Age-Related Increase in Resistance to Acute *Trypanosoma cruzi* Infection in Rats is Associated with an Appropriate Antibody Response

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Abstract

Inoculation at weaning with *Trypanosoma cruzi* in inbred 'l' rats resulted in a self-resolving acute infection characterized by marked parasitaemias, whereas challenge to adult rats revealed a mild disease with extremely low parasitaemias. To explore the mechanisms underlying such age-associated differences in disease outcome, we analysed the *in vitro* replication of *T. cruzi*, nitric oxide and tumour necrosis factor-α (TNF-α) production in peritoneal macrophages (PMs), the serum concentrations of the specific immunoglobulins (Igs) IgM and IgG, antibodies exhibiting lytic activity against bloodstream forms of *T. cruzi* and circulating levels of nitrate, TNF-α and interferon-γ (IFN-γ). Macrophages from young rats were as effective as their adult counterparts for restraining intracellular parasite replication. When stimulated with IFN-γ, culture supernatants from young PMs contained higher amounts of nitrite and TNF-α. Serum samples from 4 and 7 days post infection revealed easily detectable amounts of nitrate, with values being further augmented by day 7 post infection and significantly higher in the young group. TNF-α levels were only detected in the young group by day 7 post infection. Both groups had increased amounts of IFN-γ in their sera, although in adult rats, this trend was followed by a significant drop at day 7, with young rats showing values still higher by the same time point evaluation. In contrast, young rats presented significantly lower levels of IgM and IgG antibodies during the first week of infection. Increased resistance in adult rats seems to be the result of a more appropriate antibody production.

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Introduction

Chagas' disease, or American trypanosomiasis, is a parasitic disease caused by the protozoan *Trypanosoma cruzi*, which infects about 20 million people in Latin American countries [1]. Studies on murine hosts indicate that the control of acute *T. cruzi* infection is mediated by the coordinated presence of antibodies capable of lysing circulating forms [2] and macrophages which may kill the intracellular amastigotes. This destruction is mostly facilitated by the activating microbicidal capacity of interferon-γ (IFN-γ) [3–5] as well as that of tumour necrosis factor-α (TNF-α), which enhances the *in vitro* nitric oxide (NO)-dependent trypanocidal activity of IFN-γ-activated macrophages [6–8].

To gain some insight into the age-associated changes in the course of acute *T. cruzi* infection, a study was performed several years ago in a rat model developed in our laboratory [9, 10]. In these studies, inoculation at weaning with living *T. cruzi* in inbred 'l' rats resulted in a self-resolving acute infection characterized by marked parasitaemias and production of specific antibodies. Briefly, parasites were evident microscopically by day 7 post infection, peaked during the second week and declined gradually, as the adaptive immune response developed, to undetectable levels by the end of the first month of infection. When infecting adult rats, studies revealed a mild disease with extremely low, almost undetectable, parasitaemia [11]. Such a comparative, decreased resistance of

young rats may be because of a less-efficient orchestration of the mechanisms underlying disease resolution [12], resulting in a defective humoral response and/or an unsatisfactory macrophage ability to restrain parasite growth.

Given this background, a study was undertaken to investigate the relationship between the age-related differences in the course of acute *T. cruzi* infection and the following immunological parameters: (1) *in vitro* replication of *T. cruzi* and NO and TNF-α production by peritoneal macrophages (PMs), stimulated with recombinant IFN-γ or not; (2) serum concentrations of the specific immunoglobulins (Igs), IgM and IgG, antibodies exhibiting lytic activity against bloodstream forms of *T. cruzi*; (3) complement activity; and (4) circulating levels of NO, TNF-α and IFN-γ.

Materials and methods

Animals and parasites. Male 'l' rats raised and maintained in our breeding facilities were used for the experiments, which were conducted following the Institutional Experimental Guidelines for Animal Studies. Infective blood trypomastigotes of the Tulahuén strain of *T. cruzi* were maintained by serial passage in CBi mice.

