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Biodegradable microspheres containing leukotriene B_4 and cell-free antigens from *Histoplasma capsulatum* activate murine bone marrow-derived macrophages

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ABSTRACT

Because of the potential protective role of leukotrienes (LTs) in histoplasmosis and the therapeutic and prophylactic effects of cell-free antigens from *Histoplasma capsulatum* (CFAgs), the aim of this study was to develop and characterise biodegradable LTB_4/CFAgs-loaded microspheres (MS) that could promote cellular activation for future immunisation purposes. LTB_4/CFAgs-loaded MS that were developed through a double emulsion/extraction process were characterised according to their size, zeta potential, morphology, entrapment efficiency and *in vitro* release kinetics. We evaluated the uptake of LTB_4/CFAgs-loaded MS by bone marrow derived-macrophages (BMDM). The TNF-α and chemokines, and nitrite production, in the supernatant of BMDM cultures were analysed by enzyme-linked immunosorbent assay (ELISA) and Griess reaction, respectively. We found an instantaneous release of CFAgs and a prolonged release of LTB_4 from the poly-(D,L-lactide-*co*-glycolide) (PLGA) MS. The microencapsulation process did not alter the zeta potential nor the spherical morphology of the MS. The appropriate size of the LTB_4/CFAgs-loaded MS (smaller than 10 μm) enabled the efficient uptake by BMDM and also induced TNF-α, CXCL1/KC, CCL2/MCP-1, CCL5/RANTES and nitrite oxide release by these cells. In conclusion, the biodegradable LTB_4/CFAgs-loaded MS were able to efficiently activate murine BMDM and thereby have the potential to be used in an effective vaccine against *H. capsulatum* infection.

1. Introduction

Histoplasmosis is a pulmonary disease characterised by chronic granulomatous and suppurative inflammatory reactions. The etiological agent is the dimorphic, pathogenic fungus *Histoplasma capsulatum* that presents a yeast-like morphology in tissues (Cano and Hajjeh, 2001; Woods, 2002). Resistance to *H. capsulatum* is dependent on cellular immunity, in which is mediated by $CD4^+$ and $CD8^+$ T cells (Allendöerfer et al., 1999) and macrophages that release cytokines, such as interleukin-1 (IL-1) (Deepe and McGuinness, 2006), tumoural necrosis factor-α (TNF-α) (Deepe and Gibbons, 2006), granulocyte-monocyte colony stimulating factor (GM-CSF) (Deepe et al., 1999), interferon-γ (IFN-γ) and IL-12 (Cain and Deepe, 2000). Moreover, the production of nitric oxide (NO) by activated macrophages is essential for the control of the *H. capsulatum* infection (Lane et al., 1994). Finally, during *H. capsulatum* infection, chemokines are important for leukocyte recruitment and the formation and maintenance of the granuloma, a structure that contributes to protective immunity (Heninger et al., 2009).

Leukotrienes (LTs), which are lipid mediators derived from the metabolism of arachidonic acid (AA) by the enzyme 5-lipoxygenase (5-LO) (Murphy and Gijón, 2007), are important to host defence during infections. The effector functions involved in the innate immune responses that are influenced by LTs include direct effects on leucocyte recruitment (Ford-Hutchinson et al., 1980; Medeiros et al., 1999), phagocytosis and antimicrobial mechanisms (Medeiros et al., 2004; Peters-Golden et al., 2005; Serezani et al., 2005). Our group has previously demonstrated an important role

Abbreviations: APC, antigen presenting cells; BMDM, bone marrow-derived macrophages; CFAgs, cell-free antigens; CXCL1/KC, chemokine [C-X-C motif] ligand 1/keratinocyte chemoattractant; CCL2/MCP-1, chemokine [C-C motif] ligand 2/monocyte chemotactic protein-1; CCL5/RANTES, chemokine [C-C motif] ligand 5/regulated upon activation, normally T-cell expressed and secreted; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; LTs, leukotrienes; LTB_4, leukotriene B_4; MS, microspheres; NO, nitric oxide; PLGA, poly (D,L-lactide-*co*-glycolide); TNF-α, tumour necrosis factor-α.

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of LTs in the immune response to *H. capsulatum* because a reduction in LT synthesis by the administration of MK886, a FLAP (five-lipoxygenase activating protein) inhibitor, resulted in increased numbers of lung and spleen colony-forming units (CFUs), impaired IL-12, IFN-γ and NO production and 100% mortality of sublethally *H. capsulatum*-infected mice (Medeiros et al., 2004).

Over the last decades, the incidence of histoplasmosis has increased worldwide, which is mainly a result of the immunological alterations induced by a deficiency in cellular immunity (Kauffman, 2009; Wheat, 2006; Woods, 2002). Therefore, vaccines for the prevention or treatment of this fungal infection have been investigated (Deepe and Gibbons, 2001; Gomez et al., 1992). Our group (Sá-Nunes et al., 2005) has shown that cell-free antigens from *H. capsulatum* (CFAgs) induced potent delayed-type hypersensitivity (DTH) responses in infected mice and activated *H. capsulatum*-specific cells to produce IFN-γ. Moreover, the immunisation with CFAgs was able to protect mice against a lethal inoculum of *H. capsulatum* and significantly reduced the fungal burden in the lungs and spleen of infected mice (Sá-Nunes et al., 2005). Finally, the protection of CFAgs-immunised mice was associated with increased LTB_4 generation, the production of inflammatory mediators, and the generation and recruitment of memory T cells to the lungs (Medeiros et al., 2008).

