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Mice with Genetic Deletion of Group VIA Phospholipase $A_2\beta$ Exhibit Impaired Macrophage Function and Increased Parasite Load in *Trypanosoma cruzi*-Induced Myocarditis

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***Trypanosoma cruzi* infection, which is the etiological agent of Chagas disease, is associated with intense inflammation during the acute and chronic phases. The pathological progression of Chagas disease is influenced by the infiltration and transmigration of inflammatory cells across the endothelium to infected tissues, which are carefully regulated processes involving several molecular mediators, including adhesion molecules and platelet-activating factor (PAF). We have shown that PAF production is dependent upon calcium-independent group VIA phospholipase $A_2\beta$ ($iPLA_2\beta$) following infection of human coronary artery endothelial cells (HCAECs) with *T. cruzi*, suggesting that the absence of $iPLA_2\beta$ may decrease the recruitment of inflammatory cells to the heart to manage parasite accumulation. Cardiac endothelial cells isolated from $iPLA_2\beta$-knockout ($iPLA_2\beta$-KO) mice infected with *T. cruzi* demonstrated decreased PAF production compared to that by cells isolated from wild-type (WT) mice but demonstrated increases in adhesion molecule expression similar to those seen in WT mice. Myocardial inflammation in $iPLA_2\beta$-KO mice infected with *T. cruzi* was similar in severity to that in WT mice, but the $iPLA_2\beta$-KO mouse myocardium contained more parasite pseudocysts. Upon activation, macrophages from $iPLA_2\beta$-KO mice produced significantly less nitric oxide (NO) and caused less *T. cruzi* inhibition than macrophages from wild-type mice. Thus, the absence of $iPLA_2\beta$ activity does not influence myocardial inflammation, but $iPLA_2\beta$ is essential for *T. cruzi* clearance.**

Trypanosoma cruzi is a protozoan parasite that results in significant cardiac pathology and is the etiological agent of Chagas disease. It is estimated that over 10 million people worldwide are currently infected with *T. cruzi*, and of these, about 300,000 reside in the United States (1). Chagas disease is endemic to South and Central America, where people risk acquiring the parasite from the triatomine insect vector. Chagas disease progresses from an acute stage, which may or may not be symptomatic, to a chronic stage, in which 20 to 30% of infected individuals exhibit cardiac involvement that may lead to heart failure, arrhythmias, and death (2). The long asymptomatic period separating the two stages of the disease is known as the indeterminate phase and may last for several decades.

During the acute stage of *T. cruzi* infection, the parasites infect the myocardium, leading to an intense inflammatory response. Several proinflammatory cytokines and signaling pathways are activated to facilitate the transmigration of inflammatory cells in an attempt to control parasite invasion. Activation of the endothelium and upregulation of endothelial cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), following *T. cruzi* infection are critical for these processes (3). Our group has previously demonstrated increased expression of platelet-activating factor (PAF), in addition to the upregulation of adhesion molecules, in human coronary artery endothelial cells (HCAECs) acutely infected with *T. cruzi* (4). The role of PAF in the recruitment, transmigration, and activation of inflammatory cells is well established (5–8).

PAF is an acetylated alkyl ether glycerophospholipid that can elicit biological effects at concentrations as low as 10^{-12} M (9). Mice treated with a PAF receptor antagonist demonstrate earlier mortality and increased parasitemia, suggesting that PAF is necessary for resistance to Chagas disease (10). Further, PAF-deficient mice have increased parasitemia, increased tissue parasitism, a more intense inflammatory response in the heart, and increased mortality following infection with *T. cruzi* (11). Thus, PAF production may be a critical host defense response to *T. cruzi* infection that serves to retard the progression of Chagas disease. Earlier studies have suggested that PAF can induce nitric oxide (NO) production in macrophages infected with *T. cruzi* (10).

