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Short communication

Eosinophils increase macrophage ability to control intracellular *Leishmania amazonensis* infection via PGD_2 paracrine activity *in vitro*

Patrícia da Silva Marques [a], Alessandra M. da Fonseca-Martins [b], Monique Pacheco Duarte Carneiro [c], Natália R.T. Amorim [a], Camila R. Rodrigues de Pão [a], Claudio Canetti [a], Bruno L. Diaz [a], Herbert L. de Matos Guedes [d,e,1], Christianne Bandeira-Melo [a,1,*]

[a] Laboratório de Inflamação, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[b] Laboratório de Imunobiologia das leishmanioses, Instituto de Microbiologia Paulo de Góes, Departamento de Imunologia, Universidade Federal do Rio de Janeiro, RJ, Brazil
[c] Laboratório de Bioquímica e Biologia molecular de Proteases, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[d] Instituto de Microbiologia Paulo de Góes, Departamento de Imunologia, Universidade Federal do Rio de Janeiro, RJ, Brazil
[e] Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, RJ, Brazil

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ABSTRACT

Clinical and experimental studies have described eosinophil infiltration in *Leishmania amazonensis* infection sites, positioning eosinophils strategically adjacent to the protozoan-infected macrophages in cutaneous leishmaniasis. Here, by co-culturing mouse eosinophils with *L. amazonensis*-infected macrophages, we studied the impact of eosinophils on macrophage ability to regulate intracellular *L. amazonensis* infection. Eosinophils prevented the increase in amastigote numbers within macrophages by a mechanism dependent on a paracrine activity mediated by eosinophil-derived prostaglandin (PG) D_2 acting on DP2 receptors. Exogenous PGD_2 mimicked eosinophil-mediated effect on managing *L. amazonensis* intracellular infection by macrophages and therefore may function as a complementary tool for therapeutic intervention in *L. amazonensis*-driven cutaneous leishmaniasis.

1. Introduction

Amongst classical eosinophil functions are immunoregulatory responses to helminthic infections [1,2], yet eosinophils are now emerging as regulators of infectious conditions induced by particular types of protozoans as well [3]. Specifically regarding *Leishmania*, it is not novel knowledge that infiltrates of eosinophils are hallmarks of the inflammatory response to infection by different *Leishmania* species in both clinical and experimental conditions [3]. Specifically, eosinophils can be found strategically positioned adjacent to *L. amazonensis*-infected macrophages at sites of cutaneous leishmaniasis [4]. Although, the functional nature of the potential interaction between these granulocytes and *L. amazonensis*-infected macrophages has not been directly addressed, positive correlation between the presence of eosinophils and increased resistance to *L. amazonensis* infection has been demonstrated [4,5,6]. As the molecular and/or cellular mechanisms involved in such eosinophil/macrophage crosstalk are still elusive, we hypothesized that eosinophils may impact the development of *L. amazonensis* infection, not by direct killing of promastigotes as postulated elsewhere [5], but rather by impacting cellular activity of *L. amazonensis*-infected macrophages by improving handling of intracellular amastigote content. Noteworthy, crosstalk between eosinophils and neighboring macrophages in different pathophysiological settings has already unveiled eosinophil immunomodulatory capacity to regulate macrophage functions [1,2], including even in *L. major*-driven cutaneous infection [7]. Here, to reproduce the *in situ* encounter between macrophages and eosinophils during cutaneous leishmaniasis by *L. amazonensis* infection, we brought these two leukocytes together *in vitro* to answer the questions of whether and how eosinophils could enhance macrophage ability to control intracellular *L. amazonensis* development.

* Corresponding author at: Avenida Carlos Chagas Filho, 373 – CCS room C1-020, IBCCF, UFRJ, Rio de Janeiro, RJ 21941-902, Brazil.
E-mail address: cbmelo@biof.ufrj.br (C. Bandeira-Melo).
[1] Both senior authors contributed equally to the work.

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2. Materials and methods

2.1. Obtaining mouse macrophages and eosinophils

For *in vitro* differentiation of mouse eosinophils, bone marrow cells were collected from femurs and tibiae of wild-type BALB/c mice with RPMI 1640 (20% FBS). As previously reported [8,9], cells were cultured at 10^6 cells/mL in RPMI (20% FBS) with 100 ng/mL stem cell factor (SCF; PeproTech) and 100 ng/mL FLT3 ligand (PeproTech) from days 0 to 4. Then, SCF and FLT3-L were replaced with IL-5 (10 ng/mL; Peprotech). On day 14, freshly differentiated eosinophils were enumerated and used. Of note, eosinophil purity was about 90% for all *in vitro* differentiation cultures as attested by routine visual inspection in cells stained with fast panoptic stain.

