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Lipase of *Candida albicans* induces activation of NADPH oxidase and L-arginine pathways on resting and activated macrophages

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ABSTRACT

Candida albicans secretes various hydrolytic enzymes which are considered to be an integral part in the pathogenesis. However, the role of lipases is far from being completely understood and the direct effects of these fungal enzymes during the host–pathogen interaction remain to be established. We recently isolated and characterized an extracellular *C. albicans* lipase (CaLIP), and demonstrated the ability of this fungal enzyme to interact directly with macrophages (Mϕ) and hepatocytes and to operate as a virulence factor. Herein, we explored the effects of CaLIP on Mϕ functions such as oxidative burst and L-arginine metabolism. The study was performed in cells with different activation status: normal-resting Mϕs and Mϕs primed in vivo or in vitro with *C. albicans*. The ability of this fungal factor to modulate the above-mentioned parameters was dependent on cells status, dose, and microenvironment, where the interaction took place. These results constitute a new finding in the biology of candidiasis and could illustrate an additional evolutive advantage for the fungus in the framework of the bidirectional host–pathogen interaction.

Introduction

The pathogenicity of *Candida albicans* depends on its ability to deal effectively with the host defenses, particularly with the oxidative burst of phagocytic cells. Activated macrophages (Mϕs) and polymorphonuclear leukocytes (PMN) develop an efficient tissue reaction to control the fungus and limit its growth [1]. The reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), hypochlorous acid (HOCl), and reactive nitrogen intermediates as nitric oxide ($NO^{\cdot}$) produced at high concentrations are directly involved in the killing of this microorganism [1–3]. The powerful host-protective effects of $NO^{\cdot}$ are counterbalanced by the immunopathological role of this compound, as high levels of $NO^{\cdot}$ can affect Mϕ integrity by lytic action or apoptosis induction [4]. Consequently, a tight regulation of $NO^{\cdot}$ synthesis appears to be crucial for the host. In this respect growing interest is focused on arginase up-regulation, an enzyme that competes with NO synthase for the substrate L-arginine and is related with the alternate activation pathway in Mϕs [5].

Pathogenic fungi, including *C. albicans,* secrete various hydrolytic enzymes [6]. While secreted phospholipases are well characterized [7,8], other lipolytic enzymes such as lipases and esterases have been widely neglected [6]. The direct effects of these fungal enzymes during the host–pathogen interaction remain to be established [6,9–12]. An important contribution in this field was recently made by our group. We isolated and characterized an extracellular *C. albicans* lipase (CaLIP), and established its ability to interact with host cells actively compromised during *C. albicans* dissemination [13]. We demonstrated, for the first time, that *C. albicans*-released lipase induces cytotoxicity and promotes the deposition of lipid droplets in the cytoplasm of Mϕs and hepatocytes after 48–72 h of incubation [13]. These findings correlate with our previous reports on the induction of hepatic steatosis and the impaired Mϕ function 72 h after *C. albicans* infection [14,15].

The aim of the present study was to investigate the effects of a secreted *C. albicans* lipase on NADPH oxidase and L-arginine pathways in resting or primed Mϕs. Our in vitro results demonstrate that this fungal factor *per se* can modify the host response throughout the modulation of ROS production, $NO^{\cdot}$ release and Mϕ alternative pathway activation.

Materials and methods

Strain and lipase purification. C. albicans strain No. 387 from the collection of Mycology Division of National Clinical Hospital was used for infection and lipase purification [14–17]. Extracellular lipase was purified from cell-free supernatants obtained from 72 h-cultures in liquid nutritive broth as described [13]. The purity

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of the preparation was tested in SDS–PAGE on a 13% polyacrylamide slab gel using Miniprotean II electrophoresis apparatus (Bio-Rad, Richmon, CA), and the activity of the purified enzyme was expressed as U of lipolytic activity [18]. The secreted lipase, designed as CaLIP, exhibited its maximal activity at 37 °C and pH 7–7.5 [13].

