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Leishmania Infection Inhibits Cycloheximide-Induced Macrophage Apoptosis in a Strain Dependent Manner

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Abstract

Activation of apoptosis is one of the most ancient mechanisms to eliminate intracellular infections; the capacity to subvert this programmed cell death provides an adaptive advantage to pathogens that persist in an intracellular environment. *Leishmania* species are obligate intracellular parasites that primarily reside within host macrophages. We demonstrate here that *Leishmania* infection protects macrophages from cycloheximide induced apoptosis in a species and strain specific manner. Our data further reveal that *Leishmania* phosphoglycans and direct contact between parasites and host cells are required for the inhibitory phenotype.

Keywords

Leishmania; apoptosis; macrophage

Introduction

Apoptosis, or programmed cell death is a mechanism that cells utilize to activate intracellular pathways for terminating themselves in response to a wide range of stimuli (Kerr, et al., 1972). Traditionally apoptosis has been described as a method of maintaining homeostasis, leading to elimination of potentially harmful cells (Raff, 1998), be it auto-reactive lymphocytes or cells with potentially cancerous alterations. More recently, apoptosis has been demonstrated to aid in clearance of viruses (Dragovich, et al., 1998) and has been shown to be induced by the bacterial pathogen, *Legionella pneumophila* (Lee and Esteban, 1994). However, when considering host cell infection with an intracellular pathogen, it would be to the advantage of the invading organism to subvert the apoptotic machinery, hence not destroying its niche before egression. Several different pathogens do undermine the apoptotic progression; including Chlamydia trachomatis (Xiao, et al., 2005), Escherichia coli (Sukumaran, et al., 2004), Mycobacterium tuberculosis (Park, et al., 2006), Toxoplasma gondii (Nash, et al., 1998), Plasmodium berghei (van de Sand, et al., 2005) and *Leishmania* spp. (Moore and Matlashewski, 1994). One potential mechanism behind this inhibition has been through the activation of several different signaling cascades. *Leishmania* infection stimulates the NF-κB and the PI-3 Kinase/Akt pathways (Ruhland, et al., 2007). Both of these pathways previously have been demonstrated to not only regulate inflammatory events, such as the production of cytokines,

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the also induce anti-apoptotic proteins, such as Bcl-X_L (Chen, et al., 2000, Song, et al., 2005).

Leishmaniasis is endemic in 88 countries with approximately 12 million infected and 350 million people at risk (Alvar, et al., 2006). A variety of disease manifestations are associated with *Leishmania* infection, primarily dictated by the infecting species. In the old world, infection with *L. major* results in a cutaneous, self-healing lesion and infection with *L. donovani* leads to potentially fatal visceral infection. *Leishmania* species are transmitted by the bites of infected female *Phlebotomine* sand flies. During the course of feeding, sand flies deposit the highly infective metacyclic promatigote form. These parasites are quickly taken up by resident antigen presenting cells (APCs), primarily langerhans cells (dendritic cells of the skin) and macrophages.

Leishmania parasites are covered by a thick surface coat, composed primarily of lipid-containing molecules, lipophosphoglycan (LPG) and glycoinositol phospholipds (GIPLs). LPG has been attributed with many different biological functions, ranging from guarding the parasite from the degradative environment of the sand fly midgut (Sacks, et al., 2000), to protection from complement-mediated lysis (McConville, et al., 1992), to inhibition of phagosome maturation (Desjardins and Descoteaux, 1997). Interestingly, treatment of monocyte-like cell line, U-937, with LPG isolated from *L. infantum* inhibits actinomycin D-induced apoptosis (Lisi, et al., 2005).

LPG is polymorphic among different species and life cycle stages of *Leishmania* (Ilg, et al., 1992, McConville, et al., 1995, McConville, et al., 1990, Turco, et al., 1987). All *Leishmania* species express an LPG with a conserved region, consisting of a lipid anchor and glycan core, along with two polymorphic domains, a polysaccharide backbone and an oligosaccharide cap. LPG polymorphisms are defined by modifications made at the three position of the galactose residue on the polysaccharide backbone. *L. donovani* contain no branching sugars to a few glucose substitutions, depending on the strain (Turco, et al., 1987), *L. mexicana* contains few sugar substitutions (Ilg, et al., 1992), while *L. major* (McConville, et al., 1990) and *L. tropica* (McConville, et al., 1995) contain a variety of sugar substitutions of various lengths and compositions. The repeating phosphoglycan (PG) characteristic of LPG also is found in other molecules of the *Leishmania* glycocalyx including proteophosphoglycans (Ilg, et al., 1994, Ilg, et al., 1996, Ilg, et al., 1994), secreted PG and acid phosphatase (Shakarian and Dwyer, 2000).

As LPG plays an important role in protecting *Leishmania* from the host's immune response, LPG structural polymorphisms could explain some noted differences in host response to different *Leishmania* species. LPG is implicated in modulating host cell signaling, resulting in a lack of interleukin-12 (IL-12) synthesis in murine macrophages (Descoteaux, et al., 1992, Descoteaux, et al., 1991, Proudfoot, et al., 1996); LPG also has been shown to induce IL-12 in the same cells (de Veer, et al., 2003), a paradox possibly attributed to species and strain differences in LPG structure. In addition, LPG is implicated in modulating CD40L-dependent IL-12 synthesis in human dendritic cells, with many *L. major* subspecies eliciting bioactive IL-12 upon infection, whereas *L. donovani* fails to induce IL-12 production (McDowell, et al., 2002). It is intriguing to speculate that polymorphisms in LPG structure, and thus possible function, could contribute to some of the noted variations in disease manifestation caused by different *Leishmania* species.

