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Brief Conclusive Report

Caspase-8 and caspase-9 mediate thymocyte apoptosis in *Trypanosoma cruzi* acutely infected mice

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RECEIVED DECEMBER 2, 2011; REVISED OCTOBER 25, 2012; ACCEPTED OCTOBER 29, 2012. DOI: 10.1189/jlb.1211589

ABSTRACT

Trypanosoma cruzi **acute infection leads to thymic atrophy, largely as a result of death of immature DP T cells. In a second vein, the glucocorticoid hormone imbalance promotes DP T cell apoptosis in infected mice. Herein, we assessed the involvement of caspase signaling in thymocyte death during *T. cruzi* acute infection. BALB/c mice were infected i.p. with 10^2 trypomastigote forms of *T. cruzi* and analyzed from 7 to 19 dpi. Thymocyte apoptosis was observed in early stages of infection, increasing along with time postinfection. Immature DN and DP as well as $CD4^+$ and $CD8^+$ thymocytes from infected mice showed increased activation of caspase-8, -9, and -3. In vitro treatment of thymocytes from infected mice with a general caspase inhibitor or the combination of caspase-8- and caspase-9-specific inhibitors increased the number of living thymocytes. Intrathymic injection of the general caspase inhibitor, but not caspase-8 or -9 inhibitors individually, prevented thymic atrophy and thymocyte depletion in infected mice. Moreover, blockade of glucocorticoid receptor activity with RU486 prevented DP thymocyte apoptosis, together with caspase-8 and -9 activation. These findings indicate that DP T cell apoptosis following experimental *T. cruzi* acute infection is dependent on glucocorticoid stimulation, promoting caspase-8 and -9 activation. *J. Leukoc. Biol.* 93: 227–234; 2013.**

Introduction

Chagas disease is caused by the protozoan *T. cruzi*. Transmission to humans occurs after biting of contaminated *reduviidae* insect vectors, with blood transfusion, congenitally, with organ transplantation, orally, or from a laboratory accident. It is estimated that 14–16 million people in Latin America and one million in the United States are infected with *T. cruzi*, with 670,000 premature disabilities and deaths/year worldwide [1, 2].

T. cruzi acute infection promotes several disturbances in the immune system with strong activation of innate and adaptive immune responses. Splenomegaly and expansion of subcutaneous lymph nodes were reported in mice, with persistent T and B cell polyclonal activation [3–5]. Conversely, in the same infectious conditions, atrophy of thymus and mesenteric LNs was observed [6–11].

T. cruzi-induced atrophy of the thymus occurs with a massive depletion of DP T cells [6, 8–10, 12]. Additionally, mitogenic responses of thymocytes from acutely infected mice are reduced as a result of a decrease in IL-2 production, which in turn, is associated with high levels of IL-10 and IFN-γ [9].

Among the molecules involved in thymocyte death secondary to acute *T. cruzi* infection, parasite- and host-derived moieties have been reported, comprising trans-sialidase, galectin-3, extracellular ATP, glucocorticoids, and androgens [13–20]. Conversely, typical cytotoxic molecules, such as Fas and perforin, are not involved [7].

Apoptosis can be triggered by distinct stimuli, such as growth factor withdrawal, cytotoxic factors, and death receptor-mediated signaling [21–23]. The intracellular apoptotic signaling pathways usually involve caspase activation [24, 25]. Caspases, a family of evolutionarily conserved cysteinyl aspartate-specific proteases, mediate apoptosis and inflammation. These proteases are normally expressed as zymogens and may become activated, leading to aspartate-specific cleavage of a wide number of cellular substrates, such as other caspases, NF-κB, iCAD, BH3 interacting-domain death agonist, IL-1β, and others [26, 27]. Caspase-8 is the initiator caspase activated

Abbreviations: DN=$CD4^-CD8^-$ double-negative, DP=$CD4^+CD8^+$ double-positive, dpi=day(s) postinfection, FasL=Fas ligand, iCAD=inhibitor of caspase-activated DNAse, i.p.=intraperitoneal, LN=lymph nodes, VAD=Val-Ala-Asp, zFA-fmk=benzyl-oxycarbonyl-Phe-Ala-fmk, zIETD-fmk=benzyl-oxycarbonyl-Ile-Glu(OMe)-Thr-Asp9OMe-fmk, zLEHD-fmk=benzyl-oxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fmk

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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by the apoptotic extrinsic pathway induced by FasL and TNF-α after triggering the corresponding death receptors Fas and TNF-RI [27, 28]. Caspase-9 is involved in the mitochondrial or intrinsic apoptotic pathway and is activated upon deprivation of growth factors and release of cytochrome c into the cytoplasm [28, 29].

