Thymocyte depletion during acute *Trypanosoma cruzi* infection in C57BL/6 mice is partly reverted by lipopolysaccharide pretreatment

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Abstract

Infection with *Trypanosoma cruzi* in C57BL/6 mice leads to a progressive fatal disease accompanied by thymocyte depletion, which is not related with a higher parasite burden but with increased serum levels of tumour necrosis factor alpha (TNF-α). Because this situation may result from an excessive inflammatory syndrome, mice were now given anti-TNF-α mAbs throughout their acute infection, or subjected to a LPS desensitization protocol before parasite challenge. Treatment with anti-TNF-α mAbs failed to ameliorate thymocyte depletion but shortened survival time and increased parasite load. Pretreatment with LPS (desensitization followed by a sublethal LPS dose) prolonged survival time with a trend to reduce parasitemias and TNF-α serum concentrations. Given that pentoxifylline (PTx) interferes with in vitro LPS tolerance, experiments by administering PTx in combination with the tolerance-inducing LPS doses were also performed. Such schedule significantly reduced mortality, TNF-α and IL-6 serum concentrations, and CD4⁺ CD8⁺ thymocyte loss. LPS pretreatment allowed a better infection control and protected from the accompanying tissue damage.

1. Introduction

Exposure to the haemoflagellate *Trypanosoma cruzi* in man results in a persistent infection which can remain symptomless for the lifetime or lead to Chagas’ disease, a major public health concern in Latin American countries [1]. Such divergent disease outcome are thought to result from complex interactions between *T. cruzi* and elements of the anti-parasite immune response. Hence, murine models showing dissimilar results as to the course of their experimentally induced disease have extensively been employed as a means of investigating the pathogenesis of this trypanosomal infection.

Within this context, we have recently demonstrated that inoculation of *T. cruzi*, Tulahuen strain, into C57BL/6 and BALB/c mice led to an acute infection characterized by a marked parasitemia, myocardial inflammation and an apoptosis-mediated thymocyte depletion. C57BL/6 mice showed cachexia and a progressive fatal disease, whereas 60% of the BALB/c...
mice recovered. The greater disease severity of C57BL/6 mice was not linked to an increased parasite load, as parasitemia, myocardial parasite nests and amastigote counts in peritoneal macrophages did not differ from data recorded in BALB/c animals. Thymocyte depletion reflected in a substantial loss of double positive cortical thymocytes and was more profound in C57BL/6 mice, that also displayed higher blood levels of tumour necrosis factor-alpha (TNF-α) [2].

Because of the continuous need to replenish mature peripheral T cells that undergo normal turnover throughout life [3], preserved thymus during T. cruzi infection in the mammalian host may be essential for the development of an effective immune response against parasites. On the other hand, thymocyte loss may also impact on the tissue damage-related autoimmune phenomena thought to occur during chronic T. cruzi infection [4], since the thymus is important for the generation of T regulatory cells that prevent tissue-infection [4], since the thymus is important for the generation of T regulatory cells that prevent tissue-specific autoimmunity [5,6].

In view that TNF-α was shown to be detrimental in this trypanosomiasis [7,8] and thymocyte apoptosis may be attributed to TNF-α [9,10] experiments by using anti-TNF-α monoclonal antibodies (mAbs) would provide valuable information as to its actual and still non-explored participation in the thymic atrophy accompanying acute T. cruzi-infection. Within this context, fatal outcome in C57BL/6 may be the result of an excessive inflammatory syndrome, as their lethal disease was not related to a higher parasite burden. As such, the eventual influence of lipopolysaccharide (LPS) tolerance on this experimental infection is worth testing. Prior exposure to sublethal and repeated doses of LPS is known to result in a state of tolerance or desensitization to further LPS challenge, which seems to be an adaptive mechanism designed to protect the host from further inflammatory injury [11–13]. In this setting, it must be reminded that T. cruzi presents a glycosylphosphatidylinositol-anchor structure which is reminiscent to LPS [14].

Given this background, a study was undertaken to analyze the potential influence that procedures able to neutralize TNF-α or ameliorate inflammatory reactions may exert on the thymocyte depletion and/or fatal disease of C57BL/6 mice. In this way, C57BL/6 mice were administered anti-TNF-α mAbs throughout their acute trypanosomal infection, or subjected to a LPS desensitization protocol before challenge with T. cruzi.

