



Prostaglandin D₂-loaded microspheres effectively activate macrophage effector functions



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ABSTRACT

Biodegradable lactic-co-glycolic acid (PLGA) microspheres (MS) improve the stability of biomolecules stability and allow enable their sustained release. Lipid mediators represent a strategy for improving host defense; however, most of these mediators, such as prostaglandin D₂ (PGD₂), have low water solubility and are unstable. The present study aimed to develop and characterize MS loaded with PGD₂ (PGD₂-MS) to obtain an innovative tool to activate macrophages. PGD₂-MS were prepared using an oil-in-water emulsion solvent extraction-evaporation process, and the size, zeta potential, surface morphology and encapsulation efficiency were determined. It was also evaluated *in vitro* the phagocytic index, NF-κB activation, as well as nitric oxide and cytokine production by alveolar macrophages (AMs) in response to PGD₂-MS. PGD₂-MS were spherical with a diameter of $5.0 \pm 3.3 \mu\text{m}$ and regular surface, zeta potential of $-13.4 \pm 5.6 \text{ mV}$, and 36% of encapsulation efficiency, with 16–26% release of entrapped PGD₂ at 4 and 48 h, respectively. PGD₂-MS were more efficiently internalized by AMs than unloaded-MS, and activated NF-κB more than free PGD₂. Moreover, PGD₂-MS stimulated the production of nitric oxide, TNF-α, IL-1β, and TGF-β, more than free PGD₂, indicating that microencapsulation increased the activating effect of PGD₂ on cells. In LPS-pre-treated AMs, PGD₂-MS decreased the release of IL-6 but increased the production of nitric oxide and IL-1β. These results show that the morphological characteristics of PGD₂-MS facilitated interaction with, and activation of phagocytic cells; moreover, PGD₂-MS retained the biological activities of PGD₂ to trigger effector mechanisms in AMs. It is suggested that PGD₂-MS represent a strategy for therapeutic intervention in the lungs of immunocompromised subjects.

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1. Introduction

Alveolar macrophages (AMs) play an important role in pulmonary infections by phagocytizing pathogenic bacteria and secreting molecules such as cytokines, nitric oxide (NO), reactive

oxygen species, and lipid mediators that contribute to the control of infection (Gordon, 2007; Medeiros et al., 2012; Secatto et al., 2012). Prostaglandins (PGs) are well known lipid mediators that are synthesized from arachidonic acid by cyclooxygenase enzymes that were important during the inflammatory process (Medeiros et al., 2012; Pereira et al., 2013). Several subtypes of PGs with distinct activities have been described, including PGE₂, PGI₂, and PGD₂ (Harris et al., 2002). Among them, PGD₂ has been characterized as eosinophil chemo attractant (Monneret et al., 2001) and anti-inflammatory agent (Joo et al., 2007; Joo and Sadikot, 2012; Murata et al., 2013). Intratracheal administration of PGD₂ has been shown to decrease the bacterial load in the lungs of *Pseudomonas aeruginosa*-infected mice (Joo et al., 2007). Moreover, PGD₂ and its metabolic product 15dPGJ₂ demonstrated anti-inflammatory

Abbreviations: AMs, rat alveolar macrophages; IL-6, interleukin-6; IL-10, interleukin-10; LAL, Limulus Amoebocyte Lysate; LPS, lipopolysaccharide; MS, microspheres; PBS, phosphate-buffered saline; PGs, prostaglandins; PGD₂, prostaglandin D₂; PGD₂-MS, PGD₂ loading MS; PI, phagocytic index; PLGA, poly(lactic acid-co-glycolic acid); TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-α.

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Table 1Size and zeta potential of unloaded and PGD₂-loaded MS.

Microsphere	Size (Mean \pm SD, μ m)	Zeta potential (Mean \pm SD, mV)
Unloaded-MS	4.2 \pm 2.7	−16.5 \pm 4.7
PCD ₂ -Ms	<0 \pm 3.3	−13.8 \pm 5.7

Size particles and zeta potential were determined for five different batches, and the values represent mean \pm standard deviation of the five batches.

activity by decreasing NF- κ B activation and ICAM-1, VCAM-1, iNOS, and COX-2 expression (Castrillo et al., 2000). Therefore, administration of PGD₂ may represent an innovative therapeutic strategy for the treatment of infectious and inflammatory diseases.

Microencapsulation has been adopted as an efficient approach for the administration of lipid mediators to circumvent the problem of lipid instability, which would otherwise limit their use *in vivo* (Nicolette et al., 2007). Leukotriene B₄ (LTB₄) and PGE₂ have already been efficiently encapsulated in poly (lactic-co-glycolic acid) microspheres (PLGA-MS) with preservation of their biological activity (Dos Santos et al., 2011; Nicolette et al., 2007, 2008a, 2008c, 2009). Lactic/glycolic acid polymers produce drug delivery systems with potentially high encapsulation efficiencies, determined by the chemical characteristics of the target molecules (Cohen et al., 1994; Wu, 2004). Furthermore, they possess adequate stability in a biological environment (Lima and Rodrigues Junior, 1999) and, upon degradation, release lactic and glycolic acids, which are biocompatible (Bazile et al., 1992; Lima and Rodrigues Junior, 1999). These polymers have been shown to be efficient carriers for biomolecules and to enable sustained release of the encapsulated lipid mediators (Dos Santos et al., 2011; Eldridge et al., 1991; Lima et al., 2003; Nicolette et al., 2007, 2008a, 2008c, 2009). Moreover, microencapsulation in PLGA microspheres yields polymeric particles of an appropriate size (<10 μ m) for efficient interaction with phagocytes (Nicolette et al., 2007, 2008c), as well as for efficient intranasal, intratracheal, intramuscular, and intravenous administration (Eldridge et al., 1991; O'Hagan et al., 1993).

