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Deficient control of *Trypanosoma cruzi* infection in C57BL/6 mice is related to a delayed specific IgG response and increased macrophage production of pro-inflammatory cytokines

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Abstract

Earlier work in *Trypanosoma cruzi*-infected C57BL/6 and BALB/c mice revealed an acute disease, of lethal outcome in the former group and lesser severity in BALB/c mice. Fatal course was not accompanied by an increased parasite load, but by a substantial imbalance between pro- and anti-inflammatory cytokine serum levels. To better characterise the mechanisms allowing the host to restrain the infection, we have now studied the specific IgG production and in vitro behaviour of peritoneal macrophages (PMs) when exposed to *T. cruzi*. BALC/c mice displayed higher serum levels of specific immunoglobulins in the first weeks of acute infection. In vitro infected PMs showed no between-group differences in the number of intracellular parasites, although TNFα levels were significantly higher in culture supernatants from C57BL/6 mice. Because an LPS-based pretreatment (desensitisation protocol followed by a sublethal LPS dose) reduced disease severity of C57BL/6 mice, we next explored the features of the in vitro infection in PMs from mice subjected to such protocol. PMs from LPS-pretreated mice had a decreased production of TNFα and IL-1β, becoming more permissive to parasite replication. It is concluded that deficient control of *T. cruzi* infection in C57BL/6 mice may also involve a less satisfactory specific IgG response and increased TNFα production by PMs. Improved disease outcome in LPS-pretreated mice may be associated with the reduced inflammatory cytokine production by PMs, but the

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impaired ability of these cells to control parasite growth suggests that compensatory mechanisms are operating in the in vivo situation.

Keywords: LPS-pretreatment; Cytokine; Antibody; Macrophage; *T. cruzi*

Introduction

Chagas' disease is caused by the intracellular protozoan *Trypanosoma cruzi*. About 20 million people in Latin America are infected, and 40 million are at risk of acquiring the infection (WHO, 2000).

Control of *T. cruzi* infection is achieved through the coordinated activation of several immune effector mechanisms. Experimental studies indicate that antibodies and macrophages play an essential role in destroying circulating trypomastigotes and killing intracellular forms, respectively (Lages-Silva et al., 1987; Umekita and Mota, 2000; Pascutti et al., 2003). Macrophage trypanocidal properties involve, in part, the expression of inducible NO synthase (iNOS), which is mostly stimulated by interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNFα) leading to the subsequent nitric oxide production (NO) by these cells (Reed, 1988; Torrico et al., 1991; Revelli et al., 1995; Black et al., 1989; Golden and Tarleton, 1991; Muñoz Fernandez et al., 1992). However, NO, along with cytokines promoting its production, i.e., TNFα, may act as mediators of tissue damage when produced in excessive amounts (Moncada and Higgs, 1993; Aggarwal and Natarajan, 1996).

Experimental murine models of Chagas' disease have been helpful in getting a better insight on the course of infection and disease pathology. Earlier work by infecting C57BL/6 and BALB/c mice with the Tulahuen strain of *T. cruzi* revealed an acute disease, which was lethal in the former group, with two thirds of BALB/c mice being recovered from such episode. Fatal disease outcome in C57BL/6 mice was accompanied by a substantial imbalance between pro- and anti-inflammatory cytokine levels in sera, compatible with an excessive inflammatory reaction arising from an unfavourable parasite–host relationship (Roggero et al., 2002). Reinforcing this view, challenge with LPS during infection of C57BL/6 mice was followed by a more pronounced increase of TNFα levels in serum.

Partly because LPS desensitisation was shown to protect mice from harmful effects of LPS challenge (Erroi et al., 1993) and given that LPS tolerance conferred an increased resistance to systemic infection with *Cryptococcus neoformans* or *Salmonella enterica* (Rayhane et al., 2000; Lehner et al., 2001) we further analysed the influence of LPS-pretreatment on acutely infected C57BL/6 mice. By applying some modification to the classical protocol (desensitisation followed by a sublethal LPS dose) pretreatment with LPS prolonged survival time of C57BL/6 mice. In view that pentoxifylline (PTx) affected in vitro LPS tolerance, experiments by administering PTx in combination with the tolerance-inducing LPS doses were also performed. Such schedule was shown to partly interfere with tolerance induction causing a significantly reduced mortality accompanied by decreased TNFα and IL-6 serum concentrations (Roggero et al., 2004). It follows, that a finely tuned balance of the anti-parasite immune response was required for the host to clear the infection without excessive pathological consequences.

The more detrimental disease of C57BL/6 mice may be related to a deficient ability of parasite handling by macrophages, that turns out to be more harmful than beneficial. Initial studies in peritoneal macrophages—PMs—from C57BL/6 and BALB/c mice revealed no major differences in the amount of

intracellular forms, while showing an increased TNFα production in cells from the former group. Whether this phenomenon extends to the production of other mediators deserves further exploration. On the other hand, since LPS tolerance can be reproduced in macrophages (Ziegler-Heitbrock et al., 1992), in vitro infection of macrophages from LPS-pretreated mice would provide a better understanding on the mechanisms by which LPS-pretreatment protected C57BL/6 mice.

