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IL-10 up-regulates nitric oxide (NO) synthesis by lipopolysaccharide (LPS)-activated macrophages: improved control of *Trypanosoma cruzi* infection

F. JACOBS, D. CHAUSSABEL, C. TRUYENS*, V. LECLERQ, Y. CARLIER*, M. GOLDMAN & B. VRAY
*Laboratoire d'Immunologie Expérimentale and *Laboratoire de Parasitologie, Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium*

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SUMMARY

We examined the effects of IL-10 on tumour necrosis factor-alpha (TNF-α) and NO production by LPS-activated macrophages and on the ability of these cells to control *Trypanosoma cruzi* infection. We first observed that the addition of rIL-10 to macrophages of the J774 cell line decreased their synthesis of TNF-α but increased their release of NO in a dose-dependent manner. In parallel, treatment of J774 cells with rIL-10 resulted in a better control of *T. cruzi* infection involving up-regulation of NO synthesis, as it was not observed in presence of N-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase. The enhancing effect of rIL-10 on NO production was not observed on peritoneal macrophages from wild-type C57Bl/6 mice, but well on macrophages from IL-10 knock-out mice. The control of NO production by endogenous IL-10 was confirmed by the demonstration that neutralization of IL-10 secreted by LPS-activated macrophages from wild-type mice inhibited their production of NO and, in parallel, their ability to control *T. cruzi* infection. Taken together, these data demonstrate that both exogenous and endogenous IL-10 up-regulate the production of NO by LPS-activated macrophages and improve thereby their ability to clear *T. cruzi* infection.

Keywords IL-10 *Trypanosoma cruzi* nitric oxide lipopolysaccharide macrophages

INTRODUCTION

When suitably activated, murine macrophages release NO by catabolizing L-arginine *via* an inducible nitric oxide synthase (iNOS; [1]). NO has a major role in macrophage defences against intracellular microorganisms, including parasites such as *Trypanosoma cruzi* (reviewed in [2]). Among the agents stimulating NO synthesis by macrophages, the combination of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) is especially active as well as bacterial LPS [3,4].

IL-10 is known as a potent macrophage-deactivating cytokine [5,6]. In this context, IL-10 has been shown to inhibit IFN-γ-dependent NO production by macrophages and thereby their ability to control parasitic infections [2]. However, the effect of IL-10 on LPS-induced NO production has not been clearly defined so far [5,7–11]. The present study was therefore undertaken to determine the effects of exogenous or endogenous IL-10 on NO production in this setting. The demonstration that IL-10 up-regulates NO production by LPS-activated macrophages led us to analyse the influence of IL-10 on the ability of these cells to control *T. cruzi* infection *in vitro*.

Correspondence: F. Jacobs, Clinique des Maladies Infectieuses, Hôpital Erasme, 808 route de Lennik, B-1070 Brussels, Belgium.

MATERIALS AND METHODS

Reagents

IFN-γ was kindly provided by Dr A. Billiau and Dr H. Herremans (Catholic University of Louvain, Belgium). LPS (*Escherichia coli*, serotype 0111:B4), and N-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St Louis, MO). Recombinant murine IL-10 (rIL-10) was obtained as culture supernatants from Sf9 insect cells stably transfected with the corresponding complementary DNA, as previously described [12], using the baculoviral expression vector pBlue Bac2 (Invitrogen, San Diego, CA). It was semipurified by ionic separation chromatography. The IL-10 concentration of the preparation was determined by ELISA (Genzyme Co., Cambridge, MA). A neutralizing anti-IL-10 MoAb (JES5-2A5, a rat IgG1) was obtained in ascites form [13]. As isotype-matched control, we used ascites of LO-DNP-2 hybridoma cells (kindly provided by Dr H. Bazin, Catholic University of Louvain, Belgium), secreting a rat immunoglobulin G1 MoAb with anti-dinitrophenyl specificity. LPS levels of all the reagents and media were tested using the colourimetric Limulus Amoebocyte Lysate assay (detection limit 1 pg/ml; Coatest Endotoxin Chromogenix, Mölndal, Sweden) and were < 15 pg/ml.

