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Death of adrenocortical cells during murine acute *T. cruzi* infection is not associated with TNF-R1 signaling but mostly with the type II pathway of Fas-mediated apoptosis

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ABSTRACT

Earlier studies from our laboratory demonstrated that acute experimental *Trypanosoma cruzi* infection promotes an intense inflammation along with a sepsis-like dysregulated adrenal response characterized by normal levels of ACTH with raised glucocorticoid secretion. Inflammation was also known to result in adrenal cell apoptosis, which in turn may influence HPA axis uncoupling. To explore factors and pathways which may be involved in the apoptosis of adrenal cells, together with its impact on the functionality of the gland, we carried out a series of studies in mice lacking death receptors, such as TNF-R1 (C57BL/6-*Tnfrsf1a tm1Im*$^{-/-}$ or TNF-R1$^{-/-}$) or Fas ligand (C57BL/6 Fas-deficient *lpr* mice), undergoing acute *T. cruzi* infection. Here we demonstrate that the late hypercorticosterolism seen in C57BL/6 mice during acute *T. cruzi* infection coexists with and hyperplasia and hypertrophy of *zona fasciculata*, paralleled by increased number of apoptotic cells. Apoptosis seems to be mediated mainly by the type II pathway of Fas-mediated apoptosis, which engages the mitochondrial pathway of apoptosis triggering the cytochrome c release to increase caspase-3 activation. Fas-induced apoptosis of adrenocortical cells is also related with an exacerbated production of intra-adrenal cytokines that probably maintain the late supply of adrenal hormones during host response. Present results shed light on the molecular mechanisms dealing with these phenomena which are crucial not only for the development of interventions attempting to avoid adrenal dysfunction, but also for its wide occurrence in other infectious-based critical illnesses.

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INTRODUCTION

During infectious stress, the hypothalamic-pituitary-adrenal (HPA) axis activation leads to glucocorticoid (GC) and catecholamine secretion by adrenal glands to restore host homeostasis and to cope with the inflammatory response (Besedovsky and del Rey, 1996). Nevertheless, when infection aggravates, systemic and intra-adrenal inflammatory cytokines can also sustain the adrenal secretion of GC, resulting uncoupled from the hypothalamic-pituitary (HP) unit (Bornstein et al., 2008). Intra-adrenal inflammation may also result in adrenal cell apoptosis, which in turn may influence HPA axis uncoupling. Nevertheless, factors and pathways involved in adrenal cell apoptosis during severe inflammatory or infectious processes, along with its impact on the gland functionality, are not yet fully characterized.

Apoptosis is a form of programmed cell death which involves the elimination of individual cells within an organism, thereby preserving the overall structure of surrounding tissue. Apoptosis can be triggered by two different pathways: the extrinsic and the intrinsic ones (Kiess, 1998). The extrinsic pathway is activated upon stimulation of the trans-membrane death receptor family. Members of this family include molecules such as TRAIL-Rs (DR4 and DR5), Fas (Apo-1/CD95) and TNF-R1(p55/CD120a) (Bhardwaj and Aggarwal, 2003). These receptors transmit external apoptotic signals to the death machinery, resulting in the activation of the initiator caspase-8 and finally in the executioner caspase-3. Interestingly, certain type of cells also showed a dependency on the mitochondrial apoptotic branch to undergo apoptosis following a death receptor stimulus, requiring the cleavage of the cytosolic Bid substrate by caspase-8 (Li et al., 1998; Luo et al., 1998) Truncated Bid (t-Bid) translocates to the mitochondria where interacts with Bax to induce the mitochondrial outer membrane permeabilization (Letai et al., 2002; Luo et al., 1998), leading to the cytochrome c release. Cytochrome c stimulates the apoptosome formation, which proteolytically

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activates the procaspase-3 to finally induce apoptosis (Bhardwaj and Aggarwal, 2003; Luo et al., 1998). The t-Bid-induced permeabilization may be inhibited by members of Bcl-2 family and additionally, caspase-3-mediated apoptosis may be also abolished by inhibitors, pointing out that the cellular balance between pro- and anti-apoptotic factors is crucial for the cell fate (Beug et al., 2012).

The adrenal gland plays a central role in the control of immune reaction Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi (T. cruzi*). We have shown that of HPA axis activation and the subsequent release of GCs is a critical event for the differential susceptibility to infection (Roggero et al., 2006). The intense inflammatory response that C57BL/6 mice experience during *T cruzi* acute infection resembles, to some extent, the systemic inflammatory response seen in LPS-challenged mice, a well-known systemic reaction to bacterial components in which adrenal apoptosis is likely to occur (Kanczkowski et al., 2013). Moreover, *T. cruzi*-infected mice showed a sepsis-like dysregulated adrenal response characterized by normal levels of ACTH with raised GC secretion, indicating a regulatory shift from the HP unit towards the intra-adrenal component of stress response (Kanczkowski et al., 2015).

TNF-α is a key pro-inflammatory cytokine involved in the promotion of GC production, stimulating CRH and ACTH secretion at HP unit (Turnbull and Rivier, 1999). TNF-α and its receptors are expressed in the hypothalamus, in the anterior pituitary gland, as well as in adrenal glands (Besedovsky and del Rey, 1996). During severe inflammatory processes like endotoxemia or sepsis, TNF-α appears to be linked to adrenal apoptosis (Kanczkowski et al., 2015; Mikhaylova et al., 2007; Tkachenko et al., 2011). In addition, experimental evidence showed that Fas/FasL pathway may be involved in the apoptosis of cultured adrenocortical cells (Montanaro et al., 2005).

We and others have previously demonstrated that TNF-α is systemically increased during the acute phase of *T. cruzi* infection in C57BL/6 mice, being involved not only in parasite control

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but also in tissue pathology (Pérez et al., 2007; Roggero et al., 2006, 2004, 2002). Interestingly, while TNF-R2 (p75/CD120b) contributes to a important number of TNF-α-induced responses, most effects of TNF-α observed during Chagas disease are mediated by TNF-R1(Aliberti et al., 2001; Castaños-Velez et al., 1998; Feldman and McNamara, 2000; Pérez et al., 2007). In relation to this, we recently demonstrated that TNF-α signalling through TNF-R1 acts as a potent modulator of steroideogenesis in adrenocortical cells during experimental Chagas disease (Villar et al., 2013).

As regards to *T. cruzi* infection, while Fas apoptotic pathway plays a role in the immune response regulation (Lopes et al., 1999), the Fas/Fas-L signaling is also implicated in the induction of peripheral lymphocyte death, atrophy of mesenteric lymph nodes and the myocardial damage, but does not seem quite involved in thymic atrophy (De Souza et al., 2003; Guillermo et al., 2007; Rodrigues Jr. et al., 2007). As regards endocrine tissue Fas is expressed in all zones of adrenal glands (Leithäuser et al., 1993), but it is unknown whether the adrenal Fas/Fas-L cascade plays a role during *T. cruzi* infection.

Addressing whether adrenal apoptosis is enhanced in this model of *T. cruzi* infection and the eventually involved pathways, is important for a better characterization of the adrenal dysregulation during experimental disease and its eventual impact on disease outcome. To this end, in the present study C57BL/6 wild type mice or counterparts deficient in death-dealing receptors (TNF-R1 and Fas) were infected with *T. cruzi* and further, adrenal glands were studied for the molecular intracellular pathway underlying apoptosis. Studies revealed that the apoptosis of adrenal cells is mainly mediated by the Fas signaling pathway. Also, the type II pathway of Fas-mediated apoptosis is largely involved in the intensification of adrenocortical cell death during *T. cruzi* infection.

