Prostaglandins D_2 and E_2 have opposite effects on alveolar macrophages infected with *Histoplasma capsulatum*[s]

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Abstract Prostaglandin E_2 (PGE_2) suppresses macrophage effector mechanisms; however, little is known about the function of PGD_2 in infected alveolar macrophages (AMs). Using serum-opsonized *Histoplasma capsulatum* (Ops-*H. capsulatum*) in vitro, we demonstrated that AMs produced PGE_2 and PGD_2 in a time-dependent manner, with PGE_2 levels exceeding those of PGD_2 by 48 h postinfection. Comparison of the effects of both exogenous PGs on AMs revealed that PGD_2 increased phagocytosis and killing through the chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes receptor, whereas PGE_2 had opposite effects, through E prostanoid (EP) receptor 2 (EP2)/EP4-dependent mechanisms. Moreover, PGD_2 inhibited phospholipase C-γ (PLC-γ) phosphorylation, reduced IL-10 production, and increased leukotriene B4 receptor expression. In contrast, exogenous PGE_2 treatment reduced PLC-γ phosphorylation, p38 and nuclear factor κB activation, TNF-α, H_2O_2, and leukotriene B_4, but increased IL-1β production. Using specific compounds to inhibit the synthesis of each PG in vitro and in vivo, we found that endogenous PGD_2 contributed to fungicidal mechanisms and controlled inflammation, whereas endogenous PGE_2 decreased phagocytosis and killing of the fungus and induced inflammation. [Jlr] These findings demonstrate that, although PGD_2 acts as an immunostimulatory mediator to control *H. capsulatum* infection, PGE_2 has immunosuppressive effects, and the balance between these two PGs may limit collateral immune damage at the expense of microbial containment.—Pereira, P.A.T., P. A. Assis, M. K. B. Prado, S. G. Ramos, D. M. Aronoff, F. W. G. de Paula-Silva, C. A. Sorgi, and L. H. Faccioli. Prostaglandins D2 and E2 have opposite effects on alveolar macrophages infected with *Histoplasma capsulatum*. *J. Lipid Res.* 2018. **59**: 195–206.

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Histoplasmosis is a fungal disease caused by *Histoplasma capsulatum*, mainly affecting the respiratory tract (1). The incidence of histoplasmosis has increased worldwide, which is mostly associated with immunodeficiency, such as HIV (2–4). In the lung, the immune response begins after the uptake of the fungus by alveolar macrophages (AMs) and dendritic cells. AMs have a special importance in lung defense, where they are responsible for protecting the alveolar epithelium by clearing microorganisms by phagocytosis and intracellular killing (5). These innate immune sentinel cells are among the first and most essential participants in the successful elimination of the fungus. Furthermore, AM-derived cytokines and lipid mediators induce neutrophil and mononuclear cell recruitment (5) and activation of adaptive immune responses (6, 7), and regulate phagocytosis and antimicrobial activities of phagocytic cells (5, 7, 8).

In particular, the synthesis and signaling of lipid mediators known as leukotrienes (LTs) and prostaglandins (PGs)

are increased during histoplasmosis and further regulate host defense (8, 9). Biosynthesis of PGs is coordinated by two distinct cyclooxygenase (COX) isoforms, the constitutive COX-1 and the inducible COX-2, which convert arachidonic acid (AA) to the unstable intermediate compound PGH_2. Then, terminal synthase enzymes, including PGD and PGE synthases, generate PGD_2 and PGE_2, respectively, at sites of inflammation, resulting in either proinflammatory or anti-inflammatory effects, depending on the nature of the stimulus (10, 11). Recently, we demonstrated that, during lethal *H. capsulatum* infection, pharmacological inhibition of COX-2 by the compound celecoxib increased mouse survival and the phagocytic capacity of AMs, suggesting a contribution of PGs to pathogenesis of this infection (8). Additionally, during bacterial and other fungal infections, PGE_2 and PGD_2 production is enhanced in the lung (8, 12). However, the specific roles of PGD_2 and PGE_2 during infections, especially in histoplasmosis, remain incompletely defined.

Over time, PGE_2 has emerged as a potent endogenous modulator of innate immunity and macrophage effector functions (10, 13). Generally, PGE_2 promotes endothelial cell-mediated vasodilatation and recruitment of circulating leukocytes during inflammation to areas of infection, through mechanisms activated by PGE_2 binding to E prostanoid (EP) receptors (EP1–4) coupled to G proteins present in the cell membrane (13). On the other hand, PGE_2 inhibits macrophage effector mechanisms, such as phagocytosis, through EP2 receptor and bacterial killing through EP2–4 receptors, both coupled to Gαs proteins, leading to activation of adenylate cyclase, which increases cyclic adenosine monophosphate (cAMP) concentrations (14–16) and IL-1β production (16). PGD_2, in turn, binds to the D prostanoid receptor 1 (DP1) and to the "chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes" receptor (DP2 or CRTH2) (17). PGD_2 binds to DP1, a transmembrane receptor coupled to the G-protein subunit Gαs. This prostanoid also induces elevation of cAMP, resulting in the inhibition of effector mechanisms of macrophages and other cells (18–20). However, when PGD_2 binds to DP2, a Gαi protein subunit coupled receptor, it reduces cAMP concentrations (21). Consequently, the potentially contrasting immunoregulatory roles of PGD_2 and PGE_2 require further investigation, which is the focus of the present research.

Mammalian cells communicate with each other by exchanging signals that bind specifically to surface or intracellular receptors, followed by a cascade of events that amplify and transduce the incoming signal and eventually elicit a cellular response. The cAMP-dependent protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and nuclear factor κB (NF-κB) cascades modulate common processes in the cell, and multiple levels of cross-talk between these signaling pathways have been described (22, 23). Because activation of PG receptors/cAMP axes is important to regulate macrophage effector functions (14, 15), the putative role of PGD_2 and PGE_2 in cell signaling activation and inflammatory mediator synthesis deserves detailed examination.

Despite the fact that PGE_2 has been shown to inhibit phagocytosis and killing of pathogens by macrophages (14, 15), the role of PGD_2 in modulating AM effector functions is not known. In the present study, we determined the contribution of endogenous and exogenous PGD_2 and PGE_2 on AM effector functions after immune serum (IS)-opsonized *H. capsulatum* (Ops-*H. capsulatum*) infection. Using rat AMs, we found that endogenous and exogenous PGD_2 and PGE_2 actions result in divergent effects on phagocytosis and fungicidal activities of these cells. Treatment of Ops-*H. capsulatum*-infected AMs with exogenous PGD_2 increased phagocytosis and killing through the DP2 receptor; it also inhibited phosphorylation of phospholipase C-γ (PLC-γ) without affecting c-Jun N-terminal kinase 1/2 (JNK1/2), p38, and NF-κB. Furthermore, PGD_2 inhibited IL-10 production by infected AMs, while increasing the expression of high-affinity receptors for LTB_4 (BLT1). An opposite effect was observed for PGE_2, which reduced phagocytosis and killing through an EP2/EP4-dependent manner. Additionally, it diminished phosphorylation of PLC-γ, p38, and NF-κB, while amplifying JNK1/2, and reducing TNF-α, H_2O_2, and LTB_4 production by Ops-*H. capsulatum*-infected AMs. Finally, in vivo experiments demonstrated that inhibition of PGD_2 synthesis increased susceptibility to infection in mice, as well as increased inflammatory cytokine production, while inhibition of PGE_2 synthesis increased resistance against infection and diminished lung tissue damage. Also, endogenous PGD_2 regulated only fungicidal mechanisms, whereas endogenous PGE_2 coordinated phagocytosis and killing of the fungus by AMs. Given the opposite effects of PGD_2 and PGE_2 during AM infection, these lipids might be potential targets to treat fungal lung diseases, particularly histoplasmosis.

