Caspase inhibition reduces lymphocyte apoptosis and improves host immune responses to *Trypanosoma cruzi* infection

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In experimental Chagas' disease, lymphocytes from mice infected with *Trypanosoma cruzi* show increased apoptosis *in vivo* and *in vitro*. Treatment with a pan-caspase blocker peptide inhibited expression of the active form of effector caspase-3 *in vitro* and rescued both B and T cells from cell death. Injection of the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone, but not a control peptide, reduced parasitemia and lymphocyte apoptosis in *T. cruzi*-infected mice. Moreover, treatment with caspase inhibitor throughout acute infection increased the absolute numbers of B and T cells in the spleen and lymph nodes, without affecting cell infiltrates in the heart. Following treatment, we found increased accumulation of memory/activated CD4 and CD8 T cells, and secretion of IFN-γ by splenocytes stimulated with *T. cruzi* antigens. Caspase inhibition in the course of infection reduced the intracellular load of parasites in peritoneal macrophages, and increased the production of TNF-α and nitric oxide upon activation *in vitro*. Our results indicate that inhibition of caspases with a pan-caspase blocker peptide improves protective type-1 immune responses to *T. cruzi* infection. We suggest that mechanisms of apoptosis are potential therapeutic targets in Chagas' disease.

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Abbreviations: 7-AAD: 7-amino actinomycin D · **AICD:** activation-induced cell death · **dpi:** days post infection · **zFA:** benzyl-oxycarbonyl-Phe-Ala(OMe)-fluoromethyl ketone · **zIETD:** benzyl-oxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone · **zVAD:** benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone

Introduction

Chagas' disease or American Trypanosomiasis due to infection with *Trypanosoma cruzi* affects 16–18 million people in Latin America. Concerted innate and adaptive immune responses, effected by macrophages, B cells [1], T cells [2–4], and type 1 cytokines [5], control parasitemia during acute infection, but *T. cruzi* remains in host tissues throughout life. Chronic infection causes an inflammatory condition, affecting the heart or the autonomic nervous system in 25–30% of patients. Although it is generally accepted that T cells play a pathogenic role in chronic inflammation, whether pathology results from T cell recognition of self or parasite antigens is still controversial, and treatment remains elusive [6].

Cell death by apoptosis occurs in spleens [7], lymph nodes [8], and hearts [9] in the course of infection in experimental models of Chagas' disease, and in affected hearts from chronic patients [10]. Both T and B lymphocytes increase their rates of apoptosis in the course of *T. cruzi* infection [7, 11]. There is evidence that interactions between Fas ligand and Fas receptor [11, 12], as well as nitric oxide [13–15], are involved in lymphocyte apoptosis during *T. cruzi* infection. We have shown that stimulation with TCR:CD3 agonists triggers activation-induced cell death (AICD) in T cell cultures [7] and in *T. cruzi*-infected mice [16]. Previous *in vitro* experiments also suggest that apoptosis plays a pathogenic role in *T. cruzi* infection and that blocking apoptosis is a potential therapeutic intervention [17, 18]. Interactions between apoptotic lymphocytes and infected macrophages exacerbate parasite growth *in vitro* [17, 19], whereas the injection of apoptotic cells increases parasitemia in infected mice [17]. Therefore, it is important to investigate whether inhibition of apoptosis induced by infection affects immune responses to *T. cruzi*.

Apoptosis occurs through a sequence of events initiated by caspase activation, followed by the cleavage of target substrates by effector caspases, leading to nuclear condensation, DNA fragmentation, and cell shrinkage [20, 21]. The pharmacological manipulation of apoptosis and caspases may have clinical application and is a new frontier in the development of novel approaches to treat human diseases [22, 23].

We have previously shown increased apoptosis [7] and caspase activation [24] in T cells during *T. cruzi* infection, including caspase-8, the initiator caspase in apoptosis mediated by death receptors, such as TNF receptor I and Fas [25]. However, blockade of caspase-8 *in vivo* failed to inhibit apoptosis and negatively affected T cell-mediated immunity, by reducing CD8 T cell expansion and accumulation of memory/activated T cells [24, 26]. Additionally, inhibition of caspase-8 by transgenic viral FLIP (v-FLIP) up-regulated Th2 cytokine responses and increased susceptibility to *T. cruzi* infection [24].

In contrast, the pan-caspase blocker benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD) inhibited lymphocyte apoptosis and prevented animal death shortly after experimental induction of sepsis [27]. Treatment with zVAD inhibited pathogenic effects of apoptotic cells on the innate immune system [28]. On the other hand, despite an effective delivery of fluorescent zVAD to lymphocytes *in vivo*, zVAD did not affect the life span of antigen-specific CD8 T cells in a model of viral infection [29]. Therefore, the effects of caspase inhibition on specific immune responses are largely unknown.

To investigate whether caspase inhibition affects immune responses to chronic intracellular infection caused by *T. cruzi*, experiments were conducted with the pan-caspase inhibitor zVAD *in vitro* and *in vivo*. Our results show that zVAD blocked caspase activation and lymphocyte apoptosis, and increased resistance to *T. cruzi* infection. These results suggest that mechanisms of apoptosis are potential targets for therapeutic intervention in Chagas' disease.

Results

A pan-caspase inhibitor prevented caspase activation and lymphocyte apoptosis *in vitro*