In this study, we aimed to develop and characterize biodegradable PGD₂-MS and to evaluate their biological effects on rat AMs. Our results show for the first time that PGD₂-MS are efficiently internalized by AMs and are potent inducers of the production of cytokines and NO through NF- κ B-dependent mechanisms. This drug delivery system may therefore represent an additional tool for the treatment of pulmonary infections in patients who have impaired AM effector functions.

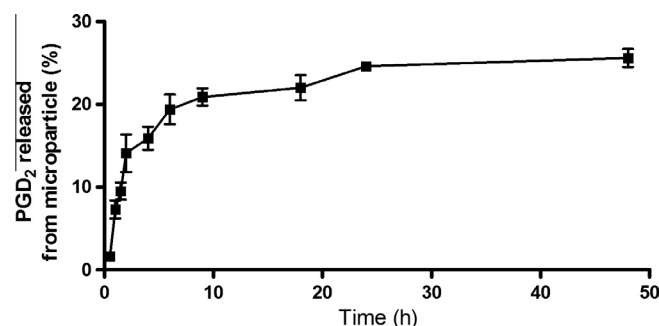


Fig. 2. *In vitro* cumulative release of PGD₂ from PGD₂-MS. PGD₂ concentration was determined by EIA over 48 h. Data are representative of three batches.

2. Material and methods

2.1. Materials

Poly-(D,L-lactide-coglycolide) (PLGA) with a 50:50 co-monomer ratio of lactic/glycolic acid (PurasorbPDLG5002[®], inherent viscosity midpoint = 0.2 dl/g) was kindly provided by CorboinPurac (Gorinchem, Netherlands). Poly (vinyl-alcohol) (Mowiol[®] 40–88) was acquired from Aldrich Chemicals (St. Louis, MO, USA). Methylene dichloride and acetonitrile were purchased from Merck. Aqua Poly/Mount was purchased from Polysciences, Inc. (Warrington, PA). Soluble PGD₂ and a commercially available PGD₂ enzyme immunoassay (EIA) kit were obtained from Cayman Chemical (Ann Arbor, Michigan 48108 USA). Panoptic stain was from Laborclin (Paraná, Brazil). The *Limulus Amoebocyte Lysate* test (LAL test, QCL-1000, Bio Whittaker) was used for endotoxin detection in formulations. Cytochalasin, LPS from *Escherichia coli* (serotype 0127:B8), and sodium nitrite (NaNO₂) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 medium, DMEM, FBS, and antibiotics (penicillin and gentamicin) were acquired from Gibco (Grand Island, NY, USA). Normocin, Zeocin and QUANTIBLue[™] were obtained from InvivoGen (San Diego, CA USA). ELISA kits for TNF- α , IL-1 β , IL-6 and TGF- β were acquired from R&D Systems (Minneapolis, MN, USA).

2.2. Animals and cell line

Specific pathogen-free Wistar rats were obtained from the animal facilities of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil. All experiments were approved by the Animal Ethics Committee of Campus of Ribeirão Preto, Universidade de São Paulo (Protocol 09.1.375.53.5). RAW-Blue[™] cells resistant to Zeocin[™] (RAW264.7 – derived

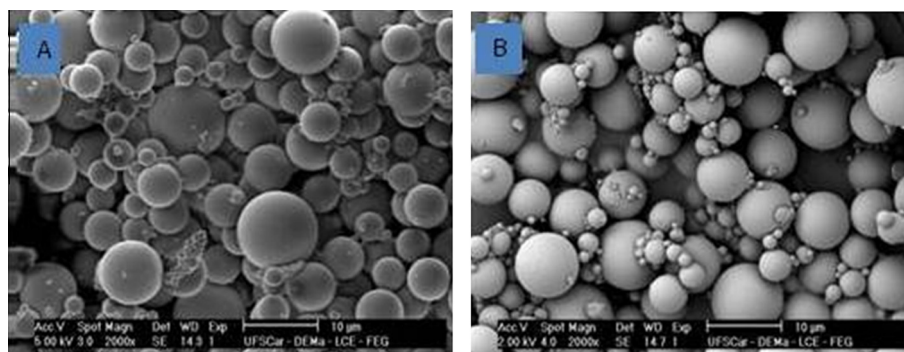


Fig. 1. (A) Unloaded-MS and (B) PGD₂-MS morphology assessed by scanning electronic microscopy (SEM).

