Molecular Pharmacology Fast Forward. Published on July 30, 2008 as doi: 10.1124/mol.108.050153
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MOL#50153

Enhanced anti-inflammation of inhaled dexamethasone palmitate using mannosylated liposomes in an endotoxin-induced lung inflammation model

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Running title page

a) Running title: Targeting of dexamethasone palmitate in a lung injury model

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c) The number of:

Text pages;	41
Tables;	1
Figures;	10
References;	38
Words in *Abstract*;	182
Words in *Introduction*;	511
Words in *Discussion*;	1265

d) List of nonstandard abbreviations: 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; NFκB, nuclear factor kappa B; p38MAPK, p38 mitogen-activated protein kinase; i.t., intratracheal

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Abstract

Inhalation of bacterial endotoxin induces pulmonary inflammation by activation of NFκB, production of cytokines and chemokines, and neutrophil activation. Although glucocorticoids are the drugs of choice, administration of free drugs results in adverse effects due to lack of selectivity for the inflammatory effector cells. Since 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 previously demonstrated 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 (DP) 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 as well as myeloperoxidase activity, and inhibited NFκB and p38MAPK 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.

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Introduction

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 (TNFα), interleukin-1 beta (IL-1β) (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 (NFκB) and p38 mitogen-activated protein kinase (p38MAPK). Subsequently, 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 pro-inflammatory cytokines and chemokines after LPS stimulation.

Glucocorticoids (GC) 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 questioned 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 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. 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 (Benameur et al., 1993).

Since 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 previously reported 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

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al., 2006). Recently, 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. Accordingly, 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.

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Materials and Methods

Materials. DP was kindly provided from 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, USA). Hydroxyprogesterone caproate was obtained from MP Biomedicals (Solon, OH, USA). 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 US 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 molar 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 acetonitrile and sonicated for 40 min. Liposomal DP was measured using an HPLC system (Shimadzu Co., Kyoto, Japan) with a 5 μm YMC-Pack ODS-A column (4.6×150 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 which was mounted with a 3500 MWCO membrane (Spectra/Por®, Spectrum Laboratories Inc., CA, USA). The release of DP into the receiver was measured by UV spectrophotometry at 236 nm. Dex was measured using HPLC systems as previous method with

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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. 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 content assayed by Lowry methods.

Isolation of Alveolar Macrophages. Alveolar macrophages were isolated as described previously (Wijagkanalan et al., 2008). Briefly, the lung was lavaged with EDTA containing buffer and the bronchoalveolar lavage (BAL) fluid was subsequently centrifuged at 200 × g 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

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at a density of 2.1 $\times$ 10^5 cells/cm^2 for 12 h before LPS challenge. In measuring the dose-response of TNFα release, alveolar macrophages were co-incubated with 1 μg/ml LPS and 1 nm – 1 μM DP formulations for 6 h. For the time-course experiments, alveolar macrophages were stimulated with 1 μg/ml LPS for 0.5 – 24 h. In the DP treatment, alveolar macrophages were co-incubated with 1 μg/ml LPS and 0.1 μM free Dex solution, DP or liposomal DP for a further 6 h. For the mannan 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 indicated times, cytokine and chemokine protein levels in the supernatant were measured by ELISA (eBiosciences Inc., San Diego, CA, USA).

Induction of LPS-Induced Lung Inflammation and Treatment. Rats were intratracheally given: 1) saline; 2) LPS at 0.5 mg/kg alone (Giraud et al., 2000); 3) free Dex (positive control) or liposomal DP with an indicated dose at the onset or 1 h-before (Pre-treatment, PreTx) LPS-induced lung inflammation; 4) co-administration 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

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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-postchallenge with 10 ml- 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 °C for further cytokine and chemokine analysis. The BAL cells were resuspended in RPMI-serum free and subjected to cytospin (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 14000 × g at 4 °C. The resulting supernatant was subjected to cytokine and chemokine measurements.

Cytokine and Chemokine Measurements. The TNFα level (eBiosciences, San Diego, CA, USA), IL-1β 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

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with an ELISA kit obtained from R&D systems (Minneapolis, MN, USA). The cytokine and chemokine levels in the lung tissue were normalized with regard to the protein content by the Lowry assay method.

MPO Activity. The MPO activity in the lung tissue was measured using an MPO Assay Kit (Cytostore Inc., Alberta, 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®, CA, USA). The nuclear protein was divided into aliquots, frozen, and stored at -80 °C until further assay. The protein concentration was determined by the Bradford assay method.

Electrophoretic Mobility Shift Assay (EMSA). The nuclear extract (50 μg lung tissue protein and 40 μg isolated alveolar macrophage protein) was mixed with binding reaction mixtures in accordance with the instructions for the LightShift® Chemiluminescent EMSA Kit (Pierce Biotechnology Ltd., Rockford, IL, USA). The protein-DNA complexes were separated on Supersep® Gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1 × TBE buffer. The separated bands were transferred to a nylon

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membrane and detected by the Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology Ltd., Rockford, IL, USA).

Western Blot Analysis. The lung tissue homogenate samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Separated protein bands were electrophoretically transferred to a nitrocellulose membrane and blocked with Tris-buffered saline containing 3% BSA. The membranes were then incubated separately with anti-p38α antibody and anti-phospho-p38MAPK (*T180/Y182*) antibody (R&D systems, Minneapolis, MN, USA) overnight at 4 °C. An anti-β-actin antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) was used as loading control. The antibody labeling of the protein bands was detected with Enhanced Chemiluminescence Reagents (Millipore Co., MA, USA).