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MATERIALS AND METHODS

Mice, parasite and infection

Wild type (WT) C57BL/6 male mice and mice lacking TNF-R1 (C57BL/6-*Tnfrsf1a tm1Imx* or TNF-R1$^{-/-}$) originally obtained from The Jackson Laboratory as well as Fas-deficient *lpr* mice (C57BL/6 background). Animals received care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington D.C., National Academy Press, 1996). All the experimental protocols were performed according to local regulations for the Care and Use of Laboratory Animals and approved by the Institutional Ethical Committee of the Medical Sciences of National University of Rosario, Argentina (Resolution 4976/2013). Sixty to 90-day-old mice (4-6/group) were used in each experiment. Infection was carried out by injecting 200 viable trypomastigotes of *T. cruzi* (Tulahuén strain) by the subcutaneous route. All evaluations were made at 17 days post infection (dpi).

Monitoring of acute infection

Bloodstream forms of *T. cruzi* were assessed under standardized conditions, by direct microscopic observation of 5 µl heparinized blood obtained from the tip of the tail, as previously reported (Roggero et al., 2002). Data were expressed as number of parasites/50 microscopic fields.

Cytokine determinations

Plasma cytokines were measured by specific two-site enzyme-linked immunosorbent assay (ELISA) according to the manufacturer´s specifications. All samples were assayed in duplicate. ELISA kits for IFN-γ, TNF-α and IL-6, were purchased from Pharmingen (San Diego, CA, USA).

For intra-adrenal detection of TNF-α mRNA, adrenals were obtained from control and infected animals at day 17 post-infection. Total RNA was isolated from cells using TRIzol

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(Invitrogen) according to the manufacture's recommendations. TNF-α mRNA levels were determined by RT-qPCR as previously described (Villar et al., 2013). Primers sequences were: TNF-α= sense 5'-ATGAGCACAGAAAGCATGATCCGCGAC-3'; antisense 5'-TCACAGAGCAATGACTCCAAAGTAGACCTG-3' and GADPH= sense 5'-AGCAATGCATCCTGCACCACCA-3'; antisense 5'-ATGCCAGTGAGCTTCCCGTTCA-3'

Corticosterone assay

Mice were housed individually and kept single-caged throughout experiments in temperature, and light-controlled rooms (light cycle from 7:00 a.m. to 7:00 p.m.). Blood samples for hormone measurements were obtained from the tip of the tail between 8:00 and 10:00 a.m., as previously reported (Villar et al., 2013). Plasma corticosterone (CT) levels were assessed by ELISA, according to manufacturer's specifications (IBL, Germany). Detection limit was 0.20 μg/dL.

Determination of cell death and proliferation

Quantitative analysis of apoptosis was performed by *in situ* specific labeling of fragmented DNA using a modified terminal deoxynucleotidyl transferase (Tdt) mediated biotin-deoxyuridine triphosphate nick-end labeling (TUNEL) method according to manufacturer's instructions (Promega, Madison, WI, USA). Slides were counterstained with methyl green. Negative controls were prepared without TdT on TUNEL reaction mixture. The development of brown color over the nuclei indicated in apoptotic cells (Gold et al., 1994). To corroborate the incidence of apoptotic bodies, serial sections were stained with hematoxylin/eosin (H&E). Each section was evaluated for the presence of apoptotic cells and apoptotic bodies. The apoptotic index (AI) was calculated as the number of apoptotic cells and apoptotic bodies, expressed as a percentage of the total number of adrenal cells counted in each case (AI= [number of apoptotic cells / total cell number] x 100), as previously reported (Gold et al., 1994; Klainguti et al., 2000).

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For proliferation studies, immunohistochemistry technique was performed on 4 μm paraffin sections. Them, were incubated with the anti-Ki-67 (clone SP6, Diagomics, dilution 1/400), followed by incubation with a streptavidin-biotin-peroxidase antibody complex (BD Pharmingen) and afterwards treated with streptavidin peroxidase reagent. Later, were incubated with 3,3'-diaminobenzidine (DAB), counterstained with hematoxylin, for observation under microscope. For the tissue evaluation of Ki-67, each slide was scored based on the percentage of positively stained nuclei.

Immunoblot assays for pro- and anti-apoptotic factors

To obtain mitochondria-enriched fractions, adrenal glands were homogenized in 4 volumes of 300 mmol/L sucrose with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin). Homogenates were centrifuged at 1000*g* to remove unbroken cells, nuclei and heavy membranes. Mitochondria-enriched fractions were obtained by centrifugation at 3000*g* at 4 C for 15min. Then, it was further centrifuged at 45000*g* for 1 h and the obtained supernatant was used as the cytosolic fraction, as previously reported (Ronco et al., 2004). Proteins were quantified according to Lowry technique. For protein detection were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electro blotted onto polyvinyl difluoride (PVDF) membranes (PerkinElmer Life Sciences, Inc., Boston, MA). Membranes were incubated with primary anti-mouse antibodies (anti-Bax, anti-Bid, anti-XIAP; anti-cytochrome c, all from Santa Cruz Biotechnology). Finally, protein levels were detected by enhanced chemiluminescence detection system (Pierce ECL, Thermo Fisher Scientific). Immunoreactive bands were quantified by densitometry using the Image J software (imagej.nih.gov).

Caspase-8 and caspase-3 activity assay

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Assessment of caspase-8 activity was carried out in cytosolic fraction using a fluorometric assay kit (Caspase-8 activity assay kit QIA71; Calbiochem®) according to the manufacturer's instructions. Briefly, adrenal glands were homogenized in lysis buffer. After the addition of caspase-8 fluorescent substrate followed by incubation at 37◦C for 2h, fluorescence was read in a DTX880 multimode detector (Beckman Coulter) at an excitation wave length of 400 nm and an emission wave length of 405 nm. The activity of caspase-3 was determined according to the manufacturer's instructions using an EnzChekTM caspase-3 assay kit (Molecular Probes, USA), as we previously reported (Francés et al., 2010). Fluorescence was measured in a DTX880 multimode detector (Beckman Coulter).

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical analysis was performed by the non-parametric analysis of variance Kruskall-Wallis followed by Dunn post-test ($k<2$) or U de Mann Whitney test ($k=2$). The GraphPad Instat 4.0 software (GraphPad, California, USA) was used for statistical analyses, and differences were considered significant when p value was <0.05.

RESULTS

Acute T. cruzi infection induces adrenal cell apoptosis

First, we performed studies to identify and quantify changes in the size, weight and the level of apoptosis in adrenal glands from infected (Tc) and uninfected (Co) C57BL/6 mice after 17 dpi. Histological studies of glands from Tc mice showed an increased adrenal size, clearly associated with the weight gain (Figure 1A). Studies to detect *in situ* fragmented DNA (TUNEL assay) in adrenal glands also showed an increase in the number of cortical apoptotic cells, reflected by the AI, (Figure 1 B). Evaluation of H&E stained slides showed the same data trend (data not shown).

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Since TNF-α was associated with both steroideogenesis and adrenal cell death, we next simultaneously estimated systemic and intra-adrenal levels of this cytokine. As shown in Figure 1C, circulating levels of TNF-α are strongly elevated after 17 dpi. In addition, the intra-adrenal amount of mRNA TNF-α assessed by RT-qPCR was also augmented, mirroring systemic findings. Confirming earlier data (Villar et al., 2013), corticosterone levels are markedly increased in Tc mice (Figure 1D), in line with weight increase and hyperplasia of the fasciculate zone.

T. cruzi infection leads to HPA axis activation and morphological changes in adrenal glands of TNF-R1$^{-/-}$ and Fas-deficient _lpr_ mice.

