

# Enhanced Anti-Inflammation of Inhaled Dexamethasone Palmitate Using Mannosylated Liposomes in an Endotoxin-Induced Lung Inflammation Model

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## ABSTRACT

Inhalation of bacterial endotoxin induces pulmonary inflammation by activation of nuclear factor  $\kappa$ B (NF $\kappa$ B), production of cytokines and chemokines, and neutrophil activation. Although glucocorticoids are the drugs of choice, administration of free drugs results in adverse effects as a result of a lack of selectivity for the inflammatory effector cells. Because alveolar macrophages play a key role in the inflammatory response in the lung, selective targeting of glucocorticoids to alveolar macrophages offers efficacious pharmacological intervention with minimal side effects. We have demonstrated previously the selective targeting of mannosylated liposomes to alveolar macrophages via mannose receptor-mediated endocytosis after intratracheal administration. In this study, the anti-inflammatory

effects of dexamethasone palmitate incorporated in mannosylated liposomes (DPML) at 0.5 mg/kg via intratracheal administration were investigated in lipopolysaccharide-induced lung inflammation in rats. DPML significantly inhibited tumor necrosis factor  $\alpha$ , interleukin-1 $\beta$ , and cytokine-induced neutrophil chemoattractant-1 levels, suppressed neutrophil infiltration and myeloperoxidase activity, and inhibited NF $\kappa$ B and p38 mitogen-activated protein kinase activation in the lung. These results prove the value of inhaled mannosylated liposomes as powerful targeting systems for the delivery of anti-inflammatory drugs to alveolar macrophages to improve their efficacy against lung inflammation.

Inhalation of lipopolysaccharide (LPS), which is a component of Gram-negative bacteria presenting as an environment pollutant, contributes to inflammation in the lung. The downstream signaling pathways after LPS stimulation include the activation of alveolar macrophages, which are key effector cells to release proinflammatory cytokines including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) (Ulich et al., 1991), chemokines such as cytokine-induced neutrophil

chemoattractant-1 (CINC-1) (Ulich et al., 1995), and activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) and p38 mitogen-activated protein kinase (p38MAPK). Thereafter, neutrophils are recruited into the lung and release protease enzymes, which trigger lung injury. Corresponding to these studies, depletion of alveolar macrophages by liposomal clodronate treatment completely suppressed the downstream signaling after LPS stimulation (Koay et al., 2002). These results indicate that alveolar macrophages play a key role in the inflammation to release proinflammatory cytokines and chemokines after LPS stimulation.

Glucocorticoids (GCs) are the drugs of choice for treatment of lung inflammation via systemic or local administration. Although inhalation of free GC is a promising therapy with less systemic toxicity, the therapeutic efficacy has been ques-

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**ABBREVIATIONS:** LPS, lipopolysaccharide; BAL, bronchoalveolar lavage; Chol, cholesterol; Dex, dexamethasone; DP, dexamethasone palmitate; DPBL, dexamethasone palmitate containing bare liposomes; DPML, dexamethasone palmitate containing mannosylated liposomes; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; Man-C4-Chol, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide; NF $\kappa$ B, nuclear factor  $\kappa$ B; MAPK, mitogen-activated protein kinase; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; CINC-1, cytokine-induced neutrophil chemoattractant-1; GC, glucocorticoid; MPO, myeloperoxidase; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; H/E, hematoxylin/eosin; Man-liposomes, mannosylated liposomes.

tioned with regard to its short half-life (O'Byrne and Pedersen, 1998), potential toxicity at high doses (Foster et al., 2006), and inability to reach alveolar macrophages (Marshall et al., 2000). For these reasons, aerosol drug delivery systems need to be developed.

Liposomes have been widely used as carriers for inhaled drugs to prolong the retention of water-soluble drugs in the lung (Fielding and Abra, 1992), facilitate intracellular delivery, and reduce the toxicity of incorporated drugs (Schreier et al., 1993; Waldrep et al., 1997). There are several reports about the application of liposomes to incorporate dexamethasone (Dex), a potent GC, for lung inflammation by intratracheal (i.t.) administration (Tremblay et al., 1993; Suntres and Shek, 2000); however, difficulties in controlling the drug incorporation and retention in liposomes have been reported (Shaw et al., 1976). Benameur et al. (1993) demonstrated that dexamethasone palmitate (DP), which is more lipophilic than Dex, was inserted in the lipid bilayer and showed significant encapsulation and retention in the liposomes.

Because alveolar macrophages play an essential role in the pathogenesis of lung inflammation, we hypothesized that targeting DP directly to alveolar macrophages could produce more effective anti-inflammation of liposomal DP than free Dex. We have reported previously macrophage-selective targeting carriers composed of novel mannosylated cholesterol derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl)formamide (Man-C4-Chol), as a ligand for mannose receptors (Kawakami et al., 2000a; Hattori et al., 2006; Higuchi et al., 2006; Yeeprae et al., 2006). We have demonstrated the efficient targeting of mannosylated liposomes (Man-liposomes) to alveolar macrophages after intratracheal administration in rats (Wijagkanalan et al., 2008). After intratracheal injection, Man-liposomes with at least 5% of Man-C4-Chol were extensively and selectively delivered to alveolar macrophages via mannose receptor-mediated uptake.

This present study aimed to evaluate the effectiveness of DP containing Man-liposomes (DPML) in the treatment of LPS-induced lung inflammation after direct pulmonary delivery. Therefore, the inflammation markers, including cytokine and chemokine release, neutrophil infiltration, myeloperoxidase (MPO) activity, and activation of downstream pathways, were determined after DPML treatment in an LPS-stimulated rat model.

## Materials and Methods

**Materials.** DP was kindly provided by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). LPS 0111:B4 and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Hydroxyprogesterone caproate was obtained from MP Biomedicals (Irvine, CA). Mannan and cholesterol (Chol) were supplied from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of the highest purity available.

**Animals.** Seven-week-old male Wistar rats were obtained from the Shizuoka Agriculture Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

**Preparation of Liposomes.** Man-C4-Chol was synthesized by the method described previously (Kawakami et al., 2000b). The lipid composition (DSPC/Chol/Man-C4-Chol) of Bare-liposomes (60:40:0) and Man-liposomes (60:35:5) was mixed with DP at a 10:1 M ratio.

The lipid mixture was dissolved in chloroform and then evaporated. The dried film was vacuum-desiccated and resuspended in water for injection. The resulting liposomes were sonicated and extruded through 200- and 100-nm polycarbonate membrane filters using an extruder device preheated to 60°C. Free DP was removed by filtration. The particle sizes and  $\zeta$  potentials of the liposomes were determined using a Zetasizer Nano ZS instrument (Malvern Instruments, Ltd., Worcestershire, UK). The concentration of the liposomes was measured using a PL test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Determination of DP and Dex.** A quantity (0.2 ml) of liposomes and hydroxyprogesterone caproate (internal standard) were lyophilized; then, DP was extracted with 1 ml of acetonitrile and sonicated for 40 min. Liposomal DP was measured using an HPLC system (Shimadzu Co., Kyoto, Japan) with a 5  $\mu$ m YMC-Pack ODS-A column (4.6  $\times$  150 mm; YMC, Kyoto, Japan). The mobile phase was acetonitrile/water (95:5) at a flow rate of 1 ml/min. The eluent was monitored at 236 nm, and the DP concentration was quantified with respect to a standard curve of DP. The incorporation efficiency of DP liposomes was determined by *in vitro* release of liposomal DP in 0.5% Tween 20 containing PBS at 37°C for 48 h using a side-by-side diffusion chamber that was mounted with a 3500 molecular weight cut-off membrane (Spectra/Por; Spectrum Laboratories Inc., Rancho Dominguez, CA). The release of DP into the receiver was measured by UV spectrophotometry at 236 nm. Dex was measured using HPLC systems according to a method described previously with modification (Schild and Charles, 1994). The HPLC analysis for biological samples was calibrated ( $R^2 > 0.998$ ).

**Biodistribution Study.** Rats were lightly anesthetized and then intratracheally administered with liposomal DP or free Dex at 0.5 mg/kg using a Microsprayer (Penn Century, Philadelphia, PA). After administration, rats were supported vertically for 1 min. The plasma, lung tissue, and alveolar macrophages were collected as described below at the indicated time and then extracted with acetonitrile before HPLC analysis. The drug concentration in lung tissue and alveolar macrophage samples was normalized with protein using a Proteostain protein quantification kit (Dojindo, Kumamoto, Japan).

**Isolation of Alveolar Macrophages.** Alveolar macrophages were isolated as described previously (Wijagkanalan et al., 2008). In brief, the lung was lavaged with EDTA containing buffer, and the bronchoalveolar lavage (BAL) fluid was subsequently centrifuged at 200g for 10 min at 4°C. The alveolar macrophages were obtained after washing once with ice-cold PBS. The cell viability was greater than 99% measured using the trypan blue exclusion method.