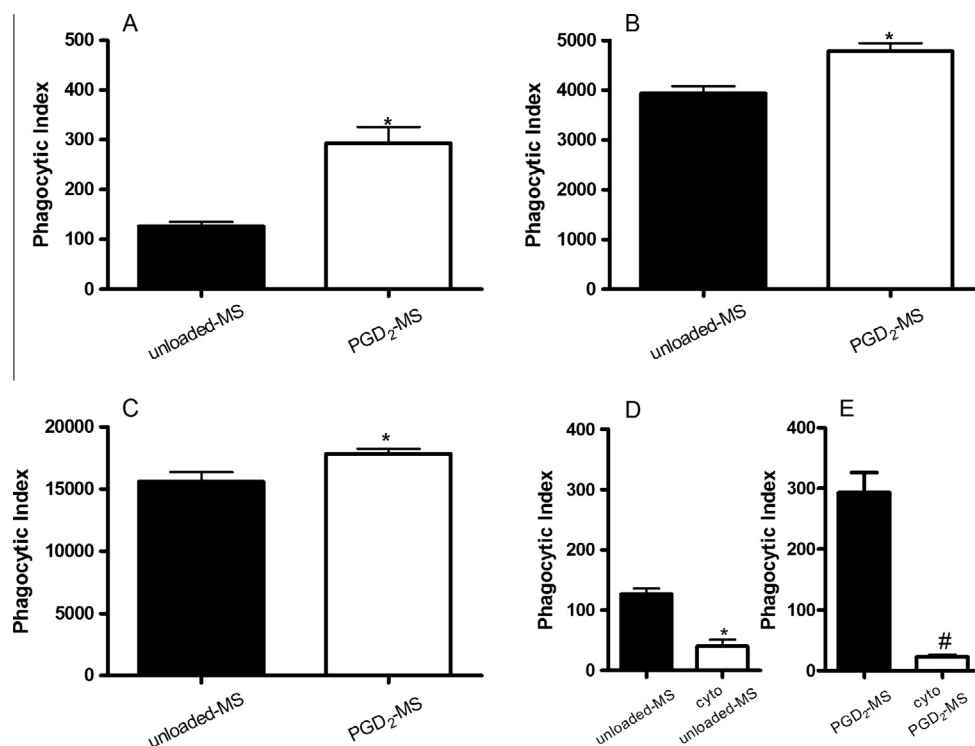


Fig. 3. Phagocytic index of AMs incubated with PGD₂-MS. The phagocytic index was calculated after 4 h (A), 24 h (B), or 48 h (C) incubation with MS. (D) Pre-treatment of cells with cytochalasin demonstrated that internalization of MS by AMs was by cytoskeleton-dependent phagocytosis. AMs were pre-treated for 30 min with cytochalasin (20 µg/mL) before MS addition. *t*-Test was used. Results are expressed as mean \pm S.E.M. and represent three independent experiments ($n = 4$ wells/experiment); * $P < 0.05$, versus unloaded-MS; # $P < 0.05$, versus PGD₂-MS.

murine macrophage cell line that stably expresses an NF- κ B/AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene), were a kind gift from Dr. Huy Ong (Université du Montréal, Canada). The cells were grown in DMEM supplemented with 10% FBS, Normocin™ (50 µg/mL) and Zeocin™ (25 µg/mL).

2.3. Preparation of PGD₂-MS

PGD₂-MS were prepared using the emulsion/solvent evaporation method, as previously described (Nicolete et al., 2008c; Trombone et al., 2007), but with some modifications. Briefly, 300 µL of a PGD₂/ethanol solution (7×10^{-3} M) was added to 10 mL of methylene dichloride containing 100 mg of polymer (PLGA 50:50). The organic phase was emulsified with 20 mL of an aqueous phase containing surfactant (PVA, 3% w/v). The resultant oil-in-water emulsion was stirred for 4 h at 600 rpm in a RW20 homogenizer (IKA, Labortechnik, Germany) to evaporate the solvent. The MS formed were centrifuged and washed three times with water. After removal of the supernatant, the MS pellet was freeze-dried, without any lyophilization additives, at -55°C and 50 mmHg during 12 h. Unloaded-MS (control) were prepared under the same conditions.

PGD₂-MS and unloaded-MS were prepared under sterile conditions, i.e., all flasks and equipment used in their preparation were previously autoclaved and manipulation always occurred under laminar flow, to avoid microbiological contamination of the samples. Even though, a *Limulus amoebocyte* (LAL) test was performed after each batch of MS was prepared to detect free LPS in samples (Dos Santos et al., 2011; Trombone et al., 2007). The maximum level of endotoxin that is considered safe according to the European Pharmacopoeia is 5 EU/kg/h.

2.4. Characterization of PGD₂-MS

All samples (1 mg) were dispersed in distilled water (0.4 mL) and analyzed at 25°C . The diameter of the particles was measured using a particle size analyzer (LS 13 320 Laser Diffraction Particle Size Analyzer; Beckman Coulter, USA). Zeta potential analysis of PGD₂-MS and unloaded-MS was conducted using a ZetaSizer Nano (Malvern instruments, England). MS were pre-coated with gold and after drying, their shape and surface were observed by scanning electron microscopy (SEM) using a ZEISS scanning microscope (ZEISS, Evo 50, Cambridge, England).

