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Splenectomy Increases Mortality in Murine *Trypanosoma cruzi* Infection

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Abstract

The spleen is a secondary lymphoid organ that harbours a variety of cells such as T and B lymphocytes and antigen-presenting cells important to immune response development. In this study, we evaluated the impact of spleen removal in the immune response to experimental *Trypanosoma cruzi* infection. C57BL/6 mice were infected with Y strain of the parasite and infection was followed daily. Mice that underwent splenectomy had fewer parasites in peripheral blood at the peak of infection; however, mortality was increased. Histological analysis of heart and liver tissues revealed an increased number of parasites and inflammatory infiltrates at these sites. Spleen removal was associated with reduction in IFN-γ and TNF-α production during infection as well as with a decrease in specific antibody secretion. Haematological disorders were also detected. Splenectomized mice exhibited severe anaemia and decreased bone marrow cell numbers. Our results indicate that spleen integrity is critical in *T. cruzi* infection for the immune response against the parasite, as well as for the control of bone marrow haematological function.

Introduction

The spleen is a secondary lymphoid organ that performs many functions. In mammals, the anatomy may vary, but in all species the spleen is divided into red pulp and white pulp [1]. The red pulp is formed by reticular fibres, fibroblasts and macrophages that remove old erythrocytes and other cells from circulation. This process results in the release of *heme* groups, so that spleen plays a major role as an iron reservoir [2, 3]. During embryogenesis and in bone marrow stress conditions such as aplastic anaemia, haematopoietic activity is reactivated in the red pulp [4], providing a compensatory mechanism to maintain the homoeostasis of blood elements.

The spleen region with immunological activity is the white pulp. It is composed of germinal centres where the proliferation of B and T cells takes place and the marginal zone where professional antigen-presenting cells, macrophages and B cells can be found [5]. Antigens that reach the circulation are captured and presented to T cells in the spleen [6, 7]. Presence and integrity of the spleen are critical for immune responses against several micro-organisms [8]. Functionally or anatomically asplenic individuals are more susceptible to bacterial infections. Splenectomy is associated with reduction in B-cell activation, in immunoglobulin secretion and in the proliferation of T cells. These defects increase the mortality of individuals infected with bacteria such as *staphylococcus* and *streptococcus* [9–11].

There are few studies on the effect of spleen removal during infection caused by non-bacterial pathogens. *Leishmania* sp. and *Trypanosoma cruzi* are parasites that induce vigorous immune responses in the spleen of their hosts [12–15]. However, the absolute requirement for this organ in immune response development upon parasite infection remains to be explored. We have shown recently that splenectomy does not change the progression of *Leishmania major* infection in BALB/c and C57BL/6 mice [12], but no studies have examined the involvement of spleen in *T. cruzi* infection.

Trypanosoma cruzi is the aetiologic agent of Chagas disease, an endemic zoonosis present in some countries of South and Central America. WHO estimates that 100 million people remain at risk of acquiring this infection yet this is one of the neglected parasite diseases in the world [16]. There are more than 10 million infected

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individuals and 5000 new cases appear every year in Latin America [17]. Infection affects many tissues including heart, spleen, bone marrow and digestive tract [18]. In particular, infection with the Y strain of *T. cruzi* is characterized by high parasitaemia and mortality in the beginning of infection [19]. This strain can infect macrophages within the heart, liver, bone marrow and spleen [20]. The development of immune response occurs primarily in the spleen resulting in the production of IFN-γ and TNF-α. Production of these cytokines is important to stimulate macrophages and to activate B cells for antibody production [21, 22].

In spite of the role of the spleen in immune response development during Chagas disease, there is no systematic study on the need for this organ during *T. cruzi* infection. In this study, we demonstrate that spleen removal before infection with Y strain of *T. cruzi* can interfere with both protective immune response and haematological homoeostasis.

Material and methods

Animals. Female C57BL/6 mice (6–8 weeks old) were obtained from our animal facility (CEBIO, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil). Animals were given water and food *ad libidum*. All animal procedures were approved by local ethical committee for animal research (CETEA – protocol number 010/2007).

Surgery procedure. For splenectomy, mice were anesthetized with 1.7 mg ketamine and 0.33 mg xylazine in physiological buffer i.p. Hair was removed in the left flank. A small incision was performed and the spleen was removed. The incision was sutured and animals were monitored until consciousness was regained at 37 °C. Control group underwent a sham surgery, and they were maintained at the same conditions. All experiments were performed 30 days afterwards when there was no sign of inflammation in the abdominal cavity.

Parasites and infection. Y strain of *T. cruzi* was used for injection. Parasites were maintained by weekly passage in Swiss mice. For experimental infection, mice were injected i.p. with 1000 blood-stage trypomastigotes. Parasitaemia levels were evaluated daily by counting the numbers of parasites in 5 μl of blood drawn from the tail vein. Mortality of infected mice was also monitored daily.

For total particulate antigen preparation, epimastigotes from Y strain of *T. cruzi* were cultured in Liver Infusion Tryptose medium (Liver infusion broth – DIFCO, Lawrence, KS, USA) with hemin (SIGMA, St. Louis, MO, USA) and glucose. During logarithmic phase, epimastigotes were collected by centrifugation and submitted to cycles of freezing and thawing. Total protein concentration was determined by Lowry method, and the extracts were stored at −20 °C.

Histology. Animals were submitted to necropsy during the acute phase of infection. Fragments of heart and liver were fixed in 4% paraformaldehyde (pH 7.2), dehydrated in alcohol and embedded in paraffin. Sections were stained with haematoxylin and eosin (HE) for standard histological procedures. All sections were analysed using 10×, 40× and 100× microscopic objectives.

Tissue extract preparation for cytokine measurements. Fragments of spleen, liver and heart were collected, washed with PBS and weighed for each 100 mg of tissue used, 1 ml of cold phosphate buffer containing 0.5% BSA and protease inhibitors. Extracts were obtained by homogenizing tissues with an electrical tissue homogenizer. Tissue samples were then centrifuged at 3500 × *g* for 15 min, and supernatants were collected and stored at 20 °C until use. Cytokines were measured as described elsewhere.