**LPS Stimulation in Isolated Alveolar Macrophages.** Isolated alveolar macrophages were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at a density of  $2.1 \times 10^5$  cells/cm<sup>2</sup> for 12 h before LPS challenge. In measuring the dose-response of TNF $\alpha$  release, alveolar macrophages were cocultured with 1  $\mu$ g/ml LPS and 1 nM to 1  $\mu$ M DP formulations for 6 h. For the time course experiments, alveolar macrophages were stimulated with 1  $\mu$ g/ml LPS for 0.5 to 24 h. In the DP treatment, alveolar macrophages were cocultured with 1  $\mu$ g/ml LPS and 0.1  $\mu$ M free Dex solution, DP, or liposomal DP for a further 6 h. For the mannan

TABLE 1

The physicochemical property, DP recovery, and incorporation efficiency of DP liposomes

Each value represents the mean  $\pm$  S.D. values ( $n = 3$ ). Incorporation efficiency was determined by *in vitro* release study for 48 h at 37°C.

Liposomes	Mean Particle Size	$\zeta$ Potential	DP Recovery	Incorporation Efficiency
	nm	mV	%	%
DPBL	100 $\pm$ 11	-7.22 $\pm$ 8.8	85 $\pm$ 30	99 $\pm$ 1.5
DPML	110 $\pm$ 6.9 <sup>†</sup>	0.07 $\pm$ 0.22*	103 $\pm$ 39 <sup>†</sup>	99 $\pm$ 1.1 <sup>†</sup>

\*  $P < 0.05$ , statistically significant difference compared with DPBL.

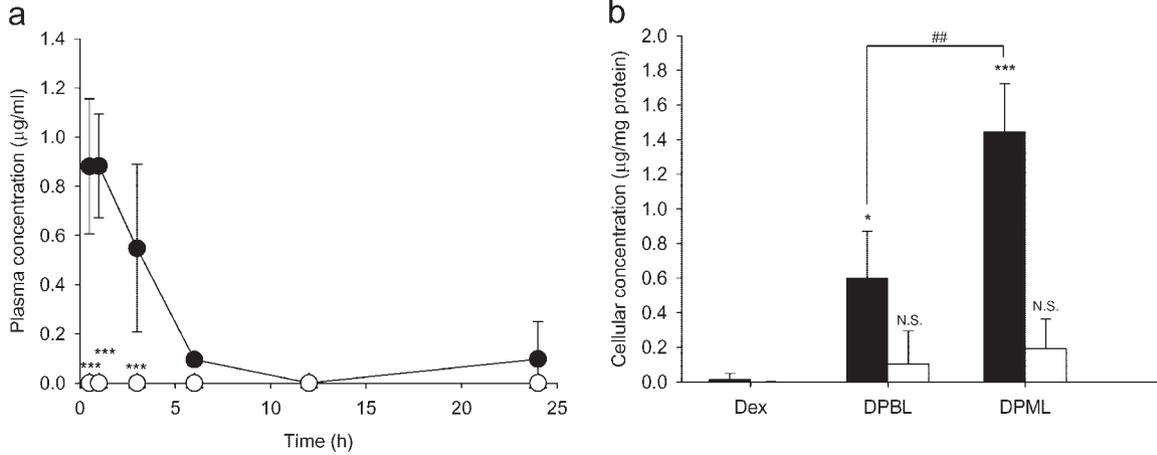
<sup>†</sup> Not significant.

inhibition study, 1 mg/ml mannan was added to the above mixture for competitive uptake mediated by mannose receptors (Hattori et al., 2006; Yeeprae et al., 2006; Wijagkanalan et al., 2008). After the indicated times, cytokine and chemokine protein levels in the supernatant were measured by ELISA (eBiosciences Inc., San Diego, CA).

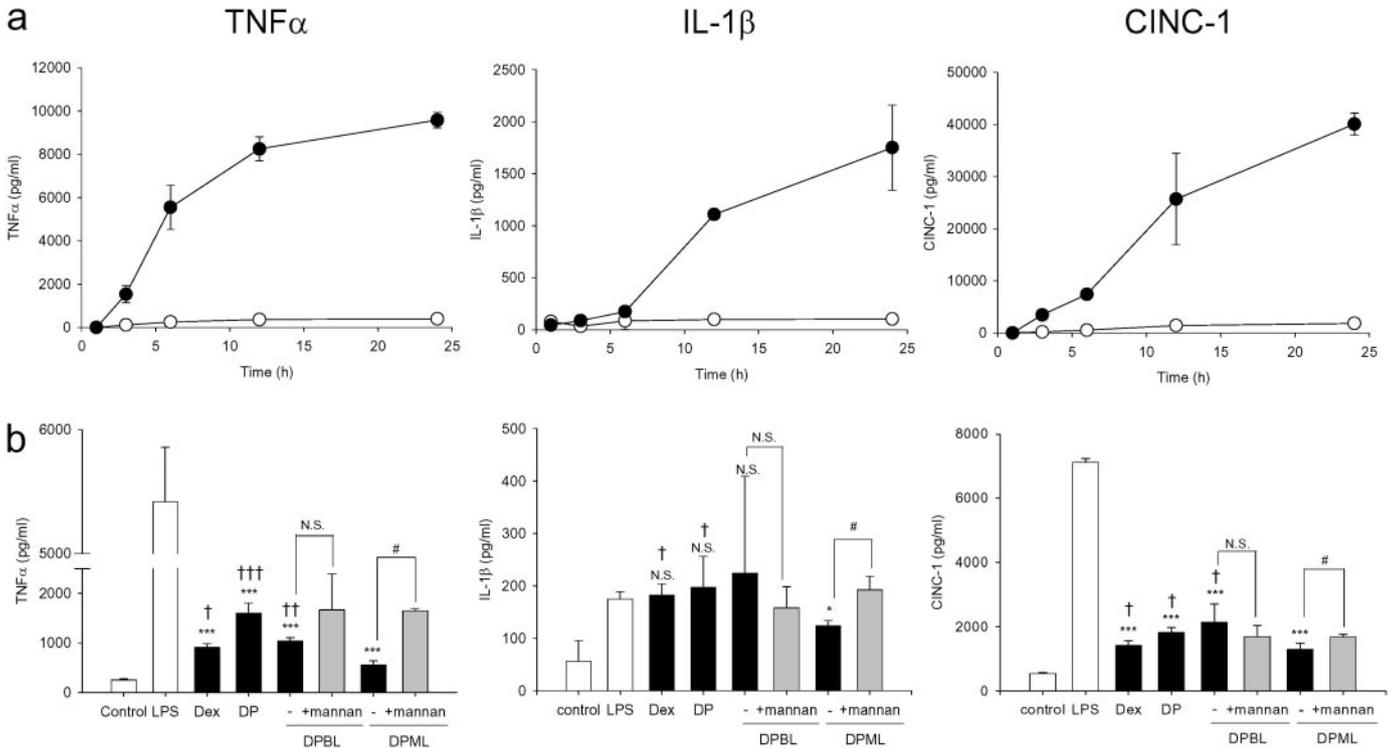
**Induction of LPS-Induced Lung Inflammation and Treatment.** Rats were intratracheally given saline; LPS at 0.5 mg/kg alone (Giraud et al., 2000); free Dex (positive control) or liposomal DP with an indicated dose at the onset or 1 h before (pretreatment)

LPS-induced lung inflammation; and coadministration of DPML with mannan, a mannose receptor ligand, at a dose of 5 mg/kg. The blood, BAL samples, and lung tissue were collected at the indicated time after challenge. To study the systemic side effects, intravenous injection of free Dex at 4 mg/kg was used as positive control, and the blood glucose levels in rats were measured using the Accu-Chek Active (Roche Ltd., Basel, Switzerland).

**Preparation of BAL Samples, Lung Tissues, and Cell Differential Counts.** The BAL fluid was collected from each rat 3 h