In order to evaluate apoptosis in lymphocytes from *T. cruzi*-infected mice, splenocytes were taken during the acute phase of infection (21 days post infection (dpi)) and stained with annexin V-FITC (Fig. 1A). A higher percentage of annexin V-positive cells was observed in lymphocytes from *T. cruzi*-infected mice, compared with splenocytes from normal mice (Fig. 1A). To study susceptibility to cell death *in vitro*, splenocytes were first depleted of adherent cells to prevent phagocytosis of apoptotic cells by macrophages. Then, we let cells lay down on the wells and proceeded with cell counts and flow cytometry after 1 and 24 h. Compared to cells counted after 1 h, there was a drop of 50% in viable cells from infected mice in 24-h cultures (Fig. 1B). By contrast, 75–80% of splenocytes from normal mice were viable after 24 h (Fig. 1B). There was a significant decrease in the recovery of viable B and T cells in cultures from infected mice (Fig. 1C). Addition of the caspase inhibitor zVAD to cultures increased cell viability, as assessed by flow cytometry with annexin V and 7-amino actinomycin D (7-AAD) in splenocytes from infected mice (Fig. 1D). Compared with splenocytes treated with diluent alone, there was a significant increase in the number of viable B cells in cultures that received zVAD (Fig. 1E). We also recovered more viable cells from T cell cultures in the presence of zVAD (Fig. 1F). Next, we investigated the expression of active caspase-3 in T cell cultures. We observed increased expression of active caspase-3 in cultured CD4 (Fig. 2A) and CD8 (Fig. 2B) T cells from *T. cruzi*-infected mice, compared with T cells from normal mice, and the treatment with zVAD significantly reduced expression of active caspase-3 in T cells from infected mice (Fig. 2C). Stimulation with anti-CD3 Ab further increased caspase-3 activation in CD4 T cells from infected mice (Fig. 2A) and increased caspase activation in T cells, as assessed by flow cytometry upon staining with VAD-FITC (Fig. 2D, E). Treatment with the irreversible inhibitor zVAD (during overnight cultures) blocked
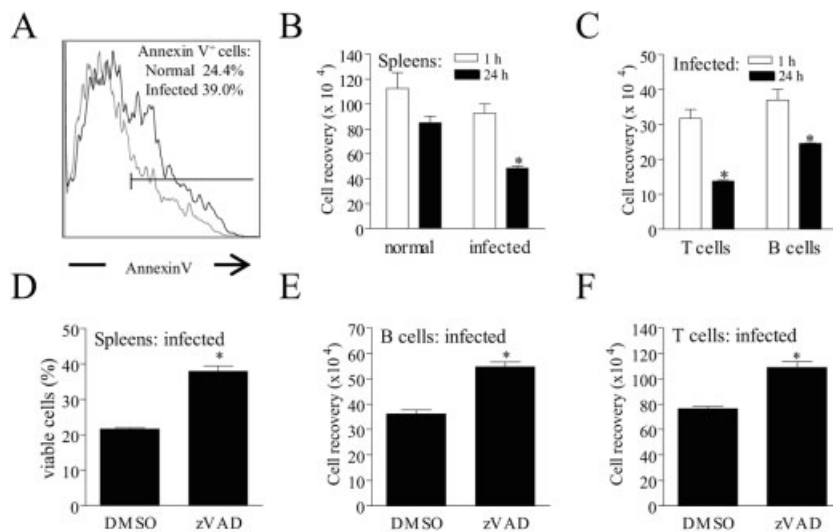
Figure 1. zVAD prevents apoptosis of lymphocytes from mice infected with *T. cruzi*. (A) Splenocytes from normal (gray line) and *T. cruzi*-infected (21 dpi; dark line) mice were stained with annexin V-FITC and analyzed by flow cytometry. (B, C) 1-h- and 24-h-cultured splenocytes from normal (B) and infected (B, C) mice were counted and stained with anti-CD19, anti-CD4, and anti-CD8 Ab for flow cytometry. Cell recovery was expressed as absolute number of cells/well. Significant differences between 1-h- and 24-h-cultured splenocytes are indicated (*) for $p < 0.05$. (D, E) Splenocytes from infected mice were cultured for 24 h with zVAD or with vehicle only, counted and stained with annexin V and 7-AAD (D) or with anti-CD19 Ab (E) for flow cytometry. In (D), annexin V^{neg}, 7-AAD^{neg} cells were considered as viable cells. (F) T cell cultures from infected mice were treated with zVAD or with DMSO for 24 h before viable-cell counts. Significant differences between cells cultured with zVAD and with DMSO only are indicated (*) for $p < 0.05$.

VAD-FITC binding in both CD4 and CD8 T cells (Fig. 2D, E) and increased recovery of viable cells in stimulated T cell cultures (Fig. 2F). The positive effects of zVAD (40 µM) on the viability of T cells from infected mice were also observed after 3 days upon stimulation with anti-CD3 Ab (increment of 53% in viable cell counts, compared with T cells stimulated in the presence of DMSO only). Therefore, zVAD prevented cell death in lymphocytes from *T. cruzi*-infected mice, by blocking caspase activation.

Treatment with zVAD reduced parasitemia and lymphocyte apoptosis *in vivo*

To test whether zVAD treatment prevents lymphocyte apoptosis and affects immune responses *in vivo*, mice were infected with *T. cruzi* and treated with zVAD 7 days later, and throughout infection. Parasitemia was followed during acute infection. Treatment with zVAD resulted in lower parasitemias, in contrast to treatment with benzyl-oxycarbonyl-Phe-Ala(OMe)-fluoromethyl ketone (zFA) control peptide, which did not differ from treatment with vehicle (DMSO) alone (Fig. 3A). In addition to reduction of parasite loads in the acute phase, treatment with zVAD improved the general aspect of mice, which did not show the ruffled fur observed in infected mice. Treatment with zVAD during infection decreased splenocyte apoptosis *in vivo* (Fig. 3B), without affecting the number of cells infiltrating the hearts