2.5. Efficiency of encapsulation and *in vitro* PGD₂ release assay

For calculating encapsulation efficiency, samples of PGD₂-MS (5 mg) were dissolved in 1 mL of acetonitrile/ethanol (7:3 v/v), to disrupt the MS structure. The solvent was then evaporated off in a vacuum concentrator centrifuge for 4 h, and the residue was dissolved in 0.5 mL of EIA buffer from the enzyme immunoassay kit. The solution was then filtered to remove precipitated PLGA, and the amount of PGD₂ in the eluate was quantified using a specific enzyme immunoassay kit, following the manufacturer's instructions. The release kinetics of PGD₂ from PGD₂-MS were monitored *in vitro*. PGD₂-MS (1 mg) was suspended in 1 mL of PBS/ethanol (50:50, v/v), pH 7.4, and incubated at 37°C on a rotating incubator. Ethanol was added to the PBS to ensure that the release experiments were conducted under sink conditions. At each time point 0, 0.5, 1, 1.5, 2, 4, 6, 9, 18, 24 and 48 h of rotation, the suspension was centrifuged and the supernatant was collected for assay of PGD₂ concentration, then 1 mL of fresh PBS/ethanol was added to the flask containing the PGD₂-MS and the experiment was continued. The concentration of PGD₂ in the supernatant was determined using a commercial EIA kit according to the manufacturer's

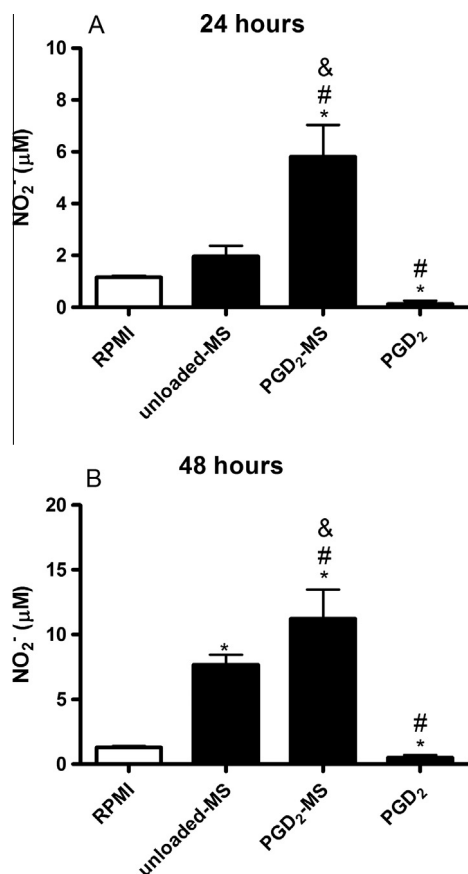


Fig. 4. NO production by AMs. Nitrite production was quantified by the Griess reaction in the supernatants of cells incubated with unloaded-MS, PGD₂-MS or soluble PGD₂. Cells incubated in medium alone were used as a negative control. Supernatants were harvested 24 h (A) and 48 h (B) after addition of the stimuli. Results are expressed as mean \pm S.E.M. and represent three independent experiments ($n = 4$ wells/experiment); * $P < 0.05$ versus RPMI medium; # $P < 0.05$, versus unloaded-MS; and & $P < 0.05$, versus soluble PGD₂. One-way ANOVA (Tukey's multiple comparisons test) was used.

instructions. Optical density was determined in a plate reader set at 450 nm. The concentration of PGD₂ was calculated based on the equation obtained from the calibration curve: $y = -16.28 \ln(x) + 158.35$; $r^2 = 0.9924$, where y is the absorbance, x is the lipid mediator concentration (pg/mL), and r is the coefficient of determination. Results are expressed as the average of three different batches of PGD₂-MS.

2.6. In vitro MS uptake by alveolar macrophages

Rat AMs were obtained by *ex vivo* lung lavage, as previously described (Serezani et al., 2007). Cells firmly adhered to a cover glass (13 mm) in 24-well plates (2×10^5 cells/well) were incubated for 4 h, 24 h and 48 h with 1 mL of a suspension of unloaded-MS or PGD₂-MS (1 mg/mL) in complete RPMI. Medium alone was used as a negative control. After incubation, the non-ingested MS were detached, and the cover glasses were removed from the plates and stained with panoptic stain. In one set of experiments, cytochalasin was used as negative control for phagocytosis. AMs adhering to a cover glass were pre-incubated for 30 minutes with cytochalasin (20 μ g/mL) before the addition of MS. MS internalized by rat AMs were visualized microscopically (Leica/ $\times 100$) and the percentage of cells that ingested at least one MS was calculated. The phagocytic index (PI) was also calculated:

$PI = \text{number of engulfed MS} \times \text{number of AMs containing at least one MS} / \text{total number of AMs}$.

2.7. NF- κ B reporter assay

RAW-Blue™ cells (1×10^5 cells/well) were cultured for 18 h at 37 °C in a humidified 5% CO₂ atmosphere in 96-well plates in DMEM medium supplemented with Normocin™ (50 μ g/mL). After this period of time, 1 mL MS suspension (1 mg/mL) was added and the cells were incubated for a further 24 h. LPS (0.5 μ g/mL) was used as a positive control, and cells incubated in medium alone were used as a negative control. After 24 h stimulation, the medium was harvested, and samples (50 μ L) were incubated overnight at room temperature in 96-well plates with QUANTIBLue™ (150 μ L, InvivoGen), to assay for SEAP. The optical density was measured at 650 nm in a micro plate reader.