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-μm sections. After removal of the paraffin with xylene and rehydration in graded alcohol, the sections were stained with hematoxylin/eosin (H/E).

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Statistical Analysis. Statistical analysis was performed using ANOVA 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.

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Results

Physicochemical Properties of DP Liposomes. The particle sizes of dexamethasone palmitate containing Bare-liposomes (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 undetectable 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 to 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 μM liposomal DP in the presence of mannan, while Dex and DP solutions were used as positive controls. Fig. 2a shows the cytokine

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release profile after LPS stimulation. Although strong inhibition of TNFα and CINC-1 ($P < 0.001$) was observed by all formulations, DPML showed significant suppression of TNFα (at least $P < 0.05$), IL-1β ($P < 0.05$) 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 following co-incubation with an excess of mannan ($P < 0.05$).

Induction and Development of LPS-Induced Lung Inflammation. In order to verify the biological profile of the airway inflammatory response after LPS challenge, rats were exposed to aerosolized LPS or saline for 0.5 – 24 h and BAL samples were collected before carrying out measurements. Fig. 3 shows the rapid increase in the TNFα level with a peak at 1 h and subsequent release of IL-1β 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 0.1 - 0.5 mg/kg (Nemmar et al., 2004) and cytokine release was measured at 1 – 24 h post-dosing. Fig. 4a shows a dose-response

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profile of liposomal DP and free Dex on the inhibition of TNFα production in BAL fluid 3 h after administration. A marked reduction on TNFα levels was observed after treatment with liposomal DP at a dose of 0.25 mg/kg compared to 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 TNFα 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, pre-treatment and co-treatment of DPML showed comparable effects. To investigate the *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-postdosing. DPML significantly attenuated TNFα ($P < 0.001$), IL-1β ($P < 0.001$) and CINC-1 ($P < 0.05$) in BAL fluid (Fig. 5a) as well as in the lung tissue (Fig. 5b) compared with no treatment, free Dex (at least $P < 0.05$), and DPBL (at least $P < 0.05$). Fig. 6a shows extensive inhibition of neutrophil migration to the lung with all treatments ($P < 0.001$); however, the more potent anti-migration effects ($P < 0.05$) as well as inhibition of MPO activity ($P < 0.01$) 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. Since DPML were used as DP-targeting carriers to

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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 co-treatment 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 (Fig. 6b, 7). However, there was no effect of mannan on the cytokine release in both control groups.

Lung Histology. To examine 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 negligible number of inflammatory cells (Fig. 8a). On the other hand, LPS-treated lungs showed moderately edematous alveolar septae and inflammatory cell recruitment indicated as arrow (Fig. 8b). Among drug-treated lungs (Fig. 8c-d), DPML-treated lungs (Fig. 8e) contained as few inflammatory cells and septal edema as control lungs. However, co-administration of mannan to DPML-treated lungs produced a marked increase in infiltration of inflammatory cells and edema in the alveoli (Fig. 8f).

NFκB Activation and Phosphorylation of p38MAPK. To examine the anti-inflammatory signaling pathways of incorporated DP, the nuclear extract from alveolar

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macrophages or lung tissue samples were subjected to EMSA and Western blot analysis. Fig. 9a-b shows an increase in NFκB activation in LPS-treated animals (Lane 3) whereas there was little detectable NFκB expression in control animals (Lane 2) as shown by both alveolar macrophage (Fig. 9a) and lung tissue samples (Fig 9b). The activation of NFκB was hardly inhibited after treatment with free Dex and DPBL (Lane 4-5). As expected, DPML significantly suppressed the activation of NFκB 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 NFκB activation was attenuated following co-treatment with mannan ($P < 0.05$). Furthermore, phosphorylation of p38MAPK was observed after LPS stimulation in rats (Fig. 9c); however, the complete down-regulation of phosphorylated p38MAPK was revealed after DPML treatment and was attenuated in the presence of mannan that corresponded to the results of the NFκB 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, only inhaled free Dex markedly increased blood glucose level identically to intravenously injected group but showed significant compared with

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saline treatment ($P < 0.01$). Interestingly, liposomal DP did not significantly alter blood glucose levels compared with control groups.

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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 NFκB and p38MAPK compared with free Dex and DPBL.

Since 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 (around 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. reported the effect of surface charge density and specific lipid headgroups 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

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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 a Microspray®. The inflammatory markers, including proinflammatory cytokines (TNFα and IL-1β), chemokine (CINC-1), and the recruitment of neutrophils (Fig. 3), were significantly up-regulated in a time-dependent manner which corresponded to the findings in previous studies when different intratracheal techniques were used (Jansson et al., 2005; Haddad et al., 2001). These results ensure the suitability of lung inflammatory conditions after aerosolized LPS exposure at 0.5 mg/kg.

In order 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 (Fig. 4 and 5). Consistent with the lung histology (Fig. 8), DPML treatment caused a more potent inhibition of neutrophil migration as well as 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* (Fig. 6b – 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.

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It has been widely accepted that the response of alveolar macrophages to endotoxin (LPS) is closely related to activation of intracellular signaling cascades including NFκB, MAPK, and phosphatidylinositol 3-kinase pathways to orchestrate production of pro- and anti-inflammatory mediators (Monick and Hunninghake, 2002). Similarly to the pathogenesis of LPS in the human alveolar macrophages, LPS-induced cytokine release and activation of NFκB (Carter et al., 1998) and p38MAPK (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. 9a-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 p38MAPK phosphorylation accompanied by the induction of MAPK phosphatase-1 (MKP-1) in control macrophages and a reversal of these effects of Dex in glucocorticoid receptor-deficient mice (Bhatacharyya 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

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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 PJ and Karin M, 1997) and induces the production of MKP-1 (Clark AR and Lasa M, 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. Since 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 JC 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 following 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.

