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ORIGINAL INVESTIGATION

Reciprocal influences between leptin and glucocorticoids during acute *Trypanosoma cruzi* infection

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Abstract Leptin and glucocorticoids (GCs) are involved in metabolic functions, thymic homeostasis and immune activity through complex interactions. We recently showed that C57BL/6 mice infected with *Trypanosoma cruzi* revealed a fatal disease associated with a dysregulated immune–endocrine response characterized by weight loss, deleterious synthesis of pro-inflammatory cytokines and GCs-driven thymus atrophy. Extending this study, we now explored the relationship between leptin and GCs, in terms of infection outcome, thymic and metabolic changes. *T. cruzi*-infected mice showed a food intake reduction, together with hypoglycemia and lipolysis-related changes. Infected animals also displayed a reduction in systemic and adipose tissue levels of leptin, paralleled by a down-regulation of their receptor (ObR) in the hypothalamus. Studies in infected mice subjected to adrenalectomy (Adx) showed a worsened course of infection accompanied by even more diminished systemic and intrathymic leptin levels, for which GCs are necessary not only to decrease inflammation but also to sustain leptin secretion. Adx also protected from thymic atrophy, independently of the reduced leptin contents. Leptin administration to infected mice aggravated inflammation, lowered parasite burden and attenuated GCs release, but did not normalize thymic atrophy or metabolic parameters. Acute *T. cruzi* infection in C57BL/6 mice coexists with a dysregulation of leptin/hypothalamic ObR circuitry dissociated from body weight and food intake control. Endogenous GCs production attempted to reestablish systemic leptin concentrations, but failed to improve leptin-protective activities at the thymic level, suggesting that the leptin/GCs intrathymic relationship is also altered during this infection.

Keywords *Trypanosoma cruzi* · Leptin · ObR · Glucocorticoids

Introduction

It is known that central and peripheral influences are reciprocally established between the immune-endocrine response and energy metabolism. During infections, these actions take place to reduce food intake or to establish a new metabolic set point, because of the high energetic cost demanded by immune response [1–6]. In cases of an excessive or prolonged pro-inflammatory response, an adverse immune–endocrine–metabolic *milieu* may be established compromising the host integrity [2–4]. Leptin, the product of the obese (*ob*) gene, glucocorticoids (GCs) and certain cytokines such as TNF-α are actively involved in this setting. Moreover, leptin and GCs display complex

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bidirectional interactions involved in metabolic and immune responses [7, 8].

Leptin is a hormone/pro-inflammatory cytokine involved in various physiological processes [7]. Leptin derives mostly from adipocytes, but it is also produced by immune cells [9, 10]. Under physiological conditions, the amount of leptin produced by the adipose tissue (AT) is directly related to its mass. Shortly after food intake, leptin levels rise, interacting with its receptors in the hypothalamus [11], being followed by a decreased food intake and increased energy expenditure [12, 13]. Leptin also seems to be a critical mediator in the host defense during infections [14], stimulating innate responses as well as inducing Th1-type responses and inhibition of T regulatory cell activity [15–20]. Leptin also influences thymic homeostasis, since mice deficient in leptin or leptin receptor showed thymic atrophy associated with a reduction in double-positive (DP) thymocytes [21, 22]. Leptin production is regulated by several factors, such as hormonal agents and inflammatory mediators, with pro-inflammatory cytokines and chronic inflammation augmenting or suppressing its production, respectively [13, 23, 24]. GCs also stimulate leptin gene expression and secretion irrespective of the effects on food intake [25–28] although an inverse relationship between leptin levels and the activity of the hypothalamus pituitary adrenal (HPA) axis has already been reported in stressful situations [29, 30]. In a non-infectious setting, leptin protects against thymic atrophy induced by an augmented GCs production [31, 32].

Recently, a potential link between metabolic abnormalities and Chagas disease was envisaged [33–35]. Studies during *Trypanosoma cruzi* infection, the causative agent of Chagas disease, have shown that diabetic mice deficient in leptin receptor are more susceptible to acute infection [36, 37]. A series of studies carried out in our laboratory in normoglycemic C57BL/6 mice undergoing an acute *T. cruzi* infection revealed a severe body weight loss, thymic involution and lethal outcome. This was associated with a deleterious synthesis of TNF-α and remarkably increased production of GCs [38–40]. GCs ablation significantly ameliorated thymic atrophy, but shortened survival time leading to a further increase in TNF-α levels in circulation [41].

The cross-regulation between leptin and GCs [7, 8] prompted us to ascertain the potential and still unexplored reciprocal influence between both compounds during acute *T. cruzi* infection, in intact animals or those subjected to adrenalectomy (Adx) or leptin administration. End-points comprised infection outcome as well as thymic and metabolic changes.

Studies indicate that the exacerbated immune activation of acutely infected animals coexists with profound alterations in the leptin/ObR circuitry and leptin/GCs relationship favoring thymic atrophy and the aggravated acute disease.

Materials and methods

Animals and experimental infection

Wild-type male C57BL/6 and deficient mice for TNF-R1 (TNF-R1 KO) were bred at the animal facilities from the School of Medical Sciences of Rosario and infected at 8–10 weeks of age. TNF-R1 KO mice, originally obtained from The Jackson Laboratory, were gently provided by Dr. Silvia Di Genaro. Animals had access to food and water ad libitum and were subcutaneously injected with 1000 viable trypomastigotes of the Tulahuén strain of *T. cruzi*. Also, uninfected animals were used as controls. Animals were allocated in temperature-controlled rooms under light–dark 12-h cycles and were handled according to institutional guidelines. Protocols for animal studies were approved by the School of Medical Sciences Institutional Ethical Committee (Resolution No. 0040/2011). Parasites were maintained by serial passages in Cbi suckling mice. Parasitemias were evaluated by the method of Brenner, as previously described [38]. Survival and food intake were monitored on a daily basis. To perform surgical procedures or to collect peritoneal exudates, animals were anesthetized with xylaxine/ketamine (2 mg/kg xylaxine + 100 mg/kg ketamine). To assess metabolic parameters after 4–6 h of fasting (i.e., glycemia), mice were killed at different days post-infection (dpi, 14, 17 or 21 days, as applicable) by CO_2 inhalation and blood samples were collected on heparin by cardiac puncture. Epididymal fat pads and thymi were collected and weighed to calculate thymus relative weight (thymus weight/body weight ×100) and the adiposity index (AI, epididymal fat pads weight/body weight ×100).

Plasma leptin, corticosterone and TNF-α assays

To evaluate plasma leptin, mice were bled by cardiac puncture. Blood was collected in sterile, endotoxin-free tubes and kept refrigerated until centrifugation. Plasma was frozen at −80 °C until used. At the same time, epididymal fat pads and thymi were removed, weighted and homogenized in 2 volumes of 300 mmol/L sucrose with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 1 μg/mL aprotinin). Homogenates were kept at −80 °C until used. Leptin and TNF-α concentrations were measured in blood or tissue samples by a specific ELISA kit, according to the manufacturer's specifications (murine leptin 900-K76, PeproTech, Mexico, and BD Pharmingen, San Diego, CA, respectively). Detection limits were 20 pg/mL for leptin and 15.6 pg/mL for TNF-α.