To obtain murine macrophages, resident peritoneal cells of naïve BALB/c mice were collected with RPMI, transferred to 24-well plates (4×10^5 cells/well) containing glass slides and incubated for 1 h at 37 °C (5% CO_2) for cell adhesion. Cells were washed (3x) with warm PBS (37 °C) and RPMI medium was added (400 μL) for 24 h incubation. Adherent macrophages were washed again before addition of fresh RPMI (10% FBS) at final working volume of 300 μL.

Protocols used in this study were approved by Animal Use Welfare Committee of Federal University of Rio de Janeiro, Brazil (CEUA/CCS/UFRJ).

2.2. In vitro co-cultures of infected macrophages and eosinophils

Adherent macrophages were first incubated with 4 or 8×10^5 (i.e. 1:1 or 1:2 ratios) stationary-growth phase L. amazonensis promastigotes (MPRO/BR/72/M1845, LV78 strain)/well for 4 h at 37 °C in 5% CO_2, as previously described [10]. After removing free parasites by washing (3x RPMI), 300 μL of RPMI (10% FBS) containing or not 2×10^6 (5:1 ratio of eosinophils/infected macrophage) freshly differentiated mouse eosinophils were added onto infected macrophages and incubated for 24 h or 72 h (cell viability ≥85%). Of note, 1:1 and 2:1 eosinophil/macrophage ratios were tested (not shown) and failed to reproducibly modulate macrophage ability to control intracellular *L. amazonensis* infection, as achieved by 5:1 ratio (*vide infra*). After removing co-cultures supernatants, each well-containing glass slide was then fixed with 4% paraformaldehyde (PFO) before staining with fast panoptic stain (Laborclin) for determination of both the percentage of infected macrophages (macrophages containing ≥ 1 amastigotes), as well as, the parasite intracellular load (total number of amastigotes counted in 100 consecutive macrophages; non-infected was considered as zero). For lipid body counting within macrophages, adherent cells were stained with 1.5% OsO_4 in 0.1 M cacodylate buffer, as previously described [9]. These analyses were performed in a blinded fashion by bright field microscopy.

Alternatively, macrophages infected with *L. amazonensis* (8×10^5 promastigotes; 1:2 ratio) were incubated/treated for 72 h with 300 μL of : (i) 2×10^6 fixed eosinophils (4% PFO for 30 min) in RPMI; (ii) eosinophil or control conditioned RPMI medium which were produced by incubating RPMI medium with or without 3×10^6 eosinophils/mL at 37 °C for 1 h, respectively; (iii) 2.5 nM of PGD_2 or DK-PGD_2 (Cayman Chemical Co.) in RPMI; or (v) 2×10^6 eosinophils pre-treated for 30 min at 37 °C with HQL-79 (10 μM; Cayman), pelleted, resuspended in 300 μL of RPMI and immediately added to infected macrophages. Infected macrophages were alternatively treated with nitric oxide synthesis inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME; 50 μM) for 30 min before addition of 2×10^6 freshly differentiated mouse eosinophils. Pre-treatment of infected macrophages with PGD_2 DP2 receptor antagonist CAY10471 (200 nM) was performed for 30 min before addition of 300 μL of eosinophil conditioned media.

TNF-α and the prostanoids PGD_2 or PGE_2 were quantified by specific ELISA and EIA kits according to the manufacturer's instructions (R&D and Cayman, respectively). Nitric oxide production was assayed indirectly by quantification of nitrites by using the Griess colorimetric method [11].

Each experimental condition was performed in duplicates and repeated at least three times with macrophages and eosinophils obtained from different mice. Results are expressed as the mean ± SEM and were analyzed by means of one-way ANOVA, followed by Student-Newman-Keuls test, with the level of statistical significance set at $p < 0.05$.

