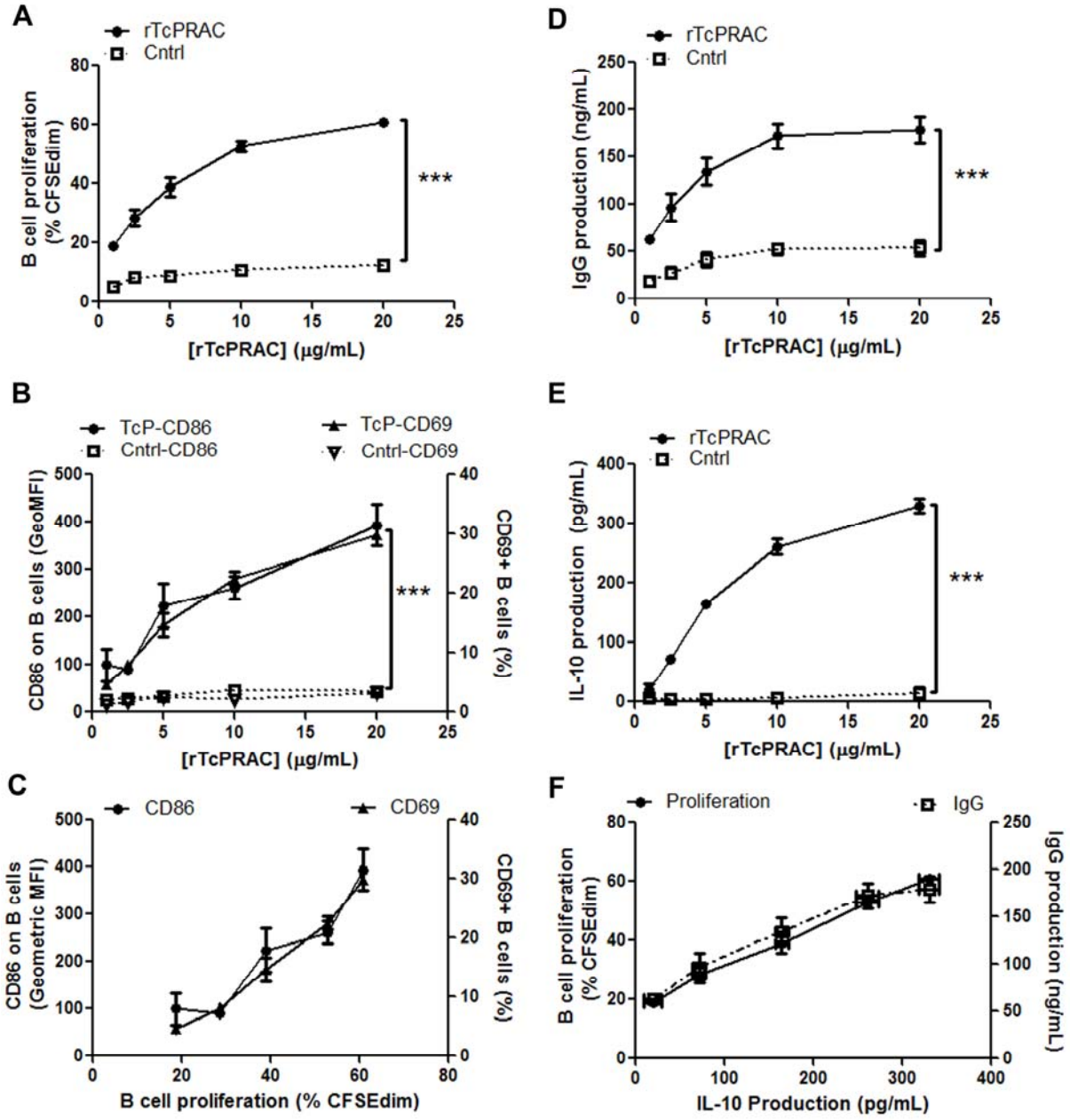

Figure 13: rTcPRAC-induced B cell proliferation, surface activation phenotype, IgG secretion and IL-10 secretion.

Balb/c splenocytes were stimulated for 72 hours with rTcPRAC. *A,* Increased B cell proliferation compared to controls ($p < 0.0001$) was dose dependent ($p = 0.02$; $R^2 = 0.86$, Pearson test). *B,* Increased expression of CD69 and CD86 on B cells compared to negative control ($p < 0.0001$) was dose dependent ($p = 0.008$; $R^2 = 0.93$ and $p = 0.009$; $R^2 = 0.92$, respectively). *C,* CD69 and CD86 expression on B cells correlated with TcPRACA-induced B cell proliferation ($p < 0.001$; $R^2 = 0.98$ and $p = 0.01$; $R^2 = 0.90$, respectively). *D,* Increased IgG secretion compared to

controls ($p < 0.0001$) was dose dependent ($p = 0.03$; $R^2 = 0.94$, Pearson test). *E*, Increased IL-10 production compared to controls ($p < 0.0001$) was dose dependent ($p = 0.02$; $R^2 = 0.88$). *F*, IL-10 production correlated with both B cell proliferation and IgG production ($p = 0.0001$; $R^2 = 0.996$ and $p = 0.0032$; $R^2 = 0.962$, respectively). Data are presented as the mean of duplicate or triplicate repeats ± SEM. 2-way ANOVA to compare treatment groups, Pearson test for correlation.

Hypergammaglobulinemia is a common attribute of B cell mitogenic response during infectious disease, and is a hallmark of acute phase Chagas' disease. To assess rTcPRAC-induced IgG secretion, post-stimulation culture supernatants were analyzed for IgG concentration by ELISA. rTcPRAC B cell stimulation resulted in a significant increase in IgG production compared to negative control stimulation ($p < 0.0001$) and correlated with rTcPRAC dose between 1 and 10 μg/mL ($p = 0.03$; $R^2 = 0.94$)(Fig 13*D*).

3.4.3 rTcPRAC-induced B cell proliferation and IgG secretion correlate with IL-10 secretion

Recent studies have highlighted non-antibody mediated B cell effector functions, such as cytokine secretion (Lund 2008). Several other investigators report IL-10 secretion from murine splenocytes after stimulation with mitogenic proteins (Ferreira, Bras et al. 1997; Dinis, Tavares et al. 2004; Madureira, Baptista et al. 2007). To evaluate rTcPRAC-induced IL-10 production, Balb/c splenocytes were stimulated with rTcPRAC or negative control, and culture supernatants were analyzed. IL-10 production correlated with rTcPRAC dose ($p = 0.02$; $R^2 = 0.88$) and was virtually undetectable for negative control stimulation (Fig 13*E*). IL-10 production also correlated with both B cell proliferation and IgG production ($p = 0.0001$; $R^2 = 0.996$ and $p = 0.0032$; $R^2 = 0.962$, respectively) (Fig 13*F*).

3.4.4 MZ B cells are more responsive to TI rTcPRAC stimulation than are FM B cells

MZ and FM B cells represent functionally and spatially distinct populations in the spleen and have been reported to differentially respond to other TI type 1 antigens (Meyer-Bahlburg, Bandaranayake et al. 2009). Therefore, we determined whether MZ and FM B cells differentially respond to rTcPRAC. To test rTcPRAC-induced MZ and FM B cell activation, splenocytes were negatively sorted to isolate an untouched mature B cell population, which was then sorted for MZ and FM mature B cells based on surface phenotype. Isolated MZ or FM B cells, or B cells in a mixed splenocytes population, were stimulated with rTcPRAC and analyzed for proliferation (Fig 14*A*) and surface marker expression (CD69, CD86, and MHC class II (MHCII)) (Fig 14*B*). After stimulation, culture supernatants were analyzed for antibody and IL-10 secretion (Fig 14*C*). MZ and FM B cells proliferated in response to rTcPRAC compared to negative controls ($p < 0.0001$ and $p = 0.0004$, respectively) (Fig 14*A*). MZ B cells proliferated to a greater extent than did FM B cells ($p = 0.0007$). rTcPRAC-induced B cell proliferation was greater in the mixed splenocytes population than in either isolated B cell population ($p < 0.001$ for both). MZ B cells proliferated more in response to LPS than did FM B cells and neither subset proliferated without stimulation (media alone). The difference between rTcPRAC-induced B cell proliferation of MZ and FM B cells was greatest at 5 μg/mL rTcPRAC, therefore activation phenotype was assessed at this concentration.

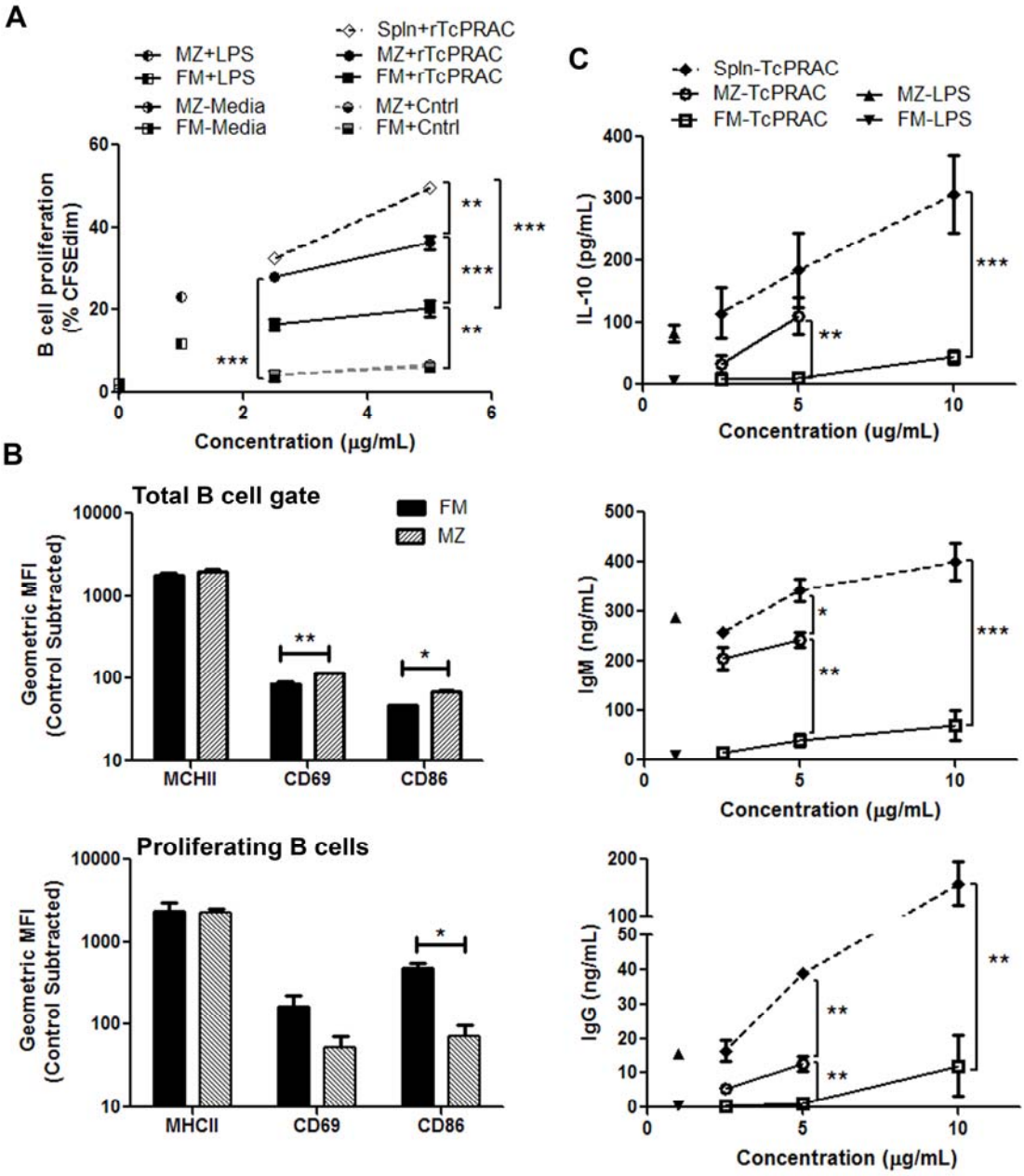

Figure 14: Differential rTcPRAC-induced TI MZ and FM B cell stimulation.

Splenocytes (spln), sort purified MZ, or sort purified FM B cells were stimulated with 2.5-10 µg/mL rTcPRAC, negative control (cntrl), 1 µg/mL LPS or left un-stimulated (media control). For flow data, the CD19+ live cell gates were analyzed. *A,* B cell proliferation was measured by CFSE diminution after stimulation. *B,* B cell activation by surface phenotypes was assessed at 5 µg/mL rTcPRAC-stimulation. *C*, rTcPRAC-induced antibody and IL-10 secretion were assessed in culture supernatants from spln, MZ, and FM B cell stimulations. Data are presented as the

mean of duplicate or triplicate repeats ± SEM *** $p < 0.0001$ ** $p < 0.001$, * $p < 0.05$ by 2-way ANOVA and Bonferroni post-test analysis.

Expression of MHCII, CD69, and CD86 increased compared to control stimulation in both subset populations ($p < 0.01$ for each) (Fig 14*B*). Analysis of the total B cell gate indicates that CD69 and CD86 levels were increased on MZ compared to FM B cells ($p = 0.009$ and $p = 0.02$, respectively)(Fig 14*A*, top). Within the proliferating cells, FM B cells displayed higher levels of CD86 ($p = 0.02$) (Fig 3*B*, bottom).

MZ B cells secreted more IL-10 than did FM B cells--twice the rTcPRAC concentration induced the same IL-10 secretion in FM versus MZ B cells (Fig 14*C*, top). MZ B cells secreted six-fold more IgM in response to rTcPRAC than did FM B cells ($p < 0.05$) (Fig 14*C*, middle). Neither FM nor MZ B cells produced significant IgG in response to rTcPRAC, whereas B cells in mixed splenocytes populations have robust IgG production (Fig 14*C*, bottom). These results indicate that rTcPRAC-induced TI activation of MZ and FM B cells, although these two subsets produce different patterns of response. MZ B cells were more sensitive than FM B cells to rTcPRAC-induced B cell proliferation, IL-10 production and antibody secretion. However, Proliferating FM B cells had an activated phenotype that was comparable or increased compared to proliferating MZ B cells. These data support the hypothesis that pathogen encoded mitogenic protein may differentially influence MZ and FM B cells during infection.

3.5 DISCUSSION

This study provides a comprehensive characterization of rTcPRAC-stimulated polyclonal B cell responses in vitro, showing that B cell proliferation correlates with activation phenotype, secretion of antibodies, and cytokine production, and that rTcPRAC differentially stimulates splenic B cell subsets. Previous reports of the TI mitogenic activity of TcPRAC were based on proliferation of T-cell depleted or athymic splenocytes (Reina-San-Martin, Degrave et al. 2000). In this study, the activation of B cells by TcPRAC was directly measured in both mixed splenocyte populations and after purification of specific B cell subsets. In addition, this study provides the first evidence that TcPRAC is not immunogenic during experimental infection.

