Host Cell Lipid Bodies Triggered by *Trypanosoma cruzi* Infection and Enhanced by the Uptake of Apoptotic Cells Are Associated With Prostaglandin E_2 Generation and Increased Parasite Growth

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Lipid bodies (lipid droplets) are lipid-rich organelles with functions in cell metabolism and signaling. Here, we investigate the mechanisms of *Trypanosoma cruzi*–induced lipid body formation and their contributions to host-parasite interplay. We demonstrate that *T. cruzi*–induced lipid body formation in macrophages occurs in a Toll-like receptor 2–dependent mechanism and is potentiated by apoptotic cell uptake. Lipid body biogenesis and prostaglandin E_2 (PGE_2) production triggered by apoptotic cell uptake was largely dependent of $\alpha_v\beta_3$ and transforming growth factor-β signaling. *T. cruzi*–induced lipid bodies act as sites of increased PGE_2 synthesis. Inhibition of lipid body biogenesis by the fatty acid synthase inhibitor C75 reversed the effects of apoptotic cells on lipid body formation, eicosanoid synthesis, and parasite replication. Our findings indicate that lipid bodies are highly regulated organelles during *T. cruzi* infection with roles in lipid mediator generation by macrophages and are potentially involved in *T. cruzi*–triggered escape mechanisms.

Chagas disease is a serious illness characterized by chronic inflammation and cardiomyopathy caused by the intracellular protozoan *Trypanosoma cruzi*. It is estimated that 15 million people are infected with or are carriers of the disease in Central and Latin America, which constitutes an important public health problem in countries in which the disease is endemic [1]. Even 100 years after the seminal discoveries of Carlos Chagas [2], many aspects of the interactions between *T. cruzi* and its mammalian host and the mechanisms of pathogenesis remain unclear. In addition, there is no vaccine against Chagas disease, and currently available drugs are toxic and have low efficacy against the chronic phase of disease. Therefore, the identification of new pathways and potential targets for intervention is necessary.

T. cruzi has evolved multiple strategies to invade and exploit many cells, including macrophages, for their survival [3]. Many biological responses developed by the host against parasite infection are exploited for their survival and growth. *T. cruzi* infection also leads to major deregulation of host lipid and glucose metabolism [4]. Recently, we and others have described the occurrence of altered lipid metabolism and increased numbers of lipid bodies (LBs; lipid droplets) in host cells infected by intracellular pathogens, which suggests that LBs may have roles in intracellular pathogen infection

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[5]. LBs are composed of a triglyceride and cholesteryl ester core with a surrounding monolayer of phospholipid, cholesterol, and a variety of associated proteins with diverse functions in cell metabolism, signaling, and inflammation [5, 6]. In leukocytes and other cells involved in infectious conditions, LB formation has been shown to occur through regulated mechanisms that are cell and stimuli specific. LB biogenesis involves both direct interactions between the pathogen and the host cell and indirect interactions mediated by bystander amplification through pathogen components and/or host-generated cytokines and chemokines [7–13]. LBs were shown to play central roles in the heightened production of eicosanoids, thus participating in the amplification of the inflammatory response and ultimately regulating the host immune response to intracellular pathogens [7, 8, 11, 14].

LB formation and association with vacuole-containing parasites has been demonstrated in infections by parasites including *T. cruzi* [7, 15, 16], *Leishmania amazonensis* [17], *Toxoplasma gondii* [18, 19], *Plasmodium falciparum* [20], and *P. berghei* [21]. Acute infection with *T. cruzi* induced a significant increase in the number of LBs observed in association with parasite-containing phagosomes in peritoneal and heart macrophages [16]. Adipocytes have been observed to be target cells during acute Chagas disease; *T. cruzi* amastigotes were observed forming clusters adjacent to LBs [4, 22]. Although the increased formation of LBs has been documented in host cells during protozoan infections, the mechanisms that govern LB biogenesis and its role to parasite pathogenesis are not well understood.

In the present study, we investigate the mechanisms involved in *T. cruzi*–induced LB formation and the functions of LBs in host-parasite interplay. We demonstrate that *T. cruzi* triggers macrophage LB formation in a Toll-like receptor 2 (TLR2)–dependent manner. *T. cruzi*–induced LB biogenesis is potentiated when macrophages ingest apoptotic cells. Moreover, we demonstrate that newly formed LBs are sites for prostaglandin E_2 (PGE_2) synthesis and that the pharmacological modulation of LB formation in the cell impacts parasite growth.

MATERIALS AND METHODS

Animals and Infection

C57BL/6, TLR2 ($Tlr2^{-/-}$), and TLR4 ($Tlr4^{-/-}$) genetically deficient mice in a homogeneous C57BL/6 background [23] were kindly donated by Dr S. Akira (Osaka University, Japan) and maintained at the Fundação Oswaldo Cruz animal facility.

Peritoneal macrophages from uninfected mice were plated in Roswell Park Memorial Institute (RPMI) medium with 1% Nutridoma-SP (Roche). Cells (1×10^6 cells/mL) were infected with 3×10^6 metacyclic trypomastigotes of *T. cruzi* clone Dm 28c induced as described [24]. After 24 hours, noninternalized parasites were removed. Extracellular motile trypomastigotes were counted after 10 days of infection.

For in vivo infection, female Holtzman rats aged 27–30 days (obtained from Universidade Federal Minas Gerais animal facility) were inoculated intraperitoneally with 3×10^5 trypomastigotes of *T. cruzi* Y strain as described elsewhere [15]. The animals were sacrificed in a CO_2 chamber on day 12 of the infection, and heart tissues were processed for transmission electron microscopy (TEM) as described below. All protocols were approved by the Fundação Oswaldo Cruz animal welfare committee.

Apoptosis Induction and Treatments

Splenocytes from naive C57BL/6 mice were incubated in RPMI medium with 1% Nutridoma-SP containing 1 μM dexamethasone (Merck) overnight to obtain apoptotic cells. Viable cells were subjected to 2 cycles of freeze/thaw to obtain necrotic cells. Cells were stained with annexin V-FITC (R&D Systems) to confirm the efficiency (>80%) of the induction of apoptosis or necrosis.

Peritoneal macrophages were treated with apoptotic, live, or necrotic cells (3×10^6 cells/well), transforming growth factor (TGF)-β1 (1–5 ng/mL) (R & D Systems), anti-mouse CD61 (integrin β_3 chain), anti-mouse CD51 (integrin α_v chain), or control immunoglobulin (Ig) G (10 μg/mL) (BD Biosciences) for 24 hours. In inhibitory studies, macrophages were pretreated with 0.1–1.0 μM desintegrin flavoridin, neutralizing antibody against TGF-β1 (50 μg/mL), aspirin (5 μM), NS-398 (1 μM), C75 (5 μg/mL), or vehicle (phosphate-buffered saline or 0.01% dimethyl sulfoxide) before stimulation with apoptotic cells for 24 hours at 37 °C. Viability was assessed by trypan blue exclusion at end of each experiment and was always >90%.

LB Staining and Counting

Macrophages were fixed with 3.7% formaldehyde for 10 minutes and stained by osmium tetroxide. The morphology of fixed cells was observed, and osmium-stained LBs were counted by light microscopy with a 100× objective lens in 50 consecutively scanned leukocytes. Alternatively, LBs were stained with 1 μm BODIPY 493/503 for 10 minutes (Molecular Probes) and analyzed by fluorescence microscopy. The nuclei of macrophages and internalized parasites were observed after 4,6-diamidino-2-phenylindole (DAPI) staining.