For corticosterone determinations, mice were housed individually for 1 week before sampling and kept single-caged throughout the experiments. Plasma samples were obtained from the tip of the tail between 8 and 10 a.m. Plasma corticosterone levels were assessed by ELISA, according to the manufacturer's specifications (IBL, Germany). Detection limit was 0.20 μg/dL.

Assessment of glucose, total cholesterol, triacylglycerol and glycerol levels in plasma

Immediately after blood extraction by cardiac puncture, plasma concentrations of glucose, total cholesterol, triacylglycerol and glycerol were assessed using diagnostic enzymatic/colorimetric kits according to the manufacturer's specifications (Wiener Lab, Rosario, Argentina). All samples were assayed in duplicate. The ratio of glycerol plasma levels (mmol/L) to the weight of epididymal fat pads (g) was also calculated to further approach lipolysis analysis [42].

Adrenalectomy and glucocorticoid supplementation

Mice were subjected to bilateral Adx performed via a dorsal approach. Two small incisions were made on each side of the back just below the rib cage, and the adrenal glands were removed with curved forceps. Sham-operated mice (Sham) were operated in a similar manner, without removing the adrenals. The animals were used 1 week after the operation for further experimentation. For GCs supplementation, mice were treated with dexamethasone at 0.6 μg/dL/day during 14 days, beginning at the moment of infection.

In vivo leptin treatment

Leptin was given in 10 consecutive injections beginning 7 days after infection. Infected mice received intraperitoneal injections of sterile PBS or recombinant murine leptin dissolved in sterile PBS at a dose of 1 μg/g body weight/day (PeproTech, Mexico). Uninfected mice also received sterile PBS or recombinant leptin.

Tissue histology and microscopy

After mice were killed, epididymal fat pads, thymi, hearts and hypothalami were removed, sliced transversally in three sections, fixed in buffered formalin or embedded in Tissue-Tek (Miles Inc., Elkhart, USA) and frozen in liquid nitrogen. Paraffin-embedded 5-μm sections were stained with hematoxylin and eosin. The area of adipocytes in epididymal fat pads (AA, pixel^2 $\times$ 10^5, n = 3–5 mice/day) was evaluated by light microscopy and subsequent digital analysis recording an area of 10 consecutive adipocytes per field (magnification 40×, Nikon Free Viewer Program). The degree of inflammatory infiltrate was determined qualitatively (absent, mild and severe) in at least three serial section of each sample by a blinded experienced pathologist. For immunofluorescence studies, 5-μm-thick cryostat sections from epididymal fat pads and hypothalamus were settled on poly-L-lysine (SIGMA)-covered glass slides, acetone fixed and blocked with PBS-BSA 1 %. Sections were incubated with 1:25 anti-ObR (goat) (sc-33981, Santa Cruz Biotechnology, INC) and 1:50 anti-leptin (rabbit) (sc-9014, Santa Cruz Biotechnology, INC), respectively, for 1 h at room temperature, washed and subjected to appropriate secondary antibody, 1:200 donkey anti-goat IgG-FITC (sc-2024, Santa Cruz Biotechnology, INC) for ObR and 1:200 goat anti-rabbit conjugated with Alexa 546 (Invitrogen) for leptin. In negative controls, the immune reaction was controlled omitting the primary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Samples were analyzed by confocal microscopy using a Nikon C1 plus Eclipse TE-2000-E2 (Nikon, Melville, NY, USA), and the images obtained were subsequently analyzed using the Image J software. An experienced pathologist blinded to the study groups examined these sections.

Flow cytometry and data analysis

For flow cytometry assays of thymic subpopulations and natural regulatory $CD4^+FoxP3^+$ T cells (nTregs), 10^6 thymocytes were resuspended in flow buffer (PBS supplemented with 10 % fetal bovine serum) and stained in one step with FITC-coupled anti-CD4 and APC-coupled anti-CD25 (mouse regulatory T cell staining kit, eBiosciences) and PerCP-coupled anti-CD8 (BD PharMingen, San Diego, CA, USA). Afterward, intracellular staining was performed using PE-coupled anti-FoxP3 and the corresponding fixation/permeabilization buffers (mouse regulatory T cell staining kit, eBiosciences). A minimum of 10^5 events was acquired using an ARIAII flow cytometer (Becton–Dickinson, New Jersey, USA). Living cells were gated on the basis of forward- and side-cell scatter. Results were analyzed using DIVA (Becton–Dickinson, New Jersey, USA).

Real-time PCR

RNA was isolated using TRIzol (Invitrogen, Life Technologies) and reverse-transcribed to cDNA using the M-MLV Reverse Transcriptase (Promega, Madison, USA). Real-time PCR was performed with the Real-Time PCR

System (Stratagene) using a Mix (Mezcla Real Kit, Biodynamics, Buenos Aires, Argentina) according to the manufacturer's instructions.

The amplification program included an initial denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s and annealing and extension at 56 °C for 1 min, for 40 cycles. Fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. The house-keeping gene GAPdH was used as an internal control. The relative gene expression levels were calculated using the comparative ΔΔCT method, where CT represents the threshold cycle and ΔCT = mean CT genes of interest−mean CT GAPDH. Results were expressed as the relative difference to the uninfected control as $2^{-\Delta\Delta CT}$. Every sample was run in three parallel reactions.

Primers used to amplify specific gene products from murine cDNA were: leptin sense, 5′-TgACATTTCACAC ACgCAgTCgg-3′; leptin antisense 5′-TTggAgAAggCCAg CAgATggAg-3′; ObR sense, 5′-CACTCTCTCTTCTgTTg ACg-3′; ObR (isoforms b and a) antisense, 5′-AAAgATg AgATggTCCCAgC-3′; TNF-α sense, 5′-ATgAgCACAg AAAgCATgATCCgCgAC-3′; TNF-α antisense 5′-TCACA gAgCAATgACTCCAAAgTAgACCTg-3′; GAPdH sense, 5′-AgCAATgCATCCTgCACCACCA-3′, GAPdH antisense 5′-ATgCCAgTgAgCTTCCCgTTCA-3′.

Phagocytosis assay

The phagocytosis assay was performed using heat-killed *Saccharomyces cerevisiae* as described previously [43]. Peritoneal macrophages (PM) were obtained from 14 dpi. Cells were centrifuged and resuspended in MEM (Sigma Chemical Co. St Louis, MO, USA) and cultured overnight in 8-well plates (Lab-Tek™ chamber slide, NUNC, Naperville, IL, USA) at a concentration of 50,000 cells/well containing the same medium supplemented with 10 % fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), gentamycin 0.2 % (10 mg/mL, GIBCO), 2 % penicillin–streptomycin and 2-mercaptoethanol. After 16 h, the culture medium was replaced and cells were exposed for 3 h to heat-killed yeast at 5:1 yeast–PM ratio at 37 °C in a 5 % CO_2 atmosphere. Briefly, after phagocytosis assay, the medium was removed and PM were washed twice with PBS, to remove yeast that might not have entered the cells. Then, samples were stained with May-Grünwald-Giemsa (SIGMA). Ten microscopic fields were randomly selected throughout the slide, and all PM in each particular microscopic field were examined. The percentage of phagocytosis was calculated as the number of macrophages with ingested yeast over total macrophages per 100.

