Pathogens and Disease, 75, 2017, ftx082

doi: 10.1093/femspd/ftx082
Advance Access Publication Date: 20 July 2017
Research Article

RESEARCH ARTICLE

Platelet-activating factor increases reactive oxygen species-mediated microbicidal activity of human macrophages infected with *Leishmania (Viannia) braziliensis*

Arissa Felipe Borges, Camila Imai Morato, Rodrigo Saar Gomes, Miriam Leandro Dorta, Milton Adriano Pelli de Oliveira and Fátima Ribeiro-Dias*†

Instituto de Patologia Tropical e Saúde Pública, Universidade Federal Goiás, Goiânia, Goiás, Rua 235 S/N, Setor Leste Universitário, Goiânia - GO 74605-050, Brazil

*__Corresponding author:__ Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235 S/N, Setor Leste Universitário, Goiânia - GO 74605-050, Brazil. Tel: (+55) 62 3209 6116; Fax: (+55) 62 3209 6363; E-mail: fatimardias@gmail.com

One sentence summary: This study demonstrates that endogenous or exogenous platelet-activating factor (PAF) increases induction of reactive oxygen species that are needed to kill *Leishmania braziliensis* in human macrophages.

Editor: Peter Timms

†Fátima Ribeiro-Dias, http://orcid.org/0000-0002-7950-3414

ABSTRACT

Platelet-activating factor (PAF) is produced by macrophages during inflammation and infections. We evaluated whether PAF is able to modulate the infection of human macrophages by *Leishmania braziliensis,* the main *Leishmania* sp. in Brazil. Monocyte-derived macrophages were incubated with promastigote forms in absence or presence of exogenous PAF. We observed that the treatment of macrophages with low concentrations of PAF prior to infection increased the phagocytosis of *L. braziliensis*. More importantly, exogenous PAF reduced the parasitism when it was added before, during or after infection. In addition, treatment with a PAF antagonist (PCA 4248) resulted in a significant increase of macrophage infection in a concentration-dependent manner, suggesting that endogenous PAF is important to control *L. braziliensis* infection. Mechanistically, while exogenous PAF increased production of reactive oxygen species (ROS) treatment with PCA 4248 reduced oxidative burst during *L. braziliensis* infection. The microbicidal effects of exogenous PAF were abolished when macrophages were treated with apocynin, an NADPH oxidase inhibitor. The data show that PAF promotes the production of ROS induced by *L. braziliensis,* suggesting that this lipid mediator may be relevant to control *L. braziliensis* infection in human macrophages.

Keywords: *Leishmania (Viannia) braziliensis*; human macrophage; platelet-activating factor; reactive oxygen species

Received: 20 March 2017; **Accepted:** 19 July 2017

INTRODUCTION

American Tegumentary Leishmaniasis (ATL) is a parasitic disease caused by several protozoan species of the genus *Leishmania*. In Brazil, the main species responsible for ATL is *Leishmania (Viannia) braziliensis*, which is associated with the development of the most severe lesions of the disease, with extensive cutaneous and/or nasopharyngeal mucosal lesions (Silveira *et al.* 2009; Alvar *et al.* 2012). *Leishmania* parasites are transmitted during blood meals of sandflies when infective metacyclic forms are inoculated into the skin. Parasites infect macrophages and multiply inside them. Thus, to control the infection is crucial that macrophage microbicidal mechanisms are induced. The initial interaction between parasites and macrophages may determine the outcome of infection (Handman and Bullen 2002).

During macrophage activation, lipid mediators such as platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocoline) are fast produced. The biological effects of PAF are mediated by binding to its G protein-coupled seven α-helices transmembrane receptor (PAFR). PAFR is expressed in several kinds of cells including macrophages. Signals transduced by PAFR promote inflammation, chemotaxis, platelet aggregation, leukocytosis and adhesion of leukocytes to the vascular endothelium. PAFR activation induces mobilization of intracellular calcium and activation of different kinases (Uhlig, Göggel and Engel 2005; Shimizu 2009; Santos, Teixeira and Souza 2016). Due to the important role of PAF in inflammation, its participation in infections has been studied and it is associated with the control and/or pathogenesis of infectious diseases, such as influenza, cerebral malaria, Chagas´ disease, meningoencephalitis and dengue (Talvani *et al.* 2003; Souza *et al.* 2009; Garcia *et al.* 2010; Lacerda-Queiroz *et al.*2012; Kamaladasa *et al.* 2016; Vilela *et al.* 2016). PAF also seems to be important for the induction of microbicidal mechanisms to control infections (Kim *et al.* 2008; Liu *et al.* 2012).

Studies on the role of PAF in leishmaniasis are scarce and restricted to murine models. PAF-treated murine macrophages are resistant to infection with *Leishmania amazonensis* (Lonardoni, Russo and Jancar 2000; Rosa *et al.* 2001). Furthermore, peritoneal macrophages of PAFR-deficient mice are more susceptible to *L. amazonensis* infection showing exacerbated lesions in comparison to infected wild-type mice. In the absence of PAFR, there was a decrease in nitric oxide (NO) production whereas arginase-1 expression was increased, contributing to the exacerbation of the infection (Santiago *et al.* 2006). In addition, treatment with a PAF antagonist increased lesion size and parasite load in wild-type mice infected with *L. amazonensis* (Lonardoni, Russo and Jancar 2000). These data suggest that PAF is produced during *L. amazonensis* infection and its inhibition is able to convert a resistant into a susceptible host. Moreover, microbicidal mechanisms involving other mechanism than NO production can also be induced by PAF during *L. amazonensis* infection (de Souza Carmo, Katz and Barbiéri 2010).

The role of PAF in human macrophages infected with *Leishmania* sp. has not been evaluated. In addition, the effects of this lipid mediator on *L. braziliensis* infection remain unknown. Considering that *L. amazonensis* is able to induce PAF-dependent mechanisms to control the infection in murine macrophages, we reasoned to investigate whether in human macrophages PAF can also contribute to control *L. braziliensis* infection. Here, we evaluated whether exogenous and endogenous PAF are important to control *L. braziliensis* infection in human macrophages.