PGE_2 and TGF-β Measurement

PGE_2 and TGF-β1 were measured in the supernatant by enzyme-linked immunoassay (EIA; Cayman Chemical) and by enzyme-linked immunosorbent assay (R&D Systems), respectively, according to the manufacturer's instructions.

COX-2 Immunolocalization and Intracellular Detection of PGE_2

Macrophages from control and stimulated groups adhered to cover slides were fixed in 3.7% formaldehyde for 10 minutes. The immunocytochemistry for cyclooxygenasep-2 (COX-2) and LBs was performed by glucose-oxidase immunostaining as

described elsewhere [8]. For immunofluorescence analysis, TritonX 0.1% permeabilized cells were incubated with goat polyclonal anti-COX-2 antibody (Santa Cruz Biotechnology) and guinea pig polyclonal anti–adipose differentiation-related protein (ADRP) (Research Diagnostics). Goat IgG was used as control. Cy2-conjugated anti-guinea pig and Cy3- conjugated anti-goat (Immuno Jackson) were used as secondary antibodies.

Immunolocalization of PGE_2 at its formation sites within stimulated macrophages was obtained using an Olympus BX-FLA fluorescence microscope performed by Eicosacell as described elsewhere [25, 26] and modified [8]. The images were obtained with a CoolSNAP-Pro CF digital camera in conjunction with Image-Pro Plus software (MediaCybernetics).

Transmission Electron Microscopy (TEM)

The morphology of macrophages in the heart, a target organ of Chagas disease [27], was evaluated in infected rats at 12 days after infection and in controls. Fragments of atria from infected animals were fixed in phosphate-buffered 1% paraformaldehyde and 1% glutaraldehyde, pH 7.3, overnight at 4°C. The samples were processed for conventional TEM as described elsewhere [28]. At least 105 electron micrographs randomly taken from macrophages were studied.

Statistical Analysis

The results were expressed as mean values ± the standard error of the mean and were analyzed statistically by means of analysis of variance followed by the Student-Neuman-Keuls test, with significance set at $P < .05$.

RESULTS

T. cruzi–Induced LB Formation Is TLR2-Mediated and Enhanced by Apoptotic Cell Uptake

By TEM using an in vivo model of *T. cruzi* infection in rats, our present findings show that apoptotic cardiomyocytes were actively phagocytosed by infiltrating macrophages (Figure 1 and Supplemental Figure 1). Remarkably, ingested apoptotic bodies led to the formation of a large number of cytoplasmic LBs (Figure 1*A* and *B*). LBs were identified surrounding morphologically typical, large apoptotic bodies containing cells in different stages of degeneration. These LBs showed distinct electron densities, as previously identified in *T. cruzi*–elicited LBs under the same model [15].

As shown in Figure 2*A*, *T. cruzi* infection leads to increased LB formation in macrophages from C57/BL6 mice, compared with LB formation in control noninfected cells. Notably, uninfected cells from the infected group, although in smaller numbers than were found in the infected cells, also exhibited increased LBs, compared with control cells, suggestive of bystander amplification of the response. The mechanism involved in LB formation within *T. cruzi*–infected cells was investigated. At 24 hours after

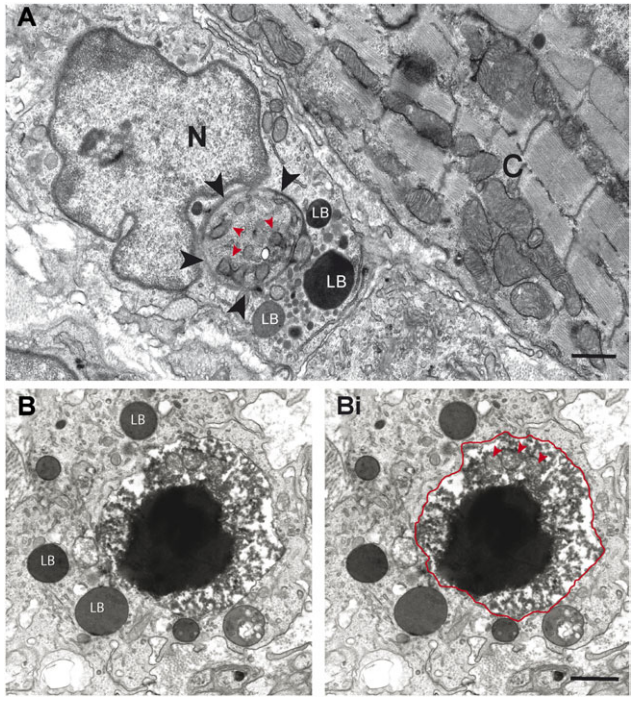

Figure 1. Uptake of apoptotic cells by macrophages induces lipid body formation during in vivo *Trypanosoma cruzi* infection. *A* and *B*, Electron micrographs of inflammatory heart macrophages from rats infected with *T. cruzi*. In *A*, apoptotic bodies show degenerating mitochondria (*red arrowheads*). In *B*, a very electron-dense nuclear chromatin is observed within the apoptotic body. Large apoptotic bodies (indicated with arrowheads in *B* and outlined in red in *Bi*) are seen in the cytoplasm surrounded by several lipid bodies (LBs). LBs exhibit distinct electron densities. C, cardiomyocyte; N, nucleus. The scale bars are 1 μm.

infection, we observed that *T. cruzi*–induced LB formation in macrophages from *Tlr2*$^{-/-}$, but not *Tlr4*$^{-/-}$, mice was drastically reduced, compared with LB formation in wild-type macrophages (Figure 2*B* and *C*).

An increased apoptosis rate among inflammatory cells, lymphocytes, and cardiomyocytes has been identified during experimental conditions and in humans with *T. cruzi* infection [15, 29–31]. Clearance of apoptotic bodies, a process termed efferocytosis, was shown to impact on host inflammatory mediator production and susceptibility to infection [29, 31, 32]. To investigate whether efferocytosis could affect infection-induced LB formation and PGE_2 synthesis, *T. cruzi*–infected cells were exposed to apoptotic lymphocytes. Efferocytosis enhanced the effects of *T. cruzi* on LB formation and PGE_2 synthesis (Figure 2*E* and *F*). *T. cruzi* failed to induce PGE_2 production in macrophages from *Tlr2*$^{-/-}$ macrophages. Although PGE_2 production induced by *T. cruzi* and apoptotic cells are decreased in *Tlr2*$^{-/-}$, compared with wild-type macrophages, *T. cruzi* and apoptotic cells induced significant PGE_2 production in *Tlr2*$^{-/-}$, compared with their respective control noninfected cells or *T. cruzi* alone (Figure 2*F*).