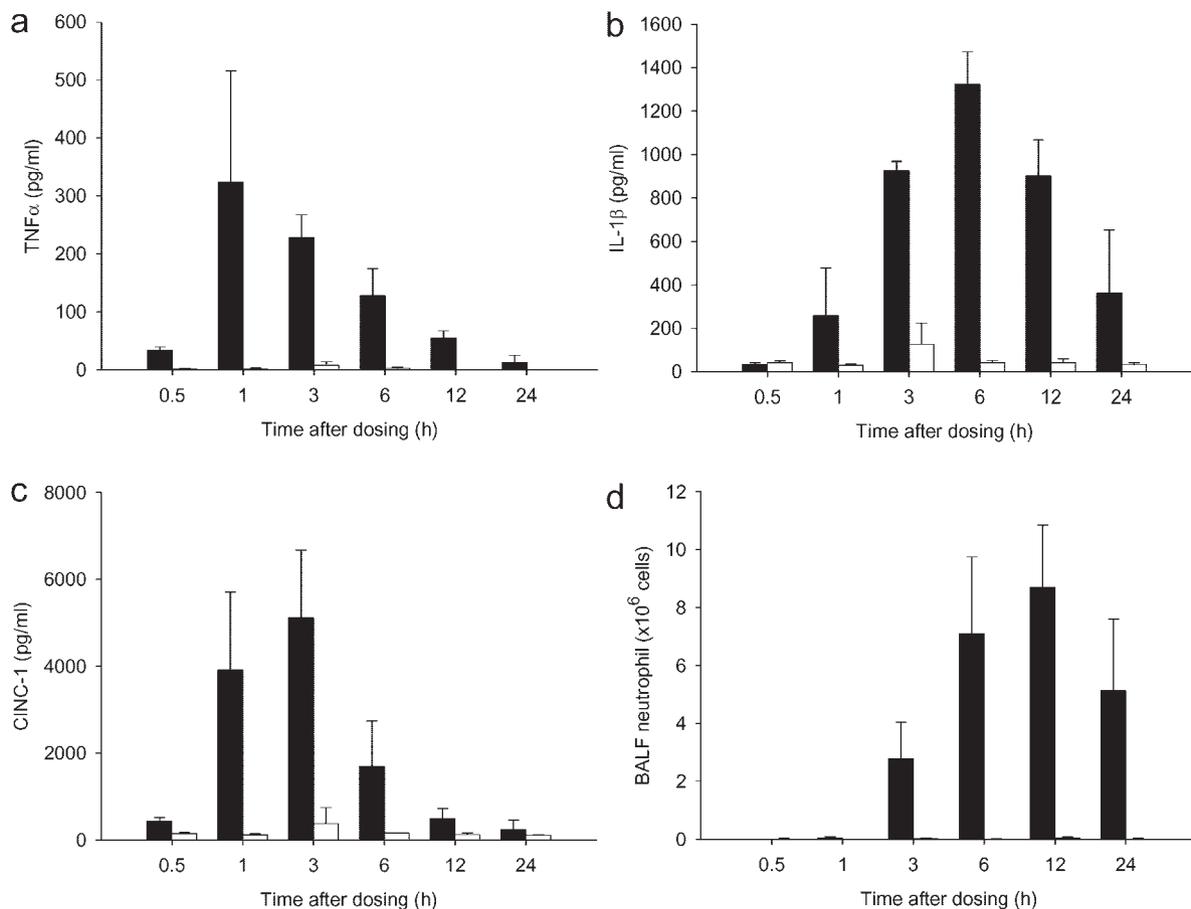
**Fig. 1.** Biodistribution of liposomal DP and free Dex after intratracheal administration at a dose of 0.5 mg/kg. a, plasma concentration time course of liposomal DP (○) and free Dex (●). b, the concentration of liposomal DP and free Dex in alveolar macrophages at 3 (■) and 24 h (□) post-dosing. Male Wistar rats were intratracheally given liposomal DP and free Dex at 0.5 mg/kg, and then plasma and alveolar macrophages were collected at indicated time points (0.5–24 h). The concentration of drugs was determined by HPLC systems. Results are expressed as mean ± S.D. of three experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ) compared with Dex treatment in each experiment (##,  $P < 0.01$ ) with each pair of treatments. N.S., not significant.



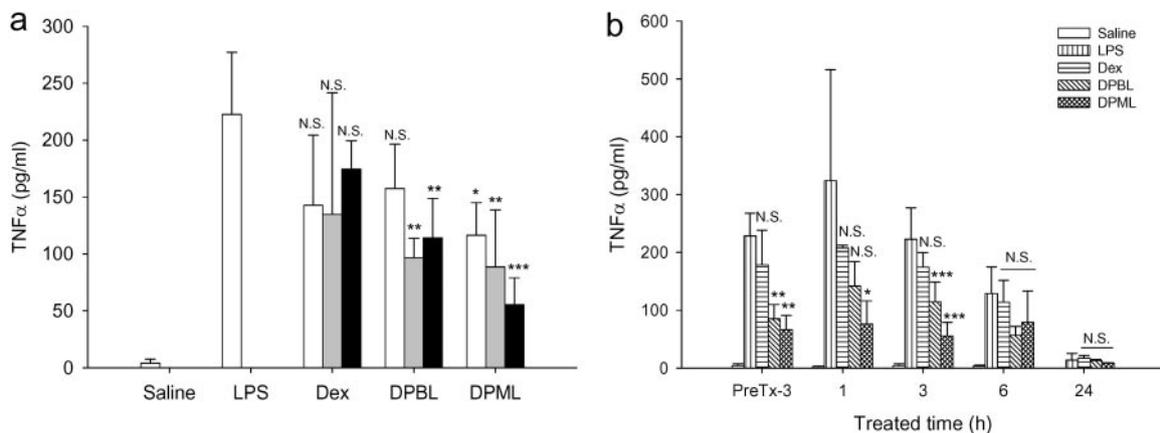
**Fig. 2.** Effects of DPML on inhibition of LPS-induced cytokine and chemokine release in vitro. a, time course of TNF $\alpha$ , IL-1 $\beta$ , and CINC-1 protein levels after LPS stimulation at 1  $\mu$ g/ml (●) and control (○). b, inhibition of TNF $\alpha$ , IL-1 $\beta$ , and CINC-1 protein levels after treatment. The alveolar macrophages were incubated with 1  $\mu$ g/ml LPS alone or coincubated with 0.1  $\mu$ M free drugs (Dex or DP) or liposomal DP (■). For the uptake inhibition, mannan was also coincubated at 1 mg/ml (□). The cytokine and chemokine levels in the supernatant were determined by ELISA after a 6-h incubation. Results are expressed as a mean ± S.D. of at least three experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ) compared with LPS treatment in each experiment (†,  $P < 0.05$ ; ††,  $P < 0.01$ ; †††,  $P < 0.001$ ) with DPML treatments (#,  $P < 0.05$ ) with each pair of treatments. N.S., not significant.

after challenge with 10 ml of PBS and repeated flushing (10 times). The BAL cells were obtained after centrifugation as described above. Small aliquots of the supernatant were frozen at  $-80^{\circ}\text{C}$  for further cytokine and chemokine analysis. The BAL cells were resuspended in RPMI serum-free and subjected to cyto spin (Shandon Scientific, Runcorn, UK) at 600 rpm for 5 min with low acceleration at room

temperature. The cells were then stained with modified Giemsa reagents (Diff-Quik, Sysmex, Japan). Four-part differential counts on 200 cells/slide were performed based on standard morphological criteria, and the number of neutrophils was determined. For the lung tissue processing, lung tissue was cut and homogenized in protease inhibitor containing PBS and then spun at 14,000g at  $4^{\circ}\text{C}$ . The



**Fig. 3.** Time course of TNF $\alpha$  (a), IL-1 $\beta$  (b), CINC-1 protein levels (c), and neutrophil infiltration (d) in the lung after aerosolized LPS challenge. Male Wistar rats were exposed to 0.5 mg/kg aerosolized LPS (■) or saline (□), and then BAL fluid and cells were collected at indicated time points (0.5–24 h). Cytokine and chemokine levels in BAL fluid were determined by ELISA and neutrophil influx in the lung was differentially counted. Results are expressed as a mean  $\pm$  S.D. of at least three experiments.



**Fig. 4.** Dose- (a) and time-response (b) profiles of DPML on the inhibition of TNF $\alpha$  release after LPS-induced lung inflammation. a, male Wistar rats were administered 0.5 mg/kg i.t. LPS alone or together with free Dex, DPBL, or DPML at a dose of 0.1 (□), 0.25 (▤), and 0.5 (■) mg/kg. The BAL fluid was collected 3 h after challenge. b, male Wistar rats were given drugs at 0.5 mg/kg i.t. together with LPS or pretreated with drugs 1 h before LPS stimulation (pretreatment, PreTx-3), and then the BAL fluid was collected at indicated time or 3 h, respectively. The TNF $\alpha$  level was measured by ELISA. Results are expressed as a mean  $\pm$  S.D. of at three experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) compared with LPS treatment in each experiment. N.S., not significant.

resulting supernatant was subjected to cytokine and chemokine measurements.

**Cytokine and Chemokine Measurements.** The TNF $\alpha$  level (eBiosciences), IL-1 $\beta$ , and CINC-1 levels (IBL Co. Ltd., Gunma, Japan) in BAL fluid were measured using an ELISA kit. These cytokine levels in the lung tissue were measured with an ELISA kit obtained from R&D Systems (Minneapolis, MN). The cytokine and chemokine levels in the lung tissue were normalized with regard to the protein content using a Proteostain protein quantification kit (Dojindo).

**MPO Activity.** The MPO activity in the lung tissue was measured using an MPO Assay Kit (Cytostore Inc., Calgary, AB, Canada).

**Nuclear Protein Extract.** A small quantity (100–150 mg) of fresh lung tissue and isolated alveolar macrophages from the same animal were extracted as described in the instructions for the Nuclear Extract Kit (Active Motif, Carlsbad, CA). The nuclear protein was divided into aliquots, frozen, and stored at  $-80^{\circ}\text{C}$  until further assay. The protein concentration was determined by the Bradford assay method (Bradford, 1976).