The impact of thymic alterations during acute infection with respect to the development of the effector immune response against the parasite remains to be properly established. Yet, it is conceivable that a decline in the generation of T cells in the thymus during acute infection might favor the parasite rather than the host. In agreement with this idea is the fact that T lymphocytes are crucial for mounting an effective anti-*T. cruzi* immune response: athymic nude mice infected with *T. cruzi* show enhanced parasitemia, as well as an increase in mortality rate and shortened survival time [30, 31].

Here, we investigated the intracellular pathways of thymocyte apoptosis during acute *T. cruzi* infection. This is an important issue to be explored, as several candidate molecules were pointed out as being involved in thymocyte death following *T. cruzi* infection, although we still lack in vivo evidence of the main apoptotic inducer of thymocyte depletion in infected mice. Herein, we evaluated the possible candidate(s) involved in *T. cruzi*-triggered thymocyte depletion by blocking apoptotic pathways in vitro and in vivo. In vivo blocking caspase activation with zVAD-fmk, but not zIETD-fmk (caspase-8 inhibitor) or zLEHD-fmk (caspase-9 inhibitor) isolated, increases the number of viable T lymphocytes within the thymus, including DN, DP, and $CD4^+$ mature thymocytes. Lastly, we found that

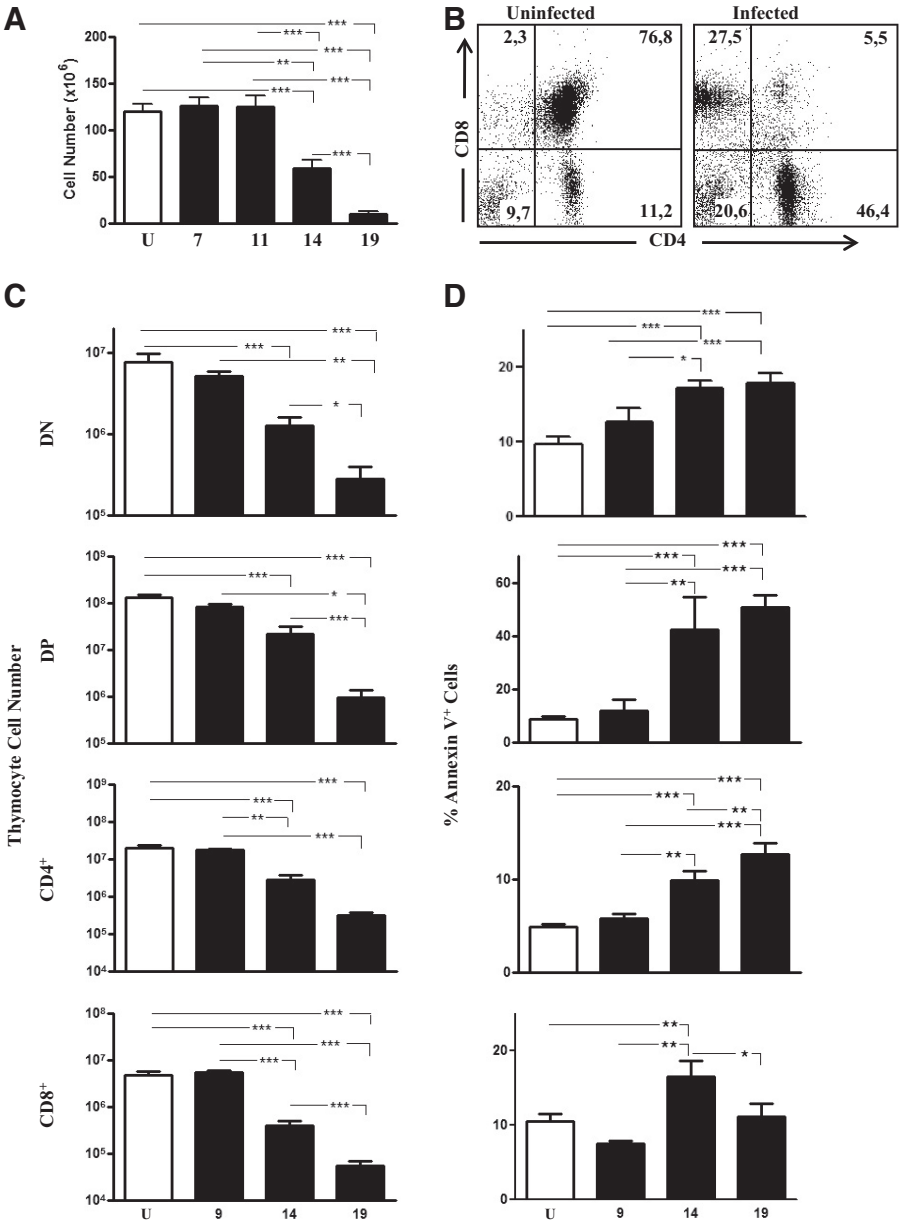

Figure 1. *T. cruzi* infection promotes thymic atrophy and thymocyte depletion by apoptosis. (A) The number of cells in the thymus in the course of infection; (B) the cytofluorometric dot plots for CD4 and CD8 in thymocytes from uninfected and 19 dpi mice. (C) The absolute numbers of DN, DP, and $CD4^+$ or $CD8^+$ single-positive thymocytes in uninfected (U) as well as 7 dpi, 11 dpi, 14 dpi, and 19 dpi. (D) We can see that the thymic changes correlate with thymocyte apoptosis-analyzed Annexin-V in CD4/CD8-defined thymocyte subsets. Percentages of apoptotic (Annexin V^+) cells in the same thymocyte subsets in the