Successful vaccination techniques stimulate immunity and protection with a minimal number of vaccine administrations. However, most current vaccination procedures require several antigen administrations and the inclusion of immunoadjuvants. These data have urged the development of new vaccine-delivery approaches, mainly using adjuvants approved for use in humans (Johansen et al., 2000). Biodegradable polymer systems using microspheres (MS) based on PLGA (poly-[D,L-lactide-*co*-glycolide]) allow for the sustained and/or controlled release of MS-encapsulated substances (Lima and Rodrigues, 1999). In this context, antigen encapsulation in MS has been used to elicit both cellular and humoral immune responses. Faisal et al. (2009) showed that the microencapsulation of an antigen derived from *Leptospira* into PLGA MS was able to confer more effective protection when compared to the antigen delivered in aluminium hydroxide. Also, PLGA MS have been used to co-encapsulate Toll-Like receptors (TLR) agonists, such as CpG oligonucleotides (CpG ODN), and antigens in order to induce a robust cytotoxic T cells (CTLs) response (Fischer et al., 2009). Our group has pioneered lipid mediator encapsulation, and we have demonstrated that PLGA MS containing LTB_4 can preserve the biological activity and induce a greater uptake rate by peritoneal macrophages (Nicolete et al., 2007). In addition, MS are able to induce greater cell activation compared with that same mediator in a solution form (Nicolete et al., 2008).

Recently, our group has demonstrated that CFAgs-loaded MS of an appropriate size are markedly engulfed by bone marrow-derived macrophages (BMDM), and these MS significantly induce BMDM to produce NO and TNF-α (dos Santos et al., 2009). Considering that LTB_4 increases cell activation (Widegren et al., 2011), cytokines production (Serezani et al., 2011), phagocytosis (Mancuso and Peters-Golden, 2000) and killing mechanisms (Serezani et al., 2005), and LTB_4 is preserved after PLGA encapsulation, the aim of this work was to develop PLGA MS containing both LTB_4 and CFAgs as a strategy to improve the induction phase of an immune response and to consequently increase the effectiveness of vaccinations that confer protection against *H. capsulatum* infection. It is expected that the presence of LTB_4 increases CFAgs uptake, and the activation of antigen presenting cells (APC), and consequently antigen presentation to T lymphocytes. Taken together, these attributes could lead to control of the infection.

In the first part of this work, we characterised LTB_4/CFAgs-loaded MS by size, zeta potential and morphology. We also determined the encapsulation efficiency and *in vitro* release profile of LTB_4 and CFAgs from the MS. In the second part of this work, we investigated if LTB_4/CFAgs-loaded MS are effective in the induction of cellular activation. For this purpose, phagocytosis, production of TNF-α, NO and chemokines (CXCL1/KC, CCL2/MCP-1 and CCL5/RANTES) *in vitro* by BMDM incubated with different MS were evaluated.

2. Materials and methods

2.1. Animals

Young adult (8 weeks old) sv129 mice were obtained from Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil, and were maintained under standard laboratory conditions. All experiments were approved by and conducted in accordance with the guidelines established by the Animal Care Committee of the University (Protocol 08.1.382.53.0).

2.2. Materials

For this study, the lipopolysaccharide (LPS) of *Escherichia coli* (serotype 0127:B8), Griess reagent mixtures, glycine, and 6-coumarin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Poly (D,L-lactide-*co*-glycolide) (PLGA), with a co-monomer ratio of 50:50 (lactic/glycolic acid) and molecular weight of 78 kDa, was obtained from Purasorb (Gorinchem, Netherlands). Poly vinyl-alcohol (Mowiol 40–88) was obtained from Aldrich Chemicals (Wankee, WI, USA). Methylene dichloride and acetonitrile were purchased from Merck (Dietikon, Switzerland), and methanol was purchased from JT Baker (Phillipsburg, USA). For the quantification of LTB_4, a commercially available LTB_4 enzyme immunoassay (EIA) kit was used (Cayman Chemical, MI, USA). Paraformaldehyde at 4% and Aqua Poly/Mount were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA) and Polysciences, Inc. (Warrington, PA, USA), respectively. Panoptic staining was purchased from Laborclin (Pinhais, PR, Brazil). Leukotriene B_4 was purchased from the Cayman Chemical Co. (St. Louis, MO, USA). For the detection of endotoxin, the *Limulus* amoebocyte lysate test (LAL) test was used (QCL-1000, Bio Whittaker, Cambrex Company, Walkersville, MD, USA). The protein content was estimated using a Coomassie Protein Assay Reagent Kit from Pierce (Rockford, IL, USA). RPMI-1640 medium, foetal bovine serum (FBS) and antibiotics (penicillin and gentamicin) were obtained from Gibco (Grand Island, NY, USA). For cell culture, non-tissue culture treated plates from Becton Dickinson were used (BD Falcon, Franklin Lakes, NJ, USA). For the analysis by flow cytometry, the mAbs specific for F4/80 coupled to allophycocyanin (APC) and anti-CD16/CD32 were purchased from BD (San Diego, CA, USA). Commercially enzyme-linked immunosorbent assay (ELISA) antibodies were used to measure TNF-α (OptEIA™, BD Biosciences Pharmingen, San Diego, CA, USA) and chemokines (CXCL1/KC, CCL2/MCP-1 and CCL5/RANTES) (R&D Systems, Minneapolis, MN, USA).

2.3. CFAgs preparation

The CFAgs from *H. capsulatum*, with a yeast-like morphology, were produced according to Sá-Nunes et al. (2005).

2.4. Microspheres preparation

The LTB_4/CFAgs-loaded MS were prepared using the double emulsion/solvent evaporation method, as previously described (Nicolete et al., 2008; Trombone et al., 2007), with some modifications. Briefly, 100 μl of a LTB_4 solution (3.0×10^{-5} M) was added to 10 ml of methylene dichloride containing 120 mg of PLGA.

Subsequently, this organic phase was emulsified with 100 μl of an inner aqueous phase containing CFAgs (10 μg), using a T25 Ultra-turrax homogeniser (IKA, Labortechnik, Germany) to produce a primary water-in-oil emulsion. This emulsion was mixed with 20 ml of an external aqueous phase containing surfactant (PVA at 3%, w/v) to form a stable water-in-oil in-water emulsion. The mixture was stirred for 4 h in an RW20n IKA homogeniser for solvent evaporation. The MS were collected and washed three times with sterile deionised water. They were then freeze-dried and stored at −20 °C until further use. The unloading MS were prepared under the same conditions but without the addition of LTB_4 and CFAgs. Fluorescent-labelled MS were prepared by adding 6-coumarin (green fluorescence) to the organic phase. The 6-coumarin/polymer was added at a concentration of 0.15 μg/mg (Trombone et al., 2007).

