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Prostaglandin D_2-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 D_2 (PGD_2), have low water solubility and are unstable. The present study aimed to develop and characterize MS loaded with PGD_2 (PGD_2-MS) to obtain an innovative tool to activate macrophages. PGD_2-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 PGD_2-MS. PGD_2-MS were spherical with a diameter of 5.0 ± 3.3 μm and regular surface, zeta potential of −13.4 ± 5.6 mV, and 36% of encapsulation efficiency, with 16–26% release of entrapped PGD_2 at 4 and 48 h, respectively. PGD_2-MS were more efficiently internalized by AMs than unloaded-MS, and activated NF-κB more than free PGD_2. Moreover, PGD_2-MS stimulated the production of nitric oxide, TNF-α, IL-1β, and TGF-β, more than free PGD_2, indicating that microencapsulation increased the activating effect of PGD_2 on cells. In LPS-pre-treated AMs, PGD_2-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 PGD_2-MS facilitated interaction with, and activation of phagocytic cells; moreover, PGD_2-MS retained the biological activities of PGD_2 to trigger effector mechanisms in AMs. It is suggested that PGD_2-MS represent a strategy for therapeutic intervention in the lungs of immunocompromised subjects.

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 PGE_2, PGI_2, and PGD_2 (Harris et al., 2002). Among them, PGD_2 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 PGD_2 has been shown to decrease the bacterial load in the lungs of *Pseudomonas aeruginosa*-infected mice (Joo et al., 2007). Moreover, PGD_2 and its metabolic product $15dPGJ_2$ 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; PGD_2, prostaglandin D_2; PGD_2-MS, PGD_2 loading MS; PI, phagocytic index; PLGA, poly(lactic acid–glycolic acid); TGF-β, transforming growth factor beta; TNF-α, tumor necrosisfactor-α.

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Table 1
Size and zeta potential of unloaded and PGD_2-loaded MS.

Microsphere	Size (Mean ± SD, μm)	Zeta potential (Mean ± SD, mV)
Unloaded-MS	4.2 ± 2.7	−16.5 ± 4.7
PCD_2-Ms	<0 ± 3.3	−13.8 ± 5.7

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

activity by decreasing NF-κB activation and ICAM-1, VCAM-1, iNOS, and COX-2 expression (Castrillo et al., 2000). Therefore, administration of PGD_2 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* (Nicolete et al., 2007). Leukotriene B4 (LTB_4) and PGE_2 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; Nicolete 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; Nicolete et al., 2007, 2008a, 2008c, 2009). Moreover, microencapsulation in PLGA microspheres yields polymeric particles of an appropriate size (<10 μm) for efficient interaction with phagocytes (Nicolete 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 PGD_2-MS and to evaluate their biological effects on rat AMs. Our results show for the first time that PGD_2-MS are efficiently internalized by AMs and are potent inducers of the production of cytokines and NO through NF-κB-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.

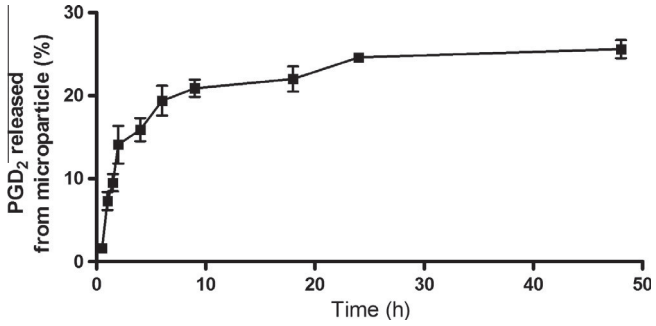

Fig. 2. *In vitro* cumulative release of PGD_2 from PGD_2-MS. PGD_2 concentration was determined by EIA over 48 h. Data are representative of three batches.