course of infection. BALB/c mice were infected with *T. cruzi*, and the thymuses were collected on indicated dpi. The number of viable thymocytes was counted in Neubauer's chamber, and exclusion of necrotic cells was performed by trypan blue. Values represent mean ± SEM, with the following numbers of mice evaluated in each group: uninfected, $n = 10$; 7 dpi, $n = 4$; 11 dpi, $n = 3$; 14 dpi, $n = 9$; 19 dpi, $n = 6$. Significant differences are indicated: $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

thymocyte depletion is glucocorticoid-dependent, inducing initiator caspase-8 and -9: infected mice treated with the glucocorticoid receptor antagonist (RU486) resulted in a reduction on thymus atrophy, DP depletion, and caspase activation.

MATERIALS AND METHODS

Mice and *T. cruzi* infection

Male BALB/c mice, aging 6–8 weeks, were obtained from the animal facility of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Animals were handled according to the rules of the Ethics Comittee for Animal Research of the Oswaldo Cruz Foundation. Mice were infected i.p. with 10^2 *T. cruzi* (Tulahuén strain), and bloodstream forms were recovered after serial passages in adult mice. Parasites were counted using Neubauer's chamber in PBS, and parasitemia was detected in 5 μL blood from tails by counting trypomastigote forms in 50 microscopic fields. Mouse parasitemia increased along with time and reached the peak on 19 dpi.

Thymocyte suspensions and in vitro caspase blockade

Thymuses were removed at 7, 9, 11, 14, and 19 dpi. Thymocyte suspensions were harvested in RPMI 1640, supplemented with 10% FCS (Cultilab, Brazil), 2 mM L-glutamine, 5×10^{-5} M 2-ME, 100 UI/mL penicillin, 1 mM sodium pyruvate, and 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), and the cells were counted using Neubauer's chamber. For in vitro blockade of caspases, 10^6 thymocytes from 14 dpi mice were maintained in triplicates for 18 h with RPMI, 10% FCS, 37°C, and 5% CO_2 and treated with the pan-caspase inhibitor zVAD-fmk, the caspase-8 inhibitor zIETD-fmk, the

