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Size effects of latex nanomaterials on lung inflammation in mice

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ABSTRACT

Effects of nano-sized materials (nanomaterials) on sensitive population have not been well elucidated. This study examined the effects of pulmonary exposure to (latex) nanomaterials on lung inflammation related to lipopolysaccharide (LPS) or allergen in mice, especially in terms of their size-dependency. In protocol 1, ICR male mice were divided into 8 experimental groups that intratracheally received a single exposure to vehicle, latex nanomaterials (250 μg/animal) with three sizes (25, 50, and 100 nm), LPS (75 μg/animal), or LPS plus latex nanomaterials. In protocol 2, ICR male mice were divided into 8 experimental groups that intratracheally received repeated exposure to vehicle, latex nanomaterials (100 μg/animal), allergen (ovalbumin: OVA; 1 μg/animal), or allergen plus latex nanomaterials. In protocol 1, latex nanomaterials with all sizes exacerbated lung inflammation elicited by LPS, showing an overall trend of amplified lung expressions of proinflammatory cytokines. Furthermore, LPS plus nanomaterials, especially with size less than 50 nm, significantly elevated circulatory levels of fibrinogen, macrophage chemoattractant protein-1, and keratinocyte-derived chemoattractant, and von Willebrand factor as compared with LPS alone. The enhancement tended overall to be greater with the smaller nanomaterials than with the larger ones. In protocol 2, latex nanomaterials with all sizes did not significantly enhance the pathophysiology of allergic asthma, characterized by eosinophilic lung inflammation and Igs production, although latex nanomaterials with less than 50 nm significantly induced/enhanced neutrophilic lung inflammation. These results suggest that latex nanomaterials differentially affect two types of (innate and adaptive immunity-dominant) lung inflammation.

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Introduction

Environmental particulate matters (PM) are well recognized to be toxic for health, including respiratory systems (Dockery and Pope, 1994). One intriguing aspect of the epidemiologic data is that health effects of PM are primarily seen in people with predisposing factors, including bronchilitis, chronic obstructive pulmonary diseases, bronchial asthma, and pneumonia (Dockery et al., 1993). In particular, diesel exhaust particles (DEP), main constituents in urban PM, experimentally not only induce lung inflammation (Ichinose et al., 1995) but facilitate several respiratory diseases such as acute lung injury and bronchial asthma (Ichinose et al., 1995; Takano et al., 2002; Yanagisawa et al., 2003). Our previous in vivo studies have demonstrated that DEP and their components (organic chemicals extracted from DEP; referred to as "DEP-OC" residual particles after extraction of chemical components; referred to as "washed DEP") facilitate both lipopolysaccharide (LPS)-related lung inflammation (Takano et al., 2002; Yanagisawa et al., 2003) and allergic airway inflammation (Takano et al., 1997; Yanagisawa et al., 2006). Furthermore, washed DEP has been shown to augment systemic inflammation and coagulatory disturbance accompanied by lung inflammation related to LPS (Inoue et al., 2006a). More recently, we have demonstrated that carbon nanoparticles (less than 100 nm in their size) exacerbate LPS-related lung inflammation with systemic inflammation and coagulatory disturbance (Inoue et al., 2006b) and allergic airway inflammation in mice (Inoue et al., 2005). Taken together, these findings demonstrate that environment-existing particles have aggravating properties on complicated lung inflammation.

The development of nano-technology has increased the exposure risk to other types of particles in addition to combustion-derived particles in the environment, namely, engineered nanomaterials (Oberdorster et al., 2005). As these materials become more widespread, many questions arise as to the consequences that nanomaterials may have on the environment. In fact, previous reports have shown that the full impact, or even partial impact, of manufactured nanomaterials on health and the environment has yet to be explored in depth (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Nel et al., 2006). In particular, nanomaterial exposure itself reportedly induces lung inflammation (Warheit et al., 2004; Shvedova et al., 2005; Chen

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et al., 2006; Warheit et al., 2006a; Warheit et al., 2006b). However, the facilitating effects of nanomaterials on the subjects with predisposing inflammatory disorders have not been fully examined. Furthermore, comparative studies of the size effects of nanomaterials via pulmonary exposure on these subjects have been less carried out.

Synthetic latex is used for variety of aims and contained in many materials such as paints, painting papers, carpet, wrapping papers, abradants, resins, gloves, etc. Besides, nano-level latex has been reported, for example, to be a useful tool for the investigation of pharmacological activity (Hasegawa et al., 2006). These facts suggest that the ambient and/or local concentrations of this type of nanomaterials mainly in manufacturing factories may increase in the future. Furthermore, its relative uniformed spherical form may allow us to estimate the size contribution, one of another important issue, to the effects. Therefore, to understand more directly the potential of latex nanomaterials to influence lung inflammation, the present study was designed to elucidate the effects of latex nanomaterials with three sizes on lung inflammation induced by intratracheal administration of LPS or allergen (ovalbumin: OVA), on which we have examined the synergistic/additive effects of environmental pollutants in vivo (Hiyoshi et al., 2005; Inoue et al., 2005; Inoue et al., 2006a; Inoue et al., 2006b; Inoue et al., 2006c; Takano et al., 1997; Takano et al., 2002).

Materials and methods

Animals. Male ICR mice 6 wk of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water ad libitum. The mice were housed in an animal facility that was maintained at 24 to 26 °C with 55 to 75% humidity and a 12-h light/dark cycle.

Latex nanomaterials. All sizes (25, 50, and 100 nm) of latex nanomaterials were purchased as fluorescent latex particles (micromer®-blue F plain) from Micromod (Rostock, Germany). Their shape is round and they are monodisperse particles from polystyrene and, are designed with carboxylic acid groups on the particle surface for the covalent binding of proteins, antibodies or other molecules. Degree of agglomeration for each size of latex nanomaterial is not disclosed. They were used as suspension in the vehicle (phosphate-buffered saline [PBS] at pH 7.4 [Invitrogen Co., Carlsbad, CA] containing 0.05% Tween 80 [Nacalai Tesque, Kyoto, Japan]) by sonication for 3 min using an Ultrasonic disrupter (UD-201; Tomy Seiko, Tokyo, Japan).