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 PGD_2 and a commercially available PGD_2 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 ($NaNO_2$) 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-1β, 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

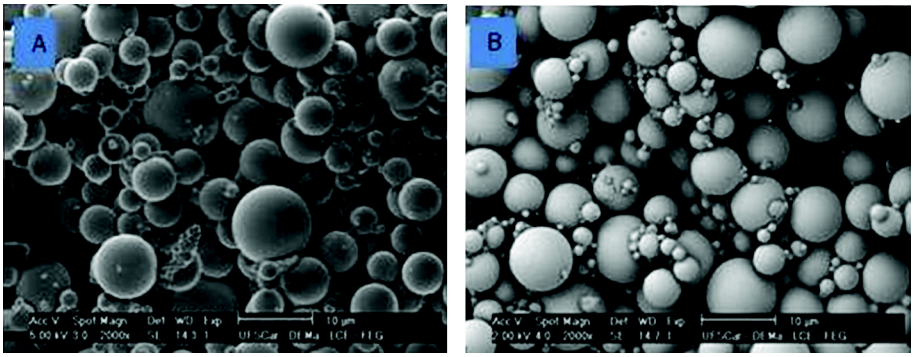

Fig. 1. (A) Unloaded-MS and (B) PGD_2-MS morphology assessed by scanning electronic microscopy (SEM).

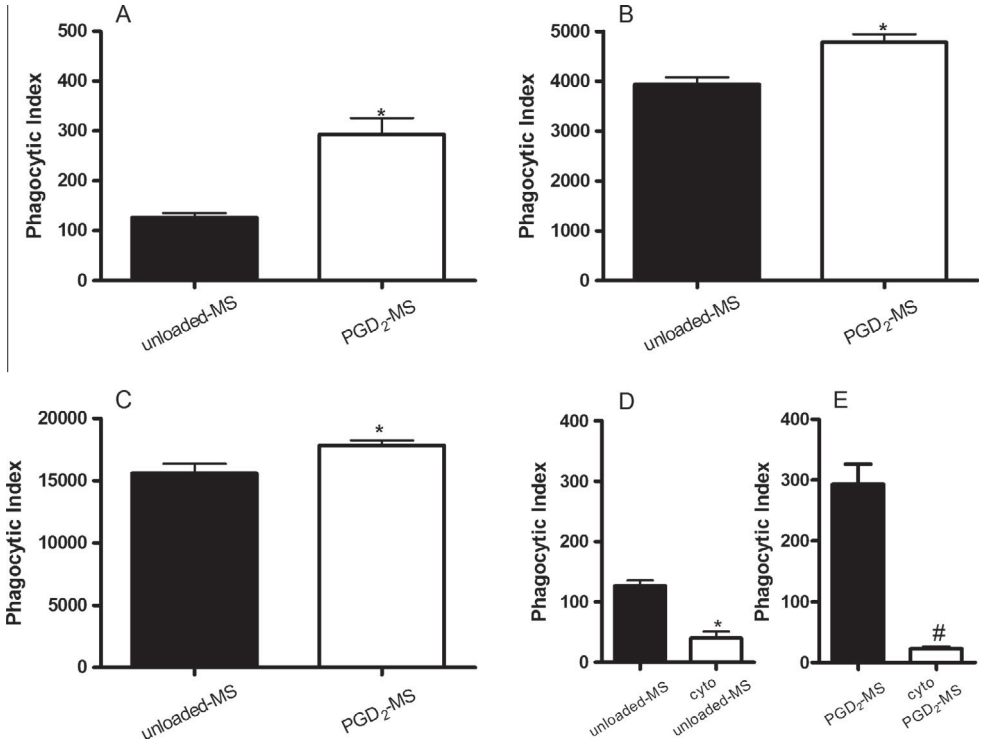

Fig. 3. Phagocytic index of AMs incubated with PGD_2-MS. The phagocytic index was calculated after 4 h (A), 24 h (B), or 48 h (C) incubation with MS. (D) Pre-treatment of cells with cytochalasin demonstrated that internalization of MS by AMs was by cytoskeleton-dependent phagocytosis. AMs were pre-treated for 30 min with cytochalasin (20 μg/mL) before MS addition. *t*-Test was used. Results are expressed as mean ± S.E.M. and represent three independent experiments ($n = 4$ wells/experiment); $^{*}P < 0.05$, *versus* unloaded-MS; $^{\#}P < 0.05$, *versus* PGD_2-MS.

murine macrophage cell line that stably expresses an NF-κB/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 PGD_2-MS

PGD_2-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 PGD_2/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.