Accordingly, PMs from both mouse strains and also from LPS-pretreated C57BL/6 mice were analysed for the in vitro features of *T. cruzi* infection, that is parasite replication and cytokine production, at different infection schedules. Given that humoral responses are mounted to contain infection, the in vivo quantitative production of specific IgG antibodies was also investigated for a better evaluation of the host response.

Materials and methods

Mice and parasites

Male BALB/c and C57BL/6 mice (6–8 weeks of age) were bred in the animal facilities at the School of Medicine of Rosario. Animals had access to food and water ad libitum, and were handled according to institutional guidelines for their handling. Trypomastigotes of the Tulahuen strain of *T. cruzi*, were maintained by serial passages in CBi suckling mice. Heparinised blood obtained from infected mice was diluted in physiological solution and washed twice. Live blood parasites were counted using a Neubauer's chamber.

Experimental design

Peritoneal macrophages from cervically dislocated BALB/c and C57BL/6 mice were obtained by lavage of the peritoneal cavity, to be further exposed to parasites. In desensitisation experiments, C57BL/6 mice were treated, as described earlier (Roggero et al., 2004). Briefly, mice were given during four consecutive days an intraperitoneal injection of LPS from *E. coli* serotype 0111-B4 (Sigma St. Louis, MO) at a dose of 2 μg/mouse/day plus pentoxifylline (PTx, 2 mg/mouse/day, Aventis Labs., Buenos Aires). On the fifth day mice received and additional sublethal dose of LPS of 200 μg/mouse/day. PMs were obtained 48 h after the 200 μg LPS dose.

For the assessment of specific antibodies, C57BL/6 and BALB/c mice were infected by subcutaneous route with 100 trypomastigote forms of *T. cruzi*. Blood was obtained by cardiac puncture at different times post-infection (pi), and serum was stored at −20 °C until tested.

Monitoring of in vivo acute infection was evaluated by assessing bloodstream forms of *T. cruzi* by direct microscopic observation of 5 μl of heparinised tail venous blood at different days pi and measured as parasites/50 fields.

Assessment of the in vitro infection

Parasite counts

Cultures of PMs at cell density of 3.10^4 cells/well in 8 well microplaques (LabTek® Chamber Slide™ System, Nalge Nunc International) were performed for counting the number of intracellular parasites by

indirect immunofluorescence. Cells were cultured at different PM : parasite ratios, 1 : 0.25, 1 : 0.5 and 1 : 1. Following 2, 4, 24 or 48 h, PMs were washed twice, fixed with ethanol 100% and stored at 4 °C. After that, PMs were covered with pooled *T. cruzi*-positive human sera diluted 1 : 30 in moist chambers during 30 min. Following washing with PBS, fluorescein isothiocyannate (FITC)-conjugated goat anti-human IgG (Sanofi Diagnostic Pasteur, France) was added to macrophage monolayers and incubated for 30 min in a moist chamber at room temperature. Autofluorescence was avoided by previous staining with Evans blue. Cells were examined under an epifluorescence microscope for estimations of the total number of amastigotes and its relative amount/infected cell from 500 cells/well. Presence of trypomastigotes in culture supernatants was also investigated from PMs obtained at 2, 4, 24 and 48 h following parasite exposure.

Culture supernatants

The peritoneal cells were recovered by lavage of the peritoneal cavity with Earle's MEM medium (PAA; Germany). Cells were washed twice and resuspended in medium supplemented with 10% foetal bovine serum (Gibco, NY, USA), 0.2% gentamycin (10 mg/ml, Gibco, NY, USA), 2% penicillin streptomycin (100 μg/ml and 100 U/ml, respectively, Gibco, NY, USA) and 0.4 mM 2-mercaptoethanol. Viability was determined by trypan blue exclusion test. Upon that, 3×10^5 cells/well were seeded in flat-bottomed 12 well plaque (Nunclon Multidishes, Nalge Nunc International, Naperville, IL, USA) and allowed to adhere 2 h. The non-adherent cells were then removed. PMs were then exposed to *T. cruzi* at 1 : 0.25, 1 : 0.5 and 1 : 1 host : parasite cell ratio. At different times post-challenge, supernatants were collected and centrifuged for the assessment of mediators. PMs were characterised through morphological appearance and by the esterase reaction.