Molecular Pharmacology Fast Forward. Published on July 30, 2008 as DOI: 10.1124/mol.108.050153
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Acknowledgment

We thank Mitsubishi Tanabe Pharma Corporation (Osaka, Japan) for providing dexamethasone palmitate.

Molecular Pharmacology Fast Forward. Published on July 30, 2008 as DOI: 10.1124/mol.108.050153
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Molecular Pharmacology Fast Forward. Published on July 30, 2008 as DOI: 10.1124/mol.108.050153
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Footnotes

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by Health and Labour Sciences Research Grants for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan.

Molecular Pharmacology Fast Forward. Published on July 30, 2008 as DOI: 10.1124/mol.108.050153
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Figure Legends

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 (filled) and 24 h (opened) 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 a 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 TNFα, IL-1β and CINC-1 protein levels after LPS stimulation at 1 μg/ml (●) and control (○). (b) Inhibition of TNFα, IL-1β and CINC-1 protein levels after treatment. The alveolar macrophages were incubated with 1 μg/ml LPS alone or co-incubated with 0.1 μM free drugs (Dex or DP) or liposomal DP (filled). For the uptake inhibition, mannan was also co-incubated at 1 mg/ml (shaded). The cytokine and chemokine levels in the supernatant were determined by ELISA after a 6 h-

incubation. Results are expressed 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.

Fig. 3. Time-course of (a) TNFα, (b) IL-1β, (c) CINC-1 protein levels and (d) neutrophil infiltration in the lung after aerosolized LPS challenge. Male Wistar rats were exposed to 0.5 mg/kg aerosolized LPS (filled) or saline (opened), 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 a mean ± S.D. of at least three experiments.

Fig. 4. (a) Dose- and (b) Time-response profiles of DPML on the inhibition of TNFα release after LPS-induced lung inflammation. (a) Male Wistar rats were i.t. administered 0.5 mg/kg LPS alone or together with free Dex, DPBL or DPML at a dose of 0.1 (opened), 0.25 (shaded) and 0.5 (filled) mg/kg. The BAL fluid was collected 3 h after challenge. (b) Male Wistar rats were i.t. given drugs at 0.5 mg/kg dose together with LPS or pre-treated with drugs 1 h before LPS stimulation (PreTx-3), then the BAL fluid was collected at indicated time or 3 h, respectively. The TNFα level was measured by ELISA.

Results are expressed a mean ± 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.

Fig. 5. Efficient inhibition of cytokine release after i.t. instillation of DPML in (a) BAL fluid and (b) lung tissue. Male Wistar rats were i.t. administered 0.5 mg/kg LPS alone (opened) or together with free Dex, DPBL or DPML (filled) 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 a mean ± 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.

Fig. 6. (a) Anti-inflammatory effects of DPML and (b) inhibition by mannan on neutrophil infiltration and MPO activity in the lung after i.t. administration. Male Wistar rats were i.t. administered 0.5 mg/kg LPS alone or together with free Dex, DPBL or DPML (filled) at a dose of 0.5 mg/kg. In the inhibition study, mannan was co-administered in each group (opened). Three hours after treatment, the neutrophil influx and MPO activity were determined in BAL samples and lung tissue, respectively.

Results are expressed a mean ± 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.

Fig. 7. Mannose-receptor mediated uptake of DPML for anti-inflammatory effects of incorporated DP after i.t. administration. (a) BAL fluid and (b) lung tissue. Male Wistar rats were i.t. administered 0.5 mg/kg LPS alone or with 0.5 mg/kg of DPML (filled) in the presence of an excess of mannan (opened). The BAL fluid and lung tissue were collected 3 h after challenge and cytokine levels were measured by ELISA. Results are expressed a mean ± 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.

Fig. 8. Lung histology after intratracheal administration of (a) Saline, (b) LPS, (c) Treatment with Dex, (d) Treatment with DPBL, (e) Treatment with DPML and (f) Treatment with DPML 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 magnification.

Fig. 9. Effect of DPML on the inhibition of NFκB activation and p38MAPK phosphorylation after i.t. instillation in rats. NFκB activation in (a) alveolar macrophages and (b) lungs. The activation of NFκB was determined as nuclear translocation of NFκB by EMSA (upper panel) and quantified by densitometry (lower panel). 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 a 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.), i.t. instillation of free Dex and liposomal DP in rats. The blood glucose level was determined 3 h after administration. Results are expressed a mean ± S.D. of at least three experiments. Statistically significant differences (** $P < 0.01$) compared with saline treatment. N.S., not significant.

Molecular Pharmacology Fast Forward. Published on July 30, 2008 as DOI: 10.1124/mol.108.050153
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Table 1. The physicochemical property, DP recovery and incorporation efficiency of DP liposomes

Liposomes	Mean particle size (nm)	Zeta potential (mV)	DP recovery (%)	Incorporation efficiency[a] (%)
DPBL	100 ± 11	-7.22 ± 8.8	85 ± 30	99 ± 1.5
DPML	110 ± 6.9 N.S.	0.07 ± 0.22 *	103 ± 39 N.S.	99 ± 1.1 N.S.