To evaluate the potential role of TNF-R1 and Fas death receptors in the development of adrenal cell apoptosis during *T. cruzi* infection, we carried out studies in WT, TNF-R1$^{-/-}$ and Fas-deficient *lpr* mice. First, we compared the course of infection between groups. As depicted in Figure 2A, Tc-TNF-R1$^{-/-}$ mice showed increased parasitemias throughout infection compared to Tc-WT and Tc-*lpr* animals, while a similar parasite load was seen in the remaining two groups. In line with this, Tc-TNF-R1$^{-/-}$ mice showed the lowest survival time. While Tc-TNF-R1$^{-/-}$ mice survived 18 days on average, Tc-WT and Tc-*lpr* animals have a mean survival of 26 and 28 days, respectively (Figure 2B). Given these differences, and for comparison purposes adrenal studies were carried out at day 17 dpi.

Moreover, all infected groups showed a significant increase in the levels of circulating inflammatory cytokines after 17 dpi. Compared with Tc-WT mice and Tc-*lpr* mice, Tc-TNF-R1$^{-/-}$ showed a 6- and 3-fold respectively increase in TNF-α levels (Figure 2C). Tc-TNF-R1$^{-/-}$ and Tc-*lpr* animals exhibited higher values of IL-6 compared to Tc-WT mice (Figure 2D). Strikingly, IFN-γ plasma levels showed a 30-fold increase in Tc-TNF-R1$^{-/-}$ animals compared to the remaining

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infected groups (Figure 1E). In essence, increased parasite load and circulating cytokines in Tc-TNF-R1$^{-/-}$ mice coexisted with a higher mortality rate.

In estimating the activation state of the HPA axis CT plasma levels were also assessed. All infected groups showed increased CT amounts compared with their uninfected counterparts (Table 1). Tc-TNF-R1$^{-/-}$ showed an even much higher increase compared to Tc-WT and Tc-*lpr* infected groups. Analysis on the weight of adrenal glands, revealed enlarged glands in all groups of infected mice in comparison with their respective uninfected counterparts (Table 1). In addition, histopathological analysis of adrenal gland sections revealed very scarce cell infiltrates and absence of parasite nests at day 17 post-infection. As depicted in Table 1 and Figure 3, the increase of the gland size was due to both hyperplasia and hypertrophy of *zona fasciculata* in all infected groups. Cortical regions were significantly increased in relation to the adrenal medulla (data not shown).

Adrenal apoptosis was prevented in *lpr* but not in TNF-R1$^{-/-}$ infected mice

It is widely known that TNF-α and FasL can initiate apoptotic events in different tissues and cells through TNF-R1 and Fas, respectively. Nevertheless, this process of cell death has been scarcely studied in the adrenal gland during *T. cruzi* infection. As such, we sought to evaluate the degree of apoptosis of cortical cells in WT, TNF-R1$^{-/-}$ and *lpr* mice during *T. cruzi* infection (Figure 4). Typical morphological events of apoptosis, including cell shrinkage, acidophilic cytoplasm, chromatin condensation and fragmentation into apoptotic bodies, were analyzed in adrenal slides by H&E staining (Figure 4A and 4B). We also performed the *in situ* TUNEL assay to identify apoptotic nuclei (Figure 4C and 4D), revealing a similar cell death pattern. Basal AI did not differ among uninfected WT, TNF-R1$^{-/-}$ and *lpr* mice (Figure 4A and 4C). *T. cruzi* infection increased the AI in the cortex in all infected groups, although in Tc-*lpr* mice, the less increased AI only attained statistical significance compared to Co-*lpr* when analyzing by the TUNEL assay (Figure 4C).

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Strikingly, AI was strongly diminished in Tc-*lpr* mice compared to Tc-WT and Tc-TNF-R1$^{-/-}$mice (Figure 4A and 4C), suggesting that Fas/Fas-L signaling is the main pathway involved in adrenal apoptosis during *T. cruzi* infection.

***T. cruzi* infection induces caspase-3 and caspase-8 activity in adrenal glands**

TNF-R1 and Fas transmit external apoptotic signals to the intracellular death machinery, resulting in the activation of the initiator caspase-8 and subsequently the downstream caspase-3.To get some insight into the mechanisms by which *T. cruzi* infection induces adrenal cell apoptosis, we analyzed both caspase-8 and caspase-3 activity in adrenal cell lysates from WT, TNF-R1$^{-/-}$ and *lpr* mice by fluorometric assays.

As seen in Figure 5A, caspase-8 activity showed a ~25% and 50% increase in adrenal glands of Tc-WT and Tc-TNF-R1$^{-/-}$ mice compared to their uninfected counterparts, respectively. Interestingly, the basal levels of caspase-8 activity remained unmodified in Tc-*lpr* mice, suggesting that the caspase-8 activation via Fas is involved in the apoptosis of adrenal cells during *T. cruzi* infection.

Caspase-3 activity was also augmented in all infected groups compared to their respective uninfected groups (Figure 5B). Since caspase-3 activity test can also detect the activity of other substrate-specific proteases like caspase-7, we performed a western blot to assess the levels of active caspase-3 (as cleaved caspase-3 fragments) in homogenates of adrenal gland (Figure 5B and 5C). In both cases, we observed that Tc-TNF-R1$^{-/-}$ mice exhibited a more increased caspase-3 activation compared to Tc-WT mice, while results in Tc-*lpr* mice did not differ from Tc-WT and Tc-TNF-R1$^{-/-}$ mice. In the Tc-*lpr* group, the unexpectedly increase in cleaved caspase-3 contrasts with the diminished AI, suggesting the presence of caspase-3 inhibitors. Caspase inhibitors, especially XIAP, can contribute to increase the apoptotic threshold, conferring resistance to Fas-mediated cell death (Bratton et al., 2002). Specifically, cleaved caspase-3 may often keep inactive due to binding of

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XIAP (Liston et al., 2003). Hence, to assess whether XIAP is involved in the survival of adrenal cells from Tc-*lpr* animals even in presence of cleaved caspase-3, we analyzed XIAP protein levels in all infected and uninfected groups. As seen in Figure 5D and 5E, we observed that XIAP expression in all Tc-groups was lower than in uninfected animals (quantified as 100%). However, Tc-*lpr* showed higher levels of XIAP protein compared with the remaining infected groups, suggesting that XIAP can prevent full activation of caspase-3 in this group. These results may partly explain, the lower apoptosis observed in Tc-*lpr* mice even with an increased caspase-3 activity.

Fas signaling is also involved in the intrinsic amplifying of apoptotic response in adrenal glands during *T. cruzi* infection

In the death receptor-mediated pathway, the apoptotic cascade may be amplified by caspase-8-mediated cleavage of Bid to give t-Bid, which engages the mitochondrial pathway triggering the cytochrome c release to increase caspase-3 activation. Therefore, t-Bid protein acts as molecular link between extrinsic and intrinsic pathways (Czabotar et al., 2014). Together with t-Bid, Bax also translocate to the mitochondria to induce the release of cytochrome c into the cytosol, shifting the balance toward apoptosis. With the aim of evaluate whether this pathway is also operative in adrenal glands during infection, we examined Bid expression in both cytosolic and mitochondrial subcellular fractions of adrenal glands. As seen in Figure 6A, in Tc-WT mice, the Bid cytosol/mitochondria ratio was strongly reduced compared to uninfected mice, indicating that during infection the protein was almost totally cleaved and translocated to the mitochondria amplifying the apoptotic cell process. In the Tc-TNF-R1$^{-/-}$ group, the decrease of Bid ratio was lower than in Tc-WT mice (Figure 6A), implying that TNF-R1 is involved to some extent in the engagement of the intrinsic pathway of apoptosis during *T. cruzi* infection. Strikingly, in *lpr* mice the Bid ratio remained unchanged regardless of whether animals were infected or not. Tc-WT and Tc-TNF-R1$^{-/-}$

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mice also displayed an increase in the mitochondrial content of Bax (Figure 6B) and the cytosolic content of cytochrome c (Figure 6D), indicating that in both groups the intrinsic pathway is operative during infection. Again, no differences were found when comparing Bax and cytochrome c contents from Tc-*lpr* mice respect their uninfected counterparts (Figure 6C and 6D). Overall, these results indicate that Fas signaling is strongly involved in the intrinsic amplification of apoptotic response triggered by caspase-8 in adrenal glands during infection.