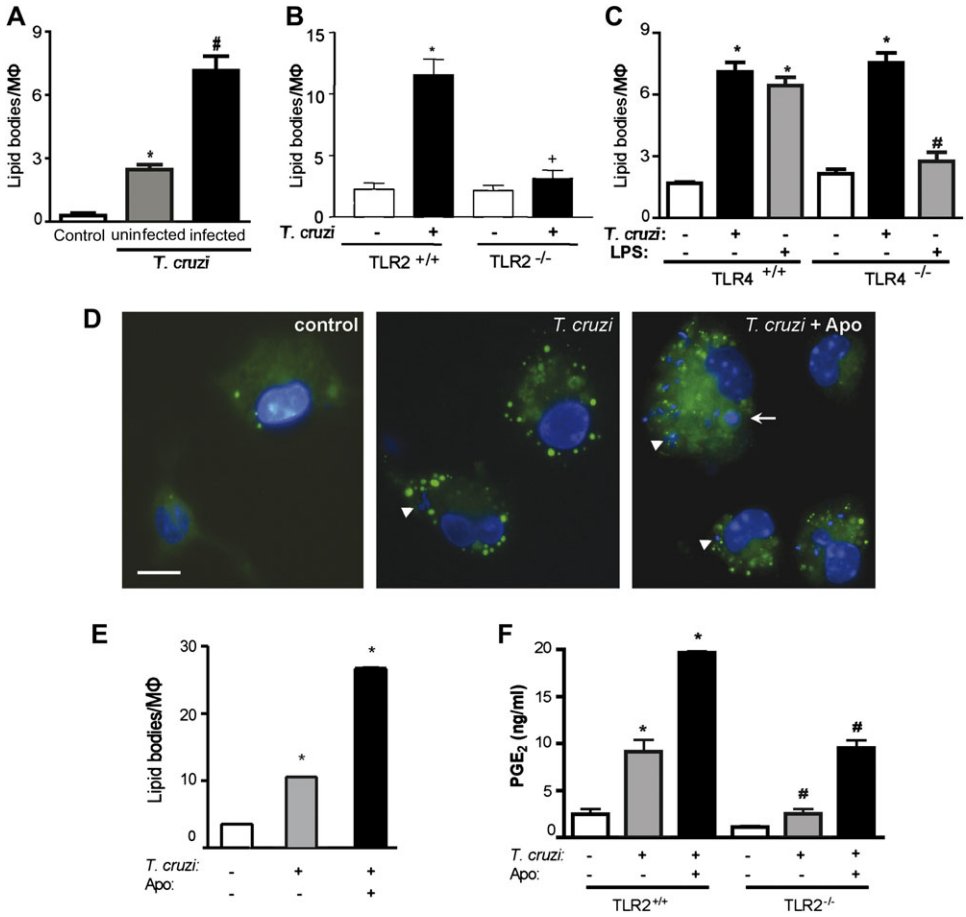

Figure 2. *Trypanosoma cruzi* infection induces macrophage lipid body formation and prostaglandin E_2 (PGE_2) synthesis in a Toll-like receptor 2 (TLR2)–dependent manner, and apoptotic cells increased this phenomenon. *A*, lipid body (LB) formation in infected and uninfected macrophages, 24 hours after infection by 3 trypomastigotes per cell. As control, the cells received vehicle. *B* and *C*, LB enumeration in wild-type ($TLR2^{+/+}$ and $TLR4^{+/+}$) and TLR2 ($TLR2^{-/-}$) or TLR4 ($TLR4^{-/-}$) knockout mice 24 hours after *T. cruzi* infection. $TLR4^{+/+}$ and $TLR4^{-/-}$ macrophages were also stimulated by lipopolysaccharide (LPS) (250 ng/mL). *D*, peritoneal macrophages from uninfected, *T. cruzi*–infected alone, or cocultured with apoptotic cells showing lipid bodies at 24 hours after infection (*green*). Nuclei of macrophages, internalized parasites (*arrowhead*), and apoptotic cells (*arrow*) are stained with DAPI (4',6-diamidino-2-phenylindole; *blue*). The scale bar is 10 μm. *E*, LB formation in macrophages from wild-type mice infected in vitro by *T. cruzi* alone or cocultured with apoptotic cells for 24 hours. *E*, PGE_2 synthesis by macrophages from $TLR2^{+/+}$ and $TLR2^{-/-}$ mice infected by *T. cruzi* and/or stimulated by apoptotic cells at 24 hours. Macrophage LBs were enumerated after osmium staining. Each bar represents the mean ± standard error of the mean (SEM) from 50 consecutively counted macrophages from at least 4 independent pools of 3 animals each. Statistically significant ($P \leq .05$) differences between control and infected or stimulated groups are indicated by asterisks; +, # represents differences between infected or uninfected cells or wild-type and knockout mice. Mϕ, macrophages.

The $\alpha_v\beta3$ Integrin Is Involved in Mechanisms of LB Formation Induced by Apoptotic Cells

The mechanisms involved in efferocytosis-driven LB formation were investigated. First, we analyzed whether efferocytosis by macrophages without concomitant infection could also induce LB formation and PGE_2 synthesis. To test this, macrophages were cocultured with living, necrotic, or apoptotic splenocytes. We observed a marked increase in the number of LBs in macrophages 24 hours after the phagocytosis of apoptotic cells. However, no increase in LBs was observed when living or necrotic splenocytes were incubated with macrophages (Figure 3*A*). In addition, we noted no differences in PGE_2 levels in the supernatant between the control cells and macrophages cocultured with living or necrotic cells. However, significantly increased levels of PGE_2 were observed in the supernatant of macrophages stimulated with apoptotic cells (Figure 3*A*).

The recognition of apoptotic cells by the $\alpha_v\beta3$ integrin (vitronectin receptor) is critical in apoptotic-cell cytoadherence and the induction of PGE_2 and TGF-β release during *T. cruzi* infections [31]. The involvement of $\alpha_v\beta3$ on LB formation and parasite replication induced by apoptotic cell uptake was evaluated. Macrophages were pretreated with flavoridin, a desintegrin that blocks binding of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [33, 34]. Flavoridin blocked LB formation (Figure 3*B*) and COX-2 expression (not shown) induced by incubation of macrophages with apoptotic cells. As shown in Figure 3*C* and *D*, the engagement and activation of $\alpha_v\beta3$ by anti-α_v or anti-β_3 monoclonal antibodies was sufficient to trigger LB biogenesis and