Study protocol. In protocol 1, mice were divided into eight experimental groups. The vehicle group received above mentioned vehicle. The LPS group received 75 μg of LPS dissolved in PBS. The latex nanomaterial groups received (125 or) 250 μg of latex nanomaterial suspension in the vehicle. The LPS+latex nanomaterial groups received the combined treatment using the same protocols as the LPS and the nanomaterial groups (n=20–21 in each group; n=8 for BAL with ELISA for lung homogenates and plasma coagulation parameters, and n=12–13 for lung water contents [n=8], lung histology [n=4–5], and ELISA for serum proinflammatory cytokines [n=10]). Vehicle, latex nanomaterials, LPS, or LPS+latex nanomaterials were suspended in 0.1-ml aliquots, and inoculated once intratracheally through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical Industries, Tokyo, Japan). We have previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on LPS-related lung inflammation using 125 or 250 μg/animal in vivo (Yanagisawa et al., 2003; Inoue et al., 2006a; Inoue et al., 2006b). Based on and referred to the previous studies from our laboratory, we chose the dosage of 125 and 250 μg/body of the nanomaterials. The animals were euthanized and studied 24 h after the intratracheal administration with BAL, pulmonary vascular permeability of water and protein, protein levels of cytokines and chemokines in the lung tissue supernatants and the sera, and parameters related to coagulatory system. In protocol 2, mice were divided into 8 experimental groups. The vehicle group received above mentioned vehicle once a week for 6 wk. The OVA group received 1 μg of OVA (Grade IV: Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 2 wk for 6 wk (Fig. 1). The latex nanomaterial groups received (50 or) 100 μg of above mentioned three sizes of latex nanomaterials suspended in the same vehicle every week for 6 wk. The OVA+latex nanomaterial groups received the combined treatment in the same protocol as the OVA and the latex nanomaterial groups (n=12–13 in each group; n=8 for BAL with ELISA for lung homogenates, n=4–5 for lung histology, and n=12–13 for ELISA for serum Ig titers in experiments using 100 μg of latex nanomaterials). Vehicle, latex nanomaterials, OVA, or OVA+latex nanomaterials were suspended in 0.1-ml aliquots, and inoculated once by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical Industries). We have previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on allergic airway inflammation at a dose of 50 or 100 μg/animal in vivo (Yanagisawa et al., 2006; Inoue et al., 2005; Inoue et al., 2006c). Based on and referred to the previous studies from our laboratory, we chose the dosage of 50 and 100 μg/body of the nanomaterials. The animals were euthanized and studied 24 h after the final intratracheal administration, with BAL, lung histology, protein levels of cytokines and chemokines in the lung tissue supernatants and Ig production. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals according to Institutional Animal Care and Use Committee (IACUC: www.iacuc.org). All animal studies were approved by the Institutional Review Board.

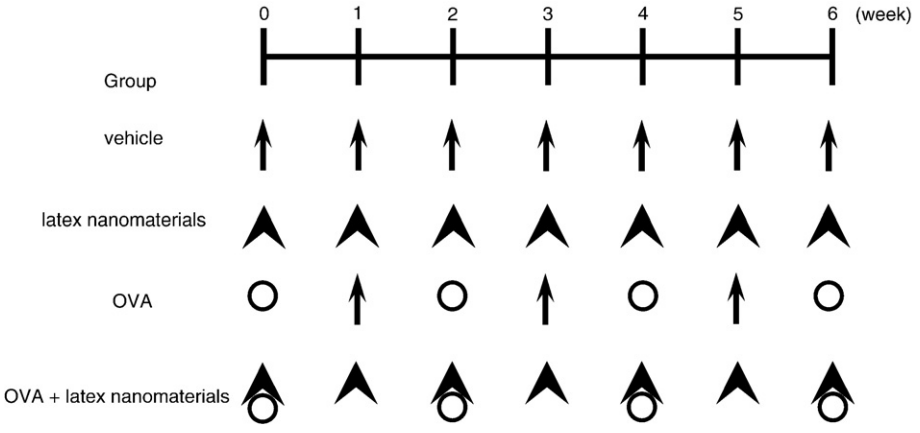

Fig. 1. Study design for protocol 2.

BAL process. BAL and cell counts were conducted as previously described (n=8 in each group at each protocol; Inoue et al., 2006a; Inoue et al., 2006b; Inoue et al., 2006c; Takano et al., 1997; Takano et al., 2002; Yanagisawa et al., 2003). After the BAL procedure, supernatants were analyzed for protein assay (n=8 in each group), and the lungs were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until assay of cytokines and chemokines.

Pulmonary vascular permeability. In protocol 1, in a separate series of experiments, the lungs were weighed and dried. The wet lung weight−the dry lung weight/body weight was calculated (ref [Ichinose et al., 1995]: n=8 in each group). In another experiment, protein concentrations in BAL fluid were determined using the commercially available Bradford protein assay (Bio-Rad Laboratory Inc., Hercules, CA: n=8 in each group) with bovine serum albumin as the standard.

Lung histology. In another experiment, the lungs were fixed and stained with hematoxylin and eosin as previously described (Takano et al., 1997; Inoue et al., 2006a). For semi-quantitative assessment in protocol 1, neutrophil infiltration was assessed by averaging the number of neutrophils enumerated in 30 randomly selected high power fields (HPFs; ×600) in each slide. Histologic sections were evaluated in a blind fashion (n=4–5 in each group at each protocol).

Quantitation of cytokine and chemokine protein levels in the lung tissue supernatants. The frozen lungs after BAL were homogenized in each protocol as described previously (Takano et al., 1997; Inoue et al., 2004; Inoue et al., 2005). ELISA for interleukin (IL)-1β(R&D Systems, Minneapolis, MN), IL-5 (Endogen, Cambridge, MA), IL-13 (R&D Systems), IL-18 (MBL, Nagoya, Japan), macrophage chemoattractant protein (MCP)-1 (R&D Systems), and keratinocyte-derived chemoattractant (KC: R&D Systems), eotaxin (R&D systems), and macrophage inflammatory protein (MIP)-1α (R&D Systems) in the lung tissue homogenates were conducted according to the manufacturer's instructions and the values were expressed as pg or ng/ml total lung supernatants (n=8 in each group at each protocol).