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM), unless otherwise stated. Comparisons were made by the nonparametric analysis of variance Kruskal–Wallis ($k > 2$ groups) followed by post hoc comparisons, when applicable. Correlation analyses were carried out using the nonparametric Spearman test. The GraphPad Instat 4.0 software (GraphPad, California, USA) was applied for graphs and statistical analysis. Differences were considered significant when p value was <0.05.

Results

Corticosterone and leptin response patterns in *T. cruzi*-infected mice

As recorded earlier, *T. cruzi*-infected mice showed a gradual increase in their systemic levels of corticosterone, which at 21 dpi were nearly sevenfold augmented compared with their baseline values. In contrast, circulating leptin levels remarkably decreased as disease progressed (nearly to 90 % at 21 dpi) (Fig. 1a). Also, leptin in epididymal fat pads practically disappeared at 21 dpi (Fig. 1b–c), which were corroborated by immunofluorescence studies of protein expression from the same samples (Fig. 1d–e). Leptin transcripts expression in epididymal fat pads correlated particularly well with the reduction in AI (r_s: 0.9076, $n = 8$, $p = 0.0013$). Deviating from the well-known effects of leptin on food intake, hypoleptinemia was positively associated with diminished food intake in infected mice (r_s: 0.7343, $n = 12$, $p < 0.004$). To discard the influence of body weight on leptin levels, the plasma leptin/body weight ratio was calculated, showing a progressive and significant decrease during the course of infection [i.e., (pg/mL)/g—Control: 11.0 ± 1.7; Infected-21 dpi: 1.9 ± 0.5, $p < 0.005$, $n = 5$/group]. Circulating levels of TNF-α and its adipose expression were inversely related to leptin levels. Nevertheless, TNF-R1-deficient mice undergoing acute *T. cruzi* infection had a much more remarkable hypoleptinemia than the one seen in their infected wild-type counterparts, suggesting that TNF-α also concurs to sustain leptin production during infection (Appendix Fig. 1 on Supplementary Material). Characteristics of the course of infection in these deficient mice are detailed in the Appendix Table 1a on Supplementary Material.

Concerning the hypothalamic ObR expression, ObR transcripts in infected mice decreased significantly compared with their uninfected counterparts (Fig. 2a). Such a decrease was more noticeable at 14 dpi and tended to

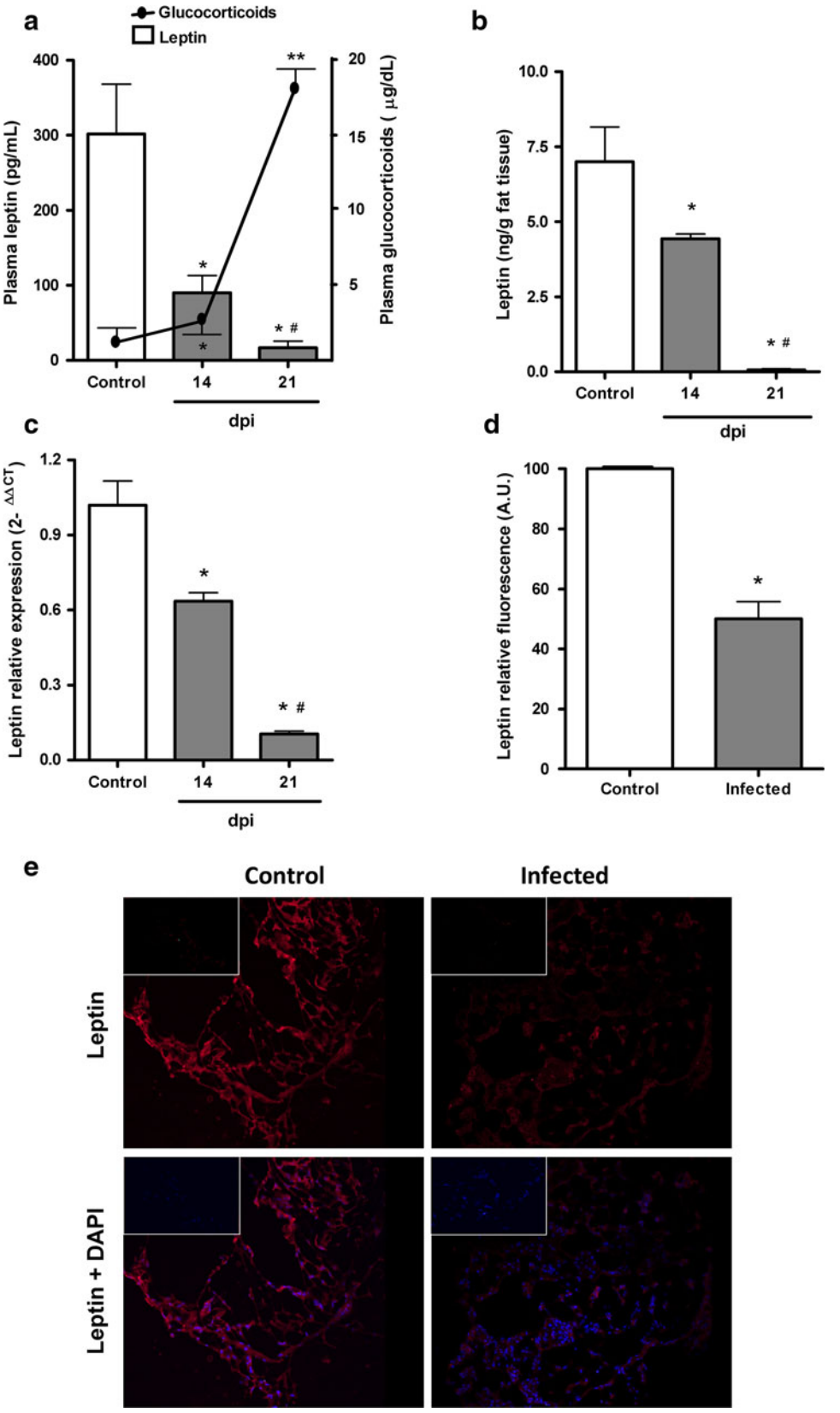

Fig. 1 Leptin and corticosterone response patterns in *T. cruzi*-infected mice. **a** Circulating leptin and corticosterone basal levels and at 14 and 21 days post-infection (dpi) detected by ELISA. **b** Leptin contents in epididymal fat pads homogenates detected by ELISA and normalized to total tissue weight at 14 and 21 dpi. **c** Leptin mRNA expression in epididymal fat pads from Control and Infected animals analyzed by real-time RT-PCR. Results are expressed as the relative difference to the uninfected Control by $2^{-\Delta\Delta CT}$ method. **d** Relative quantification of the intensity of fluorescence (arbitrary units (A.U.)) of leptin protein expression was made using Image J software from 3 to 5 microscopic fields of epididymal fat pads from Control and Infected animals (14 dpi, $n = 4$/group/day). **e** Representative immunofluorescence staining showing leptin expression in epididymal fat pads from Control and Infected mice after 14 dpi (*upper panels*, ×20 magnifications). DAPI was used for staining nuclei (*bottom panels*, ×20 magnifications). *Small boxes* correspond to negative control. For Figs. **a–d**, *bars* and *lines* represent the mean ± SEM of 6-8 mice/group/day. $^{*}p < 0.05$ versus Control group; $^{\#}p < 0.05$ versus 14 dpi. Results are representative of two or three independent experimental rounds; $^{**}p < 0.01$ versus Control group