2. Materials and methods

2.1. Mice and experimental infection

Male C57BL/6 mice (8–10 weeks of age) were bred in the animal facilities at the School of Medicine of Rosario, following institutional guidelines for their handling. Animals had access to food and water ad libitum and were subcutaneously injected with 100 viable trypomastigotes of the Tulahuen strain of T. cruzi. Parasites were maintained by serial passages in BALB/c suckling mice.

2.2. Enumeration of circulating parasites

Bloodstream forms of T. cruzi were assessed under standardized conditions, by direct microscopic observation of 5 μl of heparinized tail venous blood, at 7, 14 and 21 days post-infection (p.i.). Data were expressed as number of parasites per 50 fields.

2.3. Experimental treatment protocols

To neutralize TNF-α an anti-TNF-α mAb (rat IgG1, clone MP6-XT3, DNAX) was employed. Isotype control was obtained from ascitic fluid of mice inoculated with a rat hybridoma producing non-related IgG1. Mice were injected via the intraperitoneal (i.p.) route at days 4, 7, 10, 14 and 17 p.i. with 375 μg of purified anti-TNF-α mAb in 0.1 ml of PBS. Control mice were inoculated with a same amount of rat IgG1 or PBS only. The capacity of this mAb to bind and neutralize TNF-α effects has been described elsewhere (Pharmingen catalogue, San Diego, CA).

2.4. Pretreatment with LPS

This procedure was applied before trypomastigote challenge and consisted of four i.p. consecutive injections of LPS 2 μg/day (Escherichia coli serotype 0111:B4, SIGMA). In further experiments an additional LPS dose of 200 μg was given 24 h following the fourth 2 μg injection. In any case, mice were infected after 48 h the pretreatment was terminated.

2.5. Heart microscopic studies

Hearts were removed on days 17 p.i. (anti TNF-α treatment) or at 14 and 21 days p.i. (pretreatment with LPS), sliced transversally in three sections, and fixed in buffered formalin. Paraffin-embedded 5 μm sections were stained with haematoxylin and eosin for enumeration of inflammatory foci and evaluation of tissue parasitism, that is the number of parasite nests that were visualized in the three sections. The three sections were examined at whole by an experienced pathologist blinded to the study groups.

2.6. Thymic histology

Thymuses were removed at days 7, 14 and 17 p.i. (anti TNF-α treatment) or days 7, 14 and 21 days p.i. (LPS pretreatment). The glands were fixed either in
Bouin or 10% formaldehyde and embedded in paraffin. Sections were stained with haematoxylin–eosin. The score applied to analyze the thymic involution occurring in childhood acute diseases [15] was used to grade thymic alterations. Definitions were as follows grade 0: no alterations; grade 1: focal loss corresponding to lymphodepletion foci; grade 2: marked decrease of cortical thymocyte density with partial disappearance of cortex-medullar limit; grade 3: presence of thymic cortex in some gland sections; grade 4: absence of thymic cortex with virtual thymocyte disappearance.

2.7. Flow cytometry of thymic cell suspensions

For double staining, thymocytes (10⁶) resuspended in flow buffer (RPMI-1640 without phenol red supplemented with 3% foetal bovine serum FBS, 0.1% sodium azide and 10 mM HEPES) were stained in one step with cytochrome-coupled anti-CD4 and phycoerythrin-coupled anti-CD8a mAbs (Pharmingen). A minimum of 10⁵ events was done using a FACScan flow cytometer (Becton–Dickinson). Living cells were gated on the basis of forward- and side-cell scatter. Background staining values obtained with fluorochrome conjugate isotype controls (Pharmingen) were subtracted. Results were analyzed by using the Cell Quest software.

2.8. Serum cytokine determinations

Mice were bled by cardiac puncture at 7, 14 and 21 days p.i. Blood was collected in a sterile, endotoxin-free tube containing no anticoagulant and kept refrigerated until centrifugation. Serum was stored frozen at −20 °C until used. Murine cytokines were measured by specific plates, 3 (Sigma Chemical Co, MO, USA) and cultured in 24-well plates from R&D Systems (Minneapolis, MN, USA).