(Fig. 3C). To evaluate whether caspase inhibition was affecting CD8 T cell expansion or T cell activation, kinetic studies were conducted throughout infection. The expression of CD4, CD8, and CD44 (as a marker for T cell activation) was followed in blood samples from infected mice previously treated with DMSO or zVAD (Fig. 3D–F). Normal and infected mice were included as negative and positive controls, respectively. The proportion of CD8, but not CD4, T cells increased in the blood in the course of infection (Fig. 3D, E), independent of treatment. CD4/CD8 ratios in the blood dropped from 3.4 to 1.3 upon 14 dpi, as previously observed in spleens from infected mice [30]. We detected increased proportions of CD44^{hi} CD8 T cells (not shown), peaking at 19 dpi (59%), and CD44^{hi} CD4 T cells (Fig. 3F), peaking at 25 dpi (37%), in the blood. These results suggest that treatment with zVAD did not affect the ratio of T cell activation in infected mice. Nonetheless, inhibition of caspases increased the numbers of B and T cells in the spleens of treated mice (Fig. 4A–D). Activated CD4 (Fig. 4E) and CD8 (Fig. 4F) T cells also accumulated earlier (21 dpi) in the spleens of mice treated with zVAD. In this case, down-modulation of CD62L (Fig. 4E, F) and up-regulation of CD44 (not shown) expression were used as markers for T cell activation.
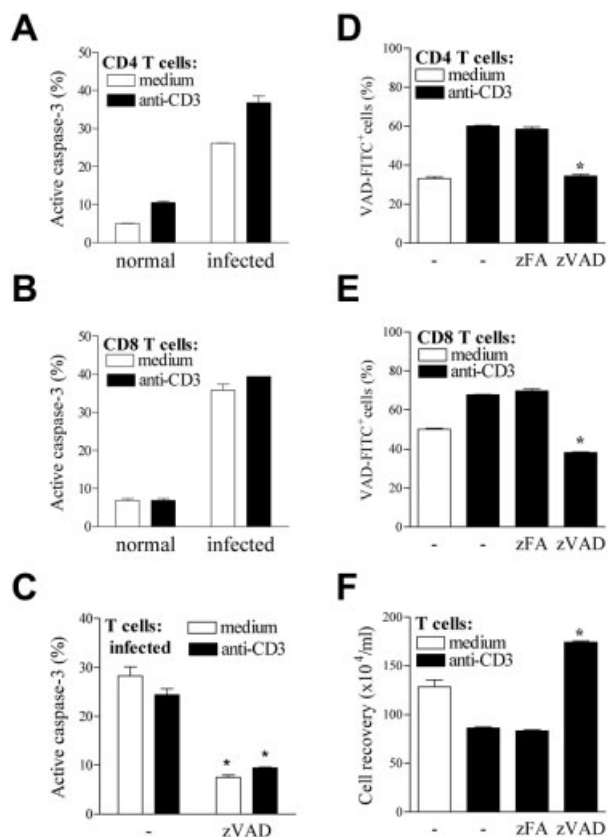
Figure 2. zVAD blocks caspase activation in T cells from *T. cruzi*-infected mice. (A–C) T cells from normal and *T. cruzi*-infected mice were cultured with medium or anti-CD3 Ab for 24 h, counted and stained for flow cytometry. (A, B) T cells were stained with anti-CD4 (A), anti-CD8 (B) and anti-active caspase-3 mAb. (C) T cells from infected mice were cultured with or without zVAD (80 µM) and stained with anti-active caspase-3 mAb. Significant differences between T cells cultured with zVAD and with medium only are indicated (*) for $p < 0.05$. (D–F) T cells from infected mice were cultured with medium only or were stimulated with anti-CD3 Ab in the presence of zVAD (40 µM) or the control peptide zFA for 24 h, before viable-cell counts (F) and staining with anti-CD4 (D), anti-CD8 Ab (E), and peptide VAD-FITC (D, E). Significant differences between stimulated T cells cultured with zVAD and with anti-CD3 Ab only are indicated (*) for $p < 0.05$.

Treatment with zVAD increased type 1 immune responses to *T. cruzi* infection

Next, we investigated how zVAD treatment altered immune responses in affected tissues. In our model, both infection and zVAD treatment were set through the i.p. route. There was an increase in the number of memory/effector (CD62L^{low}) CD8 T cells infiltrating the peritoneum in infected mice treated with zVAD (Fig. 5A). In addition, we detected an increased number of peritoneal macrophages (Fig. 5B). Compared to controls, macrophages from treated mice were larger, more spread out upon adherence (Fig. 5D–F) and showed reduced intracellular parasite loads (Fig. 5C).

These results suggest that, by blocking apoptosis, zVAD may affect immune responses to *T. cruzi in vivo*. In agreement with this conclusion, macrophages from mice treated with zVAD released higher amounts of TNF-α (Fig. 6A) and nitric oxide (Fig. 6B) in response to IFN-γ and LPS *in vitro*. There was also an increased production of IL-12 (Fig. 6C), but not IL-10 (not shown), by macrophages from mice treated with zVAD. Furthermore, splenocytes from treated mice secreted increased amounts of the type 1 cytokine IFN-γ in a recall response to *T. cruzi* antigens (Fig. 6D). Even after the interruption of treatment on day 24, parasitemia remained controlled (Fig. 3A), the numbers of activated CD8 T cells remained elevated in the spleens of treated mice, and the numbers of B cells, CD4, and CD8 T cells were increased in subcutaneous lymph nodes by day 31 of infection (not shown).

Direct effects of zVAD on parasite infection

We also investigated the effects of zVAD on intracellular infection. For that, macrophages were infected with metacyclic trypomastigotes, washed and then treated with zVAD. Under these conditions, the release of trypomastigote forms ($\times 10^4$ /mL) was not significantly affected (33.33 ± 0.72 for DMSO and 29.33 ± 1.78 for zVAD). These results suggest that zVAD treatment does not affect directly the release of parasites by infected macrophages.

Discussion

Previously, in the experimental model of sepsis, treatment with zVAD and other caspase inhibitors helped to control bacteremia and improved survival, presumably by preventing lymphocyte apoptosis [27, 31]. Here, we showed that treatment *in vivo* with the general caspase inhibitor zVAD modified host-parasite interplay in the experimental model of Chagas' disease.

Despite an intense polyclonal lymphocyte activation, *T. cruzi* infection induced defective T cell responses, associated with low IL-2 production [32] or signaling [33], CTLA-4 expression [34, 35], T cell apoptosis [7, 16], and NO production [13, 14]. This apparent paradox may be related to unbalanced ratios of proliferation and death upon T cell activation by *T. cruzi* antigens, affecting specific rather than polyclonal immune responses.