ELISA for cytokines and antibodies. To measured plasma cytokine levels, blood samples were collected in EDTA and cells were separated from plasma by 3500 × *g* centrifugation. Plasma was separated and stored at −20 °C until use. Sandwich ELISA was performed to measure cytokine concentration, using specific plates (MaxSorp; NUNC, Rochester, NY, USA), capture and detection antibodies against IFN-γ, TNF-alfa, IL-4 and IL-10. Standard curves for each cytokine were obtained using recombinant cytokines (PharMingen, San Diego, CA, USA). All reagent concentrations were used in accordance with the manufacturer's protocol. Sera were separated by blood centrifugation, and the levels of specific antibodies were detected by capture ELISA using plates coated with 20 μg/ml total *T. cruzi* (MaxSorp; NUNC, Rochester, NY, USA). For immunoglobulin detection, specific anti-mouse biotinylated antibodies were used (PharMingen). For this technique, all sera were diluted at 1:10.

Haematological parameters. Blood samples were obtained via axillary plexus and EDTA was added to avoid coagulation. Cells were counted in a haemocytometer. Differential leucocyte counts were performed on blood smears stained by the standard May-Grumwald and Giemsa solutions (Doles, Goiânia, Goiás, Brazil). Concentration of haemoglobin in the samples was determined using a haemoglobin test kit (Doles). Reticulocyte numbers were determined in blood smears stained with methylene blue based on the percentage of total erythrocytes. Bone marrow cells were obtained by flushing the femoral bone cavity with RPMI (GIBCO BRL, Grand Island, NY, USA). Total cell number was obtained using a Neubauer chamber, and cytocentrifuge smears were stained with standard May Grunwald–Giemsa solution. Differential cell counts were obtained for each 500 cells per slide per mouse.

Statistical analysis. All experiments used groups of four mice and were repeated three times. Results were represented as the mean ± standard deviation (SD). Differences between groups were calculated using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Splenectomized mice manifest increased mortality after infection with *T. cruzi*

To study the role of spleen during experimental *T. cruzi* infection, splenectomized C57BL/6 mice were infected with Y strain of *T. cruzi* and development of infection was monitored. Splenectomized C57BL6 mice exhibited significantly lower parasitaemia when compared to control mice 9 days post-infection (Fig. 1A). However, mortality rate was higher in mice in which the spleen has been removed than in mice with a spleen (Fig. 1B).

Parasitism was increased in heart and liver of splenectomized mice

Because we observed a lower parasitaemia and increased mortality in splenectomized mice, we asked whether parasites that are usually lodged in spleen would have disseminated into other organs causing damage and death. Thus, histological analyses were performed in heart and liver to analyse parasitism and inflammatory infiltration in these tissues. At day 9 after infection, we observed a higher number of amastigotes in the hearts of splenectomized mice (Fig. 2A), but there was no difference in the heart inflammatory infiltrates between splenectomized and control groups. No difference was observed either at day 16 in parasitism at heart tissue parasitism. In mice without spleens, liver sections displayed more inflammatory infiltration (delimited areas) and higher parasitism (inserts in 100×), 9 and 16 days post-infection (Fig. 2B). These results were confirmed by morphometrical analyses shown in Fig. 3.

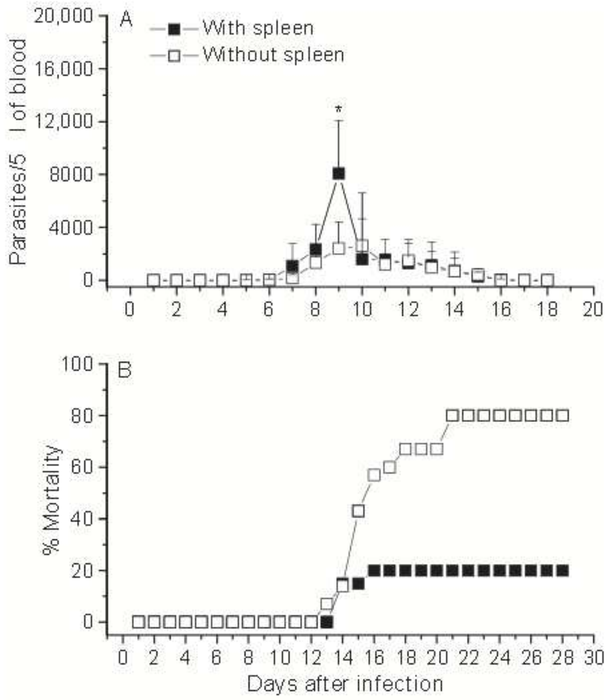

Figure 1 Parasitemia and mortality of C57BL/6 infected with Y strain of *Trypanosoma cruzi*. (A) Parasitemia. (B) Percentages of mice that succumbed to infection. Mice were injected i.p. with 1000 blood-stage trypomastigotes 30 days after splenectomy. Parasitemia levels were evaluated by counting numbers of parasites in 5 μl of blood drawn from the tail vein. Mortality of infected mice was monitored daily. Data represent mean ± SD of 10 mice from one representative experiment. *Statistical significance of $P < 0.05$ calculated by Student's *t*-test.

Alteration in tissue and plasma cytokine profiles in splenectomized mice after infection with *T. cruzi*

It is already known that cytokines are involved in the control of *T. cruzi* infection, specially IFN-γ [23]. We have shown previously that IFN-γ production is decreased in the plasma of splenectomized mice [12]. To investigate whether there were modifications in cytokine secretion that could contribute to an increase in the mortality of splenectomized mice, we measured cytokine levels in tissues affected by parasite and in plasma. Before infection, there was an increase in IFN-γ, IL-4 and IL-10 (Fig. 4A,C,D) levels in liver from mice lacking the spleen when compared to control mice. At 9 days post-infection, concentration of IFN-γ, TNF-α and IL-10 increased in the liver of mice with spleen when compared with uninfected mice. However, cytokine concentration decreased in the liver of splenectomized mice in comparison with infected mice with spleen. At this time point, there was a decrease in the liver inflammatory cytokines (Fig. 4A,B) and also in anti-inflammatory cytokines (Fig. 4C,D). This could indicate a failure in controlling both parasite lodging and inflammation in the liver. We did not observe alterations in cytokine concentrations in the heart.

Interestingly, 9 days post-infection, we also observed higher levels of IFN-γ (Fig. 5A) and increased parasitaemia (Fig. 1A) in non-splenectomized mice when compared to mice with spleen. In splenectomized mice, there were more parasites in tissues mostly in the liver, where concentrations of IFN-γ were also lower in comparison with infected controls. Liver and plasma levels of TNF-α (Fig. 5B) were also decreased 9 days post-infection in splenectomized mice.