Murine macrophage cell line

The murine macrophage cell line J774 was kindly provided by Professor G. Milon (Institut Pasteur, Paris, France). Cells were grown in RPMI 1640 supplemented with heat-inactivated fetal bovine serum (FBS; 10%), L-glutamine 2 mM, non-essential amino acids, N-2-hydroxyethylpiperazine-N′-2 ethanesulphonic acid 25 mM, penicillin 100 U/ml, and streptomycin 100 μg/ml (GIBCO BRL, Gaithersburg, MD). Macrophages were irradiated before culture (30 Gy, Mark 1-68A Irradiator; J. L. Shepherd & Associates, San Fernando, CA) to inhibit further cell replication.

Mouse peritoneal macrophages

Male C57Bl/6 and C57Bl/6 IL-10 knock-out mice (IL-10 KO), 8–12 weeks old (Jackson ImmunoResearch Labs, West Grove, PA) were maintained in our animal facilities on standard laboratory chow. They were killed by ether inhalation. Mouse peritoneal macrophages (MPM) were obtained as previously described [14]. They were allowed to adhere (4×10^5 MPM/well) in 24-well microplates (Nunc, Roskilde, Denmark) on a coverslip (13 mm diameter, Thermanox; Miles Scientific, Napierville, IL) placed in the plates for 2 h at 37°C in a 5% CO_2 water-saturated atmosphere. Non-adherent cells were removed by washing with prewarmed culture medium before adding reagents and parasites.

Trypanosoma cruzi *infection and activation of macrophages*

Trypanosoma cruzi trypomastigotes were obtained *in vitro* from infected fibroblasts as previously described [15]. They were centrifuged (15 min, 1800 ***g***, 4°C), resuspended in macrophage culture medium and added to adherent macrophages in a 5/1 parasite–cell ratio. Infected macrophages were incubated at 37°C in a CO_2 atmosphere. LPS (1 μg/ml) was added at the time of *T. cruzi* infection.

Macrophages were treated by adding either rIL-10, neutralizing anti-IL-10 MoAb (JES5-2A5) or isotype-matched control MoAb (LO-DNP-2). These reagents were added at the time of LPS activation and *T. cruzi* infection.

At 16 h post-infection (p.i.), culture supernatants were harvested for TNF-α, IL-10 or NO measurements. The cell cultures were washed with prewarmed medium to remove free parasites and medium was replaced with the respective reagents. At 48 h p.i., the macrophages were fixed with methanol, stained with Giemsa and examined by an optical microscope. The percentage of infected macrophages was recorded after examination of at least 200 cells per well [14].

Murine IL-10, TNF-α and NO assays

Murine IL-10 concentrations were determined by ELISA with a commercial kit (Genzyme, Cambridge, MA). The limit of detection of the test was 0·015 U/ml.

TNF-α was assayed, as described elsewhere [16], by sandwich ELISA using two rabbit anti-mouse TNF-α polyclonal antibodies kindly provided by W. A. Buurman (Department of Surgery, University of Limburg, Maastricht, The Netherlands). The detection limit of the assay was 20 pg/ml.

NO production was evaluated by measuring nitrite, its stable degradation product, by the Griess reaction [17]. Each culture supernatant (50 μl) was added to 50 μl of the Griess solution (1% sulfanilamide, 0·1% naphthylethylene diamine dihydrochloride, 2% H_3PO_4). The absorbance was measured at 540 nm in a microplate ELISA reader (Titertek Multiscan MCC/340, MKII EFLAB, Finland). Sodium nitrite ($NaNO_2$) diluted in culture medium was used as a standard. The detection limit of the assay was 1·0 μM.