Analysis of B cell surface markers showed that rTcPRAC simulation increased B cell surface expression of early activation marker, CD69, as well as CD86 in a dose-dependent manner that correlated with B cell proliferation. TcPRAC-induced, dose-dependent IgG secretion correlated with B cell proliferation as well. These data emphasize the potential of TcPRAC to contribute to B cell polyclonal activation during acute Chagas' disease, which is associated with increased B cell activation (Chapter 2) and is characterized by hypergammaglobulinemia as well as non-specific lymphoproliferation, both of which are thought to contribute to parasite evasion of host immunity (Reina-San-Martin, Cosson et al. 2000; Minoprio 2002).

Recent research into the function of B cells has highlighted multifunctional effector attributes of these cells (LeBien and Tedder 2008), especially the role of IL-10 cytokine-secreting B cells (Mizoguchi and Bhan 2006; Lund 2008; Madan, Demircik et al. 2009). IL-10 can be secreted from a variety of B cell subsets, including plasma cells and plasmablasts (Madan,

Demircik et al. 2009), regulatory B1 cells (B10) (Yanaba, Bouaziz et al. 2009), and potentially MZ and FM B cells (Lund 2008). We found that IL-10 was secreted by splenocytes upon stimulation with rTcPRAC. Several other B cell mitogenic proteins are known to stimulate production of IL-10, including a similar proline racemase, PrpA, from *Brucella abortus* (Ferreira, Bras et al. 1997; Dinis, Tavares et al. 2004; Spera, Ugalde et al. 2006; Madureira, Baptista et al. 2007). rTcPRAC-induced IL-10 secretion correlated with B cell proliferation and IgG secretion, suggesting that this cytokine was produced by rTcPRAC-activated B cells. IL-10 is considered a master regulator of immunity to infection and influences the fate of many cell types depending on the timing and context of IL-10 production (Couper, Blount et al. 2008). IL-10 production from rTcPRAC stimulated B cells could contribute to increased plasma cell differentiation from memory B cells (Agematsu, Nagumo et al. 1998), as well as influence other immune effector mechanisms, such as macrophage response to parasites (Abrahamsohn and Coffman 1996).

Marginal zone and Follicular B cells constitute two functionally and anatomically distinct mature B cell subsets within the spleen (Allman and Pillai 2008). MZ B cells are located at the marginal sinus of the spleen, and are considered first responders to blood stage pathogens. In addition, MZ B cells are more responsive to LPS, suggesting a greater responsiveness to TI antigens in general (Oliver, Martin et al. 1997; Meyer-Bahlburg, Bandaranayake et al. 2009). FM B cells are the predominate responders to TD antigenic stimulation (Allman and Pillai 2008). MZ B cells were activated and proliferated more in response to rTcPRAC than did FM B cells. The in vitro stimulation of MZ B cells by rTcPRAC is similar to the early and sustained expansion of MZ B cells that is associated with polyclonal B cell activation during experimental

infection (Bryan et al, unpublished data). Furthermore, analysis of purified MZ and FM B cells indicated that up-regulation of CD69 and CD86 was a TI response to rTcPRAC.

MZ B cells produce robust IgM in response to rTcPRAC stimulation, but FM B cells produce minimal IgM. These data agree with evidence from other studies showing that MZ B cells have enhanced secretory ability (Oliver, Martin et al. 1997; Gunn and Brewer 2006). IgG secretion was minimal from both MZ and FM B cells compared to the level of IgG produced by B cells in mixed splenocytes, suggesting that accessory cells, probably T cells, are necessary for driving isotype switch to IgG after rTcPRAC stimulation. These data fit with previous studies showing that polyclonal B cell activation during experimental *T. cruzi* infection can be largely abrogated by depletion of CD4 T cells (Russo, Minoprio et al. 1988). TcPRAC may be a T-independent mitogen *in vitro*, but rely more on T cell activation for full B cell mitogenic effect *in vivo*.

The correlation of rTcPRAC-induced IL-10 production with B cell proliferation and antibody secretion suggested that B cells produced IL-10 in response to rTcPRAC. rTcPRAC-induced IL-10 secretion from purified B cells confirmed this hypothesis and indicated that MZ B cells were more sensitive to rTcPRAC-induced IL-10 secretion than were FM B cells. IL-10 is secreted from splenocytes during experimental infection and has a negative impact on the development of effective anti-parasite immune responses during *T. cruzi* infection (Silva, Morrissey et al. 1992; Reed, Brownell et al. 1994). As splenic MZ B cells are poised to be first responders to blood born pathogens (Oliver, Martin et al. 1997), the ability of TcPRAC to induce

IL-10 secretion from these cells may significantly contribute to immunopathology during early *T. cruzi* infection.

The results presented here demonstrate that the *T. cruzi* B cell mitogen, TcPRAC differentially stimulates B cell subsets and primarily affects MZ B cells. B cell stimulation correlated with production of Ig and IL-10 secretion, indicating that this protein expressed by infectious trypomastigotes may contribute to the early humoral immune dysfunction seen in acute Chagas' disease.

4.0 GENETIC IMMUNIZATION GENERATES ANTIGEN SPECIFIC IMMUNITY TO THE *T. CRUZI* B CELL MITOGEN, PROLINE RACEMASE (TcPRAC)

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4.1 ABSTRACT

T. cruzi, the etiologic agent of Chagas' disease, induces polyclonal B cell activation in susceptible hosts. TcPRAC is a parasite encoded T-cell independent (TI) B cell mitogen that may contribute to this dysfunctional humoral response. TcPRAC-specific antibody responses remain undetectable during *T. cruzi* experimental infection, indicating a lack of immunogenic presentation. In contrast, intradermal genetic immunization delivered TcPRAC as an antigen. Gene-gun (GG) immunization generated high-titer TcPRAC-specific IgG without B cell dysfunction. GG immunization generated antigen-specific splenic memory B cell and bone marrow plasma cell formation, which are consistent with the presentation of TcPRAC as a T cell dependent (Td) antigen. TcPRAC-specific IgG bound mitogenic rTcPRAC, decreasing subsequent B cell activation. GG immunization with r*TcPRAC* DNA was non-mitogenic and led to generation of specific responses during co-immunization with another antigen, *T. cruzi*

complement regulatory protein (CRP). These data demonstrate the utility of genetic immunization for the conversion of a protein mitogen to an effective antigen. Furthermore, co-immunization of TcPRAC with another *T. cruzi* antigen indicates the usefulness of this approach for multivalent vaccine development

4.2 INTRODUCTION

Polyclonal B cell activation is triggered by many pathogens and contributes to evasion of host immunity through activation of non-pathogen specific B cell clones. This non-specific response often results in a dilution or delay in the generation of specific immune responses, which may contribute to the development of chronic infection (Reina-San-Martin, Cosson et al. 2000; Sangster, Topham et al. 2000; Spera, Ugalde et al. 2006). Mitogenic proteins that can contribute to this process have been identified from viruses (Rott, Charreire et al. 1996; He, Qiao et al. 2006), bacteria (Watanabe, Kumada et al. 1996; Ferreira, Bras et al. 1997; Spera, Ugalde et al. 2006), fungi (Tavares, Salvador et al. 1993) , and parasites (Reina-San-Martin, Cosson et al. 2000; Reina-San-Martin, Degrave et al. 2000; Aosai, Chen et al. 2002; Montes, Zuniga et al. 2002; Rico, Girones et al. 2002; Montes, Acosta-Rodriguez et al. 2007). Characterizations of these proteins are essential for understanding host-pathogen interaction and instrumental in the development of rational strategies for vaccination. Classical approaches to vaccine development focus on the induction of a robust secondary response to microbial epitopes and the consequences of pathogen immune evasion strategies are not often considered. Despite effective immunization, protection from challenge infection may not be optimally achieved in cases where the pathogen induces a potent polyclonal B cell response that can delay secondary responses and

dilute the existing immune effector mechanisms generated by vaccination (Reina-San-Martin, Cosson et al. 2000; Radwanska, Guirnalda et al. 2008).

While mitogenic proteins are important factors associated with immune evasion of a number of pathogens, they can also be attractive vaccine targets. Effective immune responses to these proteins can decrease immune evasion and may improve host immunity to other antigens (Tavares, Salvador et al. 1993; Tavares, Ferreira et al. 1995; Minoprio 2001; Tavares, Ferreira et al. 2003; Dinis, Tavares et al. 2004). A defining feature of a mitogen is the lack of a specific response to the mitogen itself (Coutinho, Gronowicz et al. 1974). To convert a mitogen to an immunogen requires a change in its presentation to the host immune system. Gene-gun (GG) delivery of antigen has been shown to be an effective method for generating immunity to target proteins by DNA inoculation into cells within the dermis (Robinson and Torres 1997; Payne, Fuller et al. 2002; Wang, Zhang et al. 2008). GG immunization generates a vigorous humoral and cellular response through delivery of low amounts of genetic material to a highly immunogenic organ that is relatively devoid of B cells. Therefore, GG immunization is an attractive strategy for immunogenic delivery of a pathogen encoded B cell mitogen.

4.3 MATERIALS AND METHODS

4.3.1 Gene-gun immunization.

DNA was purified from *E. coli* cells by endotoxin free Mega prep kits (Qiagen). The *CRP.daf* gene (Sepulveda, Hontebeyrie et al. 2000) was sub-cloned into pcDNA3. For eukaryotic

expression, *TcPRACA* was amplified with a 5' Kozak sequence and inserted into the pcDNA3.1/VF-his-TOPO vector (Invitrogen) and transformed into BL21(DE3) (EMD, Novagen). DNA was coated on 1.0 μm gold particles (Bio-Rad) and loaded into Tefzel tubing. DNA (8 μg) was administered by Helios Gene-gun (Bio-Rad) at 400 psi, in two shots per mouse on shaved abdomen per time-point. Immunized mice were bled after boosting and the blood processed to obtain sera. For combination experiments, mice received four shots per time-point, 2 of each immunogen. For the single immunogen controls, mice received 2 shots of DNA containing the immunogen and two shots of vector DNA to maintain the same amount of DNA delivery in all groups.

4.3.2 rTcPRAC, rCRP and whole parasite specific ELISA.

For rTcPRAC and rCRP analysis, 4 HBX Immulon ELISA plates (Thermo Scientific) were coated with 100 ng of purified protein and incubated overnight at 4°C. Plates were washed with T-PBS and blocked and stored at -20°C until used. For whole parasite ELISA, plates were coated with $2x10^5$ heat-inactivated parasites per well and incubated overnight at 4°C. Plates were washed and blocked and stored at -20°C until used. Mouse serum was diluted in block and applied to ELISA plates overnight at 4°C. Plates were washed and developed with the appropriate secondary antibody. The estimated reciprocal endpoint titer (RET) was determined graphically based on the OD_{450} values from equivalent dilution of pooled mouse pre-immunization serum samples. RET was defined as the first dilution with a value below the pre-immune OD_{450} plus SD (two or three replicates).

4.3.3 Plasma cell and memory B cell rTcPRAC specific ELISPOT.

Immunized mice were sacrificed after the third boost. BM was collected from the femur and tibula of mice and processed for single cells. BM was flushed from bones using a 32 gauge needle and mashed through a 40 μM cell strainer (BD Biosciences) with the flat head of a 3mL syringe. The resulting cell suspension was washed and the cells were resuspended in cRPMI (same as above). Spleens were processed for single cells, by gentle mashing in a 40uM cell strainer, treated with RBC lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 115uM EDTA), washed with 1 x PBS, and suspended in cRPMI, resulting in an average yield of ~$2.5x10^7$ total BM cells, resupended to $5x10^6$ cell/mL prior to diluting in the ELISPOT plates. Multiscreen HTS 96-well ELISPOT plates (BD Biosciences) were coated with 2.5 μg/mL of rTcPRAC and incubated overnight. ELISPOT plates were washed with T-PBS and blocked with cRPMI for 2 hrs. Blocking media was removed and cells (BM or splenocytes) were plated into the ELISPOT plates (6-12 wells per sample) at several dilutions (10^6, 10^5, $5x10^4$, $2.5x10^4$, $1.25x10^4$, and $1.0x10^3$ cells/well). After 5-6 hrs, the cells were washed off with PBS (3x) followed by T-PBS (3x). Secreted antibodies were detected by incubating with anti-mouse IgG conjugated to biotin (16 hrs, 4˚C), washing with T-PBS (3-4x), incubation with avidin-peroxidase complex (30 min, RT)(Vector Laboratories, Burlingame, CA), washing with T-PBS (3x) and PBS (3x), followed by incubation with AEC ELISPOT substrate (8 min, RT)(BD Biosciences). The reaction was stopped by washing with PBS. Spots were analyzed using ImmunoSpot image acquisition 4.5 and ImmunoSpot 5.0 Professional DC software (ImmunoSpot). BM plasma cells are reported as the number of spots per 10^6 splenocytes. For analysis of memory B cells, splenocytes were stimulated with 0.4 μg of R595 lipopolysaccharide (Alexis Biochemicals, Plymouth Meeting, PA) and pokeweed mitogen (PWM Emory stock, gift from Shane Crotty) for 6 days at $5x10^6$

cell/mL (37˚C, 5% CO_2) to induce differentiation into plasma cells. After stimulation, cells were washed with cRPMI, then plated on ELISPOT plates (10^6, $5x10^5$, $2.5x10^5$, $1.25x10^5$ cells/well) and incubated for 5-6 hours (37˚C, 5% CO_2). Cells were washed off the plates with PBS (3x) followed by T-PBS (3x). Expanded memory B cells were recorded as spots per 10^6 cells stimulated. This protocol was adapted from previously published studies (Slifka and Ahmed 1996; Slifka, Antia et al. 1998; Crotty, Kersh et al. 2003).

4.3.4 Binding of rTcPRAC to IgG from TcPRAC immune mice.

IgG from TcPRAC or CRP immunized mice was purified from sera via Melon column (Pierce, Thermo Scientific). 75 μg of purified IgG was bound to Protein A/G Plus resin (Pierce, Thermo Scientific) for 1 hour at 4˚C with rotation. The resin was washed with binding buffer (0.25M Tris, 0.15M NaCl) to remove unbound material. rTcPRAC protein (1-30 μg) was added to the IgG:protein A/G resin and incubated overnight at 4˚C in either 100 or 500 μL of binding buffer. Non-bound protein was spun out of the columns and concentrated to a final volume of 100uL. Non-bound protein was stored at 4˚C until used for splenocytes stimulation or SDS-PAGE/Western Blot analysis. The protein bound to the IgG:protein A/G resin was washed with conditioning buffer (Pierce) and eluted with low pH elution buffer (Pierce, Thermo Scientific) and neutralized with 1M Tris pH 9.5 and stored at 4˚C until used for stimulate splenocytes SDS-PAGE/Western Blot analysis.

4.3.5 Western Blot analysis.

Proteins diluted in sample buffer (8% SDS, 40% glycerol, 300mM Tris, 0.04% Bromophenol Blue) were separated at 110V for 1.5 hours in 10-12% SDS-PAGE. Gels were transferred to nitrocellulose using the iBlot system (Invitrogen). Memcode Blue protein stain (Pierce, Thermo Scientific) was used to visually confirm protein transfer. Membranes were blocked with block solution (3% milk, 1% BSA, 1xPBS, 0.05% Tween-20) and washed with wash buffer (1xPBS, 0.05% Tween-20). Sera were diluted into block and incubated at RT for 1 hr with rotation. Membranes were washed and diluted HRP-conjugated secondary antibodies applied in blocking buffer. Membranes were washed and developed with super signal pico (Pierce, Thermo Scientific).