Levels of anti-inflammatory cytokines such as IL-4 were decreased (Fig. 5C) in the plasma of splenectomized mice before infection, but 9 days post-infection with *T. cruzi*, splenectomized mice had a drastic increase in the levels of this cytokine. High levels of IL-4 correlated with low levels of IFN-γ at this time point in infection (Fig. 5A,C). There was no alteration in IL-10 secretion in mice without spleen (Fig. 5D).

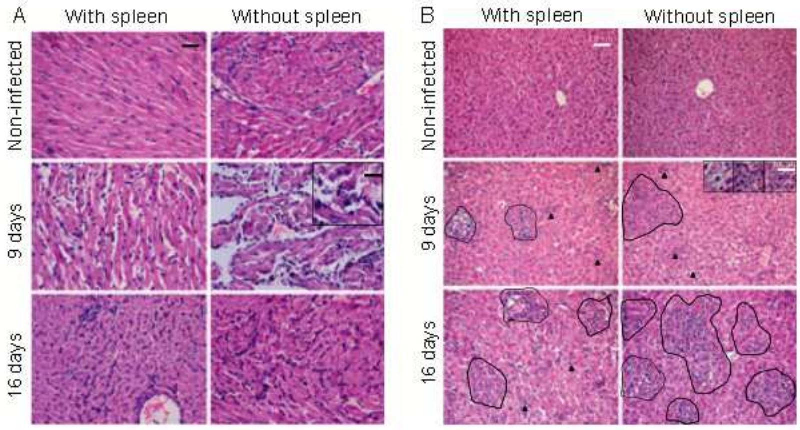

Figure 2 Histological analyses of heart and liver from mice infected with Y strain of *Trypanosoma cruzi*. Mice were injected i.p. with 1000 blood-stage trypomastigotes 30 days after splenectomy. Before infection, 9 and 16 days post-infection livers were collected and histological analysis was performed after haematoxylin and eosin staining. (A) Heart tissue of mice with or without spleen and (B) liver of mice with or without spleen (40× and inserts 100×). Time post-infection is indicated.

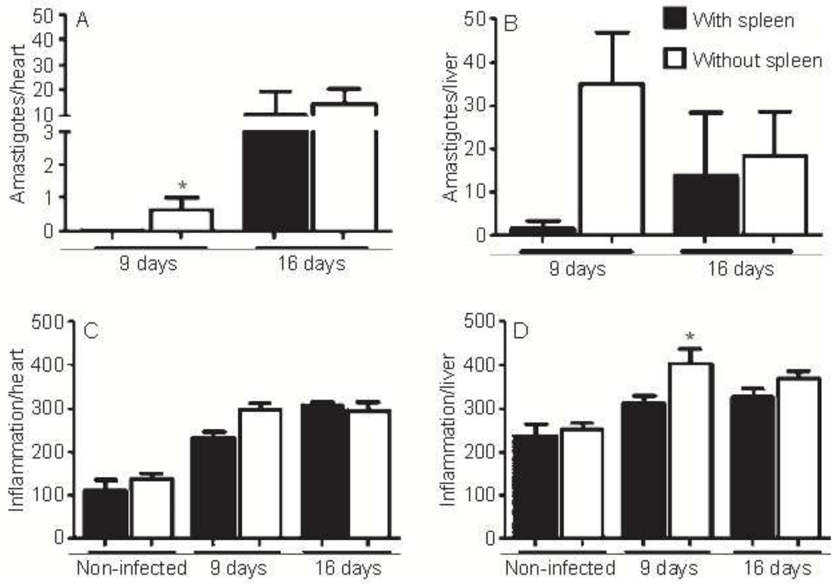

Figure 3 Morphometric analyses of inflammation and amastigotes loads in heart and liver of infected mice. Mice were injected i.p. with 1000 blood-stage trypomastigotes 30 days after splenectomy. Livers and hearts were collected and histological analysis was performed after haematoxylin and eosin staining at three time points during infection (days 0, 9 and 16 post-infection). (A and C) represent heart tissue from mice with spleen or without spleen and (B and D) liver from mice with or without spleen. Charts A and B indicate amastigote load in the tissues. Panels C and D represent counting of tissue inflammatory cells. Data result from one representative experiment with at least five samples per group. *$P < 0.05$ between mice with or without spleen calculated by Student's *t*-test.

Spleen removal is associated with low levels of specific IgG responses during *T. cruzi* infection

Antibody secretion by activated B cells is important in Chagas disease to increase the opsonization of parasites and also to activate the classical complement pathway [24]. The spleen is a major lymphoid organ for B-cell activation and immunoglobulin secretion. Levels of serum IgG, IgG1 and IgG2a were lower in splenectomized mice when compared to control animals (Fig. 6A–C) 9 and 16 days post-infection. Specific IgM was reduced in splenectomized mice (Fig. 6D) 9 but not 16 days after infection. Curiously, we observed that total non-specific IgM levels were higher in the sera of splenectomized mice on day 16 post-infection (data not shown). These results suggest that mice lacking the spleen were still able to produce immunoglobulins, but class switch activity was impaired.

Absence of spleen during *T. cruzi* infection aggravates haematological disorders

It is already known that splenectomy changes the number of circulating leucocytes [25, 26] and that spleen is an important organ for haematopoiesis [3]. Infection with *T. cruzi* can lead to alterations in haematological parameters and problems in bone marrow cell production [27, 28].

To evaluate whether mouse death was associated with changes in haematological parameters, bone marrow cells were counted before and after infection in both splenectomized and control mice. We observed a decrease in erythrocyte progenitor cells in non-infected splenectomized mice (Table 1). Moreover, 9 days after infection, splenectomized mice had a reduction in lymphocyte progenitor cell counts, and 16 days after infection, a reduction in neutrophil progenitor cells was detected