Cytokine assays and nitrite evaluation

Murine cytokines in culture supernatants were measured by specific two-site enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's specifications. ELISA kits for TNFα (limit of detection 5.1 pg/ml), IL-10 (limit of detection 4 pg/ml) and IL-1β (limit of detection 3 pg/ml) were purchased from R and D (Minneapolis, MN, USA). All samples were processed individually and assayed in duplicate, with plates being read at 450 nm. NO derivatives in PM supernatants were measured as nitrite at 24 and 48 h post-challenge with *T. cruzi*. Briefly, total nitrite was assessed by reducing nitrate to nitrite with a *Pseudomona oleovorans* (strain ATCC 8062) nitrate reductase, followed by the Griess reaction (Green et al., 1982). Equal amounts of culture supernatants and Griess reagent were combined, and incubated for 10 min at room temperature. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% naphthylethylene diamine dihydrochloride in 60% acetic acid. Absorbance was measured at 540 nm. Nitrite concentration was quantified using various $NaNO_2$ concentrations as standard and data were expressed in micromolars.

RT-PCR for TNFα

PMs (3.10^6 cells) from C57BL/6 mice subjected or not to the in vivo LPS-pretreatment were cultured in Petri dishes during 2 h before challenge with parasites. After 1 and 4 h post-exposure, the cells were washed and lysed with TRizol (Gibco, NY, USA) following instructions given by suppliers. Two micrograms of total RNA were reversely transcribed to first strand cDNA by standard procedures (Sambrook et al., 1989) using 50 μg/ml oligo-dT anchor primers, 5 mM dNTP mixture, 1 mM DTT,

50 U RNAsaOut (Gibco, NY, USA) and 200 U reverse transcriptase (SuperScript II—RNAsa H, Gibco Gibco, NY, USA) in buffer supplied. The RT mixture was amplified in a conventional PCR procedure using 4 mM $MgCl_2$, 5 mM dNTPs, 10 pmol each oligonucleotide and 1 U Taq DNA polymerase (Gibco, NY, USA) in buffer supplied. Oligonucleotides used were: TNFα sense 5′GTT CTA TGG CCC AGA CCC TCA CA 3′, TNFα antisense 5′GAT CCA CAT CTG CTG GAA GGT 3′; β-actin sense 5′GGT GAC GAG GCC CAG AGC AAG 3′, and β-actin antisense 5′ GAT CCA CAT CTG CTG GAA GGT 3′ (Bio-Synthesis Lewisville, Texas, USA). Reactions were performed in an Eppendorf thermal cycler, with the cycling parameters consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and final extension step of 10 min at 72 °C. Optimal number of amplification cycles for semi-quantification was determined experimentally to obtain the minimal detectable signal in etidiun bromide-stained gels. Expression levels of TNFα transcripts estimation was performed by standardisation with internal control of β-actin, using a densitometric scanning video camera and GELPRO 32 analysis program.

Analysis of specific IgG production

Anti-*T. cruzi* IgG levels were measured by ELISA. In brief, microtitre plates (Lockwell Modules, Nalge Nunc International, Naperville, IL, USA) were coated with 20 μg/ml of epimastigote lisate, Tulahuen strain, in 0.05 M carbonate–bicarbonate pH 9.6, blocked, and incubated with a 1 : 80 dilution of sera from either infected or uninfected mice. Specific IgG isotype was detected by incubation with goat IgG anti-mouse IgG peroxidase-conjugated (Jackson Inc) diluted 1 / 4000. Samples were assessed in duplicate and plates were read at 450 nm in an ELISA reader (Organon Tecknica) after incubation with H_2O_2 and TMB.

Statistical analysis

Differences in quantitative measurements were assessed by the Kruskall–Wallis non-parametric analysis of variance and Mann–Whitney *U* test. The data were considered significant when p value was <0.05.

Results

Studies in C57BL/6 and BALB/c mice

The first set of studies is referred to experiments carried out in the two strains of mice undergoing no in vivo interventions, before collecting PM.

Characteristics of the in vitro infection

We initially analysed the features of the in vitro infection in PMs (the visualisation of intracellular parasites is depicted in Fig. 1). Table 1 shows the number of amastigotes of 500 cells/well and the number of amastigotes/infected cell at the 1 : 1 PM : parasite ratio, 24 and 48 h following exposure. Intracellular parasite counts revealed no differences between groups, as did when cells were studied 2 and 4 h after *T. cruzi* challenge (data not shown). Differences were also insignificant

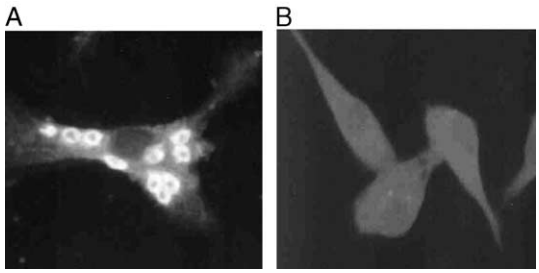

Fig. 1. Presence of amastigotes in C57BL/6 PMs detected by immunofluorescence. (A) Infected (24 h post-exposure), and (B) control PMs, respectively.

when comparing the amount of trypomastigotes in supernatants recovered at different times post-challenge, irrespective of variations in the PM: parasite cell ratios or the time point. Values (median, range trypomastigotes/ml) in culture supernatants were as follows, 24-h: BALB/c = 70 (63–101) $\times 10^3$, C57BL/6 = 98 (94–150) $\times 10^3$; 48-h: BALB/c = 5 (0–8) $\times 10^3$, C57BL/6 = 6 (0–23) $\times 10^3$. Results from cells exposed at the 1 : 0.5 and 1 : 0.25 ratios remained in the same direction (data not shown).