MATERIALS AND METHODS

Parasites

MHOM/BR/2003/IMG *Leishmania braziliensis* (IMG3) parasites were obtained from a patient with localized cutaneous leishmaniasis (*Leishbank* IPTSP/UFG) as previously described (Zauli-Nascimento *et al.* 2010). Parasites were cultured in Grace's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with inactivated 20% fetal calf serum (FCS; Gibco, Life Technologies, São Paulo, SP, Brazil), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and 100 μg/mL streptomycin (Sigma-Aldrich) at 26°C. Parasites from stationary phase of growth (6th day of culture) were washed three times with phosphate buffer saline (PBS: 1500 g, 15 min, 10°C) and diluted into PBS/0.1% formaldehyde (Synth, SP, Brazil) for quantification in hemocytometer.

Monocyte-derived human macrophages

This study was approved by Committee of Ethics in Research of Hospital das Clínicas/Federal University of Goiás (Protocol: 189/2011). Venous blood samples obtained from healthy donors ($n = 26$) were collected in EDTA-vacutainer tubes (Greiner bio-one, Vacuette, Americana, SP, Brazil). Blood was centrifuged (200 g, 5 min at room temperature) to remove the plasma and diluted into PBS containing 0.01 mM EDTA, pH 7.3 (v/v). Diluted blood was centrifuged on a density gradient (Percoll, GE Healthcare, Chicago, IL, USA). The mononuclear cells were distributed into 24-well plates (TPP, Techno Plastic Products, Trasadingen, Switzerland) over13 mm-glass coverslips (Deckglaser, Braunschweig, Germany), at the concentration of 2×10^5 cells/500 μL of RPMI 1640 medium (Sigma-Aldrich) supplemented with 15% FCS (Gibco), 11 mM sodium bicarbonate and 2 mM L-glutamine. Macrophages were derived for 5 days, with medium exchange each 48 h, at 36°C, 5% CO_2. This is the same protocol we have published before (Morato *et al.* 2014), achieving ~90% of macrophages according to cell morphology.

Treatment with exogenous platelet-activating factor or platelet-activating factor antagonist and infection of macrophages

Initial stationary phase-promastigote forms (6th day of culture) were resuspended in complete RPMI medium and added to the macrophages (10:1, parasite:cell). The plates were incubated at 36°C/5% CO_2 for 4 h, washed three times with complete RPMI medium at 37°C to remove non-internalized parasites and incubated for additional 48 h, according to Morato *et al.* (2014).

To evaluate the effects of exogenous PAF, PAF (Bachem Inc., Torrence, CA, USA) was dissolved in ethanol and further diluted in PBS with 0.25% bovine serum albumin (Sigma-Aldrich). PAF was added in different concentrations (10^{-6} M, 10^{-8} M or 10^{-10} M) 3 h before infection with the parasites, at the time of infection or 4 h post infection. When PAF was added before or during infection, it was replenished after washings and cultures were incubated for up to 48 h. To assess the effects of endogenous PAF, PAF antagonist (PCA 4248 [(2-phenylthio)ethyl-5-metoxycarbonyl-2,4,6-trimethyl-1,4-dihydropyridine-3 carboxylate] Biomol, Palo Alto, CA, USA) was dissolved in ethanol and then diluted in complete medium. PCA 4248 was added to the macrophage cultures 30 min prior to infection with the parasites and maintained for up to 48 h, at 10^{-5} M, 10^{-6} M or 10^{-7} M. In non-treated cells, ethanol was added in the highest

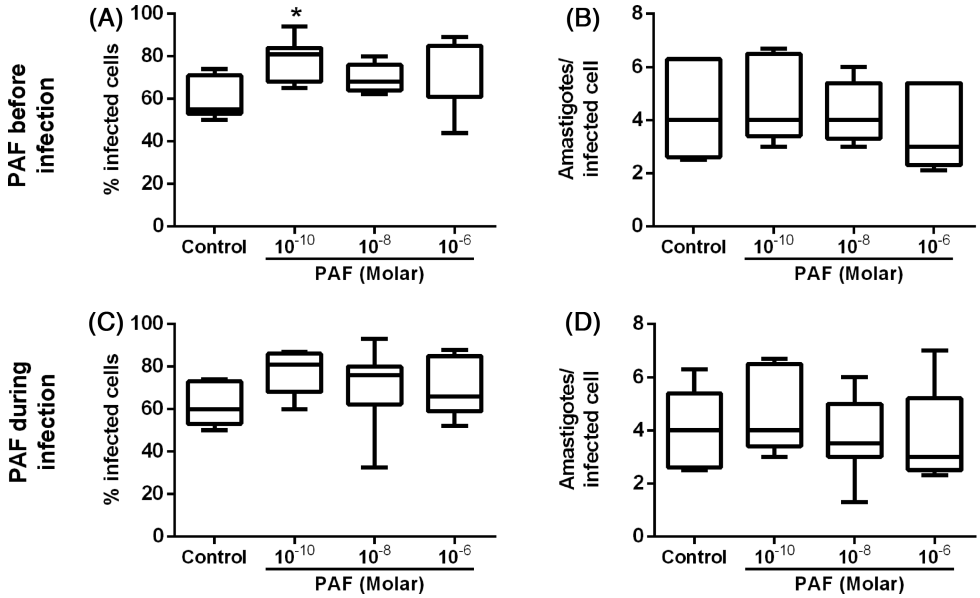

Figure 1. Pretreatment with low doses of exogenous PAF induces phagocytosis of *L. braziliensis* by human macrophages. Human macrophages were treated with different PAF concentrations 3 h before infection (**A** and **B**) or during the infection (**C** and **D**) and infected with *L. braziliensis* stationary phase promastigotes (10 parasites: 1 cell). After 4 h, parasite uptake was analyzed under light microscopy. Control corresponds to untreated cells. (A and C) Percentage of infected cells. (B and D) Number of parasites per infected cell. The data represent median, interquartile and minimal and maximal values (n = 7 – 8 donors). $^{*}P < 0.05$, compared to control, by Kruskal–Wallis/Dunn's test.

concentration used in PAF or PCA 4248 dilutions. The cytotoxicity of PAF and PCA 4248 was evaluated by MTT assay as described by Morato *et al.* (2014).