According to previous observations [7, 11], splenocytes from mice infected with *T. cruzi* were highly susceptible to undergo apoptosis *in vitro*. We observed increased expression of the active form of effector caspase-3 in T cells from infected mice. The peptide zVAD blocked the activation of caspase-3 in T cells and
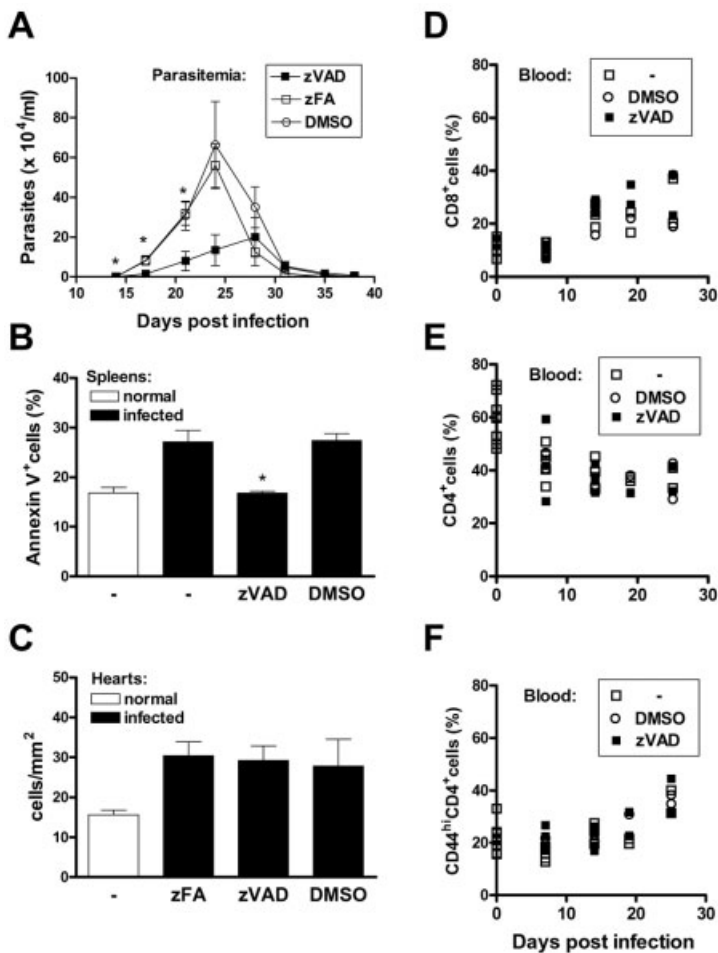
Figure 3. Blockade of apoptosis by zVAD during *T. cruzi* infection. Infected mice were injected twice a week, during 3 wk post infection, with 0.4 mg zVAD (*n* = 5), zFA (*n* = 6) or control vehicle (DMSO, *n* = 6). (A) Parasitemia was followed during the acute phase. For statistical analysis, data were transformed to ln and kinetic points, with significant differences between zVAD and control groups indicated (*) for $p < 0.05$. Results represent two independent experiments. (B) Annexin V-FITC binding in spleen cells from normal and infected (18 dpi) mice treated with zVAD or DMSO (*n* = 2–3 mice/group). (C) Inflammatory infiltrates in the hearts from normal and infected mice expressed as number of cells/mm^2 (*n* = 4–6 mice/group). Significant differences between infected mice treated with zVAD and control groups are indicated (*) for $p < 0.05$. (D–F) Flow cytometry in blood samples stained with anti-CD8 (D), anti-CD4 (E, F), and anti-CD44 Ab (F). CD44^{hi}CD4$^+$ cells were considered as activated CD4 T cells. Results obtained from individual mice were plotted for normal or infected mice without treatment (open squares) and for infected mice treated with DMSO (open circles) or zVAD (closed squares).

increased the recovery of viable B and T cells *in vitro*. Upon stimulation with anti-CD3 Ab, zVAD also prevented AICD in T cell cultures. Furthermore, treatment with zVAD decreased apoptosis and parasitemia *in vivo*, compared with mice that received either diluent only or the control peptide zFA.

The treatment with zVAD could affect immune responses to *T. cruzi* at multiple levels. First, by increasing the life span of lymphocytes, zVAD improves protective immune responses: while lymphocytes delay to die, they can act as effector cells. In agreement with this hypothesis, we found increased accumulation of both B and T cells in the spleens and lymph nodes, as well as activated CD4 and CD8 T cells in infected mice treated with zVAD. We detected increased production of

IFN-γ against *T. cruzi* antigens in splenocyte cultures. Furthermore, we found increased numbers of memory/ effector CD8 T cells and activated macrophages in the peritoneum of treated mice. Macrophages were also more responsive to treatment with LPS and IFN-γ *in vitro*, producing TNF-α and nitric oxide, and had lower intracellular parasite loads.

A second hypothesis to explain the effects of zVAD involves interactions of apoptotic cells and macrophages. We have previously shown that the phagocytosis of apoptotic cells drives *T. cruzi* growth in infected macrophages [17]. Apoptotic cells also induce TGF-β release and decrease the production of nitric oxide by macrophages responding to IFN-γ + LPS [17]. *In vivo*, injection of apoptotic cells increases parasitemia in

T. cruzi-infected mice [17] and decreases IFN-γ production in experimental sepsis [36]. Therefore, by blocking caspase activation and apoptosis, zVAD may inhibit direct negative effects of apoptotic cells on immune responses.