Levels of cytokines and NO-derived metabolites in supernatants from T. cruzi-challenged cells

As shown in Fig. 2, TNFα concentrations in supernatants from C57BL/6 mice collected at 24 and 48 h following exposure were higher than those from the BALB/c counterparts. Regarding NO-derived metabolites, there were no between-group differences in the total amount of this compound. Similar results were obtained when studying 24-h supernatants (data not shown).

Assay for specific IgG

We measured the serum levels of anti-parasite IgG throughout acute infection, since specific antibodies were shown to be effective in clearing bloodstream forms of *T. cruzi* (Brodskyn et al., 1988). As shown in Fig. 3, the amount and kinetic of specific IgG antibodies seemed to be different in the two strains of mice. Specific IgG antibodies in BALC/c mice were detected as early as day 5 pi; with day-7 and day-14 levels being significantly higher than values from C57BL/6 mice. Both strains attained similar levels of specific antibodies by day 21 pi, the time of peak parasitemias. This time point

Table 1
Amounts of intracellular amastigotes in PMs from BALB/c and C57BL/6 mice detected by immunofluorescence

	BALB/c		C57BL/6	
Time post-exposure (h)	24	48	24	48
Amastigotes/PM	2 (1–4)	7 (1–20)	5 (1–19)	10 (2–26)
Amastigotes/500 PMs	34 (1–45)	108 (50–153)	22 (9–53)	91 (33–189)

Values are expressed as median and range of 6 mice/group at the 1 : 1 PM : parasite ratio. A representative experiment from two performed.

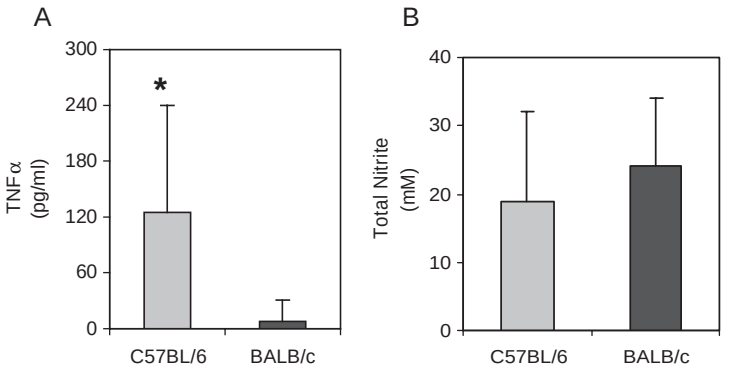

Fig. 2. Levels of TNFα (A) and NO (B) in culture supernatants from PM challenged with parasites 48 h earlier. Data are means ± SD of 4–6 mice/group and correspond to the 1:1 PM:parasite cell ratio. A representative experiment from two performed. *Statistically different from BALB/c group, $p < 0.015$.