Here we investigated the impact of *Leishmania* infection on murine macrophage apoptosis. We demonstrate that not only does *Leishmania* infection protect murine macrophages from cycloheximide induced apoptosis, it does so in a species and strain specific manner. Some

Leishmania strains completely abrogate cycloheximide-induced apoptosis and complete apoptosis inhibition requires PGs.

Materials and Methods

Mammalian Cells

RAW 264.7 macrophage cell line was grown in RPMI-1640 media supplemented with 2mM L-glutamine, 100μg/mL Penicillin/Streptomycin, and 10% fetal bovine serum, with passage every two days.

Parasites and Infection

***Leishmania major* strains —**NIH Friedlin V1 Strain (MHOM/IL/80/FN) and V1 LPG mutant, Spock (kind gift of David Sacks, National Institutes of Health), IR173 (MHOM/IR/-173), LV39 (MRHO/SU/59/P), and NIH S strain (MHOM/SN/74/Seidman). *Leishmania donovani* strains: 9515 (MHOM/IN/95/9515), 1S (MHOM/SD/62/1S), *L. donovani* LPG mutants R2D2 and C3PO (mutants of *L. donovani* 1S) (the kind gifts of Salvatore Turco, University of Kentucky), Mongi (MHOM/IN/83/Mongi-142) and *Leishmania tropica* KK27 (MHOM/AF/88/KK27), were cultured in M199 supplemented with 20% FBS, 1μg/ml Penicillin/Streptomycin, 2mM L-glutamine and incubated at 26°C. Infectious, stationary phase metacyclics were used for all infections. Metacyclic promastigotes were isolated via Ficoll (ICN, Aurora, OH) gradients (Spath and Beverley, 2001) and were complement opsonized with 5% murine serum before infecting at a concentration of 10 parasites:1 macrophage. At 16h post-infection, cells were harvested and approximately 1×10^4 cells were used for cytospins to determine infection rates. After adherence to slides, cells were methanol fixed, stained with Diff-Quick (Biochemical Sciences Inc., Swedesboro, NJ), and visualized with light microscopy. Heat killed parasites were heated for 1 hour at 65°C, and were monitored for lack of motility prior to infection. Paraformaldehyde fixed parasites were fixed in 2% paraformaldehyde for 30 minutes on ice, and subsequently washed 5 times in 0.1% BSA in PBS. Parasites were counted and used for infection. Transwell experiments were carried out using 0.4 μM pore size cell culture inserts (BD Falcon, San Jose, CA) with 2.5×10^6 macrophages per mL.

Apoptosis Assays

RAW 264.7 were fixed with 2% paraformaldehyde, washed in PBS and left for 24h at −20°C in 70% ethanol. Cells were stained using the Apo BrdU Kit (BD Pharmingen San Jose, CA) according to manufacturer's instructions. Following ethanol permeabilization, cells were washed and incubated with terminal deoxynucleotidyl transferase enzyme in the presence of BrdU and subsequently stained with a FITC-conjugated anti-BrdU antibody, and incubated in a propidium iodide/RNase solution. Cells were analyzed on an MPL500 flow cytometer (Beckman Coulter, Fullerton, CA).

Western Blot Analysis

RAW 264.7 cells were treated for 20 min with ice-cold lysis buffer (150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM pervanadate, 1 mM EDTA, 1% Igepal, 0.25% deoxycholic acid, 1 mM NaF, and 50 mM Tris-HCl (pH 7.4)). Lystates were collected and stored at −80°C until use. Samples were loaded according to cell equivalents (4×10^5 = ~100μg of protein as determined by Bradford Assay), separated by SDS-PAGE gels, and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were blocked in TBST (Tris-buffered saline with 0.1% Tween 20) supplemented with 5% powdered milk and then incubated with a 1:500 dilution of primary antibodies against Bcl-X_L (Cell Signaling, Danvers, MA) and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) (Biogenesis, Poole, England), followed by 1:2500 dilution of either HRP-conjugated anti-mouse or anti-rabbit Ig (BD Biosciences San Jose, CA). Bound antibodies were detected using SuperSignal West Pico and Fempto ECL reagents (Pierce, Rockland, IL) followed by exposure to X-ray film. Relative band intensities were determined using Adobe Photoshop CS3 (Adobe, San Jose, CA); for each experiment Bcl-X_L bands were first normalized to GAPDH and then expressed as a fold change over the uninfected sample.

Statistical Analysis

A Student's *t* test was used for comparisons of % apoptosis and infection rates. One-way ANOVA followed by Tukey's multiple comparison test was utilized to etect differences of Bcl-X_L expression. In all cases, a *P* value of less than 0.05 was considered statistically significant.