3. Results and discussion

It is now well established that eosinophils are recruited to inflamed cutaneous sites of *L. amazonensis* infected lesions [3]. Local eosinophilia appears to be a long lasting event, noticed from the very first hours of *L. amazonensis* infection to about 120 days [4], therefore indicating a potential immunoregulatory function for eosinophils throughout leishmaniasis. Of note, either potential curative [3] or deleterious [7] roles of infiltrating eosinophils have also been reported in infections triggered by other *Leishmania spp*. However, specifically for *L. amazonensis,* only direct association between increased eosinophil levels and a better control of infection has been shown [4,5,6]. For instance, in either IL-5 transgenic or toll-like receptor 2 deficient mice, increased numbers of eosinophils at the *L. amazonensis* lesions parallels the lower amastigote load and smaller cutaneous lesions [5,6]. While the positive association between eosinophilia and the control of *L. amazonensis* infection remains without characterization of the crosstalk mechanism and mediators involved, the spatio-temporal link defines a persistent co-localization between infiltrating eosinophils and the prominent population of amastigote-loaded macrophages during the course of cutaneous infection. Therefore, here we evaluated *in vitro* whether eosinophils are able to interfere with macrophage management of its intracellular load of *L. amazonensis* amastigotes. As shown in Fig. 1A, exposing infected macrophages to eosinophil presence promoted a significant decrease in (i) the percentage of infected macrophages (top graph), and (ii) the parasite load (bottom graph) in 72 h of infection, independently of the initial number of promastigotes (4 or 8×10^5; i.e. 1:1 or 1:2 ratios) given to macrophages on day 0 (Fig. 1A). Striking, in parallel to improve macrophage effectiveness to manage *L. amazonensis* intracellular infection, eosinophils did also elicit other macrophage intracellular activities, as exemplified by the presence (72 h) of a larger number of cytoplasmic lipid bodies – enhanced biogenesis of these organelles is a common feature of cellular activation of bioactive macrophages [12] – within *L. amazonensis*-infected macrophages (39.6 ± 4.3 *versus* 61.8 ± 2.9 lipid bodies/infected macrophage cultured without and with eosinophils; mean ± SEM of four experiments; $^*p < 0.05$). Such eosinophil-driven impact on macrophagic activity does not seem to depend on early events of macrophage infection by *L. amazonensis* promastigotes, since within the initial 24 h of infection eosinophils were unable to modify the number of amastigotes inside macrophages (Fig. 1B), what reflects the efficiency of both promastigote entry and differentiation into amastigotes. On the other hand, eosinophils did affect the subsequent amastigote growth observed intracellularly within macrophages between 24 and 72 h of infection (Fig. 1B), decreasing the intracellular numbers of amastigotes found within 72 h of infection. Nitric oxide-mediated leishmanicidal activity appears to be one of the effector mechanisms contributing to eosinophil-driven enhanced macrophagic ability to control intracellular amastigote burden (Fig. 1D). Even though nitric oxide levels found in supernatant of *L. amazonensis*-infected macrophages (72 h) were not modified by eosinophil presence (Fig. 2A; top left panel), the treatment with the nitric oxide synthesis inhibitor L-NAME was capable to block eosinophil impact, allowing the accumulation of enhanced numbers of intracellular amastigotes within macrophages (Fig. 1D). Of note, in L-NAME-treated co-cultures despite the eosinophil presence for 72 h, intracellular *L. amazonensis* amastigotes were able to grow/survive within infected macrophages as abundantly as in absence of eosinophils, therefore indicating that the eosinophil-driven mechanism of decreasing amastigotes growth/survival is not due to depletion of culture medium nutrients due to eosinophil presence.