4.3.6 Statistical analysis.

2-way ANOVA was used for comparing two treatments over a range of doses or after passing of time. Bonferroni post-test analysis, Student's *t* test, or Mann-Whitney tests were used for comparison of individual doses or time-points. For analysis of correlation between two treatments, the Pearson test was applied.

4.4 RESULTS

4.4.1 Experimental infection of with *T. cruzi* generates minimal anti-TcPRAC IgG response.

A defining characteristic of a mitogen is the lack of a specific response to the mitogen itself, unless it is present at very low concentration (Coutinho, Gronowicz et al. 1974). Thus, a pathogen encoded mitogen that is expressed during early infection at high enough levels to contribute to polyclonal B cell proliferation would not be expected to generate a specific immune response. To assess whether a specific response to *T. cruzi* expressed TcPRAC develops during infection, relatively susceptible (Balb/c) and resistant (C57Bl/6) mouse strains were infected with *T. cruzi*. Serial sera samples were collected and analyzed for a specific IgG and/or IgM response to rTcPRAC and a specific IgG or IgM response to *T. cruzi* whole parasites. Analysis of sera over the course of the 125 days of infection indicated that a specific IgM response did not develop to rTcPRAC in Balb/c mice and rTcPRAC specific IgG response did not develop in either model system when a specific *T. cruzi* response occurred (Fig 15*A*). Negative control coated wells were used to confirm that there was no rTcPRAC specific IgM or IgG binding (Fig 15*A*). rTcPRAC-specific IgG response remained undetectable at day 125 post-infection, when specific antibodies to CRP and several other *T. cruzi* membrane proteins were detected (Fig 15*B*). These data support the hypothesis that during experimental infection, TcPRAC protein is not presented in an immunogenic context.

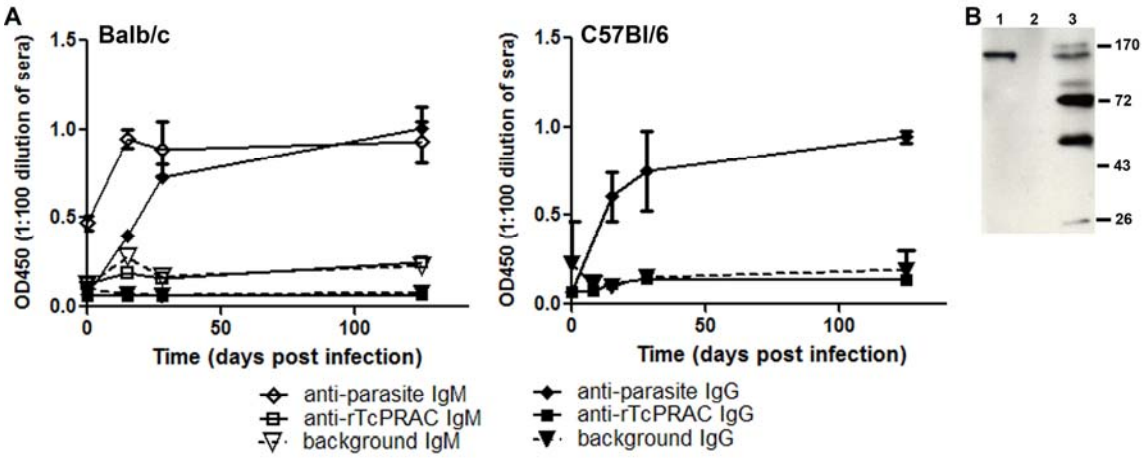

Figure 15: rTcPRAC-specific IgG is undetectable during *T. cruzi* experimental infection

Susceptible Balb/c or resistant C57Bl/6 mice were infected with *T. cruzi. A,* Serial sera samples were analyzed for response to whole parasite, rTcPRAC or negative control to establish background binding. Data represent triplicate repeats from pooled serum from five mice in each experiment. *B,* Western blot analysis of IgG reactivity to rCRP (500ng, lane 1), rTcPRAC (500ng, lane 2), and parasite membrane proteins (10ug, lane 3) with Balb/c sera 125 days post-infection (diluted 1:500).

4.4.2 *TcPRACA* DNA immunization via GG presents rTcPRAC as an immunogen

Gene-gun (GG) delivery of antigen has been shown to be an effective method for generating immunity to target proteins by DNA inoculation into cells within the dermis (Robinson and Torres 1997; Payne, Fuller et al. 2002; Wang, Zhang et al. 2008). To evaluate the potential of intradermal genetic immunization to deliver rTcPRAC in an immunogenic context, Balb/c mice were primed and boosted with *TcPRACA* DNA via GG inoculation. After each immunization, blood was collected and sera analyzed for generation of rTcPRAC-specific IgG responses. Western blot analysis demonstrated that TcPRAC-specific IgG binds to monomeric rTcPRAC protein (Fig 16*A*). Specific rTcPRAC IgG responses were evident by boost one and increased between boost one and increased after each subsequent boost (Fig 16*B).* While both IgG2 and

IgG1 were generated in response to *TcPRAC* immunization, specific IgG1 predominated with a reciprocal endpoint titer (RET) of 7.6 x 10^6 ± 2.5 x 10^6 versus an IgG2 RET of 7.0 x 10^4 ± 7.8 x 10^3 at boost 3 (average ± SEM; p = 0.04) (Fig 5*B*). Mice receiving empty vector did not mount a TcPRAC-specific IgG response that differed from naïve mice (data not shown). Furthermore, TcPRACA immune sera bound to whole parasites by ELISA with a TcPRAC-specific IgG RET of 320 ± 192 (5 mice, mean ± SEM) and flow cytometry with an significant increase in binding of IgG to parasites from TcPRAC immune sera compared to naïve controls (p < 0.01) (Fig 15*C*). These data indicate that GG immunization with *TcPRAC* generates a robust, specific humoral IgG response that is capable of binding TcPRAC on the surface of the whole parasite.

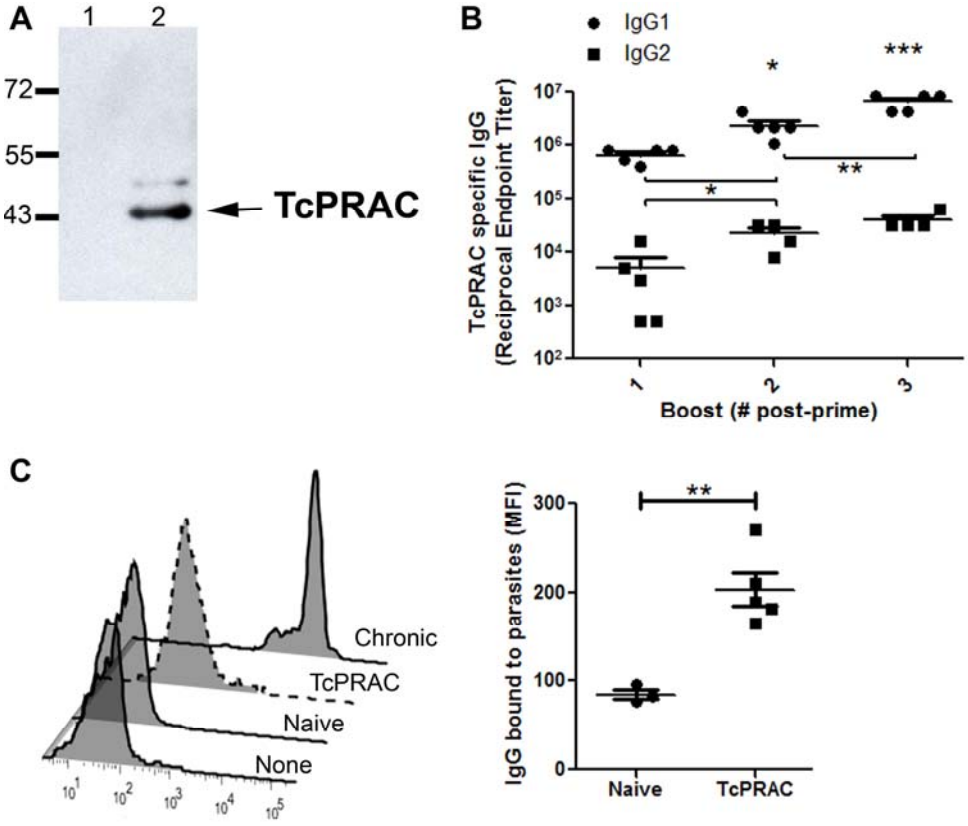

Figure 16: GG immunization with *rTcPRAC* DNA elicits an immunogenic response

Balb/c mice were primed and boosted at one month intervals. Ten days after each boost, serum was collected. *A,* TcPRAC-specific IgG (diluted 1:2000) binds to rTcPRAC (7 μg protein loaded; lane 2) without binding negative control (lane 1). *B,* Analysis of anti-rTcPRAC IgG1 and IgG2 response by ELISA. Data represent the reciprocal endpoint titer (RET) (±SEM) of five mice. *C*, Left, representative histograms showing IgG bound to the surface of live parasites: from mice after 125 days of infection (chronic), TcPRAC-immune mice, naïve mice, or with fetal bovine serum as a negative control (no mouse antibody). Right, mean florescence intensity (MFI) after treatment of parasites with naïve or TcPRAC-immune serum from five separate mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t* test.

4.4.3 *rTcPRAC* immunization generated rTcPRAC specific bone marrow plasma and splenic memory B cells

In general, TI B cell activation leads to the development of short-term plasma cells that reside in the spleen (Slifka, Matloubian et al. 1995). This response is distinct from TD antigens that induce memory B cells or long-lived plasma cells (O'Connor, Gleeson et al. 2003). To determine if *rTcPRAC* DNA immunization induces specific bone marrow plasma cells and memory B cells, mice were immunized and TcPRAC-specific IgG antibody secreting cells (ASC) were enumerated (Crotty, Kersh et al. 2003; Crotty, Aubert et al. 2004). rTcPRAC-specific bone marrow plasma cells and memory cells were detected after GG immunization but not following immunization with control antigen (Fig 17) ($p = 0.004$, Mann-Whitney Test).

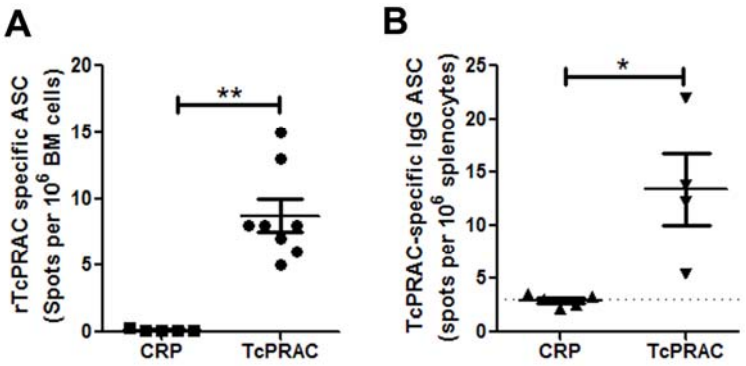

Figure 17: GG immunization generates specific bone marrow plasma cells and splenic memory B cells

A, Bone marrow (BM) cells were isolated after boost three and analyzed by rTcPRAC specific ELISPOT for antibody secreting plasma cells. Data represent ASC (±SEM) in 8 mice. *B,* Splenocytes were isolated after boost three and analyzed for rTcPRAC specific memory B cells. Data represent ASC (±SEM) in 4 mice. * $p < 0.05$, ** $p < 0.01$, Student's *t* test.

4.4.4 TcPRACA immunization did not induce mitogenic response or B cell dysfunction

A concern in using B cell mitogens as immunogens is the potential induction of polyclonal B cell activation or proliferation. To determine whether intradermal delivery of *TcPRACA* DNA led to mitogenic activation of B cells, Balb/c mice were primed with either *TcPRACA* or *CRP* DNA via GG immunization. Blood was collected prior to inoculation and up to 12 days post immunization. Blood was stained for B and T cells and analyzed by flow cytometry to determine the percentage of B and T cells within the lymphocyte gate. During the first six days post-priming with *TcPRACA* versus *CRP* there is no difference in the percentage of B cells within the peripheral blood ($p = 0.18$, 2-way ANOVA) (Fig 18A). Between days 9 and 12 post inoculation there was a small increase in the percentage of B cells in *rTcPRAC* compared to *CRP* immunized mice ($p = 0.017$, 2-way ANOVA). To test for early IgM secretion, which would indicate a mitogenic response, mouse plasma samples were analyzed for total IgM levels post prime (Fig 18*A*). There were no significant differences in the level of IgM response in mice primed with *TcPRACA* versus *CRP* ($p = 0.54$, 2-way ANOVA), although mice primed with either immunogen resulted in increased total IgM by day 12 post-prime ($p < 0.0001$). These data indicate that the delivery of rTcPRAC via GG did not result in an early non-specific polyclonal B

cell mitogenic effect. Rather, the expansion of B cells and secretion of IgM post initial priming with *TcPRACA* via GG were consistent with the generation of a specific humoral response.

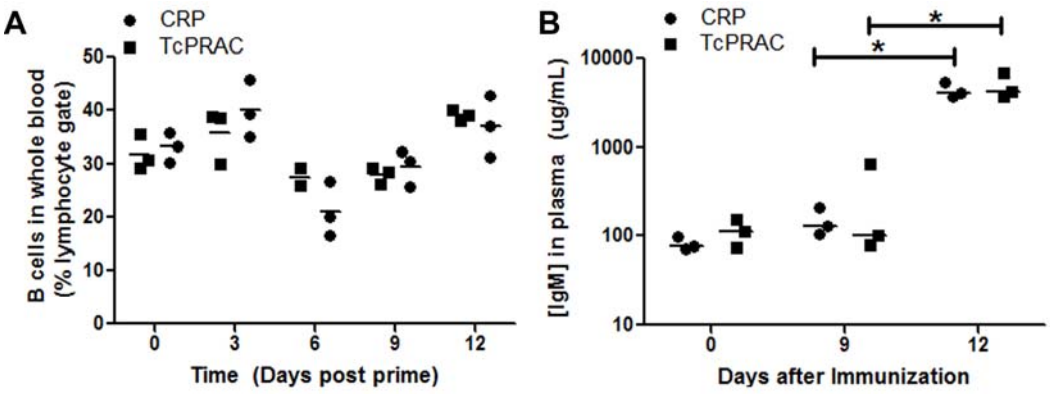

Figure 18: Inoculating mice with *rTcPRAC* DNA via GG did not induce polyclonal B cell expansion of IgM secretion.

Balb/c mice were primed by GG with *rTcPRAC* or *CRP* DNA. Serum samples were collected prior to priming and every three days for 12 days post-prime. *A,* Staining whole blood for B cells (CD19+) and T cells (CD3+) indicated that there was not a premature expansion of peripheral B cells after *rTcPRAC* prime compared to *CRP* prime. Data represent repeat measures on 3 mice. *B,* Analysis of IgM concentration in the sera of *rTcPRAC* immunized mice showed comparable levels and timing of post-prime increase compared to *CRP* immunized mice. Data represent serum samples from three mice, with the mean indicated by a line.