It should be noted that zVAD could affect other aspects of immune responses controlled by caspases. Treatment with zVAD could inhibit the killing of *T. cruzi*-infected cells by cytotoxic lymphocytes. However, this hypothesis was not directly assessed and is not supported by our findings showing reduced parasite loads upon treatment with zVAD. zVAD could also affect non-apoptotic effects of caspases. For example, caspase-8 deficiency results in defective immune responses both in humans and in experimental models [26, 37, 38]. Besides the established role of caspase-8 in death receptor-mediated death, recent studies demonstrate that caspase-8 plays a role in signaling cascades initiated by other receptors essential for immunity, such as antigen receptors and Toll-like receptors [39]. Accordingly, mice rendered defective in caspase-8 activity, either by transgenic viral-FLIP (v-FLIP) expression or by treatment with the peptide benzyl-oxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (zIETD), are more susceptible to *T. cruzi* infection [24]. Moreover, we found defective T cell expansion and increased Th2 responses upon caspase-8 blockade in *T. cruzi*-infected mice [24]. These results contrast with our findings in
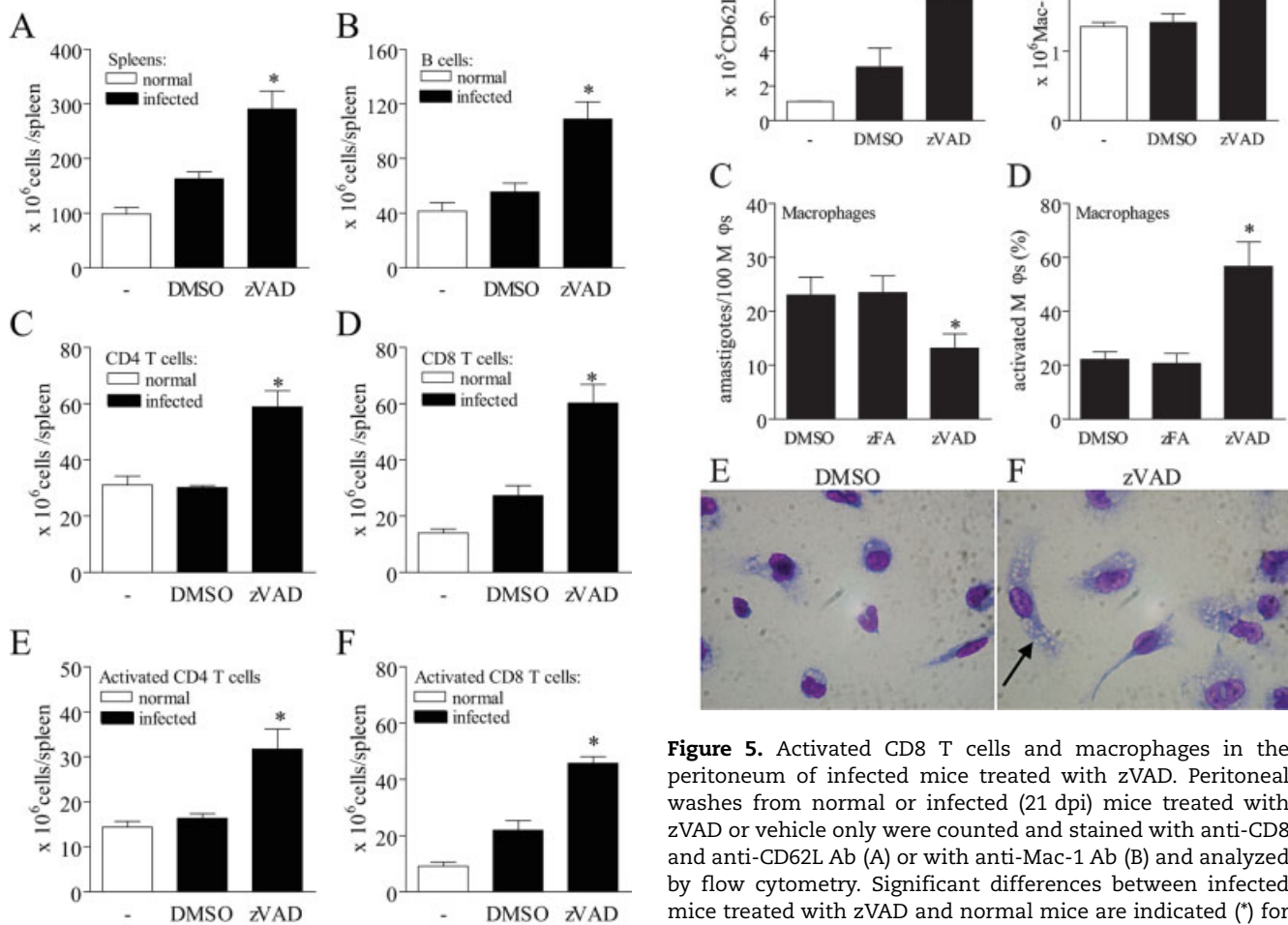

Figure 4. Treatment with zVAD increased lymphocyte life span during *T. cruzi* infection. Splenocytes from normal or infected (21 dpi) mice treated with zVAD or vehicle only were counted (A–F) and analyzed by flow cytometry (B–F). Splenocytes were stained with anti-CD19 (B), anti-CD4 (C, E), anti-CD8 (D, F) and anti-CD62L Ab (E, F). CD62L^low^CD4^+^ or CD8^+^ cells were considered as activated T cells. Significant differences between infected mice treated with zVAD and DMSO are indicated (*) for $p < 0.05$, $n = 3$ mice/group.

Figure 5. Activated CD8 T cells and macrophages in the peritoneum of infected mice treated with zVAD. Peritoneal washes from normal or infected (21 dpi) mice treated with zVAD or vehicle only were counted and stained with anti-CD8 and anti-CD62L Ab (A) or with anti-Mac-1 Ab (B) and analyzed by flow cytometry. Significant differences between infected mice treated with zVAD and normal mice are indicated (*) for $p < 0.05$, $n = 2$–3 mice/group. (C–F) Peritoneal cells from mice treated with DMSO, zFA or zVAD were cultured for 2 h, washed to remove nonadherent cells and stained with H&E. Amastigotes (C) and activated macrophages (D) were counted in H&E-stained slides. Significant differences between infected mice treated with zVAD and control groups are indicated (*) for $p < 0.05$, $n = 4$–6 slides/group. (E, F) Representative fields in H&E-stained slides from infected mice treated with DMSO (E) and zVAD (F). Arrow indicates a large, spread out macrophage. Magnification: 100 ×.
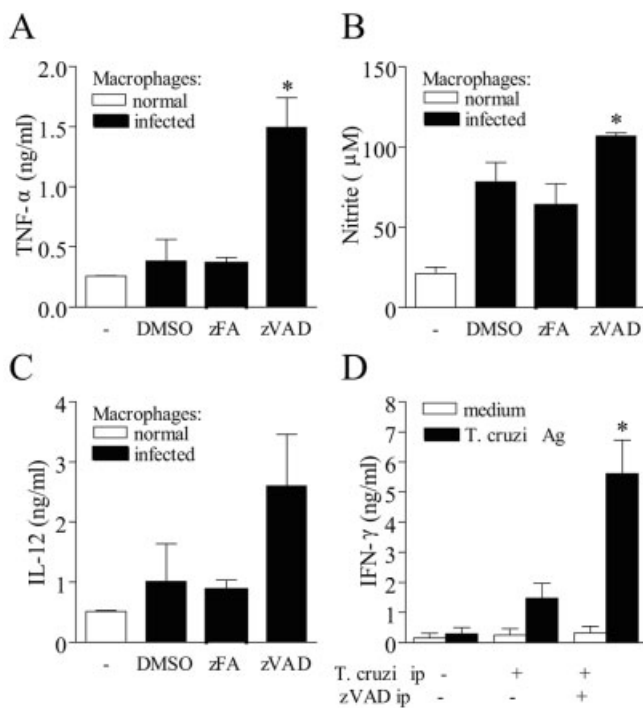
Figure 6. Increased protective immune responses to *T. cruzi* in mice treated with zVAD. Infected mice were treated with zVAD, DMSO or control peptide zFA. (A–C) Macrophages from normal or (21 dpi) infected mice were cultured with LPS and IFN-γ for 48 h and supernatants were tested for TNF-α (A), nitrites (B), and IL-12 (C). Significant differences between infected mice treated with zVAD and zFA are indicated (*) for *p* <0.05, *n* = 3 mice/group. (D) Splenocytes from normal and infected (31 dpi) mice were cultured with antigens from trypomastigotes or medium only for 72 h. IFN-γ was detected in culture supernatants by ELISA. Significant differences between infected mice treated with zVAD and control groups are indicated (*) for *p* <0.05, *n* = 4 mice/group.