2.5. Microspheres characterisation

2.5.1. Size and zeta potential

The particle diameter was determined by laser diffraction using a particle-size analyser (LS 13 320 Laser Diffraction Particle Size Analyser; Beckman Coulter, USA). The zeta potential analysis of the MS was performed using a Nano Zeta Sizer (Malvern instruments, England) (dos Santos et al., 2009). For both analyses, 5 mg of MS were dispersed in 1 ml of deionised water at 25 °C.

2.5.2. Morphology

The shape and surface of the dried MS were observed by scanning electron microscopy (SEM), using a ZEISS scanning microscope (ZEISS, Evo 50, Cambridge, England) (dos Santos et al., 2009). For this analysis, 3 mg of the dried MS was weighted and covered with gold.

2.5.3. Endotoxin measurements

To determine whether the MS were contaminated with LPS, a *Limulus* amoebocyte lysate test (LAL) was performed. Accordingly, 1 mg of unloading and LTB_4/CFAgs-loaded MS was resuspended in PBS and maintained by vortex until the homogenisation was complete. The test was performed according to the manufacturer's instructions.

2.5.4. Encapsulation efficiency

The encapsulation efficiency of LTB_4 was evaluated after the addition of 1.0 ml of acetonitrile in 3 mg of LTB_4/CFAgs-loaded MS. The sample (200 μl) was prepared by vortex (30 s) and by ultrasonic bath (15 s). Subsequently, the sample was submitted to a period of evaporation in a vacuum concentrator for 40 min. The dry sample was resuspended in 1.0 ml of ethanol and maintained by vortex (30 s) and by ultrasound bath (15 s). Aliquots of 200 μl were transferred to another tube in which they were dried and resuspended in EIA buffer for the LTB_4 quantification using the EIA kit. The optical density at 450 nm was determined using a plate reader. The sample values were calculated based on the equation obtained from the calibration curve: $y = -19.27\ln(x) + 125.66$; $r^2 = 0.9698$, where y is the absorbance, x is the lipid mediator concentration (pg/ml), and r is the coefficient of determination.

The encapsulation rate of CFAgs was determined, as previously published (dos Santos et al., 2009). The CFAgs content was assayed by using the Coomassie reagent and CFAgs standard curves. The protein concentration was determined at 595 nm using a microplate reader (μQuant; BioTek Instruments, Inc., Winooski, VT).

2.5.5. In vitro release study

The LTB_4 and CFAgs release rates from encapsulating MS preparations were assessed using a modified Franz-type diffusion cell (Microette; Hanson Research, Chatsworth, CA, USA) and a cellulose acetate membrane with a 0.45 μm pore size (Fisher, USA). Each diffusion cell was mounted by placing the cellulose membrane between the donor and the receptor compartments. The receptor compartment was filled with (i) 7 ml of ethanol/water (1:1, v/v) for the LTB_4 analysis or (ii) 7 ml of pure water for CFAgs. The receptor was also stirred at 300 rpm and thermoregulated at 37 °C by a water jacket. Ten milligrams of LTB_4/CFAgs-loaded MS were placed in the donor compartment on the membrane. The samples (2 ml) were withdrawn at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 24 and 48 h and analysed for the presence of LTB_4 or CFAgs. The LTB_4 and CFAgs concentrations in each samples were determined using the EIA kit and the Coomassie reagent with CFAgs standard curves, respectively.

2.6. Bone marrow derived-macrophages (BMDM)

Bone marrow derived-macrophages (BMDM) were obtained according to previously published methods (dos Santos et al., 2009). The bone marrow from the femurs and tibias of sv129 mice was plated on day 0 in 24-well plates at 1.0×10^6 cells/ml in RPMI-1640 medium containing 20% FBS, 30% culture supernatant L-929 (clone 929 of strain L, ATCC: CCL1) and antibiotics (10 μl/ml penicillin and 1 μl/ml gentamicin). On day 3, an equal volume of RPMI medium was added. On day 6, one half of the volume of RPMI medium was replaced. On the final day of the macrophage differentiation stage (seventh day), the cells were removed with sterile ice-cold PBS and analysed by flow cytometry for the expression of the F4/80 (phenotype of the macrophages) on the cell surface (Austyn and Gordon, 1981). Briefly, Cell suspensions (1.0×10^6 cells/ml) were preincubated with anti-CD16/CD32 (Fc Block) for 40 min at room temperature. The cells were then incubated with an APC-labelled mAb for F4/80 for 30 min at room temperature. Next, the cells were washed with PBS containing 2% FBS, resuspended in PBS containing 1% formaldehyde and analysed by flow cytometry. The analytical flow cytometry was performed using a FACSCanto (Becton Dickinson, San Jose, CA, USA), and the data were analysed using the FACSDiva software (Becton Dickinson, San Jose, CA, USA). The results are presented as adjusted percentages; i.e., the background from the appropriate isotype controls was subtracted from the positive percentage. BMDM cultures with greater than 70% of cells expressing F4/80 antigen were used in our experiments.

2.7. Uptake of MS by BMDM

The BMDM (1×10^6 per well/24-wells) were incubated for 4 h with 800 μl of a 1 mg/ml dispersion of unloading or LTB_4/CFAgs-loaded MS at 37 °C. After the incubation period, the medium was aspirated, and non-ingested MS were washed off twice with additional medium. Next, the cell suspensions were collected, cytocentrifugated and identified by panoptic staining. The same experiment was conducted at 4 °C as a control of phagocytic activity (Nicolete et al., 2007; Salman et al., 2000; dos Santos et al., 2009). The MS uptake was assessed microscopically by counting the percentage of BMDM that had ingested at least one MS. The phagocytic index (PI) was calculated according to the formula: PI = (number of engulfed MS × number of BMDM containing at least one MS)/total number of BMDM (Nicolete et al., 2007; dos Santos et al., 2009).