Each value represents the mean ± S.D. values (n = 3).

[a]Incorporation efficiency was determined by *in vitro* release study for 48 h at 37 °C.

Statistically significant differences compared to DPBL: *$P < 0.05$, N.S., not significant.

Fig. 1

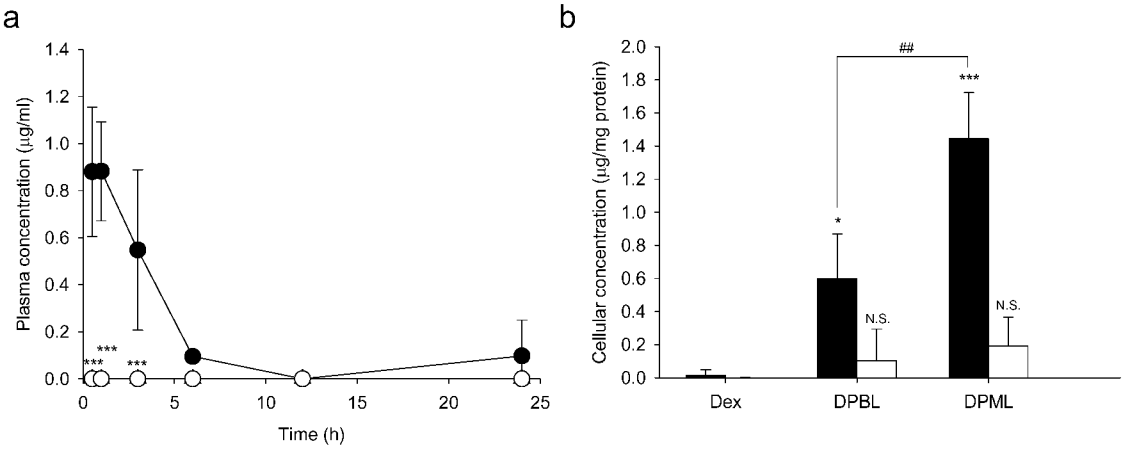

a
Plasma concentration (μg/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
0
5
10
15
20
25
Time (h)
b
Cellular concentration (μg/mg protein)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
***
N.S.
N.S.
Dex
DPBL
DPML

Fig. 2

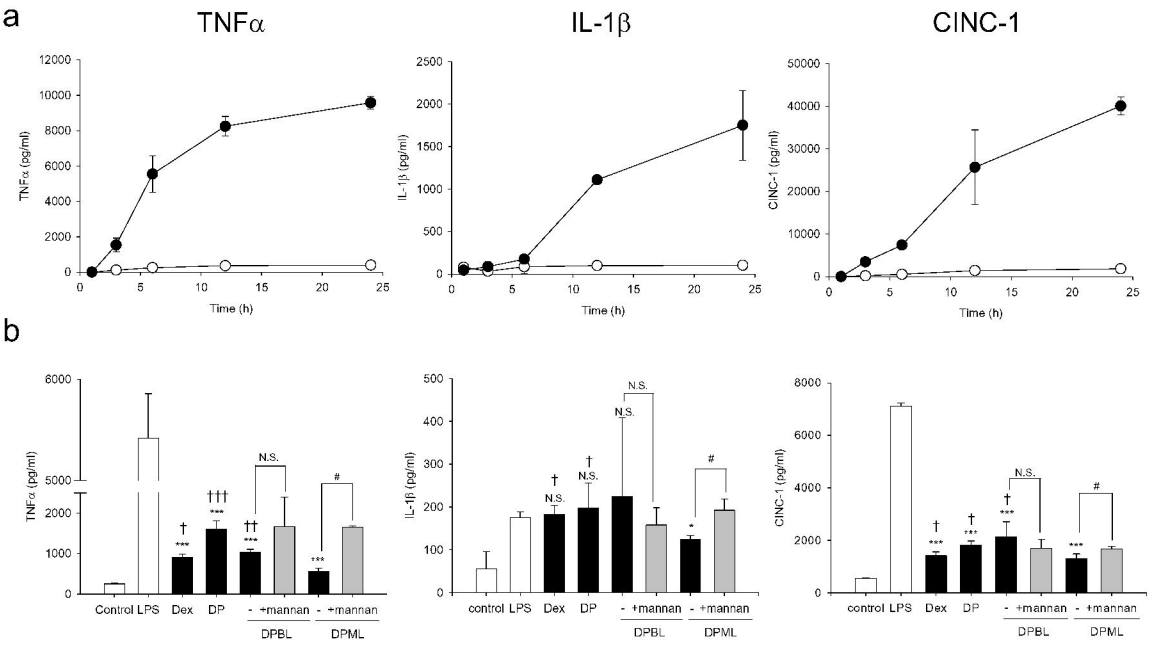

a
TNFα
IL-1β
CINC-1
TNFα (pg/ml)
IL-1β (pg/ml)
CINC-1 (pg/ml)
Time (h)
b
Control
LPS
Dex
DP
+mannan
DPBL
DPML
N.S.

