

## RESEARCH PAPER

# Liposome-encapsulated dexamethasone attenuates ventilator-induced lung inflammation

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## BACKGROUND AND PURPOSE

Systemic glucocorticoid therapy may effectively attenuate lung inflammation but also induce severe side-effects. Delivery of glucocorticoids by liposomes could therefore be beneficial. We investigated if liposome-encapsulated dexamethasone inhibited ventilator-induced lung inflammation. Furthermore, we evaluated whether targeting of cellular Fc $\gamma$ -receptors (Fc $\gamma$ R) by conjugating immunoglobulin G (IgG) to liposomes, would improve the efficacy of dexamethasone-liposomes in attenuating granulocyte infiltration, one of the hallmarks of lung inflammation.

## EXPERIMENTAL APPROACH

Mice were anaesthetized, tracheotomized and mechanically ventilated for 5 h with either 'low' tidal volumes  $\sim 7.5$  mL $\cdot$ kg<sup>-1</sup> (LV<sub>T</sub>) or 'high' tidal volumes  $\sim 15$  mL $\cdot$ kg<sup>-1</sup> (HV<sub>T</sub>). At initiation of ventilation, we intravenously administered dexamethasone encapsulated in liposomes (Dex-liposomes), dexamethasone encapsulated in IgG-modified liposomes (IgG-Dex-liposomes) or free dexamethasone. Non-ventilated mice served as controls.

## KEY RESULTS

Dex-liposomes attenuated granulocyte infiltration and IL-6 mRNA expression after LV<sub>T</sub>-ventilation, but not after HV<sub>T</sub>-ventilation. Dex-liposomes also down-regulated mRNA expression of IL-1 $\beta$  and KC, but not of CCL2 (MCP-1) in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice. Importantly, IgG-Dex-liposomes inhibited granulocyte influx caused by either LV<sub>T</sub> or HV<sub>T</sub>-ventilation. IgG-Dex-liposomes diminished IL-1 $\beta$  and KC mRNA expression in both ventilation groups, and IL-6 and CCL2 mRNA expression in the LV<sub>T</sub>-ventilated group. Free dexamethasone prevented granulocyte influx and inflammatory mediator expression induced by LV<sub>T</sub> or HV<sub>T</sub>-ventilation.

## CONCLUSIONS AND IMPLICATIONS

Fc $\gamma$ R-targeted IgG-Dex-liposomes are pharmacologically more effective than Dex-liposomes particularly in inhibiting pulmonary granulocyte infiltration. IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effectively as free dexamethasone, with the advantage that liposome-encapsulated dexamethasone will be released locally in the lung thereby preventing systemic side-effects.

## Abbreviations

ALI, acute lung injury; AP, activator protein; BE, base excess; CCL, CC chemokine ligand; Dex-liposomes, dexamethasone encapsulated in liposomes; Fc $\gamma$ R, Fc $\gamma$ -receptor; FiO<sub>2</sub>, fractional inspired oxygen concentration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; H&E, haematoxylin and eosin; HV<sub>T</sub>, high tidal volume; IgG-Dex-liposomes, dexamethasone encapsulated in IgG-modified liposomes; IL, interleukin; KC, keratinocyte-derived chemokine; LPS, lipopolysaccharide; LV<sub>T</sub>, low tidal volume; MCP, monocyte chemotactic protein;

MPO, myeloperoxidase; NF- $\kappa$ B, nuclear factor kappa B; NVC, non-ventilated controls; PaCO<sub>2</sub>, partial pressure of arterial carbon dioxide; PaO<sub>2</sub>, partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure; PMN, polymorphonuclear cell; RT-PCR, reverse transcriptase polymerase chain reaction; VILI, ventilator-induced lung injury

## Introduction

Mechanical ventilation has the potential to induce or worsen lung injury, a phenomenon referred to as ventilator-induced lung injury (VILI) (Dreyfuss and Saumon, 1998; Slutsky, 1999). VILI is characterized by enhanced inflammation, vascular leakage and impaired gas exchange (Parker *et al.*, 1993). It has been hypothesized that a ventilator-induced increase in granulocyte infiltration and inflammatory mediator expression in the lung is crucial in the development of pulmonary injury (Haitsma *et al.*, 2003; Wilson *et al.*, 2003; 2005). One of the most potent group of drugs to treat lung inflammation are the glucocorticoids, which exert their effects by binding to intracellular glucocorticoid receptors (GRs) (Brower *et al.*, 2001; Luce, 2002). After binding, the GR complex migrates from the cytosol to the nucleus where it regulates a wide range of gene activity, including inhibition of nuclear factor kappa B (NF- $\kappa$ B) and activator protein (AP)-1 driven expression of inflammatory genes (Barnes, 2006).

Previous research in experimental models of VILI showed that synthetic glucocorticoids have the potential to attenuate ventilator-induced lung inflammation (Held *et al.*, 2001; Ohta *et al.*, 2001). However, systemic administration of glucocorticoids is associated with severe side-effects like increased blood glucose levels (Weinstein *et al.*, 1995; Feldman-Billard *et al.*, 2006). Local delivery of glucocorticoids by liposomal formulations could therefore be of therapeutic importance. In this respect, we previously demonstrated that delivery of liposome-encapsulated dexamethasone (Dex-liposomes) inhibits pro-inflammatory gene expression in a murine model of glomerulonephritis without affecting blood glucose levels (Asgeirsdottir *et al.*, 2007). Liposomes are valuable drug delivery systems for treatment of VILI as they can act as a depot from which the encapsulated drug will be slowly released to enable prolonged drug exposure at low concentrations (Storm and Crommelin, 1998). Furthermore, liposomes extravasate into tissues that experience increased capillary permeability (Storm and Crommelin, 1998), which facilitates delivery at sites of inflammation or mechanical stretch.

The present study was designed to examine whether Dex-liposomes are capable of down-regulating ventilator-induced lung inflammation. Moreover, we hypothesized that conjugation of immunoglobulin G (IgG) to the Dex-liposomes would promote binding of the IgG Fc-fragment on Dex-liposomes to Fc $\gamma$ -receptors (Fc $\gamma$ Rs) on macrophages and granulocytes, thereby improving the efficacy of Dex-liposomes to inhibit granulocyte infiltration, one of the hallmarks of VILI. We investigated the effects of dexamethasone encapsulated in liposomes (Dex-liposomes), dexamethasone encapsulated in IgG-modified liposomes (IgG-Dex-liposomes) and free dexamethasone in an established murine model of VILI (Wolthuis *et al.*, 2009).