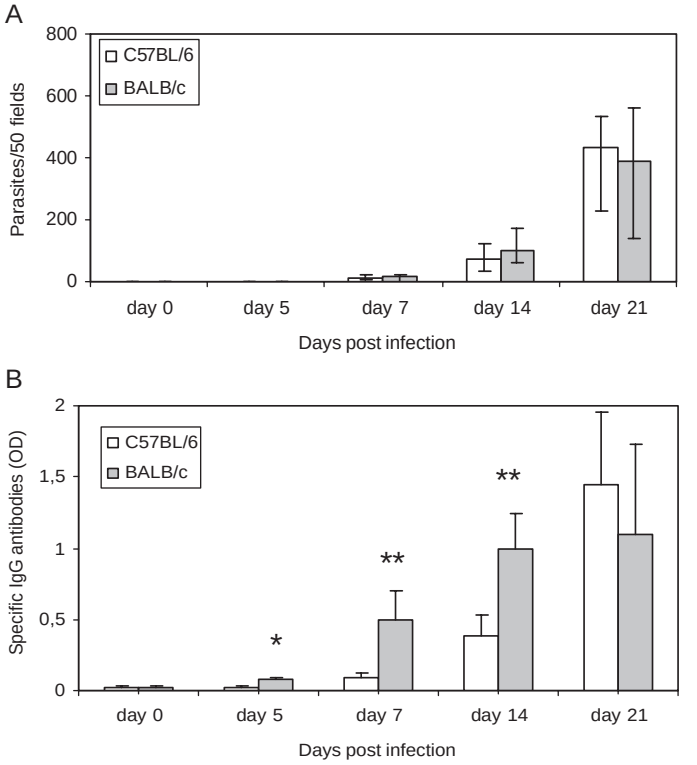

Fig. 3. Parasitemias (A) and IgG antibody responses (B) in infected C57BL/6 and BALB/c mice. Parasitemia was measured as parasites/50 fields and the data are expressed as median and range. ELISA plates were coated with 20 μg/ml of *T. cruzi*, and plasma samples were analysed at a 1:80 dilution. IgG values are presented as mean ± SD of OD (450 nm). Assessment of antibody levels at day 0 was performed in samples collected immediately before challenge with *T. cruzi*. A representative experiment from two independent rounds of 4–6 mice/group. Statistical difference between groups *$p < 0.05$ and **$p < 0.001$.

corresponded to the phase in which BALB/c mice start to control infection whereas C57BL/6 mice succumb to the acute disease.

Study in C57BL/6 mice undergoing LPS-pretreatment

In vitro infection with T. cruzi in PMs

Previous studies demonstrated that pretreatment of C57BL/6 mice with LPS plus PTx (tolerance protocol followed by a sublethal LPS dose) prolonged survival time, lowered parasitemias and diminished TNFα serum concentrations (Roggero et al., 2004). To ascertain whether such in vivo effects could involve a different pattern of response of PMs, we first compared the effect of LPS plus PTx in vivo pretreatment on the control of in vitro intracellular parasitism. Analysis of intracellular amastigote counts revealed significant differences between PMs from normal or pretreated mice, with numbers being higher in the latter group (Table 2). Disappearance of extracellular trypomastigotes in culture supernatants from pretreated mice was statistically insignificant in relation to the normal group. Values (median, range trypomastigotes/ml) in culture supernatants at 48 h post in vitro infection were as follows: control C57BL/6 38 (10–208) $\times 10^3$, LPS-pretreated C57BL/6 48 (0–103) $\times 10^3$. Similar results were obtained at the 1 : 0.5 and 1 : 0.25 ratio (data not shown).

Effect of LPS-pretreatment on cytokines and NO derivatives in cultured PMs

Cytokine production by PMs was measured at 24 and 48 h following exposure. Results deal with data recorded at 48 h, as there was a clearer pattern of group differences. Pretreatment protocol did not affect in vitro NO production, as no major differences in NO-derivative production by both types of PMs were detected (Fig. 4). However, TNFα values were diminished in supernatants of infected PMs from desensitised mice if compared with normal C57BL/6 PMs (same figure). In contrast, culture supernatants of PMs from desensitised mice contained increased amounts of IL-10 with respect to the control group, regardless of whether macrophages were infected or not ($p < 0.025$ and < 0.05, respectively, Fig. 4). Changes in IL-1β levels in culture supernatants paralleled those seen with TNFα. In PMs from pretreated mice, this cytokine fell below the values displayed by the control group (Fig. 4).

Several studies showed that the tolerant state coexists with a reduced TNFα expression (Beeson, 1946; Mathinson et al., 1990). Despite suppression forms vary between models, the most accepted one is an inhibition to a transcriptional level (Kastembauer and Ziegler-Heitbrock, 1999). Since lower

Table 2
Presence of intracellular parasite forms in PMs from C57BL/6 mice, undergoing LPS-pretreatment or not, before cell collection

	C57BL/6 normal		C57BL/6 LPS-pretreated	
Time post-exposure (h)	24	48	24	48
Amastigotes/PM	3 (1–19)	7 (2–26)	12 (1–23)*	22 (4–32)*
Amastigotes/500 PM	12 (5–29)	39 (16–67)	47 (18–78)*	125 (36–205)**

LPS-pretreatment consisted of 4 consecutive ip injections of LPS 2 μg/day plus PTx (2 mg/mouse/day), followed by and additional sublethal dose of LPS of 200 μg/mouse/day on the fifth day. PMs were obtained 48 h later to be challenged with *T. cruzi*. Values represent the median and range of 6 mice/group at the 1 : 1 MP : parasite ratio. A representative experiment from two performed.

Statistical difference from normal PMs: *$p < 0.05$, **$p < 0.014$.