MATERIALS AND METHODS

Animals

Pathogen-free male Wistar rats (125–150 g) and male C57BL/6 mice (20–22 g) were obtained from the animal facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of the University of São Paulo (Protocols 09.1.375.53.5 and 013.2016-1). The in vitro experiments with *H. capsulatum*-infected AMs and infected animals were performed in level 3 biohazard facilities at Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo.

Culture of *H. capsulatum*

The *H. capsulatum* clinical isolate was obtained from a patient at the Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. The mycelia were obtained by culturing fungi at 25°C in Sabouraud dextrose agar tubes (Difco, Detroit, MI), and the live yeast fungus was subcultured at 37°C on glutamine-cysteine-sheep blood (5%) BHI (Detroit, MI) for 15 days. Yeast cells were used when their viability was ≥90% according to fluorescein diacetate (Sigma-Aldrich, St. Louis, MO) and ethidium bromide (Sigma-Aldrich) staining (8, 9).

IS and opsonization

Rats were intraperitoneally inoculated with 1 ml containing 10^8 yeast of *H. capsulatum,* and 10 days later were submitted to a second

inoculation with an equal inoculum. After 7 days, the rats were decapitated, and blood was collected and centrifuged at 1,900 g for 10 min to obtain the IS. IS was heated at 56°C for 1 h to inactivate complement proteins and stored at $-80°C$ (24). For fungus opsonization, 1×10^8 yeast in 1 ml of PBS was incubated with 10% IS for 30 min at 37°C on a rotating platform (7), and the opsonized fungus is referred to as Ops-*H. capsulatum*. Nonopsonized fungi (*H. capsulatum*) incubated only with PBS were used for comparison as described.

AM isolation, cell culture, and treatments

Resident AMs from naïve rats were obtained via ex vivo lung lavage (24) and suspended in incomplete RPMI 1640 at 2×10^6 cells per ml. Cells were allowed to adhere to tissue culture plates for 1 h (37°C, 5% CO_2), followed by two washes with warm incomplete RPMI 1640, resulting in ± 99% of adherent cells identified as AMs by staining with Panoptic (Laborclin, Paraná, Brazil). Cells were cultured overnight in complete RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin/amphotericin B (Gibco, Grand Island, NY). The following day, cells were washed twice with warm medium (incomplete RPMI 1640) to remove nonadherent cells. AMs were preincubated with indomethacin (10 µM) (COX1/2 inhibitor; Sigma-Aldrich), or celecoxib (10 µM) (COX-2 inhibitor; Celebra®, Pfizer, SP, Brazil), or HQL-79 (1 µM) (PGD_2 synthase inhibitor); or CAY10526 (1 µM) (PGE_2 synthase inhibitor); or BWA868c (1 µM) (DP1 antagonist); or Bay-u3405 (1 µM) (DP2 antagonist); or AH6809 (1 µM) (EP2 antagonist); or AH23848 (1 µM) (EP4 antagonist), preceding fungus infection. The enzyme inhibitors or antagonists were purchased from Cayman Chemical (Ann Arbor, MI), and incubated with AMs for 30 min before infection. When necessary, cells were incubated with PGD_2 or PGE_2 for 2 min (1 µM) (Cayman Chemical), before in vitro infection. Receptor antagonist and concentrations used were previously determined by our group (16, 25) or investigated in the literature (14, 15, 26, 27). Incomplete RPMI 1640 containing the same concentrations of DMSO and/or alcohol used to dissolve the compounds was used as control and identified as vehicle. Cells treated with compounds or vehicle were used for phagocytic and fungicidal assays.

Fluorometric phagocytosis assay with FITC-labeled *H. capsulatum*

A fluorometric phagocytosis assay was performed to test the capacity of AMs to phagocytize FITC-labeled *H. capsulatum* (or Ops-*H. capsulatum*) as published previously (7). Briefly, yeast cells were labeled with FITC (Amresco, OH) for 1 h at 37°C (7). FITC-labeled Ops-*H. capsulatum* or FITC-labeled *H. capsulatum* was added, and the number of yeast cells to be used by AMs was determined through multiplicity of infection (MOI) starting from 1:1; 1:5, or 1:10, respectively. The AMs were pretreated or not with the compounds as described above before the fungus was added and then incubated in the dark (37°C, 5% CO_2). After 2 h, free yeast cells were removed by washing with warm sterile PBS, and the residual extracellular FITC was quenched with Trypan blue (250 mg/ml; Gibco) for 1 min. Fluorescence was determined by using a micro plate reader (485 nm excitation/535 nm emission, SPECTRA-Max, Molecular Devices, Sunnyvale, CA). Phagocytosis was determined by the mean of relative fluorescence units (MFI) emitted from intracellular fungi.

Fungicidal activity assay

AMs were pretreated with IFN-γ (50 ng/ml) overnight to improve their effector mechanism as described by Peck (28), and submitted or not to the above treatments. Next, cells were incubated with *H. capsulatum* (opsonized or not) at MOI 1:10, and after 2 h, cells were washed twice with warm sterile PBS to remove

the extracellular yeasts. Following another 48 h of incubation, the supernatants were collected and kept at $-80°C$ until they were used for measurements, as described. Afterward, the cells were lysed by adding 200 µl of 0.05% saponin, and an aliquot was plated on BHI agar-blood (29). After 21 days of culture at 37°C, the colony-forming units (CFU) were counted and the fungicidal activity was calculated according to the formula: [100 - (100 × CFU experimental)/CFU control].

Quantitation of cytokines

AMs treated or not with the above compounds were incubated with Ops-*H. capsulatum*, and after 48 h the cell culture supernatants or lung homogenate of *H. capsulatum*-infected mice were obtained to measure TNF-α, IL-1β, and IL-10, by using commercially available ELISA kits (R&D Systems, Minneapolis, MN). For each sample, the cytokine concentrations were obtained from a standard curve established with the appropriate recombinant cytokine. The sensitivities were >10 pg/ml.

Hydrogen peroxide production

The release of H_2O_2 from AMs was determined by phenol red oxidation, as described previously (30), in supernatant from cells pretreated or not with PGD_2 or PGE_2, and incubated with Ops-*H. capsulatum* (MOI 1:10). After 48 h of incubation, the supernatants were replaced by supplemented assay medium (RPMI 1640-containing peroxidase and phenol red) and incubated for 2 h. Then, stop solution (NaOH 1N) was added, and optical density (OD) was determined at 620 nm.

Flow-cytometry analysis of BLT1 expression in AMs

The expression of BLT1 was determined by using the flow-cytometry immune staining protocol with antibodies conjugated with fluorochromes (BD Biosciences, Franklin Lakes, NJ). The AMs (2×10^5 cells) were pretreated with the compounds described above before the fungus was added (2×10^4 cells) and incubated at 37°C, 5% CO_2. After 2 h, the supernatant was removed, and the cells were washed with 300 µl of PBS containing 2% FBS (Gibco). Cells were then suspended in polystyrene tubes and centrifuged at 400 g for 5 min. Cells were suspended in Fc block (200 µl), containing anti-CD16/CD32 antibodies at dilution 1:100 and incubated for 30 min at 4°C. Subsequently, the antibody against BLT1 was added to the AMs. After 40 min, cells were washed twice, and then fixed with PBS containing 1% (wt/vol) paraformaldehyde. A total of 20,000 events was acquired for each tube (FACSCanto™; Becton Dickinson), using the FACSDiva software.

Measurements of PGE_2, PGD_2, and LTB_4

Eicosanoids were purified from the supernatant of AMs cultures or lung homogenate (Mixer Homogenizer, IKA, Wilmington, NC) by using Sep-Pak C18 cartridges according to the manufacturer's instructions (Waters Corp.. Milford, MA). Quantifications of PGE_2 and LTB_4 (Enzo Life Science, Farmingdale, NY) and PGD_2 (Cayman Chemical) were performed by using specific enzyme immunoassay kits according to manufacturers' instructions, and the results were expressed in picograms per milliliter, or, after the data were transformed, as percentages, and infected AM was set as 100%.