## Methods

### Animals

All animal care and experimental procedures were approved by the animal use and care committees of the University Medical Center Utrecht and Academic Medical Center Amsterdam. Adult male C57Bl6 mice ( $n = 126$ ; Charles River, Maastricht, the Netherlands), weighing 20–24 g, were randomly assigned to different experimental groups.

Healthy mice ( $n = 108$ ) were exposed to mechanical ventilation as described previously (Wolthuis *et al.*, 2009). General anaesthesia was achieved with an intraperitoneal injection of 126 mg·kg<sup>-1</sup> ketamine (Eurovet Animal Health BV, Bladel, the Netherlands), 0.2 mg·kg<sup>-1</sup> medetomidine (Pfizer Animal Health BV, Capelle a/d IJssel, the Netherlands) and 0.5 mg·kg<sup>-1</sup> atropine (Pharmachemie, Haarlem, the Netherlands). Maintenance anaesthesia consisted of 36 mg·kg<sup>-1</sup> ketamine, 0.04 mg·kg<sup>-1</sup> medetomidine and 0.075 mg·kg<sup>-1</sup> atropine and was administered every hour via an intraperitoneal catheter (PE 10 tubing; BD, Breda, the Netherlands).

Groups of six mice were ventilated simultaneously for 5 h in a pressure-controlled mode, at a fractional inspired oxygen concentration (FiO<sub>2</sub>) of 50 %, inspiration-to-expiration ratio of 1:1 and positive end-expiratory pressure (PEEP) of 2 cmH<sub>2</sub>O. Mechanical ventilation was initiated with either an inspiratory pressure of 10 cmH<sub>2</sub>O (resulting in 'low' tidal volumes (V<sub>T</sub>) ~7.5 mL·kg<sup>-1</sup>; LV<sub>T</sub>) or 18 cmH<sub>2</sub>O (resulting in 'high' V<sub>T</sub> ~15 mL·kg<sup>-1</sup>; HV<sub>T</sub>). Respiratory rate was set at 100 and 50 breaths·min<sup>-1</sup> respectively. Body temperature was kept constant between 36.5–37.5°C. Non-ventilated mice ( $n = 18$ ) served as controls [non-ventilated controls (NVC)]. At the end of the 5 h experimental period, animals were killed by exsanguination.

### Synthesis of liposome-encapsulated dexamethasone

The glucocorticoid dexamethasone was encapsulated in liposomes (Dex-liposomes) or IgG-modified liposomes (IgG-Dex-liposomes) as described previously (Asgeirsdottir *et al.*, 2007). Lipids from stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000]-maleimide in chloroform/methanol (9:1), were mixed in a molar ratio of 55:40:4:1, dried under reduced nitrogen pressure, dissolved in cyclohexane, and lyophilized. The lipids were then hydrated in 10 mM HEPES and 135 mM NaCl, pH 6.7, or, when appropriate, in an aqueous solution of 75 to 100 mg·mL<sup>-1</sup> dexamethasone disodium phosphate. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar; Corning Life Sciences,

Acton, MA, USA), pore size 50 nm, using a high-pressure extruder (Lipex, Vancouver, BC, Canada). The rat IgG was thiolated by *N*-succinimidyl-*S*-acetylthioacetate and coupled to a maleimide group at the distal end of the polyethylene glycol chain. The liposomes were characterized by determining protein content, using mouse IgG as a standard (Peterson, 1977) and phospholipid phosphorus content (Bottcher *et al.*, 1961). Total liposomal lipid concentrations were adjusted for the amount of cholesterol present in liposome preparations. The amount of coupled rat IgG was 34.2 g per mol of lipid. Particle size was analysed by dynamic light scattering using a Nicomp model 370 submicron particle analyser (Santa Barbara, CA) in the volume weighing model. The diameter of Dex-liposomes was 88.6 nm and that of IgG-Dex-liposomes 103.0 nm. The content of encapsulated dexamethasone disodium phosphate was determined after Bligh and Dyer extraction in the resulting methanol/H<sub>2</sub>O phase by high-performance liquid chromatography (Melgert *et al.*, 2000). The amount of encapsulated dexamethasone phosphate was 28.0 g per mol of lipid (Dex-liposomes) or 34.8 g per mol of lipid (IgG-Dex-liposomes).

### Dexamethasone treatment

At initiation of ventilation, we intravenously administered either Dex-liposomes, IgG-Dex-liposomes (0.4 µmol of lipid per animal; 11.2 and 13.9 µg dexamethasone, respectively) or free dexamethasone (20 µg per animal). In addition, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice were intravenously treated with liposomal formulations lacking entrapped dexamethasone. Control LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice received the same volume of sterile saline (vehicle) intravenously.

### Haemodynamics and blood gas analysis

After 0, 2.5 and 5 h, systolic blood pressure and heart rate were non-invasively monitored using a murine tail-cuff system (ADInstruments, Spensbach, Germany). After 5 h, arterial blood was taken from the carotid artery for blood gas analysis (Rapidlabs 865; Bayer, Mijdrecht, the Netherlands).

### Histopathology and immunohistochemistry

The left lung was filled with Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen and cut to 5 µm cryosections using a cryostat. To assess lung histopathology, longitudinal sections were stained with haematoxylin and eosin (H&E; Klinipath, Duiven, the Netherlands). To assess granulocyte infiltration in lung tissue, longitudinal sections were incubated with rabbit antibody to polymorphonuclear cells (PMNs) (Accurate Antibodies, Westbury, NY, USA) followed by horseradish peroxidase (HRP) goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) and visualization with diaminobenzamide.

### Homogenates

Lung tissue was pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to perform multiple analyses (as described in the following).

### Myeloperoxidase (MPO) activity

MPO activity was determined as described previously (Hegeman *et al.*, 2009). In short, pulmonary tissue was

homogenized in 50 mM HEPES buffer (pH 8.0), centrifuged and pellets were homogenized again in water and 0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 mM citrate buffer (pH 5.0) and 0.22% CTAC. Substrate solution containing 3 mM 3',5,5'-tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, Steinheim, Germany), 120 µM resorcinol (Merck) and 2.2 mM H<sub>2</sub>O<sub>2</sub> in distilled water was added. Reaction mixtures were incubated for 20 min at room temperature and stopped by addition of 4 M H<sub>2</sub>SO<sub>4</sub> followed by determination of optical density at 450 nm. MPO activity of a known amount of MPO units per mL (Sigma-Aldrich) was used as reference. MPO activity was corrected for total protein (BCA protein assay; Pierce Biotechnology, Rockford, IL, USA) using BSA as standard.