Although studies have described the role of PAF in *T. cruzi* infection, much less information concerning the mechanism underlying PAF accumulation is available. We recently demonstrated that PAF production requires calcium-independent group VIA phospholipase $A_2\beta$ ($iPLA_2\beta$) and is greatly blunted in $iPLA_2\beta$-knockout ($iPLA_2\beta$-KO) mice (4). Although we have focused on $iPLA_2\beta$-mediated PAF production in the cardiovascular system, the enzyme is also involved in modulating arachidonic acid release from vascular cells and vasomotor tone (12). We have shown that the absence of endothelial cell $iPLA_2\beta$ activity is associated with a decrease in prostacyclin release. The predominant $iPLA_2$ isoform in the myocardium is the calcium-independent

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group VIB PLA_2 ($iPLA_2\gamma$), which is responsible for the production of arachidonic acid-derived eicosanoids. Although few studies to date have addressed the role of phospholipase A_2 (PLA_2) in myocarditis, several inflammatory metabolites produced following PLA_2-catalyzed hydrolysis of membrane phospholipids have been implicated in Chagas disease (10, 11, 13). Finally, previous studies have suggested that $iPLA_2\beta$ may be required for inducible nitric oxide synthase (iNOS) upregulation, increased NADP oxidase 4 (Nox4) expression, and chemotaxis in macrophages (14, 15). Here, we compared wild-type (WT) and $iPLA_2\beta$-KO mice to determine whether $iPLA_2\beta$ deficiency influences cardiac inflammation and parasite accumulation following *T. cruzi* infection.

MATERIALS AND METHODS

Parasitology. Tissue culture trypomastigotes (TCTs) from the Brazil strain of *T. cruzi* were propagated in NIH 3T3 mouse embryonic fibroblasts grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% neonatal calf serum. NIH 3T3 cells were infected with *T. cruzi* when 60% confluence was reached. Infected cells ruptured following parasite multiplication, releasing an abundant number of parasites. The supernatant containing the parasites was collected, and parasite numbers were determined using a Neubauer hemocytometer.

Mice and infections. Animal protocols were in strict accordance with the National Institutes of Health guidelines for the humane treatment of animals and were reviewed and approved by the Animal Care and Use Committee of Saint Louis University. C57BL/6 WT or $iPLA_2\beta$-KO mice were used for *in vitro* and *in vivo* studies. Mice were infected subcutaneously with 5,000 Brazil strain blood-form trypomastigotes (BFTs) and sacrificed at different time points postinfection. The hearts were collected in paraformaldehyde fixative or frozen in liquid nitrogen before processing for histological analysis. The hearts were trimmed, processed, and embedded in paraffin. Five-micrometer tissue sections were cut, and sections were stained with hematoxylin and eosin (H&E). The investigators were blind to the identity of the samples, as numerical identifiers specific to each individual mouse were used, and the samples were evaluated microscopically using a 4-point system as described previously (16, 17). The parasite pseudocysts in 20 separate bright-field regions of the ventricle were counted.

Murine endothelial cell isolation. Endothelial cells were isolated from mouse heart by collagenase digestion. The diced heart muscle was incubated in 2 mg/ml collagenase for 1 h at 37°C, and the digested tissue was passed through a cell strainer. Cells were incubated with murine immunoglobulins to block Fc receptors and then incubated with anti-mouse platelet endothelial cell adhesion molecule 1 (PECAM-1) coupled to magnetic beads. The cells obtained were cultured until they reached confluence and sorted again using ICAM-2 antibodies coupled with magnetic beads. The eluted cells were washed, resuspended in cell culture medium, and plated. Nonadherent cells were removed on the next day, and cells were grown to confluence and passaged at a 1-to-3 dilution.