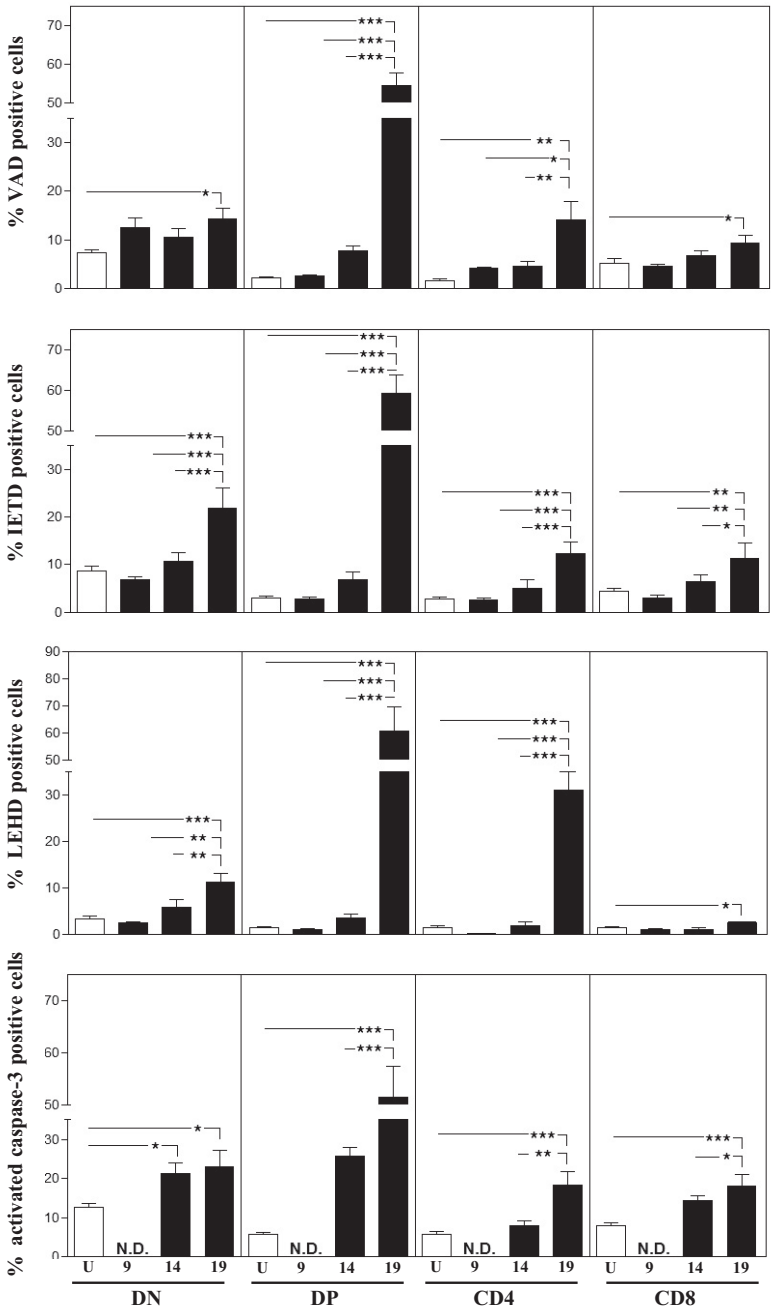

Figure 2. *T. cruzi* infection increases caspase activity in thymocytes from infected mice. Total cells from uninfected and infected mice (9, 14, and 19 dpi) were labeled with anti-CD4 and anti-CD8 mAb. The percentages of total caspases (VAD-FITC$^+$ cells), caspase-8 (IETD-FITC$^+$ cells), caspase-9 (LEHD-Red$^+$ cells), and caspase-3 (caspase-3$^+$-activated cells) cells were evaluated in DN, DP, and CD4 or CD8 thymocytes. ND, Not done. Values represent mean ± SEM of three different experiments (in each n=6 uninfected and n=6 infected mice/group). Significant differences from infected mice are observed: *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

caspase-9 inhibitor zLEHD-fmk, or the control peptide zFA-fmk (BD PharMingen, San Diego, CA, USA). Inhibitors were dissolved in DMSO (Merck, Germany), according to the manufacturer's instructions. Treatment with zIETD-fmk plus zLEHD-fmK peptide or by double doses of the respective control peptide zFA-fmk were also performed. In these experiments, thymocytes, prior to culture, were treated with one inhibitor (zIETD-fmk or zLEHD-fmk), and after incubation of 40 min in cold temperature, the other inhibitor was applied on the cells. After the addition of both inhibitors, thymocytes were cultured as described previously. Viable cells were then evaluated in Neubauer's chamber and exclusion of dead cells carried out by trypan blue.

In vivo blockade of caspases

To block caspase activity in vivo, infected animals were treated at 11 dpi with a single intrathymic injection of 100 μM zVAD-fmk, zIETD-fmk, or zLEHD-fmK peptide. In these experiments, mice were anesthetized with 5 mg/mL ketamine and 1 mg/mL xylasine (Agener União, Brazil). Intrathymic injections were performed with a single injection of the given peptide/thymic lobe in an open-chest cavity. The chest cavity was closed with surgical clips. Controls consisted of intrathymic injection of DMSO or z-FA-fmk. Four days after treatment (15 dpi), animals were killed, and thymocytes were analyzed by flow cytometry for CD3, CD4/CD8-defined subsets, and Annexin V.

In vivo blockade of the glucocorticoid receptor

T. cruzi-infected mice received daily i.p. injections of RU486 (mifepristone, Sigma-Aldrich; 1 mg/0.1 mL sesame oil) or sesame oil, starting at 10 dpi until 13 dpi. Uninfected animals received 0.1 mL RU486 or sesame oil (Sigma-Aldrich) at the same time.

Flow cytometry

Thymocytes were washed in RPMI (2% FCS) and incubated with 1 μL uninfected mouse serum, followed by addition of anti-CD4-allophycocyanin, anti-CD8-PerCP, plus anti-CD3-FITC or -PE antibodies (BD PharMingen). Thymocytes were labeled for surface molecules, fixed, and then labeled for VAD-FITC (CaspGLOW fluorescein caspase staining kit, BioVision, Milpitas, CA, USA), IETD-FITC (CaspGLOW fluorescein active caspase-8 staining kit, BioVision), or LEHD-Red (CaspGLOW red active caspase-9 staining kit, BioVision) or with PE-labeled antiactive caspase-3 mAb (BD PharMingen). Annexin V staining was performed according to the manufacturer (apoptosis detection kit, R&D Systems, Minneapolis, MN, USA). Cells were acquired using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA), with CellQuest support software (BD Biosciences). For analysis, Flow Jo software (Tree Stars, Ashland, OR, USA) was used.