### Real-time RT-PCR

Total RNA was isolated with TRIzol® reagent (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using primers for interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC) and CC chemokine ligand (CCL) 2 [monocyte chemotactic protein (MCP-1); nomenclature follows Alexander *et al.*, 2009]. PCR product size was verified on gel to confirm appropriate amplification. Data were normalized for expression of internal controls, that is, the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers: IL-6, FW ACCgCTATgAAgTTCCTCTC, RV CTCTgTgAAgTCTCCTC TCC; CCL2 FW ggTCCCTgTCATgCTTCTg, RV CATCTTgCT ggTgAATgAgTAG; for other sequences see Hegeman *et al.* (2009).

### Statistical analysis

Data are expressed as mean ± standard error of the mean. Blood gas variables (LV<sub>T</sub> vs. HV<sub>T</sub>) were analysed by independent *T*-test. All other parameters were analysed by one-way analysis of variance (ANOVA) with least significant difference (LSD) *post hoc* test. *P*-values less than 0.05 were considered as statistically significant.

### Materials

Ketamine was obtained from Eurovet Animal Health BV, Bladel, the Netherlands; medetomidine from Pfizer Animal Health BV, Capelle a/d IJssel, the Netherlands and atropine from Pharmachemie, Haarlem, the Netherlands. Dexamethasone disodium phosphate was from Bufa, Hilversum, the Netherlands; rat IgG and cholesterol from Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands and the other lipids from Avanti Polar Lipids, Alabaster, AL, USA.

## Results

### Stability of the murine model of VILI

All animals survived the ventilation procedures and were killed after 5 h of mechanical ventilation. Systolic blood pressures and heart rates were stable throughout the experiment (Table 1).

**Table 1**

Haemodynamic characteristics over 5 h of mechanical ventilation

	LV <sub>T</sub> Veh	Dex lip	IgG-Dex lip	Dex	HV <sub>T</sub> Veh	Dex lip	IgG-Dex lip	Dex
HR <sub>t=0h</sub>	370 ± 11	391 ± 19	399 ± 30	381 ± 17	370 ± 21	387 ± 16	395 ± 19	388 ± 6
HR <sub>t=2,5h</sub>	385 ± 19	408 ± 22	360 ± 17	371 ± 15	350 ± 15	358 ± 12	376 ± 14	347 ± 20
HR <sub>t=5h</sub>	409 ± 10	386 ± 28	407 ± 18	402 ± 17	367 ± 7	354 ± 12	378 ± 11	357 ± 26
BP <sub>t=0h</sub>	103 ± 5	119 ± 7	103 ± 4	112 ± 11	103 ± 6	110 ± 13	108 ± 8	113 ± 7
BP <sub>t=2,5h</sub>	64 ± 4	66 ± 4	62 ± 3	76 ± 8	69 ± 4	68 ± 5	61 ± 5	69 ± 7
BP <sub>t=5h</sub>	68 ± 7	66 ± 7	70 ± 5	64 ± 4	67 ± 5	70 ± 7	67 ± 6	78 ± 8

Data are presented as mean ± standard error of the mean (LV<sub>T</sub> *n* = 6–8, HV<sub>T</sub> *n* = 6–8).

BP, systolic blood pressure in mmHg; Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HR, heart rate in beats min<sup>-1</sup>; HV<sub>T</sub>, mice ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mice ventilated with low tidal volumes; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

**Table 2**

Arterial blood gas analysis after 5 h of mechanical ventilation

	LV <sub>T</sub> Veh	Dex lip	IgG-Dex lip	Dex	HV <sub>T</sub> Veh	Dex lip	IgG-Dex lip	Dex
PaO <sub>2</sub>	229.0 ± 20.8	230.0 ± 14.0	228.9 ± 17.9	224.6 ± 23.3	171.2 ± 15.2*	175.6 ± 14.7	177.7 ± 19.5	170.5 ± 13.5
PaCO <sub>2</sub>	31.8 ± 3.7	32.2 ± 2.7	33.6 ± 6.5	31.8 ± 4.0	33.2 ± 2.3	35.9 ± 1.3	34.1 ± 2.6	36.5 ± 1.7
pH	7.52 ± 0.03	7.50 ± 0.04	7.49 ± 0.06	7.50 ± 0.04	7.52 ± 0.03	7.48 ± 0.02	7.48 ± 0.03	7.49 ± 0.02
BE	2.52 ± 1.25	1.60 ± 1.44	0.19 ± 0.98	0.61 ± 1.38	3.70 ± 1.37	2.78 ± 1.00	1.35 ± 0.79	3.76 ± 0.86

Data are presented as mean ± standard error of the mean (LV<sub>T</sub> *n* = 8–10, HV<sub>T</sub> *n* = 8–10). \**P* < 0.05 versus LV<sub>T</sub> Veh.

BE, base excess in mmol·L<sup>-1</sup>; Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HV<sub>T</sub>, mechanically ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mechanically ventilated with low tidal volumes; PaO<sub>2</sub>, partial pressure of arterial oxygen in mmHg; PaCO<sub>2</sub>, partial pressure of arterial carbon dioxide in mmHg; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

Arterial oxygen tension (PaO<sub>2</sub>) was reduced in HV<sub>T</sub>-ventilated mice in comparison with LV<sub>T</sub>-ventilated mice (Table 2). In both ventilation groups, carbon dioxide tension (PaCO<sub>2</sub>), pH and base excess (BE) remained within the physiological range.