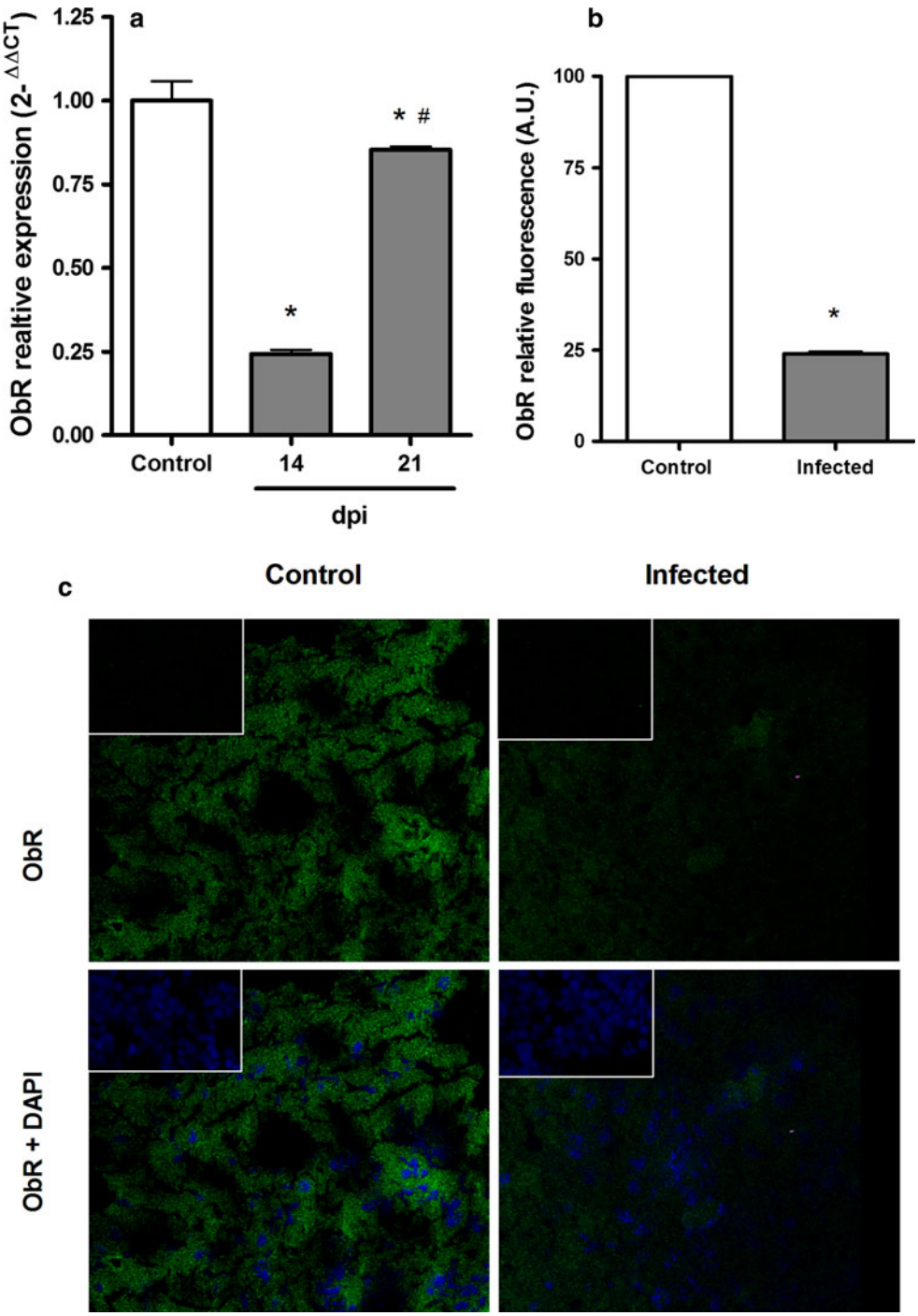

Fig. 2 Hypothalamic ObR expression **a** ObR mRNA expression levels in hypothalamus from Control and Infected C57BL/6 animals analyzed by real-time RT-PCR at 14 and 21 days post-infection (dpi). The results are expressed as the relative difference to the uninfected control by $2^{-\Delta\Delta CT}$ method. Values represent the mean ± SEM of 4-6 mice/group/day. **b** Relative quantification of the intensity of fluorescence (arbitrary units -A.U.-) of ObR protein expression was made using Image J software from 3 to 5 microscopic fields of hypothalamus from Controls and Infected animals (14 dpi, $n = 4$/group/day, mean ± SEM). **c** Representative immunofluorescence staining showing ObR expression in hypothalamus of Control and Infected C57BL/6 mice after 14 dpi (*upper panels*, ×60 magnifications) and DAPI was used for staining nuclei (*bottom panels*, ×60 magnifications). *Small boxes* correspond to negative control. Results are representative of two or three independent experimental rounds. $^{*}p < 0.05$ versus Control; $^{\#}p < 0.05$ versus 14 dpi

recover at 21 dpi, without reaching the levels seen in uninfected animals. Immunofluorescence studies also revealed a dense ObR expression in hypothalamic sections of control mice that was dramatically reduced in 14 dpi-infected counterparts (Fig. 2b–c). The low ObR central expression highly correlated with the reduced food intake (r_S: 0.8333, $n = 9$, $p < 0.002$).

Metabolic profile in *T. cruzi*-infected mice

For a more comprehensive picture of the acute infection, metabolic assessments were also performed. As seen in Table 1, C57BL/6 *T. cruzi*-infected mice showed a severe reduction in body weight and significant reduction in food intake. As well as confirming other studies reporting a

Table 1 Metabolic parameters in *T. cruzi*-infected mice during the course of infection

Parameters	Control	Infected-14 dpi	Infected-21 dpi
Body weight (g)	27.36 ± 0.33	27.33 ± 0.19	22.26 ± 0.61*#
Food intake (g/day/mouse)	3.86 ± 0.03	3.14 ± 0.11*	2.37 ± 0.16*#
Adiposity index	0.879 ± 0.06	0.576 ± 0.08*	0.236 ± 0.04*#
Glycemia (mg/dL)	156.6 ± 11.66	102.1 ± 8.04*	40.18 ± 7.57*#
Cholesterolemia (g/L)	1.72 ± 0.07	2.23 ± 0.17*	2.28 ± 0.16*
Triglyceridemia (g/L)	0.87 ± 0.36	1.73 ± 0.47*	1.78 ± 0.12*
Glycerol/mass *EFP* ratio (mmol/L/g)	4.0 ± 1.3	12.0 ± 7.0*	30.0 ± 5.5*

Metabolic parameters were evaluated in Control and Infected mice at 14 and 21 days post-infection (dpi). Values represent the mean ± SEM of 8–10 mice/group/day (one of three independent experiments). *$p < 0.05$ compared to control, #$p < 0.05$ compared to 14 dpi. Values in Control mice at the same time point evaluations showed no differences, for which they were grouped in one column

EFP epididymal fat pads

progressive decrease in fat mass and hypoglycemia during *T. cruzi* infection [37, 44–46], our mice also developed hypercholesterolemia and increased triglyceridemia. The progressive diminution in the area of adipocytes was compatible with lipolysis (Fig. 3a) and in line with the increased glycerol plasma levels/mass epididymal fat pads ratio seen throughout infection (Fig. 3b). Epididymal fat pads were progressively infiltrated by numerous mononuclear cells likely due to the presence of abundant amastigote nests (Fig. 3c). A significant local increase in TNF-α transcript expression was seen 2 weeks after infection (Appendix Fig. 1b on Supplementary Material), in coincidence with raised plasma levels of this cytokine [(pg/mL), i.e., Control: non-detectable; Infected-14 dpi: 360.3 ± 85.3, $p < 0.05$, $n = 5$/group].

