B cells modulate T cells so as to favour T helper type 1 and CD8$^+$ T-cell responses in the acute phase of *Trypanosoma cruzi* infection

Fabiola Cardillo,[1] Edilberto Postol,[2] Jorge Nihei,[1] Luiz S. Aroeira,[3] Auro Nomizo[4] and José Mengel[1]

[1]*Cellular Immunology, Autoimmunity and Experimental Chagas Disease Laboratory, Oswaldo Cruz Foundation, Gonçalo Moniz Research Center, Salvador, Bahia, Brazil,* [2]*Immunology Laboratory, Heart Institute (INCOR), University of São Paulo, São Paulo, SP, Brazil,* [3]*Unidad de Investigacion Hospital La Paz, Madrid, Spain and* [4]*Department of Clinical Analysis, Toxicology and Bromatology, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil*

doi:10.1111/j.1365-2567.2007.02677.x
Received 30 January 2007; revised 11 June 2007; accepted 11 June 2007.
Correspondence: Dr J. Mengel, Oswaldo Cruz Foundation, Centro de Pesquisas Gonçalo Moniz. R. Waldemar Falcão 121, 40296-710, Candeal, Salvador, Bahia, Brazil.
Email: jomengel@cpqgm.fiocruz.br
Senior author: Fabiola Cardillo, email: cardillo@cpqgm.fiocruz.br

Summary

In this study, we have evaluated the production of pro- and anti-inflammatory cytokines and the formation of central and effector memory T cells in mice lacking mature B cells (muMT KO). The results show that *Trypanosoma cruzi* infection in C57Bl/6mμ MT KO mice is intensified in relation to control mice and this exacerbation is related to low levels of inflammatory cytokines produced during the acute infection and the lower numbers of central and effector memory CD4$^+$ and CD8$^+$ T cells generated during the acute phase of the infection. In addition, a marked reduction in the CD8$^+$ T-cell subpopulation was observed in muMT KO infected mice. In agreement to this, the degree of tissue parasitism was increased in muMT mice and the tissue inflammatory response was much less intense in the acute phase of the infection, consistent with a deficit in the generation of effector T cells. Flow cytometry analysis of the skeletal muscle inflammatory infiltrate showed a predominance of CD8$^+$ CD45Rb low in B-cell-sufficient C57Bl/6 mice, whereas the preponderant cell type in muMT KO skeletal muscle inflammatory infiltrate was CD4$^+$ T cells. In addition, CD8$^+$ T cells found in skeletal muscle from muMT KO infected mice were less activated than in control B-cell sufficient infected mice. These results suggest that B cells may participate in the generation of effector/memory T cells. In addition and more importantly, B cells were crucial in the maintenance of central and effector memory CD8$^+$ T cell, as well as the determination of the T cell cytokine functional pattern, and they may therefore account for critical aspects of the resistance to intracellular pathogens, such as *T. cruzi*.

Keywords: *Trypanosoma cruzi*; B cells; IFN-γ; memory; CD8

Introduction

Trypanosoma cruzi is an intracellular pathogen that grows in macrophages, neurons, heart muscle cells and skeletal muscle cells among other cells.[1] Depending on the parasite strain, the growth predominates in a given cell type.[2,3] Therefore, some parasite strains may grow mainly in macrophages, whereas other strains grow, preferentially, in skeletal muscle cells.[3] Genetic differences among parasite strains and among hosts may account for this variation.[4,5] The immune response that controls the infection in peripheral tissue cells other than the macrophages requires the generation of effector T cells, as these T cells are able to migrate from lymphoid tissues to virtually any other tissue and develop a local immune response.[6,7] This immune response is usually translated as tissue inflammation. It has been shown that both CD4$^+$ and CD8$^+$ T cells are important to control the *T. cruzi* infection.[8,9] Therefore, understanding the rules and conditions for T-cell activation and full differentiation to become effectors during this infection is required to comprehend the mechanisms of resistance to *T. cruzi*. In other studies[10,11] we have shown that natural killer (NK) and/or Vγ1$^+$ γδ$^+$ T cells may be important in helping conventional T cells to become effectors. However, it has for long been recognized that antigen-presenting cells such as macrophages, dendritic cells and B cells might be of crucial importance for the generation and maintenance of effector T cells.[12,13] Besides that, it has being recognized that B cells may produce different cytokines upon stimulation, including interferon-γ (IFN-γ), interleukin (IL)-10 and IL-12.[14–16] Consequently, B cells may well contribute

to the regulation of the T-cell functional differentiation into T helper 1 (Th1) or Th2 cells. The present study was conducted in order to evaluate the role of B cells in the recruitment of T cells to establish a peripheral T-cell effector pool, during *T. cruzi* infection.

The results presented in this manuscript show that B cells participate in T-cell activation during the acute phase of *T. cruzi* infection. MuMT knockout (KO) mice were more susceptible to *T. cruzi* infection, producing less inflammatory cytokines and fewer central and effector memory T cells than wild type mice. More strikingly, infected muMT KO mice did not sustain their peripheral T cell numbers, having a considerable $CD8^+$ T cell lymphopenia. These findings were associated with a diminished capacity to mobilize inflammatory cells to infected tissues, probably resulting in the observed increase in parasite load.

Materials and methods

Animals

C57Bl/6 and C57Bl/6 muMT KO mice (1–2 months old) were from our animal house or from the Institute of Biomedical Sciences (Department of Immunology), University of São Paulo (USP), São Paulo, Brazil. The C57Bl/6 muMT KO original founding colony was kindly provided by Dr Carlos A. Martinez (Centro de Biologia Molecular, Universidad Autonoma de Madrid, Spain). The animals were kept in microisolators and were manipulated according to institutional ethical guidelines. All the protocols used in this study were approved by the Committee for Ethics in Animal Experimentation of the University of São Paulo and of the Oswaldo Cruz Foundation.

Parasites and infection

Groups of 5–10 mice were infected intraperitoneally with 5×10^2 or 5×10^3 blood-form trypomastigotes of the Tulahuem strain of *T. cruzi* in 0·2 ml of 0·15 M phosphate-buffered saline (PBS). Control mice received the same volume of PBS. The numbers of parasites were evaluated in 5 μl of blood, as previously described.[17]

In vitro *cell culture*

Splenocytes were cultured in triplicates at a density of 10^7 cells/well in 24-well plates (Nunc, Roskilde, Denmark) in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT), 50 mM 2-mercaptoethanol and 1 mM HEPES (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 37° and 5% of CO_2 for 48 hr in complete medium alone. Supernatants were kept at −70° until further experiments.

Cytokine enzyme-linked immunosorbent assay (ELISA)

The levels IL-4, IL-10, IL-12, IL-18 and IFN-γ were quantified, by two-site sandwich ELISA assays. Monoclonal and polyclonal antibodies were obtained from R & D systems (Minneapolis, MN). The ELISA assays were performed according to manufacturing instructions. The lower limit detection of IL4, IL-10 and IFN-γ in these tests was 0·01 ng/ml, 0·02 ng/ml, 0·01 ng/ml, 0·005 ng/ml and 0·1 ng/ml, respectively.

Flow cytometric analysis

The animals were analysed from day 0 to day 30 after infection. Spleen cells were isolated as described[17] and placed in ice-cold PBS supplemented with 5% FBS and 0·1% sodium azide. Staining was done as previously described.[11] The fluorochrome-conjugated monoclonal antibodies used (purchased from BD Biosciences-Pharmingen, San Diego, CA) were: fluoroscein isothiocyanate-conjugated anti-mouse CD4 and anti-mouse CD8, phycoerythrin-conjugated anti-CD44 and anti-CD45RB and biotin anti-CD62L. Biotin-conjugated antibodies were revealed by streptavidin-cychrome (Cy). After staining, the cells were fixed with 1% paraformaldehyde in PBS and analysed using a FACScan (BD Biosciences, San Jose, CA). Twenty thousand events were recorded per sample in an appropriately gated region (lymphocyte gate). Results were analysed using Flowjo software (FlowJo LLC, Ashland, OR).