To confirm the uptake of MS, the BMDM (5×10^5 cells per well/24-wells) were plated on wells containing coverslips and co-incubated with 800 μl of a 1 mg/ml of fluorescent MS dispersion (unloading or LTB_4/CFAgs-loaded MS) for 4 h. After this incubation period, the cells were washed twice with PBS, fixed for 15 min with 2% paraformaldehyde in PBS and rinsed in PBS. Cells were incubated with PBS containing glycine at 0.1 M for 5 min and then they were washed with PBS. The coverslips were mounted using Aqua Poly/Mount (Polysciences) and examined by confocal microscopy (Leica TCS SP5 AOBS – Leitz, Manheim, Germany) (dos Santos et al., 2009).

2.8. Nitric oxide, TNF-α and chemokines production by BMDM

The production of NO, TNF-α and chemokines (CXCL1/KC, CCL2/MCP-1, CCL5/RANTES) by murine BMDM was determined by Griess reaction (Green et al., 1981) and enzyme-linked immunosorbent assay (ELISA), respectively. The BMDM were incubated for 24 h with 800 μl of (i) 1 mg/ml dispersion of unloading MS, (ii) 1 mg/ml dispersion of LTB_4/CFAgs-loaded MS, (iii) LTB_4 solution at 3.0×10^{-8} M, or (iv) CFAgs solution at 50 μg/ml. BMDM incubated with medium alone or a LPS solution (500 ng/ml) were used as negative and positive controls of cell activation, respectively. The supernatants (0.1 ml) were incubated with an equal volume of Griess reagent mixtures (1% sulphanilamide, 0.1% *N*-(1-naphtyl)-ethylendiamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance was measured in a microplate reader at 540 nm and the concentrations were calculated from a sodium nitrite standard curve. The data are presented as micromoles of NO_2^- (nitrite) (mean ± S.E.M). The cytokine and chemokines were measured using commercially available antibodies with reference standard curves. The sensitivity for cytokines and chemokines was >7.5 pg/ml. The optical density of samples was determined at 450 nm in a microplate reader (μQuant; BioTek Instruments, Inc., Winooski, VT).

2.9. Statistical analysis

The data were analysed using unpaired *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Size, zeta potential and surface of the MS

The lyophilised MS (LTB_4/CFAgs-loaded MS and unloading MS) were dispersed in deionised water and submitted to a size distribution analysis. The average diameter was 6.4 μm (±4.7 μm) and 9.1 μm (±5.7 μm) for unloading and LTB_4/CFAgs-loaded MS, respectively. To evaluate whether the encapsulated CFAgs would alter the residual charges of the polymer, the analysis of the zeta potential was carried out. The average zeta potential for unloading and LTB_4/CFAgs-loaded MS was −21.8 mV (±9.5 mV) and −24.4 mV (±6.2 mV), respectively. The scanning electron micrographs of the MS are shown in Fig. 1. The unloading and LTB_4/CFAgs-loaded MS were all spherical with a regular surface.

3.2. Endotoxin measurements

The unloading and LTB_4/CFAgs-loaded MS were also assayed for the detection of endotoxin activity using the LAL test. The endotoxin contamination in all MS batches was lower than 0.1 EU/μg, and the preparations were adequate for use in accordance with the criteria of the European Pharmacopoeia (safety level for endovenous administration is 5 EU/kg/h).

3.3. Encapsulation efficiency and in vitro release of LTB_4 and CFAgs from the MS

The encapsulation efficiency of LTB_4 and CFAgs was determined with an EIA kit and the Coomassie protein assay, respectively, after the extraction of the substances from the MS. The amount of LTB_4 detected was 11.26 ng per mg of MS, whereas 37 ng of CFAgs were detected per mg MS. These values represent an encapsulation efficiency of 5.2% (LTB_4) and 44.0% (CFAgs). The *in vitro* release rates of LTB_4 and CFAgs from the PLGA MS were evaluated for up to 48 h, and the release profile of LTB_4 is shown in Fig. 2. The results demonstrated that LTB_4 release from the MS was sustained. In contrast, the CFAgs were rapidly released from the PLGA MS: up to 89% (±2%) of the total CFAgs content was detected in the receptor solution after 30 min.

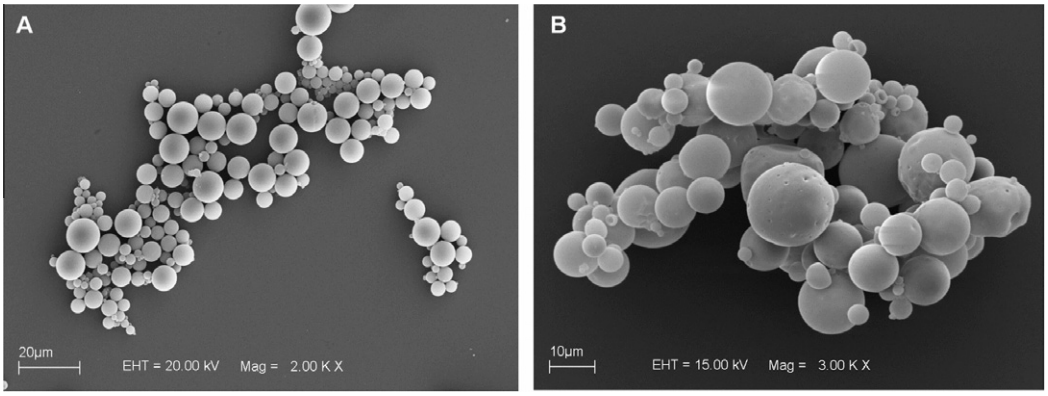

Fig. 1. (A) Unloading and (B) LTB_4/CFAgs-loaded MS morphologies assessed by scanning electron microscopy (SEM).