Leptin and ObR expression in thymus during *T. cruzi* infection

Leptin-deficient signaling in mice was shown to result in chronic thymic atrophy [22]. We have previously reported that *T. cruzi* infection in C57BL/6 mice induces a thymic depletion of double-positive $CD4^+CD8^+$ (DP) cells mainly due to GCs [38]. To evaluate the possible involvement of leptin and its receptor on *T. cruzi*-induced thymus atrophy, we assessed leptin levels in the whole thymus. Accompanying systemic findings (Fig. 1a), a significant decrease in leptin contents was seen by the time that thymus atrophy became evident (Fig. 4a–b). Expression of its mRNA at the same time point remained within the levels recorded in Control mice (Fig. 4c), suggesting a post-transcriptional regulation of its expression or an enrichment of simple positive $CD4^+$ or $CD8^+$ leptin-producing cells. By opposite, mRNA ObR expression was increased in infected whole thymus (Fig. 4d), in parallel with the onset of thymic atrophy.

Influence of glucocorticoids on leptin secretion in *T. cruzi*-infected mice

Control animals undergoing Adx had a significant increase in basal leptin levels. Conversely, Adx in infected mice resulted in an exacerbated hypoleptinemia (Fig. 5a), together with a higher increase in plasma TNF-α levels, fasting hypoglycemia, lowered parasitemias and accelerated mortality, compared with sham-operated Infected mice (Appendix Table 1b on Supplementary Material). It follows that endogenous GCs are necessary not only to improve metabolic parameters and decrease inflammation, but also to sustain leptin secretion. Confirming earlier studies, Adx reversed the thymic atrophy accompanying *T. cruzi* infection. Measurements of thymus leptin content in Infected mice subjected to Adx revealed values similar to those seen in Control animals (data not shown). Analysis on hypothalamic samples from Infected mice undergoing Adx showed that the expression of ObR continued to be down-regulated (data not shown). Parallel experiments by treating Infected animals with dexamethasone, at pharmacological doses known to cause thymic atrophy, showed further decreased levels of leptin at 14 dpi (Fig. 5b).

Effects of in vivo leptin treatment

To ascertain whether in vivo leptin supplementation favored infection resistance and improved the course of infection, *T. cruzi*-infected mice were treated for 10 consecutive days with recombinant mouse leptin. As seen also in Table 2, the markedly decreased body weight and food intake, hypoglycemia, hypercholesterolemia and triglyceridemia from *T. cruzi*-infected mice remained unchanged by leptin administration. Leptin administration induced a progressive reduction in the AI, in both uninfected and Infected animals, more remarkable in the latter ones (Table 2). Also, epididymal fat pads from Infected and Infected plus leptin-treated animals at 17 dpi showed an intense reduction in adipocyte size (Fig. 6a, upper panels), accompanied by a significantly increased glycerol plasma/mass epididymal fat pads ratio (Table 2). In Infected animals, leptin administration enhanced myocarditis (Fig. 6a, bottom panels), lowered the number of myocardial amastigote nests (data not shown) and reduced parasitemias

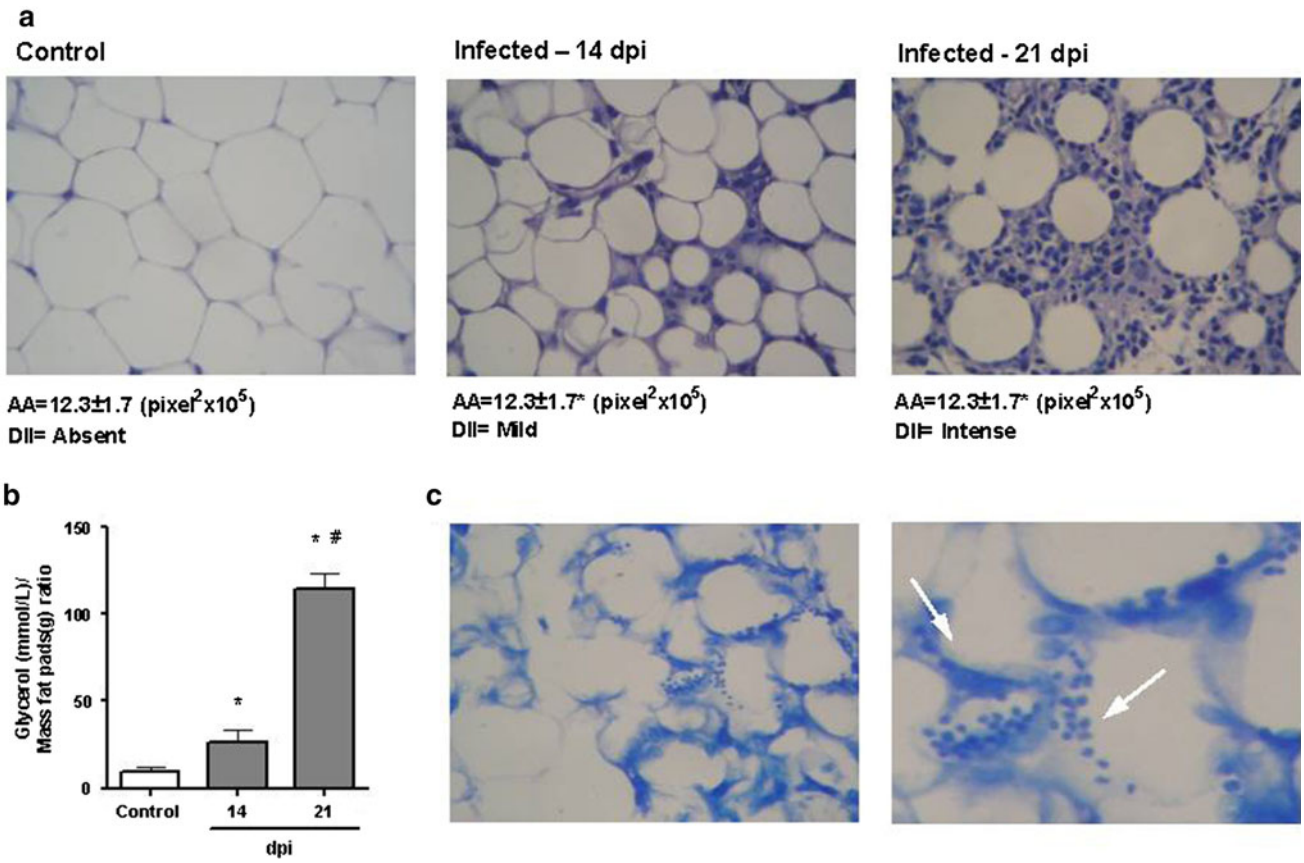

Fig. 3 Metabolic status and adipose tissue alterations during *T. cruzi* infection. **a** Representative photographs of epididymal fat pad samples stained with hematoxylin eosin at 14 and 21 days post-infection (dpi). The area of adipocytes (AA) was expressed as mean ± SEM, $n = 3$-5 mice/day. The degree of inflammatory infiltrate (DII) was analyzed in a blinded form (magnification ×40). **b** Plasma glycerol/mass of epididymal fat pads ratio during the infection. *Bars* and *lines* represent the mean ± SEM of data obtained from 4–6 mice/group. **c** Intracellular presence of the parasite nests in epididymal adipocytes at 14 dpi at different magnifications (×20 *left panel* and ×60 *right panel*). $^{*}p < 0.0001$ versus Control, $^{\#}p < 0.05$ versus 14 dpi. Results are representative of two or three independent experimental rounds