Statistical analyses

The one-way ANOVA or Student's *t*-tests were applied for the statistical analyses. *P* values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

T. cruzi infection leads to thymocyte depletion by apoptosis

Confirming previous data, we observed a progressive thymus atrophy after 11 dpi, with a significant decrease in cell number in infected mice, as seen at the 14 and 19 dpi compared with uninfected mice (**Fig. 1**). As expected, the major target cells in the thymus from *T. cruzi*-infected mice were DP thymocytes, although absolute cell counting also revealed depletion in DN, as well as CD4 and CD8 single-positive subpopulations.

Moreover, a direct correlation among DN, DP, CD4 and CD8 thymocyte depletion and Annexin V labeling was evident in the thymus from *T. cruzi*-infected mice (Fig. 1). Altogether, these data suggest that thymocyte apoptosis is involved in thymus atrophy during acute *T. cruzi* infection. Additionally, we have demonstrated that although DP thymocytes are the main target cells, the other immature and mature CD4/CD8-defined subsets also undergo apoptosis during *T. cruzi* acute infection.

T. cruzi infection induces an increase of caspase activity in thymocytes

As mentioned above, lymphocyte apoptosis can be triggered by various stimuli [22, 23] that can activate distinct caspase pathways, with caspase-8 as the initiator of the extrinsic apoptotic pathway induced by death receptors [24] and caspase-9 activated upon deprivation of growth factors and release of cytochrome c [23, 32]. To address the possibility that caspases might be involved in thymocyte death, we performed flow cytometry analysis of total caspase (VAD^+ cells), caspase-8 ($IETD^+$ cells), caspase-9 ($LEHD^+$ cells), and caspase-3 activation in the CD4/CD8-defined thymocyte subsets during acute infection. As demonstrated in **Fig. 2**, in initial stages of infection (9 dpi), we could not detect significant differences of caspase activation, neither in total caspase activity (VAD^+ cells) nor initiator caspase-8 ($IETD^+$ cells), caspase- 9 ($LEHD^+$ cells), or effector caspase-3. However, on 14 and 19 dpi, a significant increase in percentage of VAD^+, $IETD^+$, and $LEHD^+$ cells became evident in all subpopulations, especially in DP, DN, and CD4 thymocytes. Interestingly, the percentages of DP thymocytes exhibiting caspase activation in the peak of parasitemia (19 dpi) changed from $<5\%$ to 50–60%, con-

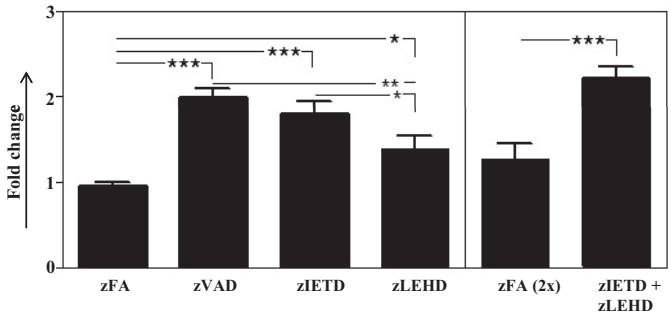

Figure 3. In vitro blockade of caspase activation prevents thymocyte death upon *T. cruzi* infection. One million thymocytes from infected mice (14 dpi) were cultured for 18 h with 100 μM zFA-fmk, zVAD-fmk, zIETD-fmk, or zLEHD-fmk. In other groups, combined treatment was done using caspase inhibitory peptides zIETD-fmk 100 μM plus zLEHD-fmk 100 μM. Treated cultures were harvested, and the number of viable thymocytes was counted in Neubauer's chamber. Exclusion of necrotic cells was performed by trypan blue. The combined treatment with zIETD-fmk plus zLEHD-fmk was also performed and compared with cells incubated with 200 μM zFA-fmk. Results are representative of three independent experiments where six wells/group were treated. Values represent mean ± SEM. Significant differences of fold change from treated cultures when compared with zFA-fmk are indicated: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$.

