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Lysophosphatidylcholine exacerbates *Leishmania major*-dendritic cell infection through interleukin-10 and a burst in arginase1 and indoleamine 2,3-dioxygenase activities

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ABSTRACT

Leishmania major is an obligate intracellular parasite hosted by phagocytes, including dendritic cells (DCs). Lysophosphatidylcholine (LPC) a pro-oxidant by-product of phospholipase A2 activity can modulate the maturation and function of DCs. However, little is known about its role in *L. major* infection.

This study examined the effects of LPC and lipopolysaccharide (LPS) in BALB/c mouse-derived DC infection by *L. major* promastigotes, *in vitro*. Our results showed early divergent effects of LPS and LPC, which lasted up to 24 h. In contrast to LPS, LPC worsened DC infection by reversing the immune balance IL-10 *vs.* TNF-α and IL-6, and inducing a sharp down regulation of CD40 and iNOsynthase activity. In addition, LPC potentiated xanthine oxidase stress, the production of kynurenine by indoleamine 2,3 dioxygenase (IDO), and arginase1 activity in the expense of iNOsynthase.

Taken together, our results highlight some biochemical events bypassing the protective Th1 response. They suggest that LPC could facilitate the proliferation of this obligate intracellular parasite by neutralizing oxidative and nitrosative stresses and sustaining both IDO and arginase1 activities.

1. Introduction

Leishmania are protozoan parasites hosted in the gut of sand flies as promastigotes. Mammalian phagocytes, including dendritic cells (DCs) are infected by the amastigote forms of the parasite [1]. The development of Th2 immune response exacerbates the disease through the down regulating effect of IL-10 and activation of arginase 1 *via* IL-4 [2,3]. Arginase enzyme hydrolyses L-arginine into ornithine, a major source of essential polyamines for parasite growth. This reduces the bioavailability of L-arginine for iNOsynthase, the nitric oxide-releasing enzyme [4].

Leishmania infection has been associated with severe cellular oxidative stress. The dehydrogenase activity mammalian xanthine oxidoreductase is readily converted to oxidase upon oxidation of critical sulfhydryl residues [5]. Xanthine oxidase (XO) generates superoxide and hydrogen peroxide, the major reactive oxygen species (ROS). The latter control the phenotypic and functional maturation of human DCs, in part through transcription nuclear factor-kappa B (NF-kB)-dependent mechanisms [6,7]. DCs play a critical role in leishmaniasis [8]. Furthermore, xanthine oxidase (XO) has been involved in innate immune response of phagocytic cells [9]. Interestingly, *Leishmania* infection is inhibited by allopurinol, a substrate analogue and selective inhibitor of XO activity, suggesting its involvement in parasite persistence [10]. However, its role in infection of DCs by *L. major* is still unknown.

Indoleamine 2,3-dioxygenase (IDO) which catalyzes the catabolism of tryptophan in many cell types including DCs, has been involved in immune response modulation [11]. In particular, IDO expressed by professional antigen-presenting cells (APC) creates local immune suppression and promotes systemic tolerance by activating Tregs. Interestingly, IDO acts as antioxidant and suppressor of Th1 protective response [11,12].

Pathogens can also mobilize lipid mediators in host cells [13]. Phosphatidylcholine, the most frequent phospholipid in cell membranes is converted into lysophosphatidylcholine (LPC) by phospholipase A2 with the concomitant release of sn-2-arachidonic acid. The latter is sequentially metabolized by cyclooxgenases and prostaglandin synthases into eicosanoids, including prostaglandin E2 (PGE2) which exerts inhibitory effects on various inflammatory cells, such as the suppression of Th1 response in infected macrophages [14–16].

Furthermore, PGE2 from *Ixodes scapularis* saliva may alter the functional maturation of murine DCs [17]. This lipid by-product of

Abbreviations: BMDC, Bone marrow-derived dendritic cells; IDO, Indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; IL-6, Interleukin-6; IL-10, Interleukin-10; kyn, kynurenine; Leish, *Leishmania*; LIPA, *Leishmania* Institut Pasteur Alger; LPS, Lipopolysaccharides; LPC, Lysophosphatidylcholine; NO, Nitric oxide; ROS, Reactive oxygen species; TNF-α, Tumor necrosis factor-α; XO, Xanthine oxidase

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phospholipases activity has been involved in various oxidative physiopathological events [18]. This component of oxidized low-density lipoprotein (LDL) has been reported as a potent atherogenic initiator by inducing macrophage polarization to foam cells [19]. LPC acts mainly through its G2A receptor, constitutively expressed on cells of innate and adaptive immune system [20]. LPC–G2A receptor has been recognized as a sensor for oxidative stress which acts as immune cell blocker [20,21]. LPC exacerbates *Trypanosoma cruzi* infection in mouse bone marrow-derived macrophages through the inhibition of IL-12 and NO production, two major mediators of *Leishmania* clearance [22]. Whereas it promotes the maturation of human DCs into a phenotype that stimulates the development of T cells producing IFN-γ [23].

This study investigated the involvement of xanthine oxidase, iNOS/arginase1 balance and IDO activity in the immune-modulatory effect of L-(1)-stearoyl-lysophosphatidylcholine in DCs infected by *L. major* promastigotes *in vitro*.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, reagents were purchased from Sigma Aldrich (St Louis MO, USA).

2.2. Ethics statement

All experiments were approved by the Ethical Committee for animal handling of the "Institut de Biologie et Médecine Moléculaires, Université Libre de Bruxelles, Gosselies, Belgique" and the "Université des Sciences et de la Technologie Houari Boumediene, Alger, Algérie".