**Electrophoretic Mobility Shift Assay.** The nuclear extract (50  $\mu\text{g}$  of lung tissue protein and 40  $\mu\text{g}$  of isolated alveolar macrophage protein) was mixed with binding reaction mixtures in accordance with the instructions for the LightShift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) Kit (Pierce Biotechnology Ltd., Rockford, IL). The protein-DNA complexes were separated on Supersep Gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in  $1\times$  Tris borate-EDTA buffer. The separated bands were transferred to a nylon membrane and detected by the Chemiluminescent Nucleic Acid Detection Module (Pierce).

**Western Blot Analysis.** The lung tissue homogenate samples were separated on a 10% SDS-polyacrylamide gel. Separated protein bands were electrophoretically transferred to a nitrocellulose membrane and blocked with Tris-buffered saline containing 3% bovine

serum albumin. The membranes were then incubated separately with anti-p38 $\alpha$  antibody and anti-phospho-p38MAPK (*Thr180/Tyr182*) antibody (R&D Systems) overnight at  $4^{\circ}\text{C}$ . An anti- $\beta$ -actin antibody (Cell Signaling Technology, Inc., Danvers, MA) was used as loading control. The antibody labeling of the protein bands was detected with Enhanced Chemiluminescence Reagents (Millipore Corporation, Billerica, MA).

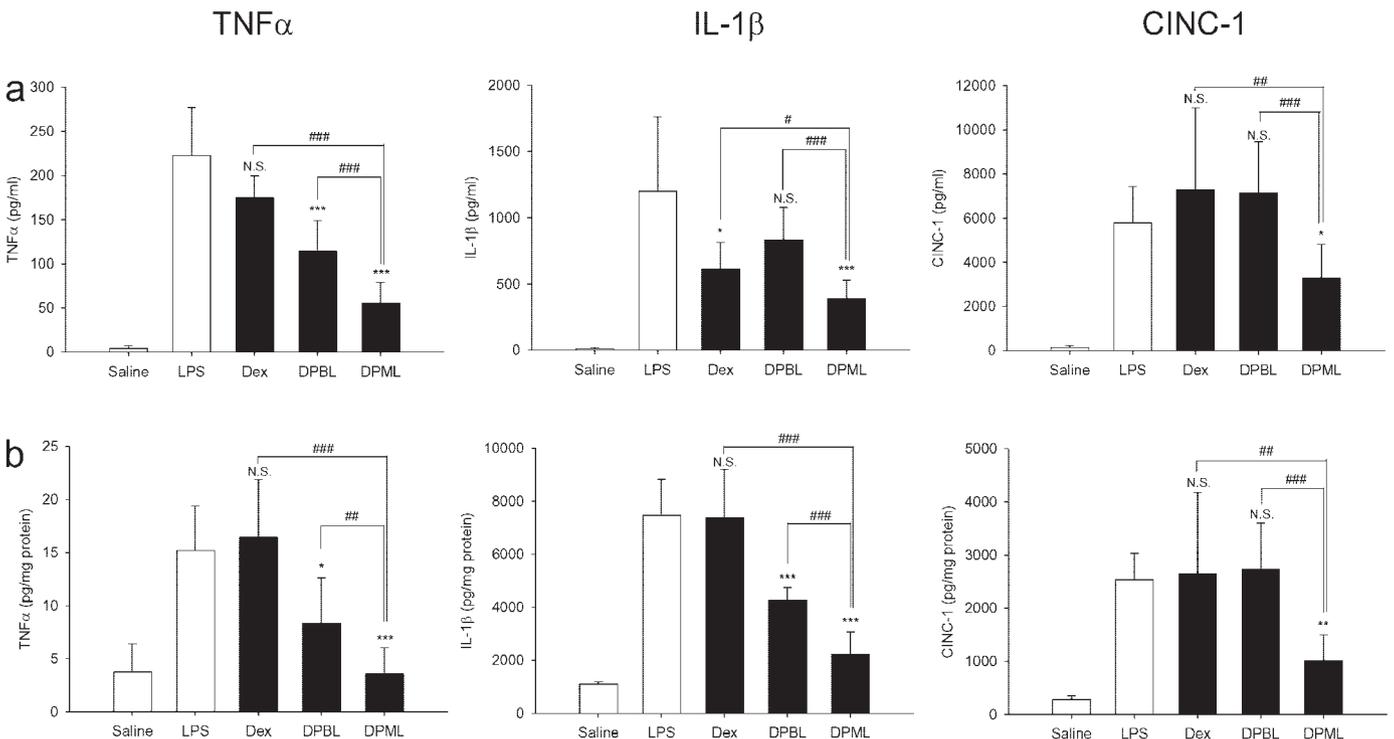
**Lung Histology.** Three hours after administration, the lung was removed en bloc and inflation-fixed with 4% formaldehyde. The inflation-fixed lung tissue samples were embedded in paraffin and cut into 5- $\mu\text{m}$  sections. After removal of the paraffin with xylene and rehydration in graded alcohol, the sections were stained with hematoxylin/eosin (H/E).

**Statistical Analysis.** Statistical analysis was performed using analysis of variance and the Turkey-Kramer test for multiple comparisons between groups or Student's *t* test for two-group comparisons.  $P < 0.05$  was considered to be indicative of statistical significance.

## Results

**Physicochemical Properties of DP Liposomes.** The particle sizes of dexamethasone palmitate containing Bareliposomes (DPBL) and DPML were comparable; however, their  $\zeta$  potentials showed significant difference ( $P < 0.05$ ). In addition, these liposomes were able to equally and stably incorporate DP (Table 1).

**Biodistribution Study.** To confirm the targeting efficiency of DP liposomes to alveolar macrophages, pulmonary and systemic distribution of liposomal DP and free Dex was determined after intratracheal administration in rats. Dex was rapidly distributed to blood circulation as early as 30 min after administration, whereas there was an undetectable



**Fig. 5.** Efficient inhibition of cytokine release after intratracheal instillation of DPML in BAL fluid (a) and lung tissue (b). Male Wistar rats were administered 0.5 mg/kg i.t. LPS alone ( $\square$ ) or together with free Dex, DPBL, or DPML ( $\blacksquare$ ) at a dose of 0.5 mg/kg. The BAL fluid and lung tissue were collected 3 h after challenge, and cytokine levels were measured by ELISA. Results are expressed as a mean  $\pm$  S.D. of at least six experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ) compared with LPS treatment in each experiment (#,  $P < 0.05$ ; ##,  $P < 0.01$ ; ###,  $P < 0.001$ ) with each pair of treatments. N.S., not significant.

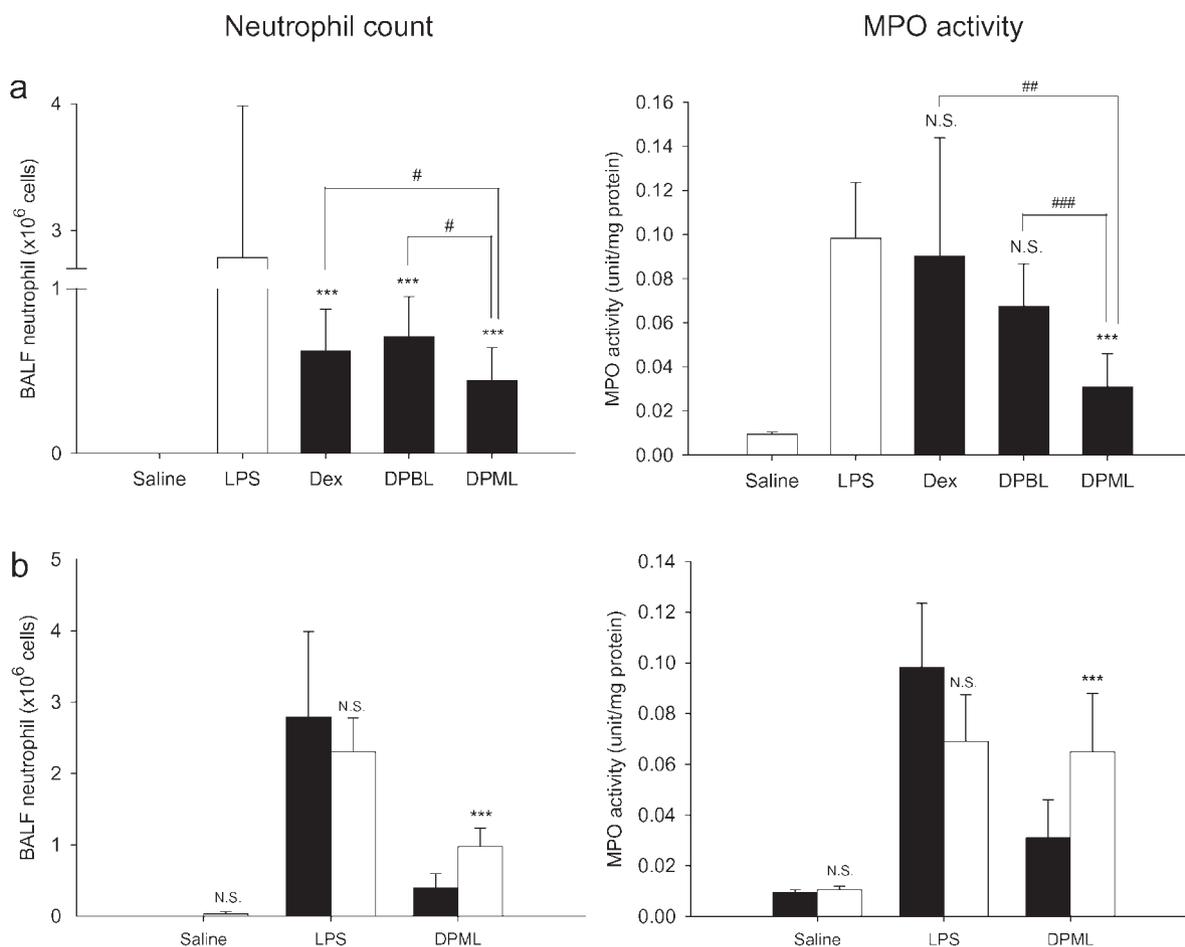
level of liposomal DP at any time point (Fig. 1a). On the other hand, liposomal DP showed a higher drug level in alveolar macrophages at 3 and 24 h after administration; however, DPML was extensively retained in alveolar macrophages compared with free Dex ( $P < 0.001$ ) and DPBL ( $P < 0.01$ ) (Fig. 1b).