Phosphoprotein detection by cytometric bead array

Samples of AMs were prepared as previously described (31). After infection and treatments, cells were washed with ice-cold PBS and then lysed with buffer containing protease and phosphatase inhibitors according to the manufacturer's protocol for adherent cells (Becton Dickinson, Heidelberg, Germany). The cell lysates were moved to Eppendorf tubes and immediately placed in

a boiling water bath for 5 min. The total protein concentration was adjusted to 1 μg/μl, and the cell lysates were stored at −80°C until measurement of kinase phosphorylation. The total protein content of the lysates was determined by using the Bradford method (Sigma-Aldrich,). Quantitative determination of phospho-JNK1/2 (T183/Y185), phospho-p38 (T180/Y182), and phospho-PLC-γ (Y783) was performed by using antibodies from the multiplex Flex Set Cytometric Bead Array (Becton Dickinson). Flow-cytometric analysis was performed using FACSCanto™, and FACSDiva was used for data acquisition and analysis (Becton Dickinson). A total of 900 events was acquired.

RAW-Blue™ cells experiment

To analyze the influence of PGE_2 and PGD_2 on NF-κB activation induced by the fungus, we used RAW-Blue™ cells (Invivogen). RAW-Blue™ is a cell line of macrophages that stably express the SEAP gene (secreted embryonic alkaline phosphatase), which is induced by NF-κB/AP-1 transcription factors and confers resistance to Zeocin™. These cells were grown in DMEM supplemented with 10% FBS, Normocin™ (50 μg/ml), and Zeocin™ (25 μg/ml). The cells were seeded in 96-well microculture plates at a density of 2×10^5 cells per well in DMEM supplemented with Normocin™ (50 mg/ml) and cultured at 37°C in a humidified 5% CO_2 atmosphere for 18 h. After this period, the cells were stimulated with PGD_2 or PGE_2 for 2 min and then incubated with Ops-*H. capsulatum* (2×10^4 yeast per well) for 24 h. After this period, the medium was collected, and samples of 50 μl were mixed in 96-well plates at 37°C for 2 h, with 150 μl of QUANTI-Blue™ (Invivogen), which is a SEAP detection medium. The OD was then measured at 650 nm by using an ELISA reader (μQuant, Biotek Instruments Inc., Winooski, VT).

Infection and in vivo pharmacological treatments

Mice were anesthetized with ketamine and xylazine (10 and 20 mg/kg, respectively) and restrained on a small board. A 30-gauge needle attached to a tuberculin syringe was inserted into the trachea, and a lethal inoculum of *H. capsulatum* in PBS (1×10^6 yeast/100 μl per mouse) was intratracheally dispensed into the lungs. Infected mice were treated by gavage with inhibitor of PGD_2 synthase (HQL-79, Cayman Chemical; 3 mg/kg/0.5 ml of water, dose chosen based on dose-response experiments ranging from 0.3 to 3 mg/kg) (32), or inhibitor of PGE_2 synthase (CAY10526, Cayman Chemical; 5 mg/kg/0.5 ml of water) (33) or water (0.5 ml), 1 h before infection and daily for 30–40 days. As a control, mice were injected with PBS and treated daily with water.

Histology

At 7 days postinfection, the lungs were removed and immediately fixed in 10% formalin for conventional histological examination. The specimens were processed, embedded in paraffin, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin (H&E) and analyzed in a blinded fashion at 100× magnification. ImageJ software (NIH, Bethesda, MD) was used to calculate the lung area damage in five random photomicrograph sections per slide, which was represented by percent lung area covered by infiltrating cells calculated for each mouse by dividing the sum of damage areas in these sections by the total area of the lung examined.

Determination of CFU

After 7 days of infection, the lungs were recovered to determine the fungal burden. *H. capsulatum* yeasts from the lung were examined as previously described (9). Two hundred microliter samples from each cell suspension were collected, and a 10-fold dilution was plated into BHI agar-blood. After incubation at 37°C for 21 days, the *H. capsulatum* CFU was counted and expressed as CFU/g lung (log 10).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Data are expressed as mean ± standard error of the mean (SEM). All experiments were conducted in triplicate unless otherwise noted, and repeated at least twice. Comparisons were performed by using ANOVA followed by Tukey's multiple comparison test as described. In all comparisons, a significance level of $P < 0.05$ was considered to be significant. Differences in survival were analyzed by using the log-rank test. Significance level was set at 5%.

RESULTS

In vitro AMs phagocytized Ops-*H. capsulatum* more efficiently and released PGE_2 and PGD_2 differently

The usual immune response to *H. capsulatum* begins in the lung with the uptake of the fungus by resident AMs that are essential cells for clearing microorganisms by phagocytosis and intracellular killing (29). In addition, it has been demonstrated that *H. capsulatum* induces a humoral immune response and leads to production of circulating immune complexes (34). Since a humoral response is enhanced during the course of infection (34), it may be relevant in the setting of reinfection, particularly in endemic areas. Therefore, we first investigated whether resident AMs produce PGD_2 and PGE_2 after in vitro infection with nonopsonized (*H. capsulatum*) or with specific IS-opsonized *H. capsulatum* (Ops-*H. capsulatum*). As expected, after 2 h, Ops-*H. capsulatum* was more efficiently phagocytized than the nonopsonized fungus (**Fig. 1A**). The phagocytosis induced PGE_2 production by AMs 2 and 48 h following infection, whereas PGD_2 production was increased only at 48 h (Fig. 1B, C). The nonopsonized fungus induced lower amounts of PGs in comparison to the opsonized fungus. Based on these data, Ops-*H. capsulatum* was selected for the next experiments to assess the effects of PGD_2 and PGE_2 on AM effector functions.

Inhibition of COX-1 and COX-2 increased phagocytosis and fungicidal activity of AMs and diminished PGD_2 and PGE_2 production