2.9. In vitro studies in peritoneal macrophages

Cells were centrifuged and resuspended in MEM (Sigma Chemical Co, MO, USA) and cultured in 24-well plates, 3 × 10⁵ cells/well (Nunc International, IL, USA) containing the same medium supplemented with 10% fetal bovine serum (Gibco-BRL, NY, USA), gentamycin 0.2% (10 mg/ml, Gibco) 2% penicillin–streptomycin and 2-mercaptoethanol. After 2 h the culture medium was replaced, and cells were exposed to T. cruzi trypomastigotes (Tulahuén strain) at 1:1, 0.5:1 or 0.25:1 parasite–host cell ratio. Culture supernatants from macrophage monolayers were obtained 24 and 48 h following parasite exposure for assessment of TNF-α.

2.10. Statistics

Differences in quantitative measurements were assessed by the Kruskal–Wallis non-parametric analysis of variance and Mann–Whitney U test. Fisher’s exact test and the χ² test were employed for comparison of categorical variables. The criterion for statistical significance was P < 0.05.

3. Results

3.1. Administration of anti-TNF-α mAbs and their effect on acute infection

To assess whether endogenous TNF-α neutralization was likely to influence acute disease outcome, particularly thymic depletion, three groups of T. cruzi-infected C57BL/6 mice were injected by the i.p. route with 375 μg of anti-TNF-α mAbs in 0.1 ml of PBS at 4, 7, 10, 14 and 17 days p.i., or non-related rat IgG1 in PBS, or PBS alone, at the same intervals. Data from an additional group of infected mice given no interventions were not presented as they overlapped with those yielded by PBS recipients. As shown in Table 1, mice treated with anti-TNF-α mAbs had higher levels of circulating parasites and a reduced survival time (P < 0.01), if compared with the IgG1 or PBS recipient groups. As shown in the same Table, data from mice administered with rat IgG1 (isotype control) did not differ from those seen in PBS recipients. Because treatment with anti-TNF-α mAbs resulted in a significantly shortened survival time, mice were sacrificed earlier, that is at 17 days p.i. with the thymus and heart being removed for histological evaluation. Data depicted in Table 1 revealed that thymus weight loss were not affected by administration of anti-TNF- mAbs since values were similar to the ones seen in the infected groups given PBS or rat IgG1. The same was true when performing the histological analysis, in which the anti-TNF-α-treated mice continued to show the same severity of thymocyte depletion, grade 3 in most cases (data not shown). Studies in myocardial sections showed that mice treated with anti-TNF-α mAbs had a fourfold increase in the number of amastigote nests with a 50% reduction in the amount of inflammatory foci by comparison with infected controls (Table 1; P < 0.05). Histological findings in hearts from mice administered rat IgG1 were quite similar to data yielded by infected mice given PBS (Table 1). Efficacy of neutralization experiments was tested by means of the cytotoxicity assay on L929 cells, showing minimal TNF biological activity in serum from anti-TNF-α-treated mice.
Table 1

<table>
<thead>
<tr>
<th>Characteristics of the acute T. cruzi infection and thymus atrophy in C57BL/6 mice treated with anti-TNF-α monoclonal antibodies</th>
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<tbody>
<tr>
<td>PBS</td>
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<tr>
<td>Parasitemia</td>
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<tr>
<td>Mortality (dead mice/total mice)</td>
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<tr>
<td>Survival time (days)</td>
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<tr>
<td>Absolute thymus weight</td>
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<tr>
<td>Relative thymus weight</td>
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<tr>
<td>Parasite nests/heart</td>
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<td>Number of heart inflammatory foci</td>
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</table>

Mice were given anti-TNF-mAbs 375 µg/injection at 4, 7, 10, 14 and 17 days p.i. Treatments employing PBS or rat IgG1 (isotypic control) were applied at the same intervals (a representative experiment from two independent series). Parasitemias correspond to day 14 p.i. and represent median parasitemias (836 ± 137, survival time 24.6 ± 0.6 days (P < 0.01, respective). Surviving mice were re- covered from their acute infection (data not shown).

*Data correspond to a group of seven non-treated non-infected mice.

b Significantly different from the three groups of infected mice, P < 0.01 (absolute) and P < 0.02 (relative).

*Significantly different from the PBS group (P < 0.05).

b Significantly different from the PBS and IgG1 groups (P < 0.01).