2.8. Induction of Cytokines and NO by MS-stimulated alveolar macrophages

Adherent AMs (2×10^5 /mL) in 24-well culture plates were incubated with 1 mL of a 1 mg/mL suspension of unloaded-MS, PGD₂-MS, soluble PGD₂ (3×10^{-6} M), or medium (negative control). After 24 h and 48 h incubation in a humidified atmosphere (37 °C, 5% CO₂), the supernatants were collected and stored at -80 °C until use. NO was determined by the Greiss reaction, and cytokines were measured using commercially available ELISA kits (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. Optical densities were measured at 450 nm in a micro plate reader (μ Quant, Biotek Instruments Inc.).

2.9. Evaluation of PGD₂-MS effects on LPS-stimulated alveolar macrophages

Rat AMs cultured as described in Section 2.8 were pre-stimulated with LPS (0.5 μ g/mL) for 2 h and then PGD₂-MS in RPMI or soluble PGD₂ (3×10^{-6} M) were added to the cells. Cells with LPS or medium alone were used as positive and negative controls, respectively. After 24 h incubation, the supernatants were collected and stored at -80 °C until use.

2.10. Statistical analysis

Results are presented as the mean \pm S.E.M. Homogeneity of variance (Levene) and normality (Kolmogorov–Smirnov) tests were run for all experimental data, and lack of homogeneity was corrected by a log transformation. Data were then analyzed by *t*-test (Fig. 3) or one-way ANOVA (Figs. 4–6), and Tukey test was employed for multiple comparisons in case of significant ANOVA results. As data from Fig. 7B did not reach homoscedasticity and there was no variance in the control group of Fig. 7D, both groups were analyzed by non-parametric Kruskal–Wallis test followed and, in case of significant effect, by the Mann–Whitney test. Statistical significance was fixed at $P < 0.05$.

3. Results

3.1. Characterization of PGD₂-MS and unloaded-MS

The diameters of lyophilized PGD₂-MS and unloaded-MS were 5.0 ± 3.3 μ m and 4.2 ± 2.7 μ m, respectively (Table 1). No differences in superficial charge were observed after PGD₂ encapsulation, i.e., both PGD₂-MS and unloaded-MS had a negative zeta potential (Table 1), which is a contributing factor in a lack of aggregation in physiological media, and also facilitates interaction with