### *Effect of Dex-liposomes and IgG-Dex-liposomes on pulmonary architecture*

Mice were intravenously treated with either saline (vehicle), Dex-liposomes, IgG-Dex-liposomes or free dexamethasone at initiation of mechanical ventilation and subsequently ventilated for 5 h. No changes in haemodynamic and blood gas variables were observed after administration of (liposome-encapsulated) dexamethasone (Tables 1 and 2). Lung sections were stained for H&E to analyse histopathological changes after mechanical ventilation (Figure 1). We observed that both LV<sub>T</sub> and HV<sub>T</sub>-ventilation (vehicle treatment) induced damage to pulmonary architecture compared with NVC, that is, thickening of the alveolar wall and cellular infiltrate. Immunohistochemical staining revealed that the infiltrated

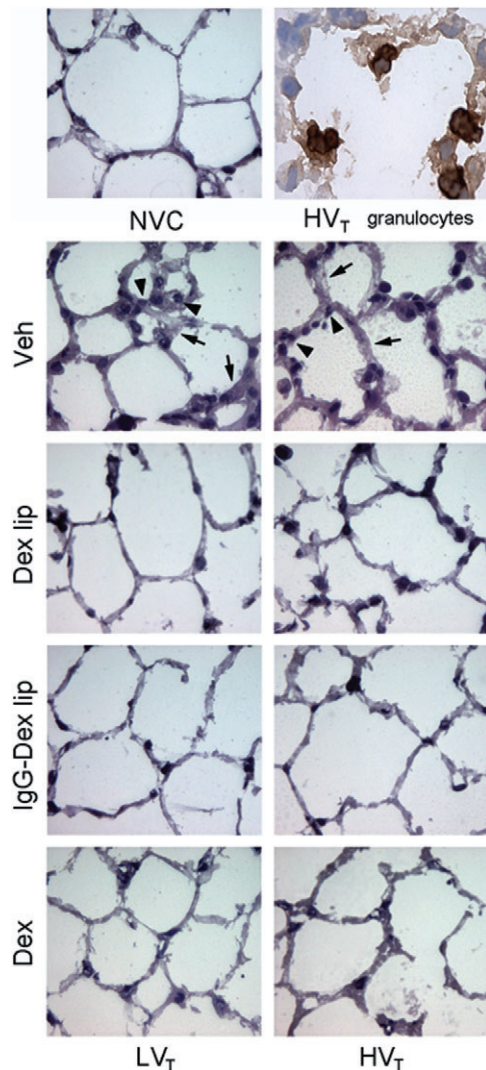
immune cells were granulocytes. Administration of Dex-liposomes, IgG-Dex-liposomes or free dexamethasone at the initiation of ventilation preserved pulmonary architecture during 5 h of mechanical ventilation.

### *Effect of Dex-liposomes and IgG-Dex-liposomes on ventilator-induced granulocyte infiltration*

To assess ventilator-induced lung inflammation, we determined influx of granulocytes into pulmonary tissue. Granulocyte infiltration was quantified by measuring MPO activity in total lung homogenates. MPO activity was significantly increased after LV<sub>T</sub> and HV<sub>T</sub>-ventilation in comparison with NVC (Figure 2), which correlated with the margination of granulocytes to the blood vessel wall observed in lung sections of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice (Figure 1).

Next, we investigated if administration of Dex-liposomes at the initiation of ventilation was capable of inhibiting lung inflammation induced by 5 h of LV<sub>T</sub> or HV<sub>T</sub>-ventilation. Treatment with Dex-liposomes attenuated MPO activity in





**Figure 1**

Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on changes in lung histology induced by mechanical ventilation. Lung sections were stained with haematoxylin and eosin (H&E) to analyse lung histopathology and presence of cellular infiltrate in pulmonary tissue. Magnification  $\times 500$ . Black arrow indicates the typical thickening of the alveolar wall and black arrowhead indicates cellular infiltrate. Lung sections were immunohistochemically stained for polymorphonuclear cells (PMNs) to assess if the cellular infiltrate consisted of granulocytes. Magnification  $\times 1000$ . Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HV<sub>T</sub>, mice ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mice ventilated with low tidal volumes; NVC, non-ventilated controls; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

mice exposed to LV<sub>T</sub>-ventilation (Figure 2A) although MPO activity was still above baseline level in these mice ( $P < 0.05$  vs. NVC). In mice exposed to HV<sub>T</sub>-ventilation, however, no inhibitory effect of Dex-liposomes on MPO activity was

observed (Figure 2B). In contrast, treatment with IgG-Dex-liposomes effectively inhibited granulocyte infiltration in both LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice. Furthermore, IgG-Dex-liposomes were as effective as free dexamethasone in down-regulating MPO activity in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice. H&E staining of lung sections confirmed the quantitative measures for granulocyte influx after treatment with Dex-liposomes, IgG-Dex-liposomes and free dexamethasone (Figure 1).

### *Effect of Dex-liposomes and IgG-Dex-liposomes on ventilator-induced cytokine expression*

To examine the effect of LV<sub>T</sub> and HV<sub>T</sub>-ventilation on cytokine expression, we determined *de novo* synthesis of the prototypic pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in total lung homogenates. Compared with NVC, both ventilation strategies induced mRNA expression of IL-1 $\beta$  (Figure 3A and B) and IL-6 (Figure 3C and D).

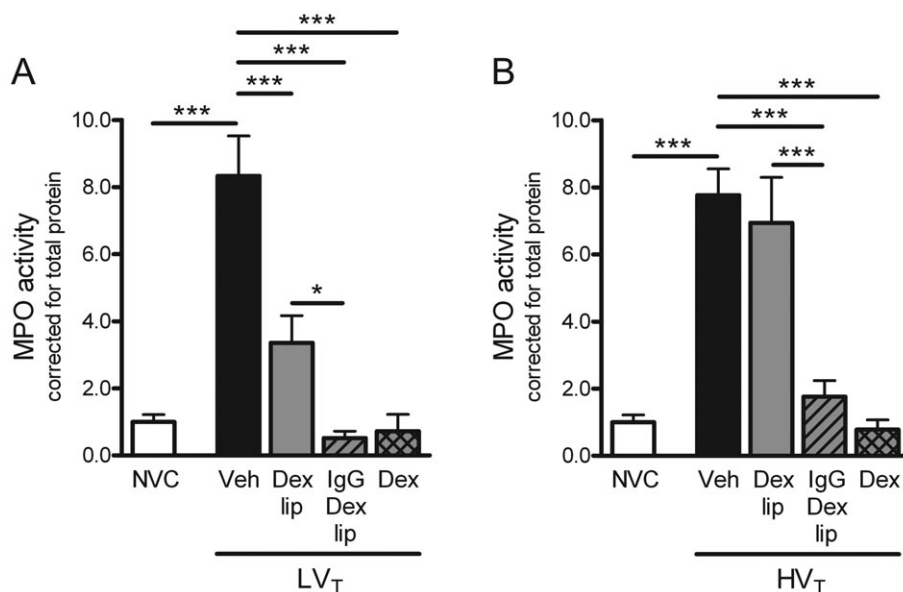
Dex-liposomes and IgG-Dex-liposomes attenuated IL-1 $\beta$  mRNA expression after LV<sub>T</sub> and HV<sub>T</sub>-ventilation. IL-6 mRNA levels were down-regulated in LV<sub>T</sub>-ventilated mice, while both liposomal formulations were fully devoid of IL-6 mRNA inhibitory activity in HV<sub>T</sub>-ventilated mice. Administration of free dexamethasone prevented the increase in IL-1 $\beta$  and IL-6 mRNA expression in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice, although IL-6 mRNA expression was still above baseline level in HV<sub>T</sub>-ventilated mice ( $P < 0.05$  vs. NVC; Figure 3D).

