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**Mice with Genetic Deletion of Group VIA Phospholipase A\textsubscript{2}\textbeta 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\textsubscript{2}\textbeta (iPLA\textsubscript{2}\textbeta) following infection of human coronary artery endothelial cells (HCAECs) with *T. cruzi*, suggesting that the absence of iPLA\textsubscript{2}\textbeta may decrease the recruitment of inflammatory cells to the heart to manage parasite accumulation. Cardiac endothelial cells isolated from iPLA\textsubscript{2}\textbeta-knockout (iPLA\textsubscript{2}\textbeta-KO) mice infected with *T. cruzi* demonstrated decreased PAF production compared to that by cells isolated from wild-type (WT) mice but demonstrated increased increases in adhesion molecule expression similar to those seen in WT mice. Myocardial inflammation in iPLA\textsubscript{2}\textbeta-KO mice infected with *T. cruzi* was similar in severity to that in WT mice, but the iPLA\textsubscript{2}\textbeta-KO mouse myocardium contained more parasite pseudocysts. Upon activation, macrophages from iPLA\textsubscript{2}\textbeta-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\textsubscript{2}\textbeta activity does not influence myocardial inflammation, but iPLA\textsubscript{2}\textbeta 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\textsubscript{2}\textbeta (iPLA\textsubscript{2}\textbeta) and is greatly blunted in iPLA\textsubscript{2}\textbeta-knockout (iPLA\textsubscript{2}\textbeta-KO) mice (4). Although we have focused on iPLA\textsubscript{2}\textbeta-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\textsubscript{2}\textbeta activity is associated with a decrease in prostacyclin release. The predominant iPLA\textsubscript{2} isoform in the myocardium is the calcium-independent
group VIB PLα2 (iPLAγ), 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

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 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 and infected with 5,000 Brazil strain blood-form trypomastigotes (BFTs) and incubated with 50 μM fluorescent-activated cell sorting (FACS) buffer and analyzed for T. cruzi infection.

Murine endothelial cell isolation. Endothelial cells were isolated from mouse heart by collagenase digestion. The diced heart muscle was exposed to 10 mM collagenase (type IV, Sigma, St. Louis, MO) for 1 h, followed by 20 mM collagenase (type I, Worthington, Lakewood, NJ) for 1 h in Ham’s F12 medium containing 10% fetal bovine serum (FBS). The cells were collected by centrifugation and suspended in fresh Ham’s F12 medium. The cell suspension was filtered through a 100-μm nylon mesh and centrifuged at 100 g for 10 min. The pellet was resuspended in serum-free Ham’s F12 medium containing 1% fetal bovine serum (FBS) before plating 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 cell 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 x 10^5 cells/well and cultured with 5% CO2 incubated for 7 days. Nonadherent cells were removed from the slides, and the slides were washed, dried, fixed, and stained with a modified Wright-Giemsa stain (Diff-Quik; I.M. Biotech, Inc., San Marcos, CA). The number of infected macrophages was determined microscopically. The percent inhibition was calculated by the following formula: \[ 1 - \frac{1}{n} \left( \frac{n}{5} \right) \text{ number of infected cells per 200 cells treated with IFN-γ} \times \left( \text{number of infected cells per 200 cells treated with medium alone} \right) \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

Pf production and adhesion molecule expression in cardiac endothelial cells. Our previous in vitro studies indicated that iPLA2β is critical for recruiting inflammatory cells to the endothelial-cell surface.
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

![FIG 1](http://iai.asm.org/)

**FIG 1** Expression of ICAM-1 and VCAM-1 and PAF production in endothelial cells isolated from the hearts of WT and iPLA₂β-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.

![FIG 2](http://iai.asm.org/)

**FIG 2** (A) Myocardial inflammation score for WT and iPLA₂β-KO mice following *T. cruzi* infection for up to 12 weeks. (B) Number of parasites in the myocardium per field for WT and iPLA₂β-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 iPLA₂β-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 iPLA₂-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 iPLA₂-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 iPLA₂-β-KO mice compared to the hearts of WT mice at 4 weeks postinfection (Fig. 2B). These data suggest that iPLA₂-β deficiency does not significantly affect the transmigration of inflammatory cells following *T. cruzi* infection but that parasite clearance is impaired in iPLA₂-β-deficient mice and that iPLA₂-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 iPLA₂-β-KO mice had an inflammation score comparable to that of the myocardium of WT mice, but the myocardium of iPLA₂-β-KO mice had an increased number of pseudocysts, which suggests that macrophages from iPLA₂-β-KO mice may be deficient in their parasite clearance ability.

To examine the role of iPLA₂-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 iPLA₂ inhibitor bromoethanol 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 coinoculation with interferon γ (IFN-γ), a known activator of the macrophage iNOS pathway (Fig. 3). Pretreatment of RAW 264.7 cells with the calcium-independent group VIB PLA₂ (iPLA₂-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 iPLA₂-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 iPLA₂-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 coinoculation 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 iPLA₂-β-KO mice released negligible amounts of NO compared to the amount released by WT mouse macrophages (Fig. 4B). These data indicate that iPLA₂-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 iPLA₂-β-KO mice (Fig. 4C). Thus, we conclude that iPLA₂-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.

**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 iPLA₂-β (4), which suggested that prolonged endothelial cell activation in chronic Chagas disease may be associated with iPLA₂-β activation and PAF production, which facilitates the recruitment and transmigration of inflammatory cells to sites of infection. However, the absence of iPLA₂-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 iPLA₂-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 iPLA₂-β. Since we did not observe a difference in inflammatory cells in the hearts of WT or iPLA₂-β-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 iPLA₂β may be required for iNOS upregulation in macrophages through activation of cyclic AMP response element-binding protein (CREB) via iPLA₂-derived lysophosphatidylcholine (23). iPLA₂β has been demonstrated to be important in macrophage chemotaxis (14), and macrophage iPLA₂β 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 iPLA₂β-deficient macrophages and tissue parasite clearance are impaired. We demonstrated here that pretreatment of RAW 264.7 cells with the iPLA₂β 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 iPLA₂β contributes to NO production. Further studies were performed using bone marrow-derived macrophages obtained from T. cruzi-infected WT and iPLA₂β-KO mice with or without IFN-γ. In agreement with previously published data (21, 22), macrophages from iPLA₂β-KO mice released less NO than cells from WT mice. Thus, the absence of iPLA₂β results in impaired parasite clearing by murine macrophages attributable to their diminished NO production, and this could explain the increased cardiac parasite load in iPLA₂β-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 iPLA₂β-dependent NO release may lead to new avenues for therapeutic interventions in Chagas disease.

In conclusion, these data indicate that iPLA₂β plays an important role in host defense against trypanosome infection by contributing significantly to macrophage NO production. The significance of endothelial cell iPLA₂β-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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