Fig. 3

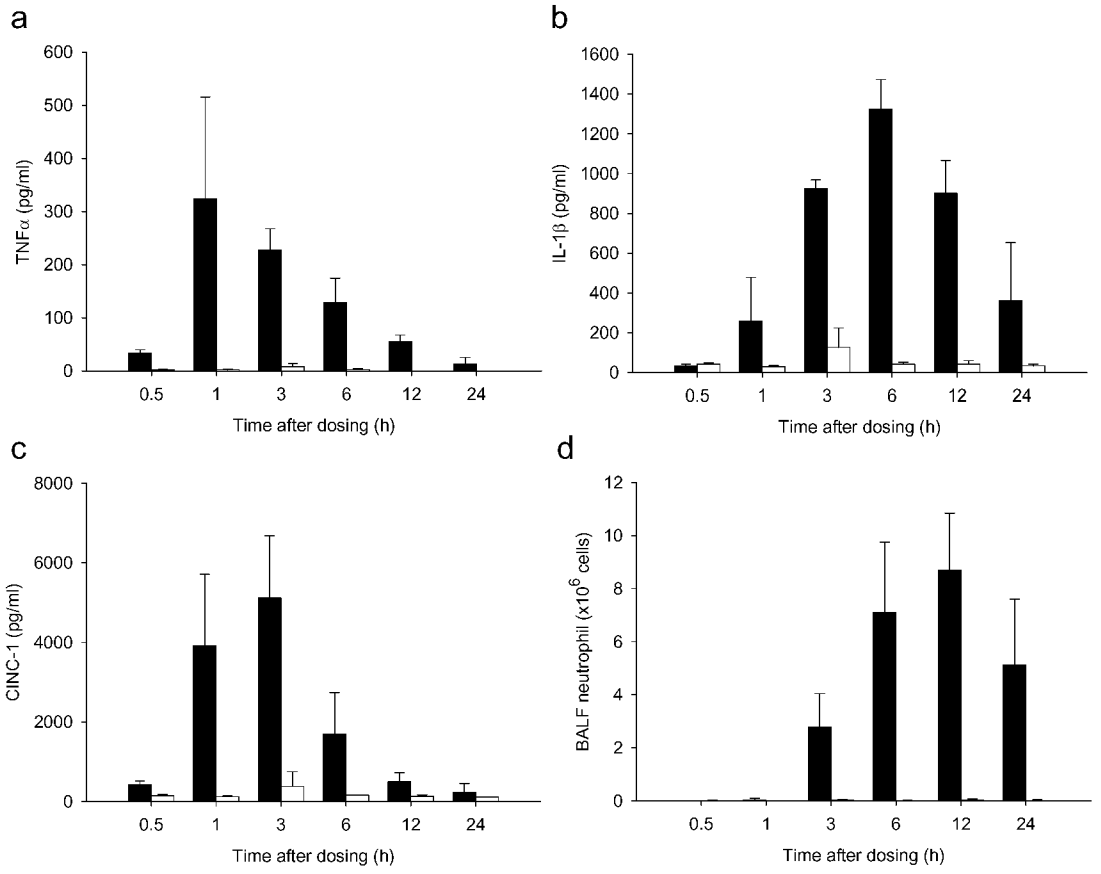

a
TNFα (pg/ml)
600
500
400
300
200
100
0
0.5
1
3
6
12
24
Time after dosing (h)
b
IL-1β (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
0.5
1
3
6
12
24
Time after dosing (h)
c
CINC-1 (pg/ml)
8000
6000
4000
2000
0
0.5
1
3
6
12
24
Time after dosing (h)
d
BALF neutrophil (x10^6 cells)
12
10
8
6
4
2
0
0.5
1
3
6
12
24
Time after dosing (h)

Fig. 4

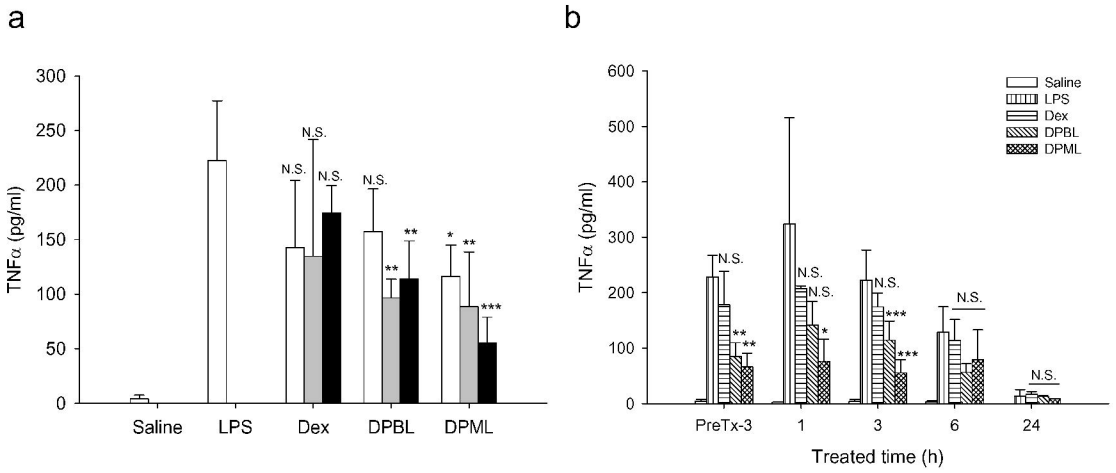

a
b
TNFα (pg/ml)
300
250
200
150
100
50
0
Saline
LPS
Dex
DPBL
DPML
N.S.
*
**
***
600
500
400
300
200
100
PreTx-3
1
3
6
24
Treated time (h)