PAF assay. Murine cardiac endothelial cells grown in 12-well culture dishes were washed twice with Hanks' balanced salt solution containing 135 mM NaCl, 0.8 mM $MgSO_4$, 10 mM HEPES (pH 7.4), 1.2 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, and 6.6 mM glucose and incubated with 50 μCi [^{3}H]acetic acid for 20 min. After the selected time interval for incubation with the appropriate agents, lipids were extracted from the cells by the method of Bligh and Dyer. The chloroform layer was concentrated by evaporation under N_2, applied to a silica gel 60 thin-layer chromatography plate, and developed in chloroform-methanol-acetic acid-water (50:25:8:4, vol/vol). The region corresponding to PAF was scraped, and the radioactivity was quantified using liquid scintillation spectrometry. The loss of PAF during extraction and chromatography was corrected for by adding a known amount of [^{14}C]PAF as an internal standard. [^{14}C]PAF was synthesized by acetylating the *sn*-2 position of lyso-PAF with [^{14}C]acetic anhydride using 0.33 M dimethylaminopyridine as a catalyst. The synthesized [^{14}C]PAF was purified by high-pressure liquid chromatography.

Endothelial cell surface expression of adhesion molecules. Murine cardiac endothelial cells were grown to confluence in 16-mm culture dishes and were incubated with *T. cruzi* (multiplicity of infection [MOI], 0.2) for up to 96 h. At the end of the incubation, the cells were fixed with 1% paraformaldehyde and incubated overnight at 4°C. The cells were then washed three times with phosphate-buffered saline (PBS) and then blocked with Tris-buffered saline–Tween 20 supplemented with 0.8% (wt/vol) bovine serum albumin and 0.5% (wt/vol) fish gelatin for 1 h at 24°C. Appropriate primary antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) was used before treatment with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin secondary antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, each well was incubated in the dark with a 3,3′,5,5′-tetramethylbenzidine liquid substrate system, and color development was measured at 450 nm.

Isolation of murine BMDMs. Bone marrow-derived macrophages (BMDMs) were isolated from the femurs of WT and $iPLA_2\beta$-KO mice. Briefly, mice were sacrificed, and the femurs were removed and cleansed of tissue. The marrows were flushed from the femurs using DMEM, disrupted by passage through a 23-gauge needle, and collected by centrifugation. The pellets were resuspended in ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.10 mM EDTA), and incubated for 10 min on ice to lyse the red blood cells. The cells were centrifuged and resuspended in bone marrow conditioned medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin devoid of macrophage colony-stimulating factor (M-CSF). Following 2 to 3 h of incubation at 37°C with 5% CO_2, nonadherent cells were decanted and resuspended in fully supplemented bone marrow conditioned medium (with M-CSF). Cells were plated in 100-mm culture dishes for 5 to 7 days, following which they were used for experiments.

Nitrite release from murine macrophages. The nitrite released into the culture supernatants obtained from RAW 264.7 cells or murine bone marrow-derived macrophages was measured. Cells were grown to confluence and infected with *T. cruzi* (MOI, 0.2) with or without interferon gamma (IFN-γ; 100 units/ml) for 24 h. The nitrite concentration was measured by mixing 50 μl of the culture supernatant with the Griess reagent system. The absorbance at 550 nm of each sample was measured, and the concentration of nitrite was determined with reference to a nitrite standard curve with nitrite concentrations ranging from 0 to 100 μM.

***T. cruzi* inhibition assay.** BMDMs were plated in 8-well tissue culture slide chambers (Nunc Lab-Tek; Thermo Scientific, Waltham, MA) at 1×10^5 cells/well in DMEM with 10% FBS. After overnight culture at 37°C, nonadherent cells were washed away and macrophages were infected with *T. cruzi* culture-derived metacyclic trypomastigotes (CMTs) at an MOI of 5 (5×10^5 parasites/well) for 3 h. The generation of CMTs was described previously (18). Extracellular parasites were then washed away, and fresh medium or medium containing purified IFN-γ (100 U/ml; Sigma Chemical Co., St. Louis, MO) was added. Two days later, the slide chambers were removed from the slides, and the slides were washed, dried, fixed, and stained with a modified Wright Giemsa stain (Diff-Quik; IMEB, Inc., San Marcos, CA). The number of infected macrophages was determined microscopically. Percent inhibition was calculated by use of the following: $\{1 - [(\text{number of infected cells per 200 cells treated with IFN-}\gamma)/(\text{number of infected cells per 200 cells treated with medium alone})]\} \times 100$.