Skeletal muscle mononuclear cells separation

Skeletal muscle from muMT KO or C57Bl/6 infected mice was sliced in small pieces (less than 2 mm diameter) and incubated in collagenase (Sigma-Aldrich) at a concentration of 1 mg/ml, diluted in RPMI for 45 min at 37°. Cell suspension and remaining tissue was further passed trough a metal mesh (70 μm pore). Recovered cell suspension was washed three times in incomplete RPMI and the pellet diluted in 40% Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A discontinuous Percoll gradient 40/80% was used to separate mononuclear cells as described before.[18] Cells at the 40/80 interface were recovered, washed three times in incomplete RPMI and used in further experiments.

Histopathological and quantitative morphological studies

These studies were performed as described before.[10,11] In brief, tissue samples were fixed in 4% neutral buffered formalin and processed for conventional paraffin embbeding on day 20 after infection. The sections (8 mm) were deparaffinized and stained with haematoxylin and eosin. A single blind evaluation of two sets of

serial sections from each tissue sample was done using histometry with the aid of an 'Integrationsplatte I' eyepice (Carl Zeiss MicroImaging Inc, Thornwood, NY). Intact parasite nests were evaluated in blinded samples by counting the number of parasites nests (mm^2) in 10 non-consecutive sections. The percentage of inflamed area per section was calculated in relation to the total area of the section. Thus, the results were expressed as percentage of inflamed area per section ± SEM (10 non-consecutive sections from each tissue sample) or nests per five mm^2 ± SD. The slide codes were revealed only after analysing the sections.

Statistical analysis

Indicated tests were used to evaluate differences among groups. The *P*-values $\leq 0{\cdot}05$ were considered significant.

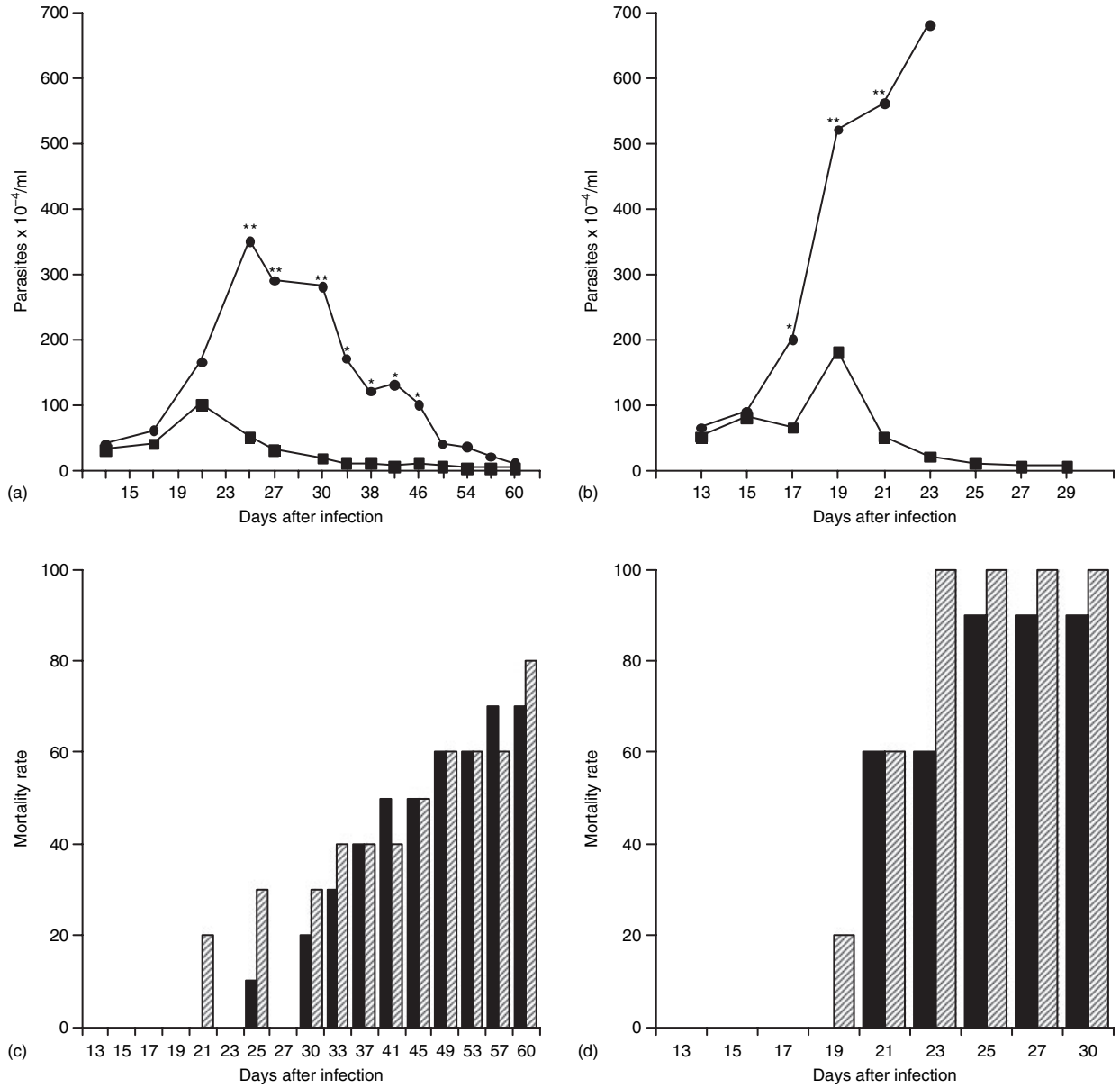

Figure 1. B cells help to control parasitaemia. In (a) and (b) the levels of parasitaemia were determined on the indicated days after an initial infection with 5×10^2 or 5×10^3 trypomastigote forms, respectively (C57Bl/6 mice, closed squares; C57Bl/6 muMT KO mice, closed circles). Each point represents the mean of the parasitaemia values from mice of the different groups. (c, d) Shows the rate of mortality in the different experimental groups: C57Bl/6 mice (filled bars) and C57Bl/6 muMT KO mice (hatched bars), $n = 10$ animals/group in the beginning of the experiment. The results shown in (a) and (b) were compared in each point using the Mann–Whitney test ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$). The mortality rates in (c, d) were compared, using Mann–Whitney *U*-test.

Results

B cells help to control parasitaemia

C57Bl/6 muMT KO mice had higher parasitaemia levels than control, B-cell competent C57Bl/6 mice, when these mice were infected with 5×10^2 parasites (Fig. 1a). In addition, there was a delay in the ability to control parasitaemia. The mortality ratio was comparable between the C57Bl/6 muMT KO mice and the control group (Fig. 1c) and about 25% of the KO mice underwent a chronic infection phase (not shown). MuMT KO mice infected with 5×10^3 parasites were not able to control parasite numbers as did the C57Bl/6 control mice (Fig. 1b). However, the mortality rate was similar in both groups of infected mice (MuMT KO and C57Bl/6). For instance, in the beginning of the fourth week of infection 100% of the MuMT KO and 90% of the C57Bl/6 mice were dead (Fig. 1d).

Spleen cells from *T. cruzi*-infected C57Bl/6 muMT KO mice produce less inflammatory cytokines (IFN-γ, IL-12), and similar or increased amounts of IL-18, in comparison with control mice

IFN-γ, IL-12 and IL-18 were spontaneously produced by C57Bl/6 cultured spleen cells after 20 days of infection