Animals, infection and macrophage purification. Adult female Wistar rats were housed in the Animal Resource Facilities, CIBICI-CONICET. For infection, rats were inoculated i.p. (3×10^8 yeasts) and after 72 h the animals were killed and peritoneal cells were recovered [15]. The cell suspension was centrifuged and washed with RPMI and adjusted to 2×10^6 cells/ml in RPMI–10% FCS–0.1% gentamicin. Mϕs were purified by adherence in six-well flat-bottom plates (Corning). After 2 h of incubation (37 °C, 5% CO_2), plates were washed with cold RPMI to remove non-adherent cells. Mϕ monolayer was >90% pure according to morphologic analysis or non-specific esterase staining (Sigma) [16]. Viability was assessed by the Trypan blue.

Experimental design. Three different Mϕ populations were used: (1) normal-resting Mϕs obtained from untreated rats (NR-Mϕs); (2) activated Mϕs obtained from infected rats 3 days after fungus inoculation (Ca-Mϕs); and (3) Mϕs obtained from normal rats and activated in vitro for 2 h with heat-killed *C. albicans* (100 °C/15 min) at a 1:10,000 ratio (NR-Mϕ + Ca). Cells were cultured with medium alone, or with 25 U or 100 U CaLIP, two doses tested previously [17]. As positive control, 1 mg/ml phorbol myristate acetate (PMA, Sigma) or 1 μg/ml LPS from *Escherichia coli* (055:B5, Sigma) was used.

Cell damage. Cell damage was measured by the release of lactate dehydrogenase (LDH) with UV-optimized method (Wiener Lab, Argentina) [13]. The release of LDH was expressed as LDH index calculated as the ratio between the LDH released by CaLIP-treated cells versus LDH released in basal conditions for each experimental protocol.

Oxidative metabolism of Mϕs. The intracellular and extracellular production of ROS were tested in 18 h cultures by the reduction of nitro blue tetrazolium (NBT) [19]. Briefly, 10^5 Mϕs in 0.1 ml HBSS were incubated with 0.1 ml of CaLIP and 0.5 ml of NBT (1 mg/ml) at 37 °C for 30 min. The reaction was stopped with 0.1 ml of 0.1 M HCl; the samples were centrifuged, and the pellet was treated with 0.4 ml dimethyl sulfoxide and 0.8 ml HBSS. Optical density was measured at 540 nm.

The extracellular ROS production was also detected by chemiluminescence (CL) produced after luminol oxidation by $O_2^{\cdot-}$ [19]. Briefly, the CL assay was assessed at 6, 18, and 24 h in polypropylene tubes containing 10^5 Mϕs, 0.1 ml of 3.36 μM luminol (Sigma), and 0.1 ml of purified CaLIP in a final volume of 0.5 ml of HBSS. CL was measured at room temperature in a BioOrbit model 1253 luminometer. The spontaneous CL was determined by incubating Mϕs without CaLIP, and the CL background of each vial was checked before use. The light emission was expressed as relative units of light (RUL) per 10^6 cells. PMA (0.1 mg/ml) and heat-inactivated (100 °C) CaLIP were used as controls [19].

Assessment of L-arginine metabolism. Mϕs were cultured with medium or exposed to different treatments. After 24 or 48 h, the supernatants and the cells were sampled for NO· measurement and arginase activity. The NO· was evaluated as nitrite by a microplate assay method using the Griess reagent and $NaNO_2$ as standard [15]. Each sample was tested by triplicate, and the results were expressed in micromolar (μM).

To assess arginase activity cell monolayers were washed with PBS and treated with 0.15 ml of 0.1% Triton X-100 containing protease inhibitors. After 30 min, cell lysates were mixed with 10 mM $MnCl_2$ 1:1, and the enzyme was activated by heating for 10 min at 56 °C. Arginine hydrolysis was conducted by adding 0.5 M L-arginine, pH 9.7, to the activated lysates [20]. The urea formed was quantified at 540 nm after the addition of 25 μl of 9% α-isonitrosopropiophenone. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute. Results are expressed as microgram (μg) urea/min.