4.4.5 rTcPRAC co-delivered with another *T. cruzi* antigen

To investigate the effect of *rTcPRAC* immunization on the generation of humoral immunity to a co-delivered antigen, mice were immunized in combination with the *T. cruzi* antigen, CRP (Sepulveda, Hontebeyrie et al. 2000). *CRP* GG immunization was evaluated with or without *rTcPRAC* DNA co-immunization (Fig 19*B*). Administration of *CRP* with or without *rTcPRAC* resulted in comparable anti-CRP IgG RET ($p = 0.345$). The boost response for *CRP* as a single

antigen versus as a combined antigen was significant (p = 0.014 for both, by Wilcoxon signed rank test; 2-way Anova p = 0.0007 for change due to time). Co-immunization resulted in rTcPRAC specific IgG response that was comparable to GG delivery of *rTcPRAC* as a single immunogen (p = 0.133). Western blot data confirms that TcPRAC immunization does not result in generation of a CRP specific response or vise versa (Fig 19*C*). The total level of IgG in serum after immunization with *rTcPRAC* was not elevated compared to serum from *CRP* immunized mice, confirming that a polyclonal immunoglobulin response was not induced by *TcPRAC* GG immunization (p = 0.4, Student's *t* test). These data demonstrate that genetic immunization with *rTcPRAC* does not interfere with the immune response to a co-delivered immunogen.

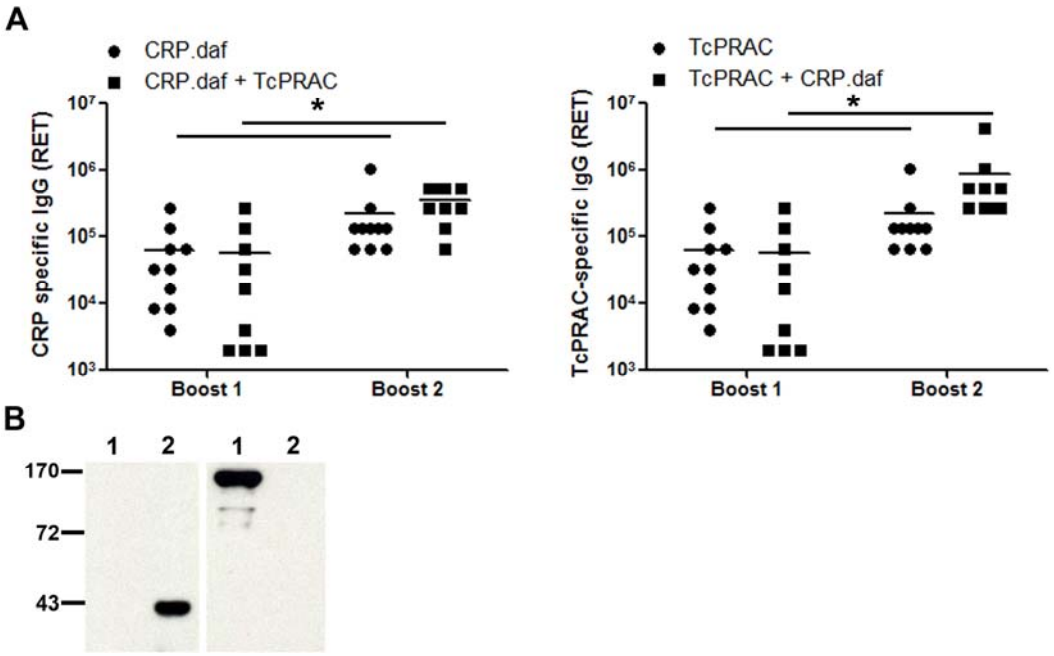

Figure 19: *rTcPRAC* DNA immunization has no negative impact on generation of antibody specific responses to a co-delivered immunogen.

A. Balb/c mice were immunized with *CRP*, *rTcPRAC,* or both immunogens in combination, and then analyzed for specific responses via ELISA. Data represent the reciprocal endpint titer (RET) from five mice for each experimental group. * p < 0.05 Student's *t* test or Bonferroni post-test from 2-way ANOVA. *C,* Western blot

analysis demonstrates lack of cross-reactivity between *TcPRAC* and *CRP* immunized mice. Lane 1 contains rCRP (500 ng) and Lane 2 contains rTcPRAC (500 ng). Blots were probed with pooled boost three sera from *rTcPRAC*-immune (left) or *CRP*-immune mice (right), diluted 1:500.

4.4.6 IgG from TcPRAC-immune mice binds to mitogenic rTcPRAC

For an antibody response against a protein mitogen to be effective in the host, it must bind to the mitogenic form of the protein. Therefore, we tested whether IgG from the sera of immunized mice could bind to mitogenic rTcPRAC, depleting mitogenic activity. To test binding, rTcPRAC-immune (TcP-imm) IgG or control CRP-immune (CRP-imm) IgG was bound to protein A/G resin and then incubated with the equivalent amounts of rTcPRAC. To test for residual mitogenic activity after IgG column depletion, an equal volume of unbound protein was recovered from each column and added to splenocytes cultures. Mitogenic rTcPRAC was depleted following treatment with TcP-Imm IgG as indicated by decreased B cell CD69 and CD86 expression, IL-10 secretion, IgM, and IgG secretion compared to CRP-imm IgG control ($p \leq 0.0001$ for each measure) (Fig 20*A*). rTcPRAC eluted from TcP-imm IgG was compared to non-specifically bound protein eluted from control CRP-imm IgG. Eluted rTcPRAC had significant mitogenic activity compared to control, inducing B cell proliferation, surface marker expression, and IL-10 secretion ($p \leq 0.0001$ for each measure)(Fig 19*B*). Western blot analysis confirmed that rTcPRAC bound to the TcP-imm IgG, whereas CRP-imm IgG did not (Fig 20*C*). These data demonstrate that TcP-imm IgG binds to functionally active, mitogenic rTcPRAC.

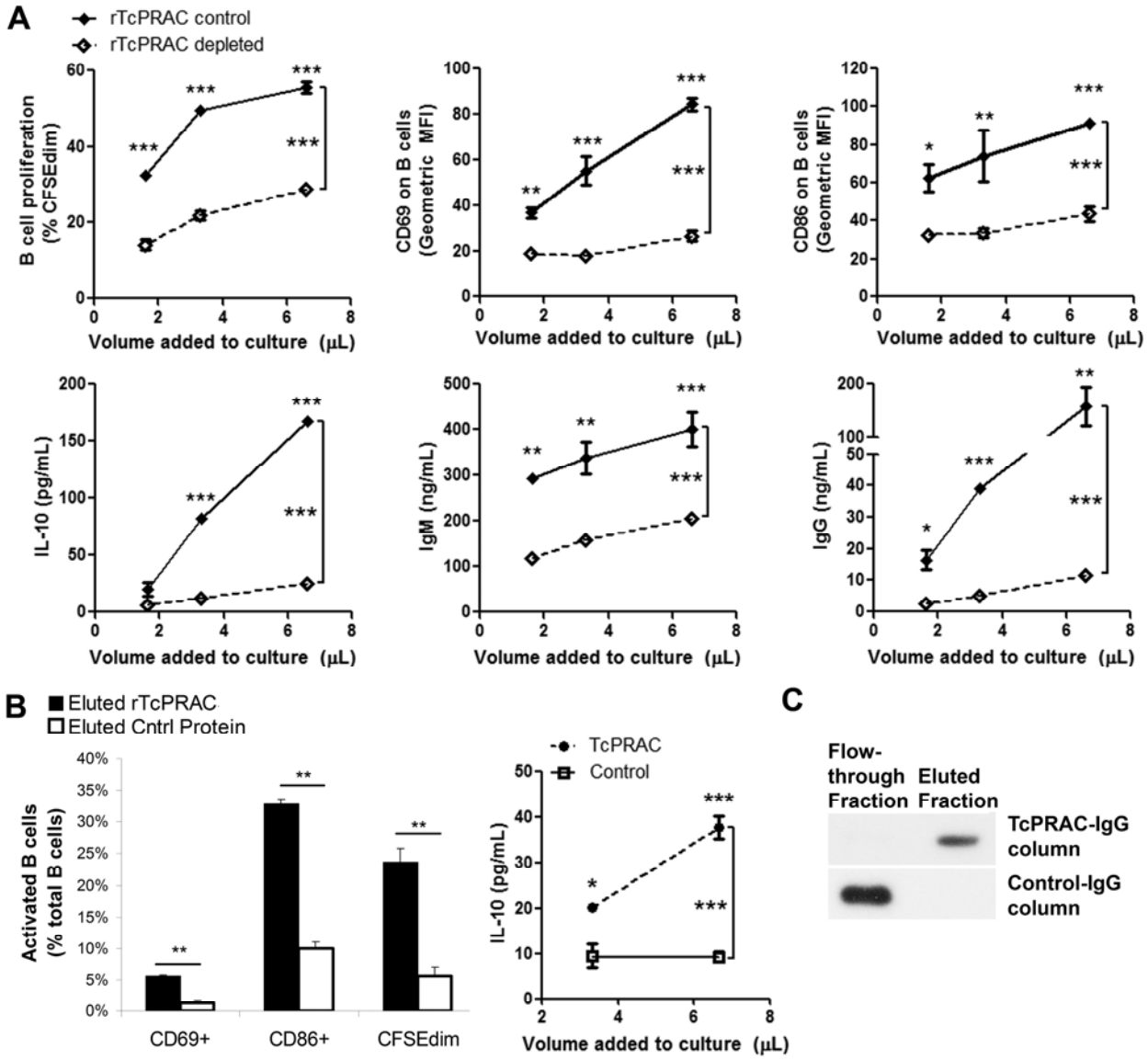

Figure 20: GG *rTcPRAC* immunization generates specific IgG that binds mitogenic rTcPRAC

rTcPRAC-immune (TcP-imm) IgG or control CRP-immune (CRP-imm) IgG was bound to protein A/G resin and then incubated with rTcPRAC. *A,* Equal volumes of non-bound protein after incubation of 30 µg rTcPRAC with TcP-imm IgG (rTcPRAC-depleted) or CRP-imm IgG (TcPRACA-control) incubation were added to splenocytes cultures. After stimulation, live cells were assessed for B cell activation in terms of B cell proliferation and surface phenotype (CD69, CD86) by flow cytometry. IL-10 and antibody (IgM and IgG) secretion were assessed by ELISA. Data represent the mean of triplicate repeats (±SEM) for each measure. *B,* rTcPRAC eluted from TcP-imm IgG was compared to non-specifically bound protein eluted from control CRP-imm IgG for ability to stimulate B cells. Left: Cells were stimulated with eluted protein. Post-stimulation, cells were analyzed for B cell proliferation and

activation (CD69, CD86) by flow cytometry. Data represent the mean of triplicate repeats (±SD) for each measure. Right: Cells were stimulated eluted protein. Post-stimulation, culture supernatant was analyzed for IL-10 secretion by ELISA. Eluted rTcPRAC induced dose-dependent IL-10 secretion compared to control eluted protein. Data represent the mean of triplicate repeats (±SEM). * $p < 0.05$, ** $p < 0.001$, *** $P < 0.0001$, Bonferroni post-tests after 2-way ANOVA or Student's *t* test. *C,* Application of rTcPRAC to TcPRAC-specific and control IgG resulted in rTcPRAC in the flow through fraction from the control IgG column and rTcPRAC in the elution fraction from the TcPRAC-specific IgG column. TcPRAC visualized by anti-TcPRAC IgG from polyclonal serum from protein immunized rabbits.

4.5 DISCUSSION

In this study we investigated the immunogenic potential of TcPRAC, a parasite encoded B cell mitogen, which is expressed by infectious parasite. We found that although TcPRAC specific antibody responses were not appreciable during experimental infection with *T. cruzi*, high titer specific IgG responses were generated through intradermal genetic immunization.

Mitogens induce a non-specific immune response and do not generally induce specific immune responses, unless modified or delivered in low doses (Coutinho, Gronowicz et al. 1974). Therefore, we investigated whether experimental infection with *T. cruzi* led to TcPRAC-specific IgG response in two mouse models of *T. cruzi* infection. In both instances, TcPRAC-specific IgG was not detected. This was not due to lack of antigen availability, as TcPRAC is expressed on the surface of and secreted by trypomastigote parasites (Reina-San-Martin, Degrave et al. 2000). TcPRAC has also been shown to be necessary for parasite differentiation and infectivity. Thus, lack of TcPRAC antigen-specific immunogenicity may help preserve this essential parasite

function (Chamond, Goytia et al. 2005). Many microbes produce mitogenic molecules that elicit polyclonal B cell activation in combination with poor specific host responses during experimental infection (Lima, Bandeira et al. 1992; Tavares, Salvador et al. 1993; Madureira, Baptista et al. 2007). Delivery of mitogenic moieties in an alternative context can diminish the mitogenic effect, producing antigen-specific responses (Soares, Ferreira et al. 1990; Tavares, Ferreira et al. 1995; Dinis, Tavares et al. 2004). For example, the *C. albicans* mitogenic protein p43, when experimentally inoculated at sub-mitogenic doses is able to partially neutralize the biological effects of this protein (Tavares, Ferreira et al. 1995; Tavares, Ferreira et al. 2003).

To test whether immunization with TcPRAC could induce an antigen-specific response, without mitogenic activation of B cells, we utilized intradermal genetic immunization with the TcPRAC gene delivered in a eukaryotic expression vector. Intradermal genetic immunization via GG uses small amounts of DNA as starting material and has highly reproducible efficacy for generation of humoral and cellular responses (Robinson and Torres 1997; Payne, Fuller et al. 2002). Delivery of rTcPRAC in this context resulted in the generation of high titer rTcPRAC-specific IgG capable of binding to rTcPRAC as well as whole parasites. Specific IgG1 RET was 100 fold greater than IgG2 RET, indicating a predominant type 2 T-cell (Th2) helper response. In addition to providing proof of concept that GG immunization can render a mitogenic protein immunogenic, these experiments further emphasize the ability of rTcPRAC to activate splenic B cells, as removal of rTcPRAC by rTcPRAC-specific IgG significantly diminished B cell activation, proliferation, and IL-10 production.

Terminally differentiated end-stage B cell plasma cells in the bone marrow and long-lived circulating memory B cells are the cellular basis for enduring antibody-mediated immunity and acquire longevity as a result of antigen-specific, CD40-dependent interactions with helper T cells (O'Connor, Gleeson et al. 2003). The generation of TcPRAC specific memory B cells and bone-marrow plasma cells by rTcPRAC GG immunization are consistent with the presentation of rTcPRAC as a TD immunogen.

To address the possibility that delivery of *TcPRAC* DNA may induce mitogenic effects in mice, we compared delivery of *TcPRAC* DNA with *CRP* DNA, a *T. cruzi* antigen (Sepulveda, Hontebeyrie et al. 2000) and found similar levels of circulating B cells and similar timing for increased circulating IgM post-prime, which was consistent with an antigenic response. Furthermore, *rTcPRAC* DNA GG co-immunization with *CRP* DNA, had no negative affect on the generation of high titer CRP-specific IgG. Conversely, delivery of TcPRAC with or without a co-immunization with *CRP* did not alter the generation of TcPRAC-specific IgG. These data further indicating that rTcPRAC was delivered as an immunogen rather than a mitogen by GG immunization and highlight the feasibility of multi-valent vaccine design with a pathogen encoded B cell mitogen.