(Fig. 6b). To check whether this was due to an improved phagocytic capacity of macrophages, the phagocytosis of heat-killed yeast in PM from Control and Infected mice given leptin or not was analyzed. As seen in Fig. 6c, PM from leptin-treated uninfected mice had an enhanced phagocytosis ($p < 0.05$). Moreover, the phagocytic capacity of PM from Infected mice was increased irrespective of leptin treatment (Fig. 6c).

Since leptin supplementation during an inflammatory setting restored thymic involution [47, 48], we also analyzed the effects of leptin treatment on the *T. cruzi*-induced thymic atrophy. In control mice, leptin induced a slight thymic hypertrophy, while resulting in a severe thymic atrophy in the infected counterparts. This was mirrored by a significant diminution of the relative thymus weight [i.e., (mg/g) Infected-14 dpi: 0.090 ± 0.007, Infected + leptin-14 dpi: 0.053 ± 0.003, $p < 0.05$, $n = 5$/group] and a slightly decreased frequency of DP cells [i.e., (%) Infected-14 dpi: 68.0 ± 4.0; Infected + leptin-14 dpi: 53.4 ± 3.0, $p < 0.05$, $n = 5$/group]. Leptin treatment did not modify the absolute number of natural $CD4^{+}Foxp3^{+}$ regulatory T cells (nTregs) in uninfected animals, but caused a noticeable decrease in them in the Infected ones ($p < 0.05$). Strikingly, infected mice exhibited a significant increase in the absolute numbers of nTregs compared with the remaining groups (Fig. 6d).

Since increased endogenous GCs levels sustain leptin expression during in vivo infection (Fig. 5a), we further analyzed whether leptin treatment, in turn, modulated GCs synthesis. As expected, GCs levels in infected animals treated with leptin during 10 consecutive days were significantly reduced (Fig. 6e), without reaching baseline values. Such diminution was not observed after 5–7 days of leptin treatment (data not shown). The less remarkabe increase in GCs in infected plus leptin-treated mice bears some relation with the increased loss of epididymal fat pad mass and the intense myocarditis (Fig. 6a bottom panels). Analysis of ObR mRNA in the hypothalamus from infected

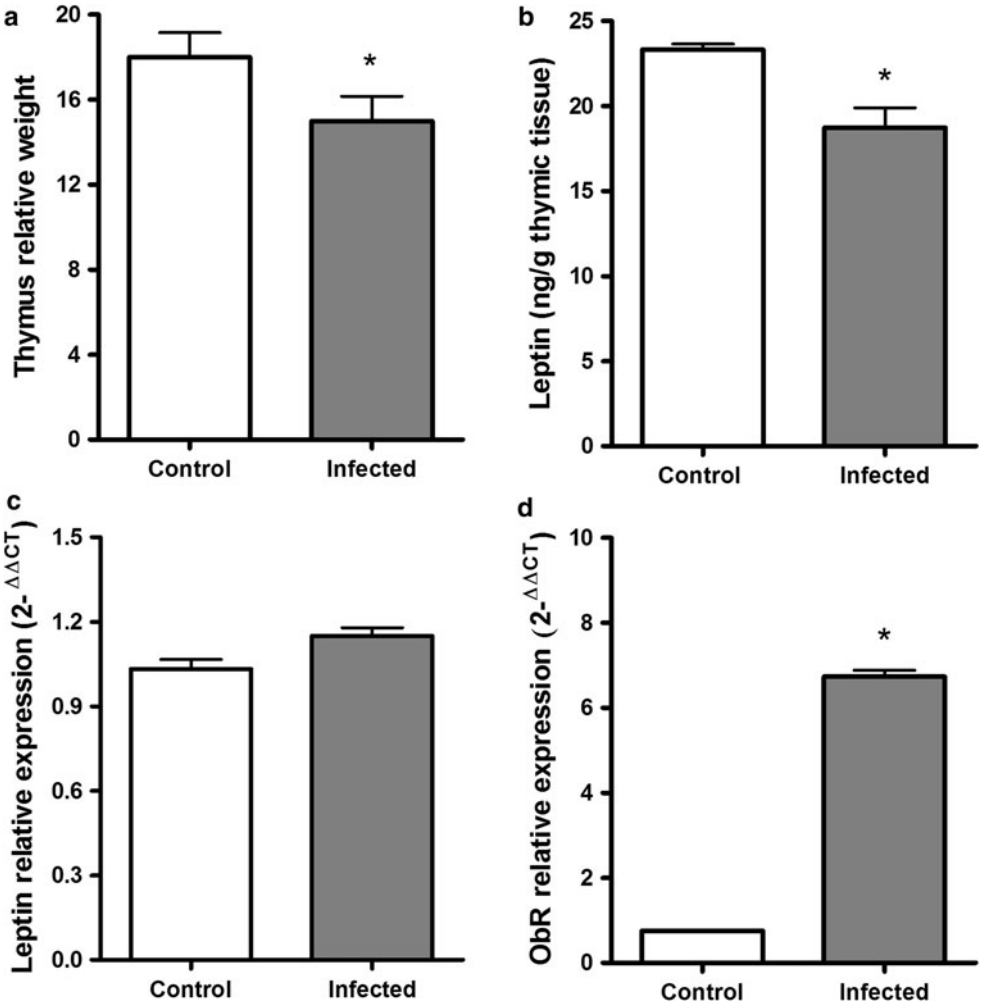

Fig. 4 Thymic expression of leptin and ObR during *T. cruzi* infection. **a** Thymuses from Control and Infected mice were removed and weighed at 14 days post-infection to calculate the relative thymus weight as a representative measurement of thymic atrophy. **b** Leptin contents were measured by ELISA and normalized by thymus weight of Infected mice. **c** mRNA expression levels of leptin in thymuses from Control and Infected animals were analyzed by real-time RT-PCR. **d** mRNA expression levels of ObR in thymuses from Control and Infected animals were analyzed by real-time RT-PCR. In all cases, the results are expressed as the relative difference to the uninfected Control by $2^{-\Delta\Delta CT}$ method. *Bars* and *lines* represent the mean ± SEM of 4–6 mice/group. $*p < 0.05$ versus Control group. Results are representative of two independent experimental rounds