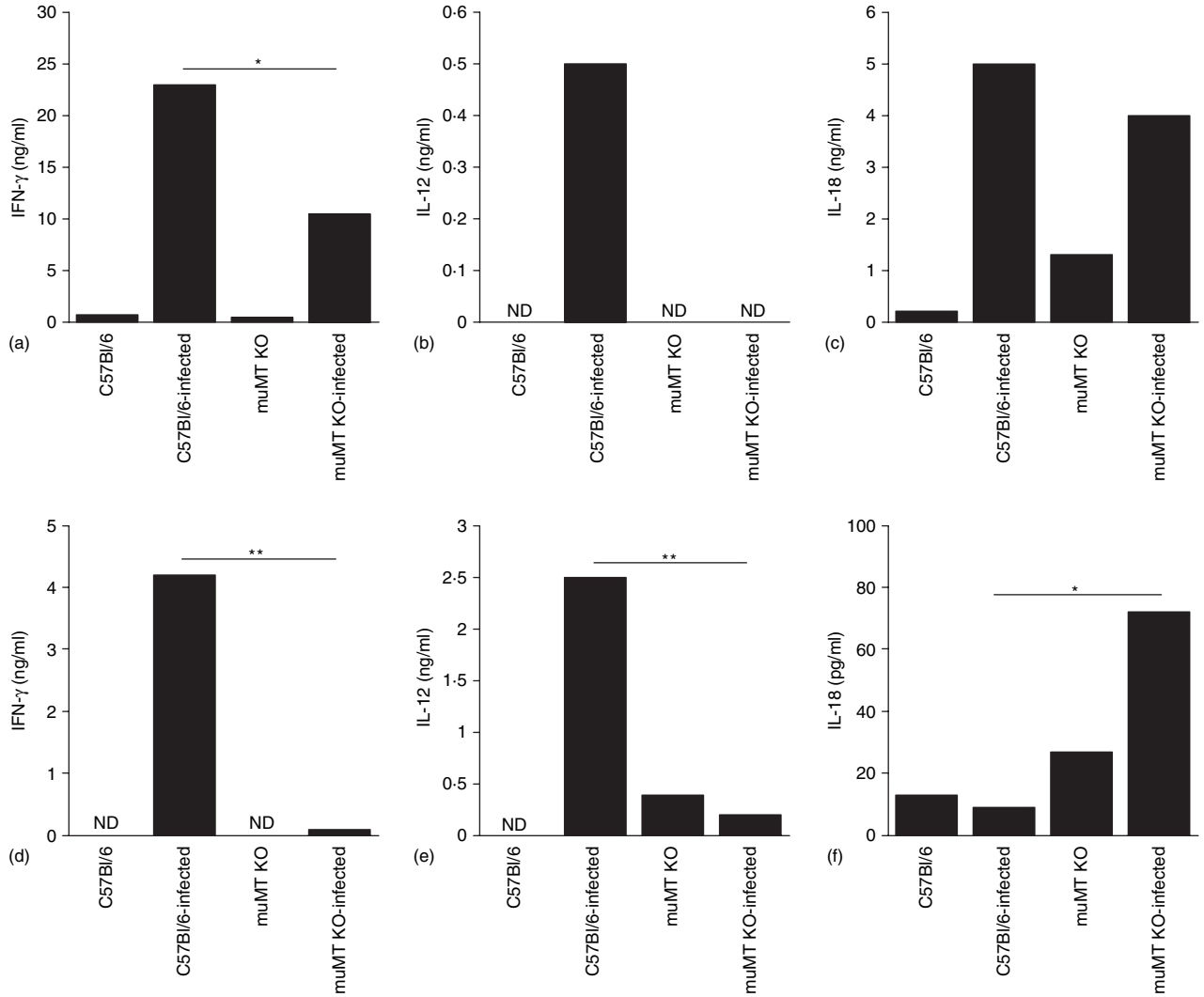

Figure 2. Spleen cells from *T. cruzi*-infected C57Bl/6 muMT KO mice produce less inflammatory cytokines (IFN-γ, IL-12), and similar or increased amounts of IL-18, in comparison with control mice. Spleen cells from control or infected C57Bl/6 or C57Bl/6 muMT KO mice were cultured *in vitro* in the presence of medium alone. Three to four spleens from each group of mice were pooled and used to prepare the cell suspensions. (a, b, c) The levels of IFN-γ (ng/ml), IL-12 (ng/ml) or IL-18 (ng/ml) production by spleen cells from the different experimental groups after 48 hr of culture. (d, e, f) The amounts of IFN-γ (ng/ml), IL-12 (ng/ml) or IL-18 (pg/ml) in the sera from the different experimental groups (day 30 after infection). Cytokines were measured by ELISA, as described in the Methods section. Columns represent the mean of the amount of cytokines of triplicate cultures or three to four different serum samples. The data are from one of three experiments with similar results. Mann–Whitney test was used. (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$; ND, not detected)

(Fig. 2). Further stimulation with soluble *T. cruzi* antigens or mitogen (concanavalin A, Con A) did not increase, and in some cases (Con A stimulation) inhibited cytokine production (not shown). Spleen cells from infected muMT KO C57Bl/6 mice produced less IFN-γ than spleen cells from B-cell sufficient C57Bl/6 mice (Fig. 2a). The same pattern was observed for IL-12, which could not be detected in supernatants of muMT KO spleen cells (Fig. 2b). IL-18 was produced in similar amounts by splenic cells from control or B-cell deficient mice (Fig. 2c). The concentration of these cytokines was also assessed in the sera. IFN-γ and IL-12 were found in higher amounts in the sera from infected C57Bl/6 mice than of muMT KO mice (Fig. 2d, e). This pattern was observed in three different experiments on days ranging from the 20th and 30th after infection (data not shown). The amount of IL-18 in sera was higher in the sera of muMT KO mice than in those of the control group after 30 days of infection (Fig. 2f).

Spleen cells from B-cell deficient mice produce higher amounts of IL-4 and similar levels of IL-10 in comparison with control mice during the acute phase of *T. cruzi* infection

The production of IL-4 was higher in supernatants obtained by cultured spleen cells from infected muMT KO mice than from infected C57Bl/6 mice (Fig. 3a). Spleen cells from infected B-cell deficient mice produced comparable amounts of IL-10 when compared to infected control mice (Fig. 3b). IL-4 and IL-10 were not detected in the sera from infected mice (data not shown).

T. cruzi-infected B-cell deficient mice have reduced numbers of $CD8^+$ splenic T cells and impaired generation of central or effector splenic memory T cells

Figure 4 shows in bar chart the numbers of splenic $CD4^+$ and $CD8^+$ T cells from the different experimental groups. There were marked increases in the numbers of $CD4^+$ and $CD8^+$ T cells in infected wild type C57Bl/6 mice. There was no change in the number of $CD4^+$ T cells, and a reduction by half in the number of splenic $CD8^+$ T cells in infected muMT KO mice in comparison with non-infected animals. Gated $CD4^+$ or $CD8^{\pm}$ T cells were analysed for the expression of markers that characterize central ($CD44^{high}$ $CD62L^{high}$) or effector ($CD44^{high}$ $CD62L^{low}$) memory T cells. Contour plots in the lower panels (Fig. 4) show that the percentages of central and effector memory $CD4^+$ and $CD8^+$ T cells were increased in non-infected B-cell deficient mice than in non-infected control mice. By day 30 after infection, the percentages of central memory $CD4^+$ T cells increased sevenfold in control C57Bl/6 mice and only three fold in B-cell deficient mice. The same pattern could be observed for effector memory $CD4^+$ T cells (fivefold increase in control C57Bl/6 mice versus less than twofold increase in muMT KO mice; Fig. 4, upper contour plot panel). Lower panel (contour plots) shows the percentages of central and effector memory $CD8^+$ T cells. These results show that B-cell deficient mice

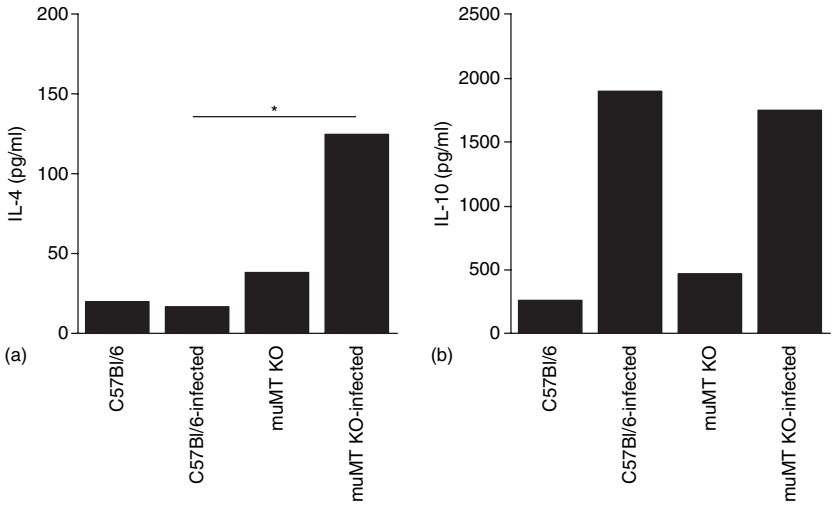

Figure 3. Spleen cells from B-cell deficient mice produce higher amounts of IL-4 and similar levels of IL-10 in comparison with control mice during the acute phase of *T. cruzi* infection. Spleen cells from control or infected C57Bl/6 or C57Bl/6 muMT KO mice were cultured *in vitro* in the presence of medium alone. Three to four spleens from each group of mice were pooled and used to prepare the cell suspensions. (a and b) The levels of IL-4 (pg/ml) and IL-10 (pg/ml) production by spleen cells from the different experimental groups after 48 hr of culture (day 30th after infection). Cytokines were measured by ELISA, as described in Methods. Columns represent the mean of the amount of cytokines of triplicate cultures. The data are from one of three similar experiments. Mann–Whitney test, $^*P < 0{\cdot}05$.