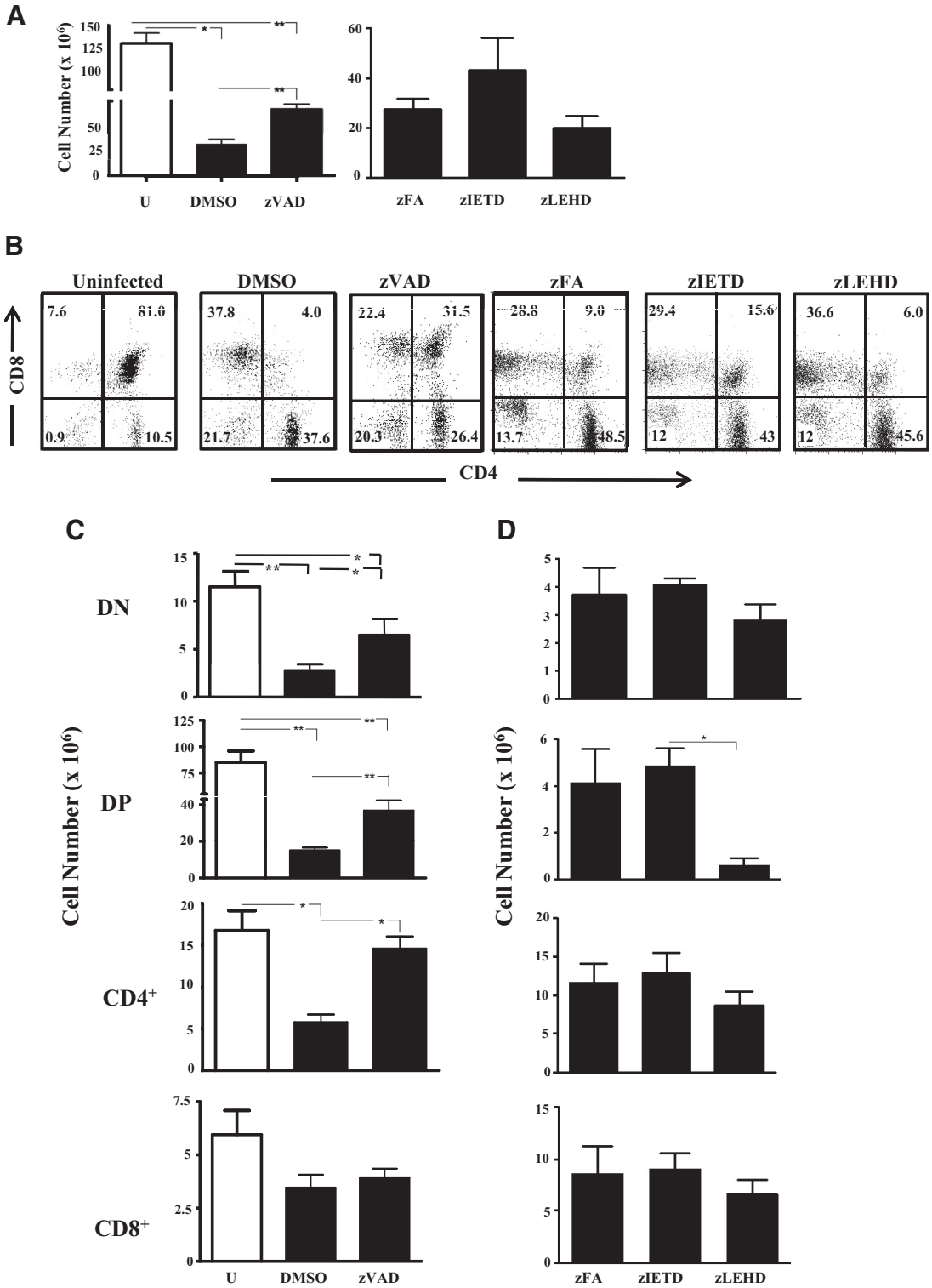

Figure 4. Intrathymic injection of zVAD-fmk modulates thymus cellularity in *T. cruzi* acutely infected mice. BALB/c mice were infected and, at 11 dpi and treated with intrathymic injection of 100 μM zIETD-fmk/lobe, zLEHD-fmk/lobe, zFA-fmk/lobe, zVAD-fmk/lobe, or DMSO/lobe. Four days later, animals were killed and the thymuses analyzed by flow cytometry for detection of CD4 and CD8. (A) The numbers of cells in the thymus from uninfected and DMSO-, zFA-fmk-, zVAD-fmk-, zIETD-fmk-, and zLEHD-fmk-treated mice; (B) the corresponding CD4/CD8-defined thymocytes subsets. (C) The absolute numbers of DN, DP, and $CD4^+$ or $CD8^+$ single-positive T cells in uninfected and infected DMSO- and zVAD-fmk-treated mice. (D) We can see the absolute numbers of DN, DP, and $CD4^+$ or $CD8^+$ T cells in infected zFA-fmk-, zIETD-fmk-, or zLEHD-fmk-treated mice. Our data demonstrated an increased number of thymocytes from infected animals treated with zVAD-fmk. Data from all panels are representative of three independent experiments, in which six to 10 animals were treated in each group. Values represent mean ± SEM. Significant differences from treated animals are indicated: $^*P < 0.05$; $^{**}P < 0.001$.

firming that these cells are more susceptible to death than other subpopulations. These results indicate that thymocyte depletion is associated with the increase of initiator caspase-8 and -9 activity and downstream caspase-3 activation following *T. cruzi* infection.