3.2. LPS pretreatment prolongs survival time in acutely infected mice

To learn whether LPS desensitization could influence the course of the acute T. cruzi infection further elicited in C57BL/6 mice, animals were initially given four daily consecutive i.p. injections of LPS 2 µg each and were infected 48 h after the latest injection. Mice undergoing this procedure showed a 10% increase in their survival time but the difference was not statistically significant when compared with data from infected control mice (data not shown). In a further step, we wished to ascertain whether additional challenge with a higher but sublethal dose of LPS was likely to modify disease outcome. In this way, mice which had been given the four consecutive LPS injections (2 µg each) received 200 µg of LPS on day 5 to be infected with T. cruzi 48 h later. Mice from the LPS pretreated-group (n = 14) had lower, but insignificant, peak median parasitemias (83, rank 42–131) and TNF-α serum concentrations (743 ± 106, means ± SEM, pg/ml), with a significantly increased survival time 24.6 ± 0.6 days (P < 0.01) if compared with non-treated mice (n = 12): parasitemia 100 (42–131), TNF-α values 836 ± 137, survival time 22.3 ± 0.4.

3.3. Combined pretreatment with LPS and PTx protects mice from lethal disease

In vitro LPS tolerance was shown to require the presence of TNF-α, with this phenomenon being inhibited provided PTx was added during the macrophage desensitization phase [16]. Hence, any eventual interference of PTxs on the LPS desensitization in vivo could help to further explore about the influence of innate immune mechanisms on the course of acute T. cruzi infection. To this end, C57BL/6 mice simultaneously received i.p. injections of LPS (2 µg) and PTx 2 mg/mice (n = 13), an immunopharmacologically active dose [17], during four consecutive days plus 200 µg of LPS on the fifth day. An additional group undergoing simultaneous LPS pretreatment with PTx injections being replaced by physiologic saline was also included (LPS + Phy, n = 7).

Groups were given four consecutive i.p. injections of LPS 2 µg/day plus an additional dose of 200 µg of LPS at day 5, in simultaneous with four i.p. injections of PTx 2 mg/mouse (LPS + PTx) or physiologic saline (LPS + Phy) at the same time point that 2 µg doses of LPS were administered. Infection was elicited 48 h following the 200 µg LPS injection (a representative experiment from two performed). Day-14 parasitemias are represented as median (rank) of parasites/50 fields from 5 to 7 mice/group. Survival time is represented as means ± SEM.

a Significantly different from non-treated mice (P < 0.005) and the LPS + Phy recipient group (P < 0.02).

b Comparison with treated groups (P = 0.05).

c Significantly different from non-treated mice (P < 0.01).

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td>Acute T. cruzi infection in C57BL/6 mice treated with LPS and PTx before infection</td>
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<tr>
<td>---</td>
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<tr>
<td>Non-treated</td>
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<tr>
<td>Parasitemia</td>
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<tr>
<td>Mortality (dead mice/total mice)</td>
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<td>Survival time (days)</td>
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</table>
Table 3
Serum levels of TNF-α in C57BL/6 mice treated with LPS and/or PTx and further infected with T. cruzi

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time point evaluations</th>
<th>Day 7 p.i.</th>
<th>Day 14 p.i.</th>
<th>Day 21 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-challenge with LPS, 200 µg</td>
<td>Day 7 p.i.</td>
<td>Day 14 p.i.</td>
<td>Day 21 p.i.</td>
</tr>
<tr>
<td></td>
<td>90 min</td>
<td>4 h</td>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>2674 ± 247a</td>
<td>724 ± 23</td>
<td>73 ± 3.7a</td>
<td></td>
</tr>
<tr>
<td>LPS + PTx</td>
<td>1322 ± 191a</td>
<td>842 ± 323</td>
<td>264 ± 7b</td>
<td></td>
</tr>
<tr>
<td>74LPS + Phy</td>
<td>921 ± 29.8</td>
<td>848 ± 68</td>
<td>227 ± 18</td>
<td></td>
</tr>
<tr>
<td>PTx</td>
<td>2611 ± 129a</td>
<td>783 ± 52</td>
<td>68 ± 3.6a</td>
<td></td>
</tr>
<tr>
<td>Non-treated and infectedd</td>
<td></td>
<td>25.1 ± 3.9</td>
<td>341 ± 17</td>
<td>1008 ± 47</td>
</tr>
<tr>
<td>Controls with no interventions</td>
<td></td>
<td>16.7 ± 3.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data represent means ± SEM (pg/ml) from 7 to 9 mice/group (a representative experiment from two independent series). Pretreated groups were given four consecutive i.p. injections of LPS 2 µg/day plus an additional dose of 200 µg of LPS at day 5, in simultaneous with four i.p. injections of PTx 2 mg/mouse (LPS + PTx) or physiologic saline (LPS + Phy) at the same time point that 2 µg doses of LPS were administered. Infection was elicited 48 h following the 200 µg LPS injection. Non-treated mice only received a 200 µg LPS dose, whereas mice from the PTx group were administered 2 mg/mice/day of PTx during the 4 previous days to the 200 µg LPS dose. Infection was induced 48 h after LPS challenge (Day 0).