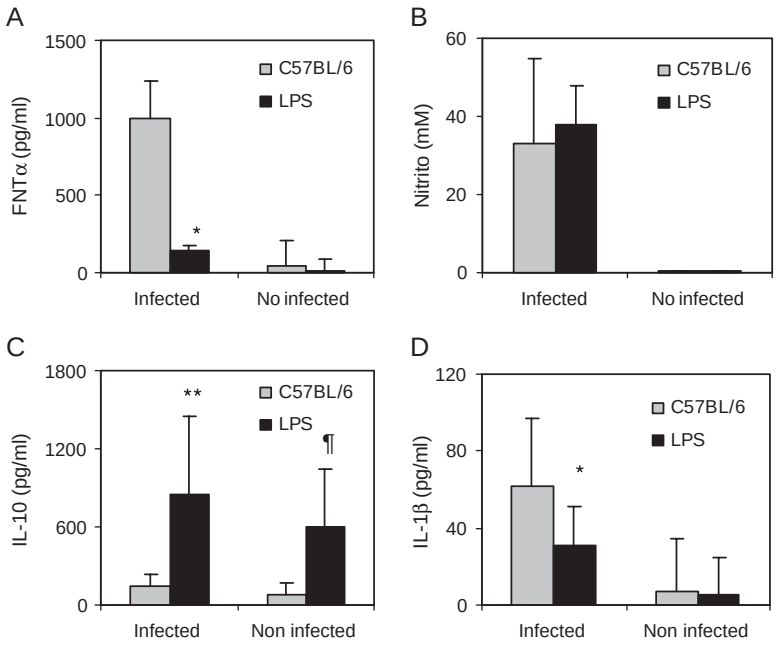

Fig. 4. Cytokine and NO production by PMs. LPS-pretreatment (LPS) consisted of 4 consecutive ip injections of LPS 2 μg/day plus PTx (2 mg/mouse/day), followed by and additional sublethal dose of LPS of 200 μg/mouse/day on the fifth day. PMs were obtained 48 h later to be challenged with *T. cruzi*. Culture supernatants of 6 mice/group were collected 48 h following parasite exposure at 1 : 1 PM : parasite cell ratio. (A) TNFα (pg/ml); (B) NO (total nitrite; μM); (C) IL-10 (pg/ml). (D) IL-1β (pg/ml). Values are means ± SD. A representative experiment from two performed. *$p < 0.01$ and **$p < 0.025$, in comparison with the non-LPS-pretreated counterparts. $p < 0.05$ in comparison with the non-LPS-pretreated counterpart.

concentrations of this cytokine were obtained in supernatants from desensitised PMs and given the relevance of TNFα in this model, we next analysed whether decreased TNFα levels were due to a transcriptional inhibitory phenomenon. To this end, total mRNA of PMs from desensitised and normal C57BL/6 mice exposed to parasites 1 and 4 h earlier, was obtained. As shown in Fig. 5 (both panels), TNFα mRNA expression at 1 or 4 h in normal PMs was remarkably augmented by comparison with data from PMs from pretreated mice.

Specific IgG response

In line with previous studies (Roggero et al., 2004), LPS-pretreated C57BL/6 mice had diminished parasitemias throughout their acute disease (Fig. 6A). In contrast, cultured PMs from similarly pretreated mice revealed comparatively increased parasite counts. Because parasite control in vivo also relies on the presence of additional effector mechanism, i.e., specific antibodies; anti-*T. cruzi* IgG levels in LPS-pretreated C57BL/6 were also measured. Infection was elicited 48 h after LPS-pretreatment was terminated. As depicted in Fig. 6B, comparisons between both groups of C57BL/6 (pretreated or not) revealed no differences in the amount of anti-*T. cruzi* IgG antibodies, during acute infection. In line with earlier experiments, 2/3 of mice from the LPS-pretreated group survived and recovered from the acute infection, whereas those undergoing no pretreatment died around day 22 pi (data not shown).

Discussion

Our preliminary study in BALB/c and C57BL/6 mice showed that these strains were an appropriate model to investigate the mechanisms accounting for the divergent disease outcome during acute *T. cruzi* infection. In fact, inoculation with Tulahuen trypomastigotes led to an acute disease, of lethal course in C57BL/6 mice, whereas most of the BALB/c mice recovered from the acute episode (Roggero et al., 2002). In view that macrophages harbour the intracellular multiplication of parasites and at the same time mediate their elimination, a further analysis of the parasite–macrophage relationship was required. The present demonstration of no between-group differences in trypomastigote numbers in culture supernatants suggests that both PMs populations are equally able to phagocytose and destroy *T. cruzi*, or to be invaded by parasites. The view of a similar strain ability of PMs to kill parasites is further supported by the lack of difference in amastigote counts, and extracellular numbers at a later stage of the in vitro infection.

With reference to macrophage-synthesised compounds, TNFα production during acute *T. cruzi* infection is clearly necessary for activation of macrophage effector function, but may also be implicated in the pathological consequences of this trypanosomiasis (Truyens et al., 1995). Studies in susceptible mice treated with TNFα revealed that while reducing intracellular parasite replication in their PMs, mortality was increased among recipients (Black et al., 1989). In our case increased presence of TNFα in