mice treated with zVAD, as we did not find evidences of increased Th2 responses (not shown) or defective establishment of T cell memory. It is possible that zVAD is not as effective in blocking T cell responses compared to specific caspase-8 inhibitors such as zIETD [40]. In agreement with this idea, quantitative differences in caspase-8 inhibition generate distinct T cell phenotypes in v-FLIP-transgenic mice [26, 41].

Here, we show that zVAD is effective in blocking effector caspases and apoptosis upon infection. Our data indicate that treatment with zVAD increases resistance to *T. cruzi* infection, due to inhibition of lymphocyte apoptosis and exacerbation of type-1 immune responses. Therefore, we suggest that treatment with caspase inhibitors may be a potential therapeutic tool to control pathogenic effects of apoptosis and down-regulation of immune responses to infection.

Materials and methods

Mice and *T. cruzi* infection

Male BALB/c mice, aging 6–8 wk, were infected i.p. with 2×10^5 *T. cruzi* (clone Dm28c) trypomastigotes obtained by chemically induced metacyclogenesis [30]. Groups of infected mice were injected i.p. at 7, 10, 14, 17, 21 and 24 days after infection with 0.4 mg/mouse of caspase inhibitor zVAD (Enzyme systems Products), the control peptide zFA, or were treated with diluent only (0.7 mL 15% DMSO/PBS). Parasitemia was detected in blood from tails, by counting trypomastigote forms. Mice in all groups remained alive upon infection and treatment. For experiments, normal or infected mice were killed during the acute phase, at 21 dpi or at the end of the acute phase (31 dpi). All experiments and animal handling were conducted according to approved institutional protocols.

Cell suspensions and cultures

Splenocytes were depleted of RBC by treatment with Tris-buffered ammonium chloride. T cell-enriched suspensions were obtained by nylon wool filtration of splenocytes. Unfractionated splenocytes or nylon wool-enriched T cells were resuspended in DMEM (Invitrogen Life Technologies), supplemented with 2 mM glutamine, 50 μM 2-ME, 10 μg/mL gentamicin, 1 mM sodium pyruvate, and 100 μM MEM nonessential amino acids (culture medium) + 10% FBS (Invitrogen Life Technologies). Splenic cells (3×10^6/mL, 0.6 mL/well) were depleted of adherent cells by 2–3 h of culture in plastic plates, followed by transfer of nonadherent cells (0.5 mL) to 48-well vessels. Cultures were set at 37°C and 7% CO_2 in a humid atmosphere for 24 h. For 30 min before the setting of cultures, splenocytes or T cells were incubated with 80 μM zVAD, or with DMSO (0.4%) as a stock diluent control. We chose to use 80 μM of zVAD as an intermediate dose (50–100 μM) [40, 42] to prevent the cleavage of caspases and caspase substrates in T cells. Cell recovery was determined by viable cell counts in a hemacytometer chamber and by flow cytometry to estimate B and T cell subpopulations. T cells (4×10^6/mL) were cultured in duplicate in medium only or stimulated with 10 μg/mL plate-bound anti-CD3 Ab (mAb 2C11; BD Pharmingen) for 24 h. For apoptosis and caspase activation, cells were washed, stained, and evaluated by flow cytometry, as described below. For inhibition of caspase activation, zVAD and zFA were used at 40 μM.

Flow cytometry

Cells were washed in sorting buffer (containing 2% FBS) and incubated with anti-CD16/CD32 Ab for Fc blocking, followed by addition of allophycocyanin-labeled anti-CD8, anti-CD4, or anti-CD19 Ab for 30 min at 4°C. For markers of T cell activation, cells were also stained with PE-labeled anti-CD62L and FITC-labeled anti-CD44 Ab (all mAb from BD Pharmingen). Cells were acquired on a FACSCalibur system, by using CellQuest software (BD Biosciences). For apoptosis detection, splenocytes were stained with FITC-annexin V (apoptosis detection kit; R&D Systems) for 20 min at room temperature in

annexin buffer; 7-AAD was added just prior to flow cytometry. For staining with PE-labeled anti-active caspase-3 mAb (BD Pharmingen), cells were first stained with FITC-anti-CD4 and allophycocyanin-labeled anti-CD8 Ab, permeabilized and then stained according to the manufacturer's instructions. For detection of caspase activation, cells were treated with 5 µM FITC-VAD-fluoromethyl ketone (Promega) for 20 min, washed, stained with allophycocyanin-labeled anti-CD8 and PE-labeled anti-CD4 Ab, and fixed with 0.5% paraformaldehyde for 30 min. For analysis, FlowJo software was used (TreeStar).

Peritoneal macrophages

Cells were washed from the peritoneum, counted and analyzed by flow cytometry, as above. Macrophages were stained with PE-labeled anti-Mac-1 Ab. Macrophages were added to slides (2×10^5/slide) and plates (3×10^5/well), and incubated for 2 h before removal of nonadherent cells. Cells were cultured on slides for a further 3 h, before staining with H&E for detection of intracellular parasites. Images were digitalized using a color digital video camera (Coolpix 995; Nikon) adapted to an Eclipse E200 microscope (Nikon). Larger and spread out macrophages were considered as activated for quantification purposes. Cultures were stimulated with 2 ng/mL IFN-γ (BD Pharmingen) and 10 ng/mL LPS (Sigma) for 48 h, for cytokine and nitric oxide production. Supernatants were collected and mixed with an equal volume of Griess reagent to determine nitrite content as described [43].