Results and Discussion

Leishmania major V1 inhibits cycloheximide-induced apoptosis

Leishmania infection has previously been demonstrated to inhibit apoptosis in mammalian cells induced with a variety of compounds, including actinomycin D (Lisi, et al., 2005, Ruhland, et al., 2007), campothecin (Ruhland, et al., 2007), and staurosporine (Akarid, et al., 2004), all inducers of the intrinsic apoptotic pathway. Here, we utilized the potent intrinsic apoptosis inducer cycloheximide (CHX); this protein synthesis inhibitor functions by halting translational elongation (Martin, et al., 1995). To assess the impact of *L. major* V1 infection on CHX-induced apoptosis, RAW 264.7 macrophages were infected with *L. major* V1 4 hours prior to treatment with ethanol (ETOH) alone (CHX carrier) or CHX dissolved in ethanol and allowed to incubate for an additional 16 hours. Apoptosis was assessed using flow cytometric based BdrU incorporation and staining (Li, et al., 1995). Numbers of apoptosis positive cells were comparable in media and ETOH treated cells in both uninfected and *L. major* infected macrophages (Fig. 1). Uninfected, CHX treated cells exhibit drastically more apoptosis; however, *L. major*-infected CHX-treated macrophages exhibit virtually no apoptosis as do non-CHX treated cells (Fig. 1), demonstrating that *L. major* V1 infection inhibits CHX-induced apoptosis.

Leishmania induced apoptosis is both species and strain dependent

Previous studies demonstrate that *Leishmania* species are able to inhibit apoptosis stimulated by a variety of compounds (Akarid, et al., 2004, Moore and Matlashewski, 1994, Ruhland, et al., 2007). Although different methods of detecting apoptosis including detection of DNA fragmentation (Moore and Matlashewski, 1994), Caspase-3 assays (Ruhland, et al., 2007), and assessing mitochodria ion potential (Akarid, et al., 2004) have been utilized, none have directly compared the ability of different species and strains to inhibit apoptosis. In addition to different *Leishmania* species-specific disease manifestations, intra-species (or strain) variations also can induce different clinical states; for example some *L. major* strains cause severe disease, rather than spontaneously resolving lesions (Neva, et al., 1979).

To assess the strain specificity of *L. major*-infection induced inhibition of apoptosis, a total of five *L. major* strains were utilized: V1, Spock, IR173, LV39, and NIH S (Fig. 2a). Three strains, V1, Spock, and IR173 completely inhibit apoptosis; however, infection of macrophages with the other two strains (LV39 and NIH S) significantly reduced apoptotic staining compared to uninfected CHX-treated macrophages, but did so to a much more limited extent (Fig 2a). The strains that completely inhibited apoptosis (V1, Spock, and IR173) upon infection of macrophages resulted in positive apoptotic staining in approximately 1% of the population, whereas infection with the other three strains (LV39 and NIH S) resulted in approximately 75% of the macrophage population staining positive for apoptosis.

Interestingly, these same strains have been shown to group in a similar fashion in different model systems. In a human monocyte derived dendritic cells, *L. major* V1 and IR173 induce CD40L-dependent IL-12p70, while neither LV39 and NIH S induce IL-12p70 secretion (McDowell, et al., 2002). Furthermore, infection of IL-4 deficient Balb/c mice with *L. major* V1 and IR173 leads to healing, however infection of this mouse strain with *L. major* LV39 results in a more severe, non-healing disease, similar to wild-type Balb/c mice (Noben-Trauth, et al., 2003, Noben-Trauth, et al., 1999). Clearly, these *L. major* strains utilize different evasion mechanisms to subvert host immune responses of both human and murine origin.

L. donovani subspecies also display a strain-dependent inhibition of CHX-induced apoptosis phenotype (Fig. 2b). *L. donovani* strain 1S completely inhibits CHX-induced apoptosis in RAW 264.7 macrophages, while two other strains, 9515 and Mongi, significantly reduce apoptosis, but to a far lesser degree. Similar to *L. major* strains, parasites that result in nearly complete apoptosis inhibition (1S) stain for a much lower incidence of apoptosis (1%) compared to 9515 and Mongi (70%). Interestingly, infection of macrophages with *L. tropica*, results in no significant inhibition of CHX-mediated apoptosis (data not shown). No significant differences in infection rates were detected (Fig. 2b and d).

L. donovani 1S, originally isolated from a patient with visceral lieshmaniasis in the Sudan differs in geographic locale compared to 9515 and Mongi, which were both isolated from visceral leishmaniasis patients in India. Interestingly, the LPG from Mongi and 1S differs (the LPG structure from 9515 has yet to be resolved); 1S displays an LPG backbone with no branching sugars attached (Thomas, et al., 1992), however Mongi has 1–2 glucose substitutions every 4–5 backbone repeat units (Mahoney, et al., 1999). Although *L. major* Spock also lacks branching carbohydrate substitutions (Butcher, et al., 1996) and completely inhibits apoptosis, this phenotype can not fully explain the absolute inhibition, as *L. major* NIH S also lacks additional sugar residues (Sacks and da Silva, 1987) and only inhibits apoptosis by 25%. Furthermore, *L. major* V1 also completely abrogates CHX-induced apoptosis and the PGs of this strain are decorated with galactose residues terminating in arabinose (McConville, et al., 1990). Nonetheless, these intra-species LPG or other PG polymorphisms potentially may contribute to differences in apoptosis inhibition.

Leishmania induced apoptosis is dependent on phosphoglycans

To address the potential role of LPG and total PGs in *Leishmania* mediated apoptosis inhibition, two mutants of *L. donovani* 1S were utilized; LPG-parasites (strain R2D2) lack only LPG and PG- parasites (strain C3PO) lack all phosphoglycans (King and Turco, 1988). *L. donovani* LPG- infection of macrophages results in nearly complete inhibition of CHX-mediated apoptosis, similar to wild type *L. donovani* 1S (Fig. 3a). Interestingly, infection of macrophages with *L. donovani* PG- results in significant inhibition of apoptosis (66%), however not nearly to the same extent as *L. donovani* 1S and LPG-(0.45% and 0.66%, respectively) (Fig. 3a). Once again, infection rates did not differ between infection groups (Fig. 3b).