Disturbed adrenal cell proliferation in absence of Fas signaling during infection

Finally, we evaluated the opposite phenomenon of cell death, the proliferative response of adrenal cells in absence of death receptors. Positive staining of the intranuclear antigen Ki-67 was used to discriminate between resting and proliferative adrenocortical cells. As seen in Figure 7, Co-WT and Co-TNF-R1$^{-/-}$ mice showed a low percentage of Ki-67 staining, with Co-*lpr* mice showing an increased but statistically insignificant proliferation when compared to the other uninfected groups. Strikingly, in the adrenal cortex of all infected groups, the proliferative response was increased compared with their uninfected counterparts. The Tc-*lpr* group showed the highest proliferation values, while Tc-TNF-R1$^{-/-}$ mice showed a more slightly increment in Ki-67 staining than the Tc-WT group.

These results indicate that during *T. cruzi* infection, the ablation of pro-apoptotic Fas-mediated signals induced a more dysregulated proliferation than the TNF-R1-mediated pathways, suggesting a Fas involvement in the control of adrenal cell turnover.

DISCUSSION

The adrenal gland plays a critical role in the host's response to infectious stress, as it constitutes the fundamental source of GC production. This response implies both dynamic and

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structural changes, including proliferation and cell death as essential processes for the integrity and functionality of this gland (Carsia et al., 1996; Wolkersdörfer et al., 1996). Therefore, pathophysiological processes that alter these processes may affect the normal functioning of this organ.

Diverse infectious stressors can induce an intense stimulation of HPA axis (Besedovsky and Del Rey, 1996). Moreover, as result of sustained and intense inflammation, HPA axis dysfunction can often occurs. Sepsis is known to present changes like adrenal insufficiency, or dysregulated adrenal response consisting of repressed ACTH with normal or elevated GC secretion (Bornstein et al., 2008). In addition to the intense inflammation accompanying acute disease (Roggero et al., 2002), our recent evidence indicates that during *T. cruzi* infection in C57BL76 mice, adrenal function is impaired, resembling some features of sepsis (Roggero et al., 2006; Villar et al., 2013). Acute *T. cruzi* infected C57BL/6 mice reach high levels of GCs towards to the end of the infection course, independently of ACTH levels. At this time, the expression of steroideogenic enzymes StAR, CYP11B1 and 11β-HSD1 is enhanced, probably by the direct effect of cytokines or parasite-derived antigens upon the gland (Corrêa-De-Santana et al., 2006; Roggero et al., 2006). Extending these observations we now report that the enlarged *zona fasciculata* reveals an increased number of apoptotic cells, indicating that during *T. cruzi* acute infection spongy cell proliferation paralleled by increased cell death takes place. While some evidence indicates that ACTH promotes adrenocortical cell survival (Ceccatelli et al., 1995; Chang et al., 2013; Wyllie et al., 1973), ACTH has also been implicated in some forms of adrenal atrophy (Fallis, 2016). Our earlier studies do not support the view of ACTH as being involved in the apoptosis of adrenal cells since their levels are diminished at the end of the course of infection; reinforcing the assumption that other factors may be implied in this phenomenon (Roggero et al., 2006). Here, analysis of adrenal cells apoptosis and its potential

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underlying mechanisms were investigated at the time in which immunoendocrine alterations were clearly established and likely to affect infection control.

Our results indicate that Fas/Fas-L signaling is likely to be the main signaling pathway involved in adrenal apoptosis during experimental acute *T. cruzi* infection. Moreover, at a molecular level, our data point out that Fas participates in the amplification of the intrinsic apoptotic pathway and the Bcl-2 family proteins like Bid and Bax, fully compatible with a typical type II pathway of Fas-mediated apoptosis (Hao and Mak, 2010; Scaffidi et al., 1998). Fas expression may be stimulated in the adrenocortical zone by pro-inflammatory cytokines secreted *in situ* and, consequently, the increased intra-adrenal expression of TNF-α and IFN-γ detected in the infected glands would represent an autocrine loop facilitating Fas-mediated apoptosis. These cytokines have been shown to increase Fas expression in a number of cell types, while the increase of Fas-mediated apoptosis depends on the cell type and particular situations (Hakuno et al., 1996; Kimura et al., 2003; Leithäuser et al., 1993; Naujokat et al., 1999; Ouaaz et al., 1999; Quirk et al., 1997; Weller et al., 1994). Moreover, a subtle but sustained occurrence of inflammatory infiltrates may trigger the apoptotic program by FasL/Fas crosslinking, as suggested by Wolkersdörfer *et al* (Wolkersdörfer et al., 1996). The intra-adrenal increase of TNF-α and IFN-γ may be the result of *T. cruzi*-derived antigen engagement to TLR-2 and TLR-4 (Carrera-Silv et al., 2010; Poncini et al., 2010), which not only are expressed in adrenal cortex but also influence steroid secretion (Bornstein et al., 2004; Kanczkowski et al., 2011).

TNF-α has been associated to adrenal apoptosis during critical severe illnesses like endotoxemia or sepsis (Kanczkowski et al., 2015; Mikhaylova et al., 2007; Tkachenko et al., 2011). Within the adrenal gland, TNF-α may be produced by immune resident cells or by the adrenocortical cells themselves (Tkachenko et al., 2011). However, our results do not support TNF-α/TNF-R1 role in the adrenal apoptosis, in line with our earlier studies discarding the possibility of TNF-α as acting

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through TNF-R2 in the adrenal glands of *T. cruzi*-infected mice (Villar et al., 2013). *In vitro* studies carried out by other laboratories, suggests that TNF-α affect positively human adrenal steroideogenesis, but in our hands, TNF-α/TNF-R1 pathway seems to be mostly involved in the control of steroideogenesis (Villar et al., 2013), displaying a clear opposite action to the one shown at hypothalamic level.

The elucidation of the adrenal apoptosis pathways in *T. cruzi* infected mice improves or current understanding of the apoptosis signaling pathways triggered in these glands during diverse infections or inflammatory conditions. Caspase-3 activity was increased in infected animals, including in Tc-*lpr* mice. It is well established that XIAP is the most potent caspase-3 inhibitor (Callus and Vaux, 2007; Liston et al., 2003; Paulsen et al., 2008). In this sense, we showed an increase of XIAP protein expression in Tc-*lpr* mice when compared to the other infected groups. This evidence may denote that higher levels of XIAP can inhibit cleaved caspase-3, resulting in lower apoptosis of adrenal cells. Reasons for differences between type I and type II pathways of Fas-mediated cell apoptosis are still unknown. Some authors propose that differences in the expression of inhibitors like XIAP may be responsible. In line with our results, hepatocytes and pancreatic β-cells also undergo type II Fas-induced apoptosis during inflammatory conditions, with the XIAP abolition turning cells resistant to Fas-mediated apoptosis (Jost et al., 2009; McKenzie et al., 2008). Remarkably, Jost *et al*, clearly demonstrated that Fas stimulation provokes a fast reduction in XIAP levels in thymocytes (type I cells), while promoting XIAP expression in hepatocytes (Jost et al., 2009). Comprehension of the molecular mechanisms underlying these phenomena may be crucial for the development of treatments tending to avoid adrenal insufficiency caused by adrenocortical cell death, which has been recognized as a problem with high prevalence in diverse critical illnesses such as shock, sepsis, or diverse type of trauma (Marik and Zaloga, 2002).