Fig. 5

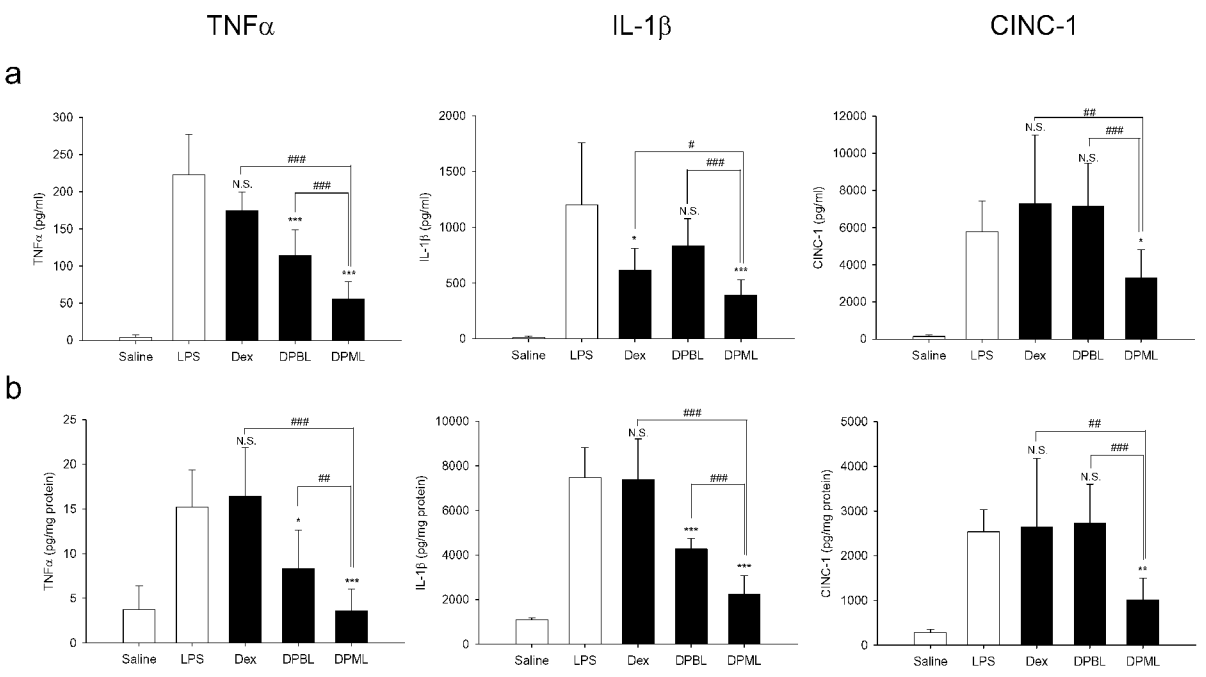

TNFα
IL-1β
CINC-1
a
b
TNFα (pg/ml)
IL-1β (pg/ml)
CINC-1 (pg/ml)
TNFα (pg/mg protein)
IL-1β (pg/mg protein)
CINC-1 (pg/mg protein)
Saline
LPS
Dex
DPBL
DPML
N.S.
*
**
***
#
##
###