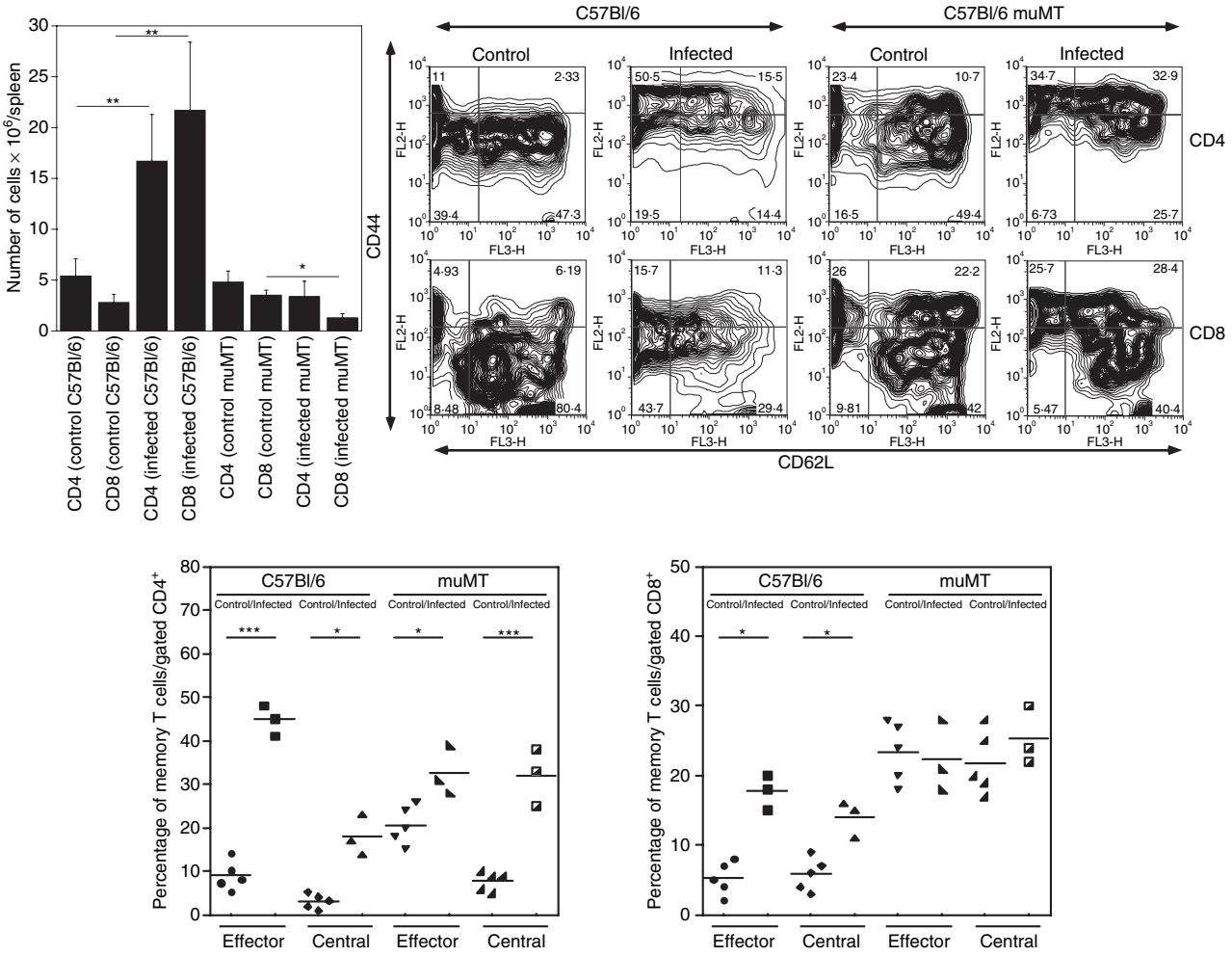

Figure 4. *T. cruzi*-infected B-cell deficient mice have reduced numbers of $CD8^+$ splenic T cells and impaired generation of central or effector splenic memory T cells. Spleen cells from control or infected C57Bl/6 and C57Bl/6 muMT KO mice, were counted, stained and evaluated by FACS as described in Methods. Frequencies of CD44 high × CD62L high T cells (central memory T cells) and CD44 high × CD62L low T cells (effector memory T cells) are shown in the upper and lower density plots inside electronically gated $CD4^+$ or $CD8^+$ T cells, respectively. Numbers inside plots represent the relative frequency of each T-cell subpopulations. Density plots are representative of one experiment where spleen cells were pooled for each experimental group (three to four spleens/group). Other two independent experiments were done where stainings were performed in individual mouse and the data was used to calculate the total number of $CD4^+$ and $CD8^+$ T cells showed in the bar chart on the right. The columns represent the mean number of each splenic T-cell subpopulation ($n = 4$–7 mice/group). Lower dot plots represent one experiment where the frequencies of splenic central and memory $CD4^+$ (left) or $CD8^+$ (right) T cells were evaluated in individual mouse. Vertical bars represent the standard deviation of the mean. Student's *t*-test was used to compare the indicated groups. Mice were infected with 5×10^2 parasites and were analysed 30 days after infection ($^*P \leq 0{\cdot}05$, $^{**}P \leq 0{\cdot}01$, $^{***}P \leq 0{\cdot}005$).

did not up-regulate, significantly, the percentage of central or effector memory $CD8^+$ T cells in relation to their previous, uninfected estate. Control C57Bl/6 mice showed an increase in the percentages of central (twofold) and effector (threefold) memory $CD8^+$ T cells along the first 30 days of infection. Lower dot plots in Fig. 4 represent one experiment where the different groups of mice were analysed individually for the splenic frequencies of central and effector memory $CD4^+$ (left) or $CD8^+$ (right) T cells. The individual analysis confirmed the observations described above for pooled spleen cells (contour plots).

B-cell deficient mice have smaller percentages of activated/memory $CD8^+$ T cells in inflammatory infiltrates

The analyses of T-cell markers in mononuclear cells derived from infected skeletal muscle tissues revealed that $CD8^+$ cells predominated over $CD4^+$ T cells (2 : 1 ratio) in wild type C57Bl/6 mice after 30 days of infection (Fig. 5; contour plots). On the other hand, $CD4^+$ T cells predominated over $CD8^+$ T cells in B-cell deficient mice. The percentage of $CD8^+$ T cells having low