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Interestingly, although caspases are most often associated with apoptosis, recent evidence points out that some of these endoproteases can also influence the proliferation cell rate. One of the earliest observations showed that treatment of T cells with caspase inhibitors led to a surprising suppression of T cell expansion (Alam et al., 1999; Kennedy et al., 1999; McIlwain et al., 2013). In other tissues, like the endometrium, the apoptosis of endometrial cells seems to be mediated by caspase-3 and the FasL/Fas system (Song et al., 2002). Nevertheless, it has been shown that caspase-3 mediates both apoptosis and cell proliferation in the equine endometrium (Roberto Da Costa et al., 2007). Since our findings in *T. cruzi*-infected glands showed a process of cell renovation coupled to an increased apoptosis in the *zona fasciculata,* caspase-3 may have a dual role to maintain adrenal homeostasis. However, further studies are necessary to elucidate the precise role of caspase-3 in adrenal gland during *T. cruzi* infection.

During *T. cruzi* infection, persistent stimuli of diverse immunoendocrine factors might be integrated by adrenocortical cells affecting central cellular responses like proliferation or survival. In this context, we cannot rule out that cortical-medullary interactions or medullary adrenaline and noradrenaline may partly account for our results. Against this background, we recently reported that *T. cruzi* infection in C57BL/6 mice do not affect adrenal catecholamine contents in the adrenal glands or plasma (Roggero et al., 2016). For which *T. cruzi*-related effects on adrenal glands seem to be more restricted to adrenal cortex.

Infection with *T. cruzi* in C57BL/6 mice is characterized by abnormal host response to infection, resulting in systemic inflammation that ultimately culminates in a life-threatening dysfunction. Part of this disadvantageous condition deals with the presence of a disrupted HPA axis, suggesting the participation of pituitary-independent factors, in which the local adrenal microenvironment appears to be crucial in this regard. Expanding our knowledge on the pathophysiology of acute *T. cruzi*-infection we now provide evidence reinforcing the view of an

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immune-adrenal crosstalk able to alter the gland microenvironment. Collectively, the late hypercorticosterolism seen in infected C57BL/6 mice is associated to hyperplasia and hypertrophy of *zona fasciculata*, which also revealed an increased number of apoptotic adrenocortical cells. Adrenal apoptosis is likely mediated by the FasL/Fas extrinsic pathway, with Fas simultaneously triggering an intrinsic amplification of apoptotic process, typical of the type II pathway of Fas-mediated apoptosis. Lastly, Fas-induced apoptosis of adrenocortical cells may be the result of exacerbated production of intra-adrenal cytokines probably involved in the late supply of adrenal hormones. Such adrenal dysfunction will ultimately affect the homeostatic restorative attempt and ensuing disease outcome.

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FIGURE LEGENDS

Fig 1

T. cruzi infection is associated to adrenal hyperplasia, apoptosis and intra-adrenal TNF-α expression

Adrenal tissue sections and serum samples were obtained at the same time from *T. cruzi*-infected wild type (Tc-WT) and uninfected (Co-WT) C57BL/6 mice. **A)** Microphotographs evidence the enlargement of adrenals during *T. cruzi infection* (H & E staining), while the bar graph shows the increase in the weight of the same glands. **B)** *T. cruzi* infection induces an increase in the apoptotic index (AI) of adrenocortical cells, evaluated by TUNEL assay. In microphotographs, apoptotic cells are evidenced by an intra-nuclear brown staining. **C)** Systemic and intra-adrenal contents of TNF-α were evaluated by ELISA and RT-qPCR respectively. **D)** Systemic corticosterone levels were evaluated using an ELISA assay. Results are shown as means ± SEM from 3–5 mice/group after 17 days post-infection (a representative experiment from 2 independent series). *p<0.05; nd = not detected

Fig 2

Characterization of _T. cruzi_ infection in Wild type, TNF-R1 knock-out and _Fas_ deficient mice

Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice were infected (Tc) with 200 trypomastigotes of Tulahuén strain or inoculated with saline (Co).

A) The parasitemia was estimated from 5 μL of infected blood as number of tripomastigotes/50 microscopic fields, magnification 40X. **B)** The survival was monitored and assessed daily. Tc-TNF-R1$^{-/-}$ mice exhibited a short-lived course of infection compared to the other groups (p<0.05 by Kaplan-Meier analysis). **C)** Plasma levels of TNF-α, IL-6 and IFN-γ were evaluated by ELISA.

Results are shown as means ± SEM from 3–5 mice/group after 17 days post-infection and correspond to a representative experiment from 3 independent series. * p<0.05; nd= not detected.

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Fig 3

Morphological changes caused by *T. cruzi* infection in adrenal glands from Wild type, TNF-R1 knock-out and *Fas* deficient mice

The adrenal cortex is subdivided into three zones (Z). The *zona glomerulosa* (the most superficial layer of the adrenal cortex), is the main site for production of mineralocorticoids. The *zona fasciculata* (the middle layer), composed by cells that contain abundant lipids, is responsible by the synthesis of glucocorticoids. Lastly, the *zona reticularis* (the inner layer), is responsible for the secretion of adrenal sex steroids. To evaluated morphological changes in these zones, adrenal tissue sections were obtained 17 days post-infection from infected (Tc)-Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice or their respective controls (Co) mice. Morphometric studies shows that in all groups of Tc-mice, the *zona fasciculata*, responsible for the corticosterone secretion, was significantly enlarged, showing by both hyperplasia and hypertrophy. The other zones showed minor changes. Microphotographs correspond to an animal illustrative of each group, obtained from one of three representative experimental rounds (H&E stain, magnification 40 X).

Fig 4

Apoptosis detection in the adrenal glands of TNF-R1 knock-out and Fas deficient mice

Adrenal tissue sections were obtained 17 days post-infection from infected (Tc)-Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice or from their respective controls (Co) mice. Apoptotic cells were quantified by H&E staining and the TUNEL methods. Bars graphs represent the apoptotic index (AI) obtained by: **A)** H&E and **C)** TUNEL staining. Microphotographs show representative stained slides of adrenal glands from all experimental groups: black arrows denote apoptotic bodies in both H&E staining samples or TUNEL positive samples (**B** and **D** respectively).

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Bars and lines represent the mean ± SEM of AI (recorded from 4 sections for each gland and 5 random fields in each section), while microphotographs are representative to an adrenal gland of each group. H&E staining magnification 100 X; TUNEL assays magnification 40X. Data correspond to a representative experiment of two independent experimental rounds. *p<0.05, (n=5 animals/group).