PGD_2-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 thought, 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 PGD_2-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 PGD_2-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 PGD_2 release assay

For calculating encapsulation efficiency, samples of PGD_2-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 PGD_2 in the eluate was quantified using a specific enzyme immunoassay kit, following the manufacturer's instructions. The release kinetics of PGD_2 from PGD_2-MS were monitored *in vitro*. PGD_2-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 PGD_2 concentration, then 1 mL of fresh PBS/ethanol was added to the flask containing the PGD_2-MS and the experiment was continued. The concentration of PGD_2 in the supernatant was determined using a commercial EIA kit according to the manufacturer's

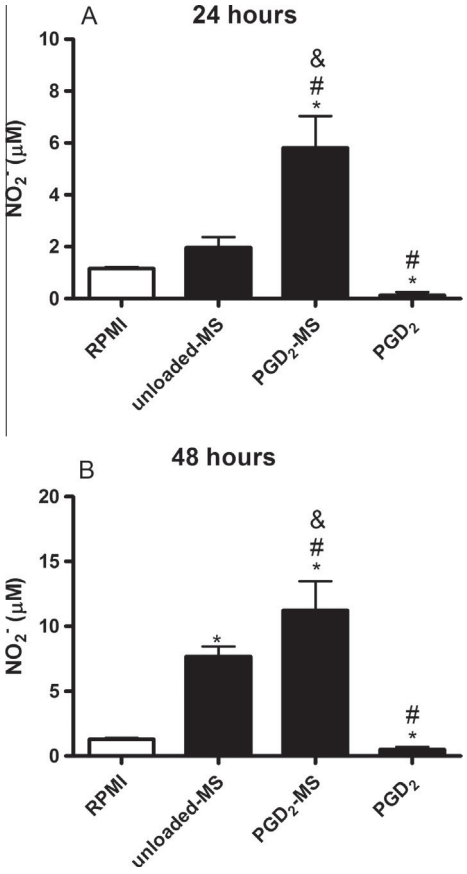

Fig. 4. NO production by AMs. Nitrite production was quantified by the Griess reaction in the supernatants of cells incubated with unloaded-MS, PGD_2-MS or soluble PGD_2. Cells incubated in medium alone were used as a negative control. Supernatants were harvested 24 h (A) and 48 h (B) after addition of the stimuli. Results are expressed as mean ± 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 PGD_2. 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 PGD_2 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 PGD_2-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 PGD_2-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 μg/mL) before the addition of MS. MS internalized by rat AMs were visualized microscopically (Leica/×100) and the percentage of cells that ingested at least one MS was calculated. The phagocytic index (PI) was also calculated: PI = number of engulfed MS × number of AMs containing at least one MS/total number of AMs.

2.7. NF-κB reporter assay

RAW-Blue™ cells (1×10^5 cells/well) were cultured for 18 h at 37 °C in a humidified 5% CO_2 atmosphere in 96-well plates in DMEM medium supplemented with Normocin™ (50 μg/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 μg/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 μL) were incubated overnight at room temperature in 96-well plates with QUANTIBlue™ (150 μL, 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, PGD_2-MS, soluble PGD_2 (3×10^{-6} M), or medium (negative control). After 24 h and 48 h incubation in a humidified atmosphere (37 °C, 5% CO_2), 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 (μQuant, Biotek Instruments Inc.).

2.9. Evaluation of PGD_2-MS effects on LPS-stimulated alveolar macrophages

Rat AMs cultured as described in Section 2.8 were pre-stimulated with LPS (0.5 μg/mL) for 2 h and then PGD_2-MS in RPMI or soluble PGD_2 (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 ± 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 Tuckey 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 PGD_2-MS and unloaded-MS

The diameters of lyophilized PGD_2-MS and unloaded-MS were 5.0 ± 3.3 μm and 4.2 ± 2.7 μm, respectively (Table 1). No differences in superficial charge were observed after PGD_2 encapsulation, i.e., both PGD_2-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