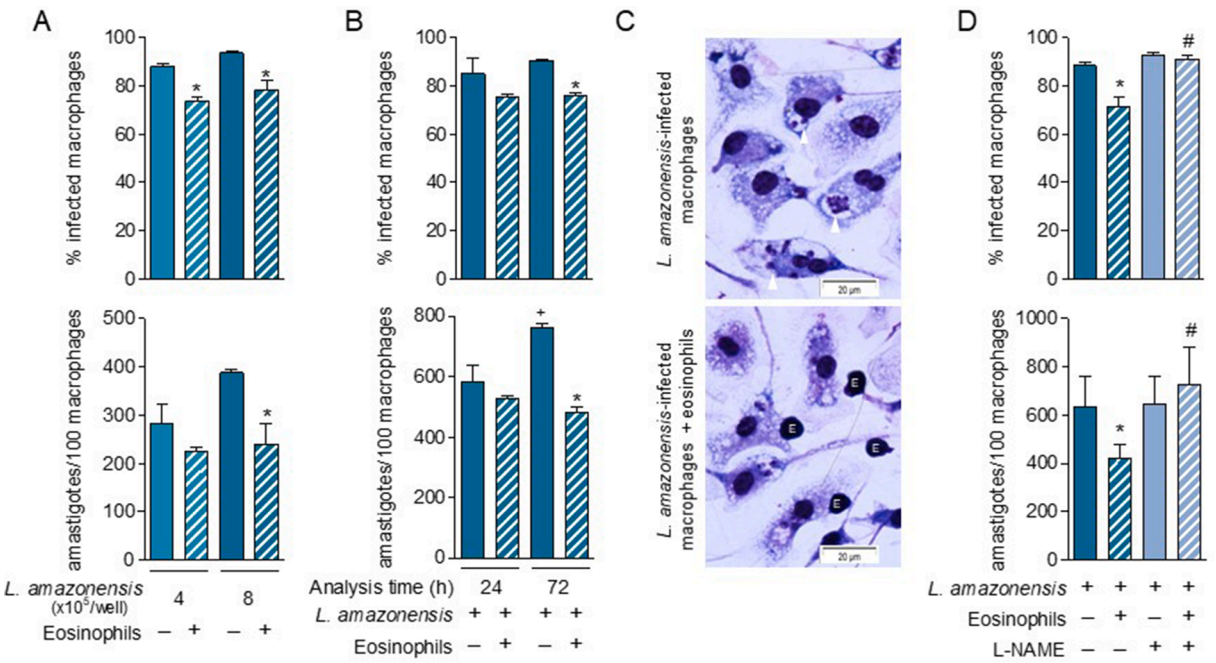

Fig. 1. Eosinophils increase macrophage ability to control intracellular *L. amazonensis* infection: effector role of nitric oxide. Adherent mouse macrophages were infected with 4 or 8 $\times$ 10^5 (1:1 or 1:2 ratios) of *L. amazonensis* promastigotes as indicated in A, or with 8 $\times$ 10^5 promastigotes/well (1:2 ratio) in B, C and D. Freshly differentiated mouse eosinophils (2 $\times$ 10^6) were added and maintained in co-culture with infected macrophages for 72 h in A, C and D, or for 24 or 72 h as indicated in B. Top graph panels show percentage of infected macrophages and bottom graph panels show parasite intracellular load as total number of amastigotes counted in 100 consecutive macrophages. In C, representative images show fast panoptic stained *L. amazonensis*-infected macrophages (top image) and co-cultures of infected macrophages and eosinophils (bottom image). In the bottom image, "E" identifies eosinophils in co-cultures. In D, *L. amazonensis*-infected macrophages were treated for 30 min with L-NAME (50 μM) before receiving eosinophils. Values display mean ± SEM of at least three experiments performed with cells prepared from distinct animals and in duplicates. *$p < 0.05$ compared to infected macrophages. #$p < 0.05$ compared to non-treated co-cultures of infected macrophages and eosinophils.

Noteworthy, a closer look at the 72 h eosinophil/infected-macrophages co-cultures revealed that eosinophils may be physically interacting with macrophages (Fig. 1C). Experiments were then designed to test the hypothesis of whether *L. amazonensis* reduction within macrophages was dependent on direct macrophage/eosinophil contact. Eosinophil stimulatory effect on infected macrophages did not seem to depend on a juxtacrine activity, but rather on a paracrine effect of mediator-secreting eosinophils, since: (i) while displaying same kind of physical interaction with infected macrophages, inactivated eosinophils (by fixation) were not able to modulate infected macrophages like active freshly differentiated ones (Fig. 2B); and (ii) the up-regulatory effect of fresh eosinophils on macrophage's leishmanistatic/leishmanicidal activity was mimicked by addition of just eosinophil conditioned medium during the 72 h of infection, while control (no eosinophil) conditioned medium caused no alteration on intracellular infection magnitude (Fig. 2C). These results indicate that the impact of eosinophils on *L. amazonensis*-infected macrophages is mediated via release of soluble bioactive mediators, rather than cell contact-induced activation, by eosinophils that act in a paracrine manner to modulate cellular functions of amastigote-loaded macrophages. Moreover, even though eosinophil paracrine activity may represent a main mechanism, one can only postulate that the close proximity between the two cell types in the co-cultures creates a micro-environment which may increase the actual proximal concentration of mediators released by eosinophils favoring the upregulation of macrophage effector functions.