2.3. Production of Leishmania major promastigotes

L. major (MHOM/DZ/06/LIPA35) *inoculum* was kindly provided by the "Laboratoire d'Eco-Epidémiologie et Génétique des Populations, Institut Pasteur (Algiers, Algeria)". The promastigotes were cultured at 25 °C in RPMI 1640 complete medium (10% heat inactivated fetal calf serum, 50 μg/ml streptomycin and 100 U/ml penicillin) containing 2 mM L-glutamine and 5 mM HEPES, pH 7.2. Metacyclic promastigotes were collected at stationary phase, washed three times in sterile phosphate buffered saline (PBS), pH 7.2, and used for dendritic cell (DC) infection.

2.4. Differentiation of bone marrow-derived DCs

BALB/c mice (6–8 weeks old) housed under pathogen-free conditions were used for bone marrow-derived dendritic cell (BMDC) isolation [24]. Briefly, bone marrow was flushed from femur and tibiae and bone marrow cells were depleted of erythrocytes with lysis buffer (150 mM NH_4Cl, 0.1 mM $KHCO_3$ and 0.01 mM Na_2 EDTA, pH 7.2), collected and cultured in 24-well plate (2.5×10^5 cells/2 ml) in RPMI-1640 complete medium containing 10% FCS, 20 ng/ml GM–CSF, 1 mM sodium pyruvate, 2 mM β-mercaptoethanol, 24 mM $NaHCO_3$ and 25 mM HEPES, pH 7.2. On days 3 and 5, bone marrow cells were transferred in fresh medium, supplemented with 10% FCS and 20 ng/ml GM–CSF. Immature DCs were collected at day 9 of culture. Bone marrow cells expressing the cell surface marker CD11c (approximately 90%), were considered BMDC.

2.5. BMDC infection and treatments

Immature DCs (1×10^6 cells/ml RPMI 1640 complete medium) were cultured overnight in 24-well plate under 5% CO_2 at 37 °C in the absence (control) or presence of 100 ng/ml lipopolysaccharides (LPS) or 40 μM of L-(1)-stearoyl-lysophosphatidylcholine (LPC). Thereafter, they were infected with *L. major* promastigotes (parasites: DC ratio, 10:1).

2.6. Evaluation of DC infection

BMDC collected from all experiments were cytospined on slides, fixed with methanol and stained with May Grünwald Giemsa. The infection index (percentage of infected DCs × the number of amastigotes per DC) was evaluated by counting 200 cells per slide under a light microscope [25].

2.7. FACS analysis of DC phenotype

DCs were washed in 1 ml FACS buffer (1% BSA, 0.01% NaN_3, PBS, pH 7.2) and incubated for 10 min with unmarked anti-FcRγ (clone 2.4G2) to prevent non-specific binding. After washing, the cells were double stained for 20 min in 50 μl of FACS buffer for CD11c *vs.* CD86, MHCII or CD40 specific monoclonal antibodies to each given marker which were coupled either with allophycocyanin (APC: anti-CD11c), phycoerythrin (PE: anti-CD86) or biotinylated (anti-CD40 and anti-MHCII) (BD Biosciences). DCs were subsequently treated with streptavidin–Cytochrome7 for 15 min. After washing for three times, DCs were stained with the live/dead fixable blue cell dye (Invitrogen). Viable cells excluding dye were analyzed in 200 μl FACS buffer using a FACS Canto II flow cytometry (Becton Dickinson). Data were analyzed using Cell Quest software.

2.8. Quantification of TNF-α, IL-6 and IL-10

The concentration of TNF-α, IL-6 and IL-10 was determined in culture supernatants using specific ELISA kits, manufacturer's protocols (eBioscience) and standard curves.

2.9. Biochemical analysis

2.9.1. Xanthine oxidase activity assay

Xanthine oxidase (XO) activity was assayed by hypoxanthine oxidation method [26]. Briefly, the reaction was initiated by the addition of 1.5 mM xanthine and the mixture was incubated at 25 °C for 5 min. Absorbance was read at 292 nm and XO activity expressed as unit enzyme. One enzyme activity is defined as the formation of 1 μmol uric acid min^{-1}.

2.9.2. Intracellular reduced glutathione assay

Reduced glutathione (GSH) was measured as described previously [27]. Cells were collected by centrifugation at 2000 g for 2 min and disrupted by freeze-thawing. Homogenates were centrifuged at 10,000 *g* for 20 min. The resulting supernatant was mixed with 5% of 5-sulfosalicylic acid for 20 min in ice and centrifuged at 10,000 *g* for 20 min. The acid-soluble fraction was allowed to react with 0.01 M Ellman's reagent (5,5′-dithiobis (2-nitrobenzoic acid)) in 0.2 M Tris-EDTA, pH 8.9. Absorbance was read at 412 nm and GSH content expressed in μM, using a GSH standard curve.

2.9.3. Nitric oxide concentration

Nitric oxide levels were determined by the measure of total nitrite (NO_2^-), the stable end product of NO [28]. Equal volumes of supernatants and Griess reagent (1% sulphanilamide and 0.1% N-(1-napthyl) ethylene diamine dihydrochloride in 2.5% H_3PO_4), were incubated for 10 min at room temperature. Absorbance was read at 540 nm and NO_2^- expressed in μM, using a sodium nitrite curve as standard.

2.9.4. Arginase1 activity assay

Arginase1 activity was measured as previously described with slight modifications [29]. Briefly, cells were disrupted with 0.1% triton X-100. Cells lysates were incubated at 37 °C for 60 min in 100 μl Tris–HCl (25 mM, pH 7.2) containing 10 mM $MnCl_2$ and 0.5 M L-arginine. After adding 9% isonitrosopropiophenone, the reaction mixture was incubated at 99 °C for 45 min. The urea formed was quantified at 540 nm and