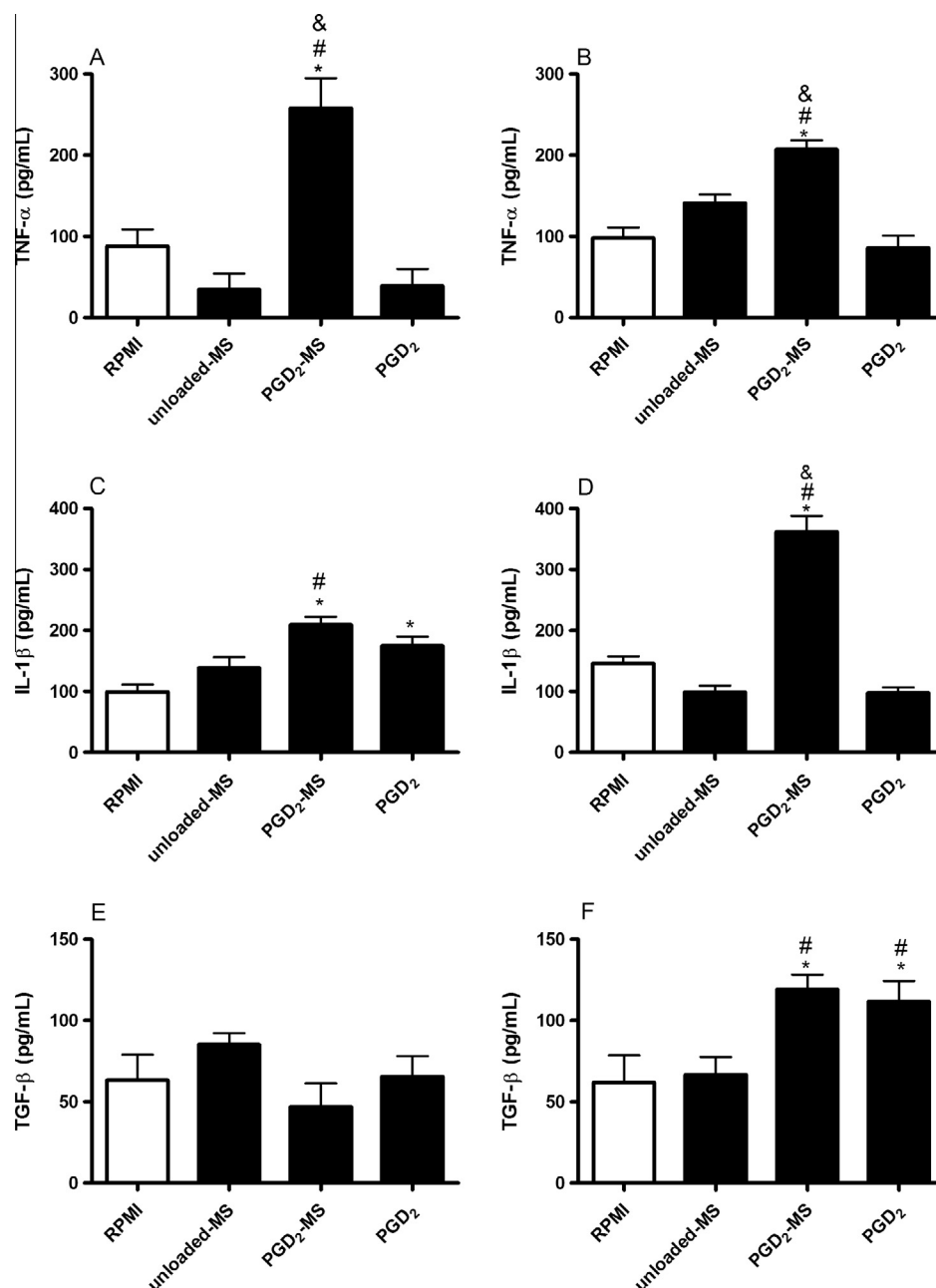


Fig. 5. PGD₂-MS-induced cytokine release by AMs. The cytokines TNF-α, IL-1β, and TGF-β were measured in AM supernatant 24 h (A–C–E) and 48 h (B–D–F) after the cells were incubated with 1 mg/mL of unloaded-MS, PGD₂-MS or soluble PGD₂. Cytokines were quantified by ELISA assay. Cells incubated in medium alone were used as a negative control. Results are expressed as mean ± S.E.M. and represent three independent experiments ($n = 5–6$ /wells/experiment); * $P < 0.05$, versus RPMI medium; # $P < 0.05$, versus unloaded-MS; & $P < 0.05$, versus soluble PGD₂. One-way ANOVA (Tukey's multiple comparisons test) was used.

cell membranes (Tabata and Ikada, 1988). Scanning electron microscopy (SEM) showed that both PGD₂-MS and unloaded-MS were spherical, with a smooth surface and no anomalies (Fig. 1).

Endotoxin levels in PLGA MS were less than 0.1 EU/μg, as detected by the LAL assay. It is important to point out that during this assay, LPS could associate with PLGA-MS surface, leaving to a false negative result. However, it is believed that it is unlikely, once all MS were prepared under GMP conditions, aiming to avoid contamination of the samples. Nevertheless, the absence of NO production by AMs incubated with loaded and unloaded-MS also indicated that the PLGA was LPS-free.

3.2. Encapsulation efficiency and in vitro release of PGD₂

The efficiency of encapsulation was determined using an EIA kit to quantify PGD₂. The amount of PGD₂ in 1 mg of PGD₂-MS was calculated to be 2.7 ± 0.1 μg, which corresponds to an encapsulation efficiency of $36 \pm 1.4\%$. The method used to recover entrapped PGD₂ from MS could under estimate the actual encapsulated drug content, as upon solvent removal, PLGA precipitated could entrap a proportion of PGD₂ again and addition of EIA buffer would dissolve only part of the PG. In this way, drug substance that is fully trapped within the PLGA material will not dissolve. Even though, the method used in this paper was the best way found to estimate PG encapsulation, as other previously tested methods interfered with the drug analysis.

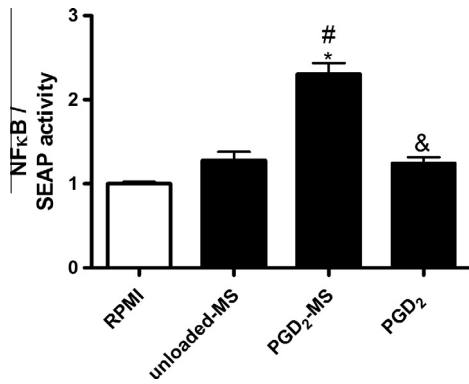


Fig. 6. PGD₂-MS activates NF-κB/AP-1. RAW-Blue™ cells were incubated for 24 h with medium, unloaded-MS, PGD₂-MS or soluble PGD₂. Values are expressed as mean ± SEM and represent two independent experiments. The ANOVA test was used. **P* < 0.05 versus medium; [#]*P* < 0.05 versus unloaded-MS; [&]*P* < 0.05 versus soluble PGD₂. One-way ANOVA (Tukey's multiple comparisons test) was used.

Analysis of PGD₂ release showed a burst release from MS at 4 h (Fig. 2), when approximately 16% of the mediator was detected in the medium. After 48 h, 26% of PGD₂ was released. These results indicate that PLGA biodegradation allows for a progressive release of PGD₂ at least over 48 h.

3.3. PGD₂-MS activate AM effector functions

To evaluate whether PGD₂-MS influence AM effector functions, we first performed phagocytosis assays using rat AMs (Fig. 3). After