Assays for circulatory levels of fibrinogen, von Willebrand factor (vWF), IL-1β, MCP-1, and KC, and activity of protein C (PC). In protocol 1, citrate plasma levels of fibrinogen and vWF, and the activity of PC (n=8 in each group) were determined using commercial kits (Diagnostica Stago, Roche, Tokyo, Japan) on STA Compact (Diagnostica Stago, Roche) as described previously (Inoue et al., 2004). Serum samples (n=10 in each group) were analyzed by ELISA for IL-1β, MCP-1, and KC (R&D Systems) according to the manufacturer's instructions as described above, and the values were expressed as pg or ng/ml.

Allergen-specific Ig determination. In protocol 2, blood was retrieved by cardiac puncture. Serum was prepared and frozen at −80 °C until assayed for allergen-specific IgG_1 and IgE. Allergen-specific IgG_1-antibody was measured by ELISA with solid-phase allergen as previously described (Inoue et al., 2005; Inoue et al., 2007: n=12–13 in each group). Allergen-specific IgE antibody was measured by commercial ELISA kit (Dainippon Sumitomo Pharmaceutical Co. Osaka, Japan) according to the manufacturer's instructions (n=12–13 in each group).

Statistical analysis. Data are reported as means±SE. Differences between groups were determined using analysis of variance (ANOVA: Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant (P<0.05) using one-way ANOVA, the Bonferroni correction was used for multiple comparison.

Results

Effects of latex nanomaterials on LPS-related lung inflammation and pulmonary vascular permeability

We examined the cellular profile of BAL fluid 24 h after the intratracheal instillation in protocol 1 and exhibited representative data (250 μg/animal of latex nanomaterials used: Table 1). Latex nanomaterials (25 or 50 nm) alone increased the number of neutrophils as compared with vehicle (P<0.01 for 25 or 50 nm latex nanomaterial). LPS exposure significantly increased the number as compared with vehicle exposure (P<0.01). The numbers in the LPS+25 or 50 nm latex nanomaterial group were identical with those in the LPS group, but greater than those in the corresponding latex nanomaterial groups (P<0.01). In contrast, the numbers were significantly smaller in the LPS+100 nm latex nanomaterial group than in the LPS group (P<0.05). Next, we examined the protein levels in BAL fluid and the lung water content 24 h after the intratracheal instillation (Table 1). Pulmonary exposure to latex nanomaterials elevated the protein level in BAL fluid (P<0.05 for 25 nm latex nanomaterial) and lung water content (P<0.01 for 25 or 50 nm latex nanomaterial, P<0.05 for 100 nm latex nanomaterial) as compared with exposure to vehicle. These values were significantly greater in the LPS group than in the vehicle group (P<0.01). These values were

Table 1
The number of neutrophils in bronchoalveolar lavage (BAL) fluid and lung histology, protein concentration in BAL fluid, and lung water content after intratracheal challenge (protocol 1)

Treatment	Neutrophils		Protein	Lung wet weight−dry weight [mg]/body weight [g]
	(×10^4 /total BAL fluid)	(cell number/HPF)	(mg/ml of BAL fluid)	
Vehicle	22.1±4.0	1.6±0.4	0.42±0.03	5.63±0.11
25 nm latex nanomaterial	69.8±12.6**	5.6±1.2*	0.74±0.03*	6.54±0.09**
50 nm latex nanomaterial	65.4±12.5**	7.3±0.8*	0.58±0.03	6.48±0.11**
100 nm latex nanomaterial	26.2±4.8	4.1±0.5*	0.59±0.05	5.93±0.13*
LPS	326.5±88.2**	94.4±20.5**	1.05±0.13**	8.43±0.15**
LPS+25 nm latex nanomaterial	338.2±44.8**,$	166.1±21.7**,##,$	1.46±0.22**,##,$	10.28±0.16**,##,$
LPS+50 nm latex nanomaterial	323.2±16.8**,$	125.5±22.7**,$	1.36±0.13**,#,$	9.61±0.26**,##,$
LPS+100 nm latex nanomaterial	212.7±34.6**,#,$	101.2±12.4**,$	1.05±0.08**,$	8.93±0.27**,#,$

Results are expressed as mean±SE (n=8 in each group for neutrophil number and protein concentration in BAL and lung water content, n=4–5 in each group for neutrophil number in lung parenchyma).
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
\# P<0.05 versus the LPS group.
\#\# P<0.01 versus the LPS group.
$ P<0.01 versus the nanomaterial group.

Table 2
Protein levels of interleukin (IL)-1β and chemokines in the lung tissue supernatants after intratracheal challenge (protocol 1)

Treatment	IL-1β	MCP-1	KC
	(ng/total lung supernatants)	(pg/total lung supernatants)	
Vehicle	0.5±0.0	16.4±4.0	59.8±13.3
25 nm latex nanomaterial	1.2±0.1*	111.9±13.3**	34.8±8.2
50 nm latex nanomaterial	1.3±0.2*	83.2±14.0**	59.8±11.3
100 nm latex nanomaterial	0.8±0.1	48.7±7.4*	43.9±12.1
LPS	41.5±4.4**	1201.5±102.5**	445.4±116.1**
LPS+25 nm latex nanomaterial	77.2±10.8**,#,$	2714.1±346.6**,##,$	1391.2±231.5**,##,$
LPS+50 nm latex nanomaterial	55.7±4.8**,#,$	2305.6±292.9**,##,$	1475.3±231.1**,##,$
LPS+100 nm latex nanomaterial	37.4±4.3**,$	1438.1±268.0**,$	668.0±164.3**,$

Results are expressed as mean±SE (n=8 in each group).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\#\# $P<0.01$ versus the LPS group.
$ $P<0.01$ versus the nanomaterial group.

further greater in the LPS+latex nanomaterial groups than in the LPS ($P<0.05$ for the LPS+50 nm latex nanomaterial group on the protein level of BAL fluid, and for the LPS+100 nm latex nanomaterial group on the lung water content; $P<0.01$ for the LPS+25 nm latex nanomaterial group on the protein level of BAL fluid and the lung water content, and for the LPS+50 nm latex nanomaterial group on the lung water content) or the corresponding latex nanomaterial ($P<0.01$) groups. Whereas in experiments using 125 μg/animal of latex nanomaterials, the data are similar as that using 250 μg/animal, although the significance did not show profoundly (data not shown).