Fig 5

Caspase-3 and caspase-8 activity in adrenal glands during *T. cruzi* infection

Adrenal glands were obtained 17 days post-infection from infected (Tc)-Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice or their respective controls (Co) mice, and processed to evaluated caspase activities by fluorometric assays. **A)** Activity of caspase-8. **B)** Activity of caspase-3. In both case, caspase activities were represented as bar graphs expressing arbitrary units of activity. Solid and dotted line represent the values obtained with Co animals **C)** Cleaved caspase-3 protein and GADPH levels were analyzed by western blot assays. **D**-E) Immunoblots of XIAP and GAPDH in adrenal gland. Bars represent densitometry values considering Co-WT, Co-TNF-R1$^{-/-}$ and Co-*lpr* as 100% relative to GAPDH. Results are expressed as mean ± SEM from 3-5 mice/group. Data correspond to one representative experiment of two independent rounds. *p<0.05

Fig 6

Bid, Bax and cytochrome c involvement in the apoptosis of adrenal cells

To evaluate the role of same Bcl-2 family proteins and also cytochrome c, adrenal glands were obtained 17 days post-infection from infected (Tc)-Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice or their respective controls (Co) mice.

A) Cytosol /Mitochondria Bid ratio. Increased presence of Bid in the mitochondria (as t-Bid) strongly diminished the Bid ratio in Tc-WT animals, mildly in Tc-TNF-R1$^{-/-}$, but this not occur in

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Tc-*lpr* mice. **B)** Representative immunoblots of Bax, cytochrome c and β-actin. **C)** Bax and **D)** Cytochrome c. In both cases, bar graphs represent densitometry values considering Co-WT, Co-TNF-R1$^{-/-}$ and Co-*lpr* as 100%. Results are expressed as mean ± SEM from 3-5 mice/group. Data correspond to one representative round of two independent experiments. *p<0.05

Fig 7

Adrenal cell proliferation in wild type,TNF-R1 knock-out and Fas deficient mice during *T. cruzi* infection

Adrenal tissue sections were obtained 17 days post-infection from infected (Tc)-Wild type (WT), TNF-R1 *knock-out* (TNF-R1$^{-/-}$) and *lpr* (Fas deficient) mice or their respective controls (Co) mice. Immunohistochemical evaluation of Ki-67 was used to estimate the proliferation in adrenocortical cells during infection. Ki-67-positive cells are detected by brown nuclear staining, the sections were slightly counterstained with H&E stain **A)** In representative microphotographs, black arrows denote Ki-67 positive cells (Magnification 40X). **B)** Quantification of Ki-67 positive cells in all experimental groups (%= number of Ki-67 positive cell/ total number of cells), recorded from 4 sections for each gland and 5 random fields in each section. Data correspond to one representative round of two independent experiments. *p<0.05 (n=3-5/group).

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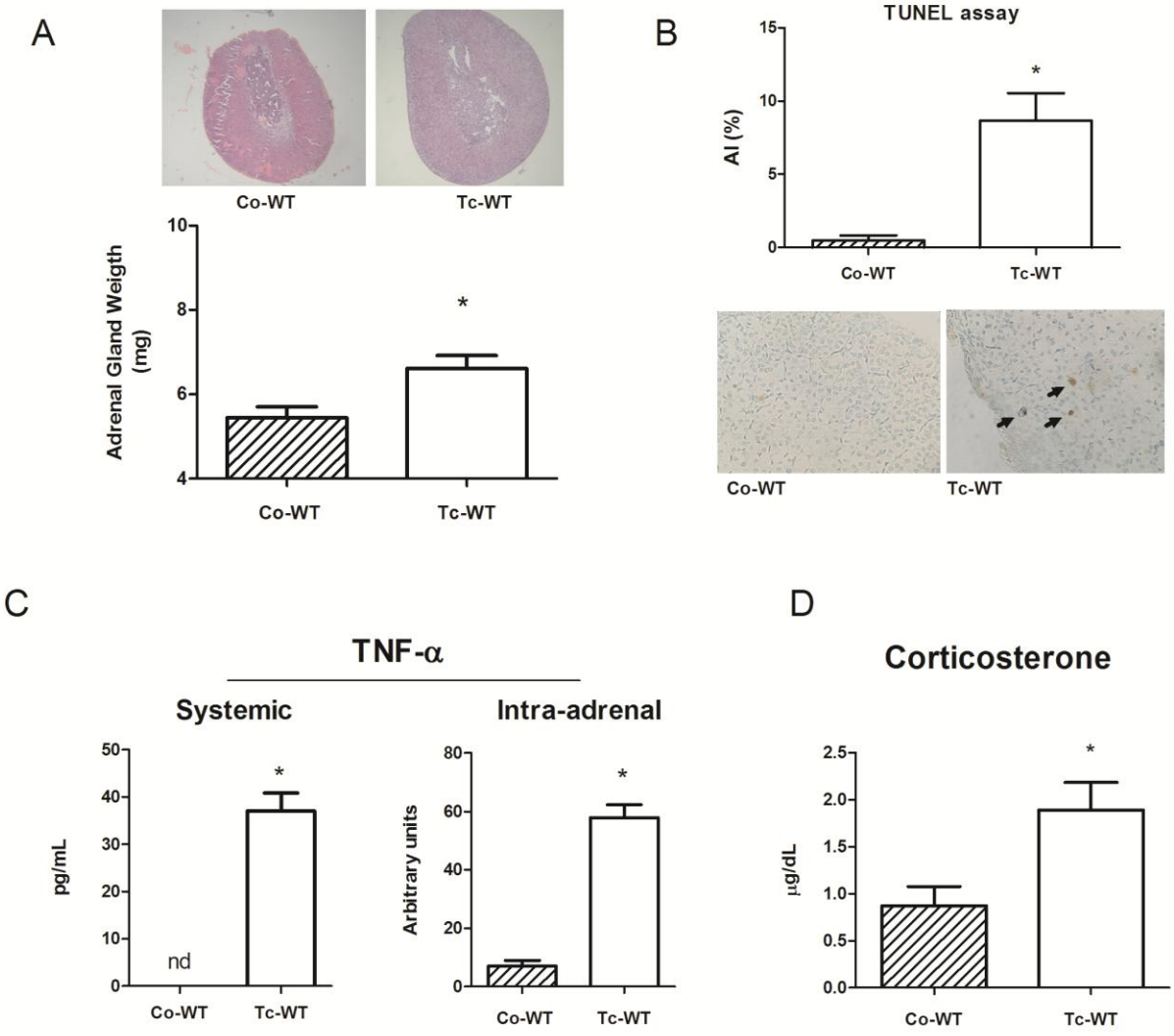
Perez et al Figura 1