As is the case with many infectious disease immunization strategies, the approach to *T. cruzi* immunization has been focused primarily on the introduction of immunodominant targets that would initiate a strong secondary response upon primary infection with parasite (Wizel, Nunes et al. 1997; Costa, Franchin et al. 1998; Wizel, Garg et al. 1998; Quanquin, Galaviz et al. 1999; Fujimura, Kinoshita et al. 2001; Schnapp, Eickhoff et al. 2002; Wrightsman, Luhrs et al.

2002; Boscardin, Kinoshita et al. 2003; Fralish and Tarleton 2003). The novel strategy presented here was to induce an immune response to a parasite-derived, immune evasion factor, in this case a B cell mitogen. This approach has the potential to combine development of immunity against immune evasion factors with traditional immunization strategies in a multi-component design, leading to improved host response to the invading pathogen. Preliminary immunization and challenge studies indicate that co-immunization with TcPRAC and CRP, as described here, improved survival and decreased parasitemia in mice infected with a lethal dose of *T. cruzi*, compared to either immunogen alone (Appendix B).

The results presented here demonstrate that while TcPRAC is apparently non-antigenic during experimental *T. cruzi* infection, it can be delivered as a potent antigen via genetic immunization. Genetic immunization with TcPRAC did not lead to detectable systemic B cell expansion and did not interfere with the immune response to a different test immunogen. These data offer the basis for further development of this novel strategy of immunoprotection in this and other infectious diseases.

5.0 THESIS CONCLUSIONS AND SIGNFICANCE

The first aim of this dissertation, presented in Chapter 2, focuses on the elucidation of parasite-specific and polyclonal B cell responses to *T. cruzi* experimental infection in relatively resistant C57BL/6 versus susceptible Balb/c mouse models. The data presented here highlight differences in humoral responses based on previously underappreciated host-parasite interactions. In particular, the association of decreased hypergammaglobulinemia with improved parasite-specific humoral response, an early Th1 response versus Th2 response, and differential rather than sustained expansion of splenic B cells of the MZ phenotype were novel findings.

The data presented in Chapter 2 draw attention to new areas of exploration needed to further understand mechanisms of parasite-specific humoral immunity versus polyclonal B cell activation during *T. cruzi* infection. The impact of cytokines and T cell help, both CD4 and NK, on the outcome of humoral responses during acute Chagas disease warrant further study. First, further investigation of cytokines responses are warranted (more cytokines, more time-points and analysis of ex vivo secretion by splenocytes), especially the B cell activating factor (BAFF) to determine the full range of cytokines that may be associated with differential humoral responses during *T. cruzi* infection (Mackay and Mackay 2002; Gorelik, Cutler et al. 2004; Sasaki, Casola et al. 2004; Acosta-Rodriguez, Craxton et al. 2007; Lindh, Lind et al. 2008). Administration of Th1 cytokines early in infection (days 5-8 post-infection) in susceptible Balb/c mice could be

used to determine whether an increased Th1 cytokine burst would be sufficient to alter the humoral responses away from polyclonal activation and toward parasite-specific responses compared to vehicle alone administration. Specific cytokine depletion at these early time points in resistant mice may have the opposite effect, leading to an increased polyclonal response. Timed depletion studies with CD4 and NK T cells might also be informative, for understanding the impact of these cells on humoral responses. Yet, these studies might be hard to interpret and require decreasing rather than ablating CD4 or NK T cells, as these immune effectors are important for control of parasitemia and it may be hard to separate out the effect on humoral responses if there are large changes in parasite kinetics.

An important step in investigating B cell interaction with T cells would be to repeat the analysis of total and parasite-specific antibody and B cell responses in C57BLKS/J (H-2d) versus C57BL/6 (H-2b) and BALB/c (H-2d) versus BALB/B10-H2b (H-2b) to determine if *T. cruzi* infected Balb/B10 H-2b haplotype mice have the same phenotype as Babl/c H-2d haplotype mice and remain more susceptible to polyclonal B cell activation than C57Bl/6 (H-2b) mice. In addition, it would be worth determining if *T. cruzi* infection of C57BlKS/J H-2d haplotype mice results in similar humoral responses as H-2b C57BL/6 mice, or whether they become more susceptible and have a pattern of humoral responses more similar to H-2d Balb/c mice. Investigation of CD40-CD40L on B cells and T cells, respectively, would also be informative in these above experiments and perhaps lead to further investigation through CD40L treatment, which has been shown to improve resistance through IFN-γ production, presumably through DC stimulation (Habib, Rivas et al. 2007). Although DC alterations were not addressed in this present study, DCs priming of B cells and direct modulation of B cell responses have been

shown in other contexts (Kushnir, Liu et al. 1998; MacPherson, Kushnir et al. 1999; Bergtold, Desai et al. 2005; Santos, Draves et al. 2008), indicating that investigation of DC-B cell interactions may help elucidate mechanisms that lead to polyclonal versus parasite-specific humoral responses during *T. cruzi* infection. Preliminary data presented in Figures 31 and 32 of Appendix C also indicate that analysis of macrophage subsets may further illuminate the differences in susceptibility and humoral responses in mice.

Increased total and CRP specific IgM were demonstrated in resistant, but not susceptible mice in this present study, indicating that IgM may be an important factor in mediating resistance to acute phase CD. Thus, the relative contribution of IgM to host resistance, through use of C57Bl/6 mice lacking sIgM, would be beneficial for further defining the crucial elements of the parasite-specific humoral response in resistant mice. If sIgM proves to be a crucial part of an effective immune response against *T. cruzi*, MZ B cells are further implicated in this effective response, as these cells can rapidly produce IgM responses (Song and Cerny 2003).

The present study provided evidence that B cells with the MZ phenotype are differentially expanded in resistant and susceptible mice, suggesting that future studies to more fully define the role of this subset during *T. cruzi* infection are warranted. The use of additional surface markers and genetic analysis, as well as immunohistochemistal analysis of the spleen, would lead to further understanding how MZ B cell population change over the course of *T. cruzi* infection. The monoclonal antibody to LFA-1 and alpha(4)beta(1) integrins can be used to deplete MZ B cells (Belperron, Dailey et al. 2007) to directly address the question of their importance in generation of polyclonal versus parasite-specific responses. MZ B cells are known

to traffic from the MZ into the follicles of the spleen to interact with T cells in germinal center reactions (Cinamon, Zachariah et al. 2008). It would be interesting to determine whether MZ B cell transport of antigen into follicles is disrupted during during *T. cruzi* infection. In addition, knock-out models are available for analysis of B cell responses in the absence of MZ B cells on the C57Bl/6 resistant background (Saito, Chiba et al. 2003; Santos, Draves et al. 2008). Thus, these mice could be used to further illuminate the role of MZ B cell during *T. cruzi* infection in resistant mice. My hypothesis would be that mice lacking MZ B cells would become more susceptible to infection due to impaired IgM response to blood-born antigen and decreased antigen transfer to follicles via MZ B cells.

Furthermore, these data have implications for vaccine design, an area of intense interest in Chagas' disease research, as this study provides evidence that host biases can result in profoundly altered host-parasite dynamics with significant differences in innate and adaptive immune responses. Therefore, multiple mouse strains should be used to fully evaluate any given vaccine. In addition, if IgM responses and/or MZ B cells are shown to be an important source of parasite-specific antibody, regimens should be developed that specifically target these B cells during immunization, as they MZ B cells can generate memory IgM responses (Song and Cerny 2003).

In the second aim, this study provides a comprehensive description of rTcPRAC-stimulated polyclonal B cell responses in vitro, showing that B cell proliferation correlates with activation phenotype, secretion of antibodies, and cytokine production, and that rTcPRAC differentially stimulates splenic B cell subsets. Previous description of TcPRAC as a TI B cell

mitogen were based on data showing proliferation of T-cell depleted or athymic splenocytes (Reina-San-Martin, Degrave et al. 2000). In this study, the activation of B cells by TcPRAC was directly measured in both mixed splenocyte populations and after purification of specific B cell subsets. These results provide evidence that MZ B cells are more responsive to TI activation by TcPRAC than are FO B cells. Furthermore, this study provides the first evidence that TcPRAC-specific responses were not detectable during experimental infection, arguing against its immunogenic presentation during experimental infection.

In the third aim, the development of TcPRAC-specific IgG, bone-marrow plasma cells, and splenic memory B cells were demonstrated after intradermal genetic immunization. These data suggest that a T-cell dependent antigen-specific response occurs during immunization, as the development of bone-marrow resident plasma cells and memory B cells have previously been shown to depend on T cell help (Slifka, Matloubian et al. 1995; Slifka, Antia et al. 1998; McHeyzer-Williams and Ahmed 1999; Fairfax, Kallies et al. 2008). Immunization generated rTcPRAC-specific IgG that were also able to bind to the surface of live parasites, demonstrating that antibodies raised to the recombinant protein were reactive against parasite derived TcPRAC. Furthermore, the TcPRAC-specific IgG generated by immunization was able to bind dimeric rTcPRAC and deplete its mitogenic activity, providing proof of concept for this immunization strategy.

The activation of B cells in vitro by rTcPRAC mirrored the activation of splenic B cells in vivo during *T. cruzi* experimental infection. In vitro, rTcPRAC-induced B cell activation increased activation marker and co-stimulatory protein expression, induced proliferation, and

resulting in IgM, IgG, and IL-10 production. In vivo, experimental infection of susceptible mice resulted in non-specific induction of CD69 and CD86 on B cells, increased B cell blast formation, hypergammaglobulinemia, and increasing circulating IL-10 over the course of acute infection. The similar patterns of polyclonal B cell activation in vitro with rTcPRAC and in vivo due to *T. cruzi* infection suggest that similar mechanisms are in play in both situations. Further experiments are needed to determine the mechanism(s) responsible for the observed polyclonal B cell activation during experimental infection in susceptible mice and to evaluate the relative contribution of TcPRAC or other parasite encoded mitogenic factors.

One possibility for the role of parasite derived mitogens, is that factor(s) act as an initial trigger of polyclonal B cell activation and that the resulting level of hypergammaglobulinemia is determined by the quality of the host response to this trigger, i.e. either amplification through production of B cell activating factors such as IL-4, IL-5, and BAFF or regulation through a contained cytokine burst that favors cellular rather than humoral activation (IFN-γ). One way to test this hypothesis would be to inoculate mice in the same manner as was done in Chapter 2, then drug treat (Benidazole) *T. cruzi* infected mice starting at day 8 post-infection, clearing parasites from theses animals, then compare them to untreated mice for the level of hypergammaglobulinemia over the course of acute infection (out to 30 days post-infection). If parasite mitogens simply trigger polyclonal B cell activation, rather than drive it, then the hypergammaglobulinemia profile would not be expected to change in treated versus untreated mice. In contrast, if parasite mitogens drive this response, the lack of stimuli would result in a lack of hypergammaglobulinemia.

In order to address the relative contribution of a specific mitogenic factor to the total polyclonal response, a method for neutralization of that factor must be used during experimental infection with wild-type parasite. The generation of TcPRAC-specific antibodies that bound to TcPRAC on the surface of parasites and were able to deplete rTcPRAC mitogenic activity should provide a useful tool for future investigation into the relative contribution of TcPRAC to polyclonal B cell activation during *T. cruzi* infection. Taking into account the evidence provided in Chapter 2 that Th2 cytokines were associated with exacerbated polyclonal B cell activation and the evidence that GG immunization skews host responses toward Th2 responses, the use of rTcPRAC-specific antibodies in a passive transfer system may be a more useful approach for determining the contribution of TcPRAC to polyclonal B cell activation. From the data presented in Chapter 2, a reasonable measure to determine this effect would be to compare the number of total IgG ASC at day 15 post-infection in Balb/c mice in mice treated with TcPRAC specific IgG versus control IgG.

The analysis of B cell subset activation during experimental *T. cruzi* infection and after in vitro stimulation with rTcPRAC both suggest that MZ B cells play an important role in the generation of polyclonal B cell responses in susceptible mice, although they may be specifically activated during experimental infection in relatively resistant mouse strains. The ability of rTcPRAC to activate MZ B cells to secrete IL-10 suggests one mechanism whereby polyclonal activation of this B cell population may lead to detrimental effects beyond hypergammaglobulinemia. Non-antibody mediated effects of B cells have not been previously investigated during *T. cruzi* infection. Further studies are necessary to determine the extent that

cytokine production by B cells during polyclonal activation could influence the course of acute phase *T. cruzi* infection.

APPENDIX A

CRP IMMUNIZATIONS

CRP DNA immunization by intramuscular administration has previously been shown to confer protection upon lethal challenge with *T. cruzi* parasite (Sepulveda, Hontebeyrie et al. 2000). This immunization route uses a large amount of DNA and results in variable generation of CRP-specific antibodies. As GG immunization uses a relatively small amount of DNA and results in highly reproducible and robust humoral and cellular responses, we investigated the ability of GG immunization to generate CRP-specific humoral immunity.

In this study, we evaluated two CRP constructs, a non-secreted (CRP-cyto) and a glycosylphosphatidylinositol (GPI) membrane bound (CRP-GPI) construct. In addition, we tested two adjuvants, CpG ODN and IL-12 in an expression plasmid, for their effect on CRP-specific IgG isotype production.

A.1 DIFFERENTIAL RESPONSE TO CRP CONSTRUCTS

To determine the relative efficacy of CRP-cyto versus CRP-GPI constructs, each was inoculated into naïve mice via intradermal GG delivery. Mice were primed, then boosted at one month intervals, three times. After boost 2, an increased CRP-specific IgG response was evident from the CRP-GPI compared to the CRP-cyto immunized mice (Fig 21). This differential CRP-specific IgG response was evident against both prokaryote and eukaryote derived rCRP (data not shown). For both constructs, IgG1 was the predominate isotype generated in response to GG immunization, indicating a predominately Th2 response to immunization. This result contrasts with the reported skewing towards Th1 responses in OVA mice immunized by GG (Morel, Falkner et al. 2004). Previous reports indicate that CpG rich oligodeoxynucleotide (ODN) delivery may act as a Th1 skewing adjuvant (Krieg 2002; Frank, Petray et al. 2003; Zhou, Zheng et al. 2003). To test the adjuvant properties of CpG ODN it was delivered to the site of GG inoculation (i.d.). Delivery of CpG ODN via needle to the site of GG immunization led to increased CRP-specific IgG2a responses, but did not significantly increase total CRP-specific IgG response.

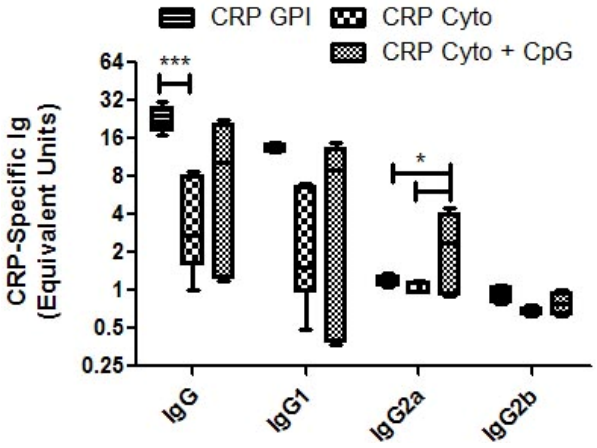

Figure 21: CRP-specific IgG responses to CRP constructs and CpG ODN.