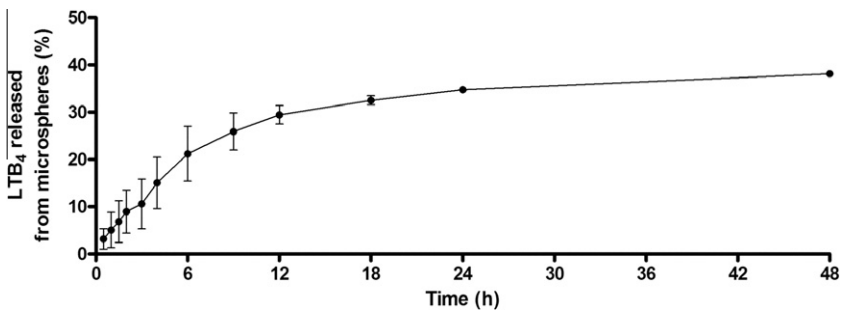

Fig. 2. *In vitro* cumulative release of LTB_4 from PLGA MS in water/ethanol (1:1), pH 7.4.

Table 1
Uptake of PLGA MS by BMDM after 4 h of incubation.

PLGA MS	% BMDM containing MS[a]	Phagocytic index
Unloading MS ($n = 5$)	35.0 ± 3.07	27.43 ± 4.56
LTB_4/CFAgs- MS ($n = 11$)	71.91 ± 4.22***	208.37 ± 35.87**

[a] Data are expressed in percentage of BMDM that engulfed at least one MS. The phagocytic index (PI) was also calculated: PI = (number of engulfed MS × number of BMDM containing at least one MS)/total number of BMDM. Results are presented as mean ± S.E.M.
** $p < 0.01$.
*** $p < 0.001$, values compared to unloading MS (unpaired t-test).

3.4. LTB_4/CFAgs-loaded MS are more efficiently captured by BMDM

After *in vitro* cellular differentiation, we confirmed the phenotype of BMDM by analysis of flow cytometry and determined that roughly 70% of BMDM had F4/80 expression on their surface. The uptake assay of MS by BMDM ($F4/80^+$ cells) revealed a greater number of phagocytosed LTB_4/CFAgs-loaded MS when compared with those incubated with unloading MS (***$p < 0.001$; Table 1). As expected, there was no particle uptake at 4 °C because the low temperature impairs macrophage activation, and therefore, reduces their phagocytotic capacity (Salman et al., 2000) (data not shown). The BMDM presented a greater phagocytic index when incubated with LTB_4/CFAgs-loaded MS than with unloading MS (**$p < 0.01$; Table 1).

The presence of MS inside the BMDM was also evaluated using a scanning confocal laser microscope. The BMDM were incubated with fluorescent unloading or fluorescent LTB_4/CFAgs-loaded MS for 4 h at 37 °C. A greater number of fluorescent LTB_4/CFAgs-loaded MS (Fig. 3B) were engulfed by BMDM as compared to engulfment of fluorescent unloading MS (Fig. 3A).

3.5. Engulfed MS induce NO, TNF-α and chemokines production by BMDM

To evaluate the effects of MS on BMDM, the production of NO, TNF-α and chemokines, which play important roles in the immune response during histoplasmosis, was measured. LTB_4/CFAgs-loaded MS stimulated relevant production of TNF-α (527.50 ± 38.99 pg/ml; ***$p < 0.001$) and NO (1.00 ± 0.13 μM; ***$p < 0.001$) by BMDM when compared with cells incubated only with medium (25.46 ± 13.42 pg/ml; 0.10 ± 0.02 μM). However, the LTB_4/CFAgs-loaded MS-stimulated BMDM did not produce statistically greater concentrations of TNF-α in comparison with those stimulated with unloading MS (445.44 ± 45.31 pg/ml), except in relation to NO production (#$p < 0.05$) (Fig. 4A and B).