ELISA

Splenocytes from each animal were cultured in triplicate (3×10^5/well) in round-bottom 96-well vessels for 72 h with *T. cruzi* antigens (metacyclic trypomastigote lysates equivalent to 3×10^5 parasites/well) or with medium alone. The presence of the cytokines IFN-γ, IL-4 and IL-10 was determined in culture supernatants. Supernatants from macrophage cultures were tested for TNF-α, IL-12 and IL-10 production. Cytokines were measured in a sandwich ELISA using pairs of specific mAb, one of which was biotinylated, and developed with avidin-HRP and substrate according to the manufacturer's instructions (BD Pharmingen).

In vitro infection

Peritoneal macrophages (3×10^5/well) added to 48-well vessels were infected overnight with metacyclic trypomastigotes (5×10^5/well), washed and treated with zVAD (60 µM) or DMSO (0.3%). Trypomastigotes released from macrophages were counted after 7 days in triplicate cultures.

Cell infiltrates in the hearts

For histopathological analysis, hearts, collected at 21 and 31 dpi, were fixed in buffered 10% formalin, dehydrated and embedded in paraffin. Sections were stained with H&E for microscopic analysis of cell infiltrates. Images were digitalized using a color digital video camera (CoolSnap cf) adapted to a BX41 microscope (Olympus, Japan). The images were analyzed using the Image Pro Program version 5.0 (Media Cybernetics) to integrate the number of inflammatory cells counted by area. The analysis was performed in ten randomly selected fields per heart from every mouse of each group.

Statistical analyses

The number (n) of animals per group is indicated in the figure legends and the symbol (*) denotes significant differences with a p value <0.05. Results are expressed as mean and SEM error bars in the figures. Data were analyzed by Student's *t*-test for independent samples using a SigmaPlot for Windows (version 4.01) package. For parasitemia, data were transformed to ln parasites/mL for statistical analyses. For *in vitro* experiments, data are expressed as average of two to three determinations per treatment, in each of at least three repeat experiments, and significant differences are indicated for p <0.05 (*).

References

1 **Kumar, S. and Tarleton, R. L.,** The relative contribution of antibody production and CD8⁺ T cell function to immune control of *Trypanosoma cruzi. Parasite Immunol.* 1998. **20:** 207–216.

2 **Tarleton, R. L., Koller, B. H., Latour, A. and Postan, M.,** Susceptibility of beta 2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature* 1992. **356:** 338–340.

3 **Rottenberg, M. E., Bakhiet, M., Olsson, T., Kristensson, K., Mak, T., Wigzell, H. and Orn, A.,** Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with *Trypanosoma cruzi* or *Trypanosoma brucei. Infect. Immun.* 1993. **61:** 5129–5133.

4 **Tarleton, R. L., Grusby, M. J., Postan, M. and Glimcher, L. H.,** *Trypanosoma cruzi* infection in MHC-deficient mice: Further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int. Immunol.* 1996. **8:** 13–22.

5 **Tarleton, R. L., Grusby, M. J. and Zhang, L.,** Increased susceptibility of Stat4-deficient and enhanced resistance in Stat6-deficient mice to infection with *Trypanosoma cruzi. J. Immunol.* 2000. **165:** 1520–1525.

6 **Kierszenbaum, F.,** Where do we stand on the autoimmunity hypothesis of Chagas disease? *Trends Parasitol.* 2005. **21:** 513–516.

7 **Lopes, M. F., da Veiga, V. F., Santos, A. R., Fonseca, M. E. and DosReis, G. A.,** Activation-induced CD4⁺ T cell death by apoptosis in experimental Chagas' disease. *J. Immunol.* 1995. **154:** 744–752.

8 **de Meis, J., Mendes-da-Cruz, D. A., Farias-de-Oliveira, D. A., Correa-de-Santana, E., Pinto-Mariz, F., Cotta-de-Almeida, V., Bonomo, A. and**

Savino, W., Atrophy of mesenteric lymph nodes in experimental Chagas' disease: Differential role of Fas/Fas-L and TNFRI/TNF pathways. *Microbes Infect.* 2006. **8:** 221–231.

9 Zhang, J., Andrade, Z. A., Yu, Z. X., Andrade, S. G., Takeda, K., Sadirgursky, M. and Ferrans, V. J., Apoptosis in a canine model of acute Chagasic myocarditis. *J. Mol. Cell Cardiol.* 1999. **31:** 581–596.

10 Tostes, S., Jr., Bertulucci Rocha-Rodrigues, D., de Araujo Pereira, G. and Rodrigues, V., Jr., Myocardiocyte apoptosis in heart failure in chronic Chagas' disease. *Int. J. Cardiol.* 2005. **99:** 233–237.

11 Zuniga, E., Motran, C., Montes, C. L., Diaz, F. L., Bocco, J. L. and Gruppi, A., *Trypanosoma cruzi*-induced immunosuppression: B cells undergo spontaneous apoptosis and lipopolysaccharide (LPS) arrests their proliferation during acute infection. *Clin. Exp. Immunol.* 2000. **119:** 507–515.

12 Lopes, M. F., Nunes, M. P., Henriques-Pons, A., Giese, N., Morse, H. C., 3rd, Davidson, W. F., Araujo-Jorge, T. C. and DosReis, G. A., Increased susceptibility of Fas ligand-deficient *gld* mice to *Trypanosoma cruzi* infection due to a Th2-biased host immune response. *Eur. J. Immunol.* 1999. **29:** 81–89.

13 Martins, G. A., Cardoso, M. A., Aliberti, J. C. and Silva, J. S., Nitric oxide-induced apoptotic cell death in the acute phase of *Trypanosoma cruzi* infection in mice. *Immunol. Lett.* 1998. **63:** 113–120.

14 Martins, G. A., Petkova, S. B., MacHado, F. S., Kitsis, R. N., Weiss, L. M., Wittner, M., Tanowitz, H. B. and Silva, J. S., Fas-FasL interaction modulates nitric oxide production in *Trypanosoma cruzi*-infected mice. *Immunology* 2001. **103:** 122–129.

15 Martins, G. A., Vieira, L. Q., Cunha, F. Q. and Silva, J. S., Gamma interferon modulates CD95 (Fas) and CD95 ligand (Fas-L) expression and nitric oxide-induced apoptosis during the acute phase of *Trypanosoma cruzi* infection: A possible role in immune response control. *Infect. Immun.* 1999. **67:** 3864–3871.