Statistical analysis. Comparison of the values for statistically significant differences was performed by Student's *t* test or one-way analysis of variance with *post hoc* analysis, performed using Dunnett's test. All results are expressed as the mean + standard error of the mean (SEM). Statistical significance was considered to be a *P* value of <0.05.

RESULTS

PAF production and adhesion molecule expression in cardiac endothelial cells. Our previous *in vitro* studies indicated that $iPLA_2\beta$ is critical for recruiting inflammatory cells to the endothe-

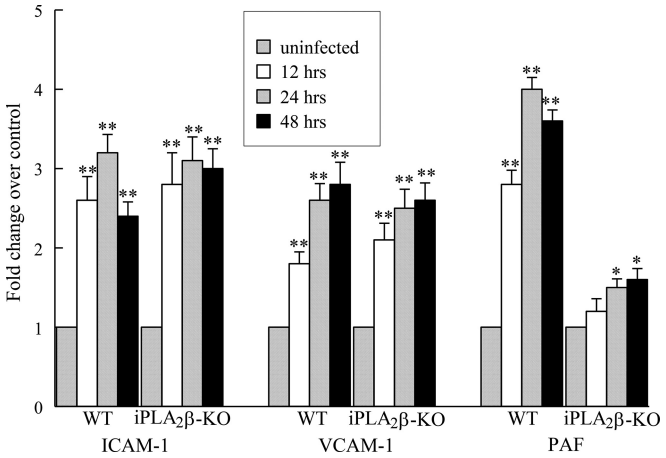

FIG 1 Expression of ICAM-1 and VCAM-1 and PAF production in endothelial cells isolated from the hearts of WT and iPLA$_2$β-KO mice infected with *T. cruzi* (MOI, 0.2) for up to 48 h. Values are normalized to those observed in the absence of infection. The values shown are means + SEMs for 4 separate cell cultures. *, $P < 0.05$ compared to untreated controls; **, $P < 0.01$ compared to untreated controls.

lium via the production of PAF (4, 6). Thus, we measured PAF production and the expression of adhesion molecules in cardiac endothelial cells isolated from WT and iPLA$_2$β-KO mice (Fig. 1). Cardiac endothelial cells isolated from WT mice demonstrated a significant increase in cell surface expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) when incubated with *T. cruzi* for up to 48 h (Fig. 1). The increase in adhesion molecules was accompanied by a significant increase in PAF accumulation in *T. cruzi*-infected WT endothelial cells (Fig. 1). When endothelial cells isolated from iPLA$_2$β-KO mice were incubated with *T. cruzi*, a significant increase in cell surface expression of ICAM-1 and VCAM-1 similar to that seen with cells isolated from WT mice was observed (Fig. 1). However, PAF production in *T. cruzi*-infected endothelial cells isolated from iPLA$_2$β-KO mouse heart was significantly reduced compared to that in endothelial cells isolated from WT mouse heart (Fig. 1). Thus, the absence of iPLA$_2$β is associated with impaired PAF production but does not affect the cell surface expression of adhesion molecules in response to *T. cruzi* infection.

Studies in iPLA$_2$β-KO mice. WT and iPLA$_2$β-KO mice were infected with the Brazil strain of *T. cruzi* (5,000 parasites), and cardiac inflammation was measured by histopathological evaluation, as described in Materials and Methods, and assigned a