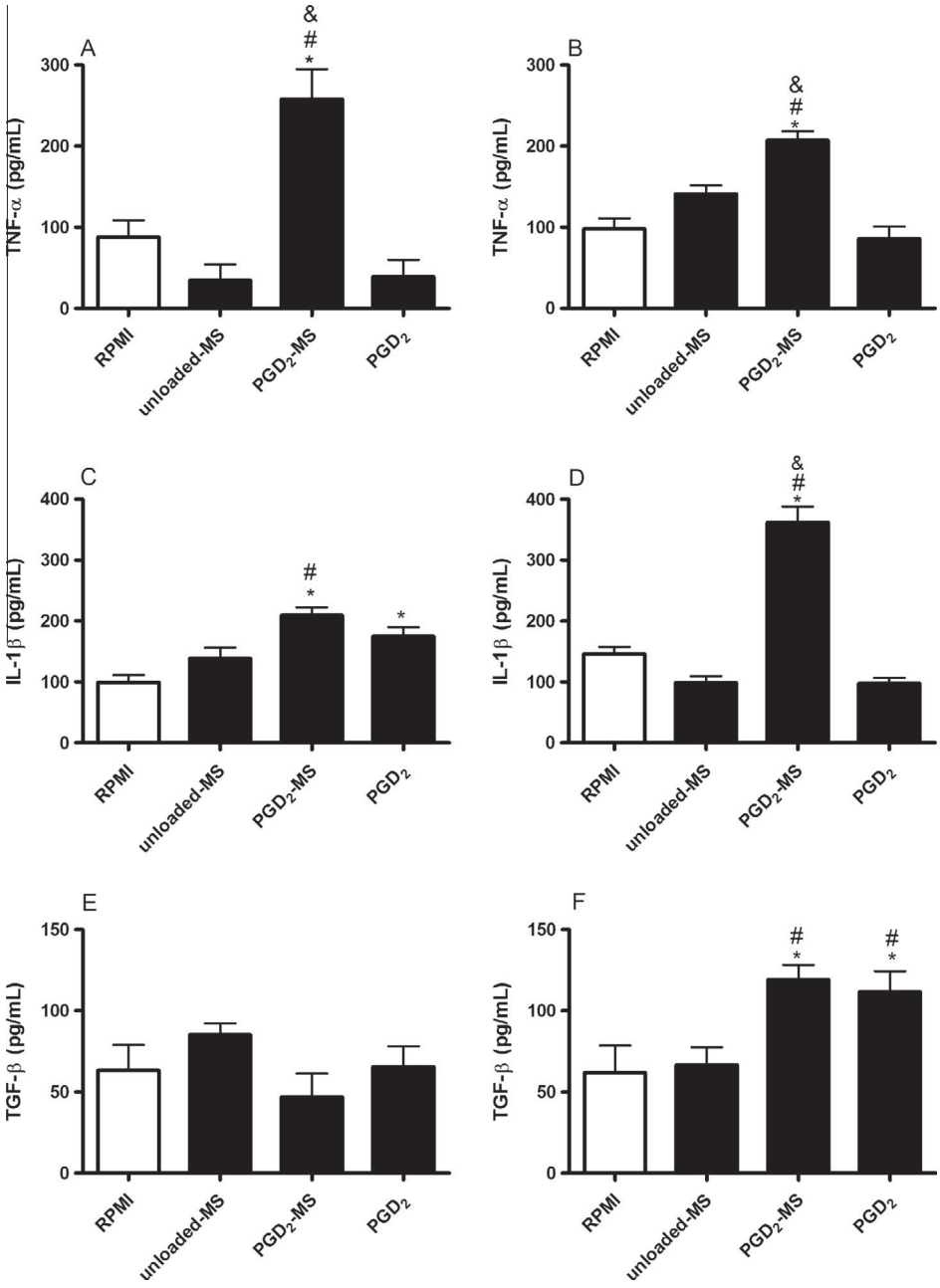

Fig. 5. PGD_2-MS-induced cytokine release by AMs. The cytokines TNF-α, IL-1β, and TGF-β were measured in AM supernatant 24 h (A–C–E) and 48 h (B–D–F) after the cells were incubated with 1 mg/mL of unloaded-MS, PGD_2-MS or soluble PGD_2. Cytokines were quantified by ELISA assay. Cells incubated in medium 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 PGD_2. One-way ANOVA (Tukey's multiple comparisons test) was used.

cell membranes (Tabata and Ikada, 1988). Scanning electron microscopy (SEM) showed that both PGD_2-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 PGD_2

The efficiency of encapsulation was determined using an EIA kit to quantify PGD_2. The amount of PGD_2 in 1 mg of PGD_2-MS was calculated to be 2.7 ± 0.1 μg, which corresponds to an encapsulation efficiency of 36 ± 1.4%. The method used to recover entrapped PGD_2 from MS could under estimate the actual encapsulated drug content, as upon solvent removal, PLGA precipitated could entrap a proportion of PGD_2 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