We next evaluated the extent to which endogenous PGs are required during FcR-mediated phagocytosis and killing of *H. capsulatum* by AMs. AMs were pretreated with indomethacin (10 μM), a dual COX-1/COX-2 inhibitor, or celecoxib (10 μM), a selective COX-2 inhibitor, and 30 min later were infected with IgG-*H. capsulatum*. Both inhibitors increased phagocytosis of Ops-*H. capsulatum* by AMs (**Fig. 2A**). Next, we evaluated the role of PGs in the fungicidal activity of AMs after 48 h of incubation with Ops-*H. capsulatum*. Nonspecific inhibition of COX enzymes or the specific inhibition of COX-2 augmented clearance of IgG opsonized fungi by AMs (Fig. 2B). As expected, both COX inhibitors suppressed the production of PGD_2 and PGE_2
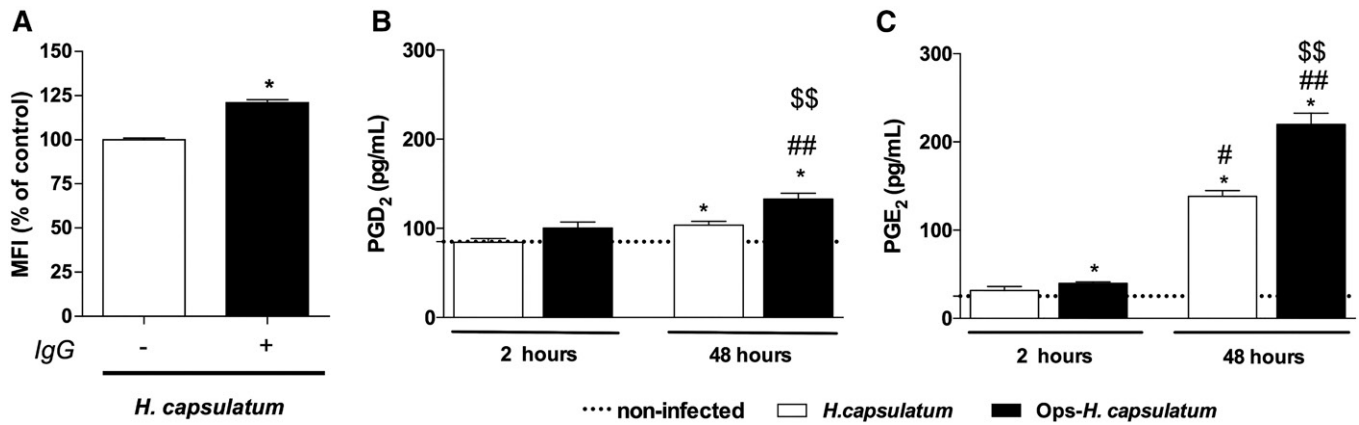
Fig. 1. Ops-*H. capsulatum* are more efficiently phagocytized by AMs and induced higher amounts of PGE_2 and PGD_2 than did *H. capsulatum*. A: AMs were incubated with *H. capsulatum* or Ops-*H. capsulatum* labeled with FITC (MOI 1:10), and phagocytosis was assessed 2 h later. Data are expressed as average of fluorescence intensity (MFI) from internalized yeast (n = 6). To determine PGD_2 (B) and PGE_2 (C) production, AMs were incubated with *H. capsulatum* or Ops-*H. capsulatum* (MOI 1:10) during 2 and 48 h, and the supernatants were collected for PG quantification by immunoassays as described in Materials and Methods (n = 3). * $P < 0.05$ [*H. capsulatum* vs. Ops-*H. capsulatum* (A); noninfected AMs vs. AMs + *H. capsulatum* or vs Ops-*H. capsulatum* (B, C)]; # $P < 0.05$ (48 h AMs + *H. capsulatum* vs. 2 h *H. capsulatum*); ## $P < 0.05$ (48 h AMs + Ops-*H. capsulatum* vs. 2 h Ops-*H. capsulatum*); $$ $P < 0.05$ (48 h AMs + *H. capsulatum* vs. 48 h Ops-*H. capsulatum*). One-way ANOVA and Tukey's multiple comparison tests were used. Data are representative of two independent experiments (±SEM).

(Fig. 2C, D). Our data demonstrate that inhibition of COX-1- or COX-2-mediated PG synthesis during fungal infection increased the phagocytic and fungicidal activity of AMs against Ops-*H. capsulatum*.

PGD_2 and PGE_2 have opposite effects on phagocytosis and fungicidal activity of AMs infected with Ops-*H. capsulatum*

It has been demonstrated that PGE_2 inhibits bacterial phagocytosis and killing by AMs (14, 15). Because we detected that both PGD_2 and PGE_2 are produced by AMs infected with *H. capsulatum* (Fig. 1), we subsequently investigated the biological effect of each PG in regulating phagocytosis and killing by AMs. First, we

showed that the inhibition of endogenous PGD_2 synthesis by HQL-79 at 1 μM (**Fig. 3A**) (and at other concentrations of 0.1 and 10 μM; data not shown) did not modify phagocytosis of Ops-*H. capsulatum* by AMs. In contrast, inhibition of endogenous PGE_2 by CAY10526 at 2 μM increased phagocytosis (Fig. 3B) (and at other concentrations of 0.2 and 20 μM; data not shown). Next, we evaluated the effects of exogenous administration of each PG on phagocytosis. The addition of exogenous PGD_2 (1 μM) increased (Fig. 3A), whereas exogenous PGE_2 (1 μM) inhibited phagocytosis (Fig. 3B) of IgG-*H. capsulatum*. Inhibition of endogenous PGD_2 or PGE_2 did not distinctively alter the effects of the exogenous mediators (Fig. 3A, B).
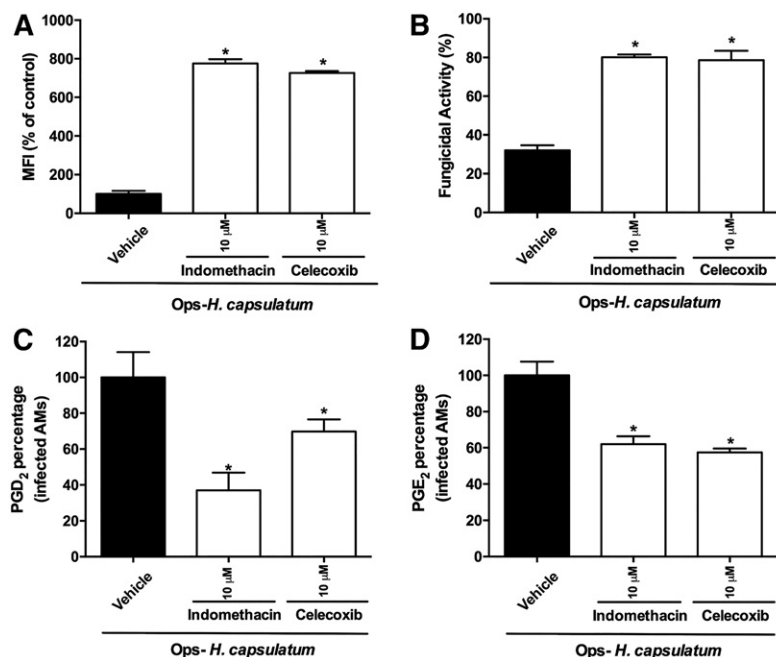

Fig. 2. Inhibition of COX-1 and COX-2 decreased PGD_2 and PGE_2 and augmented phagocytosis and fungicidal activity of AMs. A: AMs were pretreated or not with indomethacin (10 μM) or celecoxib (10 μM) for 30 min prior to addition of Ops-*H. capsulatum* labeled with FITC (MOI 1:10). Phagocytosis was assessed 2 h later. Data are expressed as average of fluorescence intensity (MFI) from internalized yeast (n = 6). B: AMs were incubated with Ops-*H. capsulatum* at MOI 1:10 for 2 h to allow yeast internalization, and, after 48 h, AMs were lysed, and live yeast was determined by CFU number. Fungicidal activity was expressed as a percentage from total internalized yeast (100%) (n = 4). Measurement of PGD_2 (C) and PGE_2 (D) by immunoassays is described in Materials and Methods. The supernatants were collected 2 h after indomethacin or celecoxib treatment and fungal infection (n = 4). Production of PGD_2 and PGE_2 was expressed as a percentage, and infected and vehicle-incubated cells were set as 100% (AMs + Ops-*H. capsulatum*). * $P < 0.05$ [AMs + Ops-*H. capsulatum* (in vehicle) vs. other groups]. One-way ANOVA–Tukey's multiple comparison tests were used. Data are representative of two independent experiments (±SEM).
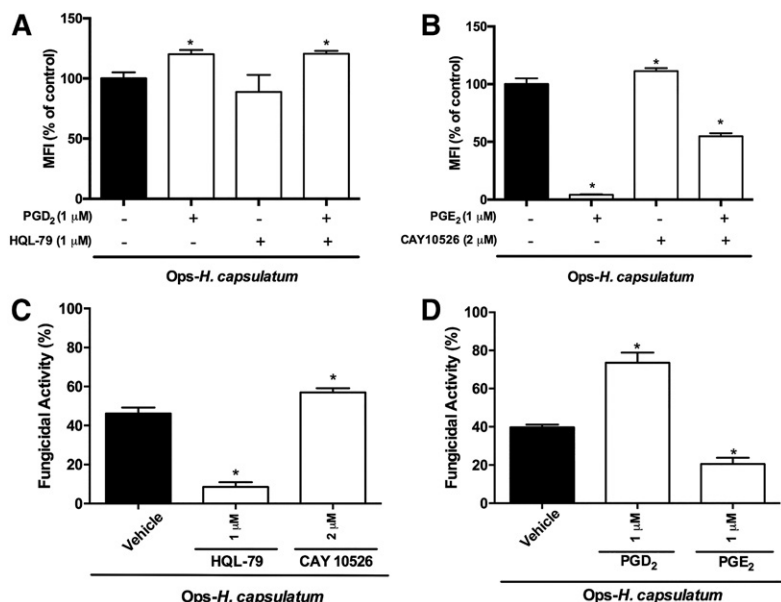
Fig. 3. PGD_2 and PGE_2 have opposite effects on phagocytosis and fungicidal activity of AMs. The effects of pretreating AMs with a specific inhibitor of PGD synthase (HQL-79) or PGE synthase (CAY10526), or soluble PGD_2 or PGE_2 on phagocytosis and fungicidal activity were assessed. AMs were pretreated with HQL-79 (1 μM) or/and PGD_2 (1 μM) (A) or CAY10526 (2 μM) or/and PGE_2 (1 μM) (B), respectively, 30 and 2 min before the addition of Ops-*H. capsulatum* (MOI 1:10) labeled with FITC. Phagocytosis was assessed 2 h later, and data are expressed as average of fluorescence intensity (MFI) from internalized yeast (n = 6). The fungicidal activity was also evaluated following treatment with HQL-79 or CAY10526 (C) or with PGD_2 or PGE_2 (D). After 48 h, AMs were lysed, and CFU number determined live yeast. Fungicidal activity was expressed as a percentage from total internalized yeast by AMs incubated with vehicle (100%) (n = 4). * $P < 0.05$ [AMs + Ops-*H. capsulatum* (in vehicle) vs. other groups]. One-way ANOVA and Tukey's multiple comparison tests were used. Data are representative of two independent experiments (±SEM).