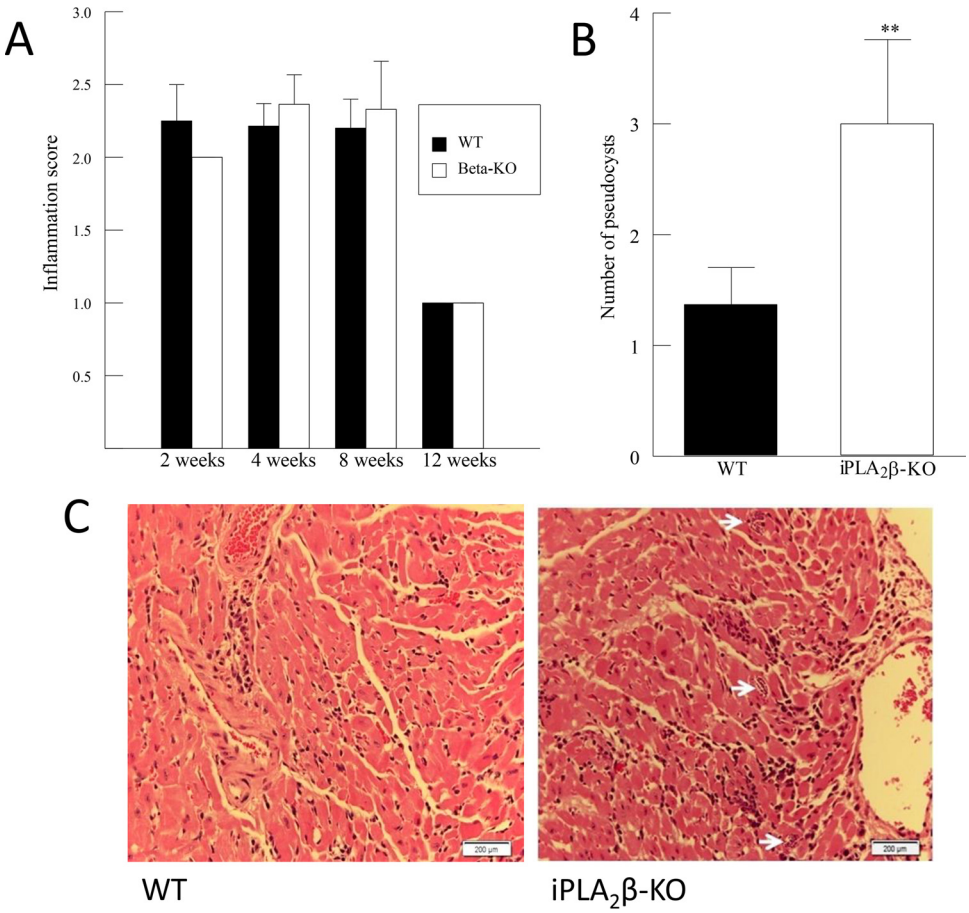

FIG 2 (A) Myocardial inflammation score for WT and iPLA$_2$β-KO mice following *T. cruzi* infection for up to 12 weeks. (B) Number of parasites in the myocardium per field for WT and iPLA$_2$β-KO mice following *T. cruzi* infection for 4 weeks. **, $P < 0.01$ compared to WT. The results in panels A and B are means + SEMs for at least 6 separate samples. (C) Hematoxylin-and-eosin-stained sections of hearts from WT and iPLA$_2$β-KO mice following 4 weeks of *T. cruzi* infection. Arrows, parasite pseudocysts in the myocardium.

numerical value on the basis of the size and severity of the inflammatory foci. Cardiac inflammation was observed in WT and iPLA$_2$β-KO mice at 2, 4, and 8 weeks postinfection but resolved by week 12 (Fig. 2A). No difference in the inflammation score between WT and iPLA$_2$β-KO mice was observed at any time point at which the score was determined following infection with *T. cruzi* (Fig. 2A), but the number of parasite pseudocysts was increased in the hearts of iPLA$_2$β-KO mice compared to the hearts of WT mice at 4 weeks postinfection (Fig. 2B). These data suggest that iPLA$_2$β deficiency does not significantly affect the transmigration of inflammatory cells following *T. cruzi* infection but that parasite clearance is impaired in iPLA$_2$β-deficient mice and that iPLA$_2$β could play a protective role in acute infection.