RESULTS

rIL-10 up-regulates NO synthesis by LPS-activated J774 macrophages

We first investigated the effect of exogenous rIL-10 on NO production by macrophages of the J774 cell line. Preliminary data showed that rIL-10 (10 U/ml) decreased basal TNF-α production by resting macrophages ($0\cdot44\pm0\cdot05$ *versus* $0\cdot25\pm0\cdot04$ ng/ml; $P<0\cdot01$, Student's *t*-test), while their production of NO

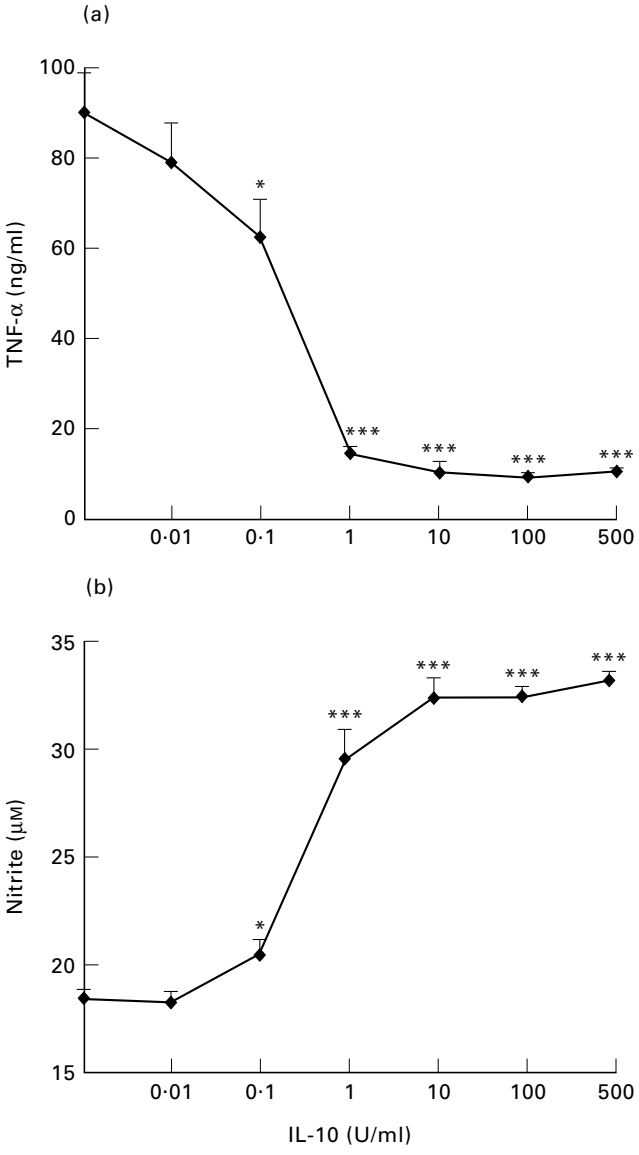

Fig. 1. Effects of rIL-10 on the production of tumour necrosis factor-alpha (TNF-α) and NO by LPS-activated J774 macrophages. J774 cells were activated with LPS (1 μg/ml) and treated with the indicated concentrations of rIL-10. TNF-α (a) and nitrite levels (b) were determined in culture supernatants collected after 16 h. Data are expressed as mean ± s.d. of three independent experiments performed in triplicate. *$P<0\cdot05$; ***$P<0\cdot001$ compared with LPS-activated J774 cells in the absence of rIL-10 (Student's *t*-test).