Interestingly, we also found that, after 48 h, inhibition of PGD synthase impaired the killing of Ops-*H. capsulatum* by AMs, whereas inhibition of PGE synthase increased this process (Fig. 3C). These findings indicate that the inhibition of endogenous PGD_2 exerted no effect on the phagocytic ability of AMs. However, these treatments regulated the fungicidal activity of infected AMs differently. Adding exogenous PGD_2 and PGE_2, we confirmed that PGD_2 increased the killing of IgG-*H. capsulatum*, whereas PGE_2 reduced the ability of AMs to eliminate the fungus (Fig. 3D). Together, these data indicate the opposite effects of PGD_2 and PGE_2 on yeast killing by AMs and demonstrate, for the first time, that phagocytosis can be enhanced only by exogenous PGD_2, which, in vivo, may be released by its own cells and other neighbors.

PGD_2 and PGE_2 receptors differently control phagocytosis and killing of Ops-*H. capsulatum* by AMs

PGD_2 and PGE_2 signaling occurs through specific G-coupled receptors to mediate effector mechanisms in macrophages (14, 35). Therefore, we investigated which receptors are driving the effects of exogenous and endogenous PGD_2 and PGE_2 on phagocytosis and killing of Ops-*H. capsulatum* by AMs. Phagocytosis of Ops-*H. capsulatum* was increased by pretreatment of AMs with PGD_2 alone or PGD_2 in presence of a DP1 receptor antagonist (BWA868c), but was reduced after blocking DP2 receptors by the compound Bay-u3405. When used in combination, DP1/DP2 antagonists did not affect PGD_2-increased phagocytosis. Interestingly, blocking DP1, DP2, or DP1/DP2 in the absence of exogenous PGD_2 did not affect the phagocytosis of the fungus (**Fig. 4A**). On the other hand, in the presence of exogenous PGE_2, an EP2 antagonist (compound AH6809) did not modify its inhibitory effects, whereas an EP4 antagonist (AH23848 compound) potentiated its inhibitory action. Unexpected simultaneous inhibition of EP2/EP4, in the presence of exogenous PGE_2, almost reversed its inhibitory

action. Nevertheless, when AMs were pretreated with EP1 or EP2 antagonists, in the absence of exogenous PGE_2, significant and similar inhibition of phagocytosis was observed, but simultaneous EP2/EP4 blocking had no effect (Fig. 4B).

When the fungicidal activity of AMs was evaluated in the presence of exogenous PGD_2, and DP2 and DP1/DP2 antagonists, its fungicidal activity was reversed, unlike the DP1 antagonist. However, blocking DP1, DP2, or DP1/DP2 in the absence of exogenous PGD_2 did not affect AM killing activity (Fig. 4C). However, only blocking the EP4 receptor potentiated exogenous PGE_2-induced reduction of fungicidal activity of AMs infected with Ops-*H. capsulatum*. Simultaneous inhibition of EP2/EP4 completely blocked the inhibitory effects of exogenous PGE_2. Similar results were observed by pretreating AMs with EP2 and/or EP4 antagonists in the absence of exogenous PGE_2 (Fig. 4D). Together, these data further demonstrate that PGD_2 increased phagocytosis and fungicidal activity through DP2 receptor activation, and PGE_2-impaired phagocytosis and fungicidal activity are mediated by the cooperation of both receptors EP2 and EP4.

PGD_2 and PGE_2 modified PLC-γ, MAPK, and NF-κB/AP-1 signaling during Ops-*H. capsulatum* infection

In order to identify cell signaling pathways activated or inhibited by PGD_2 and PGE_2 during Ops-*H. capsulatum* AM infection, we investigated the phosphorylation of PLC-γ, JNK1/2, and p38 and the activation of NF-κB. As demonstrated by protein phosphorylation, PLC-γ, JNK1/2, p38, and NF-κB/AP-1 pathways were activated in AMs in response to Ops-*H. capsulatum* infection. Although Ops-*H. capsulatum* infection induced PLC-γ phosphorilation, the addition of exogenous PGD_2 or PGE_2 reduced phospho-PLC-γ after 2 h (**Fig. 5A**). In infected cells, exclusively, PGE_2 potentiated phosphorylation of JNK1/2 at an earlier time point (Fig. 5B), abrogated phosphorylation of p38
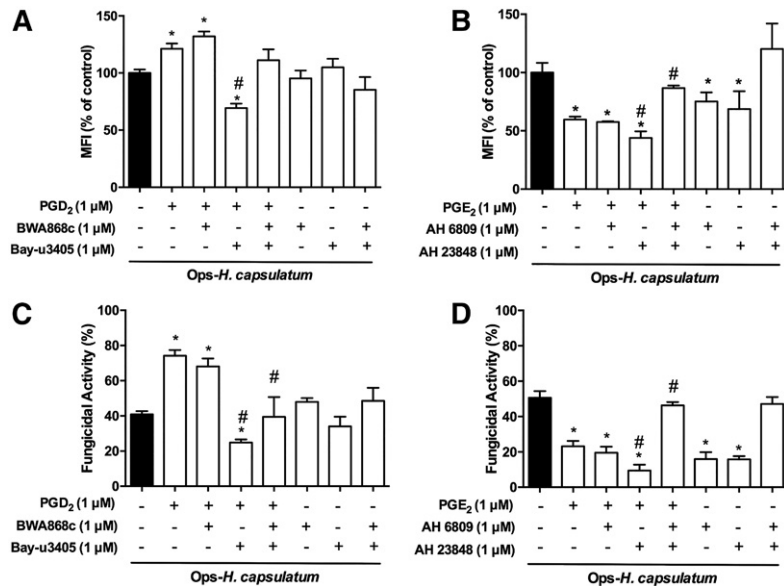
Fig. 4. PGD_2 and PGE_2 receptors antagonists differently impacted phagocytosis and killing of Ops-*H. capsulatum* by AMs. AMs were pretreated or not for 20 min with DP1 antagonist (BWA868c; 1 μM) or/and DP2 antagonist (Bay-u3405; 1 μM) before addition of vehicle or PGD_2 (1 μM) for 2 min. Subsequently, the cells were infected with Ops-*H. capsulatum* labeled with FITC (MOI 1:10), and phagocytosis (A) and fungicidal activity (C) were assessed 2 and 48 h later, respectively. In another set of experiments, AMs were pretreated or not with EP2 antagonist (AH 6809; 1 μM) and/or with EP4 antagonist (AH 23848; 1 μM) before the addition of vehicle or PGE_2 (1 μM) for 2 min. Subsequently, the cells were infected with Ops-*H. capsulatum* labeled with FITC (MOI 1:10), and phagocytosis (B) and fungicidal activity (D) were assessed 2 and 48 h later, respectively. Phagocytosis is expressed as average of fluorescence intensity (MFI) from internalized yeast by AMs in vehicle (n = 6). For fungicidal activity, after 48 h, AMs were lysed, and the fungicidal activity was determined as described in Materials and Methods. Fungicidal activity was expressed as a percentage of live yeast recovered (CFU) from cells treated with the antagonists in comparison to total yeast recuperated from AMs incubated with vehicle (100%) (n = 4). * $P < 0.05$ (AMs + Ops-*H. capsulatum* vs. other groups); # $P < 0.05$ (AMs + Ops-*H. capsulatum* PGD_2 or PGE_2 vs. treatments). One-way ANOVA and Tukey's multiple comparison tests were used. Data are representative of four independent experiments (±SEM).