* and **Significantly different from the LPS + Phy group, (P < 0.01) and (P < 0.05), respectively.

a Difference with the LPS + PTx and LPS + Phy groups (P < 0.001).

b Different from the non-treated and infected group (P < 0.001).

c Significantly different from the LPS + Phy group (P < 0.01) and non-treated and infected mice (P < 0.001).

d A parallel group of infected mice undergoing no pretreatment.

In parallel with these studies, we also measured serum TNF-α levels, at different intervals following the administration of the 200 µg LPS dose. For comparison purposes, another group – given i.p. injections of PTx 2 mg/mice during 4 days before challenge with 200 µg of LPS – was included, as well. Data depicted in the first part of Table 3 show that the LPS + Phy and LPS + PTx groups had lower peak TNF-α concentrations in response to 200 µg of LPS challenge, if compared with mice given no 2 µg LPS doses (non-pretreated group) or PTx alone (P < 0.001). Further comparison between LPS + Phy and LPS + PTx mice revealed that such a decrease of peak TNF-α levels was even lower in the former group (P < 0.01). At the time that infection was induced, that is 48 h after the 200 µg LPS challenge, TNF-α levels in LPS + Phy and LPS + PTx mice were higher than those from non-treated and PTx-pretreated groups (P < 0.001), with the LPS + PTx group showing a further increase in relation to LPS + Phy mice (P < 0.05). Mice given no tolerising doses of LPS or those treated with PTx alone were not challenged with T. cruzi as they were not fully recovered from the lethargic effects of the 200 µg LPS injection. Hence, a further group of T. cruzi-infected mice undergoing no previous manipulations was included as infected controls. As shown in the second part of Table 3, LPS + PTx mice presenting an ameliorated acute disease, displayed the lowest TNF-serum concentrations by the third week p.i., with significant differences in relation to the LPS + Phy (P < 0.01) and non-treated infected groups (P < 0.001). Although LPS + Phy mice had decreased TNF-α blood levels in relation with control infected mice, the difference did not reach statistical significance.

3.4. Peritoneal macrophages from LPS + PTx pretreated mice produce less TNF-α when exposed to T. cruzi

For a better analysis of the desensitization protocol effects, we also studied the TNF-α production of peritoneal macrophages from non-treated (n = 6) or pretreated mice upon exposure to parasites. Cells were obtained from two groups of mice (n = 6, each) that received i.p. injections of LPS (2 µg) in simultaneous with either PTx 2 mg/mice (LPS + PTx) or physiologic saline (LPS + Phy), during 4 consecutive days. Peritoneal macrophages were harvested 48 h that treatment was terminated and 2 h later were exposed to T. cruzi at 1:1, 0.5:1 or 0.25:1 parasite-host cell ratio. Culture supernatants were collected 24 and 48 h following parasite exposure. Results from a representative experiment at the 1:1 parasite:host cell ratio are presented in Table 4. It can be seen that 24- or 48-h culture supernatants from both pretreated groups contained less amounts of TNF-α in comparison with the non-pretreated groups, reflecting in a significant difference at both time points (P < 0.005). Supernatants from LPS + Phy mice had even lower TNF-α concentrations than LPS + PTx counterparts, statistically significant in 48-h cultured cells (P < 0.05, same table).