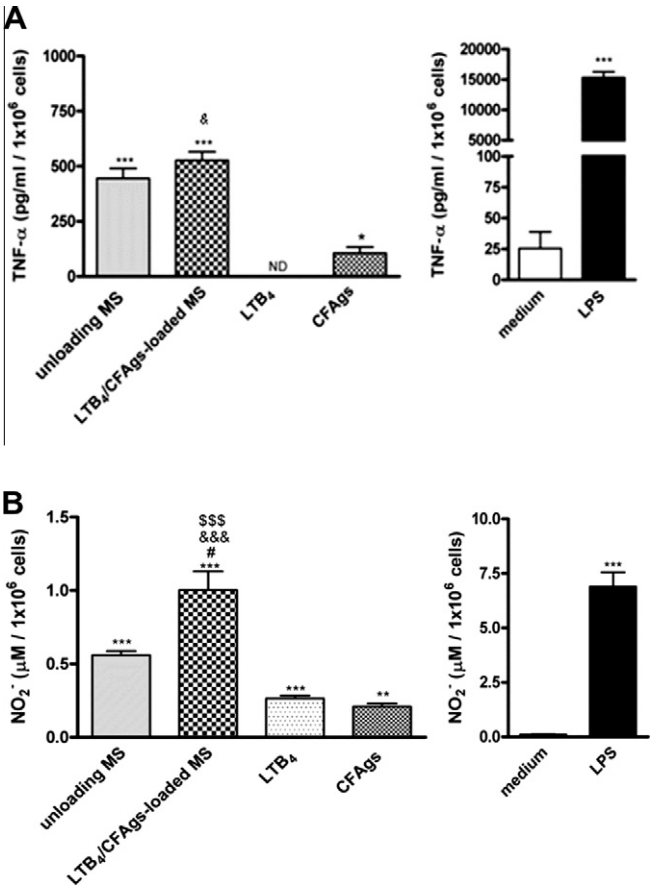

Fig. 4. (A) TNF-α and (B) nitrite (NO_2^-) production by BMDM (1.0×10^6 cells) after 24 h of incubation at 37 °C with medium, 1 mg/ml of unloading MS, LTB_4/CFAgs-loaded MS, LTB_4 (3.0×10^{-8} M) or CFAgs (50 μg/ml) in solution forms. LPS (0.5 μg/ml) was used as positive control. The cytokine ($n = 8$) and nitrite ($n = 6$) concentration in the supernatants of the BMDM culture were quantified by ELISA and Griess reaction, respectively. The results are expressed as mean ± S.E.M.; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, values compared with medium; #$p < 0.05$, values compared with unloading MS (unpaired t-test); &$p < 0.05$ and &&&$p < 0.001$, values compared with CFAgs in the solution form; $$$$p < 0.001$, values compared with LTB_4 in the solution form. ND = value not determined by assay.

In addition, we evaluated the production of CXCL1/KC, CCL2/MCP-1 and CCL5/RANTES. The BMDM stimulated with LTB_4/

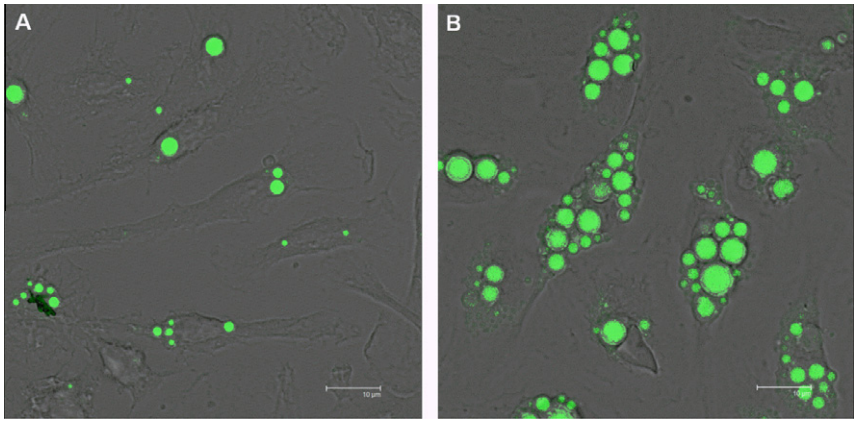

Fig. 3. Confocal images of BMDM containing (A) fluorescent unloading MS or (B) LTB_4/CFAgs-loaded MS. The BMDM were incubated with fluorescent MS for 4 h at 37 °C, and subsequently, phagocytosis was evaluated by confocal microscopy. Microphotographs were taken and examined under 100× magnification.

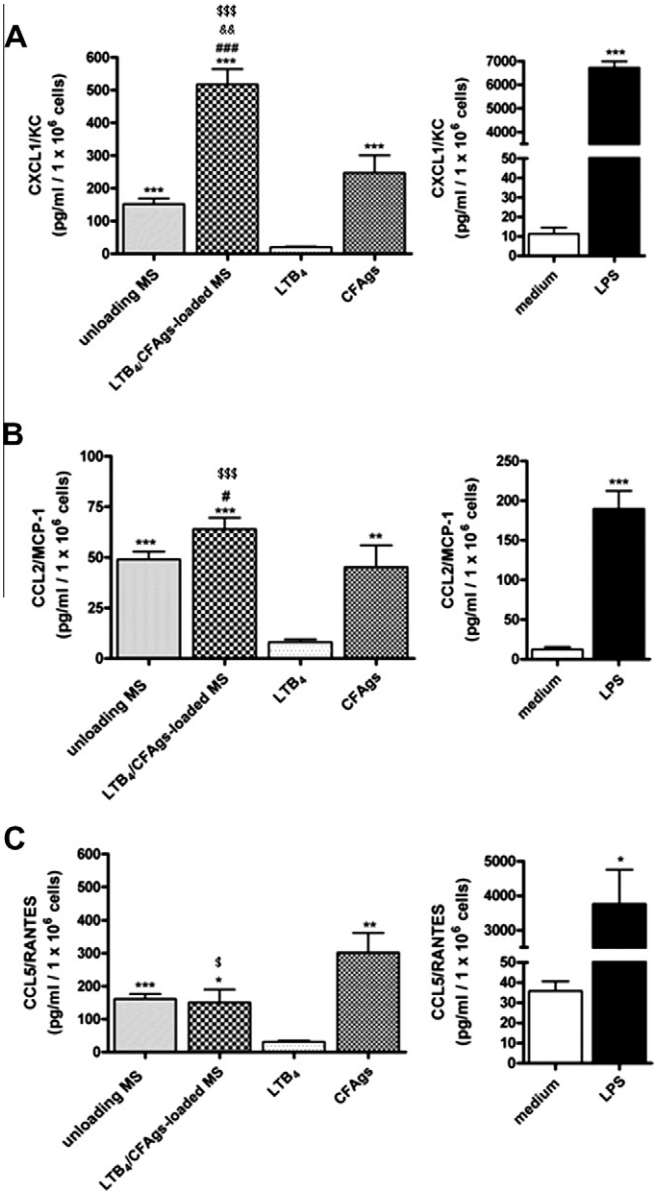

Fig. 5. (A) CXCL1/KC, (B) CCL2/MCP-1 and (C) CCL5/RANTES production by BMDM (1.0×10^6 cells) after 24 h of incubation at 37 °C with medium, 1 mg/ml of unloading MS, LTB_4/CFAgs-loaded MS, LTB_4 (3.0×10^{-8} M) or CFAgs (50 μg/ml) in solution forms. LPS (0.5 μg/ml) was used as positive control. The ELISA assay was used to quantify the chemokine concentrations in the supernatants of the BMDM culture. The results are expressed as mean ± S.E.M. ($n = 9$); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, values compared with medium; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, values compared with unloading MS; $^{\&\&}p < 0.01$, values compared with CFAgs in the solution form; $^{\$}p < 0.05$ and $^{\$\$\$}p < 0.001$, values compared with LTB_4 in the solution form (unpaired *t*-test).