### *Effect of Dex-liposomes and IgG-Dex-liposomes on ventilator-induced chemokine expression*

To study the effect of LV<sub>T</sub> and HV<sub>T</sub>-ventilation on *de novo* synthesis of chemokines, we determined KC and CCL2 mRNA expression in total lung homogenates. mRNA expression of KC (Figure 4A and B) and CCL2 (Figure 4C and D) was increased after LV<sub>T</sub> and HV<sub>T</sub> ventilation. Treatment with Dex-liposomes diminished KC mRNA expression induced by LV<sub>T</sub> or HV<sub>T</sub>-ventilation. However, Dex-liposomes did not affect CCL2 mRNA levels in LV<sub>T</sub>-ventilated mice while they enhanced CCL2 mRNA levels in HV<sub>T</sub>-ventilated mice (204.5%;  $P < 0.001$  vs. HV<sub>T</sub> vehicle; Figure 4D). IgG-Dex-liposomes diminished KC mRNA expression after both LV<sub>T</sub> and HV<sub>T</sub>-ventilation and CCL2 mRNA expression only after LV<sub>T</sub>-ventilation. Administration of free dexamethasone inhibited KC and CCL2 mRNA levels in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice.

### *Effect of empty liposomes on lung inflammation*

Liposomal formulations without entrapped dexamethasone were administered at initiation of ventilation to evaluate whether lipid carriers as such would influence the inflammatory response caused by LV<sub>T</sub> or HV<sub>T</sub>-ventilation. We used empty IgG-modified liposomes as these liposomal formulations were most efficient in down-regulating ventilator-induced lung inflammation. Empty IgG-liposomes were ineffective in alleviating the increase in MPO activity, IL-1 $\beta$ , IL-6, KC and CCL2 mRNA expression in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice (Figure 5A to J).



**Figure 2**

Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on granulocyte influx induced by mechanical ventilation. (A–B) In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. Data are expressed as mean  $\pm$  SEM, and shown relative to NVC. (A/B) NVC  $n = 18/18$ , Veh  $n = 20/14$ , Dex lip  $n = 15/14$ , IgG-Dex lip  $n = 10/9$ ; Dex  $n = 7/7$ . \* $P < 0.05$ , \*\*\* $P < 0.001$  versus NVC, LV<sub>T</sub>/HV<sub>T</sub> Veh or LV<sub>T</sub>/HV<sub>T</sub> Dex lip. Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HV<sub>T</sub>, mice ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mice ventilated with low tidal volumes; NVC, non-ventilated controls; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