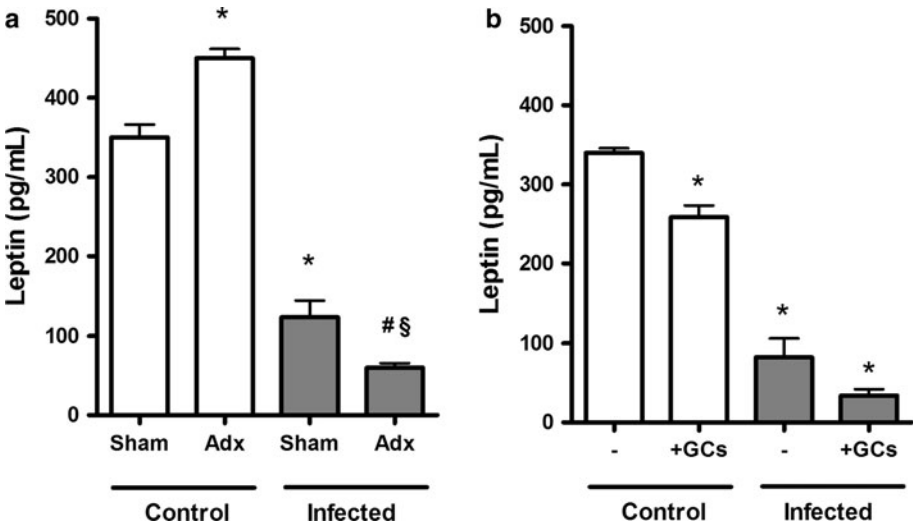

Fig. 5 Effect of glucocorticoids on leptin levels during *T. cruzi* infection. **a** Mice were adrenalectomized (Adx) or sham-operated (Sham) 7 days before infection. Blood samples from Control and Infected mice were obtained at 14 days post-infection (dpi). Plasma leptin levels were measured by ELISA. $*p < 0.05$ versus Sham group; $^{\#}p < 0.05$ versus Adx group; $^{\S}p < 0.05$ versus Infected-Sham group. **b** Infected mice were treated with dexamethasone at 0.6 μg/dL/day. Blood samples were collected at 14 dpi to assess leptin plasma levels (pg/mL, ELISA). Data represent mean ± SEM of (4–6 mice/group). $*p < 0.05$ versus Control group. Results are representative of three independent experimental rounds

Table 2 Metabolic parameters in *T. cruzi*-infected mice after leptin treatment

Metabolic parameters	Control	Infected	Control + Leptin	Infected + Leptin
Body weight (g)	28.05 ± 0.25	23.82 ± 0.72*	27.93 ± 0.44	23 ± 0.9*
Food intake (g/day/mouse)	3.73 ± 0.07	2.84 ± 0.17*	3.56 ± 0.11	2.69 ± 0.21*
Adiposity index	0.887 ± 0.03	0.479 ± 0.02*	0.242 ± 0.07§	0.189 ± 0.02$^{\lambda}$
Glycemia (mg/dL)	162.1 ± 12.2	70.3 ± 5.2*	165.6 ± 11.7	80.20 ± 10.3*
Cholesterolemia (g/L)	1.47 ± 0.09	2.26 ± 0.19*	1.50 ± 0.08	2.29 ± 0.19*
Triglyceridemia (g/L)	0.91 ± 0.20	1.75 ± 0.49*	1.22 ± 0.5	1.87 ± 0.55*
Glycerol/mass *EFP* ratio (mmol/L/g)	4.21 ± 1.20	20.41 ± 6.74*	19.71 ± 6.32§	51.46 ± 8.65*$^{\lambda}$

Values represent the mean ± SEM of 4–6 mice/group (one of three independent experiments) at 17 days post-infection. *$p < 0.05$ compared to their respective Control group, $^{\S}p < 0.05$ versus Control group, $^{\lambda}p < 0.05$ compared to Infected mice group

EFP epididymal fat pads

and leptin-treated mice showed levels as reduced as in the untreated counterparts (data not shown).

Discussion

While being increased in some acute non-lethal infections [52, 53], experimental *T. cruzi* infection resulted in leptin diminution, mirroring findings in patients with fatal sepsis or tuberculosis [49–51]. Present leptin reduction bears no relation with loss of body weight and food intake in acutely *T. cruzi*-infected mice. It means that leptin-mediated physiological mechanisms are ineffective here, and other mediators may be implicated in these alterations. A reduction in food intake is a common feature of many inflammatory diseases, with cytokines such as TNF-α, IL-6 or IL-1β playing a role in anorexia, lipolysis and body weight loss [44, 55]. The wasting seen in *T. cruzi*-infected mice may then result from the inflammatory *milieu* largely mediated by TNF-α, given its potent lipolytic and anorexigenic effects. At first sight, increased TNF-α production may be also implicated in leptin reduction, since patients with cardiac cachexia show elevated TNF-α and low leptin concentrations [56], whereas the LPS-induced anorexia in *ob/ob* mice is associated with a TNF-α increase [57]. Nevertheless, during *T. cruzi* infection, TNF-α appears to sustain leptin secretion, because *T. cruzi*-infected TNF-R1-deficient mice display even lower leptin levels, corroborating other studies in which TNF-α stimulates leptin expression [54, 58].

Weight loss is not implicated in the marked hypoleptinemia, considering the leptin/body weight ratio was also reduced during infection. Our results deviate from studies showing a hyperleptinemic state in *T. cruzi*-infected CD-1 mice paralleled by increased body weight [44]. Factors accounting for this dissimilarity not only include genetically linked differences in the metabolic background (hyperglycemic or diabetic state), but also a different experimental approach.

The biologically active form of ObR in the hypothalamus is regulated by systemic levels of leptin. During fasting, when serum leptin levels are low, the hypothalamic expression of ObR is increased [59–61], whereas leptin supplementation in stressed rats lowers such expression [62]. Hyperglycemic ObR-deficient mice (*db/db*) evidence an increased susceptibility to *T. cruzi* infection [20]. A recent study in genetically modified *db/db* mice expressing ObR only in the brain showed that these *T. cruzi*-infected mice have a normalized glycemia and improved anti-parasite response [37]. Metabolic abnormalities seen in our *T. cruzi*-infected mice are also associated with the progressive hypothalamic ObR low expression. These effects appear to be transcriptional, because equivalent changes were observed in the expression of ObR protein form. ObR down-regulation is typically observed in obese animals with leptin resistance. During *T. cruzi* infection, this may be rather related to a dysregulation of leptin/ObR circuitry. To our knowledge, this is the first report showing hypothalamic ObR expression abnormalities during *T. cruzi* infection.

During fasting, decreased leptin levels trigger GCs secretion and increase lipolysis [13], resembling findings seen in acutely *T. cruzi*-infected mice. Similar conditions have already been reported in stressful conditions [29, 30], as well as in *ob/ob* and *db/db* mice displaying higher levels of GCs [22]. Hypoleptinemia in present *T. cruzi* infection seems to favor HPA axis activation, since leptin supplementation lowered GCs synthesis. Unlike the prevailing view that corticoid treatment or chronic elevations of GCs stimulate leptin gene expression and secretion [25–28], in our hands supra-physiological GCs supplementation failed to increase leptin levels, indicating that leptin diminution during *T. cruzi* infection is independent of HPA axis activity.