Systemic infection. Rats were infected by the subcutaneous or the intraperitoneal route, at the time of weaning (21–28 days) or during adulthood (70–120 days) with 10^6 or 7×10^6 parasites, respectively. Bloodstream forms of *T. cruzi* were assessed under standardized conditions by direct microscopic observation of 5 µl of heparinized tail venous blood, at 7, 9, 14, 21 and 28 days post infection. Data were expressed as number of parasites per 50 fields.

In vitro infection of PMs. Resident macrophages were obtained from uninfected rats by lavage of the peritoneal cavity with Earle's minimum essential medium (MEM) (PAA Laboratories, Cölbe, Germany). Cell suspensions were centrifuged at $500 \times g$, resuspended in Earle's MEM supplemented with 10% fetal bovine serum, 20 µg/ml of gentamycin and 0.2 mM 2-mercaptoethanol, seeded in Nunclon Multidishes (Nalge Nunc International Naperville, IL, USA) and allowed to adhere at 37 °C for 18 h. The nonadherent cells were then removed. After the adherence phase, PMs were exposed to *T. cruzi* trypomastigotes at a 5 : 1 parasite–host cell ratio. At different times post exposure, supernatants were collected to assess the number of parasites and nitrite and TNF-α levels. To assess the number of phagocytic cells and intracellular parasite counts, PMs were cultured at a cell density of 10^5 cells/well in culture chambers specially designed for optic microscopy (LabTek® ChamberSlide™ System, Nalge Nunc International). Macrophage monolayers were exposed to the parasite for 24 h at a 5 : 1 parasite–host cell ratio, then washed to remove nonphagocytized parasites, fixed with ethanol and stained with Giemsa for evaluation of the number of phagocytic cells per 300 cells. In order to obtain activated PMs, the cells were incubated with recombinant rat (rR)-IFN-γ, from the adherence phase onwards, at the two concentrations 400 and 2000 U/ml. The rR-IFN-γ was kindly provided by Roussel Uclaf (Romainville, France). The stated endotoxin content of this compound was negligible. PMs and trypomastigotes cultured separately in the presence of the cytokine were included as controls.

Relative concentration of parasite in culture supernatants. Three hundred thousand cells per well were seeded in 12-well plates and exposed to the parasite. At 4, 24 and 48 h following exposure, supernatants were obtained, and the remaining parasites were counted with a haemacytometer. As a viability control of parasites *in vitro*, parasites were cultured under the same conditions in the absence of PMs.

Cytokine assays. For the TNF-α production, 3×10^5 cells/well were seeded in 48-well plates and exposed to the parasite. At 4, 24 and 48 h post exposure, supernatants were obtained, centrifuged and assayed for the presence of this cytokine. For the circulating levels, sera obtained at days 4, 7, 14 and 21 post infection were used. Concentrations of TNF-α and IFN-γ were measured by specific two-site enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's specifications with reference standard curves, using known amounts of the respective rat recombinant cytokines. The antibody pairs for the detection of TNF-α (detection limit 5 pg/ml) and IFN-γ (detection limit 10 pg/ml) were from R&D Systems (Minneapolis, MN, USA). All samples were processed individually and assayed in duplicate, with plates being read at 450 nm.

Nitrite measurement. Samples were those obtained for the assessment of cytokines. Endogenously synthesized NO in serum or culture supernatants was measured by the Griess reaction [13], slightly modified to detect total nitrite (nitrate + nitrite). Briefly, total nitrite concentration was quantified using various $NaNO_3$ concentrations as standard. Nitrate present in samples or standards was first reduced by incubation in the presence of *Pseudomona oleovorans* for 90 min at 37 °C, and then samples were incubated with the Griess reagent. Absorbance was measured at 540 nm, and data were expressed in µM or nmoles/3×10^5 cells for serum or culture supernatants, respectively.