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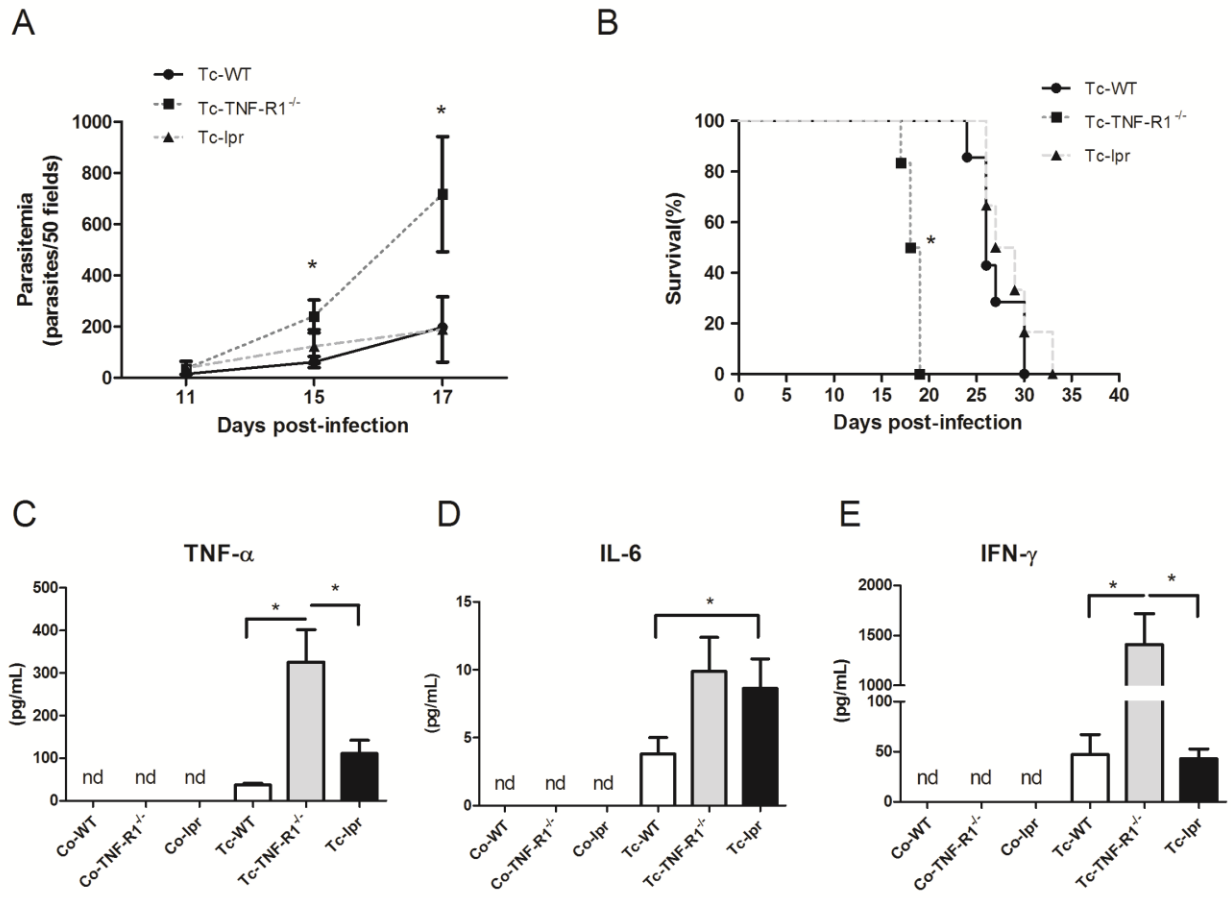
Perez et al, Figura 2



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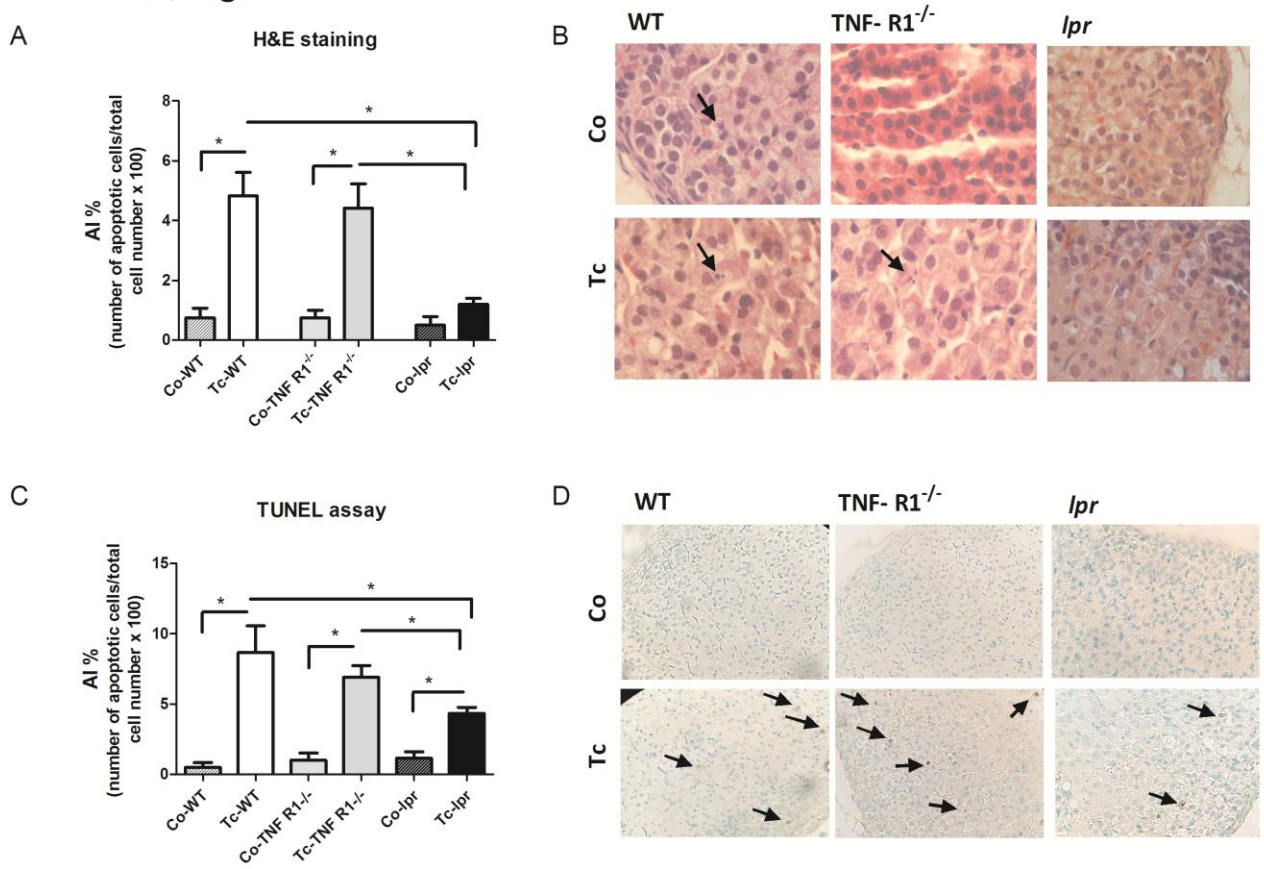
Perez et al, Figura 4