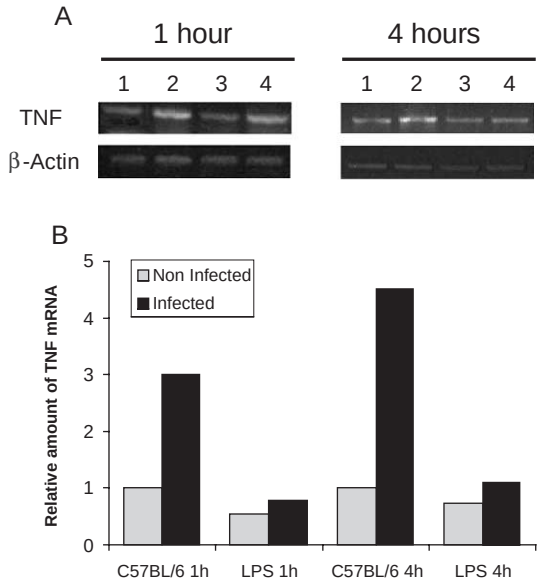

Fig. 5. (A) TNFα mRNA expression evaluated by semiquantitative RT-PCR 1 and 4 h post-exposure of PMs with *T. cruzi*. (1) C57BL/6 non-infected, (2) C57BL/6 infected, (3) C57BL/6 LPS-pretreated and non-infected, (4) C57BL/6 LPS-pretreated and infected. LPS-pretreatment (LPS) consisted of 4 consecutive ip injections of LPS 2 μg/day plus PTx (2 mg/mouse/day), followed by and additional sublethal dose of LPS of 200 μg/mouse/day on the fifth day. After 48 h, PMs (3.10^6 cells) from mice undergoing the in vivo LPS-pretreatment, or not, were cultured in Petri dishes during 2 h before challenge with parasites. After 1 and 4 h post-exposure, the cells were washed and lysed with TRizol. (B) Beta actin mRNA was used to normalise TNFα mRNA among samples. Relative values were calculated considering TNFα/β-actin ratio as 1 for non-treated, non-infected animals. Results corresponded to a representative experiment from two independent series.

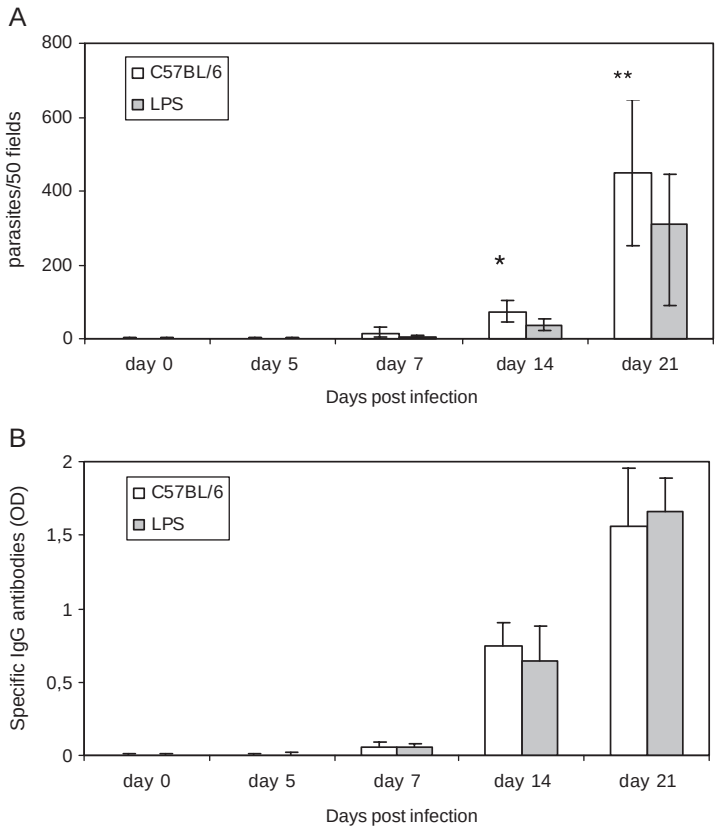

Fig. 6. (A) Parasitemias and, (B) IgG antibody response in infected C57BL/6 mice undergoing LPS-pretreatment or not. LPS-pretreatment (LPS) consisted of 4 consecutive ip injections of LPS 2 μg/day plus PTx (2 mg/mouse/day), followed by and additional sublethal dose of LPS of 200 μg/mouse/day on the fifth day. Animals were infected 48 h later. Parasitemia was measured as parasites/50 fields and data are expressed as median and range. ELISA plates were coated with 20 μg/ml of *T. cruzi*, and plasma samples were analysed at a 1 : 80 dilution. IgG values are represented as means ± SD of OD (450 nm) of 4–6 mice/group. Assessment of antibody levels at day 0 was performed in samples collected immediately before challenge with *T. cruzi*. A representative experiment from two performed. Statistical difference with the non LPS-pretreated group $*p < 0.05$ and $**p < 0.001$.

culture supernatants from C57BL/6 mice were not accompanied by a reduced parasite replication with respect to the BALB/c group. It follows that C57BL/6 PMs are more prone to TNFα synthesis upon encounter with parasites and/or additional mediators take part in the control of intracellular growth. Despite that NO was shown to be involved in parasite clearance (Petray et al., 1995) and its synthesis was stimulated by TNFα (Silva et al., 1995), NO may not be implied in the control of this in vitro infection, as no differences in NO-derivative contents were found between both PMs populations. Beyond these facts, augmented TNFα production by cells from C57BL/6 mice did help to explain our previous findings in which the systemic levels of this cytokine in acutely *T. cruzi*-infected mice were higher in this mouse strain.