Analysis of antibody responses. Individual blood samples from controls or *T. cruzi*-infected rats were collected at days 4, 7 and 14 post infection and allowed to clot; sera were prepared and stored at −20 °C. Individual sera were analysed using commercial antibody capture ELISA (Chagatest recombinant 3.0, Wiener Laboratories, Rosaric, Argentina), adapted for the detection of rat antibodies. ELISA plates, obtained already coated with recombinant *T. cruzi* antigen, were incubated with properly diluted rat serum for 90 min at 37 °C. Plates were washed three times and incubated for 90 min at 37 °C, with developing reagent diluted 1/2500 in wash buffer, that is peroxidase-conjugated goat anti-rat IgM µ-chain specific (Jackson ImmunoResearch,

West Grove, PA, USA) or peroxidase-conjugated goat anti-rat IgG, whole molecule (Sigma, St Louis, MO, USA), and then developed with H_2O_2–3,3′,5,5′-tetramethylbenzidine. The reaction was stopped after 30 min with 2 N H_2SO_4 and read at 450 nm.

Search for lytic antibodies. Serum was collected at 4, 7 and 14 days following infection. On each occasion, samples from age-matched uninfected rats were taken as well. Lytic antibodies were assessed in accordance with a published method [14] with slight modifications. One hundred microlitres of a blood trypomastigote suspension (6×10^6 parasites/ml) were incubated with 100 µl of heat-inactivated sera (30 min, 56 °C) for 15 min at room temperature. Then, 50 µl of this suspension was further incubated for 60 min at 37 °C with 50 µl of either fresh or decomplemented rat serum. Viable trypanosomes were counted in a haemocytometer, and killing was calculated according to the following formula (C = complement):

$$\%\text{lysis} = 100 - \frac{\text{number of parasites after incubation with C}}{\text{number of parasites after incubation without C}} \times 100.$$

CH_{50} assay. The haemolytic CH_{50} assay was performed according to a standard method [15], with slight modifications. Briefly, sheep red blood cells (SRBCs) were sensitized using haemolysin and then incubated at 37 °C for 30 min with different dilutions of serum in buffer, buffer alone (background) or water (total lysis). Haemolysis was assessed by measuring optical density at 530 nm. Results of the haemolytic assays were calculated by using Von Krough's equation.

Statistical analysis. Two rounds of experiments were performed. Statistical comparisons were made by the Kruskal–Wallis nonparametric analysis of variance and Mann–Whitney *U*-test.

Results

Parasitological features of systemic infection in young and adult rats

Young and adult rats that had been infected at weaning or when aged 70–120 days, respectively, were initially analysed for the parasitological features of their acute infection. As regard to subcutaneously induced infection, young rats had noticeable parasitaemia by day 7 post infection, showing peak values at day 9 post infection, which declined thereafter to undetectable levels by day 21 post infection (Fig. 1A). In contrast, circulating forms of *T. cruzi* were hardly detectable in adult rats, with a virtual disappearance from circulation on day 9 post infection. Data from rats challenged by the intraperitoneal route yielded a similar pattern of group differences (Fig. 1B).