While much of the current focus on virulence factors of *Leishmania* has centered on LPG, few studies have illustrated the importance of total PGs during *Leishmania* infection. Some of these studies have used the equivalent of LPG-and PG- parasites in *L. major*. *In vivo*, both Balb/c and SCID mice infected with PG- *L. major* (*lpg2*-) fail to develop any disease, however, infection with LPG- *L. major* (*lpg1*-) results in severe disease manifestation, similar to infection with wild type *L. major* (Spath, et al., 2003, Uzonna, et al., 2004). Similarly, using the same *L. donovani* PG and LPG deficient parasites utilized here, infection with wild type *L. donovani* and LPG- parasites results in higher nitric oxide production relative to PG- parasites in siRNA MyD88-depleted, interferon-primed, RAW 264.7 macrophages (Flandin, et al., 2006). Likely, PGs also play a vital role in establishing *Leishmania* and preventing CHX-mediated apoptosis.

Leishmania induces Bcl X_L protein expression

L. major infection is able to circumvent the apoptotic machinery in macrophages induced by both staurosporine treatment and MCSF deprivation by preventing Cytochrome C (Cyt C) release from the mitochondria (Akarid, et al., 2004). Several inhibitors of Cyt C release, including Bcl-2 and Bcl-X_L are transcribed as a result of NF-κB activation (Chen, et al., 2000, Song, et al., 2005), and NF-κB is activated as a result of *Leishmania* infection (Ruhland, et al., 2007). To address the potential that species- and strain-dependent differences in inhibition of CHX-mediated apoptosis could be mediated by Bcl X_L, infections with all of the strains and species detailed in Figure 2 were repeated and protein levels of Bcl X_L were assessed. Infection of macrophages with all the *Leishmania* strains tested (Fig. 4) results in up regulation of Bcl X_L in untreated macrophages. As expected CHX treatment lowered overall protein levels in all cells as determined by GAPDH expression. Unexpectedly, the Bcl-X_L/GAPDH expression ratios were lower in the EtOH and CHX treated groups compared to the medium control for most groups.

While Bcl-X_L is strongly up-regulated at the protein level in response to *Leishmania* infection, it does not explain the dependence on strain specificity (Fig. 4). No significant difference in Bcl-X_L expression between any of the infections in response to EtOH (carrier control) or CHX were detected. While there are statistically significant differences between *Leishmania* strains in the untreated samples (*L. donovani* PG- and LPG- samples significantly lower than *L major* V1, SPOCK, IR173, LV39 and *L. donvani* 9515 significantly lower than *L. major* IR173, LV39) is there is no detectable pattern in Bcl-X_L levels between strains that completely inhibit CHX-induced apoptosis (*L. major* V1, SPOCK, IR173, and *L donovani* 1S, LPG-) and strains that only reduce apoptosis levels (*L. major* LV39, NIH-S, and *L donovani* PG-, 9515, Mongi). Previous studies have demonstrated that *Leishmania* infection leads to Akt-dependent phosphorylation of Bad, another anti-apoptotic factor that prevents Cyt C release from the mitochondria (Ruhland, et al., 2007). Clearly, *Leishmania* infection induces the activation of more than one anti-apoptotic protein.

Leishmania infection induced inhibition of apoptosis requires contact between host and parasite

Inhibition of CHX-mediated apoptosis in *L. major* V1 infected cells is nearly total and does not significantly differ from non-apoptosis induced macrophages (Fig. 2a). While infection rates for *L. major* V1 are high (80%), not every cell is infected, however very few of the macrophages stain positive for apoptosis (about 1%), suggesting a possible paracrine mechanism. To test if a macrophage secreted factor was responsible for the observed apoptosis inhibition, a transwell system was utilized. Macrophages were separated into two distinct populations; one in a lower well and one in an upper insert, separated by a 0.4 μM membrane (Fig. 5). Macrophages in the lower chamber were harvested to dissect the importance of direct parasite contact with macrophages in inhibition of CHX-induced apoptosis. *L. major* V1 parasites added directly to the lower well resulted in little apoptosis positive staining similar to levels previously observed (Figs. 1 and 2a). However, *L. major* V1 infection in the insert did not result in inhibition of CHX-mediated apoptosis in macrophages in the lower well (Fig. 5), indicating that paracrine signaling is not sufficient to inhibit CHX-induced apoptosis.

Metabolically active parasites are required for complete inhibition of CHX-mediated apoptosis

Leishmania products, especially LPG have powerful properties, effects that can be observed in the absence of live parasites. For example, the addition of LPG alone is able to inhibit actinomycin-D induced apoptosis in U-937 cells (Lisi, et al., 2005). In order to understand the role that parasite antigen compared to live infection might have on CHX-mediated apoptosis, both heat-killed and paraformaldehyde-fixed (PFA) *L. major* V1 parasites were utilized to treat

macrophages before ETOH or CHX treatment. Infection with non-metabolically active *L. major* V1 (both heat-killed and PFA fixed) significantly inhibited CHX-mediated apoptosis, however, not nearly to the same extent as macrophage infection with live *L. major* (Fig. 6) (1% vs. 77% Heat killed vs. 55% PFA fixed). Interestingly, there were fewer apoptotic positive cells in the PFA fixed group compared to heat-killed condition.