At the end of incubations, macrophages were stained with the Instant Prov Kit (Newprov, Pinhais, PR, Brazil) and analyzed under light microscope (1000× magnification). The frequency of infected cells (%) and mean number of parasites per infected cell were evaluated. At least 300 cells were analyzed in each coverslip. Only cells with macrophage morphology were considered during the counts (Fig. S1, Supporting Information).

Evaluation of reactive oxygen species production and effects

NBT (nitro blue tetrazolium) assay was adapted from Muniz-Junqueira and de Paula-Coelho (2008) to evaluate the production of superoxide anion (O_2^-). Briefly, macrophages were incubated with PAF (10^{-10} M) 3 h prior to addition of the parasites, in the absence or presence of an inhibitor of reactive oxygen species (ROS) production, apocynin (10^{-4} M) and/or PCA 4248 (10^{-5} M) added 30 min prior infection. After 1 h of infection, macrophages were treated with a Hanks-Tris solution containing 0.05% NBT (Sigma-Aldrich) for 20 min, 36°C, 5% CO_2. The slides were then washed and the cells were fixed with methanol and stained with a solution of 1.4% safranin and 28.6% glycerol in distilled water. The percentage of macrophages that reduced NBT was evaluated under light microscopy, and the results were expressed as percentage of positive cells.

Statistical analysis

The data were evaluated by Wilcoxon paired test for analysis of two groups of paired samples and Kruskal–Wallis with Dunn's post-hoc test to compare three or more groups, using GraphPad Prism v.5 software (San Diego, CA, USA). Level of statistical significance was set at $P < 0.05$.

RESULTS

Low doses of exogenous platelet-activating factor enhance the phagocytosis of *Leishmania braziliensis* by human macrophages

PAF has been associated with increased phagocytic capacity of macrophages (Muehlmann *et al.* 2012; Lee *et al.* 2016). To assess whether PAF enhances phagocytosis of *Leishmania braziliensis*, human macrophages were treated with PAF before or during the infection. Pre-treatment with low doses of PAF (10^{-10} M) increased the percentage of infected cells evaluated after 4 h of incubation of macrophages with promastigote forms (Fig. 1A). However, the number of parasites per infected cell was not significantly altered (Fig. 1B). When PAF was added at the same time when parasites were added to macrophages, despite a similar profile of pre-treated macrophages, we did not observe significant changes in the phagocytosis of *L. braziliensis* (Fig. 1C and D).

Exogenous platelet-activating factor increases leishmanicidal activity of human macrophages

Since PAF is able to activate microbicidal mechanisms (Kim *et al.* 2008; Liu *et al.* 2012), we investigated whether exogenous PAF treatment could lead to the control of *L. braziliensis* infection in human macrophages. For this, we compared the infection rates 4 h and 48 h after the incubation of macrophages with *L. braziliensis*. We observed an increase in the percentage of infected cells in untreated macrophages from 4 h to 48 h. However, when cells were treated with low doses of PAF (10^{-10} M) before or at the time of infection, a reduction in the percentage of infected cells was observed (Fig. 2A and C). In addition, we observed a replication of the parasites in untreated cells (4 h vs 48 h) that was controlled by different concentrations of PAF, prior to or at the time of infection (Fig. 2B and D). The effect of PAF on reducing the percentage of infected cells and controlling

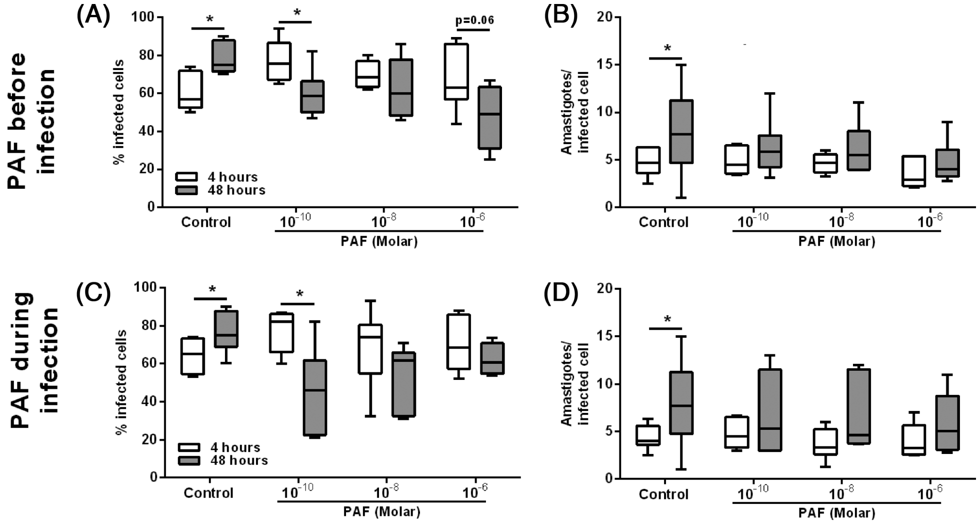

Figure 2. Exogenous PAF increases leishmanicidal activity of human macrophages. Human macrophages were treated with different PAF concentrations 3 h before infection (**A** and **B**) or during the infection (**C** and **D**). After infection with *L. braziliensis* stationary phase promastigotes (10 parasites: 1 cell), for 4 h, cells were washed to remove non-internalized parasites and the treatment was restored for additional 48 h. Control corresponds to untreated cells. (A and C) Percentage of infected cells. (B and D) Number of parasites per infected cell. The data represent median, interquartile and minimal and maximal values (n = 7 – 8 donors). *P < 0.05 (4 h vs 48 h) by Wilcoxon paired test.