Effects of latex nanomaterials on LPS-related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the intratracheal instillation in protocol 1. No pathological change was seen in the lung obtained from the vehicle group. Infiltration of neutrophils was moderately seen in the lungs from the latex nanomaterial groups and moderately seen in those from the LPS groups. Combined treatment with LPS and latex nanomaterials markedly enhanced leukocyte (mainly neutrophil) sequestration into the lung parenchyma as compared with LPS treatment alone, especially with smaller latex nanomaterials with overall trend. Furthermore, we determined the number of neutrophils in the lung (Table 1). Latex alone increased the number as compared with vehicle ($P<0.05$). LPS challenge significantly increased the number as compared with vehicle challenge ($P<0.01$). The number was further greater in the LPS+latex nanomaterial groups than in the LPS group ($P<0.01$ for LPS+25 nm latex nanomaterial) or the corresponding latex nanomaterial groups ($P<0.01$).

Effects of latex nanomaterials on the expression of LPS-related proinflammatory cytokine and chemokines in the lung

We next measured the protein levels of IL-1β, MCP-1, and KC in the lung tissue supernatants 24 h after the intratracheal instillation in protocol 1 (Table 2). Pulmonary exposure to latex nanomaterials alone elevated the levels of IL-1β ($P<0.05$ for 25 or 50 nm latex nanomaterial) and MCP-1 ($P<0.05$ for 100 nm latex nanomaterial; $P<0.01$ for 25 or 50 nm latex nanomaterial) as compared to that to vehicle. LPS challenge significantly elevated the levels of all of these proteins as compared with vehicle challenge ($P<0.01$). The levels were further greater in the LPS+25 or 50 nm latex nanomaterial group than in the LPS ($P<0.05$ for the IL-1β level; $P<0.01$ for the MCP-1 or the KC) or the corresponding latex nanomaterial ($P<0.01$) groups.

Effects of latex nanomaterials on LPS-related coagulatory parameters

We next analyzed coagulatory parameters 24 h after the intratracheal challenge in protocol 1 (Table 3). LPS challenge significantly elevated the fibrinogen level as compared to vehicle challenge ($P<0.01$). The level was further greater in the LPS+25 or 50 nm latex nanomaterial group than in the LPS group ($P<0.05$ for the LPS+50 nm latex nanomaterial group; $P<0.01$ for the LPS+25 nm latex nanomaterial group) or the corresponding latex nanomaterial groups ($P<0.01$). As compared to vehicle challenge, LPS challenge caused a significant increase in the level of vWF ($P<0.05$). The level was further greater in the LPS+25 nm ($P<0.01$) or the LPS+50 nm ($P<0.05$) latex nanomaterial group than in the LPS group or the corresponding latex nanomaterial groups ($P<0.01$). LPS significantly decreased the activity of PC as compared with vehicle ($P<0.01$). The activity was significantly lower in the LPS+latex nanomaterial groups than in the corresponding latex nanomaterial groups ($P<0.05$).

Effects of latex nanomaterials on circulatory levels of LPS-related proinflammatory cytokine and chemokines

We measured the protein levels of IL-1β, MCP-1, and KC in the sera 24 h after the intratracheal instillation in protocol 1 (Table 4). LPS challenge significantly elevated the level of IL-1β as compared with

Table 3
Plasma coagulatory parameters after intratracheal instillation (protocol 1)

Treatment	Fibrinogen	vWF	Activity of PC
	(mg/dl)	(%)	
Vehicle	336.3±6.2	73.9±6.7	6.1±0.4
25 nm latex nanomaterial	366.0±6.5	72.1±3.0	5.0±0.2
50 nm latex nanomaterial	350.3±18.2	72.8±1.1	5.1±0.1
100 nm latex nanomaterial	345.7±10.4	64.8±9.5	5.6±0.3
LPS	592.3±26.4**	87.9±6.2*	3.5±0.3**
LPS+25 nm latex nanomaterial	797.8±40.6**,##,$$	97.5±5.9**,##,$$	3.3±0.3**,$
LPS+50 nm latex nanomaterial	687.3±54.1**,#,$$	92.3±5.0**,#,$$	3.3±0.3**,$
LPS+100 nm latex nanomaterial	585.3±65.5**,$$	84.9±4.5*,$$	3.6±0.3**,$

Results are expressed as mean±SE (n=8 in each group).
* $P<0.05$ versus the vehicle group.
** $P<0.01$ versus the vehicle group.
\# $P<0.05$ versus the LPS group.
\#\# $P<0.01$ versus the LPS group.
$ $P<0.05$ versus the nanomaterial group.
$$ $P<0.01$ versus the nanomaterial group.

Table 4
Circulatory levels of interleukin (IL)-1β and chemokines after intratracheal instillation (protocol 1)

Treatment	IL-1β	MCP-1	KC
	(ng/ml)	(pg/ml)	
Vehicle	5.7±1.2	12.9±1.5	59.8±13.3
25 nm latex nanomaterial	4.7±0.9	21.4±4.3	34.8±8.2
50 nm latex nanomaterial	4.5±0.9	24.2±2.6*	59.8±11.3
100 nm latex nanomaterial	2.8±0.9	23.6±2.5*	43.9±12.1
LPS	9.2±1.8*	191.1±29.9**	445.4±116.1**
LPS+25 nm latex nanomaterial	11.7±1.9**,$$	504.9±74.3**,##,$$	1391.2±231.5**,##,$$
LPS+50 nm latex nanomaterial	13.5±2.0**,#,$$	687.1±71.7**,##,$$	1475.3±231.1**,##,$$
LPS+100 nm latex nanomaterial	4.5±1.2$	313.1±73.2**,#,$$	668.0±164.3**,$$

Results are expressed as mean±SE (n=10 in each group).
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
\# P<0.05 versus the LPS group.
\#\# P<0.01 versus the LPS group.
$ P<0.05 versus the nanomaterial group.
$$ P<0.01 versus the nanomaterial group.

vehicle challenge (P<0.05). The level was further greater in the LPS+25 nm or the LPS+50 nm latex nanomaterial group than in the LPS (P<0.05 for LPS+50 nm latex nanomaterial) or the corresponding latex nanomaterial groups (P<0.01). Latex nanomaterial (50 and 100 nm) challenge significantly elevated MCP-1 level (P<0.05 vs. vehicle). LPS challenge also significantly elevated the levels of MCP-1 and KC as compared with vehicle challenge (P<0.01). The levels were further greater in the LPS+latex nanomaterial groups than in the LPS (P<0.01 for LPS+25 or 50 nm latex nanomaterial group) or the corresponding latex nanomaterial groups (P<0.01).