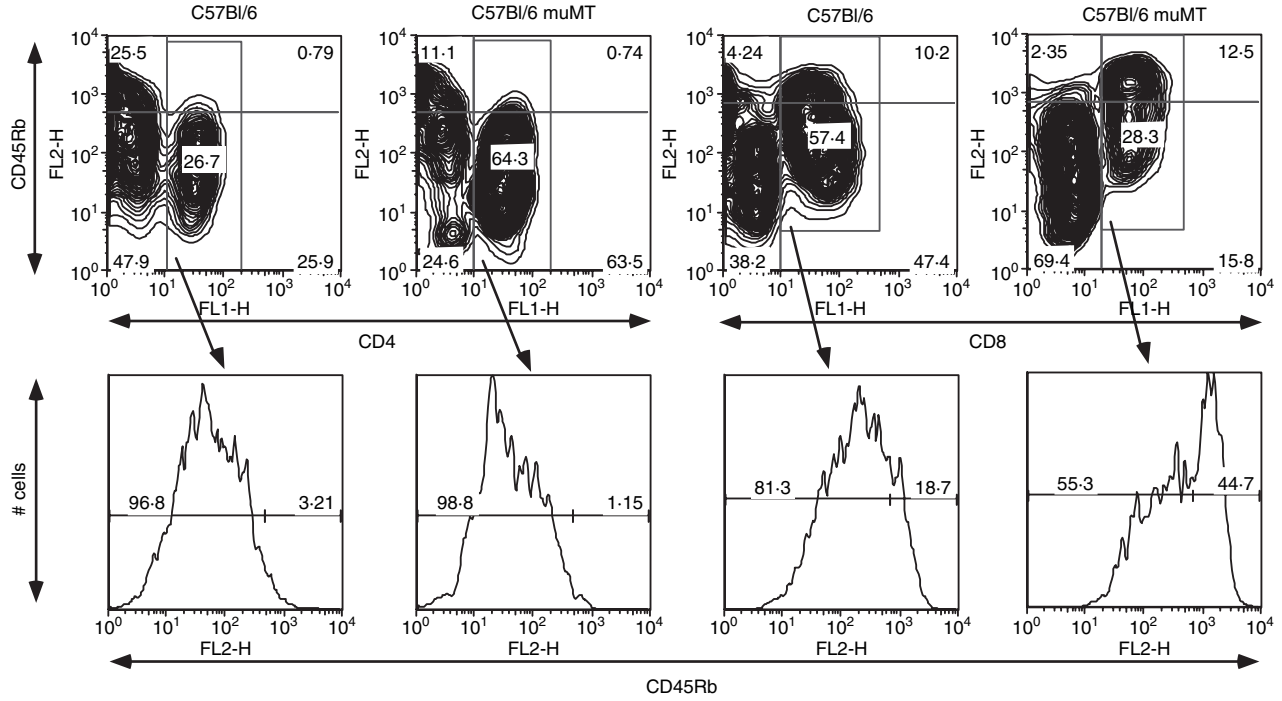

Figure 5. B-cell deficient mice have smaller percentages of activated/memory $CD8^+$ T cells in inflammatory infiltrates. Skeletal muscle mononuclear cells (SMMC) from infected C57Bl/6 and C57Bl/6 muMT KO mice were separated, stained and evaluated by flow cytometry as described in the Methods section. Upper density plots show the percentage of SMMC $CD4^+$ and $CD8^+$ T cells in the respective experimental groups, as indicated. Frequencies of CD45Rb low and high T cells are shown in the lower histograms inside electronically gated $CD4^+$ or $CD8^+$ T cells, respectively. Numbers inside plots and histograms represent the relative frequency of each T-cell subpopulations. Histograms are representative of one out of three experiments with similar results. Pooled SMMC were obtained from three to four mice/group.

levels of CD45Rb molecules was lower in mononuclear cells derived from skeletal muscle from infected muMT KO mice than in infected control C57Bl/6 mice (Fig. 5; histograms). The percentage of $CD4^+$ CD45Rb low in these cells was comparable in both groups (Fig. 5; histograms). Tissue $CD4^+$ T cells from both groups showed a remarkable down-modulation of the CD4 molecule when compared to splenic $CD4^+$ T cells (not shown).

B-cell deficient mice have decreased inflammatory infiltrate and augmented tissue parasitism during the acute phase of *T. cruzi* infection

The percentage of inflamed area in skeletal muscle tissues was larger in wild type C57Bl/6 mice than in B cell-deficient mice after 20 days of infection (Fig. 6a). Figure 6(c, d) illustrates in low magnification muscle tissues from C57Bl/6 and muMT KO infected mice, respectively. In addition, the number of intact parasite nests was increased in muMT KO mice (Fig. 6b). Nests were also larger and contained more amastigote forms in muMT KO mice than in B-cell sufficient mice (not shown).

Discussion

This study was conducted in order to investigate the role of B cells during the acute phase of *T. cruzi* infection. We showed that muMT KO C57Bl/6 mice infected with the Tulahuem strain of *T. cruzi* had increased levels of parasitaemia, but were still able to control the acute infection when infected with low numbers of the parasite (5×10^2). In addition, these mice showed equivalent mortality ratios when compared to controls during the acute phase of the infection, thus indicating that the lack of B cells may, in part, be compensated by other infection-controlling mechanism during this phase of the infection. However, infection with higher numbers of parasites (5×10^3) resulted in uncontrolled parasitaemia in B-cell deficient mice. Yet, in this case, the mortality ratio was not different from control B-cell sufficient mice. Previous studies have shown that antibodies against *T. cruzi* antigens could play an important role in the control of the infection.[19,20] It has being demonstrated that anti-*T. cruzi* antibodies of some immunoglobulin G (IgG) isotypes are very efficient to fix complement and clear the parasite,[21] thus suggesting that parasite-specific IgG is an important component of the immune response that