**Effects of DPML on Inhibition of LPS-Induced Cytokine and Chemokine Release In Vitro.** To evaluate the anti-inflammatory effects and uptake mechanism of DPML, alveolar macrophages were treated with  $0.1 \mu\text{M}$  liposomal DP in the presence of mannan, whereas Dex and DP solutions were used as positive controls. Figure 2a shows the cytokine release profile after LPS stimulation. Although strong inhibition of  $\text{TNF}\alpha$  and CINC-1 ( $P < 0.001$ ) was observed by all formulations, DPML showed significant suppression of  $\text{TNF}\alpha$  (at least  $P < 0.05$ ), IL-1 $\beta$  ( $P < 0.05$ ), and CINC-1 ( $P < 0.05$ ) compared with DPBL and free drugs after 6-h incubation (Fig. 2b). In addition, the anti-inflammatory effects of DPML were inhibited after coincubation with an excess of mannan ( $P < 0.05$ ).

**Induction and Development of LPS-Induced Lung Inflammation.** To verify the biological profile of the airway inflammatory response after LPS challenge, rats were exposed to aerosolized LPS or saline for 0.5 to 24 h, and BAL

samples were collected before carrying out measurements. Figure 3 shows the rapid increase in the  $\text{TNF}\alpha$  level with a peak at 1 h and subsequent release of IL-1 $\beta$  and CINC-1 level with a peak at 6 and 3 h, respectively. Thereafter, the neutrophil recruitment in the lung was initiated 3 h after LPS administration.

**Effect of DPML on the Inhibition of Cytokine Release and Neutrophil Infiltration after Intratracheal Instillation.** To optimize the dose and treated time of DPML on the in vivo cytokine inhibition, rats were intratracheally administered LPS with either liposomal DP or free Dex at a dose range of 0.1 to 0.5 mg/kg (Nemmar et al., 2004), and cytokine release was measured at 1 to 24 h after dosing. Figure 4a shows a dose-response profile of liposomal DP and free Dex on the inhibition of  $\text{TNF}\alpha$  production in BAL fluid 3 h after administration. A marked reduction on  $\text{TNF}\alpha$  levels was observed after treatment with liposomal DP at a dose of 0.25 mg/kg compared with the LPS-treated group ( $P < 0.01$ ); furthermore, DPML showed the most effective action at a dose of 0.5 mg/kg ( $P < 0.001$ ). In addition, significant  $\text{TNF}\alpha$  inhibition by DPML was observed as rapid as 1 h ( $P < 0.05$ ) and showed an optimal treated time at 3 h ( $P < 0.001$ ) after administration (Fig. 4b). However, pretreatment and cotreatment of DPML showed comparable effects. To investigate the



**Fig. 6.** Anti-inflammatory effects of DPML (a) and inhibition by mannan (b) on neutrophil infiltration and MPO activity in the lung after intratracheal administration. Male Wistar rats were administered 0.5 mg/kg i.t. LPS alone or together with free Dex, DPBL, or DPML (■) at a dose of 0.5 mg/kg. In the inhibition study, mannan was coadministered in each group (□). Three hours after treatment, the neutrophil influx and MPO activity were determined in BAL samples and lung tissue, respectively. Results are expressed as a mean  $\pm$  S.D. of at least six experiments. Statistically significant differences (\*\*\*,  $P < 0.001$ ) compared with LPS treatment in each experiment in a or with control in each experiment in b (#,  $P < 0.05$ ; ##,  $P < 0.01$ ; ###,  $P < 0.001$ ) with each pair of treatments. N.S., not significant.

in vivo anti-inflammatory effects of DPML, rats were intratracheally administered as described above at a concentration of 0.5 mg/kg, and BAL samples and lung tissue were subsequently collected at 3 h after dosing. DPML significantly attenuated TNF $\alpha$  ( $P < 0.001$ ), IL-1 $\beta$  ( $P < 0.001$ ), and CINC-1 ( $P < 0.05$ ) in BAL fluid (Fig. 5a) and in the lung tissue (Fig. 5b) compared with no treatment, free Dex (at least  $P < 0.05$ ), and DPBL (at least  $P < 0.01$ ). Figure 6a shows extensive inhibition of neutrophil migration to the lung with all treatments ( $P < 0.001$ ); however, the more potent antimigration effects ( $P < 0.05$ ) and inhibition of MPO activity (at least  $P < 0.01$ ) were observed after treatment with DPML compared with DPBL and free Dex.

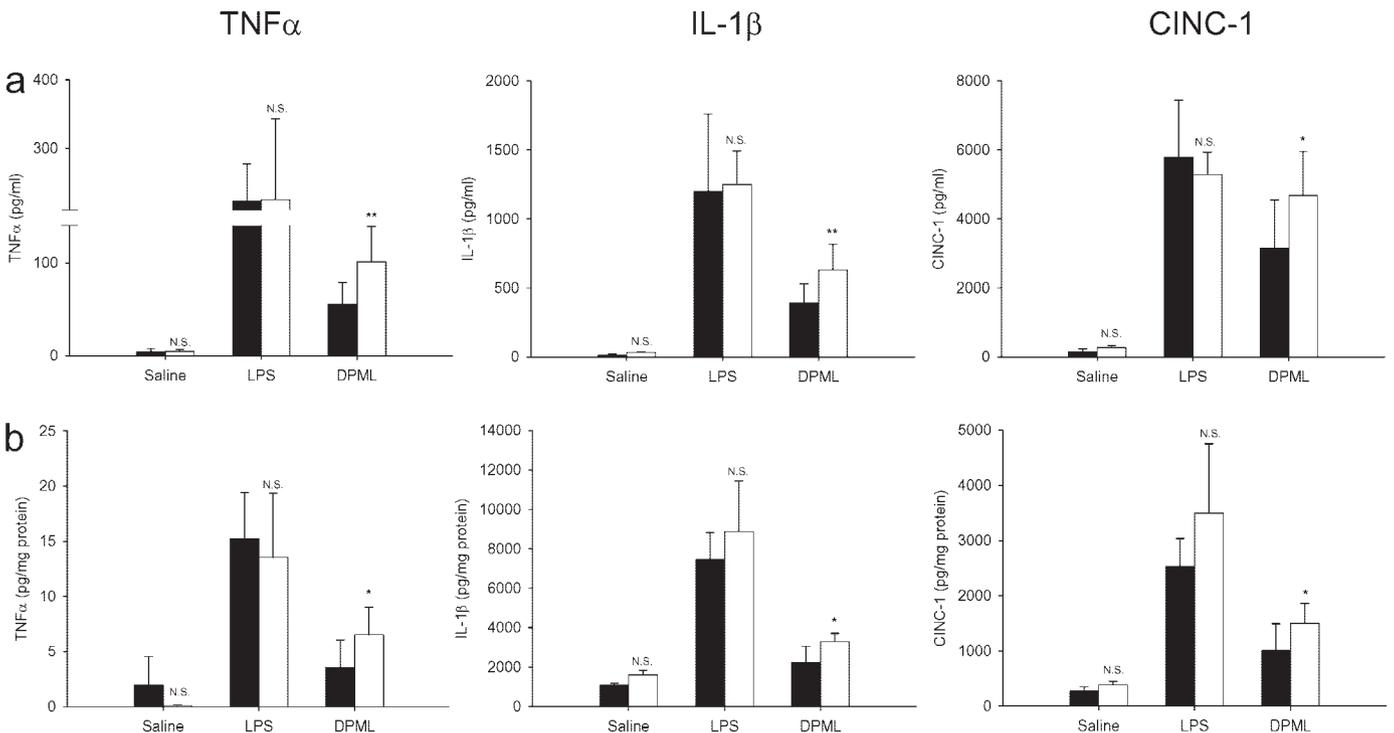
**Uptake Mechanisms of DPML by Alveolar Macrophages for Pharmacological Intervention of Incorporated DP.** Because DPML were used as DP-targeting carriers to alveolar macrophages, the uptake mechanism of DPML was investigated with the competitive mannose receptor ligand, mannan, through the anti-inflammatory effects of incorporated DP. After cotreatment with mannan, the anti-inflammatory effects of DPML, including cytokine release (at least  $P < 0.05$ ), neutrophil infiltration ( $P < 0.001$ ), and MPO activity ( $P < 0.001$ ), were significantly compromised in both BAL fluid and lung tissue (Figs. 6b and 7). However, there was no effect of mannan on the cytokine release in both control groups.