In vitro and in vivo caspase blockade prevents thymocyte death following *T. cruzi* infection

To investigate the influence of caspase activity in thymocyte depletion, BALB/c mice were infected with *T. cruzi* and sacrificed on 14 dpi. Cultured thymocytes were then treated with pan-caspase (zVAD-fmk), caspase-8 (zIETD-fmk), or caspase-9 (zLEHD-fmk) inhibitors or the controls (unrelated peptide zFA-fmk or DMSO). Considering that we have detected an increase in caspase-8 and -9 activation in thymocytes from acutely infected mice, we also included a combined treatment with zIETD-fmk plus zLEHD-fmk to confirm that these caspases are acting together in thymocyte death. Actually, inhibition of caspase-8 or -9 pathways individually, partially reduced the amounts of apoptotic cells. However, the combined treatment with zIETD-fmk plus zLEHD-fmk increased the numbers of viable cells when compared with treatment of each inhibitor alone (**Fig. 3**). Similar results were observed with zVAD-fmk-treated cells. Taken together, these data indicate that caspase blockage with zVAD-fmk or caspase-8 plus caspase-9 inhibitors is efficient in blocking in vitro apoptosis of thymocytes derived from *T. cruzi*-infected mice.

We then investigated the in vivo proapoptotic role of caspase activation in thymocytes from *T. cruzi* acutely infected mice. For that, BALB/c mice were infected and at 11 dpi, animals were submitted to an intrathymic injection of 100 μM zFA-fmk (control peptide), zIETD-fmk, zLEHD-fmk, zVAD-fmk, or DMSO (diluent) in each thymic lobe.

In vivo blockage of caspase-8 resulted in an overall increase in the number of viable thymocytes, although the differences between zFA-fmk and zIETD-fmk were not statistically significant (**Fig. 4**). Interestingly, although blockage of caspase-9 significantly increased the number of thymocytes in vitro, these results were not confirmed in vivo (Fig. 4). However, intrathymic injection with zVAD-fmk efficiently blocked thymocyte depletion, especially in DN, DP, and CD4 thymocytes from infected mice (Fig. 4). Thymus atrophy is not involved in parasite control, as thymectomized and intrathymically zVAD-fmk-treated mice showed similar parasitemia when compared with its counterparts (Supplemental Fig. 1).

Therefore, we suggest that blockade of caspase-8 or -9 activation isolated is not sufficient to prevent thymocyte apoptosis, as the other pathway is still activated, leading to thymocyte death. As such, thymocyte apoptosis in *T. cruzi* infection must be blocked by general caspase inhibition or simultaneous blockade of caspase-8 and -9 activation. Most importantly, these results were confirmed in vivo after a single intrathymic injection of z-VAD-fmk.