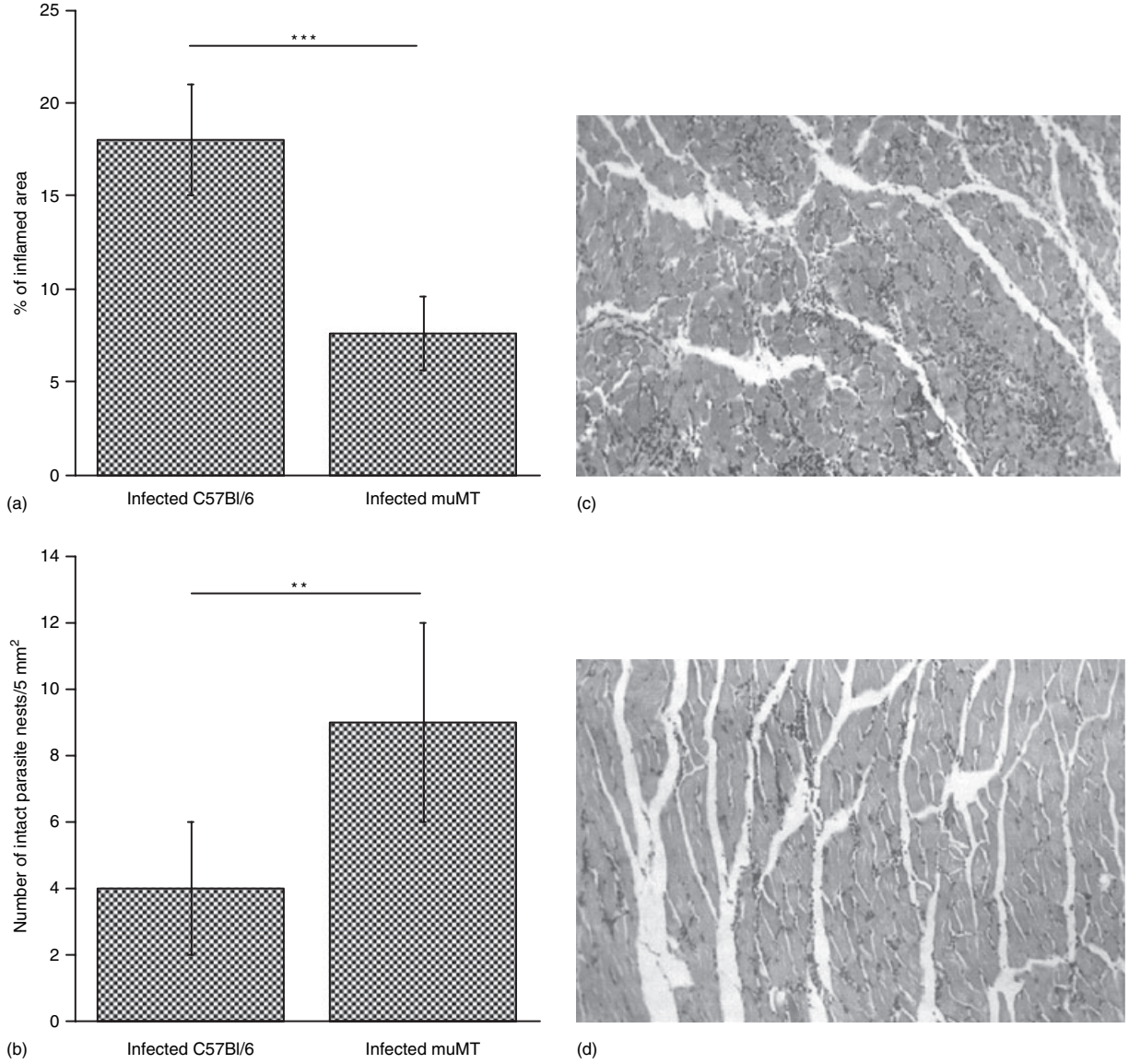

Figure 6. B-cell deficient mice have decreased inflammatory infiltrate and augmented tissue parasitism during the acute phase of *T. cruzi* infection. Wild-type C57Bl/6 or muMT KO C57Bl/6 mice were infected i.p. with 5×10^2 trypomastigote forms of Tulahuem strain of *T. cruzi*. Quantitative analysis of inflammatory infiltrates (a) and the numbers of intact *T. cruzi* parasite nests (b) in skeletal muscle tissues were evaluated in 10 non-consecutive histological sections obtained from mice at day 20 after infection, as described in Materials and Methods. Results are shown as mean ± SD. Student's *t*-test was used to compare indicated groups. Two and three asterisks indicate that differences are statistically significant ($P \leq 0{\cdot}01$ and $P \leq 0{\cdot}005$, respectively) between the indicated groups ($n = 8$–10 mice/group). Representative skeletal muscle sections from wild-type C57Bl/6 and muMT KO C57Bl/6 infected mice (c and d, respectively) (magnification × 100) are shown. Similar results were obtained in four independent experiments.

controls the infection. Although muMT mice lack B cells, we were able to detect very low levels of non-IgM/non-IgG specific antibodies to *T. cruzi* (data not shown). Therefore, we may not formally exclude the role of specific antibodies of other classes, rather than IgM and IgG, in the early control of parasitism in muMT mice infected with low numbers of *T. cruzi*. It has being shown that muMT KO mice produce IgA[22] and this may well be the case in this experimental model. In spite of that, muMT mice showed increased parasitaemia levels in all studied situations, clearly showing an important role for B cells and possibly antibodies in the early control of parasite multiplication.

The production of pro-inflammatory cytokines such as IFN-γ and IL-12 is of crucial importance to control intracellular parasites.[23,24] The T-cell functional pattern (Th1 or Th2) developed in the course of an infection usually relates to host-resistance or susceptibility.[7] Th1 cells

induce inflammatory responses, whereas the other functional pattern (Th2) has an anti-inflammatory regulatory activity with a major impact in up-regulation of antibody production.[25] For $CD8^+$ T cells this situation is less clear, but some studies suggest that this T-cell population could be divided in at least two different functional patterns depending on the production of IFN-γ or IL-10.[26] It has being shown that IL-12 produced by dendritic cells or macrophages and/or IL-18 may favour the development of T cells that secrete IFN-γ (Th1).[27] On the other hand, IL-4 production favours the appearance of Th2 immune responses which may lead to the production of large quantities of IL-10, a cytokine that down-regulates cellular immune responses by diminishing the activation of dendritic cells and the ability of macrophages to kill microorganisms.[28] Additionally, it has being shown that IL-4 promotes susceptibility in *T. cruzi* infection.[29] IL-4 may be also important in inducing the secretion of transforming growth factor-β,[30] a cytokine that also down-regulates antigen-presenting cells[31] being involved in the reduction of resistance to *T. cruzi*.[32] In fact, it was observed in the work described herein that spleen cells from *T. cruzi*-infected muMT KO mice secrete more IL-4, similar amounts of IL-10 and less IFN-γ and IL-12 during the acute phase of the infection, in comparison with spleen cell from infected control mice. In addition, a strong reduction of Th1 cytokine levels was also found in muMT sera during the acute phase of *T. cruzi* infection. IL-18 has also being related to the Th1 pattern.[33] However, infected muMT KO mice, which had an impaired Th1 response, produced equal or increased amounts of IL-18 during the acute infection, when compared to infected wild-type C57Bl/6 mice, arguing against a major role of this cytokine during the acute *T. cruzi* infection, as suggested before[34] and indicating therefore that IL-12 is the major cytokine favouring the Th1 pattern in this model. These results may point out that a different class of immune response, tending to a Th2 pattern, was preferentially mounted by infected muMT KO mice, thus justifying, at least in part, the increased susceptibility to *T. cruzi* showed by these mice and implicating B cells in the promotion of a Th1-type T-cell polarization during the infection.

The control of *T. cruzi* infection by the host immune system also requires the generation of activated/memory or effector T cells.[10,35–37] Effector T cells are able to migrate from secondary lymphoid tissues to many other tissues in the infected organism and secrete a number of different cytokines, including IFN-γ or IL-4 and IL-10.[38] However, very little is known about the conditions that could favour the generation and maintenance of fully mature effector T cells.[39] Effector T cells usually have low levels of CD45Rb and CD62L molecules and high levels of CD44[40,41] including a characteristic pattern of expression of chemokine receptors.[42] A second population of memory/activated T cells was described as also bearing high levels of CD44, but instead are CD62L high.[43] This latter population has been called central memory T cells.[44] Macrophages, dendritic cells and B cells can function as antigen-presenting cells to activate $CD4^+$ T cells.[12,13,45] It has been shown that B-cell activation by the cross-linking of their antigen receptors or through their CD40 molecules may up-regulate costimulatory molecules such as B7, which in their turn promote T-cell expansion and differentiation.[46–48] The expansion of antigen-specific B cells during an immune response allow a large number of these cells to capture and concentrate small amounts of antigens providing efficacious antigen presentation for T cells.[48,49] In fact, the relationship between $CD4^+$ T cells and B cells is relatively well known and B cells are described to be important in the generation of memory/activated effector $CD4^+$ T cells in many different models.[13,50–53] However, the activation and differentiation of $CD8^+$ T cells by B cells is less well documented and B lymphocytes are apparently important in the contraction phase of $CD8^+$ T cell responses, being implicated in the long-term survival of effector $CD8^+$ T cells.[54–56] The results presented here argues in favour of these previous data, showing that during the acute phase of *T. cruzi* infection the generation of central and effector memory $CD4^+$ and $CD8^+$ T cells was greatly diminished in muMT KO mice when compared with B-cell sufficient C57Bl/6 mice. In addition, we demonstrated that absolute numbers of splenic $CD8^+$ T cells were much lower in infected muMT KO mice, thus indicating that B cells were required for the expansion and maintenance of this T cell subset during the infection. Central and effector CD8 T cell frequencies did not increase during the acute infection in muMT mice, differently from what was observed in control C57Bl/6 mice where the percentage of central memory $CD8^+$ T cells increased two fold and effector memory $CD8^+$ T lymphocytes augmented threefold in relation to uninfected controls. Moreover, a reduction in the total numbers (twofold) of total splenic $CD8^+$ T cells was found after 30 days of infection in the muMT KO mice. Taken together, these results strongly argue that B cells are of crucial importance to the generation and maintenance of memory T cells particularly of the $CD8^+$ phenotype, during *T. cruzi* infection. In accordance with this view, it has been previously described that immunization of muMT KO mice with *T cruzi* antigens before infection did not result in protection as it did for in B-cell sufficient mice.[36,57]

The mechanism by which B cells exert these non-redundant and marked effects could involve direct interactions between T and B cells, with requirement for specific costimulatory molecules. For instance, the finding that the blocking of CD27/CD70 interaction results in a deficient generation of effector T cell memory indicates that the presence of these molecules on B cells are

required to regulate T-cell activation.[58,59] Therefore, this type of interaction could be crucial for the generation and maintenance of effetor/memory T cells during the acute phase of *T. cruzi* infection. In addition, it has being recognized that B cells produce a number of cytokines that might regulate function and/or survival of T cells.[14–16,55] Alternatively, it is possible that B cells influence the activity of other lymphoid or myeloid populations,[60,61] which could, in their turn, provide activation and/or survival signals for T cells. For instance, it was described that NK cells and Vγ1-bearing γδ T cells are involved in the generation of memory in the conventional T-cell compartment.[10,11] Recently, it was demonstrated that B cells might influence the composition of NK-cell subpopulations and therefore, their functional activities[62] thus supporting the hypothesis of an indirect effect of B cells over T-cell activation and/or survival. Evidences that B cells interact with γδ T cells are also available[63] thus suggesting the possibility that B cells could mediate their effects over conventional T cells trough γδ T cells. Finally, it remains possible that B cells, by the secretion of their major product (i.e. immunoglobulins), could maintain T cells either by trophic activities mediated by interactions between the Fc and Fc-receptors[60,61] and/or by facilitating thymic positive T-cell selection and therefore T-cell replenishing, as previously suggested.[60,64,65] Independently of the precise mechanism by which B cells mediate their activity, the data presented here demonstrate a pivotal role of B cells in the generation of memory T cells and their maintenance during *T. cruzi* infection.