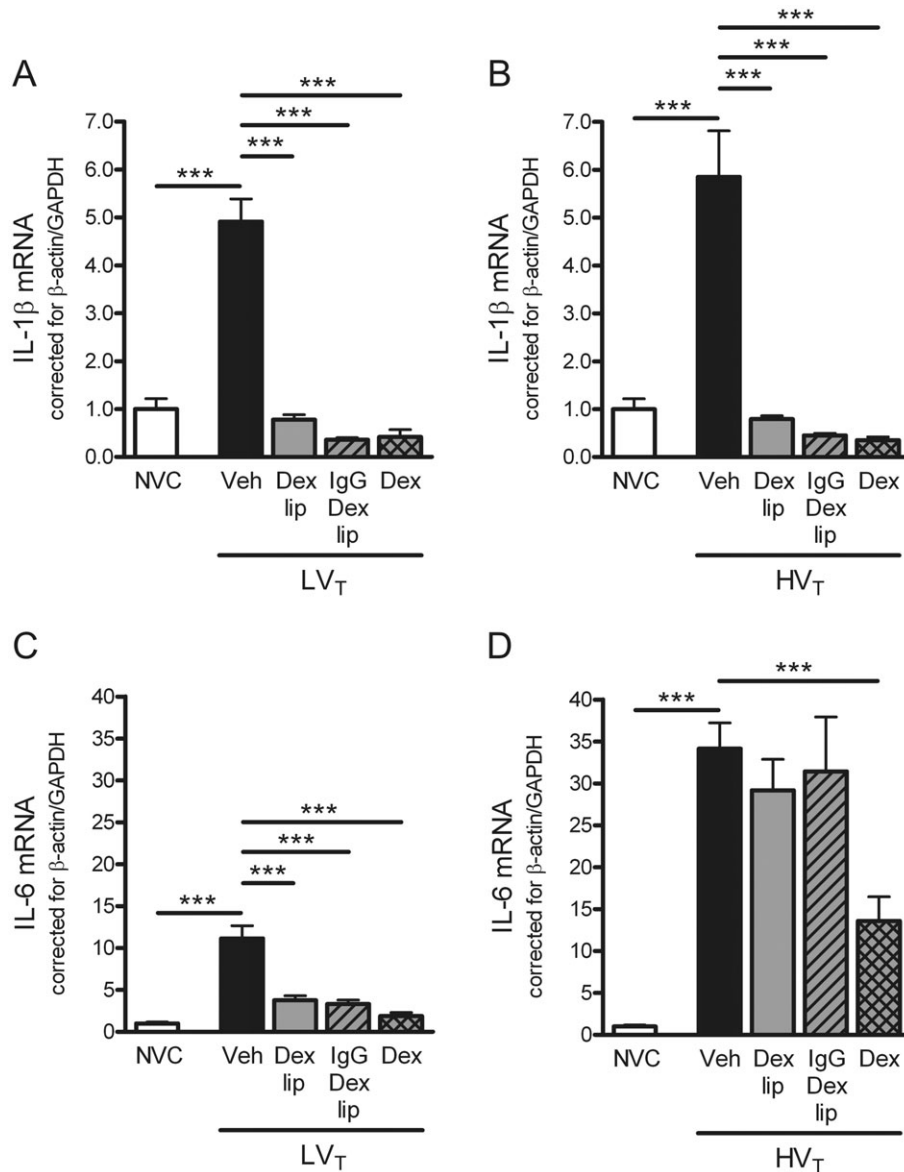
## Discussion and conclusions

The present study was designed to evaluate whether liposome-encapsulated dexamethasone attenuated lung inflammation induced by mechanical ventilation. Our major finding is that Fc $\gamma$ R-targeted IgG-Dex-liposomes were pharmacologically more effective than Dex-liposomes particularly in preventing influx of granulocytes, a major hallmark of VILI. We demonstrated that treatment with IgG-Dex-liposomes successfully protected against granulocyte influx induced by LV<sub>T</sub> or HV<sub>T</sub>-ventilation, which was accompanied by inhibition of IL-1 $\beta$  and KC mRNA expression in both ventilation groups, and IL-6 and CCL2 mRNA expression in the LV<sub>T</sub>-ventilated group. Moreover, IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effectively as free dexamethasone. In contrast, Dex-liposomes only attenuated IL-1 $\beta$  and KC mRNA during both ventilation strategies and IL-6 mRNA and granulocyte influx during LV<sub>T</sub>-ventilation. All administration formulae of dexamethasone preserved pulmonary architecture during mechanical ventilation without inducing changes in haemodynamic and blood gas variables.

Both LV<sub>T</sub> and HV<sub>T</sub>-ventilation induced inflammation in pulmonary tissue as measured by increased granulocyte infiltration and cytokine/chemokine mRNA expression. Since we used non-perfused lung homogenates, we cannot exclude that part of this enhanced cytokine/chemokine response was derived from blood cells. Previous studies described that the combined effects of high PaO<sub>2</sub> levels and

mechanical ventilation worsen VILI (Sinclair *et al.*, 2004; Li *et al.*, 2007). Since we used moderate hyperoxia (FiO<sub>2</sub> of 50 %) in our experimental model of VILI, it cannot be excluded that the higher oxygen levels may aggravate the stretch-induced inflammatory response in the lung. However, we have already shown that hyperoxia (FiO<sub>2</sub> of 100 %) by itself did not lead to pulmonary inflammation (Hegeman *et al.*, 2009). Therefore, it is tempting to speculate that the moderate hyperoxia in our present study will not be the primary cause of lung inflammation during mechanical ventilation.

In agreement with prior reports (Held *et al.*, 2001; Ohta *et al.*, 2001), we showed that free dexamethasone successfully protects against lung inflammation induced by 5 h of mechanical ventilation. It has been recognized that systemic administration of glucocorticoids may lead to unwanted side-effects like hyperglycaemia, deposition of body fat, suppressed systemic immunity and increased susceptibility to infections (Schacke *et al.*, 2002). The advantage of using liposome-encapsulated dexamethasone is that the drug will be released more locally in the lung thereby inhibiting ventilator-induced inflammatory responses without inducing hyperglycaemia, one of the first clinically relevant side-effects of free dexamethasone treatment (Weinstein *et al.*, 1995; Feldman-Billard *et al.*, 2006). Unfortunately, we were not able to investigate the effects of dexamethasone on blood glucose levels in this experimental model of VILI as the anaesthesia procedure itself was affecting blood glucose levels (Zuurbier *et al.*, 2008). However, our previous study in a murine model