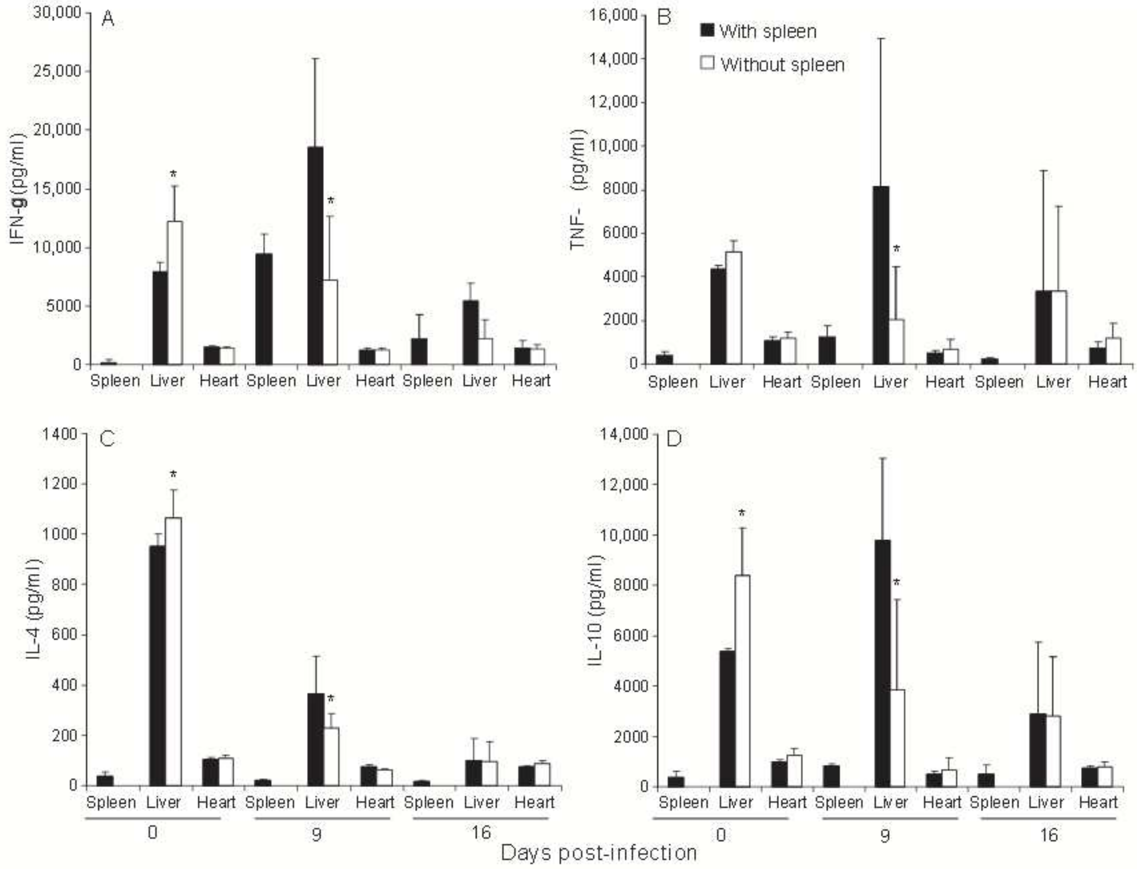

Figure 4 Cytokine production in tissues before and after infection with Y strain of *Trypanosoma cruzi*. Cytokine concentrations were determined by capture ELISA with specific antibodies to IFN-γ (A), TNF-α (B), IL-4 (C) and IL-10 (D) in the supernatants of tissue homogenates. Each point represents mean ± SD of two independent experiments with five animals per group. Assays were performed before, 9 or 16 days after parasite inoculation. *Significant differences ($P < 0.05$) between mice with and without spleen calculated by Student's *t*-test.

(Table 1). Interestingly, the progress of infection resulted in a decreased number of cells in bone marrow. On day 16 post-infection, the number of bone marrow cells was significantly reduced in splenectomized mice when compared with control mice.

The number of circulating cells was also analysed. Circulating lymphocytes and neutrophils were increased in splenectomized mice in comparison with control mice prior to infection (Table 2). However, on day 16 post-infection, there was a reduction in circulating monocytes and neutrophils. Interestingly, there was a drastic decrease in eosinophils during infection in both groups of mice (Table 2). At this time point, there was a reduction in the total number of bone marrow and peripheral blood cells in splenectomized mice (Table 2).

Because *T. cruzi* infection can lead to haematological alterations [28, 29], additional blood parameters were also analysed. No alteration was found in erythrocyte number, haemoglobin concentration or reticulocyte number in mice without the spleen before infection with *T. cruzi* (Fig. 7). Nine and 16 days after infection, a drastic reduction in red blood cell number (Fig. 7A) and haemoglobin concentration (Fig. 7B) in splenectomized mice was detected. Analysis of reticulocyte number is a reliable parameter to investigate anaemia as higher reticulocyte counts usually represent immature cells being produced by bone marrow in anaemia condition. Blood erythrocytes and reticulocytes numbers only fall together in bone marrow failure. Figure 7C demonstrates that there was no alteration in the reticulocytes of non-infected nice. However, on day 9 after infection, splenectomized mice presented higher numbers of circulating reticulocytes when compared with control mice. Sixteen days post-infection, number of reticulocytes dropped sharply in the circulation of splenectomized mice (Fig. 7C).

Discussion

In the present study, we demonstrated the importance of spleen during experimental *T. cruzi* infection. Removal of this lymphoid organ was associated with increased mor-