Immunoblot analysis. iNOS expression was assessed in Mϕs stimulated for 48 h. Whole cell extracts were prepared as described previously [17]. Equal amounts of protein (30 μg/lane) were fractionated in a 10% SDS–polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose membranes, and incubated with 2 μg/ml anti-iNOS polyclonal antibody (Santa Cruz Biotechnol). Immunodetection was performed with the enhanced chemiluminescence kit followed by exposure to Amersham Hyperfilm (Uppsala, Sweden).

Statistical analysis. Differences between means were assessed using ANOVA followed by Student–Newman–Keuls test. A p value <0.05 was considered significant.

Results

Effect of CaLIP on ROS production in normal-resting Mϕs

To determine the ability of CaLIP to induce oxygen-free radical formation in NR-Mϕs, we evaluated the production of intracellular and extracellular ROS by NBT reduction after 18 h of incubation with 25 or 100 U of CaLIP (Fig. 1A). In agreement with the resting status, NR-Mϕs exhibited a low spontaneous release of oxidant species; after the stimulation with PMA, the cells produced and released significant amounts of ROS (100% of stimulation). Interestingly, CaLIP was able to induce ROS increase at both concentrations tested, although a stronger effect was observed at 25 U. Comparatively, intracellular and extracellular host stimulated with CaLIP represent 58% and 52% for 25 U, and 40.5% and 36.2% for 100 U, of the values obtained with PMA.

To assess ROS production with a more sensitive technique, NR-Mϕs were cultured with both doses of CaLIP and evaluated by CL after 6, 18, and 24 h of culture. PMA was used as positive control. While ROS were absent at 6 h, NR-Mϕs produced significant levels of the oxygen radicals after 18 and 24 h of contact with CaLIP; the highest production was detected for 25 U at 18 h of culture (Fig. 1B). Considering the lower levels of ROS detected with 100 U of CaLIP or after prolonged treatment, we evaluated the toxic activity of the fungal factor by LDH release. Fig. 1C shows that at both doses and at different times the integrity of Mϕs was preserved.

Effect of CaLIP on NO· production, iNOS expression, and arginase activity in normal-resting Mϕs

In response to infection with several pathogens and inflammatory cytokines, noticeable changes occur in Mϕ arginine metabolism [5,20]. These include, for instance, increment in NO· synthesis via iNOS and catabolism of arginine to ornithine and urea via arginase. To evaluate the effect of CaLIP on L-arginine pathways, we treated NR-Mϕs (25 and 100 U) during 24 or 48 h. PMA and LPS triggered a significant NO· production (Fig. 2A). We found a dose-dependent stimulation of NO· production with higher levels after 48 h of culture. Accordingly, the iNOS protein (~130 kDa) evaluated by Western blot (inset) exhibited a stronger expression after the incubation with the highest dose tested.

The L-arginine metabolism regulation in cells that possess both iNOS and arginase activities is poorly understood. Then, we evaluated the effect of 25 and 100 U of CaLIP on Mϕ arginine metabolism after 48 h of culture. PMA and LPS were used as activation controls [21]. Interestingly, a significant increment in urea levels was detected after CaLIP contact and the major effect was observed with

A

Treatment		ROS	
Cell status	CaLIP (U)	Intracellular ($OD_{540\ nm}$)	Extracellular ($OD_{540\ nm}$)
NR-Mϕs	0	0.015 ±0.002	0.010 ±0.009
	25	0.062* ±0.002	0.048* ±0.006
	100	0.043* ±0.004	0.033 ±0.004
	PMA	0.106* ±0.013	0.091* ±0.009