The acute phase of *T. cruzi* infection is marked by an intense tissue parasitism surrounded by an expressive inflammatory infiltrate composed mainly by $CD8^+$ T cells and fewer $CD4^+$ T cells in C57Bl/6 mice (Figs 5 and 6[66]). However, in infected muMT KO mice the ratio $CD8^+$/$CD4^+$ T cells in skeletal muscle mononuclear cells was inverted and $CD4^+$ T cells predominate, indicating that, during the *T. cruzi* infection, the effect of B cells on these two T-cell subpopulations is not quantitatively or qualitatively identical. In fact, the activation profile of $CD8^+$ T cells, and not of $CD4^+$ T cells, disclosed differences in relation to the expression of the activation/memory marker CD45Rb. In parallel, there was a decrease in the numbers of cells infiltrating skeletal muscle tissues and an increase in the numbers and size of the parasite nests found in this tissue, thus denoting a less effective anti-parasite local immune response. These findings may reflect the lower effector memory $CD8^+$ T-cell activation and/or, as discussed above, a decrease in the production of IFN-γ.

In conclusion, this study shows that B cells have important functions during the acute phase of an intracellular parasite infection, by regulating the pattern of the T-cell mediated immune response and/or the functional phenotypes of effector/memory $CD8^+$ T cells.

Acknowledgments

We are in debt to Dr Carlos A. Martinez for providing us the C57Bl/6 muMT founding colony. We also thank Drs Lain Pontes de Carvalho for critical reading of the manuscript and Drs Thiago L. Carvalho and Vinícius N. Motta for suggestions on the manuscript. Fellowships: J.N was supported by a FAPESB fellowship (proc. 822/2005), and E.P. by a FAPESP fellowship (99/10621-3). J.M and F.C. are recipients of CNPq research scholarships. This work was supported by grants from FAPESP (95/9379-2 and 97/06225-0) and CNPq (305835/2003–3).

References

1 Andrade LO, Andrews NW. The *Trypanosoma cruzi*-host-cell interplay: location, invasion, retention. *Nat Rev Microbiol* 2005; **3**:819–23.

2 Andrade SG. *Trypanosoma cruzi*: clonal structure of parasite strains and the importance of principal clones. *Mem Inst Oswaldo Cruz* 1999; **94** (Suppl. 1):185–7.

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

4 Andrade SG, Andrade V, Brodskyn C, Magalhaes JB, Netto MB. Immunological response of Swiss mice to infection with three different strains of *Trypanosoma cruzi*. *Ann Trop Med Parasitol* 1985; **79**:397–407.

5 Andrade V, Barral-Netto M, Andrade SG. Patterns of resistance of inbred mice to *Trypanosoma cruzi* are determined by parasite strain. *Braz J Med Biol Res* 1985; **18**:499–506.

6 Kierszenbaum F, Pienkowski MM. Thymus-dependent control of host defense mechanisms against *Trypanosoma cruzi* infection. *Infect Immun* 1979; **24**:117–20.

7 Tarleton RL. The role of T-cell subpopulations in experimental Chagas' disease. *Res Immunol* 1991; **142**:130–3.

8 Ben Younes-Chennoufi A, Said G, Eisen H, Durand A, Hontebeyrie-Joskowicz M. Cellular immunity to Trypanosoma cruzi is mediated by helper T cells ($CD4^+$). *Trans R Soc Trop Med Hyg* 1988; **82**:84–9.

9 Tarleton RL. Depletion of $CD8^+$ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. *J Immunol* 1990; **144**:717–24.

10 Cardillo F, Cunha FQ, Tamashiro WM, Russo M, Garcia SB, Mengel J. $NK1.1^+$ cells and T-cell activation in euthymic and thymectomized C57Bl/6 mice during acute *Trypanosoma cruzi* infection. *Scand J Immunol* 2002; **55**:96–104.

11 Nomizo A, Cardillo F, Postol E, de Carvalho LP, Mengel J. V gamma 1 gammadelta T cells regulate type-1/type-2 immune responses and participate in the resistance to infection and development of heart inflammation in *Trypanosoma cruzi*-infected BALB/c mice. *Microbes Infect* 2006; **8**:880–8.

12 Britz JS, Askenase PW, Ptak W, Steinman RM, Gershon RK. Specialized antigen-presenting cells. Splenic dendritic cells and peritoneal-exudate cells induced by mycobacteria activate effector T cells that are resistant to suppression. *J Exp Med* 1982; **155**:1344–56.

13 Tzehoval E, De Baetselier P, Ron Y, Tartakovsky B, Feldman M, Segal S. Splenic B cells function as immunogenic antigen-

presenting cells for the induction of effector T cells. *Eur J Immunol* 1983; **13**:89–94.

14 Mengel J, Dare L, Dare GM, Delgado M, Nomizo A, Silva JS, Campos-Neto A. An activated murine B cell lymphoma line (A-20) produces a factor-like activity which is functionally related to human natural killer cell stimulatory factor. *Eur J Immunol* 1992; **22**:3173–8.

15 O'Garra A, Stapleton G, Dhar V *et al.* Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int Immunol* 1990; **2**:821–32.

16 Pang Y, Norihisa Y, Benjamin D, Kantor RR, Young HA. Interferon-gamma gene expression in human B-cell lines: induction by interleukin-2, protein kinase C activators, and possible effect of hypomethylation on gene regulation. *Blood* 1992; **80**:724–32.

17 Cardillo F, Falcao RP, Rossi MA, Mengel J. An age-related gamma delta T cell suppressor activity correlates with the outcome of autoimmunity in experimental *Trypanosoma cruzi* infection. *Eur J Immunol* 1993; **23**:2597–605.

18 Ribeiro-Dos-Santos R, Mengel JO, Postol E, Soares RA, Ferreira-Fernandez E, Soares MB, Pontes-De-Carvalho LC. A heart-specific $CD4^+$ T-cell line obtained from a chronic chagasic mouse induces carditis in heart-immunized mice and rejection of normal heart transplants in the absence of *Trypanosoma cruzi*. *Parasite Immunol* 2001; **23**:93–101.

19 Kierszenbaum F, Howard JG. Mechanisms of resistance against experimental *Trypanosoma cruzi* infection. the importance of antibodies and antibody-forming capacity in the Biozzi high and low responder mice. *J Immunol* 1976; **116**:1208–11.

20 Corsini AC, Braz R, Ciampi DB, Zucato MR. Resistance to *Trypanosoma cruzi* infection in relation to the timing of IgG humoral response. *Z Parasitenkd* 1982; **68**:15–25.

21 Lima-Martins MV, Sanchez GA, Krettli AU, Brener Z. Antibody-dependent cell cytotoxicity against *Trypanosoma cruzi* is only mediated by protective antibodies. *Parasite Immunol* 1985; **7**:367–76.

22 Macpherson AJ, Lamarre A, McCoy K, Harriman GR, Odermatt B, Dougan G, Hengartner H, Zinkernagel RM. IgA production without mu or delta chain expression in developing B cells. *Nat Immunol* 2001; **2**:625–31.

23 Plata F, Wietzerbin J, Pons FG, Falcoff E, Eisen H. Synergistic protection by specific antibodies and interferon against infection by *Trypanosoma cruzi* in vitro. *Eur J Immunol* 1984; **14**:930–5.

24 Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice and is produced by murine macrophages in response to live trypomastigotes. *Infect Immun* 1996; **64**:1961–7.

