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Trypanosoma cruzi Infection and Nuclear Factor Kappa B Activation Prevent Apoptosis in Cardiac Cells

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Studies of cardiac pathology and heart failure have implicated cardiomyocyte apoptosis as a critical determinant of disease. Recent evidence indicates that the intracellular protozoan parasite *Trypanosoma cruzi*, which causes heart disease in chronically infected individuals, impinges on host apoptotic pathways in a cell type-dependent manner. *T. cruzi* infection of isolated neuronal cells and cardiomyocytes protects against apoptotic cell death, whereas apoptosis is triggered in T cells in *T. cruzi*-infected animals. In this study, we demonstrate that the ability of *T. cruzi* to protect cardiac cells in vitro from apoptosis triggered by a combination of tumor necrosis factor alpha and serum reduction correlates with the presence of intracellular parasites and involves activation of host cell NF-κB. We further demonstrate that the apoptotic block diminishes activation of caspase 3. The ability of *T. cruzi* to prevent apoptosis of infected cardiomyocytes is likely to play an important role in establishment of persistent infection in the heart while minimizing potential damage and remodeling that is associated with cardiomyocyte apoptosis in cardiovascular disease.

Chagasic cardiomyopathy, resulting from chronic infection with the intracellular protozoan pathogen *Trypanosoma cruzi*, is a leading cause of heart failure in Latin America (50). During the acute stage of infection, *T. cruzi* can invade and replicate within a wide range of host cell types and tissues, including the heart (5). As infection progresses in immunocompetent hosts, parasites are effectively cleared from the majority of tissue sites but persist for years in cardiac and smooth muscle (34, 45, 52). While the basis of this tropism is currently unknown, it is clear that establishment of long-term myocardial infection by *T. cruzi* is critical to the pathogenesis of Chagas' disease (52, 59). Cardiac myocytes are terminally differentiated, nondividing cells that are not readily replaced following cell death (31). Accumulating evidence indicates that cardiomyocyte loss due to tissue damage and apoptosis plays a critical role in cardiovascular remodeling, fibrosis, and hypertrophy in many forms of cardiovascular disease (13, 31). Thus, protection of these specialized cells from apoptosis following exposure to extrinsic or intrinsic stress resulting from pathogen infection, ischemia, oxidative stress, or volume overload (11, 14, 25, 44, 49) is clearly a desirable outcome.

Apoptosis can be triggered by the activation of the extrinsic or "death receptor" pathway and/or the intrinsic-mitochondrial stress-induced pathway (3). The extrinsic pathway is mediated through death receptors, members of the tumor necrosis factor (TNF) receptor superfamily (28), activated by ligand binding. Upon ligand binding the cytoplasmic domain of the receptor interacts with a cascade of molecules (43), leading to cleavage of caspase 3, regarded as a central regulator of apoptosis.

Many intracellular pathogens exploit host cell apoptotic pathways as a strategy for survival and/or dissemination in the host (7, 12, 17, 21, 39, 46, 55). *T. cruzi* exhibits a complex relationship with the host apoptotic response. Intracellular infection with this pathogen can block programmed cell death in a variety of nonimmune cell types (2, 12, 43), whereas apoptosis is selectively triggered in CD4+ T cells migrating to sites of infection in *T. cruzi*-infected animals. Phagocytosis of apoptotic T cells by *T. cruzi*-infected macrophages, in contrast, fuels replication of intracellular parasites (15, 33). Thus, differential modulation of apoptotic pathways in parasitized versus noninfected immune effector cells appears to be an effective strategy for *T. cruzi* to promote its own survival and growth while limiting inflammation and histopathological changes at foci of infection.

As cardiomyocytes are end-differentiated cells which do not divide, maintenance of this cell population via apoptosis prevention would be critical for both establishment of chronic *T. cruzi* infection and host survival. The cumulative findings of several laboratories indicate that *T. cruzi* infection can block apoptosis initiated through death receptor and mitochondrial stress pathways (2, 9, 38). *T. cruzi* infection of HeLa cells protects against Fas-mediated apoptosis, as indicated by the significant inhibition of caspases 8 and 3 (38). A recent report revealed that the secreted *T. cruzi* cysteine protease cruzipain can promote cardiomyocyte survival under conditions of serum deprivation in a mechanism that involves upregulation of the mitochondrial arginase-2 gene (2). In the best studied model, *T. cruzi* protects neuronal and glial cells from death triggered by growth factor depletion in a mechanism that involves an abundant parasite surface glycoprotein, *trans*-sialidase, and activation of host cell Akt (8, 9). As infective *T. cruzi* trypomastigotes activate the phosphatidylinositol-3 (PI-3) kinase/Akt pathway in primary cardiomyocytes during the invasion process

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(57), it is possible that this pathway contributes to the mechanism of parasite-dependent protection from cell death in cardiomyocytes. In addition, recent studies in our laboratory demonstrated that *T. cruzi* infection of isolated cardiomyocytes induces a robust hypertrophic response that is mediated by the secreted proinflammatory cytokine interleukin-1β (IL-1β) via NF-κB activation (41). It is well recognized that in addition to acting as a transcription factor for proinflammatory cytokines, NF-κB also serves as a transcription factor for several anti-apoptotic molecules. Exposure of cardiomyocytes to low levels of proinflammatory cytokines in the heart can precondition the myocardium to temporarily protect cardiomyocytes from apoptotic cell death (4, 11, 18, 19, 23, 29, 44, 48, 56). Thus, it is possible that the cytokine-driven hypertrophic response may induce a prosurvival state in *T. cruzi*-infected cardiomyocytes.