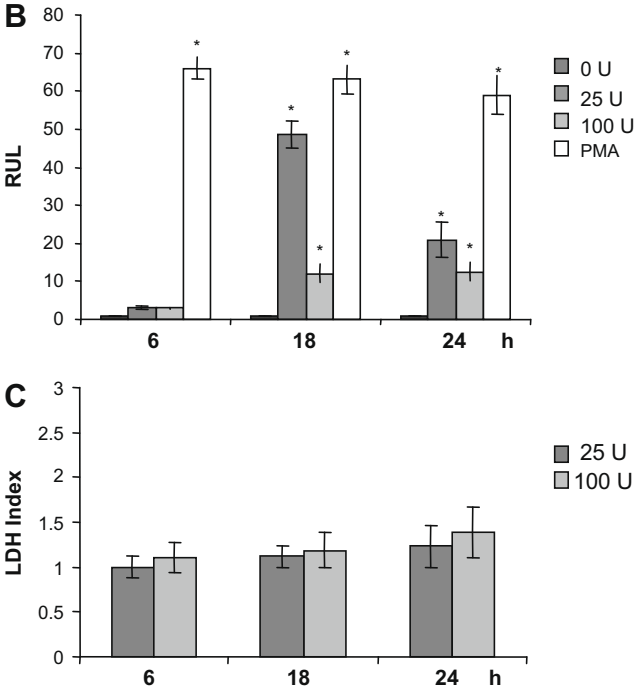

Fig. 1. Effect of *C. albicans* lipase (CaLIP) on the oxidative stress in normal-resting macrophages (NR-Mϕs). (A) Intracellular and extracellular increment of ROS by NBT assay after 18 h of incubation. (B) $O_2^{\cdot-}$ production by chemiluminescence assay expressed as RLU. (C) Cytotoxic effect of CaLIP by lactate dehydrogenase (LDH) release expressed as LDH index. One of three representative experiments is shown. The results are expressed as mean value ± SE. *Treated NR-Mϕs versus untreated NR-Mϕs at each time point $p < 0.05$.

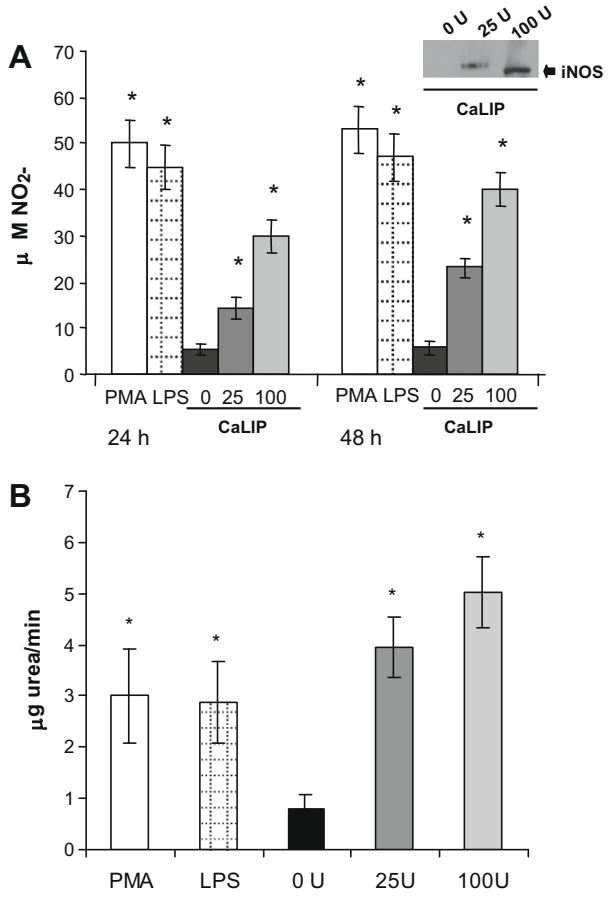

Fig. 2. Effect of CaLIP on L-arginine metabolism in normal-resting macrophages (NR-Mϕs). (A) Production of $NO^{\cdot}$ as nitrite by Griess reaction. Inset, Western blot analysis of iNOS expression in cell lysates 48 h after incubation with CaLIP. (B) The arginase activity in cell lysates (1×10^6 cells/well) expressed as microgram (μg) urea/min. PMA and LPS as positive controls. Results are means ± SE of at least three experiments. *Treated NR-Mϕs versus untreated NR-Mϕs at each time point $p < 0.05$.