(Fig. 5C), and reduced activation of NF-κB/AP-1. Together, these findings support the hypothesis that PGD_2 and PGE_2 have distinct effects on infected AMs, and that these effects can be mediated through distinct cell-signaling pathways.

Distinct effects of PGD_2 and PGE_2 on production of inflammatory mediators and BLT1 receptor expression by AMs infected with Ops-*H. capsulatum*

In tissues and cells infected by *H. capsulatum*, production of different inflammatory mediators appears to influence the success or failure of the host to control the infection (9, 36–39). Therefore, we investigated the extent to which PGs could modulate TNF-α, IL-1β, IL-10, LTB_4, and H_2O_2 production and BLT1 receptor expression by infected AMs. Ops-*H. capsulatum* infection induced production of all mediators listed above and increased the BLT1 when compared with uninfected cells. Interestingly, TNF-α, IL-10, and H_2O_2 were significantly reduced when AMs were preincubated with PGE_2 (**Fig. 6A**, C, D), whereas IL-1β was increased (Fig. 6B). In contrast, administration of PGD_2 did not modify TNF-α, IL-1β, and H_2O_2 (Fig. 6A, B, D), but inhibited IL-10 production (Fig. 6C). Specifically, regarding the cross-talk between lipid mediators, we observed that PGE_2 negatively regulated LTB_4 production, unlike PGD_2, which exerted no effect (Fig. 6E). However, only PGD_2 increased the expression of BLT1 induced by the infection (Fig. 6F). These findings reinforce the idea that PGE_2 suppresses proinflammatory mediator production, which in fact might impair fungi elimination by AMs. Alternatively, PGD_2 inhibited the production of IL-10, which represents a negative regulator of phagocytosis and killing,

while simultaneously facilitating LTB_4 beneficial effector functions.

Differential effects of inhibition of PGD_2 and PGE_2 during *H. capsulatum* infection in vivo

Previous experiments in our laboratory showed that PGs are important mediators of the pathogenesis of pulmonary histoplasmosis (8). In this study, we showed that the two PGs had different effects on activating AMs for phagocytosis and killing. To examine the physiological differences between PGD_2 and PGE_2, mice were infected with a lethal *H. capsulatum* inoculum and treated daily with selective PGD_2 or PGE_2 synthesis inhibitors. Infected mice began to die 14 days postinfection; mice treated with the selective inhibitor of PGD synthase HQL-79 showed a higher death rate, whereas mice treated with CAY10526, an inhibitor of microsomal PGE synthase activity, showed increased survival (**Fig. 7A**). The specificity effect of HQL-79 and CAY10526 was observed 7 days postinfection based on inhibition of PGD_2 and PGE_2 production (Fig. 7B). Also, inhibition of PGD_2 synthesis increased the fungal burden (Fig. 7B). *H. capsulatum*-infected mice treated with CAY10526 decreased TNF-α, IL-1β, and IL-10 production (Fig. 7C). However, HQL-79 treatment significantly increased IL-1β and IL-10 production 7 days after *H. capsulatum* infection (Fig. 7C). The lung inflammation was observed in Fig. 7D, where the inhibition of PGE_2 synthesis decreased inflammatory cell infiltrate and lung damage. These data demonstrate that inhibition of PGD_2 increases the susceptibility of mice to *H. capsulatum* infection, whereas inhibition of PGE_2 increases resistance, but reduces tissue damage.
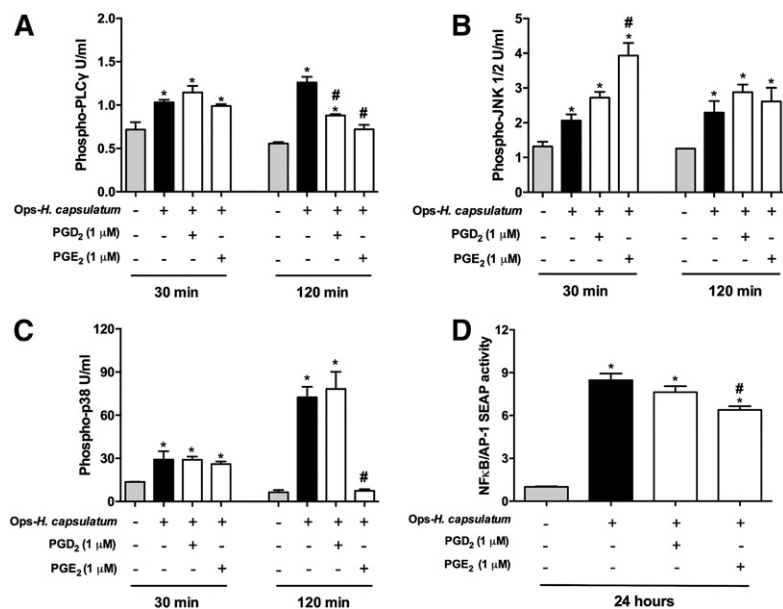
Fig. 5. MAPKs and NF-κB are involved in PG-induced macrophage activation. Quantification of phosphorylated PLC-γ (A), JNK1/2 (B), and p38 (C) were determined by cytometric bead array and expressed as units per milliliter. Phosphorylation was determined in AMs stimulated with PGD$_2$ or PGE$_2$ for 2 min. After the cells were infected with Ops-*H. capsulatum* infection (MOI 1:10) for 30 and 120 min, the cells were analyzed. Nontreated and noninfected cells were used as controls. D: Activation of NF-kB/AP-1 was measured in RAW-Blue™ cells incubated with either PGD$_2$ or PGE$_2$ for 2 min, before addition of Ops-*H. capsulatum* (MOI 1:10) for 24 h. * $P < 0.05$ (uninfected AMs vs. other groups); # $P < 0.05$ (AMs + Ops-*H. capsulatum* vs. other groups). One-way ANOVA and Tukey's multiple comparison tests were used. Data are representative of one (A–C) and two (D) independent experiments (n = 4, ±SEM).