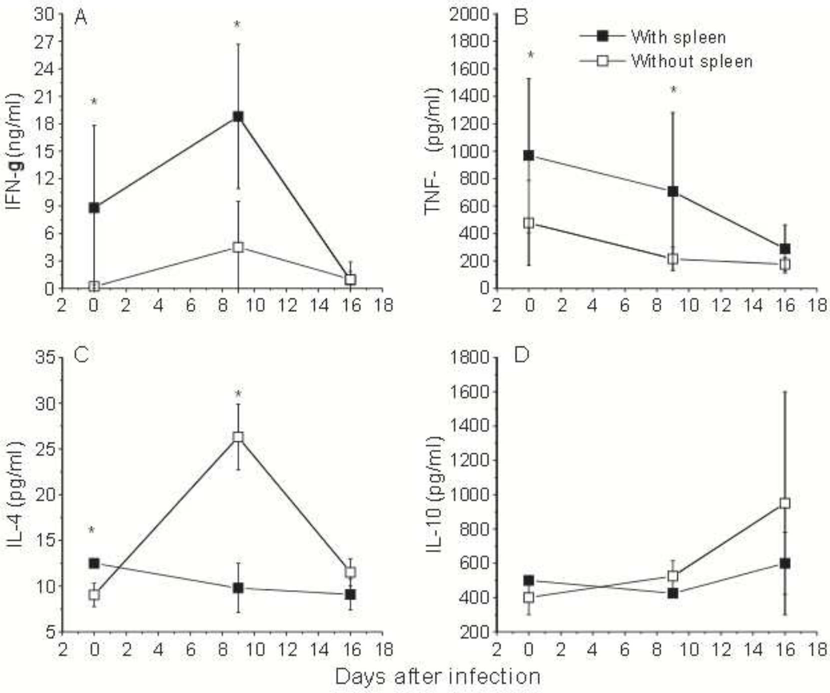

Figure 5 Cytokine levels in plasma of mice with or without spleen before infection with Y strain of *Trypanosoma cruzi*. Cytokine concentrations were determined by capture ELISA with specific antibodies to IFN-γ (A), TNF-α (B), IL-4 (C) and IL-10 (D). Each point represents mean ± SD of three independent experiments with four animals per group. Assays were performed before, 9 and 16 days after parasite inoculation. Closed squares represent mice with spleen, and open squares represent mice without spleen *Significant differences ($P < 0.05$) between mice with and without spleen calculated by Student's *t*-test.

tality rate of C57BL/6 mice infected with Y strain of *T. cruzi*. Our data suggest that two main causes might be involved in this outcome. It is plausible that decrease in the secretion of inflammatory cytokines and antibodies in splenectomized mice, specifically during the acute phase of infection, impaired protective immunity. In addition, the deleterious effects of severe anaemia installed during infection aggravated by reduction in bone marrow function that follows spleen removal may contribute to promote higher mortality.

Immunopathogenesis of experimental infection with *T. cruzi* involves many mechanisms operating during acute and chronic phases. Infection with Y strain is characterized by a high parasitaemia early after parasite inoculation [19, 20]. Because this strain of *T. cruzi* has a macrophagotropic cell tropism [20], spleen is an important organ in the establishment of infection. Our results demonstrated differences in parasitaemia between mice lacking spleen and mice with spleen. In splenectomized mice, there was a reduced parasitaemia at the peak of infection. In the absence of spleen, parasites may have lost their main proliferative site. Spleen is an important source of mononuclear cells and also an iron reservoir, which is crucial for parasite replication [30]. However, we also observed an increased mortality among splenectomized mice that could be explained by establishment of parasite amastigotes in other reticulocellular organs such as liver. Indeed, we demonstrated that there were increased number of amastigotes in the liver and heart of splenectomized mice 9 days after infection. Liver inflammatory infiltration was also augmented in mice without spleen. These results demonstrate that most of the parasites were lodged in tissues and not in the bloodstream at day 9 post-infection and this could contribute to mice death.

Strategically located between portal and systemic circulations, spleen provides a critical site where interactions between bloodborne antigens, antigen-presenting cells and lymphocytes take place. Marginal zone cells are able to capture bloodborne antigens by different receptors. The antigen presentation process that follows uptake results in the activation and proliferation of lymphocytes, antibody production and cytokine secretion [5, 31]. This large lymphoid organ has been shown to be an important organ in the development of immune responses to protozoa parasites. In *Leishmania donovani* infection, for instance, there are several modifications in the marginal zone and the white pulp of the organ. Spleen dendritic cells can migrate to lymphoid germinal centres and promote antigen presentation once Th1 cytokines are produced and a protective immune response is initiated [32].

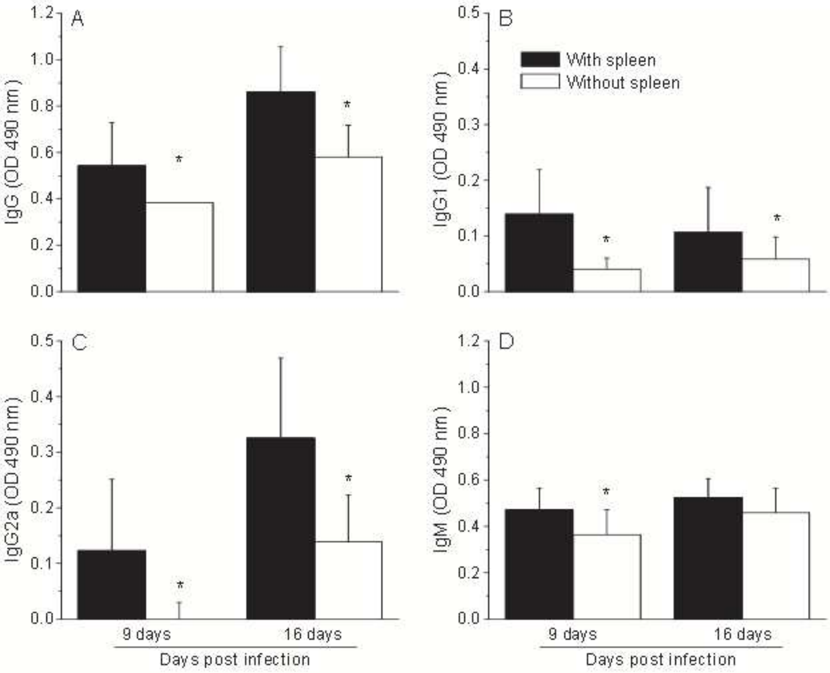

Figure 6 Antibody levels in plasma of mice with or without spleen infected with Y strain of *Trypanosoma cruzi*. Mice were injected i.p. with 1000 blood-stage trypomastigotes 30 days after splenectomy. Before infection or 9 and 16 days post-infection, blood was collected in EDTA and plasma was separated from cells by centrifugation. Plates were coated with 200 μg/ml of total epimastigote proteins. Plasma was diluted 1:10 to measure anti-*T. cruzi* antibodies. To detect IgG (A), IgG1 (B), IgG2a (C) and IgM (D), specific biotinylated antibodies were used. *Significant differences ($P < 0.05$) between control (with spleen) and splenectomized mice calculated by Student's *t*-test. Data are representative of three experiments with three animals/group.

Table 1 Differential cell count in bone marrow.