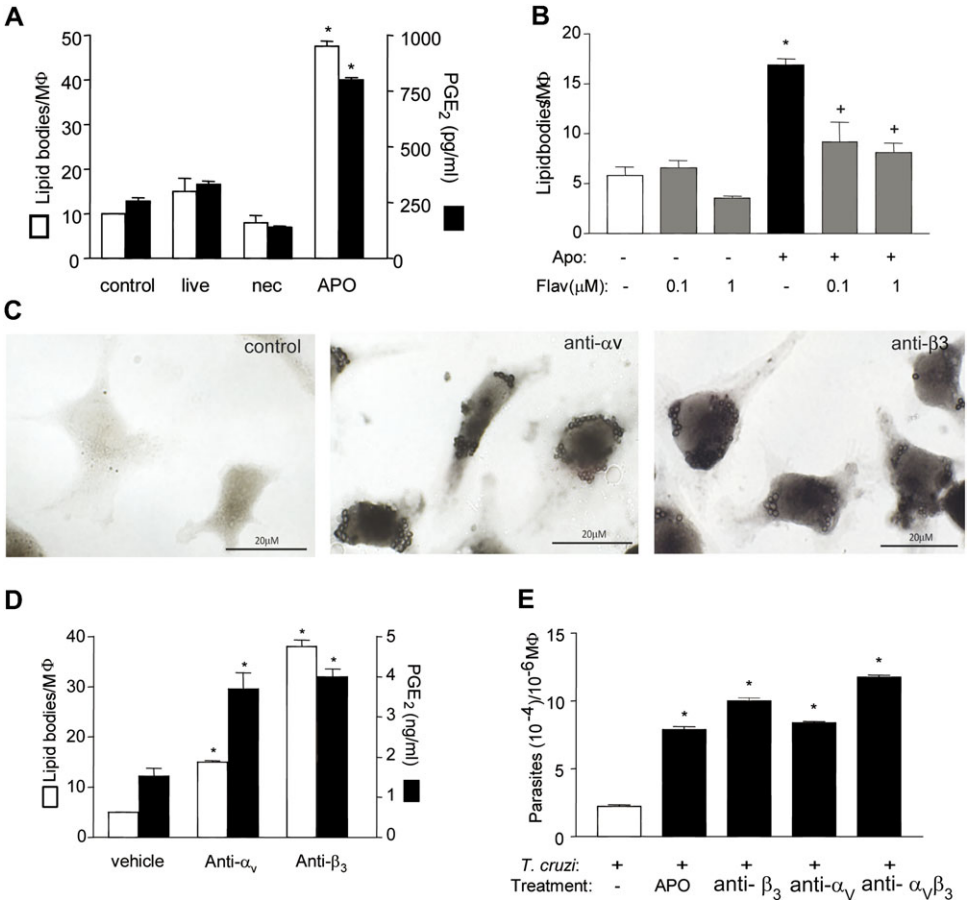

Figure 3. Signaling through $\alpha_v\beta_3$ induces lipid body formation and enhanced prostaglandin E_2 (PGE_2) generation, COX-2 expression, and parasite replication. *A*, lipid body (LB) formation (*white bars*) and PGE_2 synthesis (*black bars*) in murine macrophages cocultured with apoptotic, live, or necrotic cells. *B*, Desintegrin flavoridin (Flav) inhibited the effect of apoptotic cells on LB formation. *C* and *D*, macrophage LBs stimulated with anti-α_v and/or anti-β_3 monoclonal antibody (24 hours) were shown and enumerated after osmium tetroxide staining. The scale bars are 20 μm. *E*, parasite replication in murine macrophages infected and stimulated with anti-α_v and/or anti-β_3 monoclonal antibody. Parasites were counted after 10 days of infection. Results are the mean value ± the standard error of the mean from 3 independent pools of 3–5 animals each. Statistically significant differences ($P < .05$) between the control group and stimulated group are indicated by asterisks; + represents differences between the stimulated group and treated group.

enhanced PGE_2 synthesis in macrophages. In parallel with increased LB formation and PGE_2 synthesis, infected macrophages presented exacerbated parasite replication when cocultured with apoptotic cells for 24 hours (Figure 3*E*), even in *Tlr2-/-* macrophages (not shown). Similarly, the engagement of $\alpha_v\beta3$ by anti-α_v or anti-β_3 monoclonal antibodies markedly increased *T. cruzi* growth in macrophages in the absence of apoptotic cells (Figure 3*E*).

TGF-β Is Involved in LB Formation Triggered by Apoptotic Cells

Next, we evaluated the role of TGF-β1 in LB formation in macrophages treated with apoptotic cells and showed that macrophages produced significant levels of TGF-β in response to apoptotic, but not necrotic, cells (Figure 4*A*). Exogenous TGF-β markedly induced LB formation at levels similar to those in apoptotic cells (Figure 4*B*). Moreover, neutralization of TGF-β1 with anti–TGF-β1–neutralizing antibody inhibited LB formation induced by apoptotic cells, which demonstrated that paracrine TGF-β1 signaling is essential for efferocytosis-induced LB formation (Figure 4*C*).

LBs Are Sites for Eicosanoid-Forming Enzymes and PGE_2 Synthesis in Macrophages During *T. cruzi* Infection

As shown in Figure 5*A*, *T. cruzi*–infected macrophages were positively immunostained for COX-2, and COX-2 expression was increased when infection was in the presence of apoptotic cells. The immunostaining appeared punctate throughout the cytoplasm, which suggests that COX-2 localizes within LBs, in addition to perinuclear membrane. ADRP was used as an LB marker for colocalization purposes. COX-2 localization within LBs was confirmed by immunofluorescence that showed colocalization of COX-2 and ADRP-labeled LBs in macrophages infected with *T. cruzi* in the presence of apoptotic cells (Figure 5*B*). There was no immunoreactivity when control nonimmune IgG was used (Figure 5*A* and *B*).

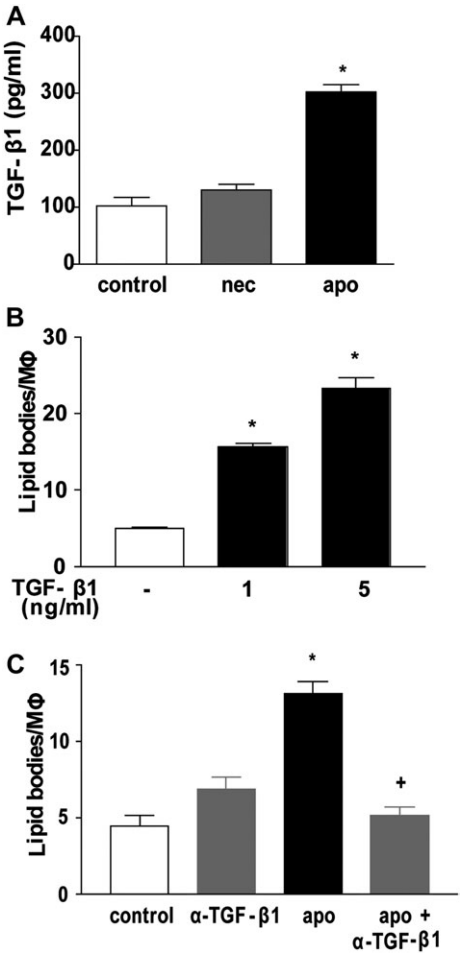

Figure 4. Transforming growth factor (TGF)-β is involved in the effects of apoptotic cell uptake on macrophage lipid body (LB) formation. *A*, TGF-β production by murine macrophages cocultured with necrotic or apoptotic cells after 24 hours. *B*, macrophage LB formation induced by TGF-β administration in vitro. *C*, LB formation induced by apoptotic cell is inhibited by pretreatment with an antibody that neutralizes TGF-β. Results are the mean ± the standard error of the mean from 3 independent pools of 3–5 animals each. Statistically significant differences ($P < .05$) between the control and the stimulated group are indicated by asterisks; + represents differences between the stimulated group and treated group.

Intracellular sites of newly formed PGE_2 were investigated by eicosacell, a newly developed strategy for direct in situ immunolocalization of eicosanoid synthesis [8, 35]. 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride was used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins, and the immobilized PGE_2 could thereafter be detected by immunofluorescence. Infected macrophages cocultured with apoptotic lymphocytes exhibited strong localized punctate or ring-shape staining for ADRP-labeled LBs and showed intense and punctate immunofluorescent staining for PGE_2 (Figure 5*C*). As shown in Figure 5*C*, intracellular sites of PGE_2 production colocalized with ADRP-labeled LBs. The specificity of PGE_2 staining was supported by the absence of immunostaining when an isotype control replaced the anti-PGE_2 (Figure 5*C*) or when there was enzymatic inhibition of PGE_2 production by indomethacin treatment (not shown).