In this study, we demonstrate the ability of *T. cruzi* to inhibit TNF-α-mediated apoptosis in primary neonatal rat ventricular cardiomyocytes (NRVMs) and the rat embryonic cardiac myoblast line H9c2. The block of apoptosis imposed by *T. cruzi*, which occurs upstream of caspase 3 activation, correlated with the presence of intracellular parasites. Treatment of cardiomyocytes with conditioned medium from parasite-infected cells, which triggers a robust cytokine-dependent hypertrophic response (41), failed to protect cells from death. Finally, while inhibition of PI-3 kinase activity failed to inhibit the ability of *T. cruzi* to protect cardiomyocytes from cell death, our data indicated a role for NF-κB-dependent signaling in this process.

MATERIALS AND METHODS

Cardiomyocyte isolation and cell culture. Primary cultures of ventricular cardiomyocytes from neonatal Sprague-Dawley rats (Charles River Laboratories) were prepared as described previously (41). H9c2 cells, an embryonic rat cardiac myoblast line (American Type Culture Collection), were maintained at less than 80% confluence in 4:1 Dulbecco's modified Eagle medium (DMEM)-M199 supplemented with 10% fetal calf serum (FCS) and incubated at 37°C in 5% CO_2. Apoptosis of cardiac cells was induced by TNF-α treatment (50 ng/ml) (Calbiochem) under conditions of reduced serum (0.5% FCS in DMEM-M199). Where indicated, cells were treated with SN-50, a competitive inhibitor peptide which prevents NF-κB activation (50 μg/ml), scrambled control peptide SN-50 M (50 μg/ml) (Calbiochem), an Iκ kinase–NEMO-binding domain (NBD) peptide (IKK-I) which inhibits IκB inactivation by binding to the IκK NEMO subunit (100 μM) and paired control peptide (IKK-IC) (Biomol), or wortmannin, a PI-3 kinase inhibitor (40 nM) (Sigma).

Parasite infection. *Trypanosoma cruzi* (Y strain) was propagated in monolayers of LLC-MK2 cells in DMEM (Gibco) with 2% fetal bovine serum, and infective trypomastigotes were harvested as described previously (51). Parasites were washed in 4:1 DMEM-M199, and the indicated number of parasites were incubated with cardiac cells for 2 h at 37°C, 5% CO_2 to allow invasion. Remaining extracellular parasites were aspirated, fresh medium was added, and cultures were incubated for the indicated times.

Parasite-conditioned media (PCM). *T. cruzi* trypomastigotes were washed three times in serum-free DMEM, and 5×10^6 parasites/ml were incubated overnight at 37°C, 5% CO_2. Parasites were pelleted, and the resultant supernatant containing secreted or shed trypomastigote molecules were collected and filtered through a 0.2-μm membrane (Millipore). PCM was then incubated with serum-depleted cardiomyocytes as indicated.

Cardiac cell-conditioned media (CCM). H9c2 cells were infected with *T. cruzi* for 48 h as described above. Culture supernatants were collected, filtered through a 0.4-μm membrane (Millipore), and incubated with serum-depleted cardiomyocytes as indicated.

Annexin V binding and FACS analysis. Following treatment, cardiac cells were trypsinized and washed with phosphate-buffered saline (PBS). Annexin V-fluorescein isothiocyanate (FITC) staining was performed according to the manufacturer's directions (Oncogene), and a minimum of 10,000 cells for each condition were analyzed by fluorescence-activated cell sorting (FACS) (FACScalibur, Becton

Dickinson) to measure FITC fluorescence (FL1-H) and propidium iodide (FL2-H). Data were analyzed using Cell Quest software (Becton Dickinson).

TUNEL staining and immunofluorescence. Following *T. cruzi* infection and other treatments, coverslips with adherent cardiac cells were fixed in 2% paraformaldehyde–PBS. TdT-mediated dUTP nick-end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche) as directed by the manufacturer. *T. cruzi* immunostaining was performed with a rabbit anti-*T. cruzi* antibody followed by Texas Red goat-anti-rabbit antibody (1:3,000) (Molecular Probes). Cell and parasite nuclei were visualized with 4′,6′-diamidino-2-phenylindole staining. Slides were visualized with a Nikon TE300, and images were captured with an Orca-100 CCD camera (Hammamatsu). A total of 300 cells for each condition were scored for *T. cruzi* infection and TUNEL staining for each coverslip. Triplicate coverslips were counted for each experimental condition.

Caspase assays. Caspase 3, 8, and 9 activities were determined using the method described by Payne et al. (40). Briefly, total cell lysate containing 2.5 or 5.0 μg of total protein was incubated with synthetic caspase 3, 8, and 9 substrates (acetyl-DEVD-MCA, LEHD-MCA, and IETD–4-methyl-coumaryl-7-amide (MCA), respectively) conjugated to the fluorescent dye 4-methyl-coumary-7-amide (MCA) (Peptides International, Louisville, KY). Caspase activity in blinded samples was determined by measuring the level of fluorescence resulting from the release of MCA via cleavage of the substrate by active caspase 3, 8, or 9. Released MCA was detected using a Perkin Elmer LS55 luminescence spectrometer (excitation, 380 nm; emission, 440 nm; cutoff filter, 430 nm [Perkin Elmer, Boston, MA]).