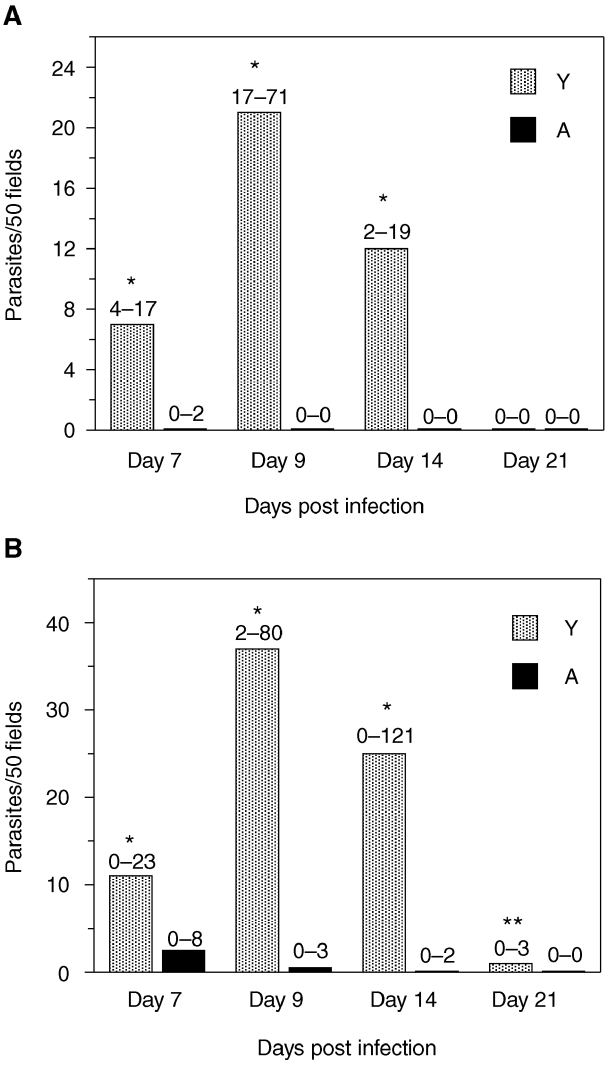

Figure 1 Parasitaemia in acutely *Trypanosoma cruzi*-infected rats of different ages. Data are median (range) values of parasites/50 fields from eight rats/group, inoculated by the subcutaneous (A) or intraperitoneal (B) route. Y and A indicate young and adult animals, respectively. Statistically different from the adult group, $^*P < 0.001$, $^{**}P < 0.01$.

In vitro infection in PMs

Macrophages are sites where *T. cruzi* replicates, and it was important to analyse the characteristics of the trypanosomal *in vitro* infection in PMs obtained from young and adult rats. Assessment on the number of extracellular parasites revealed no differences when studying culture supernatants from young or adult PMs undergoing no stimulation, irrespective of the time point. In contrast, 24 h supernatants from young PMs cultured in the presence of rR-IFN-γ contained fewer trypomastigotes than supernatants from their adult counterparts, with those stimulated with 400 U/ml being statistically significant ($P < 0.02$, data not shown).

Data from cells cultured with no rR-IFN-γ showed no between-group differences in the number of phagocytic

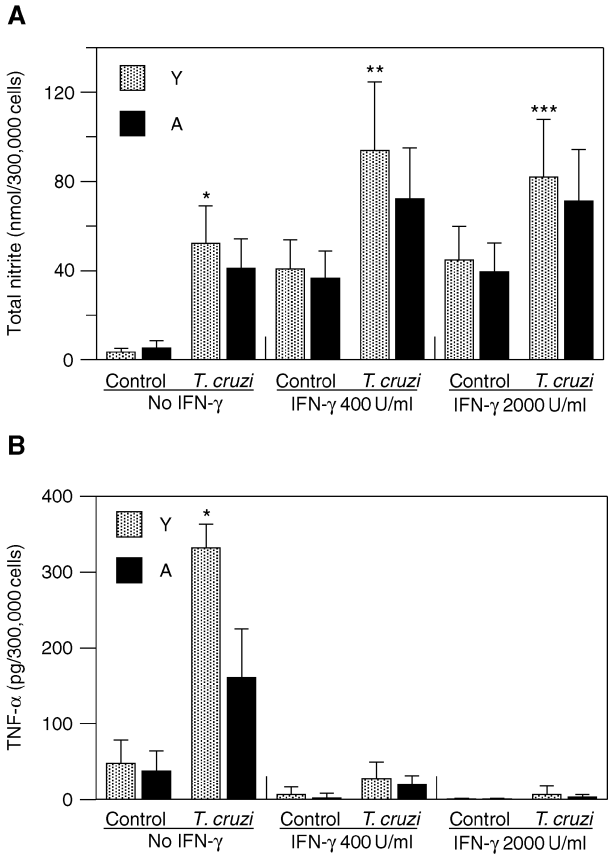

Figure 2 Nitrite and tumour necrosis factor-α (TNF-α) concentrations in culture supernatants from *Trypanosoma cruzi*-infected peritoneal macrophages (PMs). Controls, noninfected PMs; *T. cruzi*, PMs exposed to parasites. Y and A indicate young and adult animals, respectively. Data are mean ± standard deviation of eight rats/group. (A) Nitrite values – statistical difference with the adult group, $^{*}P = 0.015$, $^{**}P < 0.01$ and $^{***}P < 0.025$. (B) TNF-α concentrations – *different from the adult group, $P < 0.002$.