T. cruzi alternates between extracellular and intracellular forms and the elimination of circulating forms depends largely on antibody response (Umekita and Mota, 2000). Hence, an analysis of the

specific immunoglobulin levels was worth investigating. Our results suggested that highly susceptible C57BL/6 mice mounted a less efficient specific humoral response in the early phase of acute infection. Viewed at first sight, such a deficit would not carry major consequences for the infective process as mice did not show higher parasitemias with respect to the BALB/c group. Regardless of the fact that parasite clearance is achieved by another mechanism, the deficient antibody response of C57BL/6 mice may result in less parasite neutralisation rendering trypanosomes more able to trigger the inflammatory cascade events serving to partly explain the increased serum TNFα levels seen in our earlier study.

Considering that a more favourable course of acute infection was obtained when C57BL/6 were subjected to the LPS-pretreatment protocol (Roggero et al., 2004), further in vitro investigations were addressed to analyse the behaviour of PMs from pretreated mice when challenged with parasites. Pretreatment resulted in a clear diminution of TNFα contents in culture supernatants, which coexisted, with reduced transcriptional levels of this cytokine in the first hours post-challenge. The inhibition in the expression of TNFα in PMs, resembles findings detected at the systemic level showing a substantial TNFα decrease in LPS-pretreated mice (Roggero et al., 2004). Studies in cross-tolerisation experiments between LPS and an LPS-like molecule from *T. cruzi* membranes also revealed a reduced expression of inflammatory cytokines in PMs from the C57BL/6 strain (Ropert et al., 2001).

While the comparative increase of TNFα levels in supernatants from untreated C57BL/6 macrophages were not accompanied by reduced amounts of intracellular forms, the 80% reduction in TNFα concentrations in cultured macrophages from pretreated mice did coexist with increased amastigote counts. It implies that LPS-pretreatment promotes a different response programming in macrophages resulting in decreased TNFα production, critical enough for parasite control to become deficient. The fact that IL-10 was easily detectable in supernatants from LPS-pretreated macrophages, no matter if cultures were infected or not, reinforces the view of a distinct macrophage programming induced by LPS-pretreatment itself. Raised IL-10 concentrations may also account for rendering macrophages more permissive to parasite replication, since IL-10 was shown to down-regulate TNFα and NO production by macrophages, favouring parasite multiplication (Gazzinelli et al., 1992). Despite the IL-10 inhibitory effects on NO production, increased amounts of IL-10 in culture supernatants of PMs from pretreated mice were not accompanied by reduced nitrite concentrations, likely because TNFα production was down-regulated and this cytokine activates iNOS transcription (Baldwin, 1996; Ziegler-Heitbrock, 1995). Collectively, these findings lead to envisage a more complex interactive scenario operating on the production of biologic response modifiers. Our results are at variance with studies reporting a diminished NO production during the tolerant state (Severn et al., 1993), but in agreement with other authors (Ropert et al., 2001), who found a preserved NO synthesis in the tolerant state induced by either LPS or an LPS-like molecule from *T. cruzi* trypomastigotes.

Increased amastigote numbers in PMs from LPS-pretreated mice deviate from earlier in vivo findings wherein mice undergoing the same intervention before parasite inoculation displayed lower parasitemias throughout their acute disease. Such an ameliorated disease would not be related to a better humoral response since levels of anti-*T. cruzi* antibodies were similar in both groups of C57BL/6 infected mice, although the presence of a particular antibody isotype as playing a role in the in vivo protection cannot be ruled out. Beyond this fact, it may be that compensatory mechanisms are also operating in the in vivo situation. Our previous study revealed an earlier presence of TNFα during the initial phase of infection of LPS-pretreated mice that would have favoured a better disease control, considering the direct

trypanocidal activity of TNFα (Olivares Fontt et al., 1998). Further studies are required to establish whether the protective state is correlated with an additional mechanism depending on macrophage activity. Wirth et al., demonstrated that the ability of PMs to kill the *T. cruzi* intracellular forms was enhanced by IL-4 (Wirth et al., 1989).

Conclusions

Extending our former demonstration on the failure of C57BL/6 mice to control acute *T. cruzi* we now add evidence that such deficit might be also related with their inability to mount a satisfactory parasite-specific IgG response, coupled with an increased TNFα production by PMs. LPS-pretreatment promoted a better balance between pro- and anti-inflammatory cytokine syntheses by PMs, but impaired their ability to control intracellular parasite growth, implying that additional mechanisms are operating in the in vivo protective state.

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