Bcl-2 protein expression. Following induction of apoptosis, H9c2 cells were collected by scraping and pelleting at $1,500 \times g$ for 5 min. Cell pellets were washed in PBS and lysed using a modified radioimmunoprecipitation assay buffer. Protein concentration was determined via bicinchoninic acid protein assay (Pierce, Rockford, IL). Bcl-2 protein expression levels were determined via Western blot analysis. Five micrograms of protein per sample was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). Bcl-2 was detected via a rat anti-mouse monoclonal Bcl-2 antibody (eBioscience, San Diego, Calif.) (1:500) and secondary goat anti-mouse immunoglobulin G-horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) (1:2,500). Blots were visualized via chemiluminescence detection (Pierce Biotechnology, Inc., Rockford, IL).

Statistical analysis. The Student's *t* test was used for comparison between control and experimental samples. Values of $P < 0.05$ were considered significant.

RESULTS

Cardiomyocyte infection with *Trypanosoma cruzi* protects against apoptosis. We have previously demonstrated that isolated neonatal rat ventricular myocytes (NRVM) undergo a rapid hypertrophic response following infection with *T. cruzi*. The hypertrophic response was shown to be mediated by the proinflammatory cytokine IL-1β (41) in a TLR2-dependent manner (42). As exposure to low levels of proinflammatory cytokines can elicit a preconditioning response that temporarily protects cardiomyocytes from death (37, 47), we sought to characterize whether *T. cruzi* infection impacts apoptosis in cardiomyocytes using this pathway. Employing both flow cytometry and fluorescence microscopy to identify apoptotic cells in cardiomyocyte populations, we demonstrate that *T. cruzi* infection itself does not trigger apoptosis in NRVM (Fig. 1; see also Fig. 4). On the contrary, *T. cruzi* infection protects primary cardiomyocytes from apoptotic cell death induced by staurosporine (data not shown) or TNF-α under reduced serum conditions as determined by flow cytometric analysis of annexin V-stained cells (Fig. 1A and B). This protective effect was observed in cell populations that were infected with *T. cruzi* for 24 h prior to TNF-α treatment (Fig. 1B) or when cells were stimulated with TNF-α immediately prior to parasite invasion (Fig. 1A). For all of the following assays, cells were placed in serum-restricted media (0.5%) and TNF-α treated as indicated 24 h after infection or inhibitor treatment. Similar
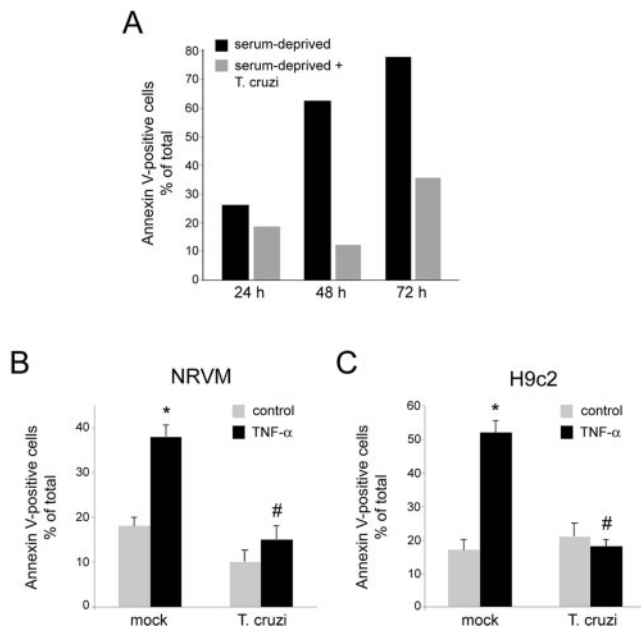
FIG. 1. *T. cruzi* prevents apoptosis in isolated cardiomyocytes and H9c2 cells. A. Isolated NRVMs were infected with *T. cruzi* and treated at the same time with 50 ng TNF-α for the amount of time indicated. Apoptosis was measured by annexin V-FITC labeling and FACS analysis of at least 10,000 cells. B. NRVMs infected with *T. cruzi* and treated with TNF-α 24 h postinfection for a total of 24 h of TNF-α treatment and 48 h of infection. Apoptosis was measured as described for panel A. C. H9c2 cells infected with *T. cruzi* and treated with TNF-α 24 h postinfection. Apoptosis was measured as described for panel A. For all experiments, data represent means of three experiments. Statistical significance was measured by a two-tailed Student's *t* test. Significant variation from untreated control (mock) ($P < 0.05$) cells is designated by an asterisk (∗). Significant variation from uninfected, TNF-α-treated cells is designated by a pound sign (#).
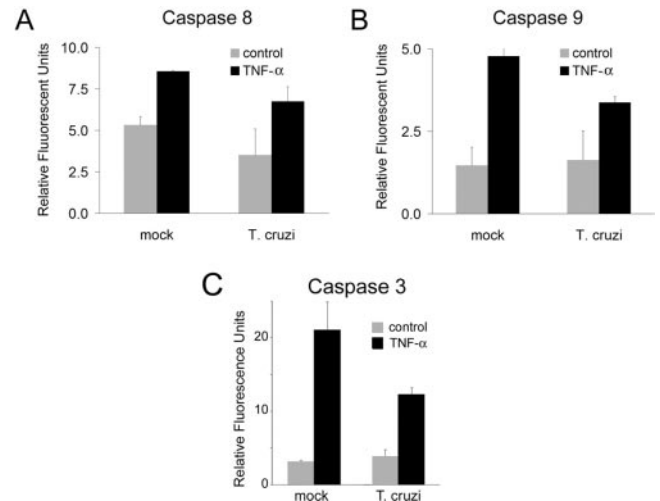