Inhibition of LB Formation Modulates LB-Derived PGE_2 Synthesis and Parasite Replication

The role of macrophage LBs in modulating *T. cruzi* and/or apoptotic cell–induced PGE_2 synthesis and parasite replication was investigated. As shown in Figure 6*A*, both aspirin (5 μM) and NS-398 (1 μM) inhibited LB formation induced by *T. cruzi* infection in macrophages in the presence or absence of apoptotic cells at 24 hours. As expected, at the concentrations of nonsteroidal anti-inflammatory drugs (NSAIDs) used, PGE_2 generation induced by *T. cruzi* with or without apoptotic cells was also significantly inhibited (Figure 6*B*). The inhibition of LB formation and PGE_2 synthesis by aspirin and NS-398 also reversed the effects of apoptotic cells on enhanced parasite replication in macrophages (Figure 6*C*). To confirm the hypothesis that PGE_2 derived from LBs favors the intracellular replication of *T. cruzi* after uptake of apoptotic cells, macrophages were treated by an inhibitor of fatty acid synthase, C75 (5 μg/mL). Treatment with C75 repressed LB formation (Figure 7*A*) in infected macrophages incubated with or without apoptotic cells. Interestingly, in parallel to LB inhibition, C75 treatment inhibited the enhanced PGE_2 production (not shown) and reversed the increased parasite replication induced by apoptotic cells, which suggests a role for LBs in mechanisms that favor intracellular parasite survival and replication (Figure 7*B*). C75 was ineffective in inhibiting *T. cruzi* growth directly (not shown).

DISCUSSION

Intracellular pathogens have evolved complex strategies to escape the immune system by manipulating the host cellular machinery. Alterations in host cell lipid metabolism are often observed, and increased numbers of LBs in leukocytes and other cells are emerging as a common phenotype in bacterial, viral, fungal, and parasitic infections [5, 7, 36]. However, the understanding of the biogenic mechanisms and functional significance of LBs in host-pathogen interactions is still limited. Our study demonstrates that *T. cruzi* is able to modulate cell lipid metabolism and accumulation through highly regulated mechanisms. Moreover, we provide data to support a role for LBs in parasite pathogenesis.

The mechanism of LB formation and PGE_2 synthesis during parasite infection was investigated. Receptor-mediated signaling has been implicated in LB biogenesis associated with inflammation and infection [5, 37]. Protozoan parasites are sensed and engage TLR-dependent signaling involved in the expression of genes that regulate immunity and cell metabolism, and these pathogens can manipulate TLR-induced signaling cascades to favor their own survival [38]. *T. cruzi* infection–induced LB

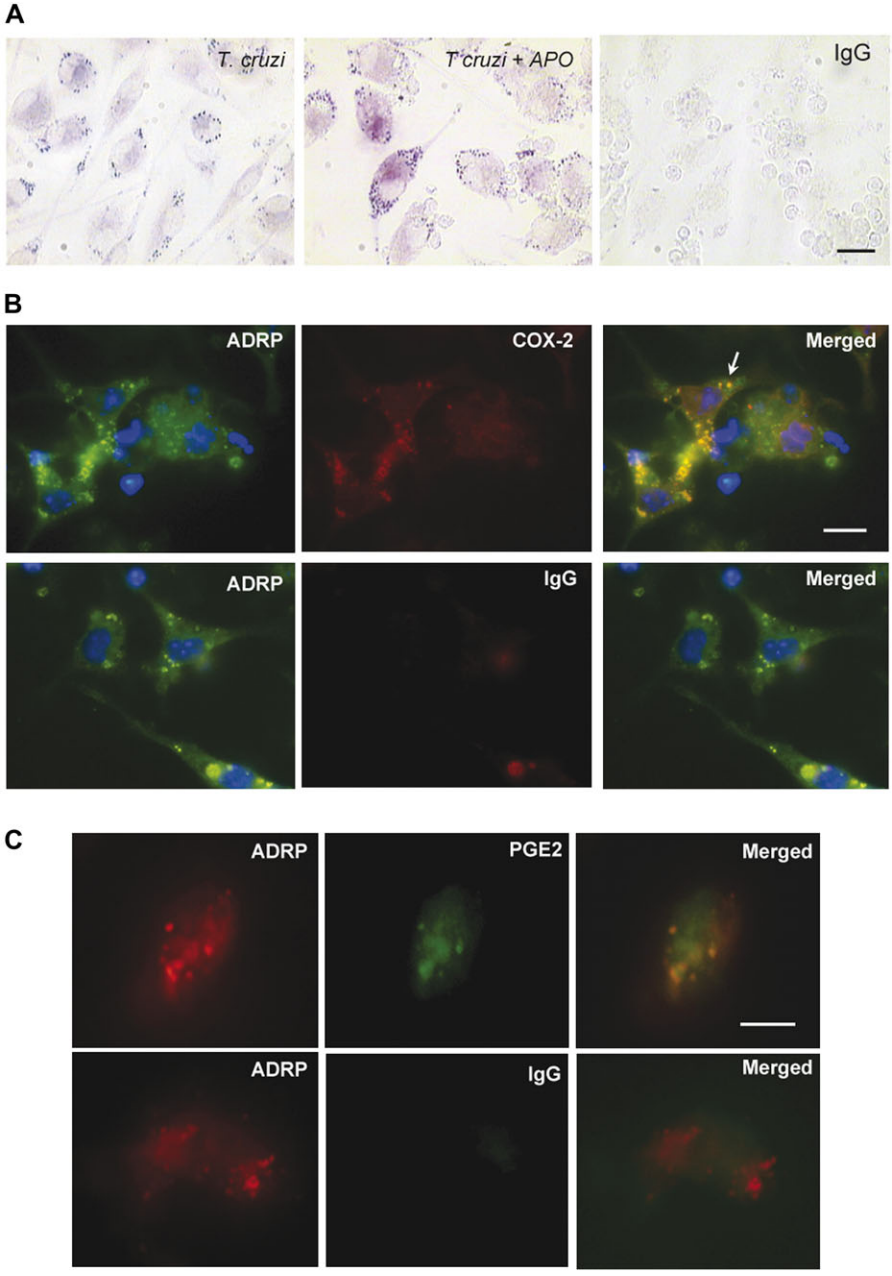

Figure 5. Lipid bodies (LBs) induced by *Trypanosoma cruzi* infection and apoptotic cell uptake are sites of cyclooxygenase-2 (COX-2) localization and prostaglandin E_2 (PGE_2) synthesis in macrophages. *A*, COX-2 localization in murine macrophages infected by *T. cruzi* (3:1) (*left panel*) and cocultured with apoptotic cells (*middle panel*) were visualized as purple punctate cytoplasmatic inclusions after glucose oxidase immunostaining; nonimmune immunoglobulin (Ig) G was used in the control micrograph (*right panel*). *B, T. cruzi*–infected macrophages cocultured with apoptotic cells were labeled for adipose differentiation-related protein (ADRP)–associated LB (*left panels*) and COX-2 localization (*upper, middle panel*). Merged image (*upper, right panel*) shows colocalization of COX-2 enzyme in ADRP-associated LB (*arrow*). Nonimmune IgG was used in the control micrograph (*lower, middle panel*). Nuclei of macrophages, internalized parasites, and apoptotic cells are stained with DAPI (4',6-diamidino-2-phenylindole; *blue*). *C, T. cruzi*–infected macrophages cocultured with apoptotic cells were labeled for ADRP-associated LB (*upper, left panel*) and for newly formed PGE_2 (*upper, middle panel*). Merged image (*upper, right panel*) shows colocalization of PGE_2 in ADRP-associated LB. Mouse IgG was used in the control micrograph (*lower, middle panel*). The scale bars are 10 μm.

formation was ablated in macrophages from *Tlr2*$^{-/-}$, but not *Tlr4*$^{-/-}$, mice, which demonstrated an essential role of TLR2 in LB formation during *T. cruzi* infection. Accordingly, LB formation in mycobacterial and *Histoplasma capsulatum* infections were also dependent on TLR2 recognition [7–9, 11, 14].