Eosinophils are known to secrete a multitude of *de novo* synthesized and release stored regulatory molecules [1,2]; therefore eosinophil conditioned media may carry several bioactive signals, including macrophage stimuli that are capable of promoting the phenomenon observed here. Indeed, from bioactive lipids to protein mediators, several candidate molecules derived from eosinophils may correspond to the paracrine signal(s) responsible for stimulating macrophages to a more effective response in controlling *L. amazonensis* intracellular infection. Although the lipid mediator PGD_2 – known to be synthesized by eosinophils [9] – is mainly recognized by its pro-inflammatory activities in allergy-related conditions [13], a growing body of data shows PGD_2 up-regulatory functions on macrophage-driven infection circuits, including: (i) PGD_2-mediated induction of nitric oxide, TNFα, IL-1β, and TGFβ production by macrophages [14]; (ii) PGD_2 ability to up-regulate secretion of nitric oxide and IL-1β in LPS-pre-stimulated macrophages [14]; and (iii) PGD_2-elicited enhanced phagocytic and killing activities of *Histoplasma capsulatum*-infected macrophages [15].

PGD_2 impact on macrophage cellular activities has not been specifically assessed for *L. amazonensis* infections, however in our study, paracrine PGD_2 participation on eosinophil-driven macrophage stimulation was investigated, considering that: (i) *in vitro* differentiated mouse eosinophils (as the ones generated and used here) are well-recognized cellular sources of PGD_2 [9]; (ii) eosinophil conditioned medium (i.e. cell-free supernatants of 3 $\times$ 10^6/mL *in vitro* bone marrow-differentiated mouse eosinophils incubated for 1 h at 37 °C) contain about 42.8 ± 3.8 pg/mL of PGD_2 (mean ± SEM of four conditioned mediums prepared with eosinophils from distinct animals; *$p < 0.05$); and (iii) increased amounts of PGD_2 were found in the supernatants of infected macrophages/eosinophils co-cultures, while no alteration was observed for PGE_2 or TNFα levels (Fig. 2A). Of note, *in vitro* differentiated mouse eosinophils (2 $\times$ 10^6/mL) rapidly respond (within 1 h) with PGD_2 synthesis, but not PGE_2 synthesis (not shown), under direct stimulation with 8 $\times$ 10^5 *L. amazonensis* promastigotes (increasing from 31.35 ± 3.9 to 82.7 ± 23.5 pg/mL of cell-free supernatant PGD_2; mean ± SEM of four experiments with eosinophils prepared from distinct animals; *$p <$ 0.05).

In line with eosinophils-driven effects on infected macrophages being mediated by PGD_2, either exogenous PGD_2 itself or the specific DP2 receptor agonist DK-PGD_2 (2.5 nM each) were capable of mimicking eosinophil effect by enhancing macrophage ability of controlling intracellular *L. amazonensis* infection (Fig. 3A). While it is not established