100 U (Fig. 2B). The fungal factor stimulated higher arginase activity than LPS and PMA. Cell viability evaluated by LDH release confirmed the non-toxic effect of CaLIP (data not shown).

Effect of CaLIP on ROS production in Mϕs activated in vivo or in vitro with C. albicans

Resting and activated Mϕs can exhibit dissimilar profiles of enzymes, receptors, and released products intimately related to their functional competence. Besides, during the first host–pathogen contact, the susceptibility threshold can be modulated to favor the invader or the receptor in a new encounter [2]. To evaluate the direct effect of the fungal virulence factor on primed cells, Mϕs purified from *C. albicans*-infected animals (Ca-Mϕs) were treated with different doses of CaLIP at 18 h; the induction of ROS was monitored (Table 1). Ca-Mϕs showed a low and spontaneous release of oxidant species similar to NR-Mϕs. After the incubation with CaLIP, intracellular and extracellular ROS were induced and the major effect was observed with 25 U. Compared with PMA, the percentages of stimulation of intracellular and extracellular ROS were 46% and 38% for 25 U, and 38% and 28% for 100 U of CaLIP.

Table 1
ROS production in Mϕs activated in vivo or in vitro with *C. albicans*.

Treatment		ROS (OD 540 nm)	
Cell status	CaLIP (U)	Intracellular	Extracellular
NR-Mϕs	0	0.015 ± 0.002	0.010 ± 0.009
Ca-Mϕs	0	0.020 ± 0.009	0.016 ± 0.005
	25	0.092* ± 0.010	0.071* ± 0.011
	100	0.064* ± 0.014	0.053* ± 0.044
	PMA	0.199* ± 0.019	0.186* ± 0.023
NR-Mϕs + Ca	0	0.025* ± 0.003	0.027* ± 0.003
	25	0.031* ± 0.005	0.044* ± 0.006
	100	0.024* ± 0.003	0.031* ± 0.004
	PMA	0.156* ± 0.033	0.191* ± 0.059

ROS production was assessed by NBT assay at 18 h of culture. One of three representative experiments is shown. The results are expressed as mean value ± SE.
* Treated Mϕs versus untreated NR-Mϕs $p < 0.05$.

Using CL assay, the ROS production in Ca-Mϕs was evaluated after 6, 18, and 24 h of incubation with CaLIP or medium alone (Fig. 3A). In the absence of fungal factor, Ca-Mϕs produced significant levels of ROS (6.1 ± 0.2 RUL) evidencing the priming during the infection. A significant production of ROS was induced after 18 and 24 h of contact with 25 U of CaLIP; at higher doses, lower