3.5. Ameliorated acute disease resulting from LPS + PTx pretreatment coexists with a decreased thymocyte depletion and cytokine production

Histological studies from a representative time point, i.e., day 21 p.i., revealed that LPS + PTx had fewer amastigote nests and inflammatory foci in their myocardial tissues, with thymocyte depletion being significantly
ameliorated (data not shown). Partial protection from thymic atrophy reflected in a significantly attenuated loss of double positive (DP) cells (Table 5; \( P < 0.005 \)). Correlatively, the percentages of single positive thymocytes values were as follows (means ± SEM, five mice/group): \( CD4^+ \) cells: non-treated 50 ± 6; LPS + PTx 38.6 ± 4.5; non-infected 10.2 ± 1; non-infected LPS + PTx 12.3 ± 1.4 (non-treated versus non-infected \( P < 0.01 \)); \( CD8^+ \) cells: non-treated 13.3 ± 1.7; LPS + PTx 12 ± 1.5; non-infected 8 ± 0.8; non-infected LPS + PTx 4.2 ± 0.5 (non-treated versus non-infected \( P < 0.02 \)).

Cytokines like IL-6 and IL-10 play a key role in inflammation, displaying pro and anti-inflammatory activities, respectively [18]. To gain a further insight into the immunological changes accompanying the ameliorated acute disease of LPS + PTx mice, serum samples taken at different times during acute infection were therefore assayed for IL-10 and IL-6 concentrations. Both pretreated groups (LPS + Phy and LPS + PTx mice) had low but detectable amounts of IL-10 and IL-6 at the time that infection was induced with no differences between them (Fig. 1). Further evaluations indicated that LPS + PTx had lower levels of both cytokines throughout their acute disease, statistically significant when compared with LPS + Phy and non-treated groups (Fig. 1; IL-10, \( P < 0.05 \) and \( P < 0.025 \); IL-6, \( P < 0.05 \) and \( P < 0.01 \)). As shown in the same figure, day-21 levels of IL-10 from LPS + Phy mice situated significantly below the values yielded by the non-treated group (\( P < 0.05 \)).

### Table 4

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-( \alpha ) levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Non-treated</td>
<td>399 ± 84a</td>
</tr>
<tr>
<td>LPS + PTx</td>
<td>91.7 ± 29</td>
</tr>
<tr>
<td>LPS + Phy</td>
<td>67 ± 16</td>
</tr>
</tbody>
</table>

Treated groups were given four consecutive i.p. injections of LPS 2 \( \mu \)g/day in simultaneous with four i.p. injections of PTx 2 mg/mouse (LPS + PTx) or physiologic saline (LPS + Phy) at the same time point that 2 \( \mu \)g doses of LPS were administered. Peritoneal macrophages were harvested 48 h that treatment was terminated and exposed to parasites, 2 h later. Culture supernatants were collected 24 and 48 h following parasite exposure. Values are mean ± SEM (pg/ml, in culture supernatants at the 1:1 parasite:host cell ratio) of 6 mice/group (one representative of two experiments performed).

aStatistical difference in relation to the remaining groups (\( P < 0.005 \)).
bSignificantly different from the LPS + PTx group (\( P < 0.05 \)).
4. Discussion

Given the thymic repercussion of *T. cruzi*-infected C57BL/6 mice and considering the deleterious effects of TNF-α in situations bearing some similarities with the present experimental model, it was important to analyze whether this cytokine was involved in this in vivo situation. Studies by employing anti-TNF-α mAbs add novel information giving no support to this view since thymic atrophy was not modified by such treatment. The same experiments indicate, that protracted neutralization of a cytokine essential to mount immune and inflammatory responses [9] leads to an immunosuppressor state that is more harmful than beneficial. The increased parasitemias and myocardial amastigote nests together with the reduced number and severity of inflammatory heart lesions seen in anti-TNF-α mAbs recipients, lend support to this assumption. Experiments in transgenic mice expressing a fusion protein (TNFR1-FcIgG3) able to neutralize TNF-α effects in vivo also showed a higher parasite load and reduced myocardial inflammatory infiltrates when infected with *T. cruzi* [19].