CFAgs-loaded MS produced significant amounts of CXCL1/KC and CCL2/MCP-1 (452.17 ± 23.73 pg/ml, 63.99 ± 5.66 pg/ml, respectively) when compared with those incubated with unloading MS ($^{\#\#\#}p < 0.001$ and $^{\#}p < 0.05$) (152.32 ± 17.37 pg/ml, 49.08 ± 3.78 pg/ml, respectively) or cells exposed to medium alone ($^{***}p < 0.001$) (11.32 ± 3.24 pg/ml, 12.3 ± 2.98 pg/ml, respectively). Nevertheless, the BMDM incubated with LTB_4/CFAgs-loaded MS produced low concentrations of CCL5/RANTES (150.98 ± 39.07 pg/ml), which was similar to the levels produced by cells exposed to unloading MS and less than the amount induced by CFAgs in solution form (Fig. 5A–C).

4. Discussion

Biodegradable polymer systems allow for the sustained release of a great range of encapsulated substances, especially antigens, and the encapsulation of antigens in PLGA MS has been an useful tool for the efficient activation of the immune system and the development of new vaccine preparations (Faisal et al., 2009; Feng et al., 2006; Men et al., 1995; dos Santos et al., 2010; Trombone et al., 2007; Lima et al., 2003). In the present study, a novel preparation of biodegradable MS containing both LTB_4 and CFAgs from *H. capsulatum* was developed and evaluated for its ability to induce cellular activation. The double emulsion/solvent evaporation method was chosen to prepare the particles because lipid LTB_4 is only soluble in organic solvents, while CFAgs are water-soluble.

In the polymeric microparticles system, obtaining a precise measurement of the encapsulated content is a critical step (Feng et al., 2006; Gupta et al., 1997). Therefore, we determined the encapsulation efficiency of LTB_4 and CFAgs in this report by extracting the encapsulated substances from the polymeric matrix and dosing them using the EIA kit and Coomassie protein assay, respectively. For the Coomassie protein assay, we adopted a careful procedure to minimise any possible interference between acetonitrile (used for dissolving MS) and the Coomassie reagent, as described in Section 2.5. Our results showed that the encapsulation rate achieved for CFAgs was higher than that found for LTB_4 (44.0% and 5.2%, for CFAgs and LTB_4, respectively). Co-encapsulation of the protein antigen seems to impair an efficient incorporation of LTB_4 in MS because our previous studies demonstrated a higher encapsulation rate of this lipid mediator by PLGA MS when LTB_4 is encapsulated alone (Nicolete et al., 2007). Nevertheless, the release studies have demonstrated a sustained *in vitro* release of LTB_4 (Fig. 2), even in the presence of CFAgs. The amount of LTB_4 in acceptor medium was constantly increased over time for up to 6 h ($r = 0.99$), after which its release rate was decreased. Nonetheless, the MS continued to release the lipid mediator after this point. In comparison, the release of CFAgs from the PLGA MS was instantaneous; nearly all of the CFAgs content reached the receptor solution after 30 min. This result is predictable because CFAgs are hydrosoluble, which facilitates their rapid expulsion from the hydrophobic PLGA MS to the aqueous receptor.

The average size of the particles (LTB_4/CFAgs-loaded MS) was 9.1 μm (±5.7 μm). Thus, the combined co-encapsulation of LTB_4 and CFAgs in the same MS increased slightly the MS diameter when compared to unloading MS (mean diameter of 6.4 ± 4.7 μm). However, this difference was considered of negligible importance because the MS diameter generated was smaller than 10 μm in each case, which is an appropriate size for their efficient phagocytosis (Champion et al., 2008). The analysis of MS zeta potential was evaluated to verify whether the encapsulation of lipid mediators (LTB_4) and proteins (CFAgs) would modify the negative charge of the polymer. Our results showed that the zeta potential of LTB_4/CFAgs-loaded MS (−24.4 ± 6.2 mV) was not significantly different from that of unloading MS (−21.8 ± 9.5 mV). Furthermore, LTB_4/CFAgs-loaded MS were largely spherical, although discreet irregularities in the shape of some particles occurred (Fig. 1). In spite of results from the literature reporting that the MS size (Champion et al., 2008; Nicolete et al., 2011) and zeta potential (Tabata and Ikada, 1988) could influence MS uptake by macrophages, our results demonstrate that neither size nor zeta potential were a major contributor to the increased phagocytosis of LTB_4/CFAgs-loaded MS by BMDM.

In this study, we demonstrated that the capture of LTB_4/CFAgs-loaded MS by BMDM occurred more efficiently than the capture of unloading MS. Importantly, both of these target molecules bore an appropriate size, zeta potential and morphology (Table 1 and

Fig. 3). Additionally, in comparison with our previously published results, the LTB_4/CFAgs-loaded MS are phagocytosed at a greater rate than the CFAgs-loaded MS (dos Santos et al., 2009). Nicolete et al. (2007) have published that PLGA MS containing LTB_4 were taken up faster by peritoneal macrophages when compared to unloading MS. Together, these results suggest that co-encapsulated LTB_4 is a major factor in the observed increase in MS phagocytosis. This increase is likely due to its release in extracellular from LTB_4/CFAgs-loaded MS, which allows it to interact with BLT1 and stimulate the MS uptake by BMDM. These results were not surprising expected as it is well-known that LTB_4 potentiates phagocytosis and the killing of microorganisms (Mancuso and Peters-Golden, 2000; Okamoto et al., 2010; Peters-Golden et al., 2005).