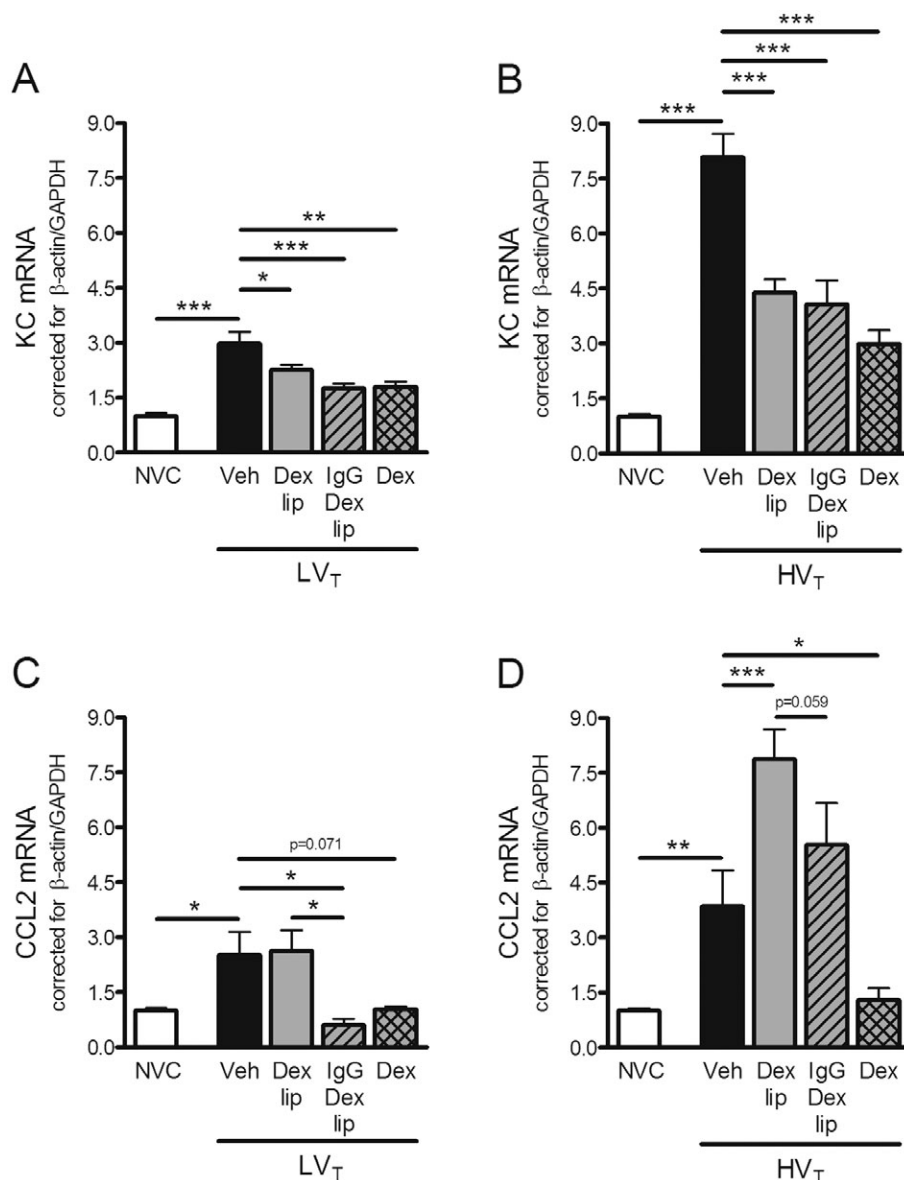
**Figure 3**

Effect of Dex-liposomes, IgG-Dex-liposomes and free dexamethasone on cytokine mRNA expression induced by mechanical ventilation. (A–B) In total lung homogenates, mRNA expression of interleukin (IL)-1 $\beta$  was determined by real-time RT-PCR. (C–D) In addition, mRNA expression of IL-6 was determined. Levels were normalized for expression of internal controls, that is, the average value of  $\beta$ -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as mean  $\pm$  SEM, and shown relative to NVC. (A/B) NVC  $n = 15/15$ , Veh  $n = 22/17$ , Dex lip  $n = 15/12$ , IgG-Dex lip  $n = 10/8$ ; Dex  $n = 7/7$ ; (C/D) NVC  $n = 15/15$ , Veh  $n = 21/18$ , Dex lip  $n = 15/13$ , IgG-Dex lip  $n = 10/10$ ; Dex  $n = 7/7$ .  $***P < 0.001$  versus NVC or LV<sub>T</sub>/HV<sub>T</sub> Veh. Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HV<sub>T</sub>, mice ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mice ventilated with low tidal volumes; NVC, non-ventilated controls; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

of glomerulonephritis clearly showed that liposome-encapsulated dexamethasone prevents the occurrence of hyperglycaemia associated with administration of the free drug (Asgeirsdottir *et al.*, 2007).

An important feature of liposomes is their preferential extravasation at sites with increased capillary permeability (Storm and Crommelin, 1998). As ventilator-induced stretch of lung tissue enhances vascular permeability (Egan, 1982;

Parker *et al.*, 1984; Dreyfuss *et al.*, 1985), liposomes may accumulate in cells of the lung and slowly release their encapsulated dexamethasone locally. Indeed, we observed that administration of Dex-liposomes significantly inhibited IL-1 $\beta$  and KC mRNA expression in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice and IL-6 mRNA expression in lungs of LV<sub>T</sub>-ventilated mice. More importantly, Dex-liposomes diminished granulocyte infiltration induced by LV<sub>T</sub>-ventilation.



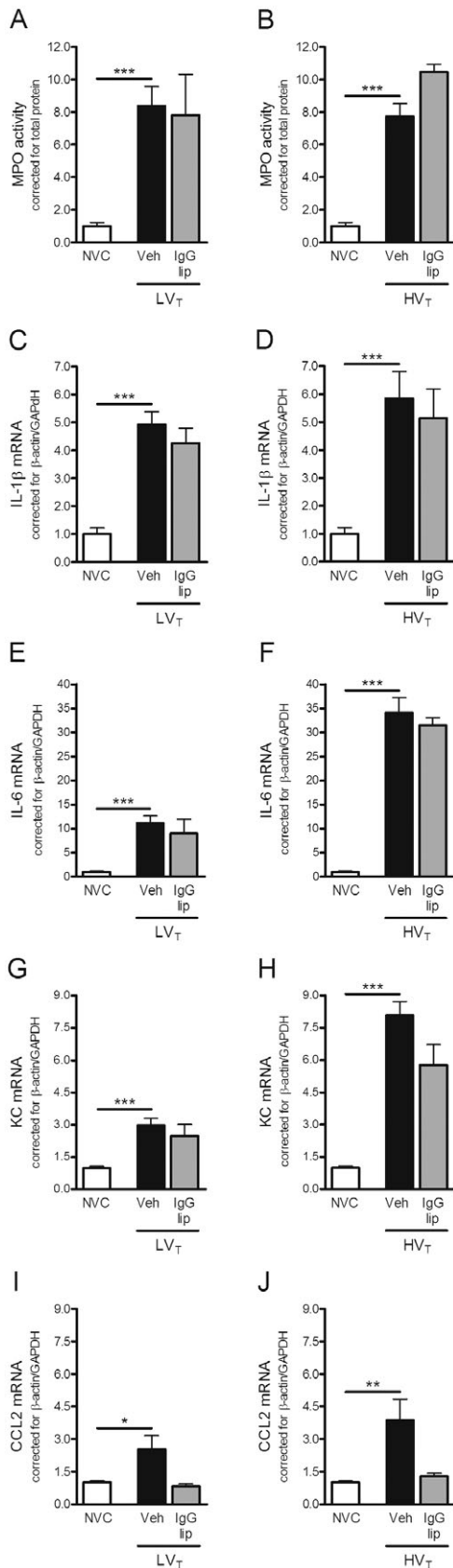
**Figure 4**

Effect of Dex liposomes, IgG-Dex-liposomes and free dexamethasone on chemokine mRNA expression induced by mechanical ventilation. (A–B) In total lung homogenates, mRNA expression of keratinocyte-derived chemokine (KC) was determined by real time RT-PCR. (C–D) In addition, mRNA expression of CC chemokine ligand (CCL) 2 was determined. Levels were normalized for expression of internal controls, that is, average value of  $\beta$ -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as mean  $\pm$  SEM, and shown relative to NVC. (A/B) NVC  $n = 15/15$ , Veh  $n = 20/17$ , Dex lip  $n = 15/13$ , IgG-Dex lip  $n = 10/9$ ; Dex  $n = 8/7$ ; (C/D) NVC  $n = 15/15$ , Veh  $n = 21/16$ , Dex lip  $n = 15/13$ , IgG-Dex lip  $n = 10/9$ ; Dex  $n = 8/7$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus NVC, LV<sub>T</sub>/HV<sub>T</sub> Veh or LV<sub>T</sub>/HV<sub>T</sub> Dex lip. Dex, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with free dexamethasone; Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with liposomes containing dexamethasone; HV<sub>T</sub>, mice ventilated with high tidal volumes; IgG-Dex lip, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with IgG-liposomes containing dexamethasone; LV<sub>T</sub>, mice ventilated with low tidal volumes; NVC, non-ventilated controls; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

Even though Dex-liposomes were capable of attenuating important parameters of VILI, especially in LV<sub>T</sub>-ventilated mice, they were not as effective as free dexamethasone in preventing granulocyte infiltration. Since granulocytes are known to be important in the pathogenesis of VILI (Kawano *et al.*, 1987), active delivery of the drug into these circulating granulocytes might be advantageous. Therefore, we hypoth-

esized that IgG-Dex-liposomes may be more efficient in inhibiting ventilator-induced lung inflammation than Dex-liposomes due to interaction with the Fc $\gamma$ R on activated granulocytes and macrophages (McKenzie and Schreiber, 1998). We found that IgG-Dex-liposomes significantly inhibited granulocyte influx in lungs of both LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice whereas Dex-liposomes only attenuated