Effects of latex nanomaterials on allergic airway inflammation

We investigated the cellular profile of BAL fluid in protocol 2 and exhibited representative data (100 μg/animal of latex nanomaterials used: Fig. 2). The number of total cells was significantly greater in the latex nanomaterial or the OVA+latex nanomaterial groups than in the vehicle group (P<0.05 for 50 nm latex nanomaterial, P<0.01 for 25 nm latex nanomaterial and OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA group (P<0.01 for OVA+25 or 50 nm latex nanomaterial). The number of macrophages was greater in the latex nanomaterial or the OVA+latex nanomaterial groups than in the vehicle group (P<0.05 for 25 nm latex nanomaterial and OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA group (P<0.05 for OVA+25 nm latex nanomaterial; P<0.01 for OVA+50 nm latex nanomaterial). Exposure to OVA or OVA+latex nanomaterials significantly increased the number of eosinophils as compared with vehicle exposure (P<0.05 for OVA or OVA+50 nm latex nanomaterial; P<0.01 for OVA+25 or 100 nm latex nanomaterial). However, the numbers were comparable among the OVA and OVA +latex nanomaterial groups. The number of lymphocytes was greater in the 25 nm latex nanomaterial group or the OVA+25 or 50 nm latex nanomaterial group than in the vehicle group (P<0.01 for 25 nm latex nanomaterial or OVA+25 or 50 nm latex nanomaterial). The number was greater in the OVA+latex nanomaterial groups than in the OVA (P<0.01) or the corresponding latex nanomaterial groups (P<0.05 for OVA+50 nm latex nanomaterial). The number of neutrophils was greater in the 25 and 50 nm latex nanomaterial groups and the OVA +25 or 50 nm latex nanomaterial group than in the vehicle group (P<0.01). The number was greater in the OVA+latex nanomaterial groups than in the OVA or the corresponding latex nanomaterial groups (P<0.01 for OVA+25 or 50 nm latex nanomaterial). Whereas in experiments using 50 μg/animal of latex nanomaterials, the data are similar as that using 100 μg/animal, although the significance did not show profoundly (data not shown).

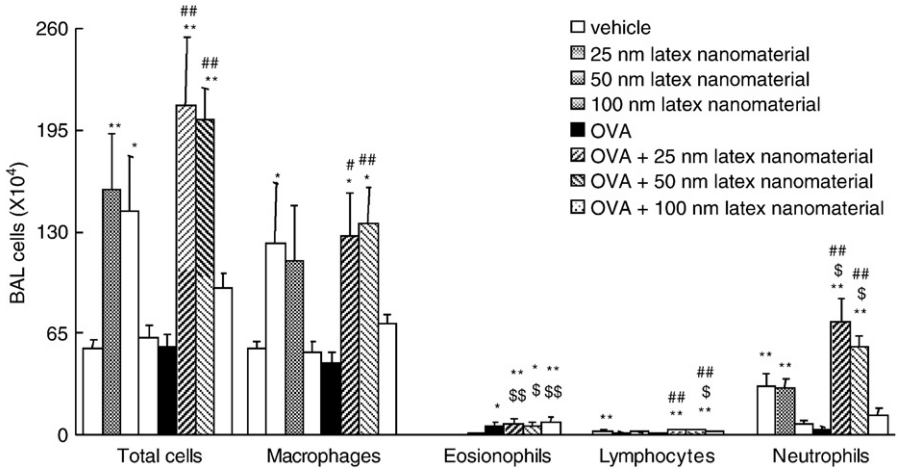

Fig. 2. Cellularity in BAL fluid after intratracheal challenge (protocol 2). Twenty four hours after the final intratracheal administration, lungs were lavaged for the analysis of BAL fluid. Count for total cells was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations stained with Diff-Quik. Results are means±SE (n=8 in each group). *P<0.05 versus the vehicle group, **P<0.01 versus the vehicle group, #P<0.05 versus the OVA group, ##P<0.01 versus the OVA group, $$P$<0.05 versus the nanomaterial group, $$$P$<0.01 versus the nanomaterial group.