	Before infection		9 days post-infection		16 days post-infection	
Bone marrow cells (cells/femur)	With spleen	Without spleen	With spleen	Without spleen	With spleen	Without spleen
Eosinophils ($\times10^5$)	6.85 ± 4.43	11.9 ± 10.2	23.4 ± 25	21.3 ± 15	0	0
Mono-macrophage ($\times10^5$)	2.31 ± 2.1	2.45 ± 0.7	22.1 ± 13.8	38.9 ± 30.5	7.60 ± 3.9	14.7 ± 8.5
Proerythroblasts + Erythroblasts ($\times10^5$)	20.8 ± 9.4	4.12 ± 4.5*	15.5 ± 15	41.7 ± 33.5	2.43 ± 1.9	2.02 ± 1.3
Neutrophils ($\times10^5$)	77.3 ± 24	111 ± 36	184 ± 99	148 ± 45	769 ± 36	10.0 ± 7.5*
Lymphocytes ($\times10^5$)	919 ± 12	167 ± 82	104 ± 50	27 ± 29*	14.5 ± 11	5.61 ± 3.5
Total ($\times10^5$/ml)	270 ± 150	320 ± 230	460 ± 180	410 ± 300	104 ± 40	44 ± 20*

*Statistically significance with differences $P < 0.05$ calculated by Student's *t*-test.

Table 2 Differential cell count in blood smears.

	Before infection		9 days post-infection		16 days post-infection	
Blood leucocytes (n/mm^3)	With spleen	Without spleen	With spleen	Without spleen	With spleen	Without spleen
Lymphocytes	4670 ± 863	7934 ± 2210*	1354 ± 376	2035 ± 620	2201 ± 1020	1435 ± 887
Monocytes	493 ± 344	316 ± 217	153 ± 127	150 ± 51	465 ± 116	98 ± 85*
Neutrophils	664 ± 127	1236 ± 278*	1044 ± 303	1311 ± 379	2156 ± 1173	1014 ± 435*
Eosinophils	293 ± 217	201 ± 162	58 ± 39	93 ± 40	32 ± 28	43 ± 58
Total	6080 ± 614	9630 ± 2260*	2600 ± 754	3590 ± 790	5000 ± 568	2750 ± 534*

*Statistically significance with differences $P < 0.05$ calculated by Student's *t*-test.

Our results indicate that spleen function is essential to immune response development during experimental *T. cruzi* infection. We observed a decrease in cytokine production in plasma and in affected organs. Liver was the main organ responsible for cytokine production in the absence of spleen. After 9 days of infection, splenec-

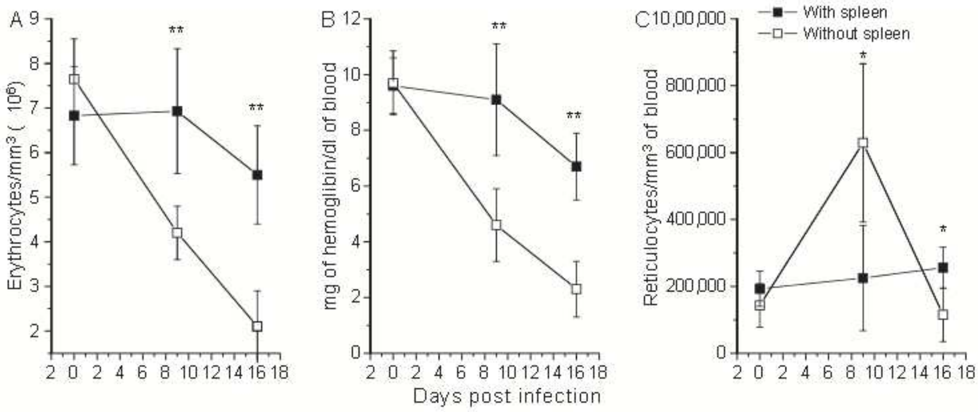

Figure 7 Haematological parameters in splenectomized mice infected with Y strain of *Trypanosoma cruzi*. Erythrocytes were counted in fresh blood collected in EDTA and diluted in acetic acid before and after infection (A). Haemoglobin concentration was also measured in fresh blood by an enzymatic kit (B). To evaluate reticulocyte numbers, blood cells were counted after staining with new methylene blue and numbers were calculated based on total erythrocyte number (C). *Significant differences ($P < 0.05$) and **$P < 0.0001$ between control (with spleen) and splenectomized mice calculated by Student's *t*-test. Data are representative of three experiments with four animals/group.

tomized mice manifested a reduced production of IFN-γ and TNF-α in liver and plasma and a decline in liver IL-10 production. Interestingly, this result suggests that spleen is important for the development of immune responses in other organs as well. Sixteen days after infection, we observed a dramatic reduction in cytokine secretion in the tissues and plasma of splenectomized mice, indicating that spleen is also important for a sustained immune response to *T. cruzi* infection.

It has been already shown that splenectomy reduces some immunological activities such as cytokine secretion [33] and reactive oxygen intermediate production [8, 34]. In addition, spleen removal increases the susceptibility to infection by intracellular pathogens, especially bacteria [35], and impairs the maintenance of immunological memory [36]. Humans who had their spleens removed are highly susceptible to streptococcus and staphylococcus infection, and often they do not survive these infections [35, 37]. Spleen also harbours memory T and B cells, being an important lymphoid organ for systemic immunization [35, 36, 38]. Control of *T. cruzi* infection is usually associated with IFN-γ [23] and TNF-α production, which promotes NO secretion [39]. In our study, splenectomized mice had a reduction in the secretion of both cytokines before and in the acute phase of infection. Our results are in agreement with another report that showed a reduction in the secretion of these cytokines and in NO production after splenectomy [40].

Host defence against bacterial infection is critically dependent on humoral immunity. Antibody-bound bacteria are usually cleared in the spleen by macrophages. In the absence of spleen, there is also a reduction in antibody secretion and a consequent rise in bacteria proliferation [35]. In *T. cruzi* infection, protective immunity also relies on specific antibody production. It requires IgM production throughout the infection and high IgG levels during the acute phase [41]. These requirements were not observed in splenectomized mice. Spleen removal was associated with a reduction in specific IgM and IgG secretion 9 days after infection and in diminished specific IgG production at all time points analysed.

A known effect of spleen removal is a rise in circulating leucocyte numbers [25, 26, 33]. *T. cruzi* infection is also associated with spleen and lymph node hyperplasia [42] and with increased numbers of blood cells during acute phase of infection. We found an increase in total circulating leucocytes in splenectomized mice before infection. Nine days after infection, there was no difference between mice with spleen and mice without spleen. In the 16th day post-infection, however, mice without spleen had fewer blood leucocytes than mice with spleen. Therefore, spleen removal interfered with leucocyte homoeostasis during infection with Y strain of *T. cruzi*, suggesting that the spleen is important in the maintenance of the leucocyte blood pool. It is likely that amastigotes also lodged in the bone marrow of splenectomized mice causing dysfunction of this organ. A study on visceral leishmaniasis showed that, when spleen is highly compromised with parasites, changes in the leucocyte blood pool are observed with a reduction in $CD5^+$ B cells and in T lymphocytes [43]. We also found that splenectomized mice had a decrease in $CD4^+$ and $CD8^+$ T cells as well as in $CD5^+$ B cells after infection. Thus, our data are in agreement with previous studies showing that spleen plays a role in the maintenance of lymphocyte cell numbers and in mounting an appropriate immune response during the development of protozoa infection.