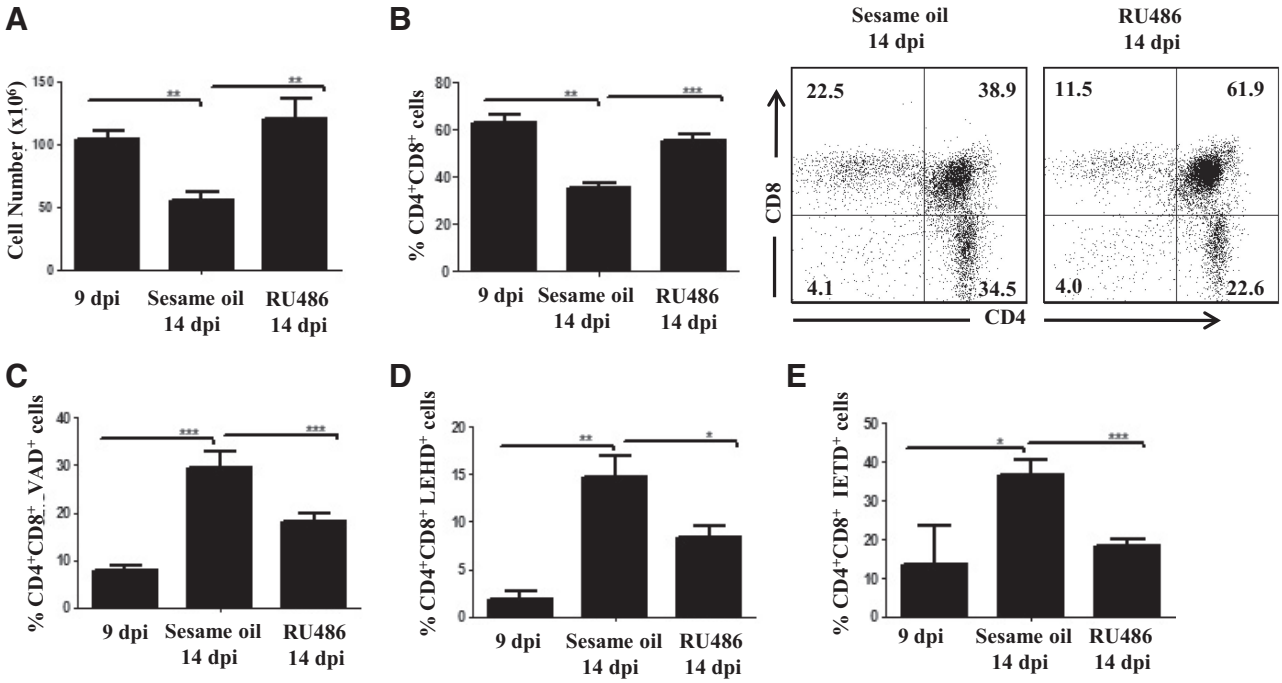

Figure 5. Blockade of the glucocorticoid receptor inhibits double-positive T cell depletion and caspase activation in thymus from *T. cruzi*-infected mice. Mice infected with *T. cruzi* received 1 mg/mL RU486 (in sesame oil) from 10 dpi to 13 dpi. Controls received sesame oil alone. On 14 dpi, animals were killed, and (A) cell number, (B) thymocyte differentiation, and (C–E) total caspase, caspase-8, and caspase-9 activation assays were performed. Percentages of total caspases (VAD-FITC$^+$ cells) and caspase-9 (LEHD-Red$^+$ cells)- and caspase-8 (IETD-FITC$^+$ cells)-positive cells were evaluated in DP thymocytes and can be seen in C–E, respectively. Values represent mean ± SEM of 11 mice treated with sesame oil and 13 mice treated with RU486. Significant differences from infected mice are observed: *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

In vivo blockade of the glucocorticoid receptor impairs caspase-8 and -9 activation in thymocytes from *T. cruzi* acutely infected mice

It is well known that glucocorticoids regulate DP thymocyte numbers by promoting thymocyte apoptosis [33–41]. Actually, once activated by ligand binding, the glucocorticoid receptor triggers thymocyte apoptosis through activation of caspase-8 and -9 [37, 42–44].

It has been shown that BALB/c mice infected with 100 Tulahuén trypomastigotes exhibit a progressive increase in corticosterone levels. Moreover, treatment with the steroid receptor antagonist RU486 from 10 dpi prevented thymus atrophy and DP thymocyte apoptosis in these infected mice [15]. In view of these data and considering other studies demonstrating that thymus atrophy in *T. cruzi* infection is mediated by glucocorticoid hormones [13, 14, 45, 46], we looked for caspase activation in infected mice treated with RU486. This treatment actually prevented thymus atrophy, DP depletion, total caspase, and caspase-8 and -9 activation in DP cells compared with sesame oil-treated mice (**Fig. 5**), whereas RU486 and sesame oil showed similar results in uninfected mice (Supplemental Fig. 2). These results are in agreement with previous reports describing glucocorticoids as strong candidates to promote in vivo thymus atrophy following acute *T. cruzi* infection [13–15]. Interestingly, DN and CD4 and CD8 T cell death was not dependent on glucocorticoid receptor signaling (data not shown). However, other molecules, such as trans-sialidase, galectin-3, extracellular ATP, and androgens, are candidates to promote thymocyte apoptosis [16–20]. The mechanisms underlying caspase activation in trans-sialidase, ATP, and androgen-mediated thymocyte death in *T. cruzi* infection warrant further investigation.

One possible explanation for the negative effect of Fas–FasL-mediated apoptosis in thymocyte apoptosis during *T. cruzi* infection is the fact that Fas stimulates activation of caspase-8 [7], and our data demonstrate that blockage of caspase-8 activation by itself in vivo is not sufficient to prevent thymocyte apoptosis and consequent thymus atrophy.

Following caspase-8 and/or caspase-9 activation, other molecules are activated, including effector caspases (caspase-3, caspase-7), iCAD, and components involved in cell integrity [47]. An increase in the downstream caspase-3 activation was also observed in thymocytes from *T. cruzi* acutely infected mice.

In conclusion, our data clearly show that in vivo thymocyte apoptosis during *T. cruzi* infection is dependent on glucocorticoid hormones, which are involved in activation of caspase-8, -9, and -3.

AUTHORSHIP

D.A.F-d-O. designed and performed flow cytometry as well as in vivo experiments, analyzed the data, and wrote the manuscript. D.M.S.V-V. performed flow cytometry and in vitro experiments and analyzed the data. P.H.N.P. participated in mouse surgery assays of intrathymic injection. D.S.d.S. participated in infection assays and flow cytometry analysis. L.R.B. participated in flow cytometry assays. W.S. participated in designing some experiments, as well as analyzing the data and writing the manuscript. J.d.M. designed the study, analyzed the data, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was funded by Rio de Janeiro State Research Foundation (FAPERJ), Brazilian National Research Council (CNPq), and Oswaldo Cruz Foundation (Brazil). We thank Dr. Agustin Zapata for critical reading of the manuscript.

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KEY WORDS:
thymus · T lymphocytes · Chagas Disease · glucocorticoid · cell death