In order to fully inactivate parasites via exposure to heat (as monitored visually), an hour incubation is necessary; in this time heat labile molecules potentially necessary for efficient inhibition of apoptosis could be denatured, whereas fixation would not necessarily cause alteration of these molecules. One such molecule, LDAA-12, a *L. donovani* antigen, known to induce colony stimulation factors, is heat labile (Singal and Singh, 2005). Also, gp63 (leishmanolysin), another major surface glycoprotein, is heat labile; after only 15 min at raised temperatures, it loses its inhibitory effect on monocyte chemotaxis (Russell and Wilhelm, 1986).

Recently, a model has been proposed which posits that *Mycobacteria*-infected macrophages undergo apoptosis and the subsequent blebs are taken up by surrounding dendritic cells. This allows for cross-presentation of *Mycobacteria* antigen to CD8+ T cells as well as lipid presentation using CD1b (Schaible, et al., 2003). Subversion of this pathway during a *Leishmania* infection could potentially have a profound effect. *Leishmania*, like *Mycobacteria*, have several lipid antigens, including glycoinositol phospholipids (GIPLs) and LPG. *Leishmania* infection down-regulates CD1 expression in human dendritic cells (Amprey, et al., 2004, Donovan, et al., 2007). Potentially, *Leishmania* could be targeting both apoptosis and lipid antigen presentation to avoid immune surveillance.

It is intriguing to speculate that *Leishmania* mediated inhibition of CHX-induced apoptosis may be a two tiered process, with actual contact with the host cell surface being the initial step. Contact between parasites and macrophages is absolutely required for protection (Fig. 5) and even metabolically inactive parasites are able to significantly reduce apoptosis compared to uninfected macrophages treated with CHX (Fig. 6). This initial interaction likely leads to the significant reduction evident in macrophages infected with all of the *L. major* and *L. donovani* strains. The early inhibition of apoptosis is independent of either PGs or LPG, as infection of macrophages with PG- parasites (which also lack LPG) significantly reduce CHX-induced apoptosis (Fig 3a). The second tier of apoptosis inhibition is likely what arises as a result of strain specificity. This step clearly is not dependent on LPG; once promastigotes are phagocytosed they halt maturation of the endosome in an LPG-dependent manner (Desjardins and Descoteaux, 1997), and transform into amastigotes. In the amastigote form, a second, unknown as of yet, signal is likely to account for the remainder of the apoptosis inhibition. This signal could potentially be a PG (or due to a PG-host interaction) that is polymorphic among different strains.

Our report demonstrates that infection of RAW 264.7 macrophages with *Leishmania* species inhibits CHX-induced apoptosis. Strain dependent immune modulation (and in turn variations in inhibition of apoptosis) are supported by our data, with similar patterns of host modulation observed in other studies (McDowell, et al., 2002, Noben-Trauth, 2000, Noben-Trauth, et al., 1999). This inhibition is due in part to PGs, independent of LPG, and requires parasite contact with the macrophage. Overall, this work demonstrates yet another manner in which *Leishmania* is able to subvert immune surveillance in a strain-dependent manner.

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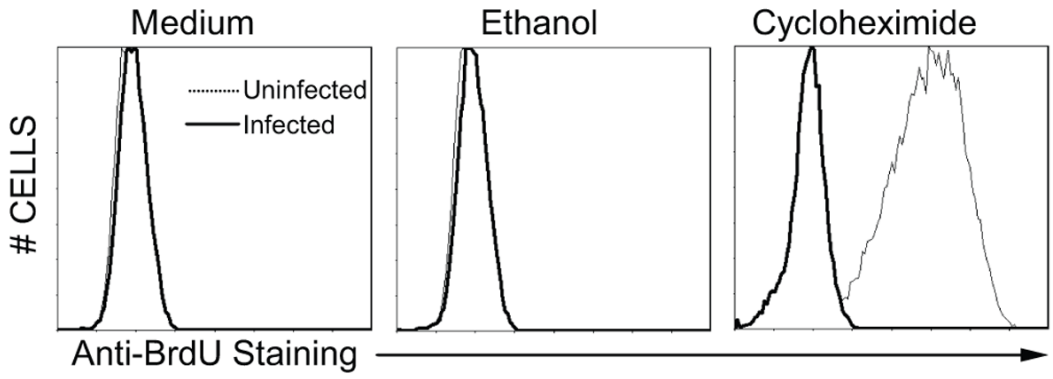

Figure 1.

L. major infection of RAW 264.7 macrophages inhibits CHX-induced apoptosis. RAW 264.7 macrophages were infected with *L. major* V1 4 hrs prior to ETOH (carrier control), CHX, or no additional (Medium) treatment for 16 hrs. Apoptosis was determined using a flow cytometry based BrdU staining. Uninfected (---) and *L. major* V1 infected (—) infected histograms were overlaid using CXP software (Beckman Coulter). One of 3 independent experiments is presented.