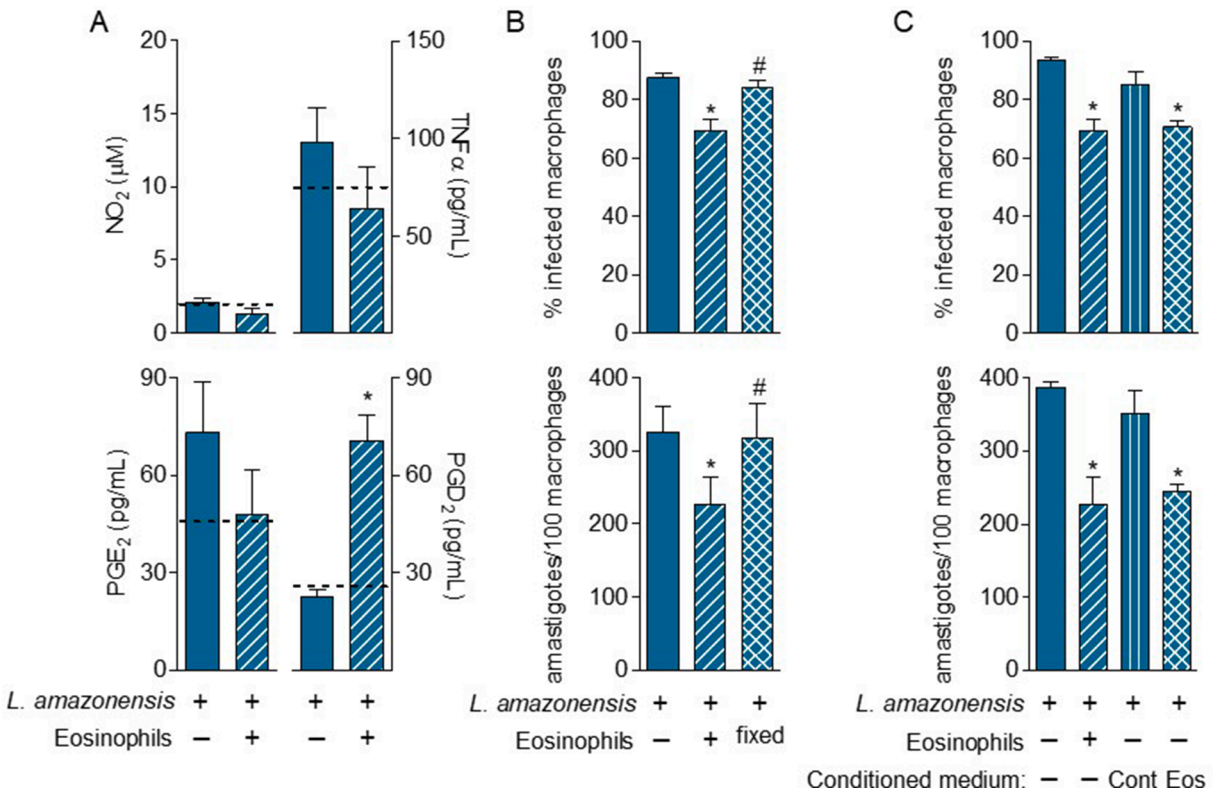

Fig. 2. Soluble molecules secreted by functionally active eosinophils increase, in a paracrine manner, macrophage ability to control intracellular *L. amazonensis* infection. Adherent mouse macrophages were infected with 8×10^5 promastigotes/well (1:2 ratio) in A, B and C. Freshly differentiated mouse eosinophils (2×10^6) were added and maintained in co-culture with infected macrophages for 72 h in A and B. In A, graphs show the levels of nitric oxide (NO), TNFα, PGE_2 and PGD_2 (as indicated) detected in 72 h supernatants of cell co-cultures (dashed lines indicate mean levels of the mediators in the supernatants of non-infected macrophages). In B, besides freshly differentiated eosinophils, inactivated PFO-fixed eosinophils (2×10^6) were added to the infected macrophage cultures for 72 h. As indicated in C, besides freshly differentiated eosinophils, control- or eosinophil-conditioned medium were incubated for 72 h with infected macrophages. In B and C, top graph panels show the percentage of macrophages infected with intracellular amastigotes, while bottom graphs display total number of amastigotes found in 100 consecutive macrophages. Values display mean ± SEM of at least three experiments performed with cells prepared from distinct animals and in duplicates. $*p < 0.05$ compared to infected macrophages. $\#p < 0.05$ compared to co-culture of infected macrophages and freshly differentiated bioactive eosinophils.

