Mice with genetic deletion of group VIA phospholipase A2β 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 A₂β (iPLA₂β) following infection of human coronary artery endothelial cells (HCAECs) with T. cruzi, suggesting that the absence of iPLA₂β may decrease the recruitment of inflammatory cells to the heart to manage parasite accumulation. Cardiac endothelial cells isolated from iPLA₂β-knockout (iPLA₂β-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 iPLA₂β-KO mice infected with T. cruzi was similar in severity to that in WT mice, but the iPLA₂β-KO mouse myocardium contained more parasite pseudocysts. Upon activation, macrophages from iPLA₂β-KO mice produced significantly less nitric oxide (NO) and caused less T. cruzi inhibition than macrophages from wild-type mice. Thus, the absence of iPLA₂β activity does not influence myocardial inflammation, but iPLA₂β is essential for T. cruzi clearance.
group VIB PLA2 (iPLA2), which is responsible for the production of arachidonic acid-derived eicosanoids. Although few studies to date have addressed the role of phospholipase A2 (PLA2) in myocarditis, several inflammatory metabolites produced following PLA2-catalyzed hydrolysis of membrane phospholipids have been implicated in Chagas disease (10, 11, 13). Finally, previous studies have suggested that iPLA2β 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 iPLA2β-KO mice to determine whether iPLA2β deficiency influences cardiac inflammation and parasite accumulation following T. cruzi infection.

**MATERIALS AND METHODS**

**Parasites.** 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.

**Murine heart and infection.** Animals were in strict accordance with the NIH guidelines on 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 iPLA2β-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 parafomaldehyde 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) conjugated to magnetic beads. The eluted cells were washed, resuspended in cell culture medium, and stained with a modified Wright Giemsa stain (Diff-Quik; IMEB, Inc., San Marcos, CA). Subsequently, cells were infected with 5,000 Brazil strain blood-form trypomastigotes (BFTs) and sacrificed at different time points postinfection. The hearts were collected in parafomaldehyde 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.

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**PAF assay.** Murine cardiac endothelial cells grown in 12-well culture dishes were washed twice with Hank’s balanced salt solution containing 135 mM NaCl, 0.8 mM MgSO4, 10 mM HEPES (pH 7.4), 1.2 mM CaCl2, 5.4 mM KCl, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, and 6.6 mM glucose and incubated with 50 μCi [3H]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 N2, applied to a silica gel 60 thin-layer chromatography plate, and developed in chloroform-methanol-acetic acid-water (50:25:8, 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 [14C]PAF as an internal standard. [14C]PAF was synthesized by acetylation of the sn-2 position of hyso-PAF with [14C]acetic anhydride using 0.33 M dimethylamino-
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 iPLA₂β-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 iPLA₂β-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 iPLA₂β-KO mouse heart was significantly reduced compared to that in endothelial cells isolated from WT mouse heart (Fig. 1). Thus, the absence of iPLA₂β 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 iPLA₂β-KO mice.** WT and iPLA₂β-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
Numerical value on the basis of the size and severity of the inflammatory foci. Cardiac inflammation was observed in WT and iPLA2β-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 iPLA2β-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 iPLA2β-KO mice compared to the hearts of WT mice at 4 weeks postinfection (Fig. 2B). These data suggest that iPLA2β deficiency does not significantly affect the transmigration of inflammatory cells following *T. cruzi* infection but that parasite clearance is impaired in iPLA2β-deficient mice and that iPLA2β 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 iPLA2β-KO mice had an inflammation score comparable to that of the myocardium of WT mice, but the myocardium of iPLA2β-KO mice had an increased number of pseudocysts, which suggests that macrophages from iPLA2β-KO mice may be deficient in their parasite clearance ability.

To examine the role of iPLA2β 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 iPLA2 inhibitor bromoenoil 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-dependent group VIB PLA2 (iPLA2γ) inhibitor (R)-BEL did not significantly affect NO release following *T. cruzi* infection with or without IFN-γ (Fig. 3), but pretreatment with the iPLA2β 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 iPLA2β-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 iPLA2β-KO mice released negligible amounts of NO compared to the amount released by WT mouse macrophages (Fig. 4B). These data indicate that iPLA2β 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 iPLA2β-KO mice (Fig. 4C). Thus, we conclude that iPLA2β 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.

**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 pro-inflammatory 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 iPLA2β (4), which suggested that prolonged endothelial cell activation in chronic Chagas disease may be associated with iPLA2β activation and PAF production, which facilitates the recruitment and transmigration of inflammatory cells to sites of infection. However, the absence of iPLA2β 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 iPLA2β-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 iPLA2β. Since we did not observe a difference in inflammatory cells in the hearts of WT or iPLA2β-KO mice infected with *T. cruzi*, our data suggest that the
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 iPLA₂ β may be required for iNOS upregulation in macrophages through activation of cyclic AMP response element-binding protein (CREB) via iPLA₂-derived lysoplasmenylcholine (23). iPLA₂ β has been demonstrated to be important in macrophage chemotaxis (14), and macrophage iPLA₂ β has recently been shown to generate lysophosphatidic acid, which is required for increased NADPH oxidase 4 (Nox4) expression and H₂O₂ generation (15). These studies suggest a possible mechanism by which NO production in iPLA₂ β-deficient macrophages and tissue parasite clearance are impaired. We demonstrated here that pretreatment of RAW 264.7 cells with the iPLA₂ β inhibitor (S)-BEL [but not with its (R)-BEL enantiomer] blunted the NO production induced by T. cruzi infection and its amplification by coinubcation with IFN-γ, which indicates that iPLA₂ β contributes to NO production. Further studies were performed using bone marrow-derived macrophages obtained from T. cruzi-infected WT and iPLA₂ β-KO mice with or without IFN-γ. In agreement with previously published data (21, 22), macrophages from iPLA₂ β-KO mice released less NO than cells from WT mice. Thus, the absence of iPLA₂ 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 iPLA₂ 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 iPLA₂ β-dependent NO release may lead to new avenues for therapeutic interventions in Chagas disease.

In conclusion, these data indicate that iPLA₂ β plays an important role in host defense against trypanosomiasis by contributing significantly to macrophage NO production. The significance of endothelial cell iPLA₂ 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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**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). (B) BMDMs isolated from WT or iPLA₂ 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 iPLA₂ β-KO mice. (C) T. cruzi inhibition in BMDMs isolated from WT or iPLA₂ β-KO mice infected with T. cruzi (MOI, 5). **, P < 0.01 compared to data between WT and iPLA₂ β-KO mice. The values shown are means ± SEMs for at least 4 separate cell cultures.