DISCUSSION

Histoplasmosis is a fungal disease that affects the lungs, especially in immunocompromised patients worldwide (1). Previously, we demonstrated that the host control of *H. capsulatum* infection was dependent on the production of cytokines and lipid mediators (8, 9). The specific importance of endogenous PGs in this lung fungal infection was highlighted by the treatment of *H. capsulatum*-infected mice with a selective inhibitor of COX-2, which primarily reduced PG synthesis while increasing LTB$_4$ production (presumably through shunting of AA metabolism), consequently reducing inflammation and increasing the resolution of the infection (8, 9). Nevertheless, a well-orchestrated pulmonary host response against infection requires activation of AMs, which themselves produce and respond to mediators that regulate critical functions such as phagocytosis and killing of microorganisms (7, 14, 15, 40, 41). The investigation of lipid mediator effects on AMs is extremely relevant (42, 43), since these cells contribute to innate and adaptive immune response triggered by microorganisms (5). It has been suggested that PGE$_2$ and PGD$_2$ can induce opposite, antagonistic actions (10), but nothing has been described about the effects of these two PGs on AM functions during *H. capsulatum* infection. Therefore, in the present study, we explored distinctive roles of PGE$_2$ and PGD$_2$ in AM effector functions after in vitro Ops-*H. capsulatum* infection.

Our results demonstrate that PGD$_2$ has a protective role, by enhancing the phagocytosis and killing of Ops-*H. capsulatum*, whereas PGE$_2$ contributes to susceptibility, by impairing phagocytosis and fungicidal capacity of AMs. Investigation of the receptors involved in PGD$_2$ actions revealed that DP2 is a key receptor in exogenous PGD$_2$, boosting AM phagocytosis and killing. DP2 is a receptor coupled to subunit Gαi of G protein (21), which is a molecule involved in elevation of intracellular calcium and reduction of cAMP production (21). The role of cAMP in

macrophage effector mechanisms is well documented; there is a strong inverse correlation between the production of this second messenger and the ability of macrophages to engage in phagocytosis and killing (15, 44, 45). Thus, we suggest that PGD$_2$-induced improvements in AM phagocytosis and killing are likely the result of decreased cAMP levels via DP2 receptors (17, 21). Conversely, we demonstrated that exogenous PGD$_2$ signaling via DP2, increased the phagocytosis and killing of Ops-*H. capsulatum*. Treatment with DP2 antagonist, in the presence of exogenous PGD$_2$, may impair the control on cAMP levels and activation of PKA, resulting in the inhibition of effector mechanisms of macrophages and other cells (18–20). Furthermore, we investigated the action of DP1 or DP2 antagonists on endogenous PGD$_2$ produced by infected AMs, and we did not observe any effects on phagocytosis and killing activity. Therefore, we suggest that DP1 and DP2 are differentially expressed in AMs, resulting in distinct and opposite cell signaling, as previously described (18, 42).

As opposed to PGD$_2$, we found that PGE$_2$ inhibited effector AM mechanisms, and its actions were mediated by both EP2 and EP4 receptors. Engagement of PGE$_2$ on EP2 and EP4 inhibits FcγR-mediated phagocytosis and bacterial killing, by mechanisms driven by elevation of cAMP (14, 15). Also, we investigated the action of EP2 or EP4 antagonists alone on endogenous PGE$_2$ produced by infected AMs and reduced on phagocytosis and fungicidal activity were observed. We speculated that blocking only one of these receptors might have resulted in compensatory increases in the endogenous signaling of the other receptor pathway. The involvement of cAMP as a key element in the actions of PGE$_2$ and PGD$_2$ on AM effector function is also supported by the impact of these two lipids on cytokine production (20, 42, 46). Other work demonstrated that cAMP upregulates IL-10 and inhibits TNF-α synthesis (47). Also, increased release of cAMP prevented the generation of reactive oxygen and nitrogen intermediates, phagosome
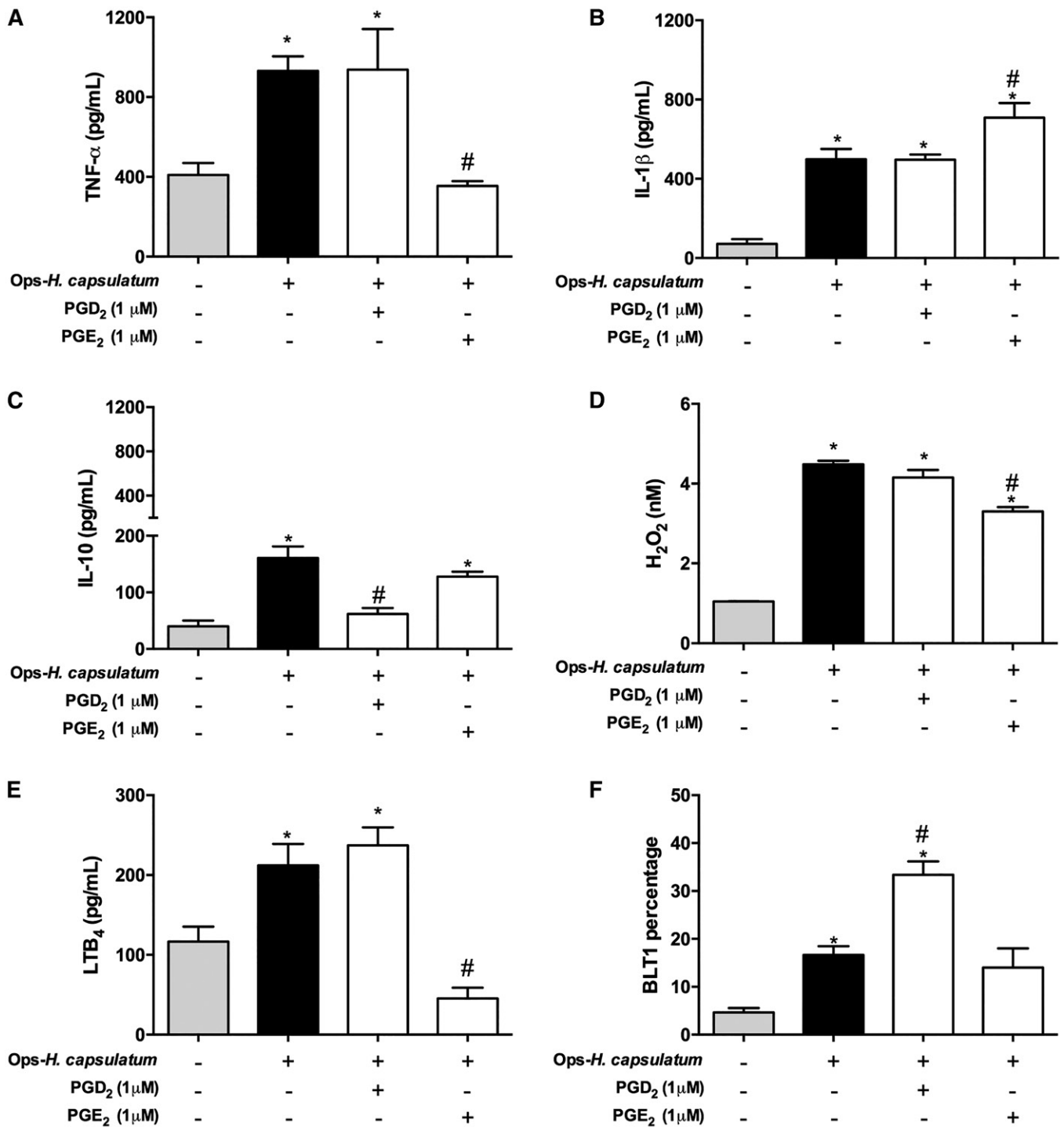
Fig. 6. TNF-α, IL-1β, IL-10, H₂O₂, and LTB₄ production and BLT1 receptor expression by infected AMs induced by exogenous PGD₂ and PGE₂. AMs were pretreated with PGD₂ (1 μM) or with PGE₂ (1 μM) for 2 min before infection with Ops-*H. capsulatum* (MOI 1:10). The supernatants were collected after 48 h of incubation and TNF-α (A), IL-1β (B), IL-10 (C), H₂O₂ (D), and LTB₄ (E) concentrations were determined by immunoassays. F: Expression of BLT1 receptor shown was analyzed by flow cytometry. * $P < 0.05$ (uninfected AMs vs. other groups); # $P < 0.05$ (AMs + Ops-*H. capsulatum* vs. other groups). One-way ANOVA and Tukey's multiple comparison tests were used. Data are representative of two independent experiments (±SEM).