Together with its immunological function, spleen is an organ where clearance of old blood cells and erythrocytes takes place [3, 44]. Old red blood cells trapped in the spleen release free iron, making this organ an important site of iron storage [2]. Y strain of *T. cruzi* has a special tropism to mononuclear cells [19], and it has been already shown that this infection is characterized by anaemia, thrombocytopenia and increase in blood leucocyte number [27, 28]. We observed similar alterations in splenectomized mice. There was a decrease in leucocyte number during infection in mice without spleens. Monocytes and neutrophils were the most affected cell types. These changes may correlate with the increase in susceptibility to infection after splenectomy. We also observed a drastic decline in eosinophils during infection in both groups. This observation is in agreement to what has been already described for experimental infection in C57BL/6 mice. There are fluctuations in eosinophil numbers as infection progresses with depletion of these cells in the bone marrow, decline in the blood and a marked rise in the peritoneal space [45]. Analysis of bone marrow cells in splenectomized mice revealed that there was no difference in monocyte production, but a decrease in neutrophil number was observed, suggesting that bone marrow could be affected by parasite lodging.

We also observed increased anaemia in mice without spleen evaluated by red blood cells count, haemoglobin concentration and number of blood reticulocytes. Reduction in haemoglobin concentration and red blood cells counts was followed by an increase in circulating reticulocytes at day 9 post-infection. However, 16 days after infection, reticulocytes declined in circulation. Failure in this feedback response of bone marrow later in infection suggests again that bone marrow function might be affected at this time point of infection by parasite invasion of the organ. There are other reports that support our results on the important role of spleen in haematological homoeostasis. Splenectomized humans are more susceptible to malaria [46], and in splenectomized monkeys infected with *Plasmodium*, anaemia is also severe during malaria progression [47].

Altogether our data suggest that spleen removal aggravated the effects of anaemia caused by infection with Y strain of *T. cruzi*, which could be one of the reasons for increased mortality in mice without spleen. In addition, *T. cruzi* infection is more severe in splenectomized mice as a result of impaired immune responses that are essential for protective immunity against the parasite.

Splenectomy is still a practice in some conditions such as visceral leishmaniasis and other protozoa parasite diseases. The present study demonstrates that this practice may result in the aggravation of anaemia and in increased susceptibility to other infections in individuals already infected with *T. cruzi*.

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References

1 Hartwig H, Hartwig HG. Structural characteristics of the mammalian spleen indicating storage and release of red blood cells. Aspects of evolutionary and environmental demands. *Experientia* 1985; 41:159 63.

2 Knutson M, Wessling-Resnick M. Iron metabolism in the reticuloendothelial system. *Crit Rev Biochem Mol Biol* 2003;38:61 88.

3 Seifert MF, Marks SC Jr. The regulation of hemopoiesis in the spleen. *Experientia* 1985;41:192 9.

4 Sasaki K, Iwatsuki H, Suda M, Itano C. Cell death and phagocytosis of haematopoietic elements at the onset of haematopoiesis in the mouse spleen: an ultrastructural study. *J Anat* 1993;183 (Pt 1):113 20.

5 Geijtenbeek TB, Engering A, van KY. DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. *J Leukoc Biol* 2002; 71: 921 31.

6 van den Eertwegh AJ, Laman JD, Noelle RJ, Boersma WJ, Claassen E. In vivo T-B cell interactions and cytokine-production in the spleen. *Semin Immunol* 1994;6:327 36.

7 Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol* 2005;5:606 16.

8 Nassif LS, Repka JC, Nassif AC, Branco Filho AJ, Olandoski M. Effects of splenectomy on peritonitis produced by a colonic injury: study in rats. *Rev Assoc Med Bras* 2004;50:268 71.

9 Wasserstrom H, Bussel J, Lim LC, Cunningham-Rundles C. Memory B cells and pneumococcal antibody after splenectomy. *J Immunol* 2008;181:3684 9.

10 Wolf HM, Eibl MM, Georgi E *et al.* Long-term decrease of CD4+CD45RA+ T cells and impaired primary immune response after post-traumatic splenectomy. *Br J Haematol* 1999;107:55 68.

11 Wara DW. Host defense against *Streptococcus pneumoniae*: the role of the spleen. *Rev Infect Dis* 1981;3:299 309.

12 Maioli TU, Carneiro CM, Assis FA, Faria AM. Splenectomy does not interfere with immune response to *Leishmania major* infection in mice. *Cell Immunol* 2007;249:1 7.

13 de MJ, Morrot A, Farias-de-Oliveira DA, Villa-Verde DM, Savino W. Differential regional immune response in chagas disease. *PLoS Negl Trop Dis* 2009;3:e417.

14 Pellegrini A, Guinazu N, Aoki MP *et al.* Spleen B cells from BALB/c are more prone to activation than spleen B cells from C57BL/6 mice during a secondary immune response to cruzipain. *Int Immunol* 2007;19:1395 402.

15 Guinazu N, Pellegrini A, Carrera-Silva EA *et al.* Immunisation with a major *Trypanosoma cruzi* antigen promotes pro-inflammatory cytokines, nitric oxide production and increases TLR2 expression. *Int J Parasitol* 2007;37:1243 54.

16 World Health Organization, 2007. New global effort to eliminate Chagas Disease. http://www.who.int/mediacentre/news/releases/2007/pr36/en/index.html (accessed on 6 June 2010).

17 Hotez PJ, Bottazzi ME, Franco-Paredes C, Ault SK, Periago MR. The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination. *PLoS Negl Trop Dis* 2008;2:e300.

18 Andrade SG. Caracterização de cepas do *Trypanosoma cruzi* isoladas no recôncavo baiano. *Rev Patol Trop* 1974;3:65 121.

19 Brener Z. The behavior of slender and stout forms of *Trypanosoma cruzi* in the blood-stream of normal and immune mice. *Ann Trop Med Parasitol* 1969;63:215 20.