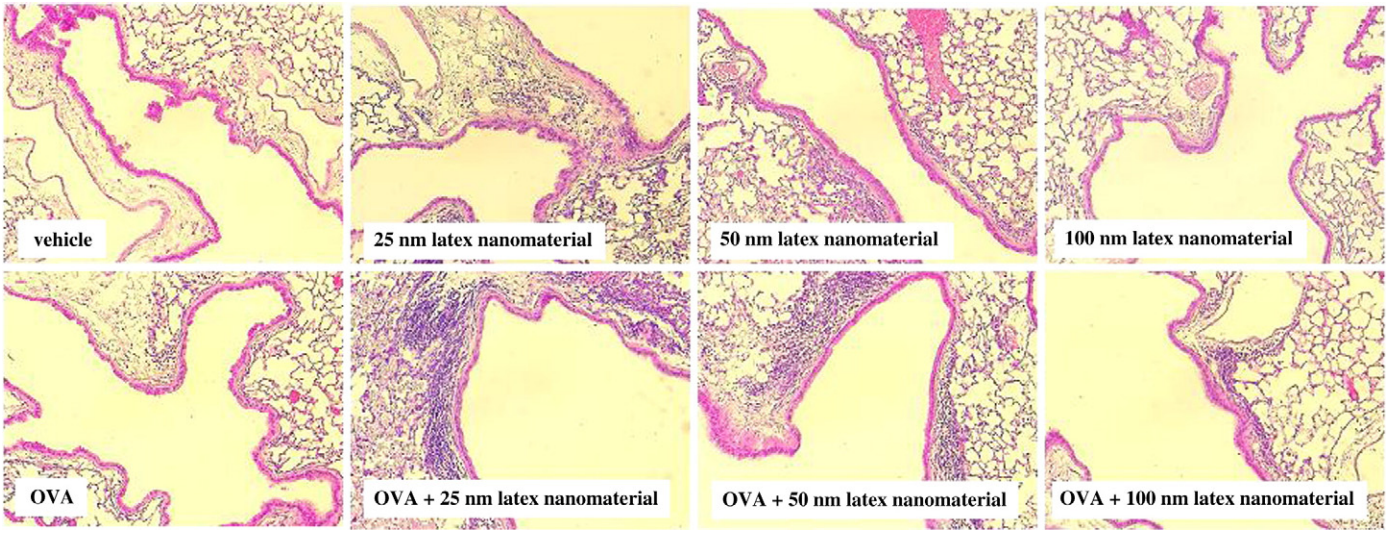

Fig. 3. Representative histological findings of the hematoxylin and eosin-stained lung obtained from the vehicle, latex nanomaterial, OVA, or OVA+latex nanomaterial groups 24 h after the final intratracheal administration. Animals received intratracheal instillation of vehicle, latex nanomaterials, OVA, or OVA+latex nanomaterials for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration (n=4–5 in each group). Original magnification ×25.

Effects of latex nanomaterials on allergen — related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the final intratracheal instillation in protocol 2 (Fig. 3). No pathological change was seen in the lung obtained from the vehicle group. Infiltration of neutrophils was slightly seen in the lungs from the latex nanomaterial groups. On the other hand, infiltration of eosinophils was moderately observed in the lung from the OVA group. Combined treatment with OVA and latex nanomaterials apparently worsened polymorphonuclear leukocyte (mainly neutrophil) sequestration into the lung parenchyma as compared with OVA treatment alone, especially with smaller latex nanomaterials with overall trend.

Effects of latex nanomaterials on local expression of cytokines and chemokines in the presence of allergen

We quantitated protein levels of IL-5, IL-13, IL-18 (Table 5), eotaxin, MIP-1α, MCP-1, and KC (Table 6) in the lung tissue supernatants. OVA challenge increased the level of IL-5 and IL-13 as compared with vehicle challenge (P<0.01). The levels were not significantly different between the OVA and the OVA+latex nanomaterial groups. The level of IL-18 was significantly greater in the latex nanomaterial groups and the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The level was further greater in the OVA+latex nanomaterial groups than in the OVA group (P<0.01). The level of eotaxin was significantly greater in the OVA and the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The level was significantly greater in the OVA+latex nanomaterial groups than in the corresponding latex nanomaterial groups (P<0.01). The level was not significantly different among the OVA and the OVA+latex nanomaterial groups. The level of MIP-1α was greater in the latex nanomaterial groups (P<0.01) or the OVA group (P<0.05) than in the vehicle group. The level was further greater in the OVA+latex nanomaterial groups than in the vehicle (P<0.01), the OVA (P<0.01 for OVA+25 or 50 nm latex nanomaterial; P<0.05 for OVA+100 nm latex nanomaterial), or the corresponding latex nanomaterial groups (P<0.05 for OVA+25 nm latex nanomaterial). The level of MCP-1 was also greater in the latex nanomaterial groups (P<0.01) or the OVA group (P<0.05) than in the vehicle group. The level was further greater in the OVA+latex nanomaterial groups than in the vehicle, the OVA, or the corresponding latex nanomaterial groups (P<0.01). The level of KC was greater in the latex nanomaterial groups than in the vehicle group (P<0.05 for 25 or 50 nm latex nanomaterial). The level was further greater in the OVA+latex nanomaterial groups than in the vehicle (P<0.01 for OVA+25 or 50 nm latex nanomaterial; P<0.05 for OVA+100 nm latex

Table 5
Protein levels of cytokines in the lung tissue supernatants after the final intratracheal challenge (protocol 2)

Treatment	IL-5	IL-13	IL-18
	(pg/ml)		
Vehicle	13.0±1.1	3.5±2.7	828.0±31.9
25 nm latex nanomaterial	12.6±0.6	3.7±1.1	2227.5±178.3**
50 nm latex nanomaterial	17.3±2.1	2.2±1.5	2196.0±201.1**
100 nm latex nanomaterial	12.6±1.0	4.1±2.2	1391.5±116.0*
OVA	120.7±32.2**	143.9±37.5**	749.1±73.4
OVA+25 nm latex nanomaterial	118.9±35.2**,$$	89.1±31.4**,$$	2786.1±283.9**,##
OVA+50 nm latex nanomaterial	150.3±19.8**,$$	139.8±41.2**,$$	2068.9±182.8**,##
OVA+100 nm latex nanomaterial	106.1±29.2**,$$	92.9±26.2**,$$	1719.4±136.8**,##

Results are expressed as mean±SE (n=8 in each group).
#P<0.05 versus the OVA group.
$$P$<0.05 versus the nanomaterial group.
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
P<0.01 versus the OVA group.
$$ P<0.01 versus the nanomaterial group.