It is well established that leptin and GCs can affect the thymic homeostasis, as leptin protects thymocytes from

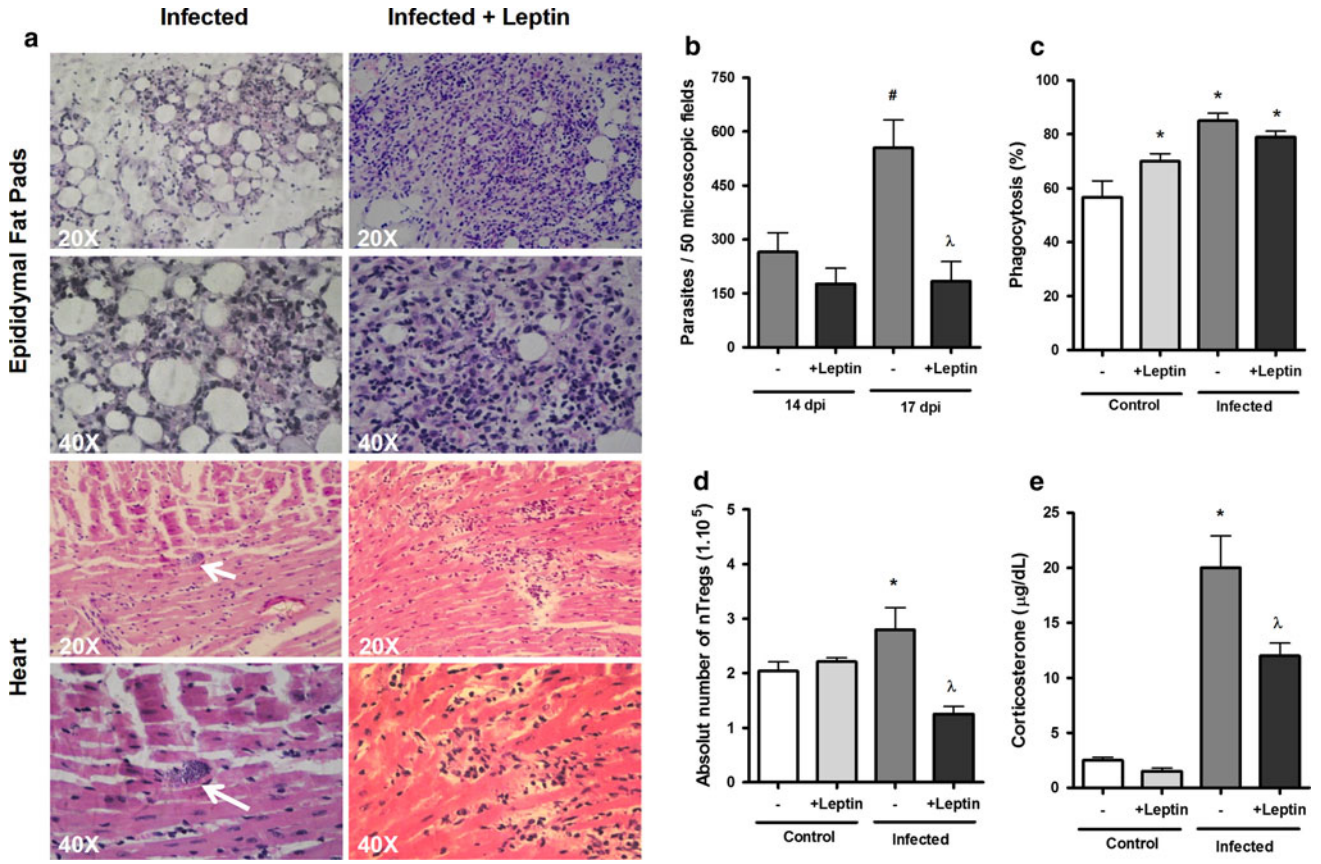

Fig. 6 Effect of leptin treatment during the acute phase of *T. cruzi* infection. **a** Epididymal fat pads (*upper panels*) and hearts (*bottom panels*) were obtained after leptin treatment (17 dpi). *Left panels* show slides from Infected mice, whereas *right panels* show slides from Infected animal treated with leptin. Pictures show at different magnifications an intense inflammation in the adipose tissue and heart, more marked in the leptin-treated group. *Arrows* indicate amastigote nests. **b** Bloodstream forms of *T. cruzi* were assessed under standardized conditions at 14 and 17 days post-infection (dpi). *Bars* and segments represent the median and upper range of parasite numbers/50 microscopic fields of 4–6 mice/group/day. **c** Peritoneal macrophages (PM) were obtained at 14 dpi from Control and Infected mice given leptin, or not. The phagocytosis assay was performed incubating PM with heat-killed yeasts during 3 h at 5:1 yeast-PM ratio. The percentage of phagocytosis was calculated as the number of macrophages with ingested yeast over total macrophages per 100. **d** Absolute numbers of $CD4^+FoxP3^+$ natural regulatory T cells (nTregs) after leptin treatment analyzed by cytofluorometry (14 dpi). **e** Corticosterone levels (µg/dL) after leptin treatment by (ELISA, 17 dpi). Except for panel "**b**", *bars* represent the mean ± SEM of individual data obtained from 4 to 6 mice/group/day. Results are representative of two independent experimental rounds. $^*p < 0.05$ versus Control non-treated; $^{\#}p < 0.05$ versus infected-14 dpi and non-treated; $^{\lambda}p < 0.05$ versus Infected and non-treated at 17 dpi. Results are representative of two or three independent experimental rounds

GCs-mediated apoptosis, whereas leptin replacement or Adx improves thymopoiesis [63, 64]. Extending our earlier studies in which thymic atrophy in *T. cruzi*-infected mice is driven by GCs [41, 65], we now show that systemic and thymic leptin deficiencies are linked to thymic atrophy. Nevertheless, leptin supplementation did not reverse DP depletion, possibly due to the strong GCs response. The increased ObR expression in thymic cells may be explained because of the already known enrichment of the $CD4^+$ or $CD8^+$ simple positive subpopulations. Nevertheless, leptin replacement in uninfected animals led to a significant increase in the thymic relative weight and absolute numbers of DP thymocytes. The well-known over-increased GCs response and apoptotic induction of DP cells seen in *T. cruzi*-infected mice are likely to override the beneficial effects of leptin at the thymic level.

The hypoleptinemia seen in *T. cruzi*-infected mice seems detrimental for parasite control considering the promoting effects of leptin on Th1-cell-mediated immunity and monocytes/macrophage activity [16–20]. Supporting this view, leptin supplementation lowered parasitemias and amastigote nests, by mechanisms other than a mere improvement in the macrophage phagocytic capacity.

In essence, acute *T. cruzi* infection in C57BL/6 mice coexists with a dysregulation of leptin/hypothalamic ObR circuitry dissociated from body weight and food intake control. Leptin diminution seems to favor HPA activation, worsening GCs-thymic atrophy and the defensive response.

Endogenous GCs production attempted to reestablish systemic leptin concentrations, but failed to improve leptin-protective activities at the thymic level, suggesting that the leptin/GCs intrathymic relationship is also altered during this infection.

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