25 Mosmann TR, Schumacher JH, Street NF *et al.* Diversity of cytokine synthesis and function of mouse $CD4^+$ T cells. *Immunol Rev* 1991; **123**:209–29.

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

27 Yoshimoto T, Takeda K, Tanaka T, Ohkusu K, Kashiwamura S, Okamura H, Akira S, Nakanishi K. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol* 1998; **161**:3400–7.

28 Vouldoukis I, Becherel PA, Riveros-Moreno V, Arock M, da Silva O, Debre P, Mazier D, Mossalayi MD. Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. *Eur J Immunol* 1997; **27**:860–5.

29 Petray PB, Rottenberg ME, Bertot G, Corral RS, Diaz A, Orn A, Grinstein S. Effect of anti-gamma-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of *Trypanosoma cruzi*. *Immunol Lett* 1993; **35** (1):77–80.

30 Kodera T, McGaha TL, Phelps R, Paul WE, Bona CA. Disrupting the IL-4 gene rescues mice homozygous for the tight-skin mutation from embryonic death and diminishes TGF-beta production by fibroblasts. *Proc Natl Acad Sci USA* 2002; **99**:3800–5.

31 Lipscomb MF, Pollard AM, Yates JL. A role for TGF-beta in the suppression by murine bronchoalveolar cells of lung dendritic cell initiated immune responses. *Reg Immunol* 1993; **5**:151–7.

32 Silva JS, Twardzik DR, Reed SG. Regulation of *Trypanosoma cruzi* infections in vitro and in vivo by transforming growth factor beta (TGF-beta). *J Exp Med* 1991; **174**:539–45.

33 Meyer Zum Buschenfelde C, Cramer S, Trumpfheller C, Fleischer B, Frosch S. *Trypanosoma cruzi* induces strong IL-12 and IL-18 gene expression *in vivo*: correlation with interferon-gamma (IFN-gamma) production. *Clin Exp Immunol* 1997; **110**:378–85.

34 Basso B, Cervetta L, Moretti E, Carlier Y, Truyens C. Acute *Trypanosoma cruzi* infection. IL-12, IL-18, TNF, sTNFR and NO in *T. rangeli*-vaccinated mice. *Vaccine* 2004; **22**:1868–72.

35 Hoft DF, Schnapp AR, Eickhoff CS, Roodman ST. Involvement of CD4 (+) Th1 cells in systemic immunity protective against primary and secondary challenges with *Trypanosoma cruzi*. *Infect Immun* 2000; **68**:197–204.

36 Burgess DE, Hanson WL. *Trypanosoma cruzi*. The T-cell dependence of the primary immune response and the effects of depletion of T cells and Ig-bearing cells on immunological memory. *Cell Immunol* 1980; **52**:176–86.

37 Paiva CN, Castelo-Branco MT, Lannes-Vieira J, Gattass CR. *Trypanosoma cruzi* protective response of vaccinated mice is mediated by $CD8^+$ cells, prevents signs of polyclonal T lymphocyte activation, and allows restoration of a resting immune state after challenge. *Exp Parasitol* 1999; **91**:7–19.

38 Zhang L, Tarleton RL. Characterization of cytokine production in murine *Trypanosoma cruzi* infection by in situ immunocytochemistry: lack of association between susceptibility and type 2 cytokine production. *Eur J Immunol* 1996; **26**:102–9.

39 Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets. Function, generation, maintenance. *Annu Rev Immunol* 2004; **22**:745–63.

40 Budd RC, Cerottini JC, Horvath C, Bron C, Pedrazzini T, Howe RC, MacDonald HR. Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J Immunol* 1987; **138**:3120–9.

41 Bradley LM, Duncan DD, Tonkonogy S, Swain SL. Characterization of antigen-specific $CD4^+$ effector T cells *in vivo*. immunization results in a transient population of MEL-14-, CD45RB- helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon gamma. *J Exp Med* 1991; **174** (3):547–59.

42 Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J Immunol* 1999; **162**:186–94.

43 Mackay CR, Marston WL, Dudler L, Spertini O, Tedder TF, Hein WR. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. *Eur J Immunol* 1992; **22**:887–95.

44 Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**:708–12.

45 Breart B, Bousso P. Cellular orchestration of T cell priming in lymph nodes. *Curr Opin Immunol* 2006; **18**:483–90.

46 Rodriguez-Pinto D. B cells as antigen presenting cells. *Cell Immunol* 2005; **238**:67–75.

47 Schultze JL, Michalak S, Seamon MJ *et al.* CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J Clin Invest* 1997; **100**:2757–65.

48 Lanzavecchia A. Antigen-specific interaction between T and B cells. *Nature* 1985; **314**:537–9.

49 Malynn BA, Romeo DT, Wortis HH. Antigen-specific B cells efficiently present low doses of antigen for induction of T cell proliferation. *J Immunol* 1985; **135**:980–8.

50 Malynn BA, Wortis HH. Role of antigen-specific B cells in the induction of SRBC-specific T cell proliferation. *J Immunol* 1984; **132**:2253–8.

51 Scott P, Natovitz P, Sher A. B lymphocytes are required for the generation of T cells that mediate healing of cutaneous leishmaniasis. *J Immunol* 1986; **137**:1017–21.

52 Rodriguez-Pinto D, Moreno J. B cells can prime naive $CD4^+$ T cells *in vivo* in the absence of other professional antigen-presenting cells in a CD154-CD40-dependent manner. *Eur J Immunol* 2005; **35**:1097–105.

53 Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation *in vivo* requires antigen presentation by B cells. *J Immunol* 2006; **176**:3498–506.

54 Asano MS, Ahmed R. CD8 T cell memory in B cell-deficient mice. *J Exp Med* 1996; **183**:2165–74.

55 Kos FJ, Mullbacher A. Enhancement of antigen-specific activation of $CD8^+$ memory cytotoxic T cells by B cell-derived factors. *Immunobiology* 1992; **186**:410–20.

56 Cauley LS, Cookenham T, Hogan RJ, Crowe SR, Woodland DL. Renewal of peripheral $CD8^+$ memory T cells during secondary viral infection of antibody-sufficient mice. *J Immunol* 2003; **170**:5597–606.

57 Kumar S, Tarleton RL. The relative contribution of antibody production and $CD8^+$ T cell function to immune control of *Trypanosoma cruzi*. *Parasite Immunol* 1998; **20**:207–16.

58 Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* 2000; **1**:433–40.

59 Lens SM, Tesselaar K, van Oers MH, van Lier RA. Control of lymphocyte function through CD27–CD70 interactions. *Semin Immunol* 1998; **10**:491–9.

60 Rodewald HR, Awad K, Moingeon P, D'Adamio L, Rabinowitz D, Shinkai Y, Alt FW, Reinherz EL. Fc gamma RII/III and CD2 expression mark distinct subpopulations of immature $CD4^-$ $CD8^-$ murine thymocytes: *in vivo* developmental kinetics and T cell receptor beta chain rearrangement status. *J Exp Med* 1993; **177**:1079–92.

61 Woelbing F, Kostka SL, Moelle K *et al.* Uptake of *Leishmania major* by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. *J Exp Med* 2006; **203**:177–88.

62 Jeannet G, Coudert JD, Held W. T and B lymphocytes exert distinct effects on the homeostasis of NK cells. *Eur J Immunol.* 2006; **36**:2725–34.

63 Spaner D, Cohen BL, Miller RG, Phillips RA. Antigen-presenting cells for naive transgenic gamma delta T cells. Potent activation by activated alpha beta T cells. *J Immunol* 1995; **155**:3866–76.

64 Joao C, Ogle BM, Geyer S. Immunoglobulin promotes the diversity and the function of T cells. *Eur J Immunol* 2006; **36**:1718–28.

65 Joao C, Ogle BM, Gay-Rabinstein C, Platt JL, Cascalho MB cell-dependent TCR diversification. *J Immunol* 2004; **172**:4709–16.

66 Sun J, Tarleton RL. Predominance of $CD8^+$ T lymphocytes in the inflammatory lesions of mice with acute *Trypanosoma cruzi* infection. *Am J Trop Med Hyg* 1993; **48**:161–9.