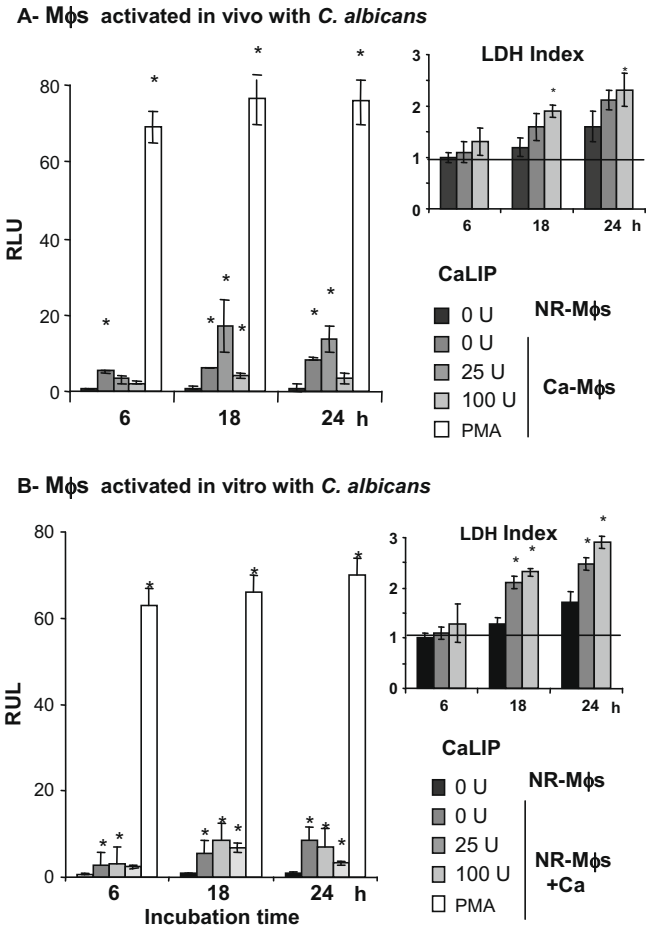

Fig. 3. Effect of CaLIP on ROS production in Mϕs activated with *C. albicans*. (A) In vivo activated Mϕs. (B) In vitro activated Mϕs. The $O_2^{\cdot-}$ production in Ca-Mϕs or NR-Mϕs + Ca by Chemiluminescence assay expressed as RLU. Cytotoxic effect of CaLIP by LDH release (LDH index). One of three experiments is shown. The results are expressed as mean value ± SE. *Treated Ca-Mϕs versus untreated Ca-Mϕs $p < 0.05$.

values were detected at both times tested. As expected, the restimulation with PMA elicited the highest ROS levels (72 ± 3.5 RUL). Cell damage evaluated by LDH index showed that Ca-Mϕs integrity was impaired after prolonged incubations with CaLIP (inset). Mϕs primed in vivo up-regulates the ROS production around threefold after restimulation with CaLIP. Major doses could have an additional toxic effect on activated Mϕs.

Next, we tested the effect of CaLIP on Mϕs activated in vitro with *C. albicans* (NR-Mϕs + Ca). As expected, after stimulation with the fungus alone a significant production of intracellular and extracellular ROS was detected, with a low additional increment after incubation with 25 U CaLIP (Table 1). The effect was also evaluated by CL in a kinetic study; in the absence of CaLIP, NR-Mϕs cultured with the whole fungus increased oxygen-free radical levels compared with resting NR-Mϕs (Fig. 3B). After 18 or 24 h, ROS production was similar with or without the addition of the fungal factor. After the CaLIP treatment, we detected LDH release, indicating that CaLIP has a toxic effect on in vitro activated Mϕs.

Effect of CaLIP on NO· production, iNOS expression, and arginase activity in Mϕs activated in vivo or in vitro with C. albicans

To explore the effect of lipase on L-arginine metabolism we evaluated the iNOS and arginase pathways in Ca-Mϕs after in vitro restimulation with CaLIP. Spontaneous release of NO· was detected in Ca-Mϕs [16] (Fig. 4A). The higher amount of NO· was