Nitrite production by RAW 264.7 macrophages. Macrophages play a critical role in clearing *T. cruzi* via the release of nitric oxide (NO). The myocardium of iPLA$_2$β-KO mice had an inflammation score comparable to that of the myocardium of WT mice, but the myocardium of iPLA$_2$β-KO mice had an increased number of pseudocysts, which suggests that macrophages from iPLA$_2$β-KO mice may be deficient in their parasite clearance ability.

To examine the role of iPLA$_2$β in macrophage responses to *T. cruzi* infection, RAW 264.7 cells were infected (MOI, 0.2; 48 h) in the absence or presence of the (*R*)- or (*S*)-enantiomer of the iPLA$_2$ inhibitor bromoenol lactone (BEL; 0.5 μM). NO release was measured by colorimetric determination of the amount of nitrite, a stable breakdown product of NO, produced. Infection with *T. cruzi* resulted in a significant increase in NO release (Fig. 3), and this was significantly augmented by coincubation with interferon gamma (IFN-γ), a known activator of the macrophage iNOS pathway (Fig. 3). Pretreatment of RAW 264.7 cells with the calcium-independent group VIB PLA$_2$ (iPLA$_2$γ) inhibitor (*R*)-BEL did not significantly affect NO release following *T. cruzi* infection with or without IFN-γ (Fig. 3), but pretreatment with the iPLA$_2$β inhibitor (*S*)-BEL significantly reduced NO release in response to *T. cruzi* infection in the presence or absence of IFN-γ (Fig. 3).

Nitrite production and parasite intracellular inhibition by mouse BMDMs. Bone marrow-derived macrophages (BMDMs) were isolated from WT and iPLA$_2$β-KO mice, and their NO production was determined by measurement of the amount of nitrite after incubation with *T. cruzi* with or without IFN-γ. As with RAW 264.7 cells (Fig. 4), *T. cruzi* infection stimulated NO production by WT mouse BMDMs, and this was amplified by coincubation with IFN-γ (Fig. 4A). Pretreatment with (*S*)-BEL but not (*R*)-BEL significantly inhibited the WT mouse BMDM NO production stimulated by *T. cruzi* infection with or without IFN-γ (Fig. 4A). BMDMs isolated from iPLA$_2$β-KO mice released negligible amounts of NO compared to the amount released by WT mouse macrophages (Fig. 4B). These data indicate that iPLA$_2$β is important for NO production by macrophages, which is critical for parasite clearance in the acute stage of *T. cruzi* infection. Finally, inhibition of intracellular replication of *T. cruzi* was significantly greater in BMDMs derived from WT mice than BMDMs derived from iPLA$_2$β-KO mice (Fig. 4C). Thus, we conclude that iPLA$_2$β deficiency impairs macrophage NO production in acute *T. cruzi* infection and that this results in an increased parasite content in the hearts of infected mice.