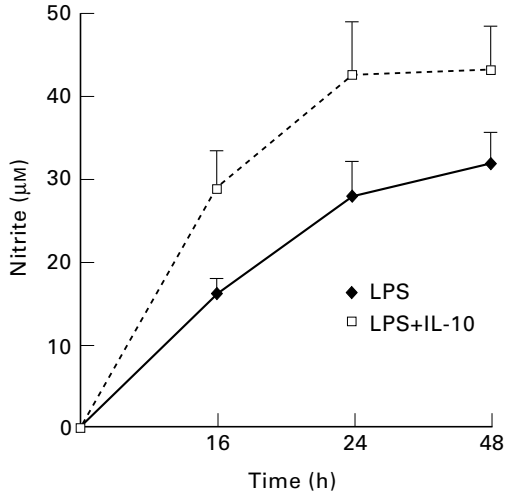

Fig. 2. Kinetics of nitrite production by LPS-activated J774 macrophages. J774 cells were activated with LPS (1 μg/ml) in the absence or presence of rIL-10 (10 U/ml) and nitrite levels were determined in supernatants collected at the indicated time points. Data are shown as mean ± s.d. of three independent experiments performed in triplicate. $P = 0{\cdot}01$, 0·02 and 0·04 after 16, 24 and 48 h, respectively, for macrophages treated with LPS and IL-10 compared with macrophages treated with LPS alone (Student's *t*-test).

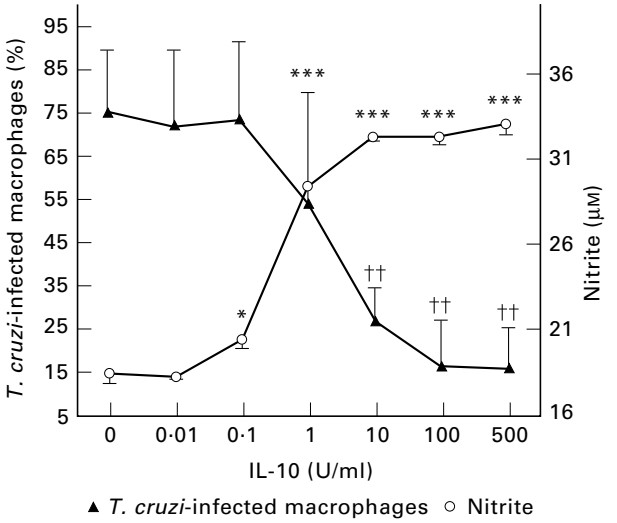

Fig. 3. Effects of rIL-10 on LPS-activated J774 macrophages infected with *Trypanosoma cruzi*. *Trypanosoma cruzi*-infected cells were activated with LPS (1 μg/ml) and incubated with the indicated concentrations of rIL-10. Nitrite levels were determined in culture supernatants collected after 16 h and the percentages of infected macrophages were determined after 48 h. Data are expressed as mean ± s.d. of three independent experiments performed in triplicate. $*P < 0{\cdot}05$; $***P < 0{\cdot}001$ compared with cells not treated with rIL-10, for nitrite concentrations; $††P < 0{\cdot}01$ for percentages of infected macrophages (Student's *t*-test).

remained below detection levels in the absence as well as in the presence of rIL-10. J774 macrophages were then activated with LPS (1 μg/ml) in the presence of graded concentrations of rIL-10 (0–500 U/ml) and culture supernatants were collected after 16 h for determination of NO and TNF-α levels. As expected, TNF-α production induced by LPS was inhibited by rIL-10 (Fig. 1a). In contrast, rIL-10 enhanced NO production in a dose-dependent manner (Fig. 1b). The activating effect of rIL-10 on NO production was already observed with 0·1 U/ml rIL-10 and was maximal at 10 U/ml rIL-10 (80% increase compared with LPS-activated J774 macrophages in the absence of rIL-10). As control, we verified that control supernatant of untransfected Sf9 insect cells had no effect in this system (data not shown). Moreover, we found that addition of 1 μg/ml of neutralizing anti-IL-10 MoAb completely inhibited rIL-10-induced NO up-regulation, whereas an isotype-matched irrelevant MoAb had no effect.