**Lung Histology.** To examine the histopathology of lung inflammation, the treated lungs were sectioned and stained with H/E. The lungs from control animals had normally thin alveolar septae with a negligible number of inflammatory cells (Fig. 8a). On the other hand, LPS-treated lungs showed moderately edematous alveolar septae and inflammatory cell

recruitment indicated with arrows (Fig. 8b). Among drug-treated lungs (Fig. 8, c–e), DPML-treated lungs (Fig. 8e) contained as few inflammatory cells and septal edema as control lungs. However, coadministration of mannan to DPML-treated lungs produced a marked increase in infiltration of inflammatory cells and edema in the alveoli (Fig. 8f).

**NF $\kappa$ B Activation and Phosphorylation of p38 MAPK.** To examine the anti-inflammatory signaling pathways of incorporated DP, the nuclear extract from alveolar macrophages or lung tissue samples were subjected to EMSA and Western blot analysis. Figure 9, a and b, shows an increase in NF $\kappa$ B activation in LPS-treated animals (lane 3), whereas there was little detectable NF $\kappa$ B expression in control animals (lane 2) as shown by both alveolar macrophage (Fig. 9a) and lung tissue samples (Fig. 9b). The activation of NF $\kappa$ B was hardly inhibited after treatment with free Dex and DPBL (lanes 4 and 5). As expected, DPML significantly suppressed the activation of NF $\kappa$ B in both types of samples (lane 6) compared with the LPS-treated group (at least  $P < 0.05$ ) and DPBL ( $P < 0.05$ ). In parallel with the above studies, the inhibition of NF $\kappa$ B activation was attenuated after cotreatment with mannan ( $P < 0.05$ ). Furthermore, phosphorylation of p38 MAPK was observed after LPS stimulation in rats (Fig. 9c); however, the complete down-regulation of phosphorylated p38 MAPK was revealed after DPML treatment and was attenuated in the presence of mannan that corresponded to the results of the NF $\kappa$ B activation study.

**Systemic Side Effects of DPML.** To examine the systemic side effect of inhaled DPML, an increase in blood glucose detected at the early stage of systemic side effect of Dex was measured after intratracheal instillation of DPML compared with free Dex (Fig. 10). Among the treatment groups,



**Fig. 7.** Mannose-receptor mediated uptake of DPML for anti-inflammatory effects of incorporated DP after intratracheal administration. BAL fluid (a) and lung tissue (b). Male Wistar rats were administered 0.5 mg/kg i.t. LPS alone or with 0.5 mg/kg of DPML (■) in the presence of an excess of mannan (□). The BAL fluid and lung tissue were collected 3 h after challenge, and cytokine levels were measured by ELISA. Results are expressed as mean  $\pm$  S.D. of at least six experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ) compared with control in each experiment. N.S., not significant.

only inhaled free Dex markedly increased blood glucose level identically with intravenously injected group but showed significant compared with saline treatment ( $P < 0.01$ ). It is interesting that liposomal DP did not significantly alter blood glucose levels compared with control groups.

## Discussion

In this study, we successfully demonstrated the targeting delivery of DP by Man-liposomes to alveolar macrophages in LPS-induced lung inflammation after intratracheal administration via mannose receptor-mediated endocytosis. This is the first report describing the efficient anti-inflammatory effects of DP at a suboptimal dose in this inflammation model. More precisely, the selectively delivered DP by Man-liposomes strongly suppressed proinflammatory cytokines, chemokine, and inflammatory signaling factors, including NF $\kappa$ B and p38 MAPK compared with free Dex and DPBL.

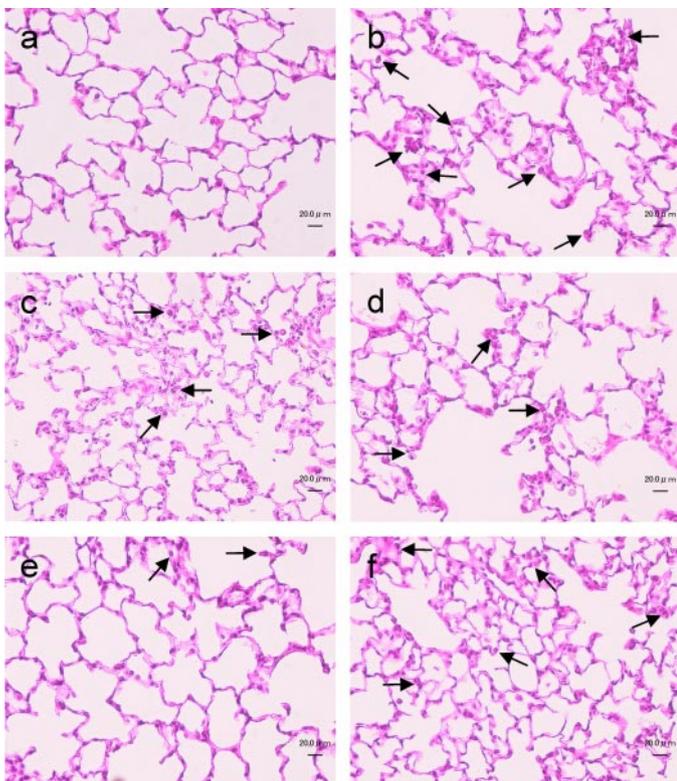
Because the physicochemical properties of liposomes influence the aerosol deposition and incorporation of drugs, the particle sizes,  $\zeta$  potentials, and incorporation efficiency of liposomal DP were investigated (Table 1). These liposomal formulations showed a small size (approximately 100 nm) allowing them free access to the alveolar space (Schreier et al., 1993; Wijagkanalan et al., 2008), and avoiding breakdown of the liposomal membrane during aerosolization (Niven et al., 1991). The particle sizes and surface charge of liposomes influence the in vivo uptake by alveolar macrophages (Fidler et al., 1980). Lee et al. (1992) reported the effect of surface charge density and specific lipid head groups

on the recognition of liposomes by the murine macrophages cell line (J774). Neutral liposomes and negatively charged liposomes showed similar cellular association to J774 macrophages; however, only high-density phosphatidylserine containing negatively charged liposomes showed significantly high uptake (Lee et al., 1992). Therefore, the effect of charge difference of these neutral liposomes DPBL and DPML, which is due to the positive charge of the Man-C4-Chol structure itself, on their uptake is excluded. Furthermore, the incorporated DP was stably retained in these liposomal formulations after a 48-h incubation correlated with a previous study involving the high retention of steroid palmitate in liposomes (Shaw et al., 1976, Teshima et al., 2004). These results confirm the stability of the liposomal formulations of DP for in vitro studies and intratracheal application in rats.

To characterize the pathology of inhaled LPS on lung inflammation, LPS was intratracheally instilled into the lung at a dose of 0.5 mg/kg using Microsprayer (PennCentury). The inflammatory markers, including proinflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ), chemokine (CINC-1), and the recruitment of neutrophils (Fig. 3), were significantly up-regulated in a time-dependent manner that corresponded to the findings in previous studies when different intratracheal techniques were used (Haddad et al., 2001; Jansson et al., 2005). These results ensure the suitability of lung inflammatory conditions after aerosolized LPS exposure at 0.5 mg/kg.

To elucidate the therapeutic efficiency of DPML after LPS stimulation, rats were given DPML by the intratracheal route in LPS-induced lung inflammation. At as low a dose as 0.5 mg/kg, the cytokine levels were markedly attenuated in both BAL fluid and lung tissue within several hours (Rocksén et al., 2000; Haddad et al., 2001) after treatment with DPML (Figs. 4 and 5). Consistent with the lung histology (Fig. 8), DPML treatment caused a more potent inhibition of neutrophil migration and inhibition of MPO activity, a protease expressed in neutrophils (Fig. 6a). These results are in agreement with the ability of inhaled free Dex at a dose of 0.5 mg/kg to cause strong inhibition of neutrophil accumulation in the lung after stimulation with diesel particles (Nemmar et al., 2004) and pneumonitis antigen (Tremblay et al., 1993). It has been accepted that selective delivery of the therapeutic agents into the target site increases drug efficacy and decreases side effects (Takakura and Hashida, 1996). Corresponding to our previous study involving the effective targeting of mannosylated liposomes to alveolar macrophages (Wijagkanalan et al., 2008), the enhanced anti-inflammatory effects of DPML in this study could be explained by the significant increase in cellular DP concentration at 3 h and prolonged drug localization in alveolar macrophages after targeting of DP by using mannosylated liposomes (Fig. 1b). Furthermore, these effective anti-inflammatory effects of DPML were significantly attenuated in the presence of mannan in vitro (Fig. 2b) and in vivo (Figs. 6b and 7), despite partial effect. These results indicate the improved anti-inflammatory effects of DPML by targeted delivery to alveolar macrophages via mannose receptor-mediated endocytosis.