FIG. 2. *T. cruzi* inhibits caspase 3 activity in infected H9c2 cells. H9c2 cells were infected with *T. cruzi* and treated with TNF 24 h postinfection. Twenty-four hours following TNF-α treatment, cells were lysed and 5 ng total cell protein was assessed for caspase 3, 8, and 9 activity. Fluorometer measurements were taken for each well at 6 h, and relative fluorescence units were calculated for each sample. (C) Data represent means of three experiments. (A and B) Data represent the means of a single experiment done in triplicate.

results were obtained with the rat embryonic cardiomyoblast line H9c2 (Fig. 1C). Since H9c2 cells are more sensitive than NRVM to apoptotic killing induced by TNF-α and a greater proportion of H9c2 cells become infected with *T. cruzi* compared to NRVM, H9c2 cells were included in subsequent analysis of this parasite-driven prosurvival response.

Inhibition of apoptosis usually induces a change in caspase activation. To identify whether *T. cruzi* alters these mediators of apoptosis, the activity of caspases 3, 8, and 9 was measured in TNF-α-treated H9c2 cells in the presence and absence of parasite infection. We find that the activity of the central mediator of apoptosis, caspase 3, was increased following induction of apoptosis with TNF-α (Fig. 2C, mock, black). Caspase 3 activity was reduced in TNF-α-stimulated *T. cruzi*-infected cells (Fig. 2C, *T. cruzi*, black). A similar but less robust trend was found in caspase 9 activity (Fig. 2B) and not caspase 8 (Fig. 2A). These findings indicate that following *T. cruzi* infection, a block in the progression of apoptosis occurs as evidenced by a reduction of caspase 3 activation.

Protection from apoptosis correlates with the presence of intracellular parasites. To assess the role of soluble factors released by parasites or by infected cardiomyocytes in protecting cells from death, NRVMs were stimulated with parasite-conditioned medium or conditioned medium harvested from infected cardiomyocytes at 24 h postinfection in the presence of TNF-α. In contrast to the ability of *T. cruzi* infection to protect cells from TNF-α-stimulated death (Fig. 1), treatment of NRVM with PCM or CCM failed to protect cells from apoptosis (Fig. 3A). Pretreatment of NRVM with antibodies to TLR2 that block signaling through this pathway (14) failed to inhibit the prosurvival effects associated with *T. cruzi* infection (Fig. 3B). These findings suggest that neither parasite-shed factors, TLR-2 signaling, nor cardiomyocyte-released factors appear to play a primary role in initiating the inhibition of apoptosis, a difference from *T. cruzi*-mediated hypertrophy. These results may instead indicate that, once inside cardiac cells, the parasite promotes intracellular signals to stimulate this protective response.

To characterize the relationship between parasite infection and protection from apoptotic cell death, TUNEL staining and immunofluorescence microscopy were employed to score for apoptosis in both parasite-infected and uninfected cells in culture. Using an infecting dose of *T. cruzi* that results in ~50% infection, we examined the effect of TNF-α treatment on both parasite-containing and uninfected cells in the same cultures. Consistent with results for annexin-V staining, *T. cruzi* infection alone did not increase the proportion of apoptotic cells compared to mock-infected controls (data not shown). Upon stimulation of *T. cruzi*-infected cultures with TNF-α, an increase in the number of TUNEL-positive cells was observed only in the population of cells that lacked intracellular parasites (Fig. 4A, no intracellular parasites). In contrast, no significant increase in the number of TUNEL-positive cells was observed among the cells harboring intracellular parasites (Fig. 4A, parasite-containing).