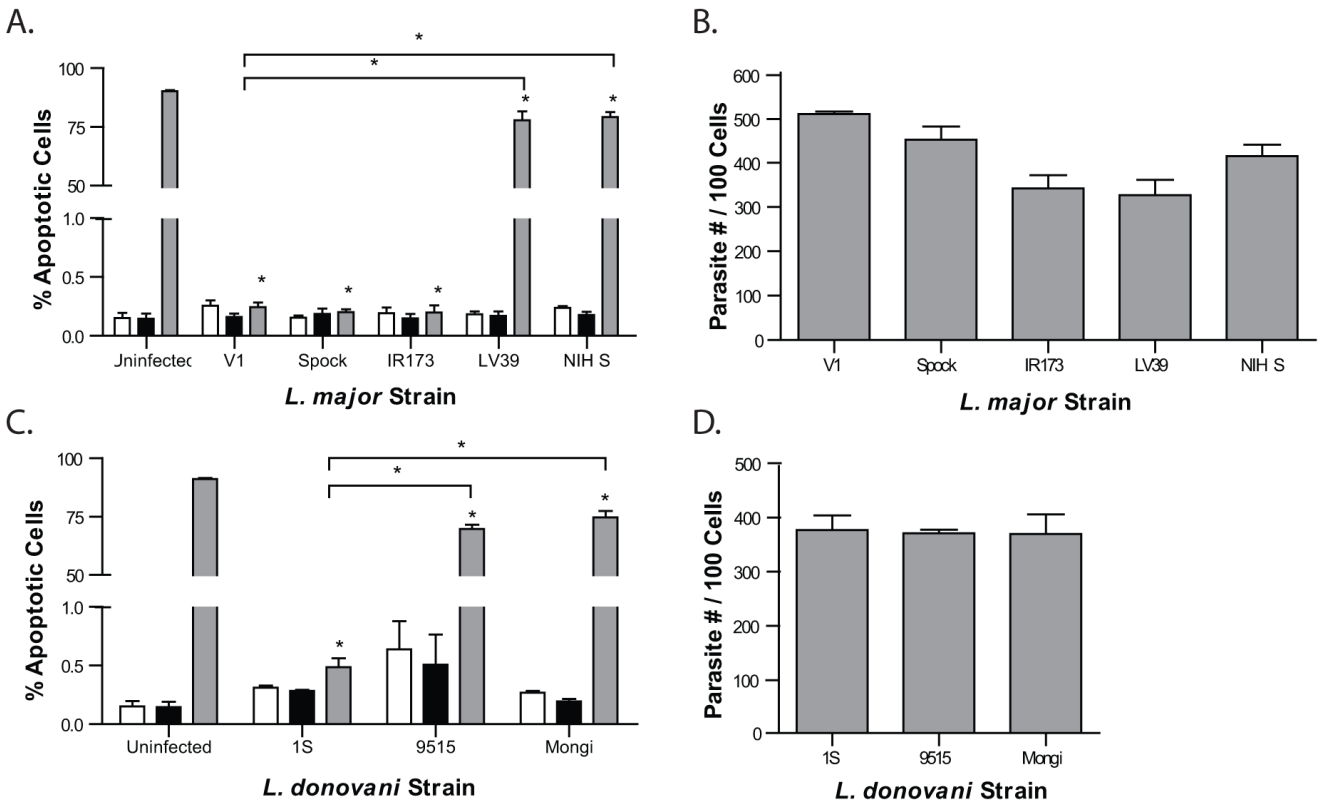

Figure 2.

Inhibition of apoptosis is species and strain dependent. RAW 264.7 macrophages were infected with *L. major* strains (V1, Spock, IR173, LV39, and NIH S) (a) or *L. donovani* strains (1S, 9515, and Mongi) (c) 4 hrs prior to ETOH (■), CHX (▩), or no additional (□) treatment for 16 hrs. Apoptosis was determined using a flow cytometry based BrdU staining. Mean ± SEM of % apoptosis from 3 independent experiments is presented. *$p \leq 0.05$ compared to uninfected CHX treatment unless noted. (b & d) Infections were quantified to ensure equal parasite infection and presented as number of parasites per 100 cells. No statistically significant differences of infection rates were detected.

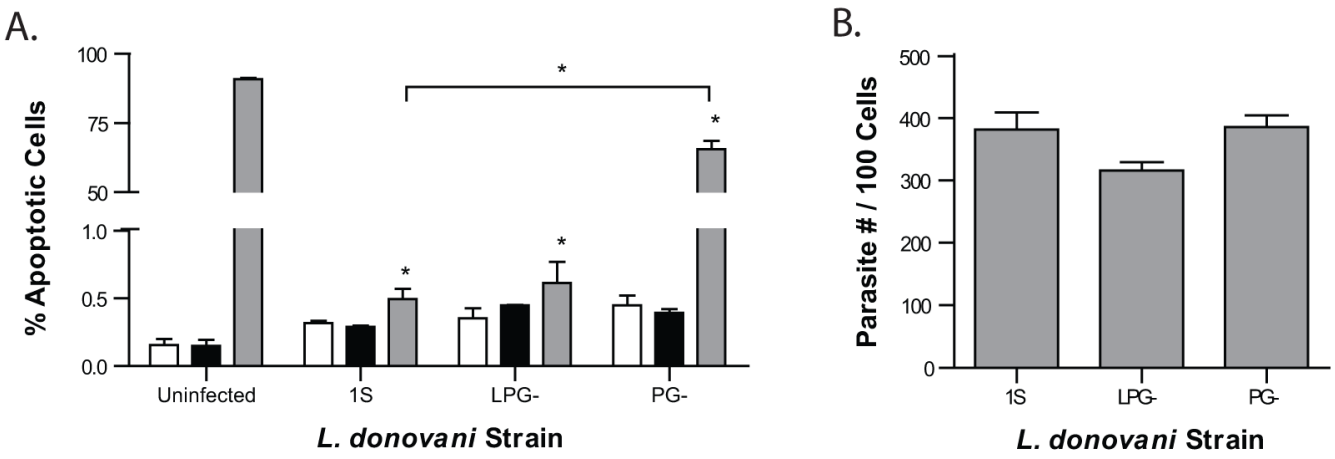

Figure 3.