20 Melo RC, Brener Z. Tissue tropism of different *Trypanosoma cruzi* strains. *J Parasitol* 1978;64:475 82.

21 Brener Z, Gazzinelli RT. Immunological control of *Trypanosoma cruzi* infection and pathogenesis of Chagas' disease. *Int Arch Allergy Immunol* 1997;114:103 10.

22 Rodrigues V Jr, Agrelli GS, Leon SC, Silva Teixeira DN, Tostes S Jr, Rocha-Rodrigues DB. Fas/Fas-L expression, apoptosis and low proliferative response are associated with heart failure in patients with chronic Chagas' disease. *Microbes Infect* 2008;10: 29 37.

23 Romanha AJ, Alves RO, Murta SM, Silva JS, Ropert C, Gazzinelli RT. Experimental chemotherapy against *Trypanosoma cruzi* infection: essential role of endogenous interferon-gamma in mediating parasitologic cure. *J Infect Dis* 2002;186:823 8.

24 Mussalem JS, Vasconcelos JR, Squaiella CC *et al.* Adjuvant effect of the *Propionibacterium acnes* and its purified soluble polysaccharide on the immunization with plasmidial DNA containing a *Trypanosoma cruzi* gene. *Microbiol Immunol* 2006;50:253 63.

25 Bessler H, Bergman M, Salman H, Beilin B, Djaldetti M. The relationship between partial splenectomy and peripheral leukocyte count. *J Surg Res* 2004;122:49 53.

26 Drew PA, Kiroff GK, Ferrante A, Cohen RC. Alterations in immunoglobulin synthesis by peripheral blood mononuclear cells from splenectomized patients with and without splenic regrowth. *J Immunol* 1984;132:191 6.

27 Marcondes MC, Borelli P, Yoshida N, Russo M. Acute *Trypanosoma cruzi* infection is associated with anemia, thrombocytopenia, leukopenia, and bone marrow hypoplasia: reversal by nifurtimox treatment. *Microbes Infect* 2000;2:347 52.

28 Santiago HC, Feng CG, Bafica A *et al.* Mice deficient in LRG-47 display enhanced susceptibility to *Trypanosoma cruzi* infection associated with defective hemopoiesis and intracellular control of parasite growth. *J Immunol* 2005;175:8165 72.

29 Lalonde RG, Holbein BE. Role of iron in *Trypanosoma cruzi* infection of mice. *J Clin Invest* 1984;73:470 6.

30 Arantes JM, Pedrosa ML, Martins HR *et al. Trypanosoma cruzi*: treatment with the iron chelator desferrioxamine reduces parasitemia and mortality in experimentally infected mice. *Exp Parasitol* 2007;117: 43 50.

31 Engwerda CR, Ato M, Cotterell SE *et al.* A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during *Leishmania donovani* infection. *Am J Pathol* 2002;161:429 37.

32 Melby PC, Yang J, Zhao W, Perez LE, Cheng J. *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. *Infect Immun* 2001;69:4719 25.

33 Eibl M. Immunological consequences of splenectomy. *Prog Pediatr Surg* 1985;18:139 45.

34 Brandt CT, Leite CR, Manhaes-de-Castro R, Brandt FC, Manhaes-de-Castro FM, Barbosa-de-Castro CM. Evaluation of the effect of splenectomy with autologous spleen tissue implantation in some monocyte functions in children with hepatosplenic schistosomiasis mansoni. *Rev Soc Bras Med Trop* 2005;38:38 42.

35 Okabayashi T, Hanazaki K. Overwhelming postsplenectomy infection syndrome in adults - a clinically preventable disease. *World J Gastroenterol* 2008;14:176 9.

36 Mrusek S, Vallbracht S, Ehl S. The impact of splenectomy on antiviral T cell memory in mice. *Int Immunol* 2005;17:27 33.

37 Waghorn DJ. Overwhelming infection in asplenic patients: current best practice preventive measures are not being followed. *J Clin Pathol* 2001;54:214 8.

38 Uzonna JE, Wei G, Yurkowski D, Bretscher P. Immune elimination of *Leishmania major* in mice: implications for immune memory, vaccination, and reactivation disease. *J Immunol* 2001;167:6967 74.

39 Abrahamsohn IA, Coffman RL. Cytokine and nitric oxide regulation of the immunosuppression in *Trypanosoma cruzi* infection. *J Immunol* 1995;155:3955 63.

40 Teixeira FM, Fernandes BF, Rezende AB *et al. Staphylococcus aureus* infection after splenectomy and splenic autotransplantation in BALB/c mice. *Clin Exp Immunol* 2008;154:255 63.

41 Garcia GA, Arnaiz MR, Esteva MI *et al.* Evaluation of immune responses raised against Tc13 antigens of *Trypanosoma cruzi* in the outcome of murine experimental infection. *Parasitology* 2008;135: 347 57.

42 Carneiro CM, Martins-Filho OA, Reis AB *et al.* Differential impact of metacyclic and blood trypomastigotes on parasitological, serological and phenotypic features triggered during acute *Trypanosoma cruzi* infection in dogs. *Acta Trop* 2007;101:120 9.

43 Guerra LL, Teixeira-Carvalho A, Giunchetti RC, Martins-Filho OA, Reis AB, Correa-Oliveira R. Evaluation of the influence of tissue parasite density on hematological and phenotypic cellular parameters of circulating leukocytes and splenocytes during ongoing canine visceral leishmaniasis. *Parasitol Res* 2009;104:611 22.

44 Hara H, Ogawa M. Erythropoietic precursors in mice under erythropoietic stimulation and suppression. *Exp Hematol* 1977;5:141 8.

45 Nakhle MC, de Menezes MC, Irulegui I. Eosinophil levels in the acute phase of experimental Chagas'disease. *Rev Inst Med Trop Sao Paulo* 1989;31:384 91.

46 Demar M, Legrand E, Hommel D, Esterre P, Carme B. *Plasmodium falciparum* malaria in splenectomized patients: two case reports in French Guiana and a literature review. *Am J Trop Med Hyg* 2004; 71:290 3.

47 Carvalho LJ, Alves FA, de Oliveira SG *et al.* Severe anemia affects both splenectomized and non-splenectomized *Plasmodium falciparum*-infected Aotus infulatus monkeys. *Mem Inst Oswaldo Cruz* 2003; 98:679 86.