whether *L. amazonensis* infection modulates it, biologically active DP2 receptors are known to be expressed by mouse peritoneal macrophages [16]. Indeed, it has been shown that the specific activation of DP2 receptors mediates PGD_2 ability to enhance control of intracellular *H. capsulatum* infection by macrophages [15]. To ascertain that an eosinophil-derived PGD_2 represents the molecule mediating eosinophil-driven up-regulation of macrophage leishmanistatic/leishmanicidal activity, first eosinophils were pretreated with HQL-79 – specific inhibitor of hematopoietic PGD synthase, known to block PGD_2 synthesis by *in vitro* differentiated eosinophils [9]. In contrast to non-treated eosinophils, HQL-79-pretreated eosinophils fail to promote the reduction in the numbers of amastigotes within co-cultured *L. amozonensis*-infected macrophages (Fig. 3B). The lack of effect of eosinophils with disrupted PGD_2 synthesis by HQL-79 pre-treatment does indicate that eosinophil-derived PGD_2 is needed to improve macrophage cellular activity controlling intracellular *L. amazonensis* infection. Even though clearly required, we assume that eosinophil-derived PGD_2 may not be sufficient to promote full eosinophil effect, since: (i) distinct to nM concentration of exogenous PGD_2, stimulation of infected macrophages with pM concentrations of PGD_2 – comparable to PGD_2 amounts found in eosinophil/macrophage co-cultures or eosinophil conditioned medium – failed to promote enhancement of macrophage ability to control intracellular *L. amazonensis* infection (not shown); (ii) HQL-79 treatment, although capable to significantly inhibit eosinophil effect on infected macrophages, did not block it entirely (Fig. 3B); and (iii) eosinophils and macrophages are known to be able to release a multitude of mediators. We postulate that to control the cellular activity of *L. amazonensis*-infected macrophages, a collaboration/synergism between eosinophil-derived PGD_2 and other(s) mediator(s) takes place in the eosinophil- or eosinophil conditioned medium-driven effects. Characterization of additional molecular candidates are future goals; for instance, analyzing potential roles of IL-1β and LTB_4 or revisiting still possible functional impacts of TNFα and PGE_2 in PGD_2-orchestrated effect, although eosinophil presence did not modulate their co-culture levels (Fig. 2A). Of note, such synergistic missing factor(s) may not be necessarily eosinophilic products, but soluble or exosome-contained mediators derived from macrophages and/or even *L. amazonensis* themselves [17].

While studies are now being carried out for identification of such additional eosinophil-derived agents, the role of eosinophil-derived PGD_2 was definitively confirmed, since pre-treatment of infected macrophages with CAY10471, a selective antagonist of PGD_2 DP2 receptor, blocked the effect of PGD_2-containing eosinophil conditioned medium on infected macrophages (Fig. 3C).

Recently, eosinophil biological significance has evolved from disease-driven inflammatory deleterious cells to housekeepers of tissue physiological functions [1,2]. In line with this new understanding, our findings unveil a mechanism involved in the eosinophil role of improving protection against *L. amazonensis* infection: the enhancement of macrophages competence in dealing with intracellular growth of *L. amazonensis* (Fig. 3D). Our study also identifies a lipid – PGD_2, rather than a cytokine – as a mediator of eosinophil/macrophage cross-talks (Fig. 3D) and, therefore experimental and clinical studies investigating the impact of PGD_2 or selective DP2 agonists as putative adjuvant therapy candidates in the treatment of *L. amazonensis* cutaneous