Fig. 6

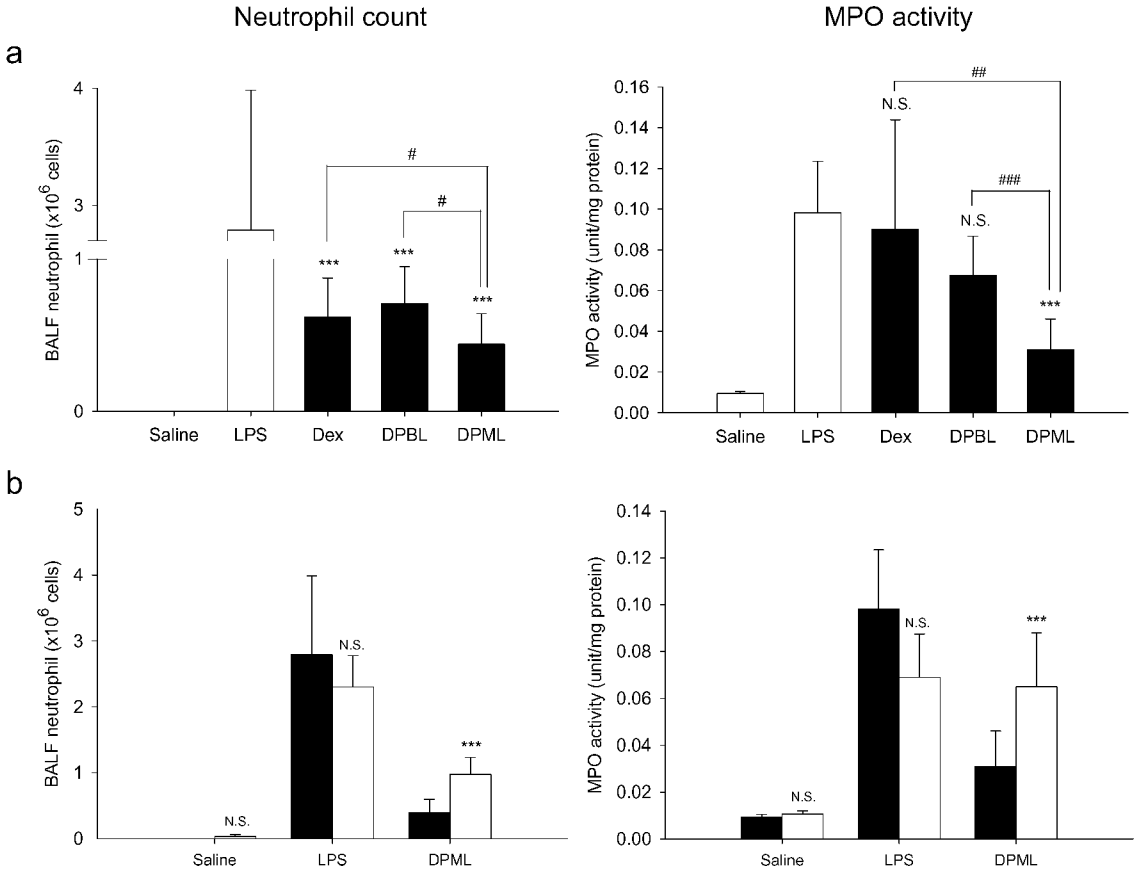

a
Neutrophil count
MPO activity
BALF neutrophil (x10^6 cells)
MPO activity (unit/mg protein)
Saline
LPS
Dex
DPBL
DPML
N.S.
#
##
###
***
b

Fig. 7

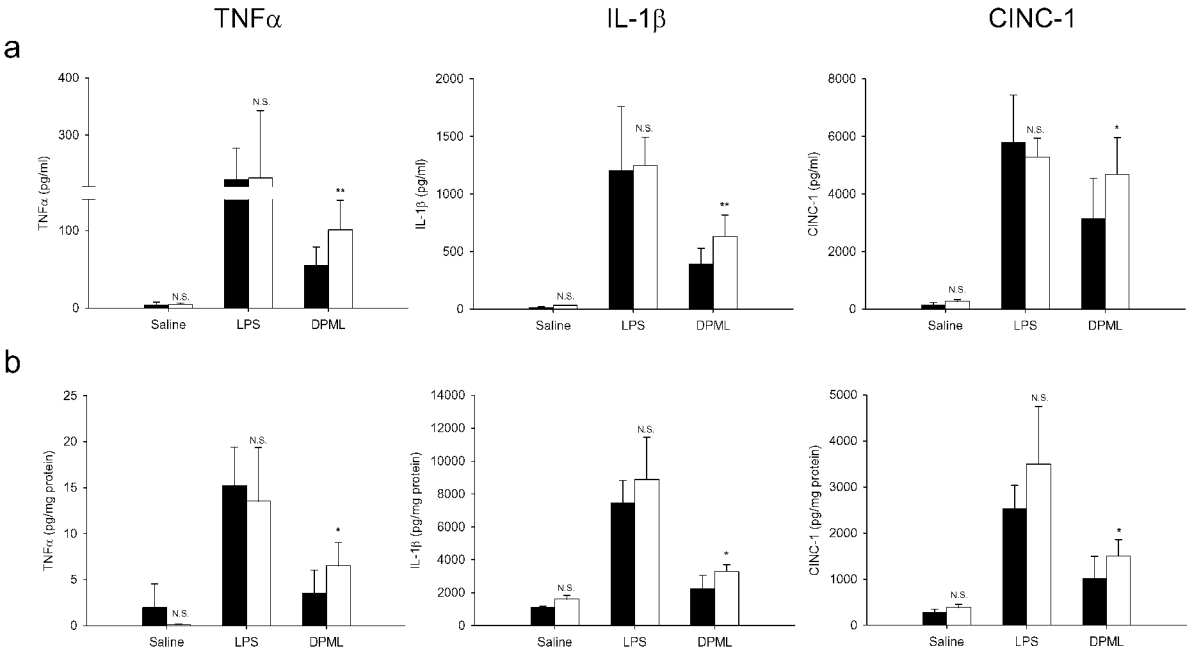

TNFα
IL-1β
CINC-1
a
b
TNFα (pg/ml)
IL-1β (pg/ml)
CINC-1 (pg/ml)
TNFα (pg/mg protein)
IL-1β (pg/mg protein)
CINC-1 (pg/mg protein)
N.S.
*
**
Saline
LPS
DPML