Table 6
Protein levels of chemokines in the lung tissue supernatants after the final intratracheal challenge (protocol 2)

Treatment	Eotaxin	MIP-1α	MCP-1	KC
	(pg/ml)			
Vehicle	74.7±5.0	2.0±1.4	25.2±9.5	156.7±113.9
25 nm latex nanomaterial	111.2±7.4	237.9±16.6**	363.1±46.2**	505.2±73.0*
50 nm latex nanomaterial	126.9±12.3	188.0±21.7**	314.9±44.2**	510.1±44.8*
100 nm latex nanomaterial	108.4±10.6	73.3±11.1**	126.7±13.9**	237.5±28.0
OVA	513.2±116.4**	16.3±4.5*	94.7±15.0*	142.4±40.4
OVA+25 nm latex nanomaterial	522.7±177.2**,$$	496.0±71.9**,##,$	830.4±143.6**,##,$$	934.5±180.4**,##,$$
OVA+50 nm latex nanomaterial	828.1±175.0**,$$	206.2±24.6**,##	855.1±150.6**,##,$$	802.5±149.8**,##
OVA+100 nm latex nanomaterial	877.0±357.5**,$$	66.7±17.0**,#	364.6±139.8**,##,$$	342.0±110.5#

Results are expressed as mean±SE (n=8 in each group).
* P<0.05 versus the vehicle group.
** P<0.01 versus the vehicle group.
\# P<0.05 versus the OVA group.
\#\# P<0.01 versus the OVA group.
$ P<0.05 versus the nanomaterial group.
$$ P<0.01 versus the nanomaterial group.

nanomaterial), the OVA (P<0.01 for OVA+25 or 50 nm latex nanomaterial), or the corresponding latex nanomaterial groups (P<0.01 for OVA+25 nm latex nanomaterial).

Effects of latex nanomaterials on allergen-specific production of IgG_1 and IgE

We measured allergen-specific IgG_1 and IgE (Fig. 4). The allergen-specific IgG_1 and IgE were significantly greater in the OVA or the OVA+latex nanomaterial groups than in the vehicle group (P<0.01). The titers of both IgG_1 and IgE were comparable among the OVA and the OVA+latex nanomaterial groups.

Discussion

The present study has demonstrated that latex nanomaterials instilled intratracheally enhance neutrophilic lung inflammation with pulmonary vascular permeability related to LPS resulted from activated innate immunity. The enhancement is concomitant with the increased local (lung) expression of proinflammatory cytokine

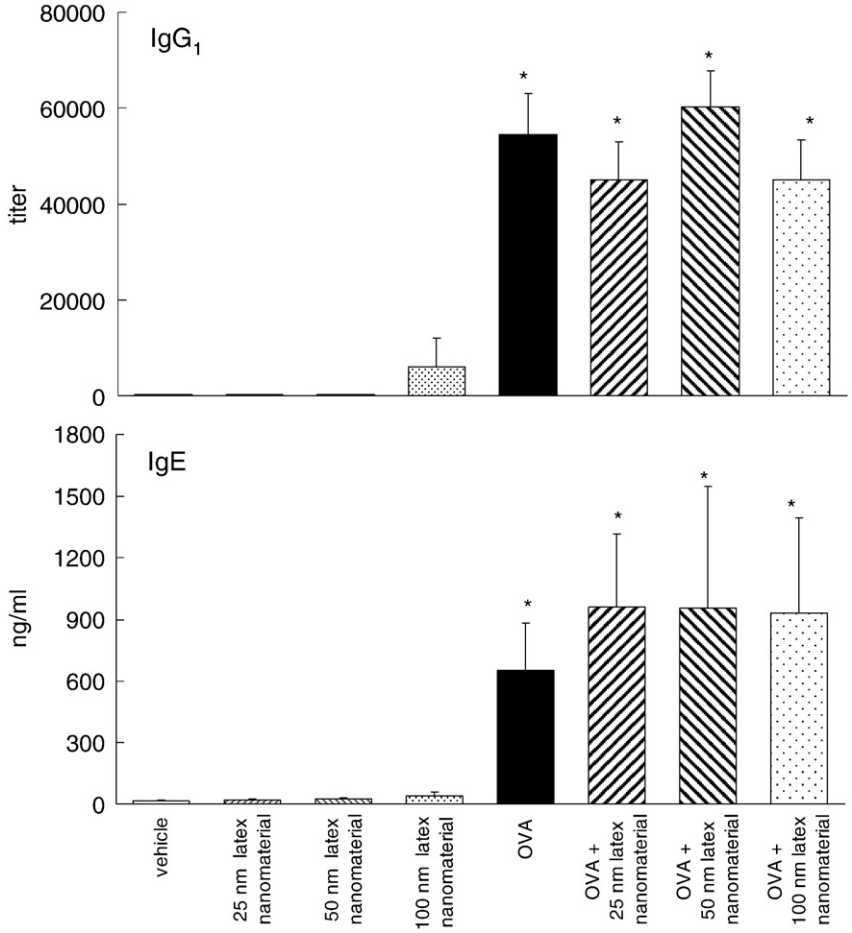

Fig. 4. Allergen-specific IgG_1 and IgE titers. Eight groups of mice were intratracheally administered vehicle, latex nanomaterials, OVA, or the combination of OVA+latex nanomaterials for 6 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_1 and IgE were analyzed using ELISA. Results are expressed as mean±SE (n=12–13 in each group). *P<0.01 versus vehicle.

such as IL-1β, and chemokines such as MCP-1 and KC. In addition, combined challenge with LPS and nanomaterials further increases circulatory levels of fibrinogen, vWF, IL-1β, MCP-1, and KC, as compared with challenge with LPS alone. The enhancing effects of nanomaterials on the pathology tend to be greater with the smaller nanomaterials than with larger ones in overall trend. In contrast, repetitive exposure to the latex nanomaterials does not significantly exacerbate the hallmarks of allergic airway inflammation characterized by eosinophilic lung inflammation with Ig productions predominant consequence of activated adaptive immunity.

Regarding the effects of PM including nanoparticles on LPS-related lung inflammation, we have previously demonstrated that DEP, important constituents in PM, enhance the lung inflammation (Takano et al., 2002; Yanagisawa et al., 2003) and the accompanying coagulatory disturbance (Inoue et al., 2006a) using a similar protocol to that in the present study. Furthermore, we have demonstrated that carbon nanoparticles, used as a relevant type of ambient PM, also deteriorate the pathophysiology (Inoue et al., 2006b). In that study, smaller (14 nm) nanoparticles have more markedly aggravated the features than larger (56 nm) ones (Inoue et al., 2006b). On the other hand, previous reports have shown the full impact, or even partial impact, of manufactured nanomaterials on health and the environment (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Hardman, 2006; Nel et al., 2006). For example, a single pulmonary exposure to nano TiO_2 particles (19–21 nm) reportedly induces emphysema-like lung injury (Chen et al., 2006). Also, single-walled carbon nanotubes (1–4 nm in a diameter) instilled through the airways (pharynges) induce lung inflammation, which is characterized by fibrogenic changes with granuloma formation (Shvedova et al., 2005). However, all these studies have examined the effects of (nano) materials on physical (normal) conditions. The present results have, for the first time to our knowledge, shown that exposure to latex nanomaterials can additively/synergistically augment both local (lung) and systemic inflammation with coagulatory disturbance related to LPS, and that the effects are greater with the smaller materials than with the larger ones with overall trend, which is similar to the effects of carbonaceous nanoparticles found in our previous study (Inoue et al., 2006b).