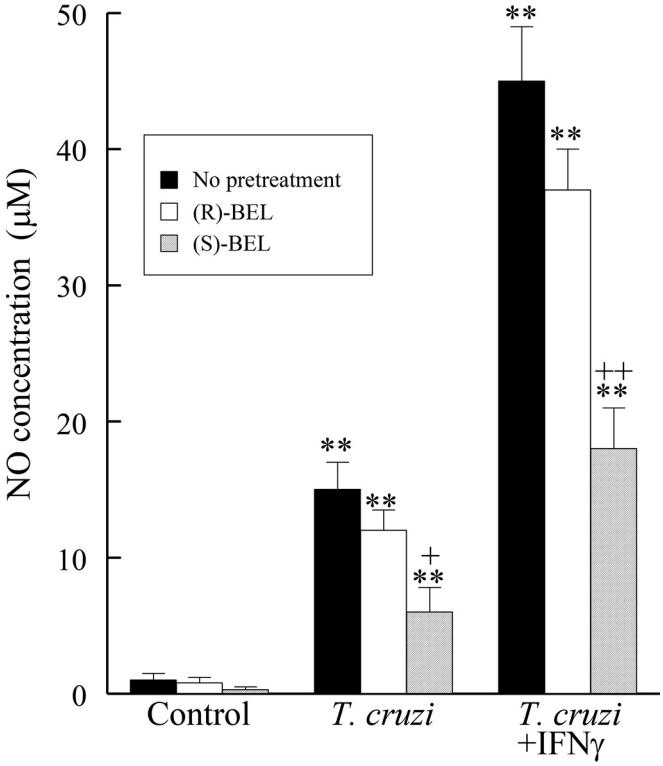

FIG 3 NO release from cells of the murine macrophage cell line RAW 264.7 following infection with *T. cruzi* (MOI, 0.2) with or without IFN-γ (100 units) for 24 h. **, $P < 0.01$ compared to untreated cells; +, $P < 0.05$ compared to treated or infected cells in the presence or absence of BEL (0.5 μM); ++, $P < 0.01$ compared to treated or infected cells in the presence or absence of BEL (0.5 μM). The values shown are means + SEMs for 4 separate cell cultures.

DISCUSSION

A characteristic pathological feature in Chagas disease is cardiac inflammation during both the acute and chronic stages. Acute parasitemia elicits the transmigration of inflammatory cells across the endothelial cell barrier to the myocardium, and this process involves the increased expression of adhesion molecules and proinflammatory cytokines when *T. cruzi* infects endothelial cells (19, 20). We and others have demonstrated previously that infection of endothelial cells with *T. cruzi* results in significant PAF production and the upregulation of cell surface adhesion molecules that can directly contribute to inflammatory cell recruitment to infected sites (4, 19, 20). We have also recently shown that endothelial cell PAF production following *T. cruzi* infection requires iPLA$_2$β (4), which suggested that prolonged endothelial cell activation in chronic Chagas disease may be associated with iPLA$_2$β activation and PAF production, which facilitates the recruitment and transmigration of inflammatory cells to sites of infection. However, the absence of iPLA$_2$β did not result in a significant decrease in inflammatory cells in the myocardium, despite the significant decrease in PAF production in *T. cruzi*-infected endothelial cells from the iPLA$_2$β-KO mouse heart (Fig. 1). In this study, we observed a significant increase in cell surface expression of adhesion molecules in response to *T. cruzi* infection that was not significantly affected by the absence of iPLA$_2$β. Since we did not observe a difference in inflammatory cells in the hearts of WT or iPLA$_2$β-KO mice infected with *T. cruzi*, our data suggest that the