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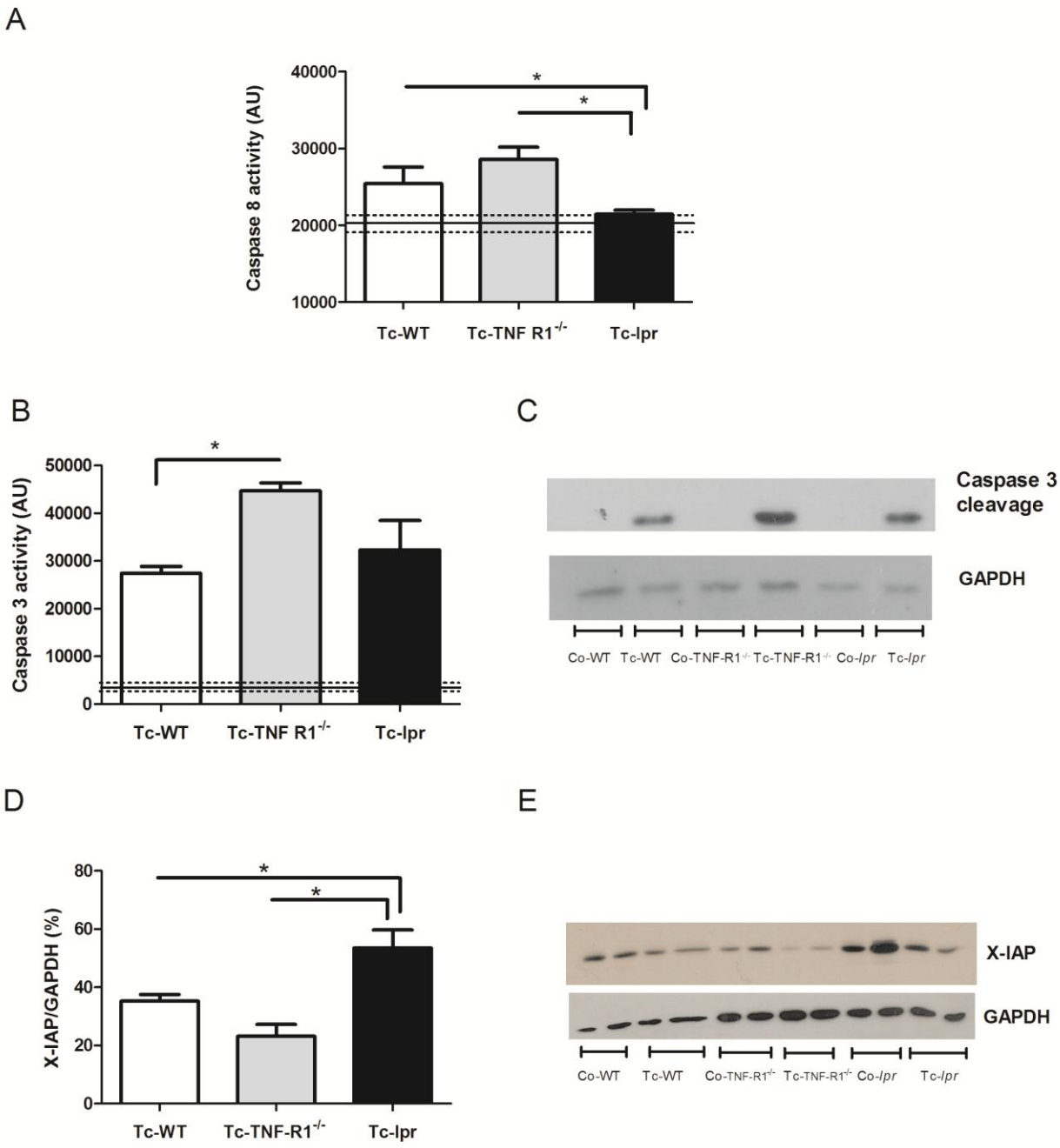
Perez et al, Figura 5



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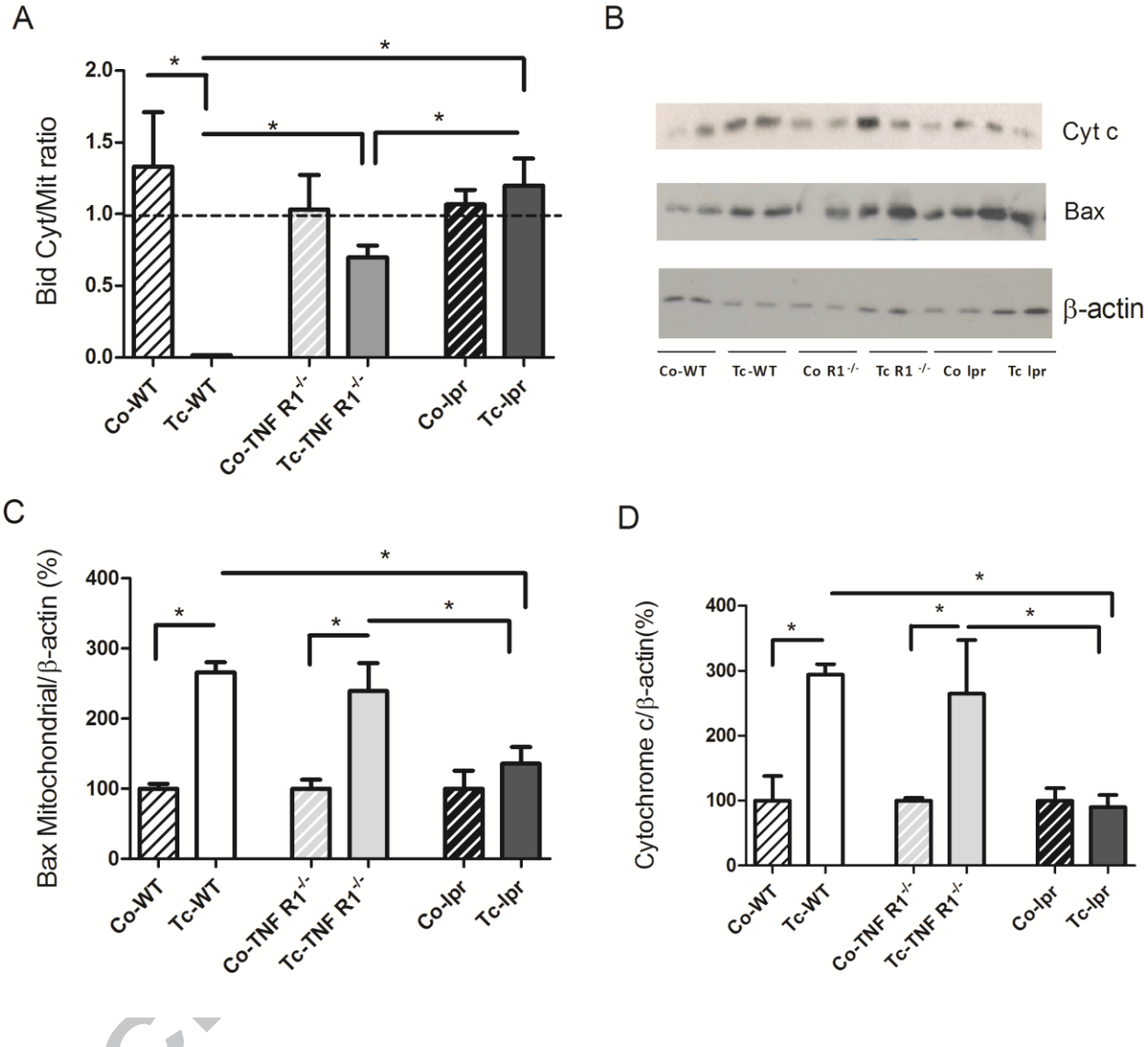
Perez et al, Figura 6



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Perez et al, Table 1

.	WT	Tc-WT	TNF R1$^{-/-}$	Tc-TNF R1$^{-/-}$	*lpr*	Tc-*lpr*
Adrenal gland weight (mg)	5,0 ± 0.8	6,2 ± 0.1*	5.3 ± 0.6	6,6 ± 0,9*	5,5 ± 0,5	7,2 ± 0,2*
Corticosterona (ug/dl)	0,87 ± 0,20	1,89 ± 0,29*	0,47 ± 0,18	4,29 ± 0,50*, #	0,55 ± 0,9	1,89 ± 0,84*,&
Cortex area (pixel^2)	589960±39069	754250±13408*	586340±35014	772975±213835*	466970±86247	674590±93528*
Z. Fasciculata (pixel^2)	243740±32784	326900±62449*	256800±43023	385263±126143*	26590±46477	382200± 62238*

*p,0.05 vs uninfected counterparts

Values represent mean ± sem of six mice/group, at 17 days p.i.

WT (wild type), TNF-R1$^{-/-}$ (TNF receptor deficient) and lpr (FAS receptor deficient) control mice; Tc-WT, Tc- TNF-R1$^{-/-}$ and Tc-lpr infected counterparts

A representative experiment from 2 independent series is shown.

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Highlights

Adrenal apoptosis is likely mediated by the FasL/Fas pathway in *T.cruzi*-infection

T cruzi-Infection is related with an exacerbated production of intra-adrenal cytokines

ACTH as not being involved in the apoptosis of adrenal cells

During *T. cruzi* infection the immune endocrine alterations affect infection control

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