The enhanced NO production induced by rIL-10 was observed early after LPS activation (Fig. 2). Indeed, the overproduction of NO by macrophages treated with LPS and rIL-10 compared with macrophages cultured with LPS alone was more pronounced after 16 h of culture (77% increase) than after 48 h (36% increase).

rIL-10 enhanced T. cruzi *control by LPS-activated J774 macrophages through an increase of NO production*

To evaluate the relevance of rIL-10-induced NO overproduction in terms of macrophage defences, we investigated the ability of LPS-activated macrophages to control *T. cruzi* infection in the presence or absence of rIL-10. Irradiated J774 macrophages were treated with LPS (1 μg/ml) and graded concentrations of rIL-10 (0–500 U/ml) and incubated with *T. cruzi* trypomastigotes at a 5/1 parasite–cell ratio. Culture supernatants were collected after 16 h for determination of NO levels and macrophages were stained after 48 h to detect the presence of intracellular parasites. As shown in Fig. 3, the increased production of NO in the presence of rIL-10 was associated with a dose-dependent decrease in the percentages of infected macrophages, a maximal effect being achieved with 100 U/ml of rIL-10. These experiments were repeated on macrophages stimulated with a lower dose of LPS (10 ng/ml) and similar effects of rIL-10 were observed (data not shown).

To evaluate the role of NO overproduction in the enhanced control of *T. cruzi* infection induced by rIL-10, J774 infected macrophages were cultured with LPS (1 μg) and rIL-10 (10 U/ml) in the presence or absence of L-NAME (5 mM), a competitive inhibitor of NO synthase. The major decrease in NO production induced by L-NAME was associated with a four-fold increase in the percentage of infected macrophages (Table 1), indicating that the improved control of *T. cruzi* by rIL-10-treated macrophages involved NO overproduction.

Table 1. NO production and control of *Trypanosoma cruzi* infection by LPS-activated J774 cells

Culture conditions†	Nitrite (μM/10^5 cells)‡	Infected cells (%)‡
Medium	8·6 ± 1·7‡	54 ± 8·6‡
rIL-10	18·3 ± 3·4*	15·8 ± 11·2*
rIL-10 + L-NAME	4·2 ± 0·8**	66·5 ± 4·9 **

† LPS-activated J774 cells were treated with rIL-10 (10 U/ml) in the presence or absence of N-nitro-L-arginine methyl ester (L-NAME) (5 mM); nitrite levels in supernatants were measured after 16 h and the percentages of infected cells were determined after 48 h.

‡ Data are expressed as mean ± s.d. of three independent experiments performed in triplicate.

$*P < 0{\cdot}01$ compared with LPS-activated macrophages; $**P < 0{\cdot}01$ compared with LPS/IL-10-treated J774 cells (Student's *t*-test).

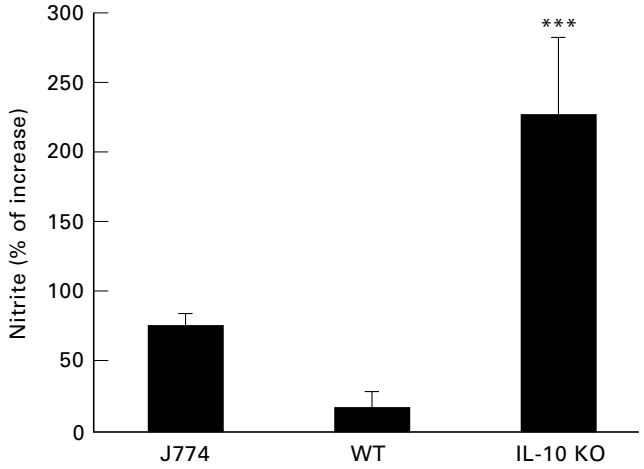

Fig. 4. rIL-10 up-regulates NO production by peritoneal macrophages of IL-10-deficient but not wild-type mice. J774 cells and peritoneal macrophages from either IL-10-deficient mice (IL-10 KO) or wild-type control C57Bl/6 mice (WT) were treated as described in the legend of Fig. 3 in absence or presence of rIL-10 (10 U/ml); nitrite levels were measured in culture supernatants after 16 h. Results are expressed as percentages of increase compared with macrophages not treated with rIL-10 (mean ± s.d. of three independent experiments performed in triplicate). ***$P < 0{\cdot}001$ compared with macrophages from wild-type mice (Student's *t*-test).