cells; median (range) of eight rats per group were as follows, young = 15.8 (12.1–19.5), adult = 13.7 (9.8–17.4). In contrast, more phagocytic cells were found in rR-IFN-γ-treated PMs from young rats (28.6 (23–40.8), $n = 8$) than from their adult counterparts (19.5 (16.5–23.1), $n = 8$, $P < 0.01$). There were no differences in the intracellular parasite counts, irrespective of whether the cells were stimulated with rR-IFN-γ or not (one to four amastigotes per macrophage, data not shown).

We next investigated the concentrations of a couple of macrophage-synthesized compounds which may be present in culture supernatants collected at different times following parasite exposure. Nitrite concentrations are depicted in Fig. 2A. Culture supernatants from infected, but unstimulated, PMs had detectable nitrite levels, with values being significantly higher in the young group. Studies in culture supernatants from rR-IFN-γ-treated PMs (400 or 2000 U/ml) revealed a further increase in nitrite concentrations more evident in the young group, these concentrations being significantly different from those in adult animals. Culture supernatants from control PMs treated with rR-IFN-γ showed measurable nitrite amounts, with no differences between the young and adult groups. Abundant TNF-α was recorded in culture supernatants from infected, but unstimulated, PMs, particularly in the young group reflecting a significant difference compared with the adult group (Fig. 2B). Measurements in culture supernatants from rR-IFN-γ-treated PMs revealed minimal quantities of TNF-α.

Levels of circulating cytokines and NO-derived metabolites

In line with the study objectives, we then analysed the circulating levels of mediators, which are likely to play a role in the protective responses elicited by *T. cruzi* infection, that is nitrate, TNF-α and IFN-γ. Assessment focused on the earlier phase of acute infection, as parasitological differences were clearer during this period. Data analysis of nitrate measurements indicated that this compound was present in the circulation, although levels appeared to be clearly augmented at day 7 post infection, and significantly higher in the young group (Fig. 3A). Nitrite concentrations in these rats decreased to normal values by the third week post infection. As regards to TNF-α, measurable levels of this cytokine were only detected in the young group by day 7 post infection (data not shown). When studying IFN-γ, both groups of *T. cruzi*-infected rats had increased amounts of this cytokine in their sera by day 4 post infection. In the case of adult rats, this trend was followed by a significant drop at day 7, with the young group showing values still higher by the same time point evaluation (Fig. 3B). In this group, IFN-γ remained increased for an additional week (data not shown).

Serum-specific antibodies and complement activity

Attempts were also made to analyse the serum levels of anti-*T. cruzi* antibodies during acute infection. As shown in Fig. 4, young rats had lower levels of IgM antibodies at 4 and 7 days post infection, which were statistically significant if compared with the adult group. Analysis of IgG serum concentrations at day 7 post infection revealed a similar pattern of group differences. Both groups showed similar amounts of specific Igs by day 14 post infection.

In qualitative terms, the presence of complement-mediated lytic antibodies in the sera from the two experimental groups was distributed as follows: day 14, young = 1/5; adult = 0/5 (not statistically significant, Fisher's exact test).