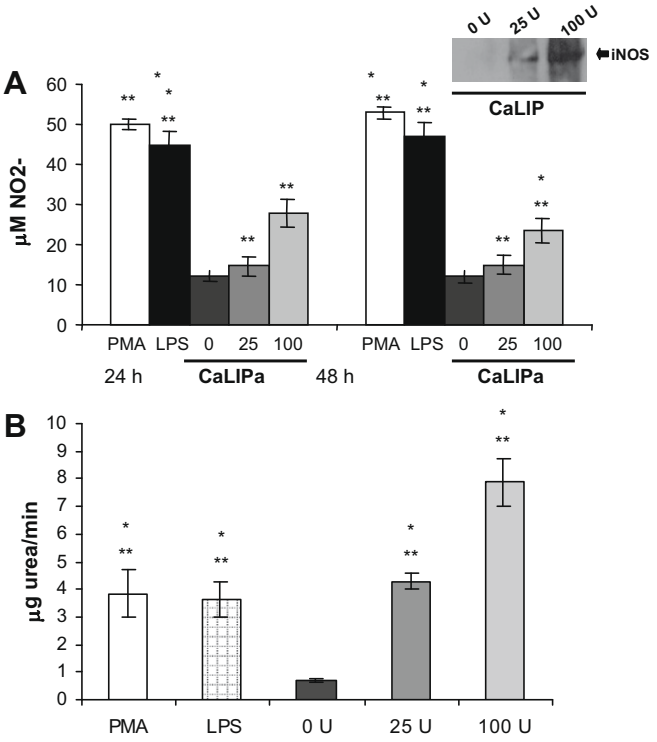

Fig. 4. Effect of CaLIP on L-arginine metabolism in Mϕs activated in vivo with *C. albicans* (Ca- Mϕs). (A) The NO· release was evaluated by Griess assay. Inset, Western blot analysis of iNOS (130 kDa) expression in cell lysates after 48 h of incubation with CaLIP. (B) Arginase activity was assessed as described in Fig. 2B. Results are means ± SE of at least three experiments. *Ca-Mϕs versus NR-Mϕs $p < 0.05$; **treated Ca-Mϕs versus untreated Ca-Mϕs $p < 0.05$.

detected after 24 h of culture with 100 U of CaLIP ($p < 0.05$). With a longer incubation (48 h) the same profile and similar levels were detected (Fig. 4A). The Western blot assay performed at 48 h revealed the up-regulation of iNOS expression in a dose-dependent way (inset of panel B). The higher expression of iNOS and the reduced production of NO· observed at this time could be related to the lower viability of Ca-Mϕs after exposure to CaLIP (Fig. 3A, see LDH index).

The spontaneous activity of arginase in Ca-Mϕs was absent (Fig. 4C), but after 24 h of incubation with 25 U of CaLIP these cells produced similar amounts of urea than NR-Mϕs treated with the fungal factor (3.9 ± 0.8 versus 4.2 ± 0.7 mU urea/protein). Interestingly, 100 U of CaLIP up-regulated significantly the arginase activity on primed cells; in that condition, the values were even higher than those obtained with LPS and PMA. These results demonstrate that after the initial priming, subsequent exposure to this fungal virulence factor favors the Mϕ alternative activation.

Finally, we also evaluated the effect of CaLIP on L-arginine pathways in NR-Mϕs activated in vitro with the whole fungus (data not shown). The exposure to heat-killed fungus induced iNOS expression and NO production (2.2 ± 0.2). While equal or lower levels of nitrogenate metabolites were detected after CaLIP treatment (25 and 100 U), an elevated toxic effect was observed (LDH index – 48 h = 6.2 ± 0.3). Although significant level of urea was detected in NR-Mϕs activated in vitro with *C. albicans* at 48 h ($p < 0.05$), the elevated toxicity of CaLIP on these cells precluded the analysis of arginase pathway in that condition.

Discussion

Phagocytes appear as crucial elements in fungal control and resistance to mucosal and systemic candidiasis. Optimal fungal

killing requires the generation of ROS and nitrogen intermediates. Furthermore, activated Mϕs enhance their effector function through the expression of coestimulatory molecules, the activation of classic and alternative L-arginine pathways, the release of cytokines, and regulation of Th1 profile. During the first interaction with the host, this opportunistic yeast displays several strategies to modulate the colonization/infection balance [1,3]. While the role of phospholipases in *C. albicans* pathogenesis has been established [7,10], the contribution of lipases is far from being completely understood [6].