It has been widely accepted that the response of alveolar macrophages to endotoxin (LPS) is closely related to the activation of intracellular signaling cascades including NF $\kappa$ B, MAPK, and phosphatidylinositol 3-kinase pathways to orchestrate the production of pro- and anti-inflammatory mediators (Monick and Hunninghake, 2002). Similarly to the



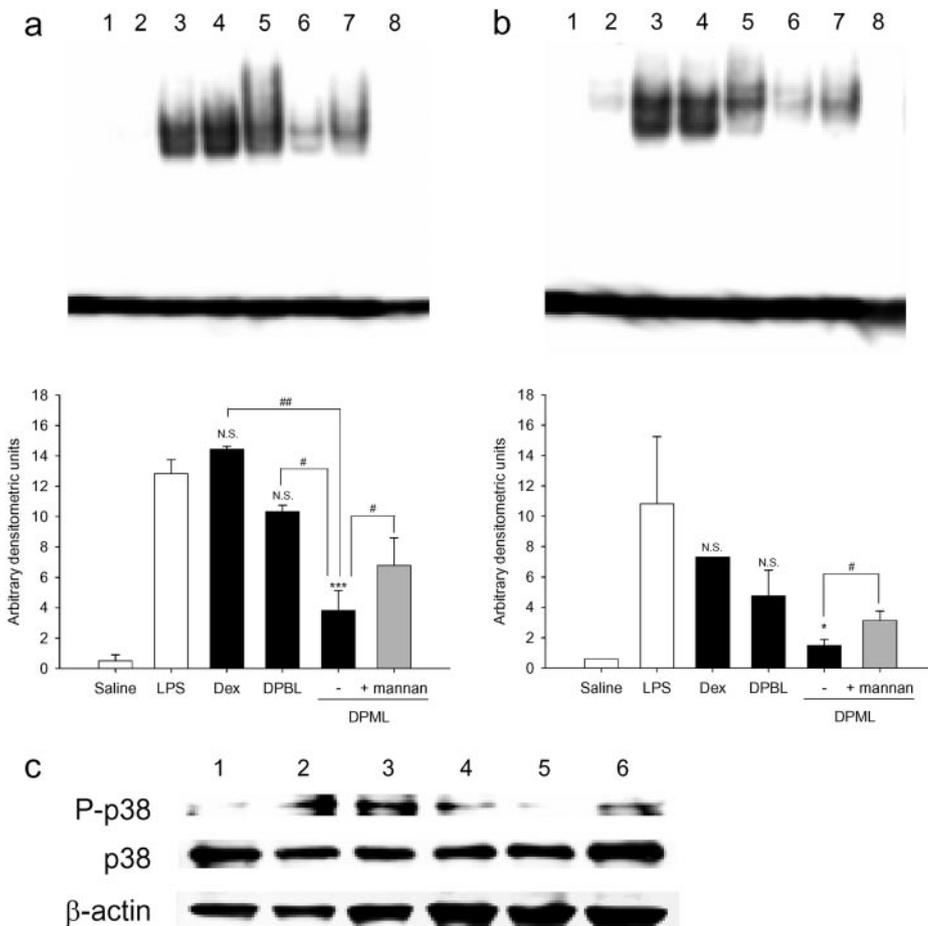
**Fig. 8.** Lung histology after intratracheal administration of saline (a), LPS (b), treatment with Dex (c), treatment with DPBL (d), treatment with DPML (e), and treatment with DPML (f) in the presence of an excess of mannan. After treatment, the lungs were embedded in paraffin, sectioned, and stained with H/E. Neutrophil accumulation in the lung was indicated as arrow. All images are at 400 $\times$  magnification.

pathogenesis of LPS in the human alveolar macrophages, LPS-induced cytokine release and activation of NFκB (Carter et al., 1998) and p38 MAPK (Carter et al., 1999) were enhanced in the adult respiratory distress syndrome-like in vitro model. To investigate the effects of DPML on the intracellular signaling pathways, the activation of NFκB and p38MAPK were determined after LPS induction. Corresponding to a previous study (Kim et al., 2006), inhaled LPS activated NFκB (Fig. 9, a and b) and phosphorylation of p38 (Fig. 9c) by which this active form is translocated to the nucleus for transcription, although the p38 protein level was similar to that of the control treatment 3 h after challenge. In parallel with the cytokine release study, DPML also markedly down-regulated NFκB and p38 phosphorylation compared with free Dex and DPBL. These results agree with the recent study showing that Dex selectively inhibits p38 MAPK phosphorylation accompanied by the induction of MAPK phosphatase-1 in control macrophages and a reversal of these effects of Dex in glucocorticoid receptor-deficient mice (Bhattacharyya et al., 2007). The effects of DPML on NFκB and p38 regulation could be explained by the fact that DP (esterified DP) is hydrolyzed to release Dex after uptake of DPML in alveolar macrophages, and then the released Dex forms a complex with glucocorticoid receptors. This complex directly interacts with the NFκB complex (Barnes and Karin, 1997) and induces the production of MAPK phosphatase-1 (Clark and Lasa, 2003) to suppress the downstream transcription of NFκB and p38MAPK, respectively.

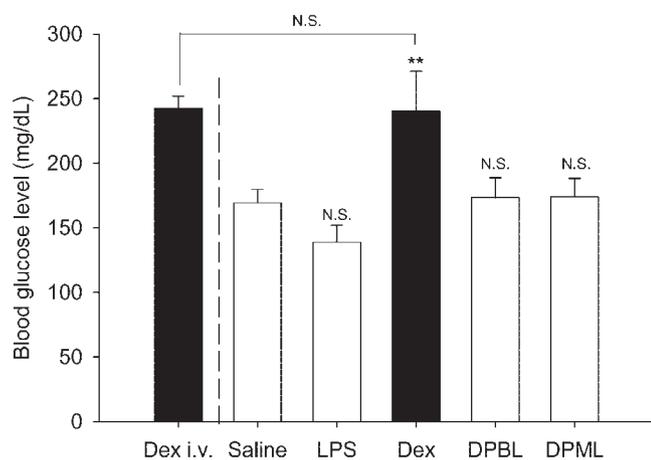
As far as therapeutic efficiency is concerned, the systemic

side effects of inhaled liposomal DP could not be ruled out, although DPML was found to be an effective treatment of lung inflammation. Because an increase in glucose level is clearly apparent as early as the first day of Dex treatment in healthy volunteers, suggesting Dex-induced hyperglycemia (Beard et al., 1984), the blood glucose level was measured after inhaled DPML (Fig. 10). There was no significant increase in blood glucose after liposomal DP treatment, whereas this effect was found after both inhaled and intravenous free Dex. Corresponding to the short absorption half-life of Dex after inhalation (Schreier et al., 1993), the systemic side effect was correlated to the rapid distribution of inhaled Dex into the blood circulation (Fig. 1a) and caused the induction of blood glucose. These results lead us to believe that DPML is a highly efficient and safe treatment of lung inflammation.

In this study, dexamethasone palmitate was used as the incorporated drug for the treatment of LPS-induced lung inflammation; however, inhaled mannosylated liposomes can be applied to a wide variety of not only anti-inflammatory drugs but also drugs used to treat alveolar macrophage-associated diseases. In conclusion, we have demonstrated that DPML is highly effective in improving lung function with regard to reduction of proinflammatory cytokines, suppression of neutrophil infiltration, and down-regulation of NFκB and p38MAPK signaling pathways after direct pulmonary delivery at a suboptimal dose. These observations suggest the improved anti-inflammatory effects of DP by mannosylated liposomal delivery to alveolar macrophages after



**Fig. 9.** Effect of DPML on the inhibition of NFκB activation and p38MAPK phosphorylation after intratracheal instillation in rats. NFκB activation in alveolar macrophages (a) and lungs (b). The activation of NFκB was determined as nuclear translocation of NFκB by EMSA (top) and quantified by densitometry (bottom). Lane 1, labeled control NFκB; lane 2, saline; lane 3, LPS alone; lane 4, Dex treatment; lane 5, DPBL treatment; lane 6, DPML treatment; lane 7, DPML treatment with mannan; lane 8, LPS (as lane 3) with unlabeled control NFκB. c, phosphorylation of p38MAPK. The protein bands of phosphorylated p38 (P-p38), p38, and β-actin were determined by Western blot analysis. Lane 1, saline; lane 2, LPS alone; lane 3, Dex treatment; lane 4, DPBL treatment; lane 5, DPML treatment; lane 6, DPML treatment with mannan. Results are expressed as mean ± S.D. of two experiments. Statistically significant differences (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ) compared with LPS treatment in each experiment, (#,  $P < 0.05$ ; ##,  $P < 0.01$ ) with each pair of treatments. N.S., not significant.