LPS-induced NO production by MPM was partially due to endogenous IL-10

In the next series of experiments, we analysed the effect of rIL-10 on MPM activated with LPS (1 μg/ml) and simultaneously infected with *T. cruzi* trypomastigotes. On these cells, rIL-10 (10 U/ml) did not significantly influence either NO production measured in culture supernatants after 16 h ($11{\cdot}2 \pm 2{\cdot}2\,\mu$M *versus* $13{\cdot}2 \pm 2{\cdot}9\,\mu$M in absence of rIL-10) or the percentages of *T. cruzi*-infected cells determined after 48 h ($36{\cdot}8 \pm 9{\cdot}8\%$ *versus* $21{\cdot}1 \pm 6{\cdot}3\%$ in absence of rIL-10). The difference between the responses of J774 cells and peritoneal macrophages to rIL-10 led us to compare their production of cytokines upon LPS activation. After 16 h of LPS stimulation (1 μg/ml), J774 cells did not produce detectable levels of IL-10 but produced high levels of TNF-α ($90{\cdot}1 \pm 8{\cdot}9$ ng/ml), whereas significant levels of IL-10 ($3{\cdot}5 \pm 0{\cdot}2$ U/ml) and low levels of TNF-α ($5{\cdot}2 \pm 0{\cdot}8$ ng/ml) were detected in supernatants of peritoneal macrophages. This led us to consider that endogenous IL-10 might control NO production in peritoneal macrophages. Indeed, we found that rIL-10 induced a major increase in peritoneal cells from IL-10-deficient mice, whereas it had no significant effect on peritoneal cells from wild-type mice of the same background (C57Bl/6) (Fig. 4).

Finally, we further analysed the regulatory role of endogenous IL-10 on NO production by neutralizing endogenous IL-10 produced by normal peritoneal macrophages. As shown in Fig. 5, addition of a neutralizing anti-IL-10 MoAb (JES5-2A5; 10 μg/ml) to peritoneal macrophages incubated with LPS (1 μg/ml) and infected with *T. cruzi* significantly reduced their NO production and their ability to control *T. cruzi* infection, whereas an irrelevant isotype-matched MoAb had no effect on either parameter.

DISCUSSION

Although the deactivating effects of IL-10 on monocytes/macrophages and its ability to inhibit Th1-type responses are well established, there is growing evidence that IL-10 cannot be simply considered as an anti-inflammatory and immunosuppressive cytokine. As a matter of fact, IL-10 was found to enhance cytolytic activities of $CD8^+$ cells and natural killer (NK) cells and to favour antibody-dependent cellular cytotoxicity [18–22].

It appears that the effects of IL-10 on NO production are highly dependent on the experimental system considered, as both suppression and up-regulation of NO production by IL-10 were reported in