Host cell apoptosis, mostly lymphocyte apoptosis, is often observed in the course of parasitic infections and plays an important immune regulatory role in Chagas disease [39]. We demonstrated that *T. cruzi*–induced LB formation is highly enhanced in the presence of apoptotic cells. Apoptotic cells are

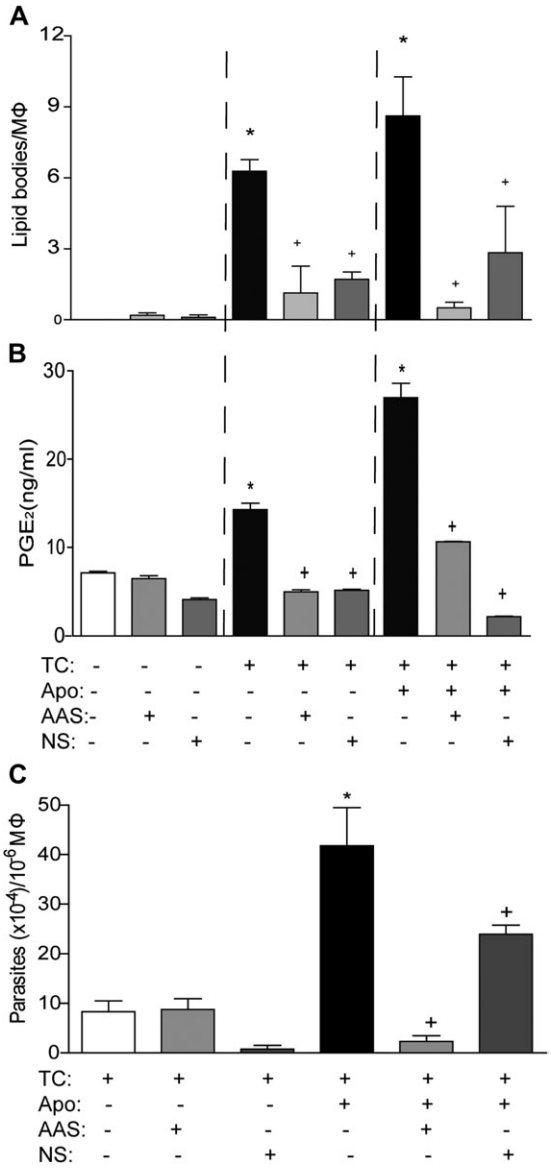

Figure 6. Nonsteroidal anti-inflammatory drugs, aspirin, and NS-398 inhibited lipid body formation, prostaglandin E_2 (PGE_2), and parasite replication. Lipid body (LB) enumeration (*A*) PGE_2 production (*B*) and parasite replication (*C*) in murine macrophages infected in vitro by *Trypanosoma cruzi* (multiplicity of infection, 3:1) for 24 hours. After infection, the cells were treated with 5 μM aspirin (ASA) or 1 μM NS-398 for 24 hours at 37°C. Vehicle alone (0.01% dimethyl sulfoxide) was used as a control. LB were enumerated after osmium staining, and PGE_2 was measured by enzyme immunoassay. Parasites were counted after 10 days of infection. Results are the mean ± the standard error of the mean from 3 independent pools of 3 to 5 animals each. Statistically significant differences ($P < .05$) between the control group and stimulated group are indicated by asterisks; + represents differences between the stimulated group and treated group.

known to induce an anti-inflammatory response mediated in part by integrins and the induction of active TGF-β in macrophages [31, 40]. Freire-de-Lima et al [31] demonstrated that stimulation with antibodies agonistic to $\alpha_v\beta_3$ induced PGE_2 synthesis in the absence of apoptotic cells. Interestingly, this phenomenon was accompanied by LB formation, enhanced COX-2 expression, and increased parasite replication. To confirm the involvement of integrins in LB formation, treatment with flavoridin, a desintegrin that blocks binding via $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [33, 34], remarkably inhibited LB formation and COX-2 expression induced by incubation of macrophages with apoptotic cells. These results demonstrate that signaling to induce LB formation, PGE_2 production, and COX-2 expression in macrophages during efferocytosis requires recognition via integrin receptors. Because $\alpha_5\beta_1$ receptors may also participate in efferocytosis [41] and the desintegrin used in this study inhibits both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [33, 34], we cannot rule out the participation of other integrins in addition to $\alpha_v\beta_3$ in LB biogenesis. It has been demonstrated that the interaction with apoptotic neutrophils leads macrophages to secrete TGF-β and also renders phagocytic cells permissive to *T. cruzi* infection [42, 43]. Earlier demonstrations have delineated a role of TGF-β in eicosanoid production, including PGE_2 release [31, 40]. Here we observed that apoptotic, but not necrotic, cells induced TGF-β production, and this mediator directly induced LB formation. Effects of efferocytosis on eicosanoid metabolism were shown to be mediated through TGF-β release [44]. Our results confirm this proposed model and show that the LBs are the site of eicosanoid-enzyme compartmentalization and increased PGE_2 production. The inhibition of released TGF-β by an anti-TGF-β1 neutralizing antibody reversed this mechanism by blocking LB formation. Although the involvement of other coreceptors cannot be ruled out, the present results indicate that uptake of apoptotic cells by macrophages through interaction with $\alpha_v\beta3$ triggers TGF-β1–dependent increase in LB formation and a subsequent increase in PGE_2 synthesis.

The hypothesis that the formation of new LBs elicited by *T. cruzi* infection and apoptotic cell uptake provides a distinct intracellular domain for regulated eicosanoid production is supported by multiple lines of evidence. First, in this study, we observed a significant correlation between LB formation and PGE_2 generation when the infected cells were cocultured with apoptotic cells. Second, immunolocalization of COX-2 demonstrated that COX-2 is localized at the *T. cruzi* and apoptotic cell–induced LBs. Moreover, through the direct assessment of intracellular sites for PGE_2 production, we were able to demonstrate that LBs are the main intracellular domain for PGE_2 synthesis. Additionally, these data reinforce previous findings [31, 44] and indicate that cyclooxygenase metabolites are crucial for the ability of apoptotic cells in macrophages to increase parasite growth in vitro.