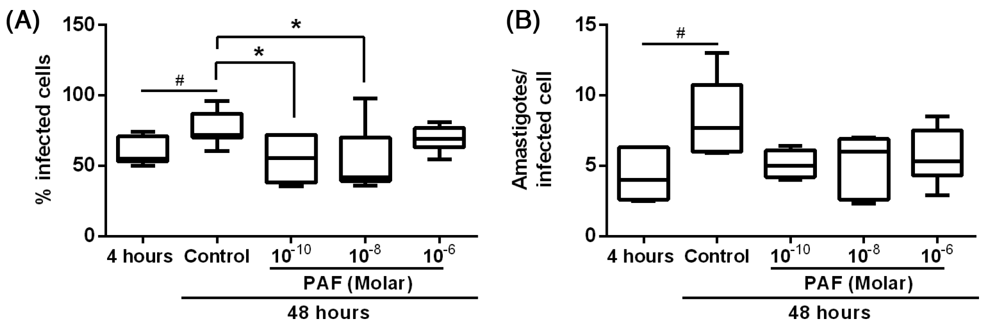

Figure 3. Treatment with exogenous PAF after infection control *L. braziliensis* within human macrophages. Human macrophages were infected with *L. braziliensis* (10 parasites: 1 cell) for 4 h. The cells were washed to remove non-internalized parasites and treated with different PAF concentrations for additional 48 h. Control corresponds to untreated cells. (**A**) Percentage of infected macrophages. (**B**) Number of amastigotes per infected macrophage. The data represent median, interquartile and minimal and maximal values (n = 7 – 8 donors). *P < 0.05 (4 h vs control 48 h) by Wilcoxon paired test; **P < 0.05 compared to control 48 h, by Kruskal–Wallis/Dunn's test.

parasite replication was also demonstrated by the infection rate at 48 h:4 h (Fig. S2, Supporting Information)

To assess whether treatment with PAF after establishment of infection would reduce parasitism in human macrophages, we infected the cells with *L. braziliensis* during 4 h and then PAF was added. We observed that treatment with low concentrations of PAF reduced the 48 h-increased percentage of infected cells indicating that some cells were activated to eliminate the parasites (Fig. 3A). The 48 h-increased number of parasites per infected cell was also reduced after treatment with different concentrations of PAF but the differences were not statistically significant (Fig. 3B).

Endogenous platelet-activating factor plays a role in the control of human macrophages infection with *Leishmania braziliensis*

We have observed that pre- or post-treatment with PAF increases anti-*L. braziliensis* activity of human macrophages. On the other side, it is possible that human macrophages produce PAF after infection with *L. braziliensis* and this lipid mediator can interfere with the establishment of the infection. Thus, to investigate whether endogenous PAF is important in infection control, we pretreated human macrophages with PCA 4248, a PAF antagonist. Inhibition of PAF with all concentrations of PCA 4248 increased the percentage of infected cells compared to the untreated control (Fig. 4A). In addition, when cells were treated with 10^{-5} M PCA 4248 we observed that the number of parasites per infected cell was increased in relation to the control (Fig. 4B).

Both treatments with exogenous PAF or PCA 4248 were not toxic to parasites or to human cells (Fig. 5).

Platelet-activating factor controls *Leishmania braziliensis* infection in human macrophages through reactive oxygen species production

It is known that PAF can induce the production of ROS by macrophages (Huang *et al.* 1988) and ROS are important to control *Leishmania* infections, especially in human macrophages (Novais *et al.* 2014; Carneiro *et al.* 2016). Thus, we evaluated whether exogenous PAF induces ROS production in *L. braziliensis*-infected human macrophages. As showed in Fig. 6,

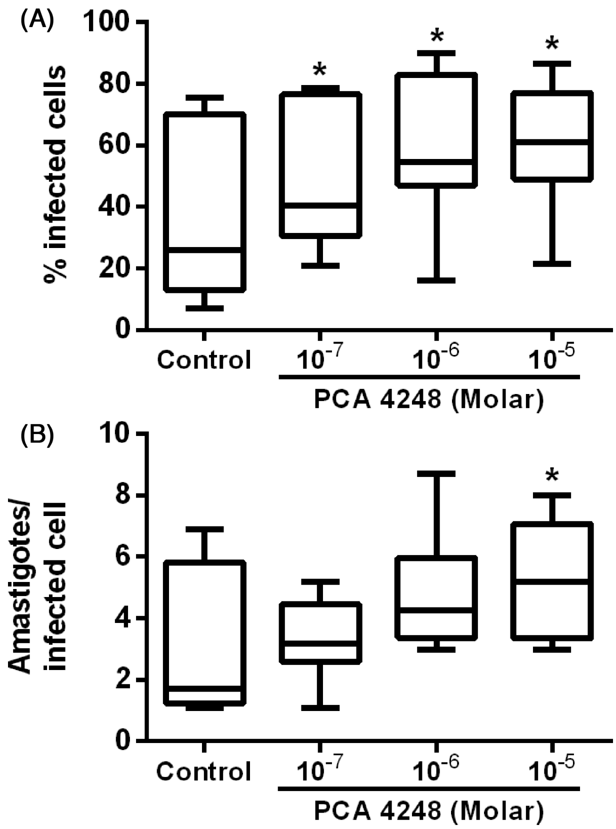

Figure 4. Endogenous PAF plays a role in the control of *L. braziliensis* infection in human macrophages. Human macrophages were treated with different concentrations of the PAF antagonist (PCA 4248) 30 min prior to infection. Cells were infected with *L. braziliensis* (10 parasites: 1 cell) for 4 h, washed to remove non-internalized parasites and the treatment was restored for additional 48 h. Control corresponds to untreated cells. (**A**) Percentage of infected macrophages. (**B**) Number of amastigotes per infected macrophage. The data represent median, interquartile and minimal and maximal values (n = 7 – 8 donors). $^{*}P < 0.05$, compared to control, by Kruskal–Wallis/Dunn's test.

L. braziliensis was able to induce ROS production, and pre-treatment with PAF increased ROS production by infected cells. In addition, inhibition of endogenous PAF by treatment with PCA 4248 partially reversed the production of ROS induced by *L. braziliensis*.

To confirm that the effects of PAF are dependent on ROS production, we treated the cells with apocynin, an NADPH oxidase inhibitor, which reduces ROS production by phagocytes (Fig. 7A). We showed that the inhibition of ROS production by apocynin reverses the effects of exogenous PAF in reducing the percentage of infected cells and the number of parasites per infected cell, after 48 h of incubation (Fig. 7B and C). That apocynin decreased the PAF-induced ROS in infected cells is shown in Fig. 7D.