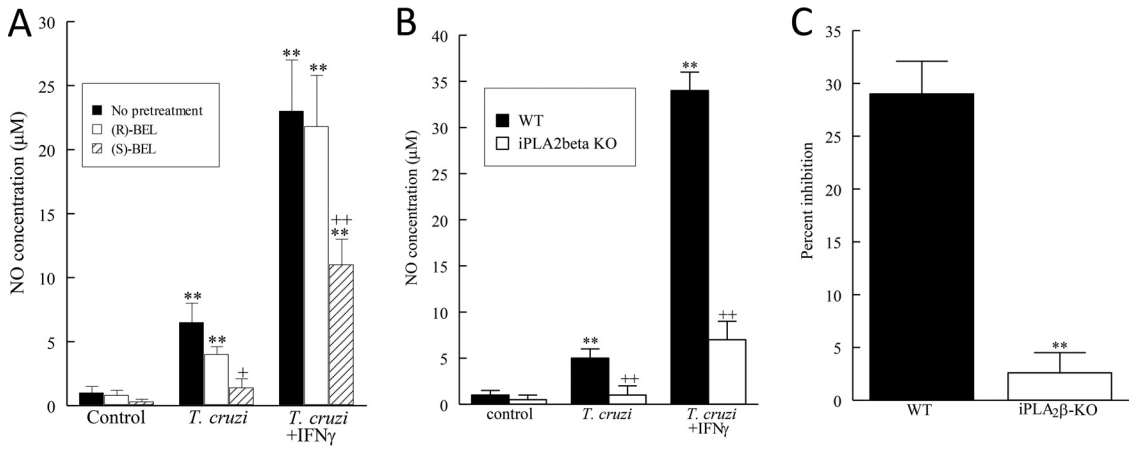

FIG 4 NO release from murine BMDMs and inhibition of *T. cruzi* intracellular parasites. (A) NO release from WT mouse BMDMs incubated with *T. cruzi* (MOI, 0.2) with or without IFN-γ (100 units) for 24 h. **, $P < 0.01$ compared to untreated cells; +, $P < 0.05$ compared to treated or infected cells in the presence or absence of BEL (10 μM); ++, $P < 0.01$ compared to treated or infected cells in the presence or absence of BEL (10 μM). (B) BMDMs isolated from WT or iPLA$_2$β-KO mice were treated with *T. cruzi* (MOI, 0.2) or IFN-γ (100 units) for 24 h. **, $P < 0.01$ compared to untreated cells; ++, $P < 0.01$ compared to NO release between WT and iPLA$_2$β-KO mice. (C) *T. cruzi* inhibition in BMDMs isolated from WT or iPLA$_2$β-KO mice infected with *T. cruzi* (MOI, 5). **, $P < 0.01$ compared to data between WT and iPLA$_2$β-KO mice. The values shown are means + SEMs for at least 4 separate cell cultures.

upregulation of adhesion molecules is sufficient to recruit inflammatory cells across the endothelium in the presence of decreased PAF production.

Macrophages comprise key immune cells found in chagasic lesions and play an important role in parasite clearance via NO production (21, 22). Previous studies have suggested that iPLA$_2$β may be required for iNOS upregulation in macrophages through activation of cyclic AMP response element-binding protein (CREB) via iPLA$_2$-derived lysoplasmenylcholine (23). iPLA$_2$β has been demonstrated to be important in macrophage chemotaxis (14), and macrophage iPLA$_2$β has recently been shown to generate lysophosphatidic acid, which is required for increased NADP oxidase 4 (Nox4) expression and H_2O_2 generation (15). These studies suggest a possible mechanism by which NO production in iPLA$_2$β-deficient macrophages and tissue parasite clearance are impaired. We demonstrated here that pretreatment of RAW 264.7 cells with the iPLA$_2$β inhibitor (*S*)-BEL [but not with its (*R*)-BEL enantiomer] blunted the NO production induced by *T. cruzi* infection and its amplification by coincubation with IFN-γ, which indicates that iPLA$_2$β contributes to NO production. Further studies were performed using bone marrow-derived macrophages obtained from *T. cruzi*-infected WT and iPLA$_2$β-KO mice with or without IFN-γ. In agreement with previously published data (21, 22), macrophages from iPLA$_2$β-KO mice released less NO than cells from WT mice. Thus, the absence of iPLA$_2$β results in impaired parasite clearing by murine macrophages attributable to their diminished NO production, and this could explain the increased cardiac parasite load in iPLA$_2$β-KO mice infected with *T. cruzi*. An increased tissue parasite load could eventually result in greater myocardial damage and more adverse clinical outcomes in patients with chronic Chagas disease. The knowledge that parasite clearance by macrophages requires iPLA$_2$β-dependent NO release may lead to new avenues for therapeutic interventions in Chagas disease.

In conclusion, these data indicate that iPLA$_2$β plays an important role in host defense against trypanosome infection by contributing significantly to macrophage NO production. The significance of endothelial cell iPLA$_2$β-dependent PAF production in cardiac inflammation during acute Chagas disease remains uncertain, but recruitment of inflammatory cells is enhanced in *T. cruzi*-infected endothelial cells. Because inflammation is a carefully regulated process involving several mediators with some redundancy in function, impaired PAF production may only minimally affect the recruitment of inflammatory cells across the endothelium following *T. cruzi* infection *in vivo*, and this might be offset in part by the concomitant increase in cell surface expression of endothelial adhesion molecules.

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