Non-LPG PGs are vital for inhibition of CHX-induced apoptosis. (a) RAW 264.7 macrophages were infected with *L. donovani* strains (1S, LPG-, and PG-) 4 hrs prior to ETOH (■), CHX (▩), or no additional (□) treatment for 16 hrs. Apoptosis was determined using a flow cytometry based BrdU staining. Mean ± SEM of % apoptosis from 3 independent experiments is presented. *$p \leq 0.05$ compared to uninfected CHX treatment unless noted. (b) Infections were quantified to ensure equal parasite infection and presented as number of parasites per 100 cells. No statistically significant differences of infection rates were detected.

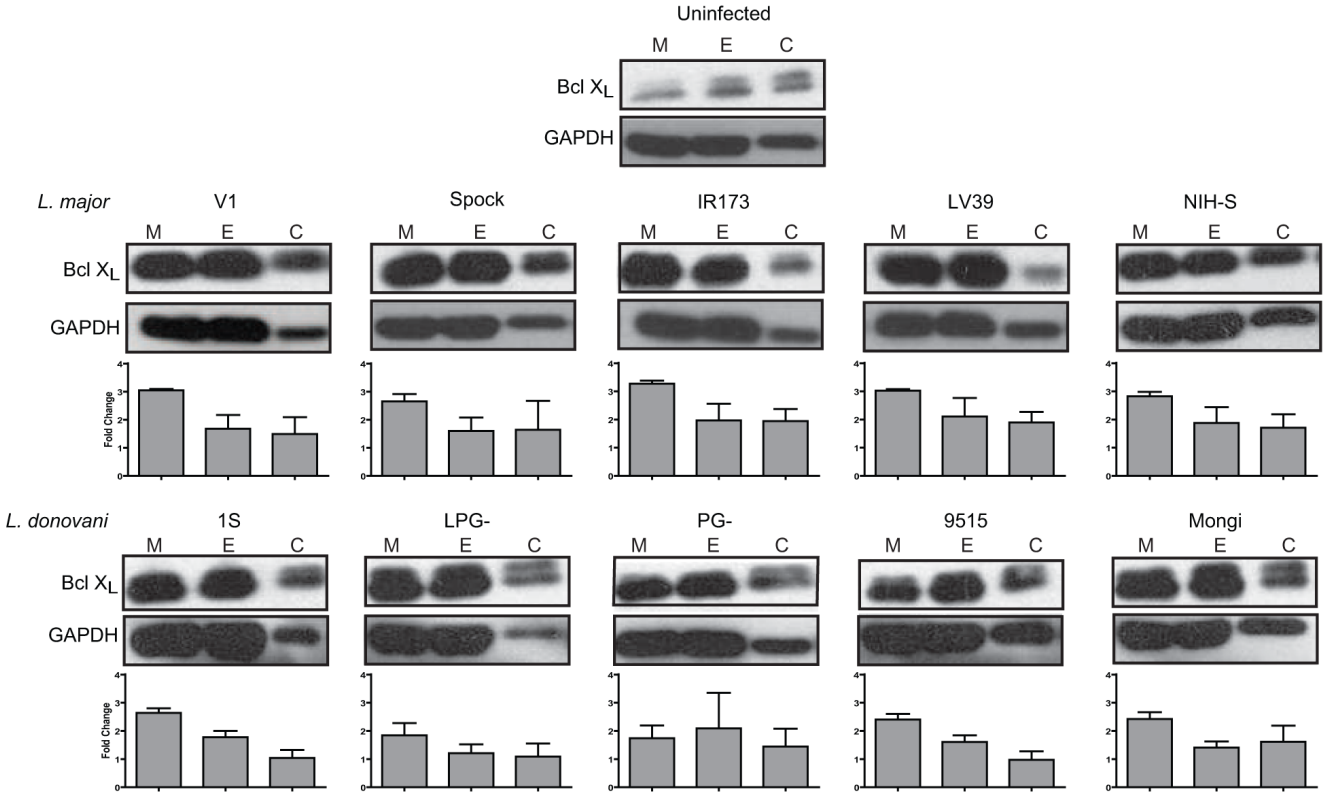

Figure 4.

Leishmania infection induces Bcl-X_L protein expression, but is not the factor contributing to strain dependent inhibition of apoptosis. RAW 264.7 macrophages were infected with *L. major* strains (V1, Spock, IR173, LV39, and NIH S) and *L. donovani* strains (1S, LPG-, PG-, 9515, and Mongi) 4 hrs prior to ETOH (E), CHX (C), or no additional (M) treatment for 16 hrs. Lysates were separated via SDS-PAGE by loading 4×10^5 cell equivalents and assessed by western blotting with anti-Bcl-X_L and GAPDH antibodies. Blots from 1 of 3 independent experiments are presented. Relative band intensities were determined for each experiment, normalized to GAPDH, and expressed as fold change over the uninfected sample. Mean fold change ± SEM is presented.