acidification and lysosomal enzyme release, and consequently microorganism killing (48). In histoplasmosis, effective host defense requires the production of cytokines, such IL-12, IFN-γ, TNF-α, IL-1β (20, 36, 37, 39), and LTB₄ (9), which, acting via BLT1 receptors, contribute to the elimination of the fungus (49). In contrast, IL-10 is a

negative regulator that inhibits phagosome maturation, leading to profound intracellular proliferation of microorganisms (50). Production of these cytokines is under the control of the transcription factor NF-κB, which translocates to the nucleus after activation of MAPK pathway in response to pathogens (51). Infection of AMs with
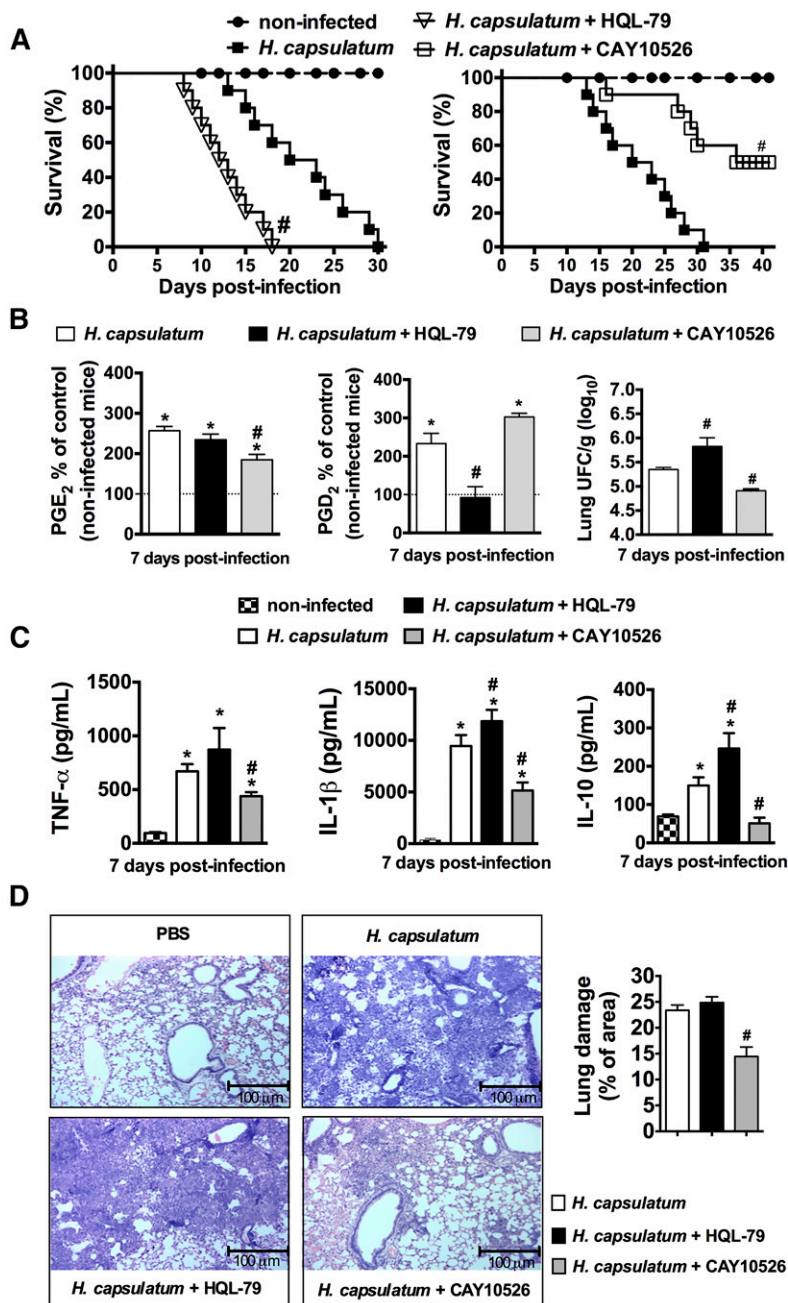
Fig. 7. PG synthesis inhibition differently affects the survival of mice infected with lethal inoculum of *H. capsulatum* and inflammatory parameters. A: Mice were treated daily orally with water or HQL-79 (inhibitor of PGD_2 synthesis: 3 mg/kg/0.5 ml) for 30 days (n = 10) or treated daily with CAY10526 (inhibitor of PGE_2 synthesis: 5 mg/kg/0.5 ml) for 40 days (n = 10). A group of noninfected mice was used as controls (n = 10). B: Lipid mediators PGD_2 and PGE_2 in the lung parenchyma were measured by enzyme immunoassay and fungal burden in the lungs. C: Lung cytokine concentrations (TNF-α, IL-1β, and IL-10) were determined at 7 days following infection by ELISA. D: Lung tissues were also processed and stained with H&E to detect leukocyte infiltration after 7 days. Percent of cell infiltrated area corresponding to extent of lung damage. # $P < 0.05$ (*H. capsulatum* + H_2O vs. *H. capsulatum* + HQL-79 or *H. capsulatum* + CAY10526 treatment); * $P < 0.05$ [PBS (non-infected mice, dashed line) vs. *H. capsulatum* + H_2O *or H. capsulatum* + HQL-79 or *H. capsulatum* + CAY10526 treatment]. A: Log rank test for survival analysis was used. B–D: One-way ANOVA and Tukey's multiple comparison test were used. Data are representative of two independent experiments (±SEM).

Ops-*H. capsulatum* induced phosphorylation of p38 MAPK, JNK1/2, and PLC-γ. Interestingly, PGE_2 treatment in AMs within 30 min of infection increased the phosphorylation of JNK1/2, with subsequent inhibition of phosphorylation of p38 after 120 min of infection and reduced NF-κB/AP-1 activity and TNF-α production after 24 h. In this regard, it was previously demonstrated that p38 signaling is important for TNF-α production and macrophage activation (52), whereas PKA activation is necessary for downregulation of TNF-α by lipopolysaccharide (53). Activation of JNK can be negatively regulated by NF-κB (54). Based on these findings, we hypothesized that activation of the MAPK, PLC-γ, and NF-κB/AP-1 pathways by PGE_2 and PGD_2 in Ops-*H. capsulatum*-infected AMs contributed differently to phagocytosis and fungicidal activity. Further studies are needed to investigate the pivotal role of these pathways in AM effector functions and the cross-talk between them. Moreover, we demonstrated that PGD_2 reduced IL-10 production by AMs, and inhibition of PGD_2 production in vivo enhanced IL-10 release in the *H. capsulatum* infected lung, which correlated with increased phagocytosis and killing of *H. capsulatum*. In contrast, PGE_2 treatment inhibited the production of TNF-α and H_2O_2, which are important mediators of macrophage activation and killing (55, 56). Finally, we demonstrated the effects of PGD_2 that were opposite to those of PGE_2 during *H. capsulatum* infection in mice. While PGD_2 showed protective effects in infected mice, PGE_2 showed suppressive action in the lung during infection. On this model, inhibition of PGE_2 production diminished inflammatory cell infiltrate and lung damage, accompanied by reduced production of TNF-α and IL-1β.

To our knowledge, the present study describes, for the first time, the role of PGE$_2$ and PGD$_2$ in AM effector mechanisms in the context of *H. capsulatum* infection. We demonstrated a new role for PGD$_2$ as a key mediator for effective phagocytosis and fungicidal functions of AMs. On the other hand, these studies revealed that PGE$_2$ impairs phagocytosis and killing of the fungus. Notwithstanding, these findings shed light on novel perspectives for the treatment of fungal diseases using PGD$_2$ as a potential pharmacological immunomodulatory agent to control the infection.

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