**Fig. 10.** Blood glucose level after intravenous injection of free Dex (Dex i.v.), intratracheal instillation of free Dex, and liposomal DP in rats. The blood glucose level was determined 3 h after administration. Results are expressed as mean  $\pm$  S.D. of at least three experiments. Statistically significant differences (\*\*,  $P < 0.01$ ) compared with saline treatment. N.S., not significant.

intratracheal administration in LPS-induced lung inflammation. Hence, the selective delivery of drugs to alveolar macrophages, inflammatory effector cells, improves the pharmacological effects at a low dose and minimizes systemic side effects. This study provides valuable information to help in the design of delivery systems for clinical application to treat inflammatory lung diseases.

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#### References

- Barnes PJ and Karin M (1997) Nuclear factor- $\kappa$ B: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336**:1066–1071.
- Beard JC, Halter JB, Best JD, Pfeifer MA, and Porte D Jr (1984) Dexamethasone-induced insulin resistance enhances B cell responsiveness to glucose level in normal men. *Am J Physiol Endocrinol Metab* **247**:E592–E596.
- Benamer H, De Gand G, Brasseur R, van Vooren JP, and Legros FJ (1993) Liposome-incorporated dexamethasone palmitate: chemical and physical properties. *Int J Pharm* **89**:157–167.
- Bhattacharyya S, Brown DE, Brewer JA, Vogt SK, and Muglia LJ (2007) Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase. *Blood* **109**:4313–4319.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Carter AB, Monick MM, and Hunninghake GW (1998) Lipopolysaccharide-induced NF- $\kappa$ B activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am J Respir Cell Mol Biol* **18**:384–391.
- Carter AB, Monick MM, and Hunninghake GW (1999) Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am J Respir Cell Mol Biol* **20**:751–758.
- Clark AR and Lasa M (2003) Crosstalk between glucocorticoids and mitogen-activated protein kinase signaling pathways. *Curr Opin Pharmacol* **3**:404–411.
- Fidler IJ, Raz A, Fogler WE, Kirsh R, Bugelski P, and Poste G (1980) Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. *Cancer Res* **40**:4460–4466.
- Fielding RM and Abra RM (1992) Factors affecting the release rate of terbutaline from liposome formulations after intratracheal instillation in the guinea pig. *Pharm Res* **9**:220–223.
- Foster JM, Aucott L, van der Werf RH, van der Meijden MJ, Schraa G, Postma DS, and van der Molen T (2006) Higher patient perceived side effects related to higher daily doses of inhaled corticosteroids in the community: a cross-sectional analysis. *Respir Med* **100**:1318–1336.
- Giraud O, Seince PF, Rolland C, Leçon-Malas V, Desmots JM, Aubier M, and Dehoux M (2000) Halothane reduces the early lipopolysaccharide-induced lung inflammation in mechanically ventilated rats. *Am J Respir Crit Care Med* **162**:2278–2286.
- Haddad EB, Birrell M, McCluskie K, Ling A, Webber SE, Foster ML, and Belvisi MG

- (2001) Role of p38 MAP kinase in LPS-induced airway inflammation in the rat. *Br J Pharmacol* **132**:1715–1724.
- Hattori Y, Kawakami S, Nakamura K, Yamashita F, and Hashida M (2006) Efficient gene transfer into macrophages and dendritic cells by in vivo gene delivery with mannoseylated lipoplex via the intraperitoneal route. *J Pharmacol Exp Ther* **318**:828–834.
- Higuchi Y, Kawakami S, Oka M, Yabe Y, Yamashita F, and Hashida M (2006) Intravenous administration of mannoseylated cationic liposome/NF $\kappa$ B decoy complexes effectively prevent LPS-induced cytokine production in a murine liver failure model. *FEBS Lett* **580**:3706–3714.
- Jansson AH, Eriksson C, and Wang X (2005) Effects of budesonide and N-acetylcysteine on acute lung hyperinflation, inflammation and injury in rats. *Vascul Pharmacol* **43**:101–111.
- Kawakami S, Sato A, Nishikawa M, Yamashita F, and Hashida M (2000a) Mannose receptor-mediated gene transfer into macrophages using novel mannoseylated cationic liposomes. *Gene Ther* **7**:292–299.
- Kawakami S, Wong J, Sato A, Hattori Y, Yamashita F, and Hashida M (2000b) Biodistribution characteristics of mannoseylated, fucosylated, and galactosylated liposomes in mice. *Biochim Biophys Acta* **1524**:258–265.
- Kim HJ, Lee HS, Chong YH, and Kang JL (2006) p38 mitogen-activated protein kinase up-regulates LPS-induced NF- $\kappa$ B activation in the development of lung injury and RAW264.7 macrophages. *Toxicology* **225**:36–47.
- Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, and Christman JW (2002) Macrophages are necessary for maximal nuclear factor- $\kappa$ B activation in response to endotoxin. *Am J Respir Cell Mol Biol* **26**:572–578.
- Lee KD, Hong K, and Papahadjopoulos D (1992) Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density. *Biochim Biophys Acta* **1103**:185–197.
- Marshall BG, Wango A, Harrison LI, Young DB, and Shaw RJ (2000) Tumour necrosis factor- $\alpha$  production in human alveolar macrophages: Modulation by inhaled corticosteroid. *Eur Respir J* **15**:764–770.
- Monick MM and Hunninghake GW (2002) Activation of second messenger pathways in alveolar macrophages by endotoxin. *Eur Respir J* **20**:210–222.
- Nemmar A, Hoet PH, Vermeylen J, Nemery B, and Hoylaerts MF (2004) Pharmacological stabilization of mast cells abrogates late thrombotic events induced by diesel exhaust particles in hamsters. *Circulation* **110**:1670–1677.
- Niven RW, Speer M, and Schreier H (1991) Nebulization of liposomes. II. The effects of size and modeling of solute release profiles. *Pharm Res* **8**:217–221.
- O'Byrne PM and Pedersen S (1998) Measuring efficacy and safety of different inhaled corticosteroid preparations. *J Allergy Clin Immunol* **102**:879–886.
- Rocksén D, Lilliehöök B, Larsson R, Johansson T, and Bucht A (2000) Differential anti-inflammatory and anti-oxidative effects of dexamethasone and N-acetylcysteine in endotoxin-induced lung inflammation. *Clin Exp Immunol* **122**:249–256.
- Schild PN and Charles BG (1994) Determination of dexamethasone in plasma of premature neonates using high-performance liquid chromatography. *J Chromatogr B Biomed Appl* **658**:189–192.
- Schreier H, Gonzalez-Rothi RJ, and Stecenko AA (1993) Pulmonary delivery of liposomes. *J Control Release* **24**:209–223.
- Shaw IH, Knight CG, and Dingle JT (1976) Liposomal retention of a modified anti-inflammatory steroid. *Biochem J* **158**:473–476.
- Suntres ZE and Shek PN (2000) Prophylaxis against lipopolysaccharide-induced lung injuries by liposome-entrapped dexamethasone in rats. *Biochem Pharmacol* **59**:1155–1161.
- Takakura Y and Hashida M (1996) Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. *Pharm Res* **13**:820–831.
- Teshima M, Kawakami S, Nishida K, Nakamura J, Sakaeda T, Terazono H, Kitahara T, Nakashima M, and Sasaki H (2004) Prednisolone retention in integrated liposomes by chemical approach and pharmaceutical approach. *J Control Release* **97**:211–218.
- Tremblay GM, Thérien HM, Rocheleau H, and Cormier Y (1993) Liposomal dexamethasone effectiveness in the treatment of hypersensitivity pneumonitis in mice. *Eur J Clin Invest* **23**:656–661.
- Ulich TR, Howard SC, Remick DG, Wittwer A, Yi ES, Yin S, Guo K, Welpy JK, and Williams JH (1995) Intratracheal administration of endotoxin and cytokines. VI. Antiserum to CINC inhibits acute inflammation. *Am J Physiol* **268**:L245–L250.
- Ulich TR, Watson LR, Yin SM, Guo KZ, Wang P, Thang H, and del Castillo J (1991) The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. *Am J Pathol* **138**:1485–1496.
- Waldrep JC, Gilbert BE, Knight CM, Black MB, Scherer PW, Knight V, and Eschenbacher W (1997) Pulmonary delivery of beclomethasone liposome aerosol in volunteers. Tolerance and safety. *Chest* **111**:316–323.
- Wijagkanalan W, Kawakami S, Takenaga M, Igarashi R, Yamashita F, and Hashida M (2008) Efficient targeting to alveolar macrophages by intratracheal administration of mannoseylated liposomes in rats. *J Control Release* **125**:121–130.
- Yeeprae W, Kawakami S, Yamashita F, and Hashida M (2006) Effect of mannose density on mannose receptor-mediated cellular uptake of mannoseylated O/W emulsions by macrophages. *J Control Release* **114**:193–201.

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