DISCUSSION

In this study, we show for the first time that PAF plays important effects on the control of human macrophage infection by *Leishmania braziliensis*. We observed that low doses of exogenous PAF increased the amount of human macrophages that phagocytosed promastigotes of *L. braziliensis* without alteration of the number of parasites captured by cells (Fig. 1). Phagocytosis is the first mechanism used by macrophages to control *Leishmania* infection and involves membrane receptors present on immune cells (Liu and Uzonna 2012; Ueno and Wilson 2012). Thus, our results suggest that PAF increased receptors involved in *Leishmania* phagocytosis in some cells, increasing the number of cells that are able to uptake the parasites. In accordance with our results, previous studies have demonstrated that exogenous PAF induces phagocytosis of several microorganisms by macrophages (Ichinose *et al.* 1994; Soares *et al.* 2002; Lee *et al.* 2016). It is important to note that high doses of exogenous PAF had a limited effect on the percentage of cells phagocytosing *L. braziliensis* but seems to reduce number of parasites per infected cell (Fig. 1), indicating the dependence of PAF concentrations to exert its biological effects. That PAF acts depending on its concentration has been demonstrated in some studies (Kroegel *et al.* 1989; Hwang 1991; Pustynnikov *et al.* 1991; O'Flaherty, Jacobson and Redman 1992) and this is probably due to the differences in PAFRs affinities (LeVan *et al.* 1997; Yost, Weyrich and Zimmerman 2010; Santos, Teixeira and Souza 2016). It has been shown that the pre-treatment of murine macrophages with low doses of PAF reduces the capture of *Leishmania amazonensis* (Rosa *et al.* 2001). Together with our data, this suggests that different levels of activation of macrophages and/or PAFRs can lead to different consequences in the phagocytosis of *L. braziliensis* by human macrophages, which requires further investigation.

After the uptake of parasites, macrophage microbicidal effects are essential for infection control. We show that exogenous and endogenous PAF induces *L. braziliensis* death in human macrophages (Figs 2–4). Previously, it was shown that PAF induces the killing of *L. amazonensis* by murine macrophages (Lonardoni, Russo and Jancar 2000; Rosa *et al.* 2001).

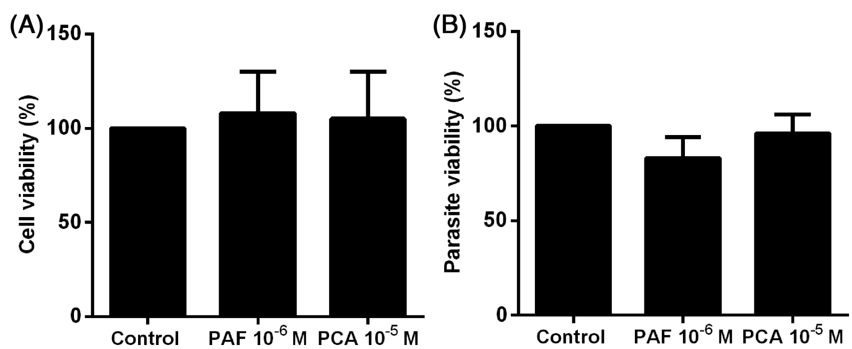

Figure 5. PAF or PCA 4248 treatments are not cytotoxic to macrophages or parasites. Human macrophages (**A**) and *L. braziliensis* promastigotes from stationary phase of growth (**B**) were incubated with PAF 10^{-6} M or PCA 4248 10^{-5} M for 24 h. Cell viability was assessed by MTT assay. The data represent median and interquartile (n = 3).

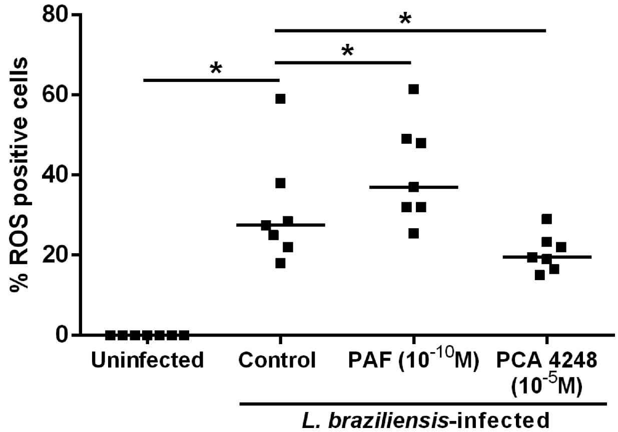

Figure 6. Exogenous or endogenous PAF increases ROS production by human macrophages infected with *L. braziliensis*. Human macrophages were pretreated with PAF 10^{-10} M (3 h before infection) or PCA 4248 10^{-5} M (30 min before infection) and infected with *L. braziliensis* (10 parasites: 1 cell) for 1 h. ROS production was analyzed by NBT assay as described in Materials and Methods. Control corresponds to untreated cells. Data represent median and individual values (n = 7 donors). $^{*}P < 0.05$, by Kruskal–Wallis/Dunn's test.

Furthermore, PAFR-deficient mice are more susceptible to *L. amazonensis* infection, suggesting that PAFR signaling is essential for murine infection control (Santiago *et al.* 2006). In accordance, our study improves the knowledge about the role of PAF in leishmaniasis by showing that PAF also induces microbicidal activity in *L. braziliensis*-infected human macrophages.

