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Caspase-8 Activity Prevents Type 2 Cytokine Responses and Is Required for Protective T Cell-Mediated Immunity against Trypanosoma cruzi Infection


During Trypanosoma cruzi infection, T cells up-regulate caspase-8 activity. To assess the role of caspase-8 in T cell-mediated immunity, we investigated the effects of caspase-8 inhibition on T cells in viral FLIP (v-FLIP) transgenic mice. Compared with wild-type controls, increased parasitemia was observed in v-FLIP mice infected with T. cruzi. There was a profound decrease in expansion of both CD4 and CD8 T cell subsets in the spleens of infected v-FLIP mice. We did not find differences in activation ratios of T cells from transgenic or wild-type infected mice. However, the numbers of memory/activated CD4 and CD8 T cells were markedly reduced in v-FLIP mice, possibly due to defective survival. We also found decreased production of IL-2 and increased secretion of type 2 cytokines, IL-4 and IL-10, which could enhance susceptibility to infection. Similar, but less pronounced, alterations were observed in mice treated with the caspase-8 inhibitor, zIETD. Furthermore, blockade of caspase-8 by zIETD in vitro mimicked the effects observed on T. cruzi infection in vivo, affecting the generation of activated/memory T cells and T cell cytokine production. Caspase-8 is also required for NF-κB signaling upon T cell activation. Blockade of caspase-8 by either v-FLIP expression or treatment with zIETD peptide decreased NF-κB responses to TCR/CD3 engagement in T cell cultures. These results suggest a critical role for caspase-8 in the establishment of T cell memory, cell signaling, and regulation of cytokine responses during protozoan infection.

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2 E.M.S. and L.V.C.G. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Marcela de Freitas Lopes, Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Bloco G, Ilha do Fundão, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21944-970, Brazil. E-mail address: marcelal@biofis.uff.br


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4 Abbreviations used in this paper: AICD, activation-induced cell death; c-FLIP, cellular FLIP; c-FLIPL, c-FLIP long; Fasl, Fas ligand; v-FLIP, viral FLIP; WT, wild type.

American trypanosomiasis or Chagas’ disease affects 20 million people in Latin America and is caused by the intracellular protozoan parasite Trypanosoma cruzi. T. cruzi elicits strong innate and adaptive immune responses that control parasitism, but do not eradicate infection (1–3). Both CD4 and CD8 effector T cells are required to reduce parasitism, prevent animal death, and establish immunological memory against T. cruzi infection (4–6). Cytokines secreted by T cells play a central role in host immune responses to T. cruzi infection (3, 7–9) by inducing macrophages to kill parasites (10, 11). The type 1 cytokine IFN-γ up-regulates macrophage trypanocidal activity, whereas the type 2 cytokine IL-10 inhibits IFN-γ effects (10, 11). Resistance to T. cruzi infection depends on a balance between type 1 and type 2 cytokine responses (3, 8, 9).

Apopoptosis of T cells occurs in the course of T. cruzi infection and could have a deleterious role, either by compromising immune responses (12, 13) or by exacerbating parasite replication (14, 15). CD4 T cells activated by T. cruzi infection die by activation-induced cell death (AICD) (12). AICD is effected through Fasl (FasL)/Fas interactions and is absent in FasL-deficient gld mice infected with T. cruzi (16).

Caspase-8 is an initiator caspase in apoptotic signaling triggered by Fas and TNF receptor I (TNF-RI) (17–19). However, recent studies demonstrated that caspase-8 activity may also be required for some aspects of T cell activation and IL-2 production (20–23). Oligomerization and cleavage of procaspases into active caspases-8 initiate apoptosis through activation of effector caspases (24, 25). Caspase-8 activation is regulated by short and long isoforms of cellular FLIP (c-FLIP), which interfere with Fas-mediated apoptosis (26). In addition, caspase-8 induces nonapoptotic signaling through the NF-κB pathway upon heterodimerization with c-FLIP long (c-FLIPL), cleavage of c-FLIPL into p43 subunit, and recruitment of TNF receptor-associated factor-2 (27).