Because endotoxin tolerance is known to result in a down regulated production of some proinflammatory cytokines, i.e., TNF-α [11–13], it deemed of interest to analyze the eventual effects of LPS desensitization on this lethal acute infection. Endotoxin tolerance was initially defined as a reduced febrile response of rabbits given low LPS doses before challenge with a lethal dose of this compound [20]. LPS tolerance can be reproduced in vitro since prior exposure of macrophages to LPS leads to a lowered production of TNF-α, IL-6 and NO when cells are further stimulated with endotoxin [13,21]. Such a tolerant state implies a different response programming. In fact, studies wherein LPS-pretreated mice showed decreased levels of TNF-α, IL-6 and IFN-γ upon challenge with LPS, revealed no gross modifications in other cytokine concentrations, i.e., IL-12 and IL-18 [22]. Regarding its functional repercussion, although LPS tolerance might impair antimicrobial immunity, a series of experimental studies demonstrate that LPS-tolerance not only ameliorates the lethal effects of Shiga 2 toxin [23,24], but also confers an increased resistance to systemic infection with *Cryptococcus neoformans* or *Salmonella enterica* [25,26]. In the case of cryptocephalosporin, protection continued to be observed even when in addition to prior LPS desensitization a higher LPS dose was administered the day after infection was induced.

Although in our case conventional LPS desensitization failed to cause substantial disease modifications, it was sensible to find out whether challenge with a higher but sublethal LPS dose before parasite inoculation was likely to prime the host for a better antiparasite response. Experiments by employing such a pretreatment schedule showed a longer survival time with lower, but statistically not significant, parasitemias and peak TNF-α serum concentrations. This demonstration along with the fact that LPS and glycosylphosphatidylinositol from *T. cruzi* interact with functionally similar macrophage Toll-like receptors [27], suggest the existence of a cross tolerance mechanism as partly accounting for a less harmful course of acute infection. This assumption is reinforced by the lower TNF-α synthesis of peritoneal macrophages from LPS-desensitized mice, when cultured in presence of *T. cruzi*.

Based on a demonstration that in vitro macrophage tolerance to LPS was inhibited by PTx [16], we next wished to ascertain to what extent PTx may modify our in vivo results by administering PTx in combination with the tolerance-inducing LPS doses. Such schedule resulted in a substantial improvement of the acute infection-associated biological parameters, causing a significantly reduced mortality. To get some insight into the potential mechanisms underlying this phenomenon a detailed analysis of the TNF-α concentrations was performed. Measurements upon challenge with the 200 μg LPS dose revealed that peak TNF-α levels were reduced in tolerant mice with those given no PTx showing even lower concentrations of this cytokine. It follows that in our model, PTx may partly interfere with tolerance induction. Supporting this view, studies in peritoneal macrophages from mice subjected to LPS + PTx or LPS-Phy desensitization protocols revealed a decreased TNF-α production when exposed to *T. cruzi*, which was less marked in cells of the former group. Analysis by the time that infection was induced revealed that groups subjected to LPS pretreatment, alone or combined with PTx, had significantly higher levels of TNF-α if compared to those undergoing no interventions. Presence of TNF-α during the early phase of infection was likely to favour disease control [28] because of its direct trypanocidal activity [29] and/or macrophage activating effects [30,31]. Such an improved protective state may also have determined a lower TNF-α production during ensuing infection, explaining the diminished concentrations seen in pretreated mice, particularly the LPS + PTx group, by the later phase of their acute disease. To some extent, LPS + PTx pre-treatment may also have downregulated TNF-α production during infection, given the lower amounts of this cytokine in culture supernatants of peritoneal macrophages from mice undergoing this intervention upon in vitro exposure to *T. cruzi*.

With regard to thymic changes, despite the ability of TNF-α to induce thymocyte apoptosis [10], reduced TNF-α presence may not account for the ameliorated thymic atrophy and DP cell loss, since thymus involution was not inhibited by anti-TNF-α. It suggests that compounds other than TNF-α are implied in this phenomenon. Studies in other mouse model of acute
T. cruzi infection failed to relate thymocyte loss with an increased glucocorticoid production, since similar changes were found in adrenalectomized counterparts [32]. Our demonstration that LPS + PTx mice had decreased levels of serum IL-6 suggests a down regulated synthesis of a broader spectrum of inflammatory mediators, likely implied in thymus atrophy, and hence responsible for its partial reversion and decreased presence of IL-10, as well. Within this setting it is worth mentioning that IL-6 was found to cause thymic atrophy with loss of CD4+ , CD8+ thymocytes [33].

The fact that this partly modified LPS pretreatment protected the host from the accompanying tissue damage while promoting a better infection control provides a stimulating ground for further exploring the influence of such innate-associated immune mechanisms on the immunopathogenesis of this trypanosomiasis.

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References


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