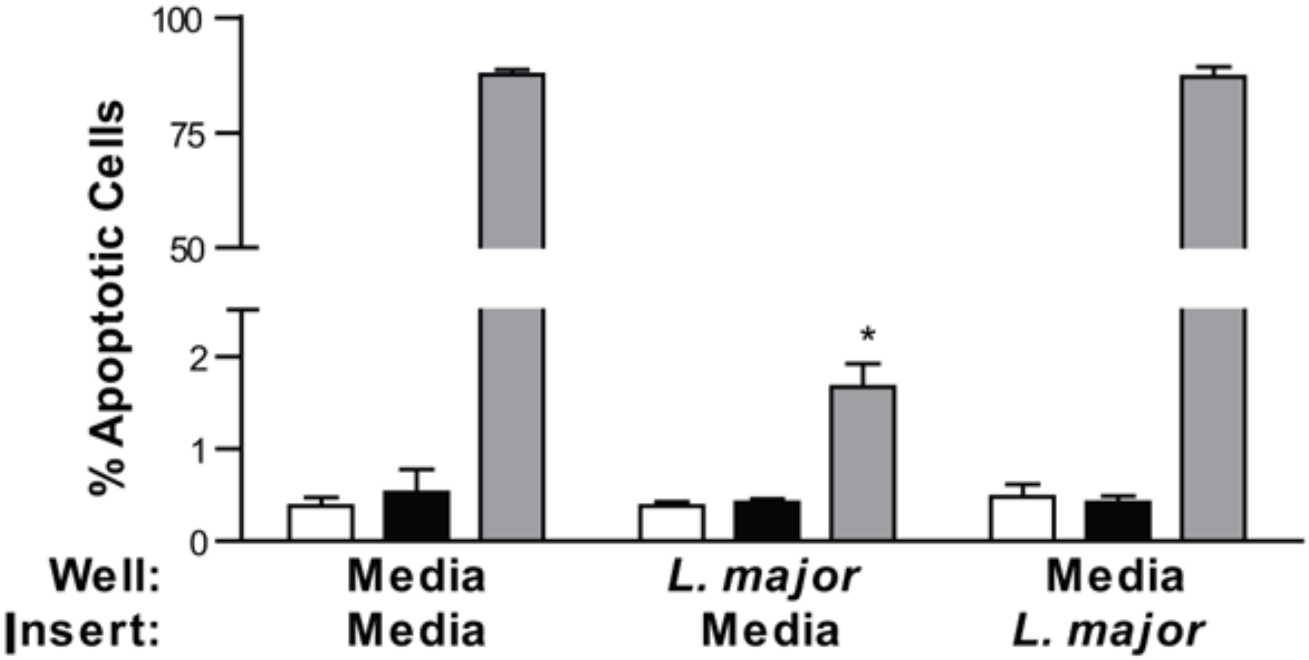

Figure 5.

Inhibition of apoptosis requires direct contact between *Leishmania* and macrophage. RAW 264.7 macrophages were plated both in a lower chamber and a 0.4μM cell culture insert in a 6 well plate and either the macrophages in the well or insert were infected with *L. major* V1 4 hrs prior to ETOH (■), CHX (▩), or no additional (□) treatment for 16 hrs. Apoptosis was determined using a flow cytometry based BrdU staining. Mean ± SEM of % apoptosis from 3 independent experiments is presented. *$p \leq 0.05$ compared to uninfected CHX treatment. No statistically significant differences of infection rates were detected.

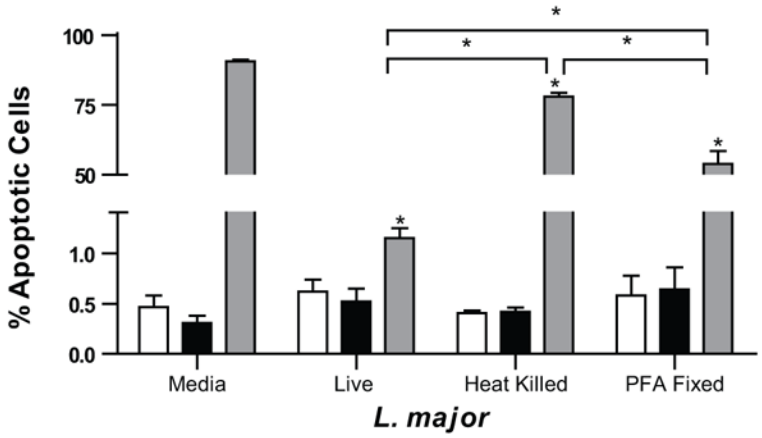

Figure 6.

Inhibition of apoptosis requires infection with metabolically active *Leishmania*. RAW 264.7 macrophages were infected with one of three different conditions, live *L. major* V1, heat killed *L. major* V1 (heated at 65°C for 1 hour) or paraformaldehyde fixed *L. major* V1 4 hrs prior to ETOH (■), CHX (▩), or no additional (□) treatment for 16 hrs. Apoptosis was determined using a flow cytometry based BrdU staining. Mean ± SEM of % apoptosis from 3 independent experiments is presented. *$p \leq 0.05$ compared to uninfected CHX treatment unless noted. No statistically significant differences of infection rates were detected.