Viral FLICE/caspase-8 inhibitory proteins (v-FLIPs) mimic c-FLIP in the ability to block caspase-8 activation, suppressing death effector caspase cascades (28–30). Recently, T cell function was investigated in mice engineered to block T cell caspase-8 activity by transgenic v-FLIP (MC159) expression (31). Transgenic v-FLIP inhibited Fas-mediated T cell death in vitro, but unexpectedly impaired CD8 T cell memory in vivo and affected immunity
to T. cruzi and viral infections (31). These effects correlated with defective survival of memory/activated T cells (31) and mirrored similar findings in caspase-8-deficient mice (32).

In this study we show that both CD4 and CD8 T cell immune responses are impaired in T. cruzi-infected v-FLIP mice, with deficiency accumulation of memory/activated T cells and exacerbated type 2 cytokine responses. Furthermore, treatment of nontransgenic mice with the caspase-8 inhibitor zIETD peptide in vivo increased parasitemia and type 2 cytokine responses while reducing numbers of memory/activated T cells during T. cruzi infection. These results place caspase-8 as an essential component of T cell signaling that induces efficient immunity and immune regulation in protozoan infection.

Materials and Methods

Mice and T. cruzi infection

Male v-FLIP transgenic (v-FLIP) and C57BL/6 wild-type (WT) mice were obtained from the National Institutes of Health. Mice, aged 13 wk, were infected i.p. with T. cruzi (clone Dm28c) trypomastigotes derived from cell cultures (Fig. 2A only) (31) or with metacyclic trypomastigotes obtained by chemically induced metacyclogenesis (see Fig. 2B and following figures with v-FLIP mice) (33). Male BALB/c mice, aged 6–7 wk, were obtained from the Oswaldo Cruz Institute (FIOCRUZ). Mice were infected with metacyclic trypomastigotes and treated at 4, 7, 11, 13, 16, 19, and 22 days after infection with 0.4 mg/injection of the caspase-8 inhibitor peptide zIETD-fmk, the caspase-9 inhibitor zLEHD-fmk, or the control peptide zFA-fmk (Enzyme Systems Products) diluted in 0.7 ml of DMSO/PBS. zIETD, for inhibition of caspase-8 and caspase-9, respectively, or with zLEHD, for inhibition of caspase-8 and caspase-9, or with DMSO (0.4%) only and cultured in 24-well vessels. T cells from normal or infected mice were treated with DMSO only or were cultured with anti-CD3 and zIETD (40 μM) or DMSO (0.4%) for 4 h. Total extracts were tested by Western blots with Abs against IκBa and actin as a control (Santa Cruz Biotechnology).

ELISA

For cytokine production, T cells or CD4 T cells from each infected mouse were cultured in triplicate in 96-well vessels (2 × 10^6 cells/well) with plate-coated anti-CD3 or with medium alone, as described above. Levels of IFN-γ, IL-4, IL-5, and IL-10 were determined in culture supernatants harvested after 48 h. Cytokine levels were measured in a sandwich ELISA format from specific mouse Abs (supplied by Sigma-Aldrich) which was biotinylated (R&D Systems or BD Pharmingen) and developed with streptavidin-alkaline phosphatase (BD Pharmingen) and p-nitrophenylphosphate substrate (Sigma-Aldrich).

IκB degradation

T cells (1 × 10^6) from infected BALB/c mice were either fresh or stimulated with immobilized anti-CD3 (10 μg/ml) for 1, 2, or 4 h in 48-well vessels. T cells from normal or infected mice were treated with DMSO only or were cultured with anti-CD3 and zIETD (40 μM) or DMSO (0.4%) for 4 h. Total extracts were tested by Western blots with Abs against IκBa and actin as a control (Santa Cruz Biotechnology).