We therefore evaluated the impact of modulation of the LB formation on PGE_2 production and parasite growth in macrophages exposed to apoptotic cells. First, we tested the effect of 2 NSAIDs, aspirin (dual COX-1 and COX-2 inhibitor) and NS-398 (COX-2 inhibitor) that, in addition to their ability to inhibit

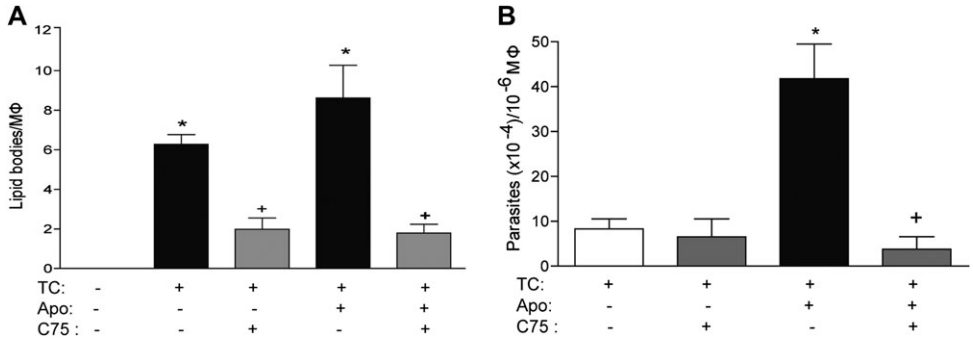

Figure 7. Fatty acid synthase inhibitor, C75, partially blocked the lipid body (LB) formation and parasite replication. Enumeration of LB *A*, and extracellular motile trypomastigotes *B*, from murine macrophages infected in vitro by *Trypanosoma cruzi* (multiplicity of infection, 3:1) for 24 hours. After infection, the cells were treated with C75 (5 μg/mL) for 24 hours at 37°C. Vehicle alone (0.01% dimethyl sulfoxide) was used as a control. LBs were enumerated after osmium staining. Parasites were counted after 10 days of infection. Results are the mean ± the standard error of the mean from 3 independent pools of 3–5 animals each. Statistically significant differences ($P < .05$) between the control group and stimulated group are indicated by asterisks; + represents differences between the stimulated group and treated group.

cyclooxygenase, also have COX-independent inhibitory effects on LB biogenesis [45, 46]. Interestingly, both drugs inhibited the LB formation in infected macrophages in the presence or absence of apoptotic cells, suppressed apoptotic cell effects on LBs-derived PGE_2 production, and reversed the effects of apoptotic cells on parasite replication. In different cell systems, LB biogenesis was shown to require new lipid synthesis in a mechanism tightly regulated by fatty acid synthase [47–49]. Accordingly, the fatty acid synthase inhibitor C75 significantly inhibited LB formation induced by *T. cruzi*, with or without the presence of apoptotic cells. Through a mechanism independent of inhibition of the COX-2 enzyme, LB inhibition by C75 leads to inhibition of PGE_2 production in cancer cells [47], mycobacteria-infected macrophages [10, 28], and *T. cruzi*–infected macrophages (not shown). Strikingly, we demonstrated that treatment with C75, in parallel to LB inhibition, reversed the parasite replication induced by apoptotic cells. This suggests a role for newly formed LBs in intracellular parasite survival. In support of the hypothesis that LBs are involved in intracellular parasite growth, pathogen-induced host LB formation has been associated with parasite survival advantages in hepatitis C virus [50], dengue [49], and mycobacterial infections [8, 10]. Although the precise mechanisms by which host-derived LBs favor the intracellular growth of *T. cruzi* deserve further study, our findings, together with evidence from the literature, indicate that LBs have immunomodulatory effects on parasitized cells through the production of PGE_2 and may also have roles as nutrient-rich source of lipids for parasite growth.

In conclusion, *T. cruzi* infection induces TLR2-dependent macrophage LB formation, which is amplified by the uptake of apoptotic cells in a mechanism dependent on integrins and TGF-β synthesis. Macrophage LBs formed during *T. cruzi* infection with apoptotic cell stimulation directly impact the capacity of macrophages to generate increased amounts of PGE_2. Indeed, LBs are the main intracellular sites for PGE_2 synthesis during intracellular pathogen infection, which may have impact on the ability of the cell to fight infection. Our findings support the concept that *T. cruzi* induces and exploits host-derived LBs to prolong and sustain its own survival. Furthermore, relevant to the need for new treatment strategies against *T. cruzi*, we report that pharmacologic intervention of LB biogenesis inhibits *T. cruzi* survival and replication in macrophages.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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References

1. Coura JR, Dias JC. Epidemiology, control and surveillance of Chagas disease: 100 years after its discovery. Mem Inst Oswaldo Cruz **2009**; 104(Suppl 1):31–40.
2. Chagas C. Nova tripanozomiase humana. Estudos sobre a morfolojía e o ciclo evolutivo de Schizotrypanum cruzi n.gen., n.sp., ajente etiolójico de nova entidade morbida do homen. Mem Inst Oswaldo Cruz **1909**; 1:159–218.
3. Andrews NW, Schenkman S, Ley V, Whitlow MB, Robbins ES, Nussenzweig V. *Trypanosoma cruzi*: mechanisms of cell-invasion and intracellular survival. Mem Inst Oswaldo Cruz **1988**; 83(Suppl 1): 452–5.
4. Nagajyothi F, Desruisseaux MS, Weiss LM, et al. Chagas disease, adipose tissue and the metabolic syndrome. Mem Inst Oswaldo Cruz **2009**; 104(Suppl 1):219–25.
5. Bozza PT, D'Avila H, Almeida PE, et al. Lipid droplets in host-pathogen interactions. Clin Lipidol **2009**; 4:791–807.
6. Farese RV Jr, Walther TC. Lipid droplets finally get a little R-E-S-P-E-C-T. Cell **2009**; 139:855–60.