Mice primed and boosted twice with CRP-GPI or CRP-Cyto via GG. One group of CRP-cyto immunized mice were also injected with CpG ODN at the site of GG inoculation. Serum samples were analyzed for CRP-specific IgG responses by ELISA using 5 mice per group. * $p < 0.05$, *** $p < 0.001$, Student's *t* test.

A.2 USE OF IL-12 AS AN ADJUVANTS DURING GG IMMUNIZATION

Use of IL-12 has previously been reported as an effective adjuvant during GG immunization with a parasite derived immunogen (Sakai, Hisaeda et al. 2003). To investigate the effect of IL-12, naïve mice were primed and boosted at one month intervals with CRP alone, using the CRP-GPI construct described above, or in combination with IL-12. IL-12 was delivered as precipitated plasmid DNA on gold beads via gene-gun along with the CRP expression plasmid. IL-12 in combination with CRP GG immunization resulted in higher titer CRP-specific IgG responses and a decreased IgG1/IgG2a ratio compared to CRP administration alone (Fig 22). These changes demonstrate the utility of IL-12 immunization in boosting the CRP-specific humoral responses as well as shifting the balance of the Th response towards a more mixed response. Immune sera from IL-12 adjuvanted CRP GG immunization had increased ability to inhibit CRP activity on the surface of parasites, leading to increased parasite lysis in the presence of complement (Figure 23).

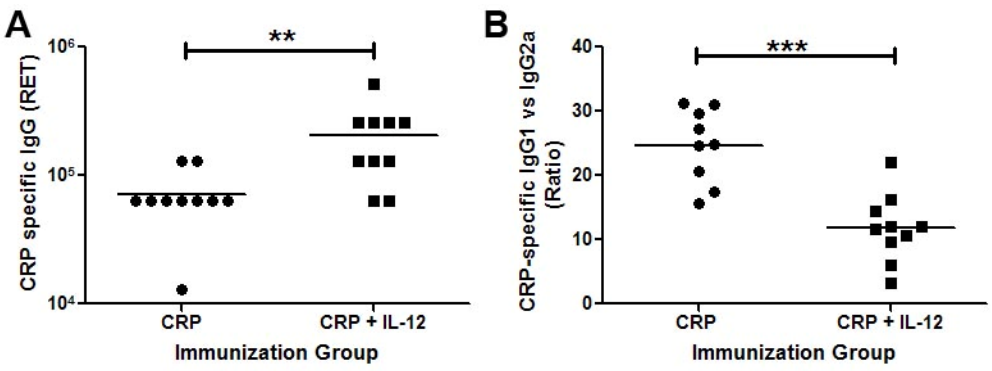

Figure 22: IL-12 boosts CRP-specific responses and led to decreased IgG1/IgG2a ratio.

Mice were primed and boosted at one month intervals with CRP or CRP and IL-12 via intradermal genetic immunization (GG). Serum was collected after the third boost for analysis of CRP-specific response by ELISA. *A.* CRP-specific IgG reciprocal endpoint titers (RET) were determined using reactivity compared to pre-immune control serum. Five mice were analyzed per group. ** $p < 0.01$. *B*, CRP-specific IgG1 and IgG2a were determined by ELISA and the IgG1 response compared to IgG2a response is reported as the ratio of the reactivity to each. Five mice were analyzed per group. *** $p < 0.001$.

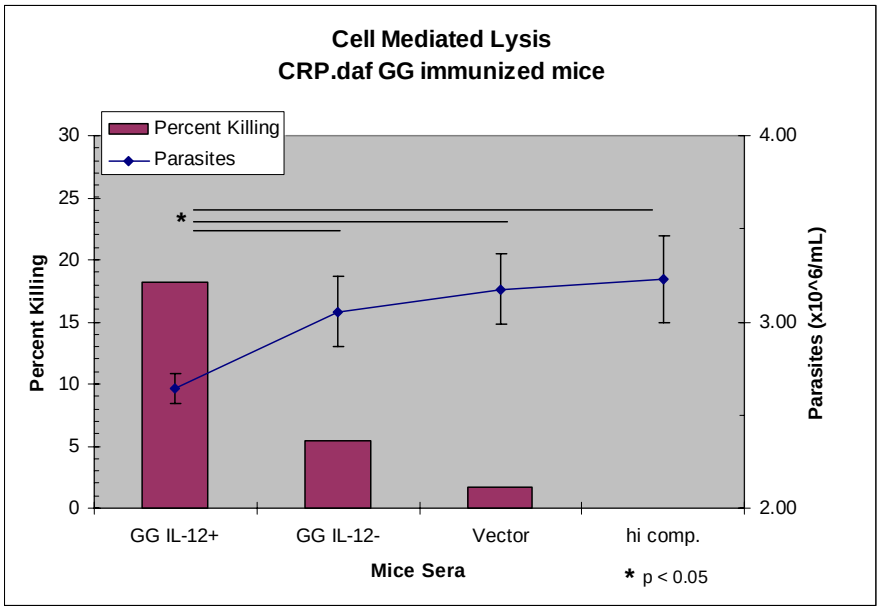

Figure 23: IL-12 administration improves parasite specific anti-CRP reactivity in serum from immunized mice.

Immune serum from mice immunized with CRP in combination with IL-12 were compared with immune serum from CRP alone or vector immunized mice for ability to facilitate complement mediated lysis of tissue culture derived parasites.

A.3 CRP IMMUNIZATION CONCLUSION

Intradermal genetic immunization with CRP constructs via gene-gun delivery results in high titer antibody responses. The magnitude of these responses depends on the type of construct generated. GG immunization with the membrane expressed, GPI anchored, construct produced higher titer antibody response with greater reproducibility than did the non-secreted CRP construct. Use of an adjuvant, CpG ODN, with the non-secreted CRP construct was able to increase the magnitude of the specific response to levels comparable with the membrane tethered construct. In addition, the use of CpG ODN led to a less pronounced IgG1 / IgG2a response, indicating a shift away from a predominate Th2 and towards a more mixed Th response. Use of IL-12 as an adjuvant during GG immunization with the membrane tethered CRP-GPI construct had a similar effect with improved CRP specific IgG titers and a more mixed Th response. This effect had functional consequence, as immune serum from mice receiving the IL-12 adjuvant had higher complement mediated lytic activity than did serum without IL-12. These results suggest that membrane presentation is optimal for the generation of humoral immunity against CRP during GG immunization and that the type of helper T cell response generated during immunization can be altered by delivery of an appropriate adjuvant.

APPENDIX B

PARASITE CHALLENGE OF IMMUNIZED MICE

Challenge models are necessary for fully testing vaccine efficacy. Therefore, experimental infection of mice immunized with TcPRAC, CRP, or combined regiment have been performed and evaluated. Here, we present data that indicate that GG immunization with TcPRAC, CRP, or a combined regimen may increase protection of immunized mice during parasite challenge. These are preliminary data that must be repeated to further validate the observed effects and to determine correlates of protection.

B.1 CHALLENGE OF TCPRAC IMMUNE MICE

Inoculation of naïve mice with TcPRAC DNA via gene gun resulted in high titer antibody response by boost three with a TcPRAC-specific IgG RET of 153600 ± 72407 (mean ± SD). Two weeks after the final boost, these mice were challenged with a highly virulent isolate of Y strain *T. cruzi* (50 Y-Br, i.p.). Mice were monitored for fitness and sacrificed when they became moribund. Parasites were enumerated in whole blood after RBC lysis over the course of patent parasitemia. These data show a trend toward increased survival and significant decline in peak

parasitemia in TcPRAC immune mice, suggesting that TcPRAC immunization improves disease outcome upon challenge with virulent parasite (Fig 24).

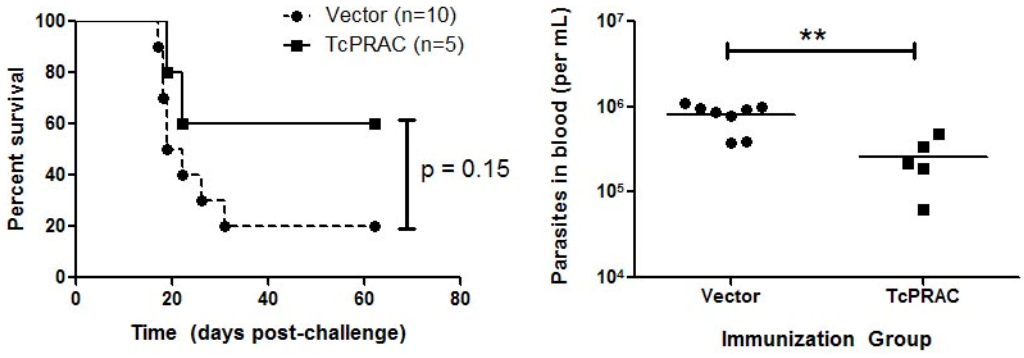

Figure 24: Challenge of TcPRAC immune mice leads to decreased peak parasitemia.

Mice were primed and boosted twice with TcPRAC DNA or vector alone. Two weeks after the final boost, mice were inoculated with 50 Y-Br parasites (i.p.). Left: TcPRAC mice show a non-significant trend towards increased survival. The numbers of mice are indicated on the plot. Right: Parasites were enumerated after RBC lysis of whole blood. The numbers of parasites in the blood at peak parasitemia are reported on this graph. ** $p < 0.01$ by Student's *t* Test.

B.2 CHALLENGE OF CRP IMMUNIZED MICE

Mice were primed and boosted three times with CRP DNA or vector alone. After the third boost, these mice were challenged with a highly virulent isolate of Y strain *T. cruzi* (50 Y-Br, i.p.). Mice were monitored for fitness and sacrificed when they became moribund. Survival analysis indicated that CRP immune mice experienced increased survival compared to vector controls (Figure 24A). Survival was associated with increased levels of CRP-specific IgG prior to challenge (Figure 25B). There was no significant difference in parasitemia curves or magnitude of peak parasitemia in CRP-immune versus vector control mice (data not shown).

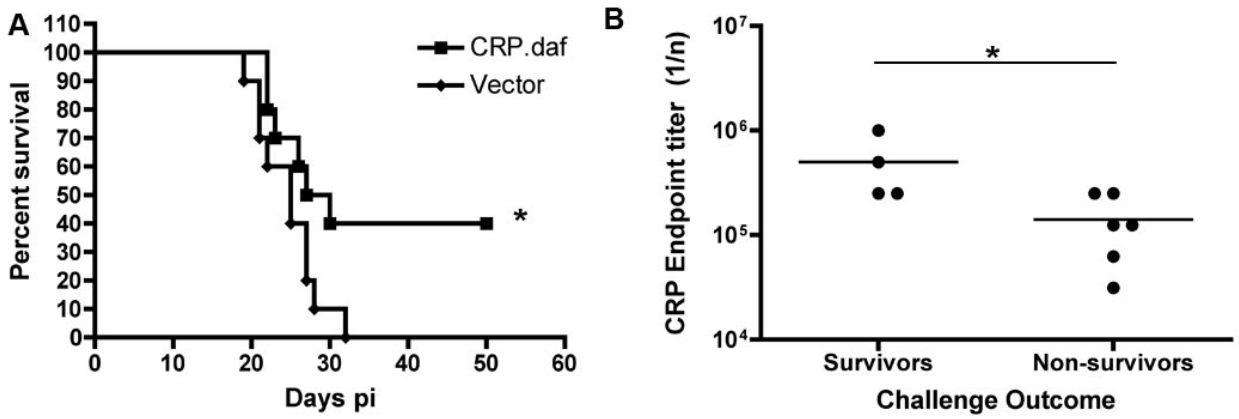

Figure 25: CRP protection from challenge associated with increased CRP-specific IgG

Mice were primed and boosted three times with CRP or vector DNA. Two weeks after the final boost, mice were inoculated with 50 Y-Br parasites (i.p.). *A,* CRP immunized mice show a significant increase in survival compared to vector control mice. * $p < 0.05$. *B,* Analysis of CRP endpoint titers after the final boost indicates that survivors had a significantly higher titer CRP-specific response. * $p < 0.05$.

B.3 CHALLENGE OF MICE WITH CRP/TcPRAC COMBINED IMMUNIZATION

Mice were primed and boosted three times with vector, CRP, or TcPRAC DNA or with both CRP and TcPRAC. After the third boost, these mice were challenged with a highly virulent isolate of Y strain *T. cruzi* (50 Y-Br, i.p.). Mice were monitored for fitness and sacrificed when they became moribund. Parasites were enumerated in whole blood after RBC lysis over the course of patent parasitemia. Co-immunization with TcPRAC and CRP, as described (section 4.3.2), improved survival and decreased parasitemia in mice infected with a virulent *T. cruzi*, compared to either immunogen alone (Figure 26).

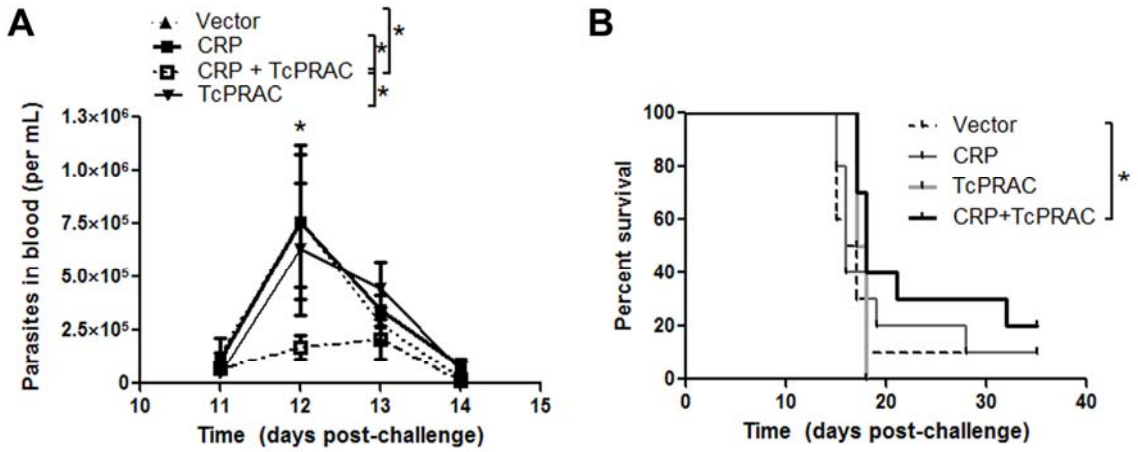

Figure 26: Immunized with both CRP and TcPRAC indicates improved survival and decreased parasitemia. Mice were primed and boosted three times with CRP, TcPRAC, CRP and TcPRAC, or vector DNA. Two weeks after the final boost, mice were inoculated with 50 Y-Br parasites (i.p.). *A*, Parasitemia was monitored from day 11 to 14 post-infection. * $p < 0.05$, 2-way ANOVA. *B*, Survival analysis after infection. Nine-ten mice were used per group. * $p < 0.05$, Gehan-Breslow-Wilcoxon Test.