EMSA

T cells (5 × 10^6/ml) from normal or infected BALB/c mice were treated for 1 h with 40 μM zIETD or DMSO (0.4%) only and cultured in 24-well vessels with 10 μg/ml immobilized anti-CD3 or medium. After 15 h, culture supernatants and cells were collected for ELISA and EMSA, respectively. T cells from v-FLIP or WT mice were activated with 5 μg/ml anti-CD3 and anti-CD28 for 24 h. Nuclear extracts were obtained and analyzed for NF-κB activation by EMSA, as previously described (34). Binding reactions were performed using 2 μg of nuclear protein in the presence of 40,000 cpm of 32P-end-labeled double-stranded consensus NF-κB oligonucleotide (sequence, 5′-AGT TGA GGC GAC TTG CCC AGG C-3′; Santa Cruz Biotechnology) and 1 μg of poly(dI-dC):(dI-dC) (Amersham Biosciences) for 30 min at room temperature and were analyzed by EMSA.

Statistics

Results are expressed as the average and SEM in the figures. Data were analyzed by Student’s t test for independent samples using a SigmaPlot for Windows (version 4.01) package. Differences with a p < 0.05 were considered significant. The number (n) of animals per group is indicated in figure legends, and an asterisk denotes significant differences between groups of infected mice. For in vitro experiments, data were expressed as the average of three determinations for most experiments, and significant differences were indicated for p < 0.05.

Results

Up-regulation of caspase activity in T cells from T. cruzi-infected mice

By using a combination of flow cytometry and enzymatic colorimetric assays, we evaluated caspase activity in lymphocytes in the course of T. cruzi infection (Fig. 1). Compared with T cells from uninfected mice, both CD4 and CD8 T cells expressed increased caspase activation during acute infection (Fig. 1A). Expression of activated caspases was also observed in B cells during infection (not shown). T cells from T. cruzi-infected mice expressed increased caspase-8 activity compared with T cells from normal mice (Fig. 1B). Expression of activated caspases further increased in T cells upon activation with anti-CD3 in vitro (Fig. 1C) and
correlated with increased AICD in T cell cultures from infected mice (Fig. 1D). Next, we evaluated the effects of the caspase-8 inhibitor zIETD and the caspase-9 inhibitor zLEHD. Although the specificity of caspase blockers has been questioned (21), the use of distinct caspase inhibitors (zIETD and zLEHD) and a control peptide (zFA) resulted in different outcomes in our in vivo and in vitro models. Incubation with zIETD partially prevented the induction of activated caspases by anti-CD3 and blocked AICD in T cell cultures from infected mice (Fig. 1D). In contrast, no significant differences were observed in T cells treated with zLEHD or control peptide zFA. Therefore, T cell activation may contribute to increased caspase activity in the course of T. cruzi infection.

Blockade of caspase-8 activity increases susceptibility to T. cruzi infection

To assess the role of caspase-8, we investigated the outcome of T. cruzi infection in transgenic mice expressing the caspase-8 inhibitor v-FLIP in T cells (v-FLIP mice). As previously reported (31), v-FLIP mice were highly susceptible to high doses of virulent (culture-derived) T. cruzi trypanosomatids, with increased parasitemia (Fig. 2A) and mortality (31). To mimic natural human infection and prevent mortality, we infected transgenic mice with low doses of metacyclic forms of T. cruzi, which we have shown to be comparable to insect-derived metacyclic trypanosomatids (33). Again, v-FLIP mice had higher parasitemia and delayed resolution of acute infection compared with WT mice (Fig. 2B). Nontransgenic BALB/c mice were also infected with metacyclic forms of T. cruzi in the presence or the absence of treatment with caspase inhibitors. Parasitemia was increased in mice treated with the caspase-8 inhibitor zIETD compared with the control peptide zFA or with zLEHD, which did not differ from the control (Fig. 2C). These results demonstrate that blockade of caspase-8 activity, either by transgenic expression of v-FLIP in T cells or by administration of zIETD, increased susceptibility to T. cruzi infection.