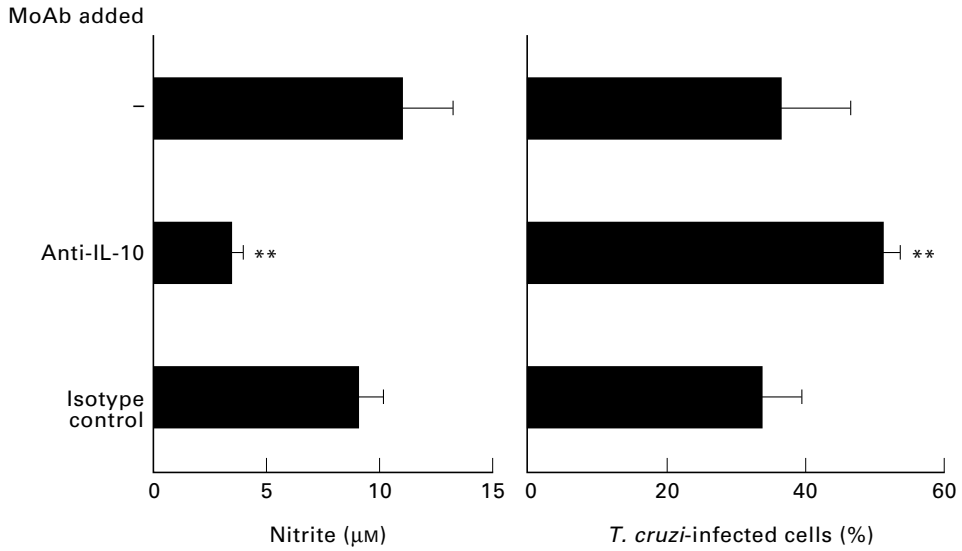

Fig. 5. Effects of endogenous IL-10 neutralization in LPS-activated peritoneal macrophages infected with *Trypanosoma cruzi*. Mouse peritoneal macrophages from wild-type C57Bl/6 mice were activated with LPS (1 μg/ml), infected with *T. cruzi* and cultured either in medium alone or in the presence of either neutralizing anti-IL-10 MoAb (JES5-2A5; 10 μg/ml) or the same concentration of its isotype-matched control (LO-DNP-2; 10 μg/ml). Nitrite levels were determined in culture supernatants after 16 h and the percentages of infected macrophages were determined after 48 h. Data are shown as mean ± s.d. of triplicate determinations in three different experiments. **$P < 0{\cdot}01$ compared with isotype control MoAb (Student's *t*-test).

previous studies. Indeed, although IL-10 down-regulates the production of NO by IFN-γ-activated macrophages, it increases NO production when macrophages are activated with IFN-γ and TNF-α [23], and also when macrophages are treated by IL-10 during 3 days and then activated by high doses of LPS [24]. In the present study, we found that exogenously added as well as endogenously produced IL-10 up-regulated NO production by LPS-activated macrophages. In both settings, the ability of macrophages to control *T. cruzi* infection was enhanced as a consequence of the increased NO synthesis.

Our observations that both exogenous and endogenous IL-10 are protective in an *in vitro* model of *T. cruzi* infection of LPS-activated macrophages are in contrast with other *in vitro* studies showing that IL-10 impairs the macrophage defences against intracellular microorganisms in the case of IFN-γ activation. As for *in vitro* studies, the role of IL-10 during *in vivo* infections is not clear. Although IL-10 was shown to promote the outgrowth of *Streptococcus pneumoniae* [25], group B *Streptococcus* [26], *Chlamydia trachomatis* [27], *Klebsiella pneumoniae* [28], *Salmonella* [29], *Candida* [30,31], *Legionella pneumophila* [32], *Mycobacterium avium* [33,34], a protective effect of IL-10 was recently described in *Pseudomonas aeruginosa* pneumonia [35]. The role of endogenous IL-10 in the course of experimental *T. cruzi* infections was investigated in IL-10 knock-out mice: IL-10-deficient mice had lower parasite burden than their wild-type counterparts but their mortality rate was higher, most likely because of the toxicity associated with the overproduction of IL-12, IFN-γ and TNF-α [36,37]. Similar results have been observed in *Toxoplasma* [38,39] and *P. chabaudi* [40] infections. Moreover, *in vivo* injection of rIL-10 in *T. cruzi*-infected mice resulted in higher levels of parasitaemia, but did not affect either the mortality rate or the histopathology [40].

In parallel with its stimulating effect on the production of inflammatory mediators, administration of LPS during *T. cruzi* infection resulted in early mortality [41,42]. In this setting, rIL-10 administration might not only protect against the acute cytokine-mediated pathology as indicated by previous studies in experimental endotoxaemia [12,43], but also promote parasite clearance by enhancing NO synthesis, as suggested by the *in vitro* observations reported in the present study.

In conclusion, we demonstrated that both exogenous and endogenous IL-10 up-regulate the production of NO by LPS-activated macrophages, which results in a better control of *T. cruzi* infection. These data confirm that IL-10 can up-regulate or down-regulate NO production by macrophages according to the experimental model.

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