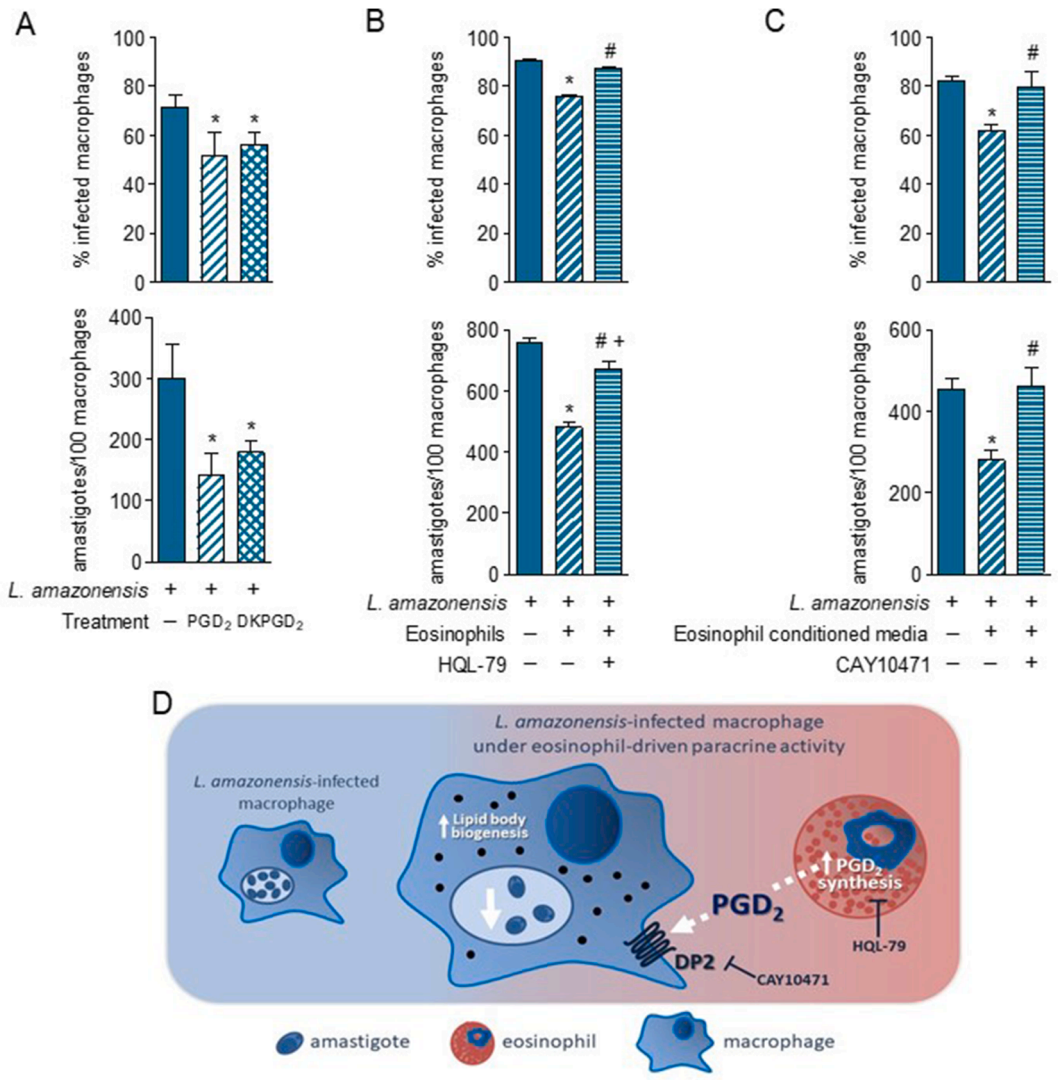

Fig. 3. Eosinophils-derived PGD_2 increases macrophage ability to control intracellular *L. amazonensis* infection via activation of DP2 receptors on macrophages. Adherent mouse macrophages were infected with 8×10^5 promastigotes of *L. amazonensis* (1:2 ratio). In A, instead of eosinophils, the lipid molecules PGD_2 itself (2.5 nM) or DK-PGD_2 (2.5 nM) were added for 72 h, as indicated, to cultures of only infected macrophages. In B, freshly differentiated mouse eosinophils were pre-treated for 30 min with an inhibitor of PGD_2 synthesis (HQL-79; 10 μM). Non-treated or treated eosinophils (2×10^6) were then added and maintained in co-culture with infected macrophages for 72 h. In C, *L. amazonensis*-infected macrophages were treated or not for 30 min with CAY10471 (200 nM) before addition of eosinophil conditioned medium which was maintained with infected macrophages for 72 h. All top graphs show the percentage of macrophages infected with intracellular amastigotes, while bottom graphs display total number of amastigotes found in 100 consecutive macrophages. Values display mean ± SEM of at least three experiments performed with cells prepared from distinct animals and in duplicates. * and +, $p < 0.05$ compared to infected macrophages. #$p < 0.05$ compared to co-culture of infected macrophages and freshly differentiated non-treated eosinophils. D shows a schematic representation of the collective body of data demonstrating that eosinophil-derived PGD_2 activates macrophages which display improved capability to control intracellular *L. amazonensis* infection.

leishmaniasis may be of interest.

4. Disclaimer

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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CRediT authorship contribution statement

Patrícia da Silva Marques: Investigation, Methodology, Writing - review & editing. **Alessandra M. da Fonseca-Martins:** Investigation, Methodology, Writing - review & editing. **Monique Pacheco Duarte Carneiro:** Investigation, Methodology, Writing - review & editing. **Natália R.T. Amorim:** Investigation, Methodology, Writing - review & editing. **Camila R. Rodrigues de Pão:** Investigation, Methodology, Writing - review & editing. **Claudio Canetti:** Conceptualization, Writing - review & editing. **Bruno L. Diaz:** Conceptualization, Supervision, Formal analysis, Writing - review & editing. **Herbert L. de Matos Guedes:** Conceptualization, Supervision, Formal analysis, Writing - review & editing, Funding acquisition. **Christianne Bandeira-Melo:** Conceptualization, Supervision, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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