Here we studied the effects of CaLIP on two biochemical pathways of Mϕs, such as oxidative burst and L-arginine metabolism. Experiments were performed in cells with different activation status: normal-resting Mϕs and Mϕs primed in vivo or in vitro with *C. albicans*. The first contribution is that CaLIP was able to trigger significant production of ROS and NO· and to activate the alternative Mϕ pathway. The second is the ability of fungal factor to display an early cytotoxic effect in host cells primed with the fungus. These results constitute a new finding in connection with the pathogen and its virulence factors, and could represent an additional evolutive advantage for the fungus in the framework of the bidirectional host–pathogen interaction.

Several virulence factors can trigger the NADPH oxidase pathway and induce supraoptimal levels of oxygenate metabolites as part of the damage mechanism. In many conditions, ROS production can also injure or sensitize the host cells to other factors [22,23]. Our findings show that CaLIP operates as a potent stimulus for ROS generation in NR-Mϕs, but triggers dissimilar profiles in activated cells. While in Ca-Mϕs the CaLIP induced an important increase in ROS production, at the same dose the effect on in vitro activated cells was considerably low and associated to decrease of viability. The specificity of this phenomenon is supported by the fact that the NADPH oxidase enzymatic complex was still operative in both types of primed cells, with residual capacity of ROS production after PMA stimulation. Previously we reported that CaLIP is able to induce lipid deposition, viability reduction, and progressive apoptosis of resting Mϕs after 48–72 h of culture [13]. Here we observed that activated Mϕs were more susceptible to cytotoxic effects of fungal virulence factor. Although the knowledge on the effect of fungal lipases in candidiasis pathogenesis is incipient [6,8–11], in *Pseudomonas aeruginosa* infection, lipases are considered as potent virulence factors that modulate Mϕ function [24,25]. These enzymes can operate alone or can induce harmful effects together with other bacterial factors [26]. The *P. aeruginosa* toxin ExoU, with activity on neutral lipids and phospholipids, participates in the mobilization of lipid body, release of inflammatory mediators, promotion of oxidative stress and cytotoxic effects in airway epithelial cells [24].

Several interesting points related to CaLIP effects on L-arginine metabolism can be highlighted from our study; first, overexpression of iNOS and high NO· production in NR-Mϕs upon CaLIP stimulation; to our knowledge this effect has been never described for *Candida* lipases. Second, differences according to cell activation status: Mϕs from infected animals exhibited moderated iNOS expression and spontaneous release. However, the restimulation with high doses of CaLIP increased moderately the NO· release, while it provoked the progressive impairment of cell viability. Besides, cells exposed to killed *C. albicans* and high doses of CaLIP showed manifest damage. An incomplete activation of host cells due to the variation of pathogen surface components after heat treatment, or the absence of physiological microenvironment [3,15,27], could explain the different susceptibility of Mϕs to deleterious effects of CaLIP.

The alternative Mϕ activation induced by parasites such as *Schistosoma, Leishmania*, *Trypanosoma brucei,* and *Trypanosoma cruzi* favors pathogen survival and influences the outcome of the disease [8,28]. Challenging the paradigm of reciprocal regulation of iNOS versus arginase described in many infections, we reported that *C. albicans* triggers the two metabolic pathways on Mϕs [15], as described for LPS [21]. In *C. albicans* infection, Th1 response and classic Mϕ activation are associated with pathogen control, while Th2 profile favors growth and establishment of the fungus [1,3]. The fact that one fungal virulence factor strongly triggers the arginase pathway on resting and activated Mϕs could constitute an additional estrategy of the pathogen to modulate the host immune response.

Taken together, the present results extend our previous observation that CaLIP is a virulence factor released by the pathogen, with potent direct effect on pivotal host cells [17]. The evaluated mechanisms suggest that the fungal factor can simultaneously activate NADPH oxidase and L-arginine pathways and modulate the host response with its own benefit. Our in vitro findings contribute to understand the role of virulence factors in *C. albicans* infection immunopathogenesis.

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