7. D'Avila H, Maya-Monteiro CM, Bozza PT. Lipid bodies in innate immune response to bacterial and parasite infections. Int Immunopharmacol **2008**; 8:1308–15.
8. D'Avila H, Melo RCN, Parreira GG, Werneck-Barroso E, Castro-Faria-Neto HC, Bozza PT. *Mycobacterium bovis* bacillus Calmette-Guerin induces TLR2-mediated formation of lipid bodies: intracellular domains for eicosanoid synthesis in vivo. J Immunol **2006**; 176: 3087–97.
9. D'Avila H, Almeida PE, Roque NR, Castro-Faria-Neto HC, Bozza PT. Toll-like receptor-2-mediated C-C chemokine receptor 3 and eotaxin-driven eosinophil influx induced by *Mycobacterium bovis* BCG pleurisy. Infect Immun **2007**; 75:1507–11.
10. Almeida PE, Silva AR, Maya-Monteiro CM, et al. *Mycobacterium bovis* bacillus Calmette-Guerin infection induces TLR2-dependent peroxisome proliferator-activated receptor {gamma} expression and activation: functions in inflammation, lipid metabolism, and pathogenesis. J Immunol **2009**; 183:1337–45.
11. Mattos KA, D'Avila H, Rodrigues LS, et al. Lipid droplet formation in leprosy: Toll-like receptor-regulated organelles involved in eicosanoid formation and *Mycobacterium leprae* pathogenesis. J Leukoc Biol **2010**; 87:371–84.
12. Pacheco P, Bozza FA, Gomes RN, et al. Lipopolysaccharide-induced leukocyte lipid body formation in vivo: innate immunity elicited intracellular loci involved in eicosanoid metabolism. J Immunol **2002**; 169:6498–506.
13. Pacheco P, Vieira-de-Abreu A, Gomes RN, et al. Monocyte chemoattractant protein-1/CC chemokine ligand 2 controls microtubule-driven biogenesis and leukotriene B4-synthesizing function of macrophage lipid bodies elicited by innate immune response. J Immunol **2007**; 179: 8500–8.
14. Sorgi CA, Secatto A, Fontanari C, et al. *Histoplasma capsulatum* cell wall β-glucan induces lipid body formation through CD18, TLR2, and dectin-1 receptors: correlation with leukotriene B4 generation and role in HIV-1 infection. J Immunol **2009**; 182:4025–35.
15. Melo RCN, D'Avila H, Fabrino DL, Almeida PE, Bozza PT. Macrophage lipid body induction by Chagas disease in vivo: putative intracellular domains for eicosanoid formation during infection. Tissue Cell **2003**; 35:59–67.
16. Melo RCN, Fabrino DL, Dias FF, Parreira GG. Lipid bodies: structural markers of inflammatory macrophages in innate immunity. Inflamm Res **2006**; 55:342–8.
17. Pinheiro RO, Nunes MP, Pinheiro CS, et al. Induction of autophagy correlates with increased parasite load of *Leishmania amazonensis* in BALB/c but not C57BL/6 macrophages. Microbes Infect **2009**; 11: 181–90.
18. Charron AJ, Sibley LD. Host cells: mobilizable lipid resources for the intracellular parasite *Toxoplasma gondii*. J Cell Sci **2002**; 115: 3049–59.
19. Nishikawa Y, Quittnat F, Stedman TT, et al. Host cell lipids control cholesteryl ester synthesis and storage in intracellular *Toxoplasma*. Cell Microbiol **2005**; 7:849–67.
20. Jackson KE, Klonis N, Ferguson DJ, Adisa A, Dogovski C, Tilley L. Food vacuole-associated lipid bodies and heterogeneous lipid environments in the malaria parasite, *Plasmodium falciparum*. Mol Microbiol **2004**; 54:109–22.
21. Rodriguez-Acosta A, Finol HJ, Pulido-Mendez M, et al. Liver ultrastructural pathology in mice infected with *Plasmodium berghei*. J Submicrosc Cytol Pathol **1998**; 30:299–307.
22. Combs TP, Nagajyothi, Mukherjee S, et al. The adipocyte as an important target cell for *Trypanosoma cruzi* infection. J Biol Chem **2005**; 280:24085–94.
23. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity **1999**; 11:443–51.
24. Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S. In vitro differentiation of *Trypanosoma cruzi* under chemically defined conditions. Mol Biochem Parasitol **1985**; 16:315–27.
25. Liu LX, Buhlmann JE, Weller PF. Release of prostaglandin E2 by microfilariae of *Wuchereria bancrofti* and *Brugia malayi*. Am J Trop Med Hyg **1992**; 46:520–3.
26. Bandeira-Melo C, Phoofolo M, Weller PF. Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are the sites of chemokine-enhanced leukotriene C_4 production in eosinophils and basophils. J Biol Chem **2001**; 276:22779–87.
27. Melo RCN. Acute heart inflammation: ultrastructural and functional aspects of macrophages elicited by *Trypanosoma cruzi* infection. J Cell Mol Med **2009**; 13:279–94.
28. D'Avila H, Roque NR, Cardoso RM, Castro-Faria-Neto HC, Melo RCN, Bozza PT. Neutrophils recruited to the site of *Mycobacterium bovis* BCG infection undergo apoptosis and modulate lipid body biogenesis and prostaglandin E production by macrophages. Cell Microbiol **2008**; 10:2589–604.
29. DosReis GA, Freire-de-Lima CG, Nunes MP, Lopes MF. The importance of aberrant T-cell responses in Chagas disease. Trends Parasitol **2005**; 21:237–43.
30. Zhang J, Andrade ZA, Yu ZX, et al. Apoptosis in a canine model of acute Chagasic myocarditis. J Mol Cell Cardiol **1999**; 31:581–96.
31. Freire-de-Lima CG, Nascimento DO, Soares MB, et al. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature **2000**; 403:199–203.
32. Erwig LP, Henson PM. Clearance of apoptotic cells by phagocytes. Cell Death Differ **2008**; 15:243–50.
33. Pfaff M, Tangemann K, Muller B, et al. Selective recognition of cyclic RGD peptides of NMR defined conformation by alpha IIb beta 3, alpha V beta 3, and alpha 5 beta 1 integrins. J Biol Chem **1994**; 269: 20233–8.
34. Lu X, Lu D, Scully MF, Kakkar VV. Snake venom metalloproteinase containing a disintegrin-like domain, its structure-activity relationships at interacting with integrins. Curr Med Chem Cardiovasc Hematol Agents **2005**; 3:249–60.
35. Bandeira Melo C, Weller PF, Bozza PT. EicosaCell—an immunofluorescent-based assay to localize newly synthesized eicosanoid lipid mediators at intracellular sites. Methods Mol Biol **2011**; 689:163–81.
36. van der Meer-Janssen YP, van Galen J, Batenburg JJ, Helms JB. Lipids in host-pathogen interactions: pathogens exploit the complexity of the host cell lipidome. Prog Lipid Res **2010**; 49:1–26.
37. Bozza PT, Magalhaes KG, Weller PF. Leukocyte lipid bodies: biogenesis and functions in inflammation. Biochim Biophys Acta **2009**; 1791:540–51.
38. Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol **2006**; 6:895–906.
39. DosReis GA, Lopes MF. The importance of apoptosis for immune regulation in Chagas disease. Mem Inst Oswaldo Cruz **2009**; 104(Suppl 1): 259–62.
40. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest **1998**; 101:890–8.
41. D'Mello V, Birge RB. Apoptosis: conserved roles for integrins in clearance. Curr Biol **2010**; 20:R324–7.
42. Silva JS, Twardzik DR, Reed SG. Regulation of *Trypanosoma cruzi* infections in vitro and in vivo by transforming growth factor beta (TGF-beta). J Exp Med **1991**; 174:539–45.
43. Ming M, Ewen ME, Pereira ME. Trypanosome invasion of mammalian cells requires activation of the TGF beta signaling pathway. Cell **1995**; 82:287–96.
44. Freire-de-Lima CG, Xiao YQ, Gardai SJ, Bratton DL, Schiemann WP, Henson PM. Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. J Biol Chem **2006**; 281:38376–84.
45. Bozza PT, Pacheco P, Yu W, Weller PF. NS-398: cyclooxygenase-2 independent inhibition of leukocyte priming for lipid body formation

and enhanced leukotriene generation. Prostaglandins Leukot Essent Fatty Acids **2002**; 67:237–44.
46. Bozza PT, Payne JL, Morham SG, Langenbach R, Smithies O, Weller PF. Leukocyte lipid body formation and eicosanoid generation: cyclooxygenase-independent inhibition by aspirin. Proc Natl Acad Sci U S A **1996**; 93:11091–6.
47. Accioly MT, Pacheco P, Maya-Monteiro CM, et al. Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E_2 synthesis in colon cancer cells. Cancer Res **2008**; 68:1732–40.
48. Schmid B, Rippmann JF, Tadayyon M, Hamilton BS. Inhibition of fatty acid synthase prevents preadipocyte differentiation. Biochem Biophys Res Commun **2005**; 328:1073–82.
49. Samsa MM, Mondotte JA, Iglesias NG, et al. Dengue virus capsid protein usurps lipid droplets for viral particle formation. PLoS Pathog **2009**; 5:e1000632.
50. Boulant S, Targett-Adams P, McLauchlan J. Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. J Gen Virol **2007**; 88:2204–13.