There were no between-group differences in complement haemolytic activity at any time point evaluation To illustrate the point, mean ± SD of CH_{50} units/ml of eight rats/group were as follows: day 4,

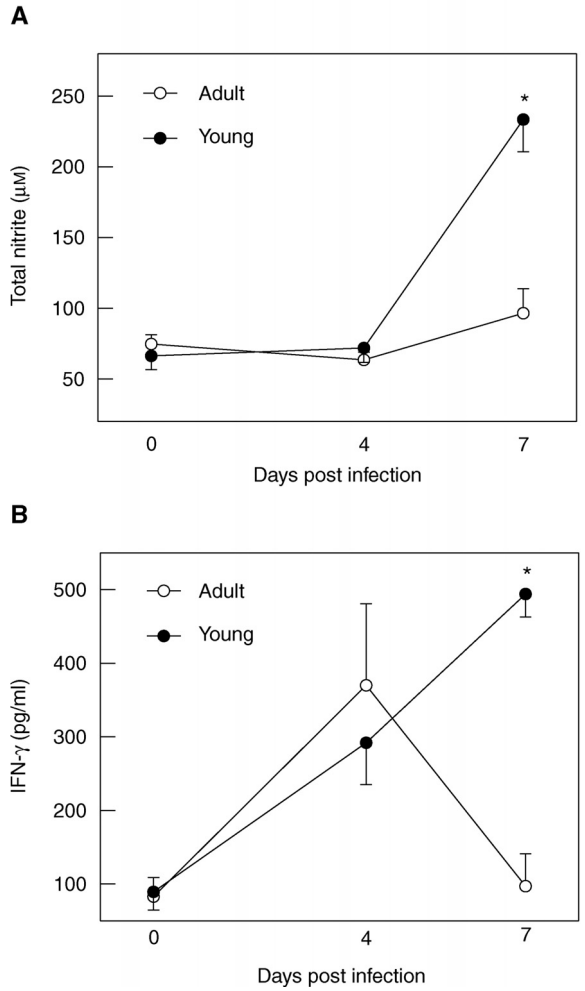

Figure 3 Serum nitrate (A) and interferon-γ (IFN-γ) (B) concentrations during experimental *Trypanosoma cruzi* infection. Y and A indicate young and adult animals, respectively. Values represent mean ± standard deviation (μM nitrite and pg/ml IFN-γ) of eight rats/group. *Different from the adult group, $P < 0.001$

young = 37.6 ± 5.4, adult = 37.2 ± 5.7; day 7: young = 40.1 ± 5.4, adult = 39.2 ± 10.8.

Discussion

Responses induced by immunization early in life are known to differ from those elicited in adults. Thus, some studies indicate that quantitative responses in young animals develop more slowly and are weaker, whereas qualitative responses are distinctly Th2 biased with predominant antibody production and poor cellular reactions [16–18]. While results presented herein showed that adult rats were more capable of controlling the acute *T. cruzi* infection induced in them, this phenomenon was not associated with a more effective macrophage activity but with an earlier appearance of specific antibodies in serum.

The complex interactions that occur between cells and humoral factors during *T. cruzi* infection are thought to play a critical role in the resolution of this protozoan disease. Macrophages may be involved in this regard through phagocytic and secretory activities, the latter being enhanced as a result of endocytosis or factors from activated lymphocytes, like IFN-γ. Results from the present study indicate that macrophages from young rats were as effective as their adult counterparts in terms of restraining parasite growth, as judged by the number of amastigotes recorded in both groups. When adding IFN-γ, it was clear that young PMs showed an increased number of phagocytic cells, with their culture supernatants containing fewer trypomastigotes and higher amounts of nitrite. This implies that in the presence of an activating cytokine, young macrophages may be more capable of phagocytosing and/or destroying parasites. With regard to this, NO is thought to be involved in the *in vitro* and *in vivo* clearance of *T. cruzi* [19–22]. TNF-α was also shown to protect against *T. cruzi* [23, 24] via the activation of NO production