Fig. 8

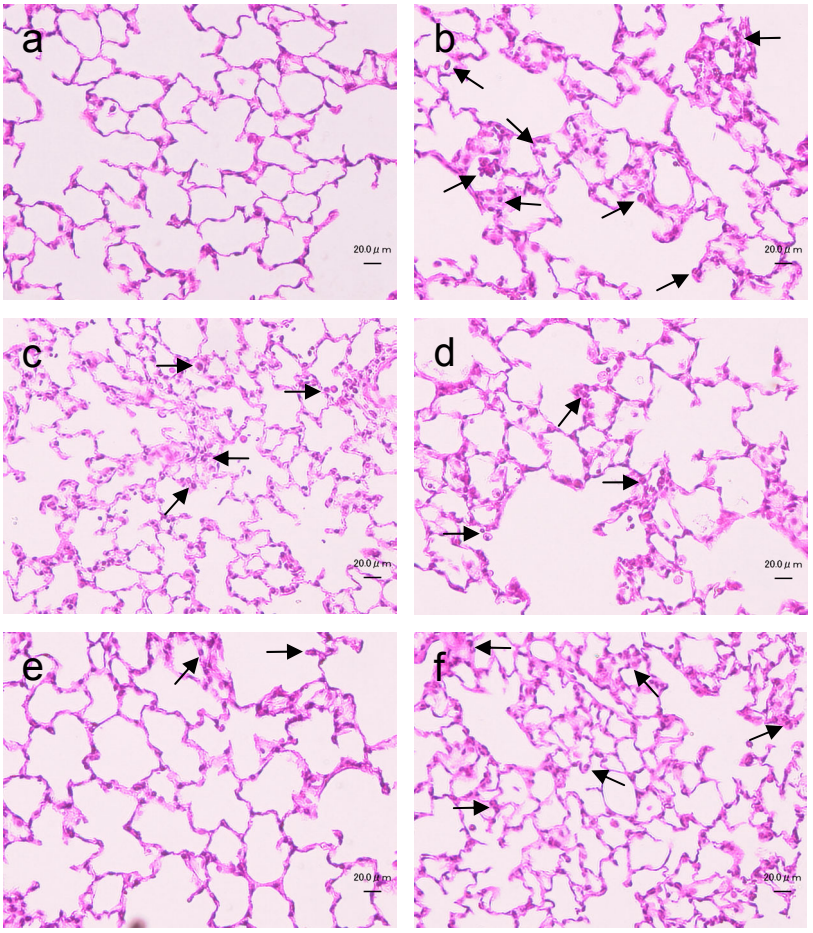
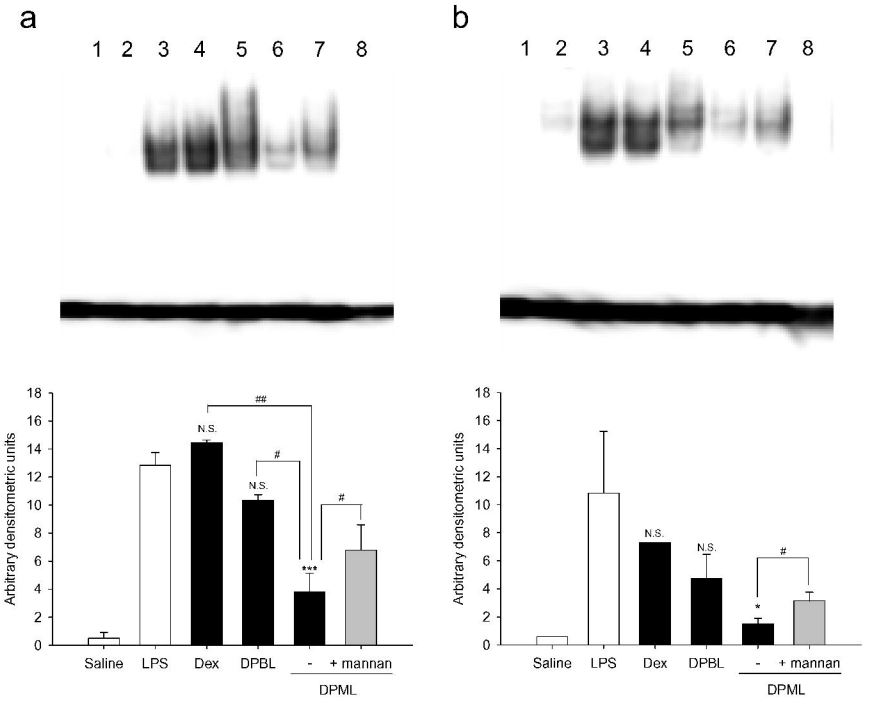


Fig. 9


a
1 2 3 4 5 6 7 8
Arbitrary densitometric units
18
16
14
12
10
8
6
4
2
0
##
#
#
N.S.
N.S.
***
Saline
LPS
Dex
DPBL
-
+ mannan
DPML
b
1 2 3 4 5 6 7 8
Arbitrary densitometric units
N.S.
N.S.
#
*
Saline
LPS
Dex
DPBL
-
+ mannan
DPML

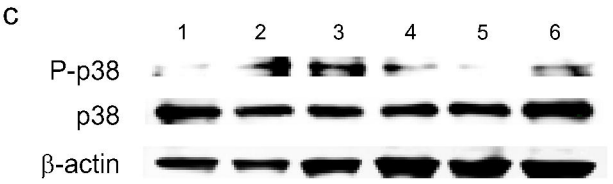
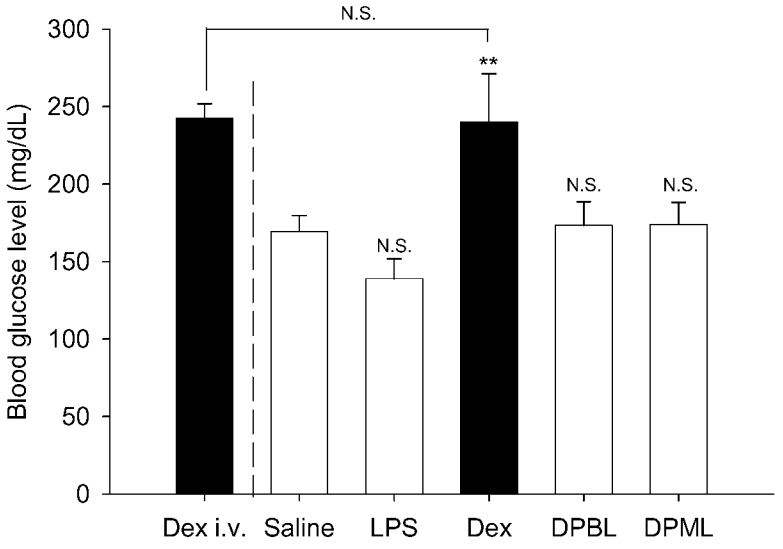

c
1 2 3 4 5 6
P-p38
p38
β-actin

Fig.10


N.S.
**
N.S.
N.S.
N.S.
300
250
200
150
100
50
0
Blood glucose level (mg/dL)
Dex i.v.
Saline
LPS
Dex
DPBL
DPML