TNF-α is a pleiotropic inflammatory cytokine that is secreted by different cells. It participates in the inflammatory response and presents a great variety of functions, including cell activation (Aggarwal, 2003). Although TNF-α could play a deleterious role during systemic inflammation, as seen in sepsis (Spooner et al., 1992), it also contributes to the development of an innate and adaptive response. It was previously demonstrated that the pretreatment of human monocytes with TNF-α enhances the capacity of these cells to present soluble antigens to T lymphocytes (Zembala et al., 1990). Moreover, TNF-α increases MHC class I expression in murine cells (Israël et al., 1989) and TNF-α synergises with IL-12 to induce IFN-γ and a Th1 immune response (Ahlers et al., 1997), thereby inducing monocytes to become dendritic cells and promoting adaptive immunity (Chomarat et al., 2003). Moreover, TNF-α is essential for the development of host defence mechanisms during *H. capsulatum* infection (Allendöerfer and Deepe, 1998; Deepe and Gibbons, 2006). To evaluate the stimulatory actions of LTB_4/CFAgs-loaded MS on BMDM, we quantified the amount of TNF-α in the supernatant following cell culture. LTB_4/CFAgs-loaded MS and CFAgs in solution induced a significant increase in TNF-α concentrations in the supernatant of BMDM cultures (Fig. 4A), confirming that PLGA MS function as particulate adjuvants (Jones, 2008). In contrast to our published results (dos Santos et al., 2009), cells stimulated with unloading MS also produced significant quantities of TNF-α. We believed that this difference may be due to the use of PLGA from different manufacturers (see in materials and Section 2), as was recently reported by Liechty et al. (2010). Conversely, TNF-α was not detected in the supernatants of BMDM cultures incubated with the LTB_4 solution alone.

Nitric oxide (NO) is an effector molecule present during infections, as it is mainly induced by intracellular pathogens, such as *H. capsulatum* (Lane et al., 1994). Although NO has been implicated in certain pathological conditions, such as rheumatoid arthritis (Vladutiu, 1995) and septic shock (Thiemermann, 1994), its immunoregulatory role has been described. Low concentrations of NO produced by APC promote Th1 cell differentiation, but not Th2, and inhibit T cell apoptosis (Niedbala et al., 1999, 2006). Moreover, NO regulates the maturation of dendritic cells and their MHC class II expression (Wong et al., 2004). Notably, NO and TNF-α are important for the control and prevention of histoplasmosis (Allendöerfer and Deepe, 1998; Lane et al., 1994). Our results demonstrating that LTB_4/CFAgs-loaded MS were effective in inducing low but significant concentrations of NO and TNF-α by BMDM, as compared to unloading MS (Fig. 4B), suggest that LTB_4/CFAgs-loaded MS may be an important tool to increase protection against *H. capsulatum* infection and can be used as a vaccine.

Chemokines are produced by inflammatory and resident cells, and play a role in both innate and adaptive immune responses and are chemoattractants for granulocytes, macrophages, lymphocytes and immature or mature dendritic cells (Sallusto and Baggiolini, 2008). Neutrophils and macrophages are pivotal cells involved in the efficient clearance of invading pathogens via their ability to perform phagocytosis and killing of microorganisms, such as *H. capsulatum* (Sá-Nunes et al., 2007; Newman, 1999; Kroetz and Deepe, 2011). Moreover, macrophages and dendritic cells recruited to the site of infection or site of vaccine administration are fundamental for antigen uptake and subsequent presentation to T cells, an essential first step in the generation of an adaptive immune response against fungal pathogens (Cutler et al., 2007). With regard to chemokine production, it was verified that all preparations, except the LTB_4 in solution form, potentiated the release of CXCL1/KC, CCL2/MCP-1 and CCL5/RANTES, when compared with medium only cultures. Nevertheless, in comparison with unloading MS, LTB_4/CFAgs-loaded MS only induced greater CXCL1/KC and CCL2/MCP-1 production by BMDM (Fig. 5A). The chemokines, along with other mediators, are important for leukocyte recruitment and the formation and maintenance a granuloma, which contributes to protective immunity (Heninger et al., 2009). The micro co-encapsulation of LTB_4 and CFAgs was able to increase chemokine release by BMDM. This fact suggests that the LTB_4 released outside the cell may stimulate MS uptake through the same mechanism involved in the enhanced phagocytosis of microorganisms. Also, released LTB_4 can influence chemokine production. Kroetz and Deepe (2010) demonstrated that CCR5 and its ligands, such as CCL5 (RANTES), participate in the recruitment of regulatory T cells (Tregs) to the sites of *H. capsulatum* infection. Thus, discrete RANTES production could avoid the recruitment of Tregs and contribute to the control of infection (Kroetz and Deepe, 2010).

Our results suggest that LTB_4/CFAgs-loaded MS are an efficient tool to be used as vaccine and can prevent *H. capsulatum* infection for several reasons. First, LTB_4/CFAgs-loaded MS induced chemokine release, which are essential chemoattractants for macrophages and dendritic cells that promote uptake and antigen presentation. Second, MS contain CFAgs, antigens that confer efficient immune protection (Sá-Nunes et al., 2005). Third, the presence of LTB_4 provides an additional stimulus to support cell activation and increase uptake by BMDM (dos Santos et al., 2009; and Table 1). Fourth, LTB_4/CFAgs-loaded MS induced a significant, albeit limited, release of TNF-α and NO (Fig. 4), when compared to LPS (30 times less). The low concentration of these mediators may be relevant for cell activation and antigen presentation to lymphocytes, without unwanted side effects.

In this context, we can suggest that LTB_4/CFAgs-loaded MS have the potential to be used as a vaccine for histoplasmosis. However, further *in vivo* studies with LTB_4/CFAgs-loaded MS must be performed in order to evaluate its potential for immunisation and protection against disease.

5. Conclusions

The PLGA MS containing LTB_4/CFAgs developed in this study exhibit promising vaccine potential that may help prevent *H. capsulatum* infection in endemic areas. Further studies are being carried out to investigate the effect of LTB_4/CFAgs-loaded MS in animal models of immunisation processes, which will facilitate an evaluation of the conferred protection against histoplasmosis.

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