The mechanisms used by human macrophages to eliminate *Leishmania* parasites have not yet been fully elucidated. In this study, whereas treatment with exogenous PAF increased ROS production inhibition of endogenous PAF caused a reduction in ROS induced by *L. braziliensis* in human macrophages (Fig. 6). Furthermore, inhibition of ROS production by apocynin reversed the PAF-induced microbicidal effects in these cells, indicating that ROS induction is essential for leishmanicidal effect of PAF in humans (Fig. 7). It has been demonstrated that ROS, but not NO, participates in the control of *L. braziliensis* infection both in monocytes from healthy donors and in lesions of patients with ATL (Novais *et al.* 2014). In addition, inhibition of ROS in monocytes of patients with cutaneous leishmaniasis caused by *L. braziliensis* favors the growth of the parasites in these cells (Carneiro *et al.* 2016). Priming with PAF increases the production of ROS inhuman neutrophils and macrophages (Hartung *et al.* 1983; Gardner, Laskin and Laskin 1993; Partrick *et al.* 1997; Verouti *et al.* 2011; Aquino *et al.* 2016). In agreement, in this study priming with PAF and the fast PAF production after *Leishmania* infection led to an increase of macrophage ROS production during the first hour of infection, which was crucial for controlling parasite infection. In experiments in which the PAF antagonist was added after 4 h of infection, no significant effect was observed after 48 h of incubation (Fig. S3, Supporting Information) corroborating the suggestion that PAF is need during the first

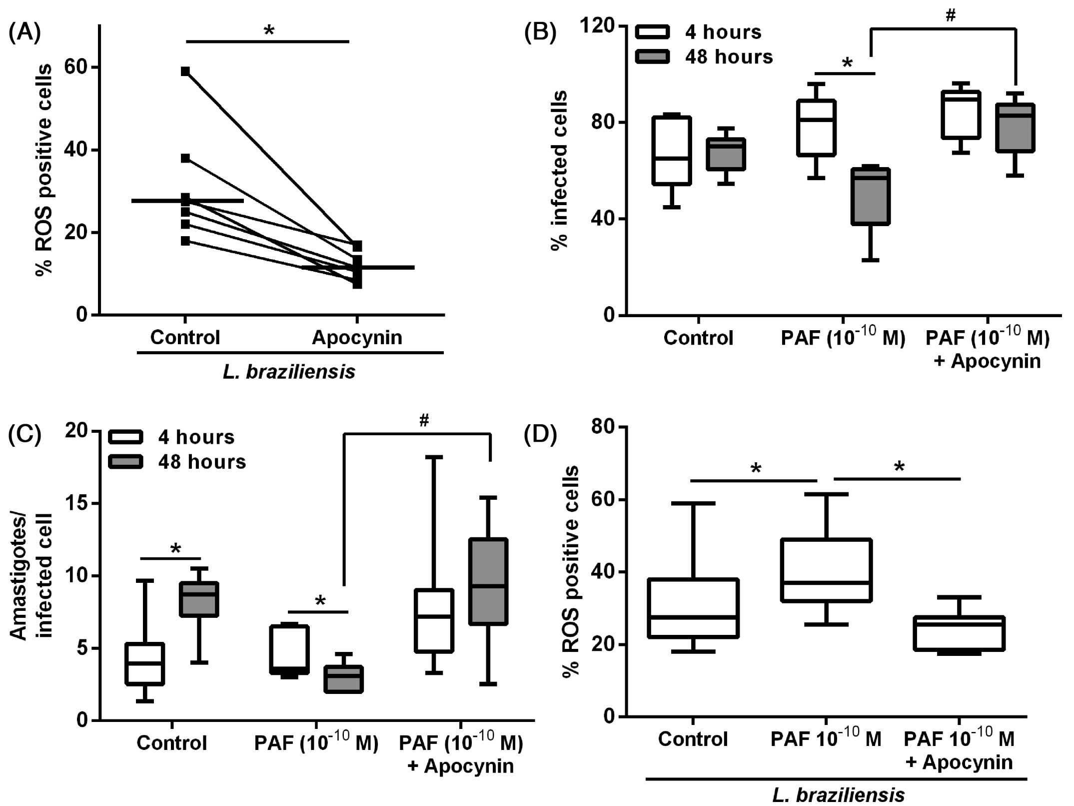

Figure 7. Inhibition of ROS production reverses the PAF-microbicidal effect. Human macrophages were pretreated with PAF 10^{-10} M (3 h before infection) and/or apocynin 10^{-4} M (30 min before infection) and infected with *L. braziliensis* (10 parasites: 1 cell) for 1 h for NBT assay, as described in Materials and Methods, or 4 h and 48 h for assessment of infection rates. Control corresponds to untreated cells. **(A)** Percentage of ROS positive cells in apocynin-treated macrophages infected with *L. braziliensis*. **(B)** Percentage of infected macrophages. **(C)** Number of amastigotes per infected macrophage. **(D)** Percentage of ROS positive cells in apocynin-treated macrophages infected with *L. braziliensis*. In A, the data represent median and individual values (n = 7 donors); $^{*}P < 0.05$, compared to control, by Wilcoxon paired test. In B–D, the data represent median, interquartile and minimal and maximal values (n = 7 donors); $^{*}P < 0.05$, by Wilcoxon paired test and $^{**}P < 0.05$, by Kruskal–Wallis/Dunn's test.

hours to contribute for *L. braziliensis* death. Considering NO as a microbicidal molecule produced by macrophages, it is important to stress that human macrophages produced very low levels of NO after *Leishmania* infection (Carneiro *et al.* 2016). Nevertheless, in murine models, NO is known to play a key role in the control of *Leishmania* spp. Infection (Liew *et al.* 1990; Rocha *et al.* 2007; Gomes *et al.* 2016) and PAF increases macrophage microbicidal activity against *L. amazonensis* in an NO-dependent manner (Lonardoni, Russo and Jancar 2000; Santiago *et al.* 2006). In addition, in mice with NADPH oxidase deficiency, the control of *L. braziliensis* infection is not affected, whereas iNOS deficiency leads to an increase of lesions, suggesting that NO, but not ROS, is required to kill *L. braziliensis* in mouse models (Rocha *et al.* 2007). These results highlight the differences between mice and humans concerning mechanisms to kill *Leishmania* spp., which are present in different levels of relevance in human and murine macrophages, but both are upregulated by PAF. How PAF improves the production of ROS has been investigated. It is known that ROS production is upregulated by increased intracellular Ca^{2+}concentration (Nunes and Demaurex 2010) and by activation of protein kinase C (Chakraborty, Ghosh and Basu 2000). Ca^{2+} is involved in the activation of the NADPH complex, acting as a second messenger in ROS pathway. Since PAF activates intracellular Ca^{2+} and PKC (Huang *et al.* 1988), superoxide anion production is expected to increase after PAF stimulation, as previously demonstrated in murine macrophages (Huang *et al.* 1988) and in equine macrophages and neutrophils (Muehlmann *et al.* 2012). Thus, we can assume that these mechanisms can explain ROS increase in *L. braziliensis*-infected macrophages after PAF stimulation, but these mechanisms still remain to be investigated.