determining the number of AMs that phagocytized at least one MS, we calculated the phagocytic index by counting the numbers of MS inside the cells after 4, 24, and 48 h of incubation. Our results show that PGD₂-MS were more efficiently phagocytized than unloaded-MS at each time point evaluated (Fig. 3A–C). To analyse only internalized MS and exclude those that were attached to the cell surface, AMs were pre-treated with cytochalasin, which prevents phagocytosis. Fig. 3D and E shows that cytochalasin treatment significantly reduced the uptake of unloaded-MS and PGD₂-MS (by 68% and 92%, respectively), indicating that the majority of the MS were phagocytized by AMs. Viability assays using MTT (data not shown) demonstrated that neither PGD₂-MS nor unloaded-MS were cytotoxic at the time-points used in the study. We next measured the production of NO, TNF-α, IL-1β, and TGF-β by AMs after internalization of PGD₂-MS. Fig. 4 shows that PGD₂-MS stimulated AMs to release NO. After 24 h and 48 h incubation, PGD₂-MS induced more NO than unloaded-MS or soluble PGD₂. Similarly, after 24 and 48 h incubation with PGD₂-MS, AMs released significantly higher levels of TNF-α and IL-1β (Fig. 5A–D) than cells incubated with unloaded-MS or soluble PGD₂. TGF-β production increased only after 48 h stimulation with PGD₂-MS. Unloaded MS did not stimulate AMs to release TGF-β (Fig. 5E and F). Taken together, these results indicate that PGD₂-MS efficiently activates AM effector functions.

3.4. PGD₂-MS efficiently activate NF-κB/AP-1

Our results show that NF-κB was activated in RAW-Blue™ cells after 24 h incubation with PGD₂-MS (Fig. 6). Unloaded-MS or soluble PGD₂ did not activate NF-κB signaling.

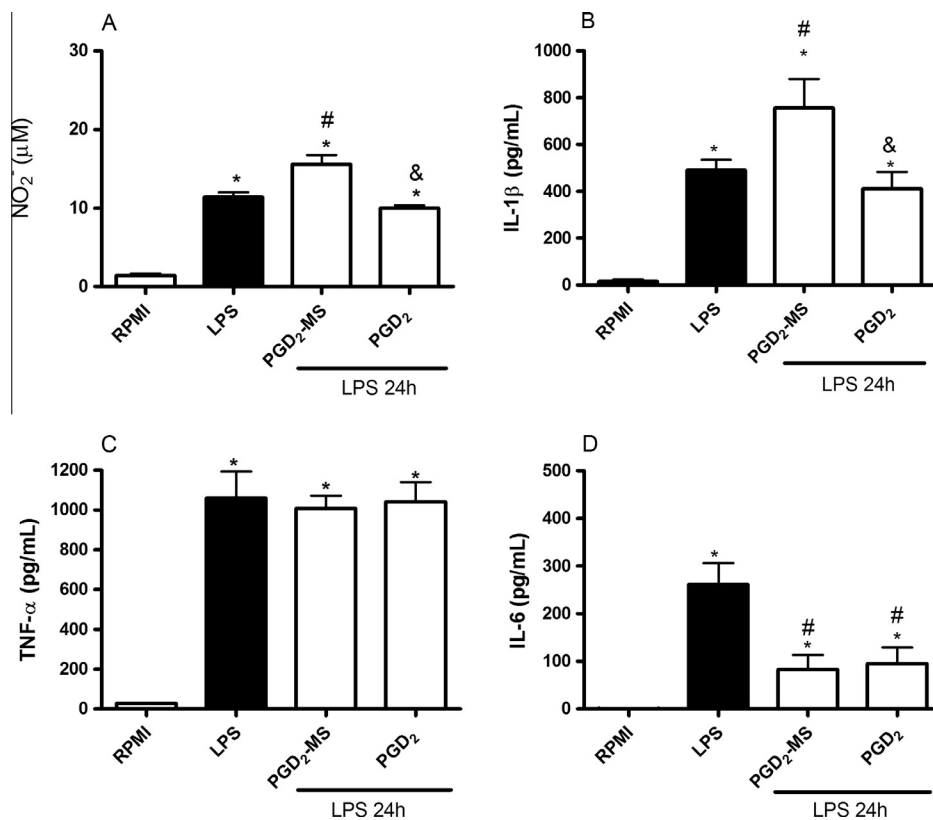


Fig. 7. PGD₂-MS effects on NO, TNF-α, IL-1β, and IL-6 release by LPS-stimulated AMs. Adherent AMs were pre-stimulated for 2 h with LPS (0.5 μg/mL) then 1 mg/mL PGD₂-MS or soluble PGD₂ was added. Cell supernatants were collected after 24 h and NO levels were determined by the Griess reaction (A), while the concentrations of IL-1β (B), TNF-α (C), and IL-6 (D) were determined by ELISA assay. Cells incubated in medium alone were used as a negative control. Values are expressed as mean ± SD (n = 6) and represent two independent experiments. **P* < 0.05 versus medium; [#]*P* < 0.05 versus LPS-stimulated cells. [&]*P* < 0.05 versus soluble PGD₂. One-way ANOVA or Kruskal–Wallis followed by Tukey's multiple comparison test or Mann–Whitney tests were used.

3.5. Effects of PGD₂-MS on LPS-stimulated AMs

We next investigated the effects of PGD₂-MS treatment on AMs stimulated by LPS to produce NO and cytokines. We measured NO, IL-1 β , TNF- α , and IL-6 in cell supernatants, 24 h after LPS addition, 22 h after addition of test samples. As expected, LPS induced AMs to release significant amounts of NO, IL-1 β , TNF- α , and IL-6 (Fig. 7). Interestingly, addition of PGD₂-MS increased the levels of NO and IL-1 β production compared to cells treated with only LPS or soluble PGD₂ (Fig. 7A and B), but, like soluble PGD₂, it did not inhibit IL-6 production (Fig. 7D). The level of TNF- α production by LPS-stimulated AMs was unaltered by treatment with either PGD₂-MS or soluble PGD₂ (Fig. 7C).