To limit parasite internalization into H9c2 cells, infections were carried out for 2 h at 20°C prior to aspirating extracellular parasites and incubating infected cells for an additional 24 h at
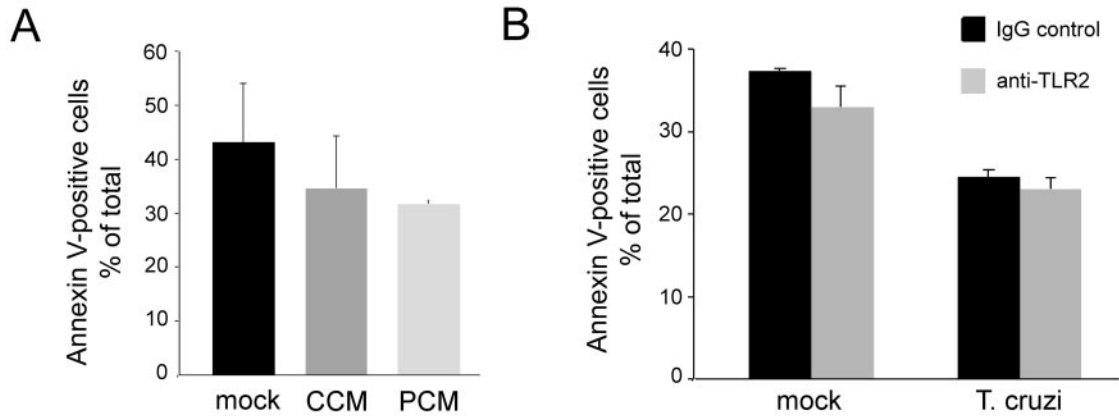
FIG. 3. Soluble factors do not contribute to prevention of apoptosis. A. H9c2 cells were treated with conditioned medium from infected cardiac cells (CCM, dark gray), conditioned medium from parasites (PCM, light gray), or were left untreated (mock, black) for 24 h after TNF-α infection. B. H9c2 cells infected with *T. cruzi* and either a TLR-2 blocking antibody (anti-TLR2, gray) or an isotype control (immunoglobulin G [IgG] control, black) were subsequently treated with 50 ng/ml TNF-α 24 h postinfection. Apoptosis was measured by annexin-V-FITC labeling as described in the legend to Fig. 1. Data represent means of three experiments ± standard deviation of the mean.

37°C. As a control, cells were incubated with the same infecting dose of *T. cruzi* at 37°C, an optimal temperature for invasion, and were processed similarly thereafter. Infections carried out under these conditions resulted in a reduction in the percent-
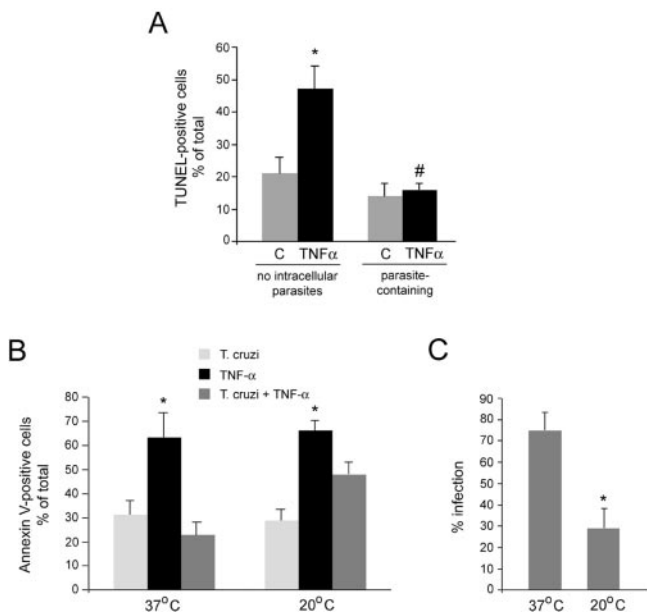

FIG. 4. Effect of *T. cruzi* infection on apoptosis of H9c2 cardiac cells. A. H9c2 cells were plated on coverslips and infected with *T. cruzi*; TNF treatment followed infection by 24 h. Cultures of H9c2 cells underwent TUNEL labeling and immunofluorescence for *T. cruzi*. Cells were counted for infection and TUNEL positivity. B. H9c2 cells were infected at either 37°C or 20°C for 2 h, treated with TNF-α, and assessed for apoptosis via annexin V-FITC and FACS analysis 24 h after TNF-α treatment. C. *T. cruzi* infection was identified via a polyclonal anti-*T. cruzi* antibody and calculated per 300 cells for infection occurring at both 37°C and 20°C. Data represent means of three experiments plus standard deviations of the means. Significant variation from untreated control cells (C) or from the 37°C temperature of infection (panel C) ($P < 0.05$) is designated by an asterisk (∗). Significant variation from uninfected, TNF-α-treated cells is designated by a pound sign (#).

age of infected cells from 72% at the permissive temperature (37°C) to 29% at the nonpermissive temperature (20°C) (Fig. 4C). Despite exposure of cells to similar numbers of extracellular parasites, decreased internalization at 20°C diminished the ability of *T. cruzi* to protect cells from TNF-α-induced death (Fig. 4B). Together with observations that negate the potential role of host cell- or parasite-derived soluble mediators (Fig. 3A), these data strongly suggest that the presence of intracellular parasites or the continuous generation of a labile factor is required to protect cells from TNF-α-mediated cell death.