Caspase-8 is required for T cell expansion and memory

We investigated alterations in immune responses that could compromise the control of parasite infection. Compared with WT mice, expansion of T cell compartments was impaired in infected v-FLIP mice, and both CD4 and CD8 T cell numbers were reduced (Fig. 3, A and B). B cells were not significantly affected (not shown). Transgenic blockade of caspase-8 activity by v-FLIP did not reduce the levels of CD4 (Fig. 3C) or CD8 (Fig. 3D) T cell apoptosis in infected mice. In fact, there was a significant increase in CD8 T cell death compared with that in WT mice (Fig. 3D). We also observed increased spontaneous cell death in T cell cultures from infected v-FLIP mice (not shown). The numbers of naïve T cells were comparable in uninfected v-FLIP and WT mice, but naïve T cells were depleted to 50% in infected v-FLIP mice compared with WT mice (not shown). However, absolute numbers of memory/activated CD4 and CD8 T cells were more drastically reduced in infected v-FLIP compared with WT mice (Fig. 4C). Most CD4 and CD8 T cells (70–90%) were generated, but did not accumulate as memory cells in
were infected with 1 × 10^5 cell culture trypomastigotes (A) or 3 × 10^5 metacyclic trypomastigotes (B). A and B, n = 5 v-FLIP mice; n = 6 (A) or 5 (B) WT mice. C, BALB/c mice were infected with 1 × 10^5 metacyclic trypomastigotes and treated during the acute phase with zIETD to block caspase-8 activity, with zLEHD (caspase-9 inhibitor), or with zFA as the control peptide (n = 6 mice/group). Parasitemia (average and SEM) was followed during the acute phase and expressed as ln parasites per milliliter for statistical analysis. Kinetic points with significant differences between infected groups were indicated for p < 0.05 (*). C. Significant differences are indicated for zIETD vs zFA groups (**) and for zIETD vs zLEHD groups (**). The zIETD group was significantly different from both zLEHD and zFA groups.

v-FLIP mice, possibly due to increased cell death, as previously suggested (31). Changes in memory/activated T cells were also investigated in infected BALB/c mice treated with caspase-8 inhibitor. Although the effects of zIETD treatment in vivo were not as potent as transgenic v-FLIP expression, numbers of memory/activated T cells were significantly lower in infected mice treated with zIETD compared with control zFA peptide (Fig. 5, A and B). These results indicate that caspase-8 inhibition reduced memory/activated CD4 and CD8 T cells in T. cruzi infection.

**Blockade of T cell caspase-8 activity in vitro**

To evaluate the direct effects of zIETD on T cells, T cell cultures from normal and infected v-FLIP and WT mice were infected with 10^5 metacyclic trypomastigotes (A) or 3 × 10^5 metacyclic trypomastigotes (B). A and B, n = 5 v-FLIP mice; n = 6 (A) or 5 (B) WT mice. C, BALB/c mice were infected with 1 × 10^5 metacyclic trypomastigotes and treated during the acute phase with zIETD to block caspase-8 activity, with zLEHD (caspase-9 inhibitor), or with zFA as the control peptide (n = 6 mice/group). Parasitemia (average and SEM) was followed during the acute phase and expressed as ln parasites per milliliter for statistical analysis. Kinetic points with significant differences between infected groups were indicated for p < 0.05 (*). C. Significant differences are indicated for zIETD vs zFA groups (**) and for zIETD vs zLEHD groups (**). The zIETD group was significantly different from both zLEHD and zFA groups.