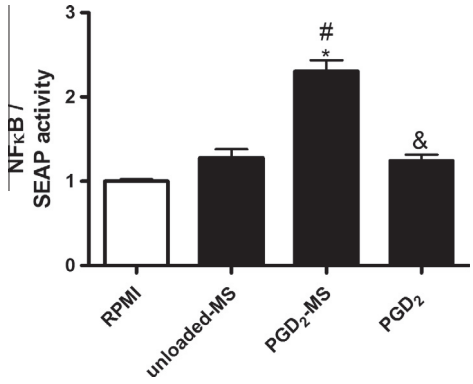

Fig. 6. PGD_2-MS activates NF-κB/AP-1. RAW-Blue™ cells were incubated for 24 h with medium, unloaded-MS, PGD_2-MSor soluble PGD_2. Values are expressed as mean ± SEM and represent two independent experiments. The ANOVA test was used. $^{*}P < 0.05$ *versus* medium; $^{\#}P < 0.05$ *versus* unloaded-MS; $^{\&}P < 0.05$ *versus* soluble PGD_2. One-way ANOVA (Tukey's multiple comparisons test) was used.

Analysis of PGD_2 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 PGD_2 was released. These results indicate that PLGA biodegradation allows for a progressive release of PGD_2 at least over 48 h.

3.3. PGD_2-MS activate AM effector functions

To evaluate whether PGD_2-MS influence AM effector functions, we first performed phagocytosis assays using rat AMs (Fig. 3). After determining the number of AMs that phagocytised 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 PGD_2-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 PGD_2-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 PGD_2-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 PGD_2-MS. Fig. 4 shows that PGD_2-MS stimulated AMs to release NO. After 24 h and 48 h incubation, PGD_2-MS induced more NO than unloaded-MS or soluble PGD_2. Similarly, after 24 and 48 h incubation with PGD_2-MS, AMs released significantly higher levels of TNF-α and IL-1β (Fig. 5A–D) than cells incubated with unloaded-MS or soluble PGD_2. TGF-β production increased only after 48 h stimulation with PGD_2-MS. Unloaded MS did not stimulate AMs to release TGF-β (Fig. 5E and F). Taken together, these results indicate that PGD_2-MS efficiently activates AM effector functions.

3.4. PGD_2-MSefficiently activate NF-κB/AP-1

Our results show that NF-κB was activated in RAW-Blue™ cells after 24 h incubation with PGD_2-MS (Fig.6). Unloaded-MS or soluble PGD_2 did not activate NF-κB signaling.