Involvement of host cell NF-κB in *T. cruzi*-dependent protection against apoptosis. Early in the infective process, *T. cruzi* activates a number of host cell signaling pathways (6), including PI-3K/Akt (57) and NF-κB (22, 26, 27), both of which can activate prosurvival responses in mammalian cells (24, 36, 46, 53). To determine if either of these *T. cruzi*-activated pathways contributes to the overall ability of *T. cruzi* to protect cardiac cells from apoptosis, *T. cruzi*-infected H9c2 cells were treated with wortmannin or the NF-κB inhibitor peptides SN-50 and Iκ kinase-NBD (IKK-I) immediately following infection and prior to induction of apoptosis by addition of TNF-α. Whereas wortmannin treatment of *T. cruzi* infected cells failed to reverse the protection against apoptosis afforded by this parasite (data not shown), SN-50 treatment resulted in partial abrogation of protection (Fig. 5A and C). This was demonstrated in both NRVM (Fig. 5A) and H9C2 cells (Fig. 5A and C) using annexin V-FITC staining (Fig. 5A) or TUNEL staining (Fig. 5C). As expected, addition of SN-50 or IKK-I alone caused a slight increase in the number of apoptotic cells in NRVM and H9c2 populations and did not inhibit TNF-α-triggered apoptosis (Fig. 5). Detection of apoptotic cells in cell populations by annexin-V binding revealed that inhibition of NF-κB activation in *T. cruzi*-infected cells by SN-50 resulted in partial block of the apoptotic protection, whereas addition of the control peptide had no effect (Fig. 5A). Inhibition of IκB inactivation resulted in a partial block of apoptotic protection in *T. cruzi*-infected H9c2 cells as well (Fig.
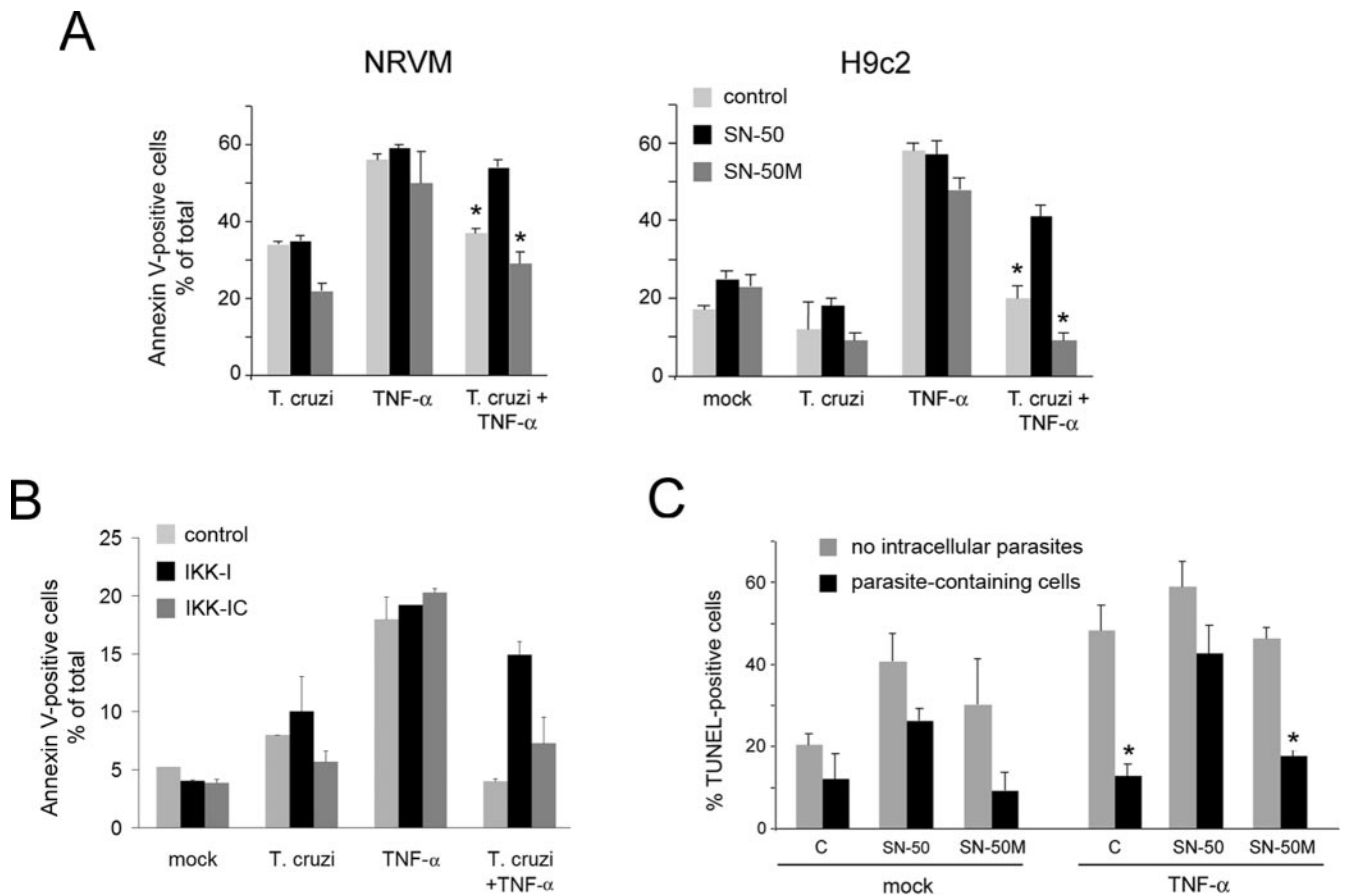
FIG. 5. Contribution of host cell NF-κB and PI-3K-dependent pathways to *T. cruzi*-mediated prevention of apoptosis. A. NRVM or H9c2 cells were infected with *T. cruzi* and treated with SN-50 (50 ng/ml) or the scrambled peptide SN-50 M (50 ng/ml) and then treated with TNF-α 24 h postinfection. B. H9c2 cells were infected with *T. cruzi* and treated with IKK-I (100 μM) or the control peptide IKK-IC (100 μM) and then treated with TNF-α 24 h postinfection. C. H9c2 cells were plated onto coverslips and infected with *T. cruzi* and treated with TNF and SN-50 (50 ng) or the scrambled peptide SN-50 M (50 ng) 24 h postinfection. Apoptosis was measured by annexin V-FITC labeling (A and B) or TUNEL immunofluorescence (C). Significant variation from uninfected TNF-α-treated cells ($P < 0.05$) designated by an asterisk. (A and C) Data represent the means of three experiments plus standard deviations of the means. (B) Data from one experiment done in triplicate plus standard deviations of the means.