B.4 CHALLENGE OUTCOME CONCLUSIONS

Challenge of TcPRAC, CRP, or combined TcPRAC and CRP GG immunized mice with highly virulent *T. cruzi* parasite shows a trend towards improved outcome with single and combined immunization, as measured by parasitemia and survival, although these results were variable. Potential sources of the variability include variance in the actual dose of parasite administered, as inoculation with 50 parasites requires a several log dilution from the counted stock concentration of parasite. Furthermore, the relative degree of Th2 skewing that occurs during immunization, as the experiments reported above were performed in slightly different fashions (the degree of overall Th2 skewing was not analyzed in these mice, but should be considered in future experiments). Data from aim 1 of the present study indicates that Th2 response is associated

with exacerbated polyclonal B cell activation, which would be expected to decrease the efficacy of immunization. The most promising challenge result shown here is the decreased parasitemia and trend towards increased survival in the combined immunization with CRP and TcPRAC. Further experiments are needed to fully determine the effect of these immunization strategies. Future studies should include investigation of Th1 skewing adjuvants in the context of the combined immunization regimen (as described in Appendix A).

APPENDIX C

ADDITIONAL PRIMARY DATA

C.1 EXPERIMENTAL INFECTION WITH Y STRAIN DERIVITIVES

Two derivatives of the Y strain parasite were used in these studies. The Y-US derivative has been used in the Norris laboratory for many years and has been maintained by passage through mice. This deriviative was used to clone the CRP gene used for DNA immunization and protein production (Norris, Schrimpf et al. 1997; Norris 1998; Sepulveda, Hontebeyrie et al. 2000; Meira, Galvao et al. 2002; Beucher, Meira et al. 2003). Another Y strain derivative (Y-Br), which had been cultured in dogs prior to the studies in the Norris laboratory was given to Dr. Norris by Dr. Wendell Meira and was found to have higher virulence in Balb/c mice than did the Y-US deriviative. In order to attempt to establish non-lethal doses for analysis of *T. cruzi* infection, a series of experimental infection of Balb/c mice with Y-US and Y-Br strains was performed (Figure 26). Y-US inoculation into Balb/c causes death of approximately half the mice at a dose of 10^6 TCT (i.p.). Analysis of Balb/c mice with Y-Br indicated that it was highly virulent, with an LD50 of approximately 10-25 parasites injected i.p. (Figure 27*A*). The infection of C57Bl/6 mice with various doses of Y-Br were also evaluated to establish another experimental model. Inoculation with higher doses of parasite leads to an earlier peak

parasitemia in an given model system (data not shown), and equivalent inoculation in terms of LD50 of Y-Br in Balb/c versus C57Bl/6 mice leads to different parasitemia kinetics, with delayed peak parasitemia (~ days 12-14 pi) in the Balb/c mice compared to the C57Bl/6 mice (~ day 7-8 pi). Lethal doses of Y-Br in Balb/c mice result in a median time to death of 22 days post-infection (50-100 parasites i.p.), whereas lethal doses of Y-Br in C57Bl/6 mice results in a median time to death of 11-12 days post-infection. These results indicate that survival and parasitemia kinetics are both influenced by the initial number of parasites injected.

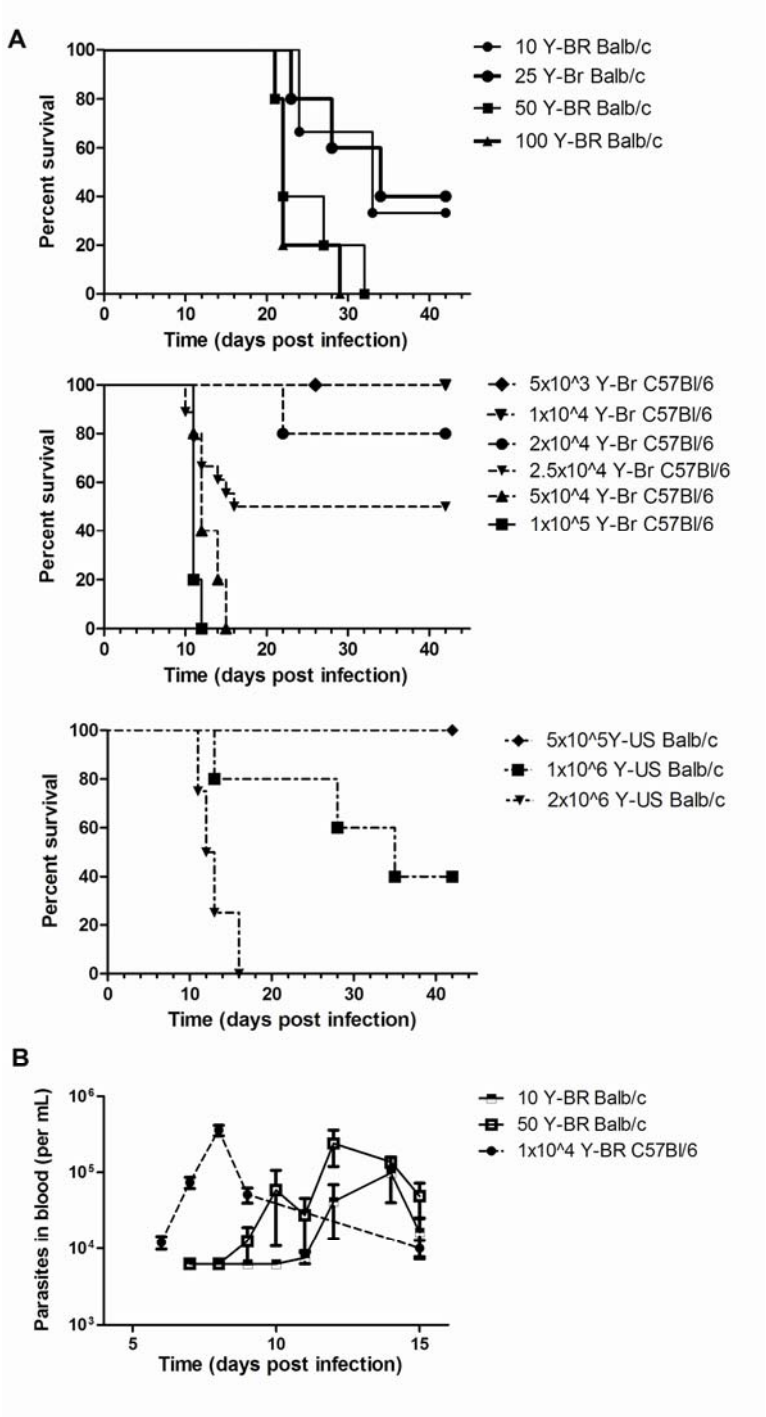

Figure 27: Survival curves and parasitemia profile of mice infected with Y strain variants.

Mice (5-10 per dose) were injected i.p. with indicated number of TCT derived parasites in PBS + 1% glucose and monitored for survival and for parasite numbers in tail blood. A, Top: Balb/c mice inoculated with the indicated doses of Y-Br variant. Middle: C57Bl/6 mice inoculated the indicated doses of Y-Br variant. Bottom: Balb/c mice

inoculated with the indicated doses of Y-US variant. B, Parasitemia profiles for Balb/c mice inoculated with 10 (~LD50) or 50 (2-3xLD50) Y-Br variant parasites, or C57Bl/6 mice with 10,000 Y-Br parasites (~0.5LD50).

C.2 DISTINGUISHING B CELLS AND B CELL BLASTS

To generate the plots in Figures 7 and 8, total splenocytes numbers were determined and splenocytes were stained for analysis by flow cytometry, as described in the methods (2.3.8). Flow cytometry data were then analyzed by FlowJo for determining the percentage of these splenocytes that were positive for CD19, indicating B cells, or CD3, indicating T cells (Figure 28). Within the B cell (CD19+) gate, these cells were further distinguished as blasts based on their granularity (side-scatter) and size (forward-scatter) (Figure 29).

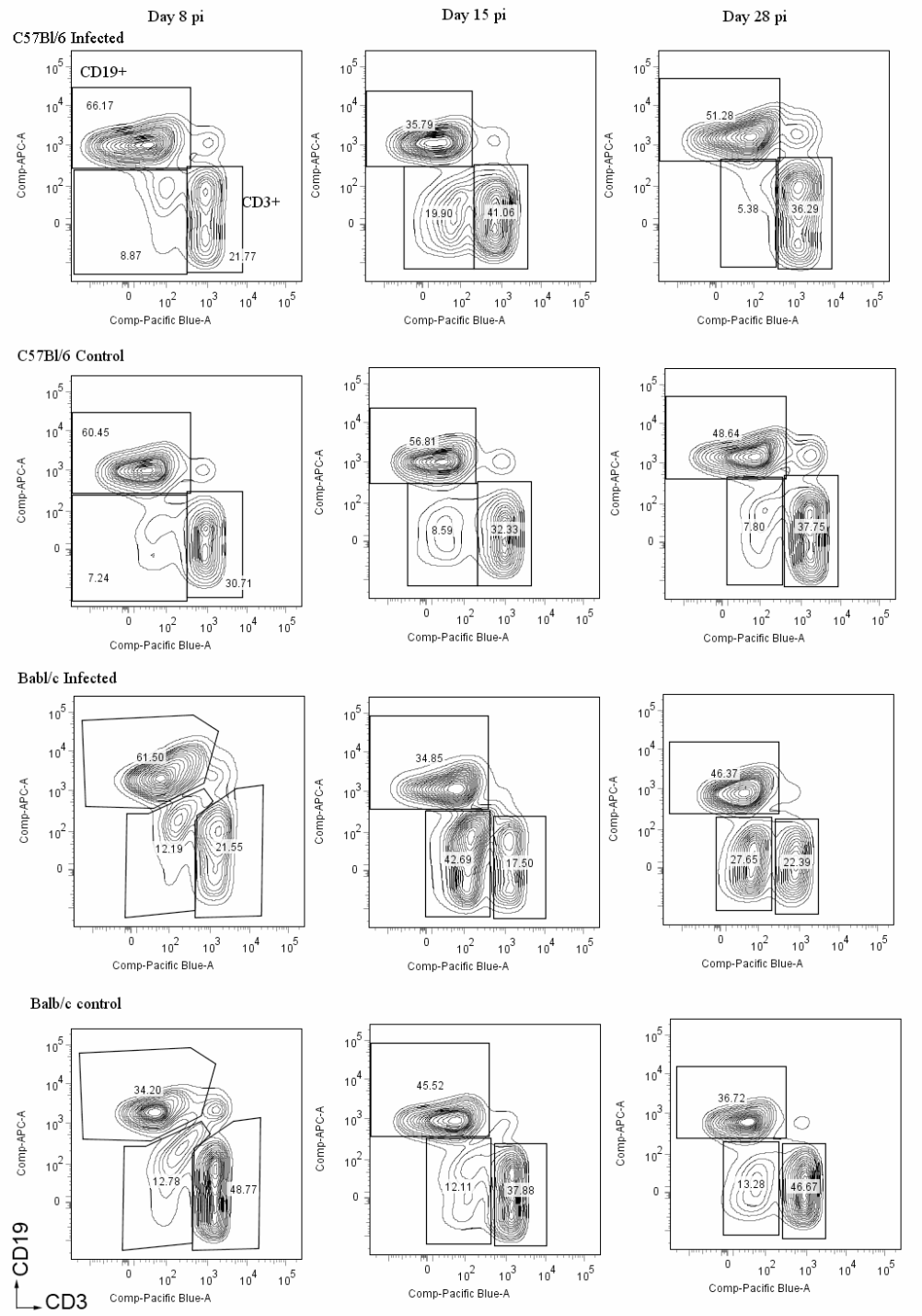

Figure 28: Analysis of CD19 and CD3 in C57Bl/6 and Balb/c splenocytes.

Splenocytes harvested and stained with CD3 (PacBlue) and CD19 (APC) for flow analysis at days 8, 15, and 28 days after inoculation with live parasite (infected) or heat-inactivated parasite (control). Shown here are representative contour plots from five mice per group per time-point.

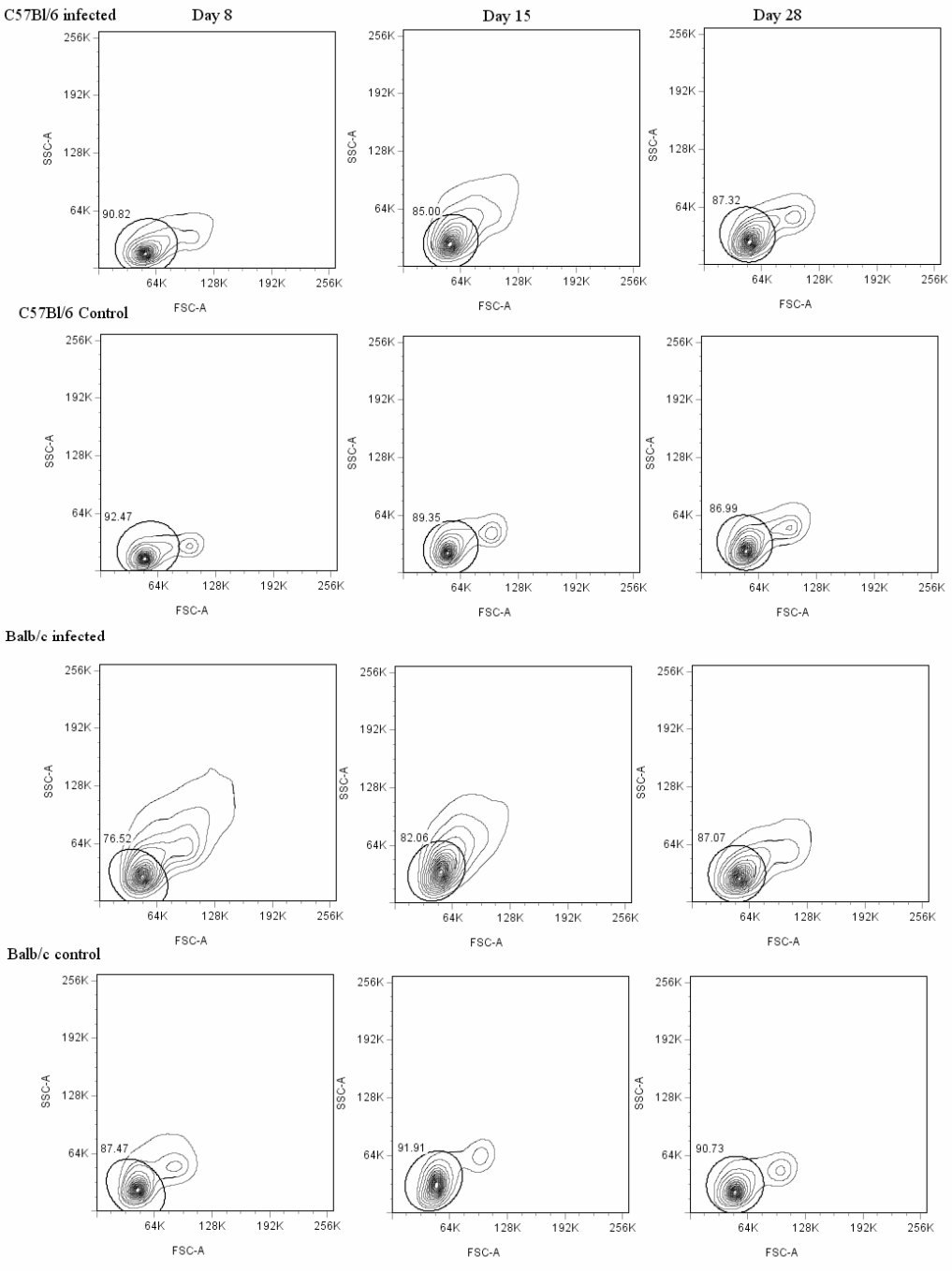

Figure 29: Representative plots showing B cell blast formation after *T. cruzi* infection.