4. Discussion

In this study, PLGA microspheres containing PGD₂ were prepared under conditions designed to preserve the biological activity, and the efficacy of PGD₂-MS in activating AM effector functions was evaluated. The effect of PGD₂ on the lung is complex because this lipid mediator is involved in lung inflammation as part of the pathology of asthma (Monneret et al., 2001), but may also be associated with a resolution of inflammation (Gilroy et al., 1999). In this context, the effect of PGD₂ on alveolar macrophages is unclear. However, PGD₂ is unstable with a short half-life *in vivo* and it is therefore necessary to develop strategies to safeguard the physical and chemical activities of such lipid mediators in a physiological environment. The average diameter and morphology of the prepared microspheres were appropriate for *in vitro* studies and facilitate interactions with the plasma membranes of cells, such as macrophages (Champion et al., 2008; Nicolette et al., 2007; Tabata and Ikada, 1988). In fact, the formulation described in this study has the optimum diameter (between 5 and 10 μ m) to increase phagocytosis by macrophages. The efficiency of PGD₂ encapsulation and its release kinetics reinforce the hypothesis that these MS are suitable for the activation of cells. The results reported here are similar to those previously reported by our group for the arachidonic acid-derived lipid mediators LTB₄ and PGE₂, showing that lipid mediators could be efficiently encapsulated (Dos Santos et al., 2011; Nicolette et al., 2007).

The ability of PGD₂-MS to activate rat AMs was evaluated. The data presented in this paper show that microencapsulation of PGD₂ increased the phagocytic index and cytochalasin inhibition of phagocytosis showed that the PGD₂-MS were indeed internalized. As expected, increased phagocytosis of PGD₂-MS was accompanied by the activation of the transcription factor NF- κ B, and increased production of NO, TNF- α , and IL-1 β . This is in contrast to previous results which reported that exogenous PGD₂ increased the levels of IL-1 β but decreased NO and TNF- α production (Bellows et al., 2006; Sandig et al., 2007; Zayed et al., 2010). It is hypothesized that these contradictory data are due to the different cell types used in the studies: AMs, the macrophage-like cell line RAW, and peritoneal macrophages. Another possible explanation for the differences observed is the route of PGD₂ delivery which may elicit different patterns of activation, i.e. intra or extracellular. After 48 h of stimulation with PGD₂-MS, AMs also secreted higher levels of the regulatory cytokine TGF- β , which is known to inhibit macrophage activation (Letterio and Roberts, 1998). Data presented in this paper indicate that PGD₂-MS activate different types of intracellular signaling overtime starting with the pro-inflammatory cytokines, TNF- α and IL-1 β , and followed later by the subsequent release of the regulatory cytokine TGF- β which may explain the dual pro- and anti-inflammatory effects of PGD₂ described in the literature (Arima and Fukuda, 2008; Monneret et al., 2001; Murata et al., 2013). Based on these results, PGD₂-MS

are proposed to be an effective tool for manipulating the immune response, given that they induce early cell activation via NF- κ B, resulting in NO, TNF- α , and IL-1 β release (Andreakos et al., 2004; Mandal et al., 2004; Perkins, 2007), as well as late cell deactivation via TGF- β secretion (Letterio and Roberts, 1998). Therefore, the use of PGD₂-MS is a feasible strategy to improve host defense in a controlled manner.

The potential of PGD₂-MS to modulate the production of cytokines and NO in LPS-stimulated AMs *in vitro* was also investigated. PGD₂-MS were shown to potentiate LPS-induced NO and IL-1 β production by AMs, but at the same time, IL-6 release was decreased, highlighting a potential role in host defense against pulmonary infections. Together with TNF- α , IL-1 β is considered essential for host defense mechanisms (Andreakos et al., 2004), by contributing to the generation of Th1 immune responses (Jayaraman et al., 2013) and the synthesis of opsonizing antibodies (Nakae et al., 2001). Besides these cytokines, NO also contributes to bacterial killing, at least in murine models (Gomes et al., 2013). Decreased IL-6 production in response to PGD₂ administration, as shown here, has been previously demonstrated by our group in a model of joint inflammation (Maicas et al., 2012), and may have important implications for the control of exacerbated inflammation. On the other hand, reduced IL-6 production has also been reported in mice deficient in H-PGDs, the enzyme responsible for PGD₂ synthesis (Murata et al., 2013).

In conclusion, the results, presented here, indicate that the microencapsulated PGD₂ formulation is efficiently internalized by AMs and induces NF- κ B activation, as well as the production of cytokines and NO. Thus, PGD₂-MS represent a viable strategy for lung therapeutic intervention in patients with compromised macrophage effector functions, and, in addition may have a potential role in the treatment of infectious diseases.

5. Conclusions

In conclusion, we demonstrate here for the first time that the characteristics of PGD₂-MS, including the spherical shape and negative zeta potential, equip them to interact with and to activate phagocytic cells. Moreover, encapsulation in PLGA microspheres preserved the biological activities of PGD₂, as evidenced by the capacity of PGD₂-MS to activate phagocytosis as well as induce the production of immune mediators.

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