5B). A more detailed analysis of this at the single-cell level was carried out by quantitation of TUNEL-positive cells. Simultaneous examination of *T. cruzi*-containing and uninfected cells in infected H9c2 cultures revealed that, similar to our previous observation, TNF-α preferentially triggers apoptosis in uninfected cells (Fig. 5C). SN-50 treatment resulted in a significant increase in the number of parasite-infected cells that were TUNEL positive as well as an increase in the number of apoptotic cells in the noninfected cell population (Fig. 5C). Treatment of cells with the scrambled peptide control did not significantly alter the number of TUNEL-positive cells (Fig. 5C). Overall, these data suggest that in cardiac cells, *T. cruzi* relies on NF-κB-dependent signaling and not PI-3 kinase-dependent events to protect against TNF-α-induced cell death.

In an attempt to identify potential downstream antiapoptotic effector molecules regulated via NF-κB, we analyzed Bcl-2 protein expression in H9c2 cells infected with *T. cruzi* and treated with TNF-α. Compared to either mock-treated or TNF-α-treated cells, *T. cruzi*-infected, TNF-α-treated cells expressed eight times more Bcl-2 protein as identified via West-

ern blot analysis (Fig. 6). Inhibition of NF-κB via IKK-I downregulated this expression by half, whereas treatment with a control peptide caused only a minimal decrease in Bcl-2 protein expression in these *T. cruzi*-infected cells (Fig. 6). In response to NF-κB activation, production of Bcl-2 and other antiapoptotic molecules by *T. cruzi*-infected cardiac cells, in response to TNF-α treatment, provides these cells protection against induced cell death.

DISCUSSION

Previous data from our laboratory has demonstrated that NF-κB is vital for *T. cruzi*-mediated IL-1β production and stimulation of cardiomyocyte hypertrophy (41, 42). NF-κB has been linked to a variety of antiapoptotic responses (30, 54) and has been shown to serve as a transcription factor for antiapoptotic molecules, including inhibitors of apoptosis proteins (IAPs) and *bcl-2*. Beyond NF-κB, several other antiapoptotic signaling pathways, including PI-3 kinase/Akt, are activated by *T. cruzi* invasion and/or infection (6, 57). Use of biochemical
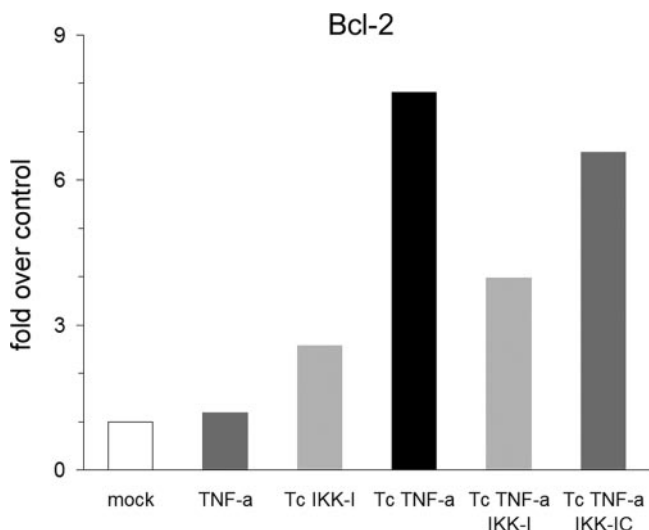
FIG. 6. *T. cruzi*-infected TNF-α-treated cells upregulate *bcl-2* via NF-κB. H9c2 cells were infected with *T. cruzi* and/or IKK-I and treated with TNF 24 h postinfection. Twenty-four hours following TNF-α treatment, cells were lysed and 5 ng total cell protein was assessed for *bcl-2* expression. Data represent fold over control by β-actin-normalized densitometric analysis of blots. Tc, *T. cruzi*.

inhibitors showed that NF-κB but not PI-3K/Akt was involved in the antiapoptotic response in cardiac cells. This finding contrasts from *trans*-sialidase prevention of apoptosis, which was found to initiate signaling resulting in autophosphorylation and activation of PI-3 kinase/Akt (10). These findings perhaps indicate that PI-3 kinase-mediated prevention of apoptosis occurs at earlier time points after infection in response to trypanomastigote invasion and shed parasite factors, whereas NF-κB-based survival responses are mediated through intracellular amastigote-mediated signaling after 24 h of infection.