In summary, we have shown that both endogenous and exogenous PAF increase the microbicidal activity of *L. braziliensis*-infected human macrophages in an ROS-dependent manner. The knowledge about the role of PAF in increasing ROS and inhibiting the growth and/or induction of death of parasites in human cells is central to improve therapeutic strategies for ATL. PAF signaling has been shown to be essential for murine visceral leishmaniasis control induced by miltefosine, an important drug in the leishmaniasis treatment (Croft, Seifert and Yardley 2006; Soto and Toledo 2007). In those studies, PAF receptor-deficient or PAF antagonist-treated macrophages were less responsive to miltefosine losing their effects on *Leishmania* killing (Gangalum *et al.* 2015). Thus, our data pave the way for the study of the role of PAF/PAFR signaling in human leishmaniasis caused by *L. braziliensis* and other species.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

ACKNOWLEDGEMENTS

Authors would like to thank Dr Célio de Oliveira Santos and all workers of Blood Bank of Hospital das Clínicas/Universidade Federal de Goiás, and also Dr Yuri Vasconcelos Pinheiro and all workers of Blood Bank of INGOH (Instituto Goiano de Oncolologia e Hematologia) for helping us with blood donors.

FUNDING

This work was supported by FAPEG (Fundação de Amparo à Pesquisa do Estado de Goiás (Universal 002/2007), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento do Ensino Superior; Procad NF 179/2010-2015). FR-D and MAPO are fellow researchers of CNPq; AFB and RSG were fellows of CAPES and CNPq, respectively.

Conflict of interest. The authors declare no conflict of interests.

REFERENCES

Alvar J, Vélez ID, Bern C *et al.* Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 2012;**7**:e35671.

Aquino EN, Neves ACD, Santos KC *et al.* Proteomic analysis of neutrophil priming by PAF. *Protein Pept Lett* 2016;**23**:142–51.

Carneiro PP, Conceição J, Macedo M *et al.* The role of nitric oxide and reactive oxygen species in the killing of *Leishmania braziliensis* by monocytes from patients with cutaneous leishmaniasis. *PLoS One* 2016;**11**:e0148084.

Chakraborty P, Ghosh D, Basu MK. Macrophage protein kinase C: its role in modulating membrane microviscosity and superoxide in leishmanial infection. *J Biochem* 2000;**127**:185–90.

Croft SL, Seifert K, Yardley V. Current scenario of drug development for leishmaniasis. *Indian J Med Res* 2006;**123**:399–410.

de Souza Carmo EV, Katz S, Barbiéri CL. Neutrophils reduce the parasite burden in *Leishmania (Leishmania) amazonensis*-infected macrophages. *PLoS One* 2010;**5**:e13815.

Gangalum PR, de Castro W, Vieira LQ *et al.* Platelet-activating factor receptor contributes to antileishmanial function of miltefosine. *J Immunol* 2015;**194**:5961–7.

Garcia CC, Russo RC, Guabiraba R *et al.* Platelet-activating factor receptor plays a role in lung injury and death caused by Influenza A in mice. *PLoS Pathog* 2010;**6**:e1001171.

Gardner CR, Laskin JD, Laskin DL. Platelet-activating factor-induced calcium mobilization and oxidative metabolism in hepatic macrophages and endothelial cells. *J Leukoc Biol* 1993;**53**:190–6.

Gomes CM, Ávila LR, Santos JC *et al.* *Leishmania (Viannia) braziliensis* amastigotes from patients with mucosal leishmaniasis have increased ability to disseminate and are controlled by nitric oxide at the early stage of murine infection. *Pathog Dis* 2016;**74**:ftw023.

Handman E, Bullen DV. Interaction of *Leishmania* with the host macrophage. *Trends Parasitol* 2002;**18**:332–4.

Hartung HP, Parnham MJ, Winkelmann J *et al.* Platelet activating factor (PAF) induces the oxidative burst in macrophages. *Int J Immunopharmacol* 1983;**5**:115–21.

Huang SJ, Monk PN, Downes CP *et al.* Platelet-activating factor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate stimulates the production of reactive oxygen intermediates in macrophages. *Biochem J* 1988;**249**:839–45.

Hwang SB. High affinity receptor binding of platelet-activating factor in rat peritoneal polymorphonuclear leukocytes. *Eur J Pharmacol* 1991;**196**:169–75.

Ichinose M, Hara N, Sawada M *et al.* A flow cytometric assay reveals an enhancement of phagocytosis by platelet activating factor in murine peritoneal macrophages. *Cell Immunol* 1994;**156**:508–18.

Kamaladasa A, Gomes L, Jeewandara C *et al.* Lipopolysaccharide acts synergistically with the dengue virus to induce monocyte production of platelet activating factor and other inflammatory mediators. *Antiviral Res* 2016;**133**:183–90.

Kim HA, Kim SH, Ko HM *et al.* Nitric oxide plays a key role in the platelet-activating factor-induced enhancement of resistance against systemic candidiasis. *Immunology* 2008;**124**:428–35.

Kroegel C, Yukawa T, Westwick J *et al.* Evidence for two platelet activating factor receptors on eosinophils: dissociation between PAF-induced intracellular calcium mobilization degranulation and superoxides anion generation in eosinophils. *Biochem Biophys Res Commun* 1989;**162**:511–21.

Lacerda-Queiroz N, Rodrigues DH, Vilela MC *et al.* Platelet-activating factor receptor is essential for the development of experimental cerebral malaria. *Am J Pathol* 2012;**180**:246–55.

Lee JJ, Simborio HL, Reyes AW *et al.* Influence of platelet-activating factor receptor (PAFR) on *Brucella abortus* infection: implications for manipulating the phagocytic strategy of *B. abortus*. *BMC Microbiol* 2016;**16**:70.