Caspase-8 inhibition on IL-2 production. Treatment with zIETD, but not zLEHD, decreased IL-2 production in T cells from normal (Fig. 6A) or infected (Fig. 6B) BALB/c mice. To test whether caspase-8 directly affects the cell signaling required for cytokine expression (27, 35), we investigated the effects of the caspase-8 inhibitor zIETD on NF-κB induction by stimulated T cells (Fig. 6). Treatment with zIETD partially inhibited IκB degradation (Fig. 6C) and nuclear translocation of p65 NF-κB subunit (not shown) upon T cell activation with anti-CD3. Moreover, zIETD decreased NF-κB activation in stimulated T cells from normal (Fig. 6D) and infected (Fig. 6E) mice. Defective NF-κB responses were also observed in stimulated T cells from v-FLIP compared with WT mice (Fig. 6F). These results indicate that caspase-8 is required in early T cell activation, after TCR:CD3 engagement.

**Caspase-8 inhibition exacerbated type 2 cytokine responses to infection**

We investigated the effects of caspase-8 inhibition on T cell cytokine secretion upon infection. Activated CD4 T cells from both infected v-FLIP and WT mice produced similar amounts of IFN-γ (Fig. 7B). However, CD4 T cells from infected v-FLIP mice produced decreased levels of IL-2 (Fig. 7A) and increased levels of IL-4 (Fig. 7C) and IL-10 (Fig. 7D). The data suggest that CD4 T cells from v-FLIP mice up-regulate type 2 cytokine responses to T. cruzi infection. We also investigated the effect of caspase-8 inhibition in nontransgenic T cells. T cells from infected BALB/c mice treated with zIETD also produced higher levels of IL-4 and IL-10 without significant changes in IFN-γ production compared with mice treated with control zFA peptide (Fig. 8, A–C). Finally, we investigated the effect of caspase-8 inhibition in nontransgenic T cells in vitro. We used T cells from infected BALB/c mice activated with anti-CD3. Addition of zIETD increased IL-4 and IL-10 production without affecting IFN-γ levels (Fig. 8, D–F), mimicking results observed in vivo. These results indicate that blockade of
T cell caspase-8 activity, either by transgenic v-FLIP or by treatment with zIETD, enhanced type 2 cytokine responses to infection.

**Discussion**

Recent studies demonstrated that besides signaling apoptosis, caspases play additional critical roles in lymphocyte activation (20–22). FasL/Fas receptor interactions, followed by recruitment of the adapter Fas-associated death domain protein and caspase-8 activation, lead to apoptosis in activated T cells (18, 36–38). Viral FLIPs block Fas-mediated apoptosis by interfering with Fas-associated death domain protein-caspase-8 death-effector domain interactions (28, 30). Although lpr and gld mice deficient in Fas/FasL are viable (39, 40), caspase-8 deficiency results in developmental defects (41). To circumvent this problem, transgenic mice were engineered to direct the expression of the caspase-8 inhibitor v-FLIP to T cells (31). Similar to mice with T cell-specific caspase-8 deletion (32), v-FLIP transgenic mice are immune deficient, showing defective T cell responses to viral infections (31). In contrast, lpr and gld mice express B and T cell accumulation, presumably due to defective Fas-mediated apoptosis (36, 39, 40, 42). In agreement with this, caspase-8 (22) and Fas (25, 43) deficiencies in humans lead to immunodeficiency or to lymphoproliferative autoimmune syndrome, respectively. It is likely that some of these disparities arise because caspase-8 deficiency may affect signaling through multiple receptors (41).

In this study we show that T cells up-regulate caspase-8 activity during *T. cruzi* infection. However, inhibition of caspase-8 activation by transgenic v-FLIP expression, impaired CD8 T cell expansion, and increased susceptibility to *T. cruzi* infection, as previously reported (31). In addition, we demonstrated defects in CD4 T cell expansion and sustained memory in *T. cruzi*-infected v-FLIP mice, suggesting that caspase-8 is also required for long term CD4 T cell-mediated immunity. Blockade of caspase-8 activation by v-FLIP did not affect CD4 or CD8 T cell activation ratios based on CD44 or CD62L expression profiles. These results agree with previous observations that other activation markers and cell cycling deficiency may affect signaling through multiple receptors (41).