**Figure 5**

Effect of empty IgG-liposomes on granulocyte infiltration, cytokine and chemokine mRNA expression induced by mechanical ventilation. (A–B) In total lung homogenates, myeloperoxidase (MPO) activity was determined as a measure of granulocyte infiltration. Levels were normalized for total protein levels. (C–F) In total lung homogenates, mRNA expression of the cytokines interleukin (IL)-1 $\beta$  and IL-6 was determined by real-time RT-PCR. G–J: In addition, mRNA expression of the chemokines keratinocyte-derived chemokine (KC) and CC chemokine ligand (CCL) 2 was determined. Levels were normalized for expression of internal controls, that is, the average value of  $\beta$ -actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as mean  $\pm$  SEM, and depicted relative to NVC. (A/B) NVC  $n = 18/18$ , Veh  $n = 20/14$ , IgG-lip  $n = 4/2$ ; (C/D) NVC  $n = 15/15$ , Veh  $n = 22/17$ , IgG-lip  $n = 4/2$ ; (E/F) NVC  $n = 15/15$ , Veh  $n = 21/18$ , IgG-lip  $n = 4/2$ ; (G/H) NVC  $n = 15/15$ , Veh  $n = 20/17$ , IgG-lip  $n = 4/2$ ; (I/J) NVC  $n = 15/15$ , Veh  $n = 21/16$ , IgG-lip  $n = 4/2$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus NVC. HV<sub>T</sub>, mice ventilated with high tidal volumes; LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with empty IgG-liposomes; LV<sub>T</sub>, mice ventilated with low tidal volumes; NVC, non-ventilated controls; Veh, LV<sub>T</sub> or HV<sub>T</sub>-ventilated mice intravenously treated with vehicle (sterile saline).

granulocyte infiltration in lungs of LV<sub>T</sub>-ventilated mice. Based on this result, we propose that IgG-Dex-liposomes interact more efficiently with granulocytes in the systemic circulation or in the marginal zone of vessels, thereby preventing granulocyte activation and infiltration into lung tissue. The observation that glucocorticoids suppress granulocyte activation and recruitment into the alveolar space (Ohta *et al.*, 2001) supports our current findings. Moreover, (IgG)-Dex-liposomes may also be internalized more efficiently by macrophages in the lung, where they primarily prevent *de novo* synthesis of IL-1 $\beta$  and KC. It should be noted that the lipid carriers themselves were ineffective in attenuating lung inflammation, as IgG-liposomes without entrapped dexamethasone did not affect the ventilator-induced increase in inflammatory mediator expression.

Previously, Suntutres and Shek described that liposome-encapsulated dexamethasone diminished neutrophil activation and infiltration in a rat model of lipopolysaccharide (LPS)-induced acute lung injury (ALI) (Suntutres and Shek, 2000). Moreover, these authors demonstrated that liposome-encapsulated dexamethasone was even more effective in down-regulating LPS-induced lung inflammation than free dexamethasone. Here we show that IgG-Dex-liposomes inhibited granulocyte influx in lungs of LV<sub>T</sub> and HV<sub>T</sub>-ventilated mice as efficiently as free dexamethasone. An explanation for this discrepancy in efficacy might be that Suntutres and Shek investigated the effects of liposome-encapsulated and free dexamethasone at 24 h after LPS-challenge (Suntutres and Shek, 2000). Because liposomes are considered as slow-release systems (Suntutres and Shek, 1998), it may well be that liposomes become more effective than free dexamethasone after longer periods of time. Moreover, it is important to note that the underlying mechanisms of LPS-induced and ventilator-induced lung inflammation are likely to be different as well, especially with respect to the consequences of mechanical stretch on lung tissue. Taken together, our present data show that liposome-encapsulated dexamethasone may effectively prevent infiltration of granulocytes

and *de novo* synthesis of pro-inflammatory cytokines and chemokines, in particular during LV<sub>T</sub>-ventilation.

Interestingly, Dex-liposomes and IgG-Dex-liposomes did not influence IL-6 and CCL2 mRNA expression induced by HV<sub>T</sub>-ventilation. These data may indicate that the target cells of our liposomal formulations may not be the only source of IL-6 and CCL2 production. *In vitro* studies have demonstrated that alveolar epithelial and capillary endothelial cells are also activated by mechanical stretch, in a stretch-amplitude dependent manner, thereby releasing inflammatory mediators into the surrounding pulmonary tissue (Vlahakis *et al.*, 1999; Iwaki *et al.*, 2009). Iwaki *et al.* demonstrated that high cyclic stretch of microvascular endothelial cells led to increased production of IL-6 and CCL2 (Iwaki *et al.*, 2009). In agreement with these previous findings, our study shows that IL-6 and CCL2 levels were elevated in HV<sub>T</sub>-ventilated mice which were exposed to high levels of mechanical stretch. It is therefore tempting to speculate that alveolar epithelial and capillary endothelial cells will not internalize the liposomal formulations and therefore be responsible for the lack of IL-6 and CCL2 down-regulation after liposome treatment. The fact that liposome-encapsulated dexamethasone was effective in inhibiting IL-1 $\beta$  and KC, which are primarily produced by alveolar macrophages (Bhatia and Mochhala, 2004), supports this explanation. Future studies are needed to obtain further insights into the working mechanism of Dex-liposomes and IgG-Dex-liposomes with respect to the enhancement of CCL2 mRNA expression in HV<sub>T</sub>-ventilated mice after liposome treatment. Our present data, however, suggest that the increase in CCL2 is not caused by the liposomes themselves, as treatment with empty IgG-liposomes did not enhance CCL2 mRNA expression.

In conclusion, our study shows for the first time that liposomal formulation of the glucocorticoid dexamethasone prevents important parameters of ventilator-induced lung inflammation. Importantly, we observed that the selective targeting of Dex-liposomes to cells expressing Fc $\gamma$ Rs, by conjugating IgG to the liposomes, markedly improved their efficacy. In this respect, IgG-Dex-liposomes inhibited most parameters of ventilator-induced lung inflammation as effectively as free dexamethasone with the advantage that liposome-encapsulated dexamethasone will be released locally in the lung thereby preventing systemic side-effects. In view of our data, we suggest that IgG-Dex-liposomes may be an attractive therapeutic strategy to treat critically ill patients diagnosed with serious inflammatory lung diseases such as VILI.

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## Conflicts of interest

None.

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