Mechanistically, the pathogenesis of acute lung inflammation reportedly involves amplified lung expression of proinflammatory cytokines including IL-1β and chemokines such as IL-8 and MCP-1 (Standiford et al., 1995; Martin, 1999; Puneet et al., 2005). In our previous studies, indeed, we have confirmed the lung expression of proinflammatory cytokine and chemokines including IL-1β, MCP-1, and KC in the lung 24 h after the intratracheal administration of LPS, DEP, washed DEP, or carbon nanoparticles is concomitant with the aggravated lung injury (Takano et al., 2002; Sanbongi et al., 2003; Yanagisawa et al., 2003; Inoue et al., 2006b). In the present study, likewise, the lung expression of these molecules paralleled the lung inflammation with overall trend (Tables 2 and 4). Thus, the exaggerating effects of latex nanomaterials on this lung inflammation might be mediated, at least in part, through the enhanced lung expression of IL-1β, MCP-1, and KC (a murine homologue of IL-8).

Nano-sized particles and materials are reportedly able to penetrate deeply into the respiratory tract and may even pass the lung to reach the systemic circulation (MacNee and Donaldson, 2000; Nemmar et al., 2001), implying that they can affect the circulatory system in the context of systemic inflammation with coagulopathy. In accord with these studies, we have previously demonstrated that pulmonary exposure to carbon nanoparticles enhance systemic inflammation with coagulatory disturbance in the same model in the present study (Inoue et al., 2006b). In the present study, LPS combined with nanomaterials, specifically with those with a diameter of less than 50 nm, significantly elevated the levels of fibrinogen, MCP-1, and KC compared to LPS alone. Additionally, the elevated level of vWF induced by LPS was further increased by the combination of LPS with nanomaterials, especially with latex with a diameter of less than 50 nm. These findings suggest that pulmonary exposure to nanomaterials, in particular to smaller ones, can directly/indirectly facilitate systemic inflammation with coagulatory disturbance accompanied by lung inflammation, like combustion-derived nanoparticles (Inoue et al., 2006b), although evidence of translocation remains unaddressed.

Overall, the (above exhibited) response to 25 or 50 nm (and partial one to 100 nm) latex nanomaterials plus LPS was almost greater than the sum of the individual responses. Thus, it is noted that these observations could be considered as synergistic effects of two inflammation-inducing agents such as latex nanomaterials and LPS.

Independently, we have previously reported that PM including DEP and carbon nanoparticles experimentally enhance allergic asthma, another lung inflammation, characterized by eosinophilic inflammation and airway hyperresponsiveness (Takano et al., 1997; Ichinose et al., 1998; Takano et al., 1998; Inoue et al., 2005; Inoue et al., 2006c), rising the possibility that nano-leveled materials can act on the pathophysiology with similar fashion to PM. In fact, TiO_2 nanomaterials (14–29 nm in size) have more prominent adjuvant effects on allergen-specific response with Ig production than fine (250–260 nm in size) ones in vivo (de Haar et al., 2006). In the present study, however, repetitive exposure to latex nanomaterials did not exacerbate the murine model of allergic asthma in the context of eosinophilic lung inflammation and Ig production in the same protocol as our previous studies (Takano et al., 1997; Inoue et al., 2005; Yanagisawa et al., 2006). The reason of this different observation from our previous ones remains puzzled. It is possible that the differences in facilitating effects on this condition between PM and latex nanomaterials might be due to those in characteristics of the materials (particles), in particular, on their surface. In support of this concept, we have shown that chemical components extracted from DEP augment this model in the same protocol as the present study (Hiyoshi et al., 2005; Yanagisawa et al., 2006; Inoue et al., 2007). Alternate, the high absorption activity of PM, which is a characteristic feature of carboneous materials might be important for the deterioration of allergic lung inflammation.

On the other hand, in the present study, the latex nanomaterials apparently exacerbated neutrophilic lung inflammation also related to allergen in a size dependent-manner. Besides, they clearly amplified lung expression of KC, a potent chemotactic factor for neutrophils, and MIP-1α, also chemotactic factors for various leukocytes, in paralleled with the neutrophilic lung inflammation. These results may suggest that latex nanomaterials mainly induce/enhance neutrophilic inflammation in vivo. To date, neutrophilic asthma has been recognized to be a distinct phenotype of asthma (Gibson et al., 2001; Douwes et al., 2002; Simpson et al., 2006; Macdowell and Peters, 2007; Simpson et al., 2008). Thus, latex nanomaterials may relate to development/exacerbation of this type of asthma. The observation that lung expression level of IL-18 was paralleled to neutrophilic lung inflammation in the present study should support the concept, since IL-18 reportedly activates neutrophils in vitro (Leung et al., 2001) and induces neutrophil-dominant allergic airway inflammation in vivo (Sugimoto et al., 2004).

In conclusion, this study demonstrated that pulmonary exposure to latex nanomaterials enhanced lung inflammation induced by LPS. The enhancement was mediated through the increased local expression of proinflammatory cytokines and chemokines. The enhancing effects were more prominent with smaller nanomaterials than with larger ones in overall trend. Smaller nanomaterials also enhanced systemic inflammation and coagulatory disturbance accompanying the lung inflammation. Conversely, the nanomaterials did not exacerbate eosinophilic lung inflammation but did neutrophilic lung inflammation related to allergen. These results suggest that nanomaterials can differentially facilitate two types of lung inflammation (innate- or adaptive immunity-dominant). These effects of nanomaterials should

shed light on the understanding of nanotoxicity, especially for sensitive populations with lung inflammation.

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