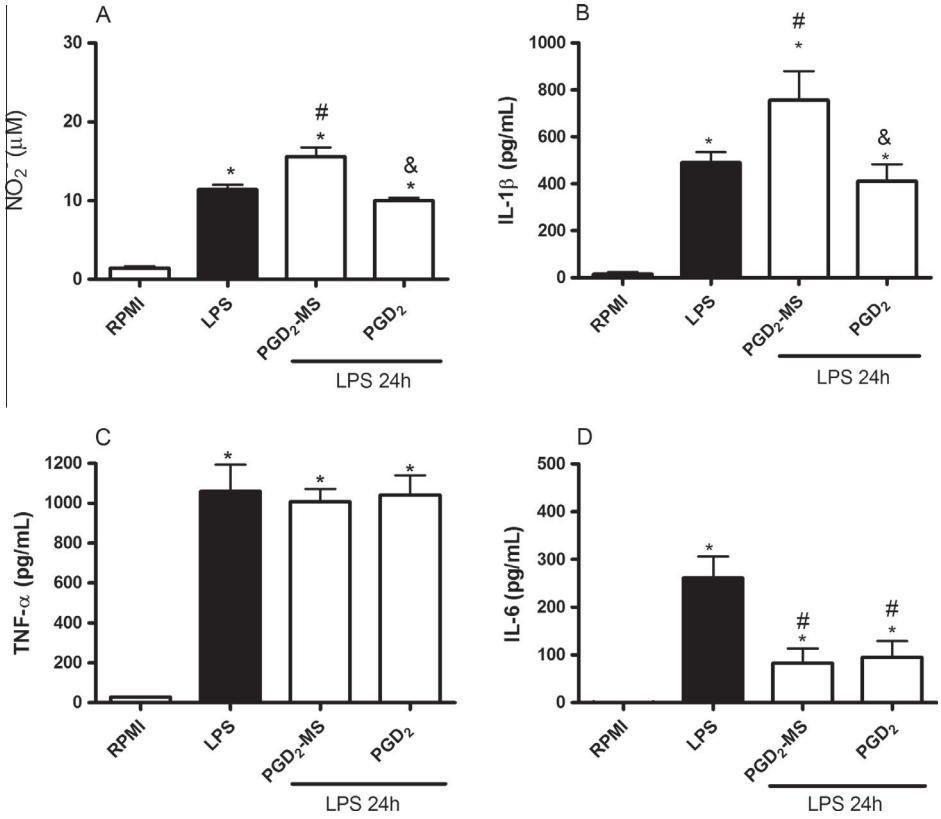

Fig. 7. PGD_2-MS effects on NO, TNF-α, IL-1β, and IL-6 release by LPS-stimulated AMs. Adherent AMs were pre-stimulated for 2 h with LPS (0.5 μg/mL) then 1 mg/mL PGD_2-MSor soluble PGD_2 was added. Cell supernatants were collected after 24 h and NO levels were determined by the Griess reaction (A), while 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 PGD_2. One-way ANOVA or Kruskal–Wallis followed by Tukey's multiple comparison test or Mann–Whitney tests were used.

3.5. Effects of PGD_2-MSon LPS-stimulated AMs

We next investigated the effects of PGD_2-MS treatment on AMs stimulated by LPS to produce NO and cytokines. We measured NO, IL-1β, 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-1β, TNF-α, and IL-6 (Fig. 7). Interestingly, addition of PGD_2-MS increased the levels of NO and IL-1β production compared to cells treated with only LPS or soluble PGD_2 (Fig. 7A and B), but, like soluble PGD_2, 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 PGD_2-MS or soluble PGD_2 (Fig. 7C).

4. Discussion

In this study, PLGA microspheres containing PGD_2 were prepared under conditions designed to preserve the biological activity, and the efficacy of PGD_2-MS in activating AM effector functions was evaluated. The effect of PGD_2 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 PGD_2 on alveolar macrophages is unclear. However, PGD_2 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; Nicolete et al., 2007; Tabata and Ikada, 1988). In fact, the formulation described in this study has the optimum diameter (between 5 and 10 μm) to increase phagocytosis by macrophages. The efficiency of PGD_2 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 LTB_4 and PGE_2, showing that lipid mediators could be efficiently encapsulated (Dos Santos et al., 2011; Nicolete et al., 2007).

The ability of PGD_2-MS to activate rat AMs was evaluated. The data presented in this paper show that microencapsulation of PGD_2 increased the phagocytic index and cytochalasin inhibition of phagocytosis showed that the PGD_2-MS were indeed internalized. As expected, increased phagocytosis of PGD_2-MS was accompanied by the activation of the transcription factor NF-κB, and increased production of NO, TNF-α, and IL-1β. This is in contrast to previous results which reported that exogenous PGD_2 increased the levels of IL-1β 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 PGD_2 delivery which may elicit different patterns of activation, i.e. intra or extracellular. After 48 h of stimulation with PGD_2-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 PGD_2-MS activate different types of intracellular signaling overtime starting with the pro-inflammatory cytokines, TNF-α and IL-1β, and followed later by the subsequent release of the regulatory cytokine TGF-β which may explain the dual pro- and anti-inflammatory effects of PGD_2 described in the literature (Arima and Fukuda, 2008; Monneret et al., 2001; Murata et al., 2013). Based on these results,PGD_2-MS are proposed to be an effective tool for manipulating the immune response, given that they induce early cell activation via NF-κB, resulting in NO, TNF-α, and IL-1β 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 PGD_2-MS is a feasible strategy to improve host defense in a controlled manner.

The potential of PGD_2-MS to modulate the production of cytokines and NO in LPS-stimulated AMs *in vitro* was also investigated. PGD_2-MS were shown to potentiate LPS-induced NO and IL-1β 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-1β 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 PGD_2 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 PGD_2 synthesis (Murata et al., 2013).

In conclusion, the results, presented here, indicate that the microencapsulated PGD_2 formulation is efficiently internalized by AMs and induces NF-κB activation, as well as the production of cytokines and NO. Thus, PGD_2-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 PGD_2-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 PGD_2, as evidenced by the capacity of PGD_2-MS to activate phagocytosis as well as induce the production of immune mediators.

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