16 Lopes, M. F. and DosReis, G. A., *Trypanosoma cruzi*-induced immunosuppression: Selective triggering of CD4+ T-cell death by the T-cell receptor-CD3 pathway and not by the CD69 or Ly-6 activation pathway. *Infect. Immun.* 1996. **64:** 1559–1564.

17 Freire-de-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., DosReis, G. A. and Lopes, M. F., Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* 2000. **403:** 199–203.

18 DosReis, G. A., Freire-de-Lima, C. G., Nunes, M. P. and Lopes, M. F., The importance of aberrant T-cell responses in Chagas disease. *Trends Parasitol.* 2005. **21:** 237–243.

19 Nunes, M. P., Andrade, R. M., Lopes, M. F. and DosReis, G. A., Activation-induced T cell death exacerbates *Trypanosoma cruzi* replication in macrophages cocultured with CD4+ T lymphocytes from infected hosts. *J. Immunol.* 1998. **160:** 1313–1319.

20 Thornberry, N. A. and Lazebnik, Y., Caspases: Enemies within. *Science* 1998. **281:** 1312–1316.

21 Nicholson, D. W., Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* 1999. **6:** 1028–1042.

22 Lavrik, I. N., Golks, A. and Krammer, P. H., Caspases: Pharmacological manipulation of cell death. *J. Clin. Invest.* 2005. **115:** 2665–2672.

23 Green, D. R. and Kroemer, G., Pharmacological manipulation of cell death: Clinical applications in sight? *J. Clin. Invest.* 2005. **115:** 2610–2617.

24 Silva, E. M., Guillermo, L. V. C., Ribeiro-Gomes, F. L., De Meis, J., Pereira, R. M. S., Wu, Z., Calegari-Silva, T. C. et al., Caspase-8 activity prevents type 2 cytokine responses and is required for protective T cell-mediated immunity against *Trypanosoma cruzi* infection. *J. Immunol.* 2005. **174:** 6314–6321.

25 Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C. et al., FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996. **85:** 817–827.

26 Wu, Z., Roberts, M., Porter, M., Walker, F., Wherry, E. J., Kelly, J., Gadina, M. et al., Viral FLIP impairs survival of activated T cells and generation of CD8+ T cell memory. *J. Immunol.* 2004. **172:** 6313–6323.

27 Hotchkiss, R. S., Tinsley, K. W., Swanson, P. E., Chang, K. C., Cobb, J. P., Buchman, T. G., Korsmeyer, S. J. and Karl, I. E., Prevention of lymphocyte cell death in sepsis improves survival in mice. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 14541–14546.

28 Qin, S., Wang, H., Yuan, R., Li, H., Ochani, M., Ochani, K., Rosas-Ballina, M. et al., Role of HMGB1 in apoptosis-mediated sepsis lethality. *J. Exp. Med.* 2006. **203:** 1637–1642.

29 Nussbaum, A. K. and Whitton, J. L., The contraction phase of virus-specific CD8+ T cells is unaffected by a pan-caspase inhibitor. *J. Immunol.* 2004. **173:** 6611–6618.

30 Lopes, M. F., Cunha, J. M., Bezerra, F. L., Gonzalez, M. S., Gomes, J. E., Lapa e Silva, J. R., Garcia, E. S. and DosReis, G. A., *Trypanosoma cruzi*: Both chemically induced and triatomine-derived metacyclic trypomastigotes cause the same immunological disturbances in the infected mammalian host. *Exp. Parasitol.* 1995. **80:** 194–204.

31 Hotchkiss, R. S., Chang, K. C., Swanson, P. E., Tinsley, K. W., Hui, J. J., Klender, P., Xanthoudakis, S. et al., Caspase inhibitors improve survival in sepsis: A critical role of the lymphocyte. *Nat. Immunol.* 2000. **1:** 496–501.

32 Harel-Bellan, A., Joskowicz, M., Fradelizi, D. and Eisen, H., T lymphocyte function during experimental Chagas' disease: Production of and response to interleukin 2. *Eur. J. Immunol.* 1985. **15:** 438–442.

33 Beltz, L. A., Sztein, M. B. and Kierszenbaum, F., Novel mechanism for *Trypanosoma cruzi*-induced suppression of human lymphocytes. Inhibition of IL-2 receptor expression. *J. Immunol.* 1988. **141:** 289–294.

34 Graefe, S. E., Jacobs, T., Wachter, U., Broker, B. M. and Fleischer, B., CTLA-4 regulates the murine immune response to *Trypanosoma cruzi* infection. *Parasite Immunol.* 2004. **26:** 19–28.

35 Martins, G. A., Tadokoro, C. E., Silva, R. B., Silva, J. S. and Rizzo, L. V., CTLA-4 blockage increases resistance to infection with the intracellular protozoan *Trypanosoma cruzi*. *J. Immunol.* 2004. **172:** 4893–4901.

36 Hotchkiss, R. S., Chang, K. C., Grayson, M. H., Tinsley, K. W., Dunne, B. S., Davis, C. G., Osborne, D. F. and Karl, I. E., Adoptive transfer of apoptotic splenocytes worsens survival, whereas adoptive transfer of necrotic splenocytes improves survival in sepsis. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 6724–6729.

37 Chun, H. J., Zheng, L., Ahmad, M., Wang, J., Speirs, C. K., Siegel, R. M., Dale, J. K. et al., Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature* 2002. **419:** 395–399.

38 Salmena, L., Lemmers, B., Hakem, A., Matysiak-Zablocki, E., Murakami, K., Au, P. Y., Berry, D. M. et al., Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev.* 2003. **17:** 883–895.

39 Su, H., Bidere, N., Zheng, L., Cubre, A., Sakai, K., Dale, J., Salmena, L. et al., Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science* 2005. **307:** 1465–1468.

40 Kennedy, N. J., Kataoka, T., Tschopp, J. and Budd, R. C., Caspase activation is required for T cell proliferation. *J. Exp. Med.* 1999. **190:** 1891–1896.

41 Woelfel, M., Bixby, J., Brehm, M. A. and Chan, F. K., Transgenic expression of the viral FLIP MC159 causes *lpr/gld*-like lymphoproliferation and autoimmunity. *J. Immunol.* 2006. **177:** 3814–3820.

42 Alam, A., Cohen, L. Y., Aouad, S. and Sekaly, R. P., Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J. Exp. Med.* 1999. **190:** 1879–1890.

43 Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R., Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* 1982. **126:** 131–138.