NF-κB activation has recently been demonstrated in cardiomyocytes after *T. cruzi* infection (49) as well as in whole hearts following in vivo *T. cruzi* infection (27), although activation was previously shown not to occur in isolated cardiomyocytes via electrophoretic mobility shift assay (22). There are multiple models of intracellular pathogens initiating NF-κB activation, including viral (58), bacterial (20), and protozoal (35). *T. gondii* has been shown to produce a novel parasite kinase which activates NF-κB, leading to a survival response mediated via known antiapoptotic, cardioprotective genes, including *bcl-2* and IAPs (35, 46). We identify that in our system, *T. cruzi*-infected cells upregulate production of *bcl-2* following induction of apoptosis. Therefore, NF-κB-mediated production of these antiapoptotic proteins could play a key role in the intracellular *T. cruzi* antiapoptotic response in isolated cardiomyocytes.

A *Trypanosoma cruzi* antiapoptotic effect was previously shown to be initiated in infected murine cells derived from both the nervous system and heart. Protection from *T. cruzi* was shown to be mediated by secreted proteins, *trans*-sialidase and cruzipain, respectively (2, 8, 9). In contrast, our present studies in cardiac cells indicate that a *T. cruzi*-mediated response could not be reproduced by the use of conditioned

medium obtained from either parasites or infected cardiac cells. In addition, the antiapoptotic response in NRVMs and H9c2 cells was found only in *T. cruzi*-infected cells, as shown by dual TUNEL and anti-*T. cruzi* immunofluorescence, and not noninfected cells within the same culture. Together, these findings argue against a role for soluble factors in this model system. In addition, we show further studies using inhibition of infection by temperature depression of invasion which indicate that actual cell invasion is required, and mere cell-surface interactions between the parasite and cardiac cell are also not sufficient to prevent apoptosis. This evidence, taken as a whole, leads us to believe that this response in cardiac cells is mediated by *T. cruzi* parasitism and not by secreted factors from either parasites or infected cells.

In the present studies, two mechanisms were utilized at the same time to induce apoptosis in cardiac cells, TNF-α treatment and serum depletion. These stimuli are known to target the "death receptor" and stress-induced pathways, respectively. These apoptotic pathways are highly integrated and overlapping, indicating that the *T. cruzi* mechanisms used to prevent cell death must function over many different means of apoptotic induction. Our present data suggest that the *T. cruzi*-induced decrease in caspase activation and therefore apoptosis appears to occur prior to activation of executioner caspase 3 in cardiac cells. The exact mechanism of *T. cruzi* apoptosis prevention remains unknown, warranting further study. The contribution of NF-κB in inhibition of apoptosis could implicate a role for other NF-κB-activated gene products, in addition to *bcl-2*, including IAPs which are found to block the apoptosis machinery between caspases 9 and 3.

Depending on the context of infection, *T. cruzi* can either promote or prevent apoptosis. Macrophages infected by *T. cruzi* have been found to promote apoptosis in T cells (15, 32) and undergo apoptosis themselves (16). In vivo studies of infected dogs showed apoptosis in immune effector cells as well as endothelial cells and cardiac myocytes (59). Apoptosis in nonimmune cells in this study was attributed to the profound inflammatory response. Alternately, in vitro studies of neuro-endocrine and Schwann cells (8, 9), as well as other nonimmune cell types (1, 2), have shown that *T. cruzi* prevents apoptosis in these cells. We have previously demonstrated that *T. cruzi* promoted cardiomyocyte hypertrophy via an NF-κB-dependent signaling pathway (42), which promotes cell survival in other systems (30). Here we show that isolated NRVMs and H9c2 cells are protected from apoptosis after *T. cruzi* infection. This process is NF-κB dependent as well, but unlike *T. cruzi*-mediated cardiomyocyte hypertrophy, it is not correlated with soluble factors. Through the differential regulation of apoptosis, *Trypanosoma cruzi* appears to have developed a strategy to avoid the immune system while preventing death of a preferred host cell type. This could influence persistence in cardiac muscle (52, 59) and transmission to other hosts, and it may result in the development of chronic cardiac Chagas' disease. The resultant effective balance that occurs between parasite persistence, immune control, and cardiovascular damage may be integrally related to differential modulation of host apoptotic functions directly or indirectly following *T. cruzi* infection.

Here we describe the mechanism of the *T. cruzi*-induced antiapoptotic effect in NRVMs and H9c2 cells. This is the first time that intracellular *T. cruzi* parasites are shown to promote

an antiapoptotic effect, implicating a role for intracellular molecules in promoting cytoprotective NF-κB activation. During *T. cruzi*-mediated prevention of cardiac cell apoptosis, NF-κB activation may lead to production of antiapoptotic molecules, inhibition of caspase 3 activation, and prevention of cell death. This prosurvival response triggered by *T. cruzi* infection indicates the importance of parasite-induced responses in manipulating the host cell environment to promote maintenance of infection. Due to this parasites' particular tissue trophism, a prosurvival response in cardiomyocytes may also permit host recovery from acute inflammation early after *T. cruzi* infection, allowing for *T. cruzi* vector transmission and progression of Chagas' disease from the acute into the indeterminate phase of disease.

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