Splenocytes harvested and stained for flow analysis at days 8, 15, and 28 days after inoculation with live parasite (infected) or heat-inactivated parasite (control). Within the CD19+ (B cell gate) cells were further analyzed for size (FSC) and granularity (SSC) Shown here are representative contour plots from five mice per group per time-point.

C.3 ANALYSIS OF CD3 SPLENOCYTES FOR CD4 AND CD8

Analysis of CD4 and CD8 positive CD3 T cells was done to determine the relative change in the T helper (CD4) and cytotoxic T cell (CD8) populations at day 15 post-infection in Balb/c and C57Bl/6 mice (Figure 30). The absolute numbers of T cell subsets were then calculated based on the original number of splenocytes. These data were reported in Figure 10.

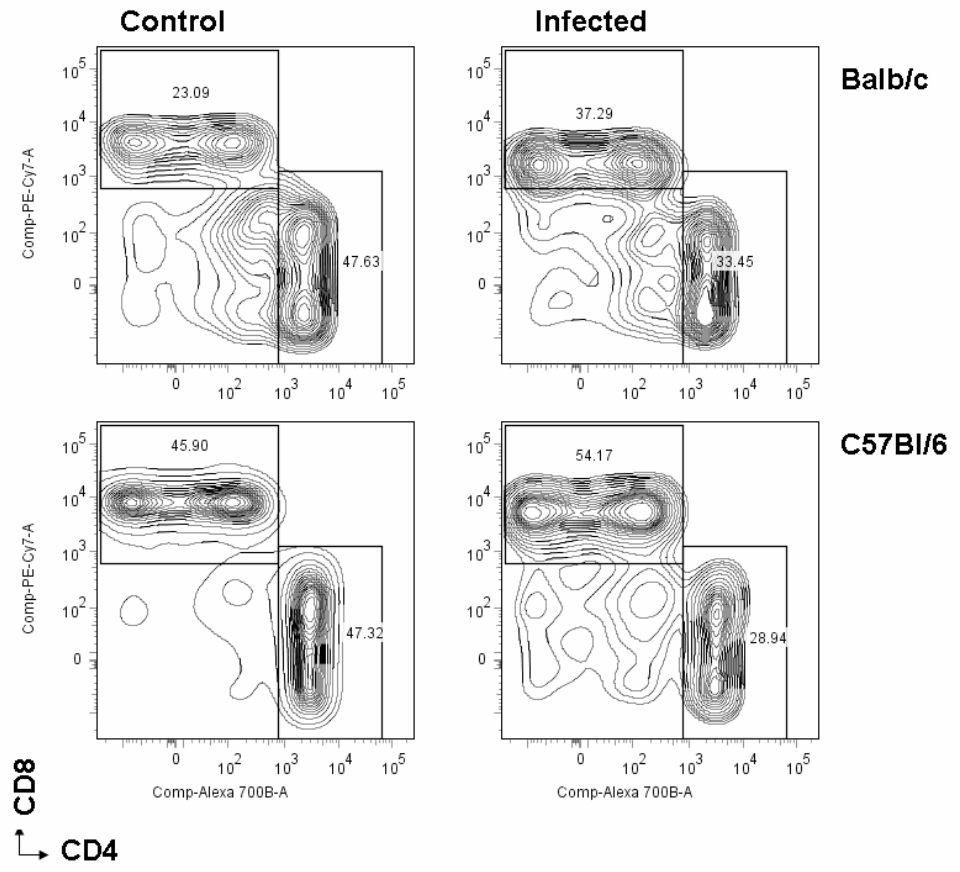

Figure 30: Representative plots showing CD8 and CD4 T cell subsets post-infection with *T. cruzi.*

Splenocytes were harvested at day 15 post inoculation with live parasites (infected) or heat-inactivated parasites (control). Cells were counted, washed, and frozen at -80˚C until analysis. To analyze, cells were thawed and stained with CD19, CD3, CD8, and CD4; data was collected by flow cytometry and analyzed with FlowJo.

C.4 ANALYSIS OF CD19 AND CD3 NEGATIVE SPLENOCYTES

Analysis of B and T cells by CD19 and CD3 at day 15 post infection in both Balb/c and C57Bl/6 models indicated a large expansion of splenocytes that did not express either of these markers. Further analysis of these populations at day 15 demonstrates that these populations contain phenotypically distinct populations, and that the composition of these populations differs between these two mouse models (Figures 31 and 32).

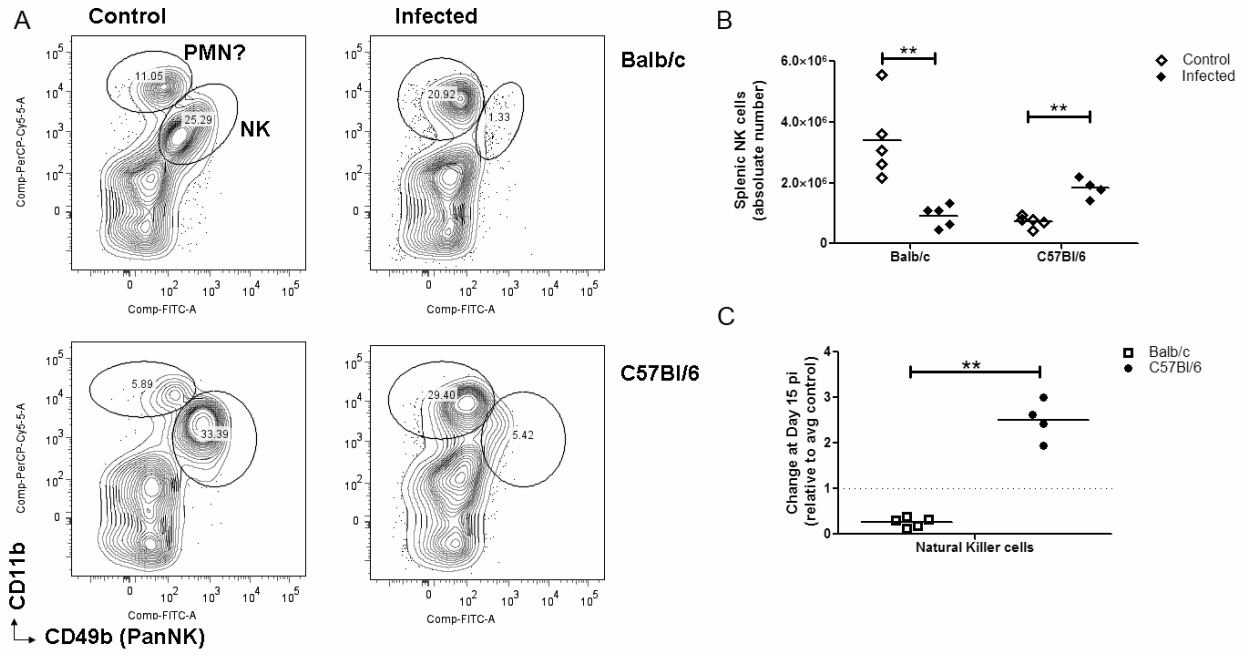

Figure 31: NK cells during infection with *T. cruzi* in Balb/c vs. C57Bl/6 mice.

Splenocytes were harvested at day 15 post inoculation with live parasites (infected) or heat-inactivated parasites (control). Cells were counted, washed, and frozen at -80˚C until analysis. To analyze, cells were thawed and stained with CD19, CD3, CD11b, and CD49b (PanNK). A, Representative dot plots showing analysis of $CD19^{neg}CD3^{neg}$ splenocytes. B, Analysis of absolute number of splenocytes demonstrates significant differences between infected and control mice for both mouse models. C, C57Bl/6 and Balb/c mice have significantly different changes in NK cell numbers within the spleen at day 15 pi.

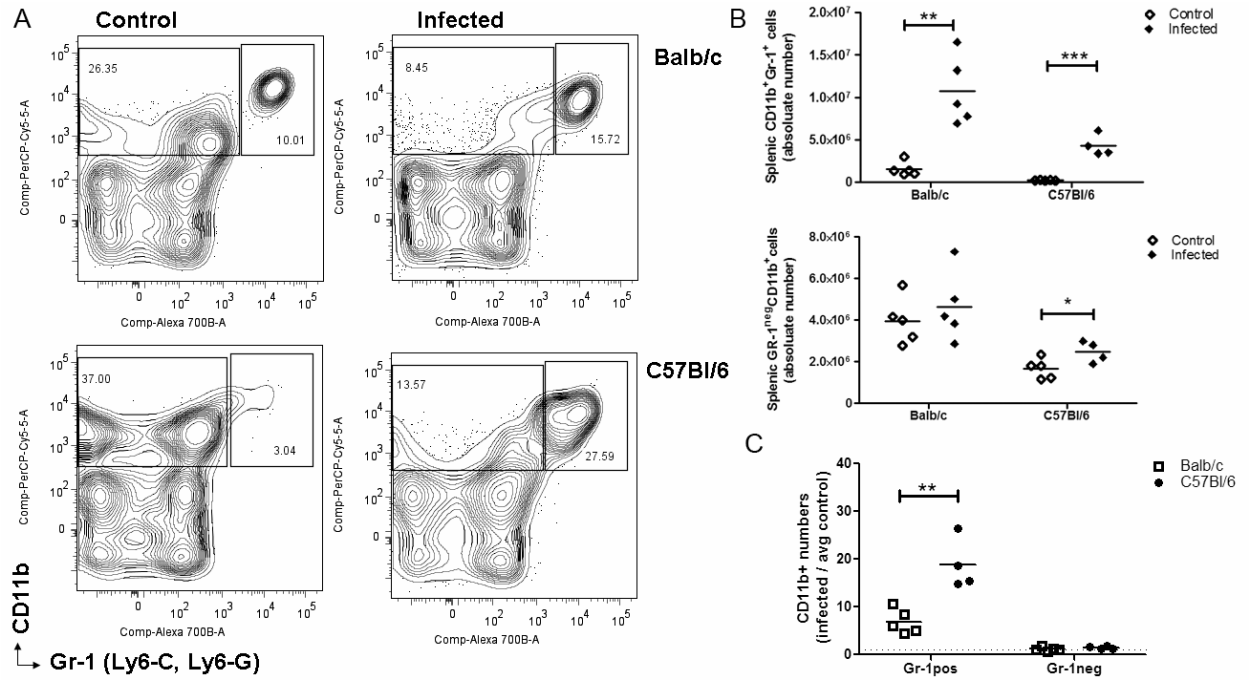

Figure 32: Expansion of CD11b^{+}GR-1^{+}CD19^{-}CD3^{-} splenocytes at day 15 post-infection with *T. cruzi.*

Splenocytes were harvested at day 15 post inoculation with live parasites (infected) or heat-inactivated parasites (control). Cells were counted, washed, and frozen at -80˚C until analysis. To analyze, cells were thawed and stained with CD19, CD3, CD11b, and GR-1. A, Representative dot plots showing analysis of CD19^{neg}CD3^{neg} splenocytes. B, Analysis of absolute number of splenocytes demonstrates significant differences between infected and control mice for both mouse models. C, C57Bl/6 and Balb/c mice have significantly different changes in CD11b^{+}GR-1^{+} (immature myloid suppressor cells) at day 15 pi.

C.5 ANALYSIS OF NATURAL VERSUS INDUCED IGM RESPONSES

After infection with *T. cruzi* with approximately 0.25 x LD50 of Y-US and Y-Br into Balb/c and C57Bl/6 mice, respectively, as outlined in Chapter 2, the IgM and IgG response was analyzed at day 15 post-infection and compared to naïve mouse serum (Figure 33). These data indicate that both strains of mice have naturally occurring IgM, but not IgG, that are reactive to tissue culture

trypomastigotes (TCT). The more resistant C57Bl/6 mice have a slightly lower (n.s.) natural IgM, but mount a more robust anti-TCT response than do Balb/c mice by day 15 post-infection. In addition, the C57Bl/6 mice have a higher IgG anti-TCT response at day 15 post-infection. Thus the improved humoral responses in C57Bl/6 versus Balb/c mice is unlikely to be due to a difference in parasite specific natural antibodies in these genetically diverse hosts.

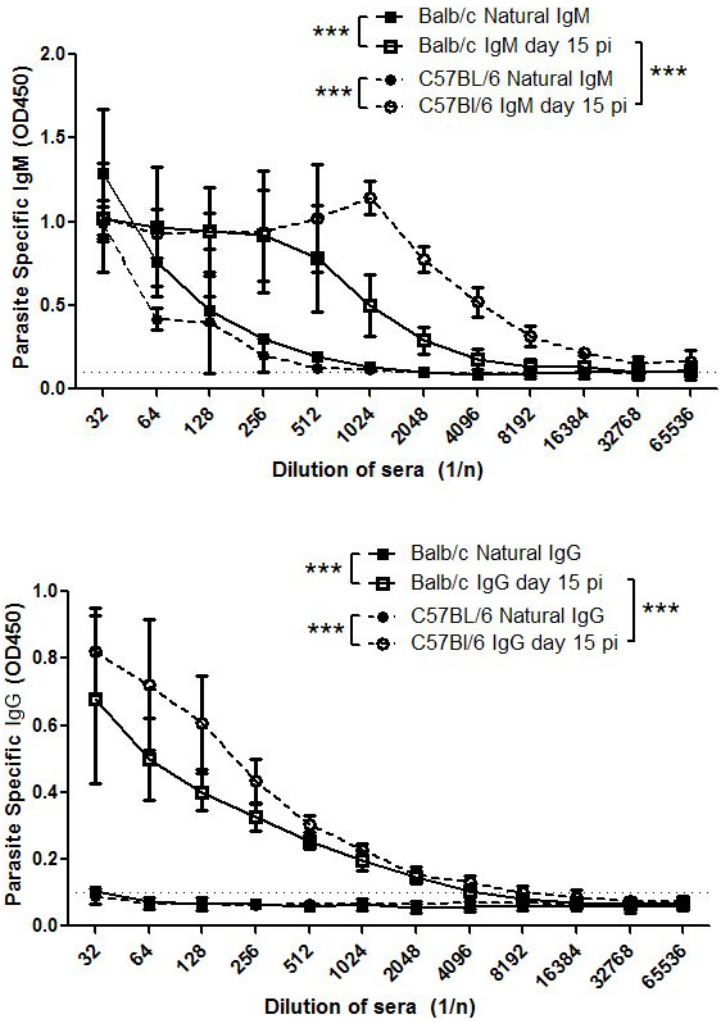

Figure 33: Natural versus induced antibody responses to *T. cruzi* trypomastigotes.

Serum samples were collected from naïve or infected (day 15 post-infection) Balb/c and C57Bl/6 mice and analyzed for reactivity to whole *T. cruzi* tissue culture derived trypomastigotes.

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