In this study, we show that the percentage of CD44high expression (for C57BL/6 mice) and increased susceptibility to infection by transgenic v-FLIP expression, impaired CD8 T cell expansion and downstream events of caspase-8 activation, lead to apoptosis in activated T cells (18, 36–38). Viral FLIPs block Fas-mediated apoptosis by interfering with Fas-associated death domain protein-caspase-8 death-effector domain interactions (28, 30). Although lpr and gld mice deficient in Fas/FasL are viable (39, 40), caspase-8 deficiency results in developmental defects (41). To circumvent this problem, transgenic mice were engineered to direct the expression of the caspase-8 inhibitor v-FLIP to T cells (31). Similar to mice with T cell-specific caspase-8 deletion (32), v-FLIP transgenic mice are immune deficient, showing defective T cell responses to viral infections (31). In contrast, lpr and gld mice express B and T cell accumulation, presumably due to defective Fas-mediated apoptosis (36, 39, 40, 42). In agreement with this, caspase-8 (22) and Fas (25, 43) deficiencies in humans lead to immunodeficiency or to lymphoproliferative autoimmune syndrome, respectively. It is likely that some of these disparities arise because caspase-8 deficiency may affect signaling through multiple receptors (41).

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defective during immune responses to viral infection in v-FLIP mice (31). These protective effects of caspase-8 on T cells may be mediated by prevention of autophagic cell death (44) or by induction of NF-κB (27, 45–47).

Previous studies indicated that FasL-deficient gld mice were more susceptible to T. cruzi infection due to increased Th2 cytokine production (16). In this study we found that CD4 T cells from infected v-FLIP mice and T cells from infected mice treated in vivo with zIETD also produced increased levels of IL-4 and IL-10 despite normal levels of IFN-γ production. Together, these data suggest that both FasL/Fas signaling (16) and caspase-8 are able to regulate cytokine responses to T. cruzi infection by preventing type 2 cytokine production. In contrast, IL-2 production is decreased in CD4 T cells from infected v-FLIP mice and in T cells from normal or infected mice treated in vitro with zIETD. Similarly, production of IL-2 is defective in stimulated T cells from caspase-8-deficient humans (22) and in caspase-8 conditional knockout mice (32). In contrast, transgenic c-FLIPL expression had a variable effect on IL-2 production by T cells (48–50) depending on the level of protein expressed in different transgenic lineages of mice (49).

Recent studies indicated that c-FLIPL transgenic mice are biased toward experimental allergy due to increased type 2 and decreased IFN-γ and NF-κB responses (50). In contrast, c-FLIPL transgenic mice were resistant to Th1-driven experimental autoimmune encephalomyelitis (51). It was suggested that c-FLIPL transmits signals that down-regulate NF-κB and IFN-γ responses (50). An alternative explanation is that overexpression of c-FLIPLp expression had a variable effect on IL-2 production by T cells (48–50) depending on the level of protein expressed in different transgenic lineages of mice (49).

Our results also indicate that caspase-8 activity is involved in the balance of Th1/Th2 responses. It is likely that inhibition of caspase-8 allows CD4 T cells to acquire a Th2 phenotype and rescues previously activated T cells from AICD during infection. Signaling requirements differ for Th1 and Th2 subsets (54), and Th2, but not Th1, responses persist in mice expressing a dominant IκB transgene, which blocks NF-κB activation in T cells (55). Therefore, down-regulation of NF-κB responses upon inhibition of caspase-8 may negatively affect Th1, but spare Th2 cytokine responses.

The multiple effects of caspase-8 on early activation signaling, regulation of cytokine responses, and induction of death receptor-mediated apoptosis may affect resistance to T. cruzi infection. However, the negative effects of caspase-8 inhibition on critical
immune responses seem to overcome the potential benefits of blocking apoptosis during infection. Therefore, in addition to sustained T cell memory, we described a novel role for caspase-8 in immune regulation. By preventing type 2 cytokine responses, caspase-8 may contribute to protective immune responses and resistance to intracellular parasites.

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Disclosures
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References