LeVan TD, Dow SB, Chase PB *et al.* Evidence for platelet-activating factor receptor subtypes on human polymorphonuclear leukocyte membranes. *Biochem Pharmacol* 1997; **54**:1007–12.

Liew FY, Millott S, Parkinson C *et al.* Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. *J Immunol* 1990;**144**:4794–7.

Liu D, Uzonna JE. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. *Front Cell Infect Microbiol* 2012;**2**:83.

Liu M, Zhu H, Li J *et al.* Group A *Streptococcus* Secreted esterase hydrolyzes platelet-activating factor to impede neutrophil recruitment and facilitate innate immune evasion. *PLoS Pathog* 2012;**8**:e1002624.

Lonardoni MV, Russo M, Jancar S. Essential role of platelet-activating factor in control of *Leishmania (Leishmania) amazonensis* infection. *Infect Immun* 2000;**68**:6355–61.

Morato CI, da Silva IA, Borges AF *et al.* Essential role of leukotriene B4 on *Leishmania (Viannia) braziliensis* killing by human macrophages. *Microbes Infect* 2014;**16**:945–53.

Muehlmann LA, Michelotto PV, Nunes EA *et al.* PAF increases phagocytic capacity and superoxide anion production in equine alveolar macrophages and blood neutrophils. *Res Vet Sci* 2012;**93**:393–7.

Muniz-Junqueira MI, de Paula-Coelho VN. Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-α production, but only via TNF-α it indirectly increases nitric oxide production by phagocytes of healthy individuals, in vitro. *Int Immunopharmacol* 2008;**8**:1633–8.

Novais FO, Nguyen BT, Beiting DP *et al.* Human classical monocytes control the intracellular stage of *Leishmania braziliensis* by reactive oxygen species. *J Infect Dis* 2014;**209**:1288–96.

Nunes P, Demaurex N. The role of calcium signaling in phagocytosis. *J Leukoc Biol* 2010;**88**:57–68.

O'Flaherty JT, Jacobson DP, Redman JF. Regulation of platelet-activating-factor receptors and the desensitization response in polymorphonuclear neutrophils. *Biochem J* 1992:241–8.

Partrick DA, Moore EE, Moore FA *et al.* Lipid mediators upregulate CD11b and prime for concordant superoxide and elastase release in human neutrophils. *J Trauma* 1997;**43**:297–302.

Pustynnikov MG, Porodenko, NV, Makarova, OV *et al.* Platelet-activating factor stimulates receptor-mediated formation of reactive oxygen intermediates in human monocytes. *Lipids* 1991;**26**:1214–7.

Rocha FJ, Schleicher U, Mattner J *et al.* Cytokines, signaling pathways, and effector molecules required for the control of *Leishmania (Viannia) braziliensis* in mice. *Infect Immun* 2007;**75**:3823–32.

Rosa MS, Vieira RB, Pereira AF *et al.* Platelet-activating factor (PAF) modulates peritoneal mouse macrophage infection by *Leishmania amazonensis*. *Curr Microbiol* 2001;**43**:33–7.

Santiago HC, Braga Pires MF, Souza DG *et al.* Platelet activating factor receptor-deficient mice present delayed interferon-γ upregulation and high susceptibility to *Leishmania amazonensis* infection. *Microbes Infect* 2006;**8**:2569–77.

Santos PC, Teixeira MM, Souza DG. Opportunities for the development of novel therapies based on host-microbial interactions. *Pharmacol Res* 2016;**112**:68–83.

Shalit M, Von Allmen C, Atkins PC *et al.* Platelet activating factor increases expression of complement receptors on human neutrophils. *J Leukoc Biol* 1988;**44**:212–7.

Shimizu T. Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu Rev Pharmacol Toxicol* 2009;**49**:123–50.

Silveira FT, Lainson R, De Castro Gomes CM *et al.* Immunopathogenic competences of *Leishmania (V.) braziliensis* and *L. (L.) amazonensis* in American cutaneous leishmaniasis. *Parasite Immunol* 2009;**31**:423–31.

Soares AC, Pinho VS, Souza DG *et al.* Role of the platelet-activating factor (PAF) receptor during pulmonary infection with gram negative bacteria. *Br J Pharmacol* 2002;**137**:621–8.

Soto J, Toledo JT. Oral miltefosine to treat new world cutaneous leishmaniasis. *Lancet Infect Dis* 2007;**7**:7.

Souza DG, Fagundes CT, Sousa LP *et al.* Essential role of platelet-activating factor receptor in the pathogenesis of dengue virus infection. *Proc Natl Acad Sci U S A* 2009;**106**:14138–43.

Talvani A, Santana G, Barcelos LS *et al.* Experimental *Trypanosoma cruzi* infection in platelet-activating factor receptor-deficient mice. *Microbes Infect* 2003;**5**:789–96.

Ueno N, Wilson ME. Receptor-mediated phagocytosis of *Leishmania*: implications for intracellular survival. *Trends Parasitol* 2012;**28**:335–44.

Uhlig S, Göggel R, Engel S. Mechanisms of platelet-activating factor (PAF)-mediated responses in the lung. *Pharmacol Rep* 2005;**57**:206–21.

Verouti SN, Fragopoulou E, Karantonis HC *et al.* PAF effects on MCP-1 and IL-6 secretion in U-937 monocytes in comparison with OxLDL and IL-1β effects. *Atherosclerosis* 2011;**219**:519–25.

Vilela MC, Lima GK, Rodrigues DH *et al.* Platelet activating factor (PAF) Receptor deletion or antagonism attenuates severe HSV-1 meningoencephalitis. *J Neuroimmune Pharmacol* 2016;**11**:613–21.

Yost CC, Weyrich AS, Zimmerman GA. The platelet activating factor (PAF) signaling cascade in systemic inflammatory responses. *Biochimie* 2010;**92**:692–7.

Zauli-Nascimento RC, Miguel DC, Yokoyama-Yasunaka JK *et al.* In vitro sensitivity of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* Brazilian isolates to meglumine antimoniate and amphotericin B. *Trop Med Int Health* 2010;**15**:68–76.