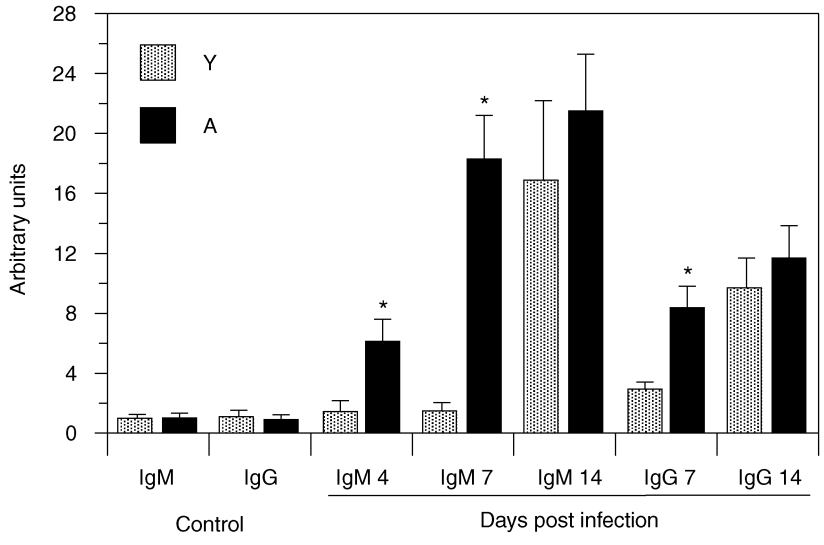

Figure 4 Specific antibodies in sera from rats undergoing *Trypanosoma cruzi* infection. Values represent mean ± standard deviation of arbitrary units from eight rats/group. Y and A indicate young and adult animals, respectively. *Difference with adult rats, $P < 0.001$.

[7, 8], although a direct effect cannot be discounted, given the parasiticidal activity of TNF-α on trypomastigotes [25]. Augmented NO and TNF-α synthesis by PM from young rats was paralleled by an increased presence of both compounds in the circulation, and likewise for IFN-γ levels. It follows that the increased *in vitro* secretory activity of young PMs may also operate *in vivo*, promoting a greater release of NO and TNF-α in circulation and stimulus for IFN-γ secretion. Taken together, these results may be interpreted as an attempt of cell-mediated mechanisms to compensate for the immaturity-associated defect of young rats in the resolution of acute infection. Parasite persistence may be responsible, at the same time, for increased NO and TNF-α production, as the synthesis of macrophage-derived proinflammatory cytokines can be triggered by a glycosylphosphatidylinositol-anchor structure of *T. cruzi* [26]. As an additional possibility, abundant presence of NO in young rats may be detrimental for infection control, considering its reported suppresor effects on lymphoproliferation to mitogens and parasite antigens in *T. cruzi*-infected mice [27]. Beyond the precise implications, the present results are in partial agreement with those reported by Russo *et al.*, demonstrating that TNF-α secretion by macrophages was significantly higher in susceptible mice than in resistant ones [28].

Although macrophages are major effector cells in inhibiting microbial multiplication, in the case of *T. cruzi*, the presence of specific antibodies was also shown to be effective in conferring protection [2]. Therefore, for a better evaluation of the protective immune mechanisms, studies were also aimed at measuring IgM and IgG anti-*T. cruzi* antibodies. Our results clearly indicated that adult rats had a better response in both antibody isotypes. This phenomenon may account for the attenuated infection seen in adult rats because anti-*T. cruzi* antibodies can either induce lysis of parasites or facilitate trypomastigote removal from the circulation [29]. The former possibility is unlikely, as our results revealed no differences in the presence of lytic antibodies, with complement activity being virtually the same in the two groups. Protection may be then explained by assuming that antibodies acted to opsonize parasites directly or indirectly rendering them more susceptible to intracellular destruction or removal. In this regard, studies carried out by Scott and Moyes demonstrated that the liver was a critical site for the immune clearance of *T. cruzi* [30].

B cells are well known for its ability to present the antigenic determinant in the context of class II molecules to specific helper T cells, which in turn selectively activate B cells by secreting cytokines like interleukin-2 (IL-2) and IL-4 [31, 32]. In the sense of an age-associated variation in antibody formation during *T. cruzi* infection, our results offer a new possibility for exploring the T–B cooperation differences underlying such phenomena and hence provide a better understanding of disease control mechanisms.

Acknowledgments

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