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Ultrafine but not fine particulate matter causes airway inflammation and allergic airway sensitization to co-administered antigen in mice

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Clinical and Experimental Allergy

Summary

Background Airborne particulate matter (PM) is an important factor associated with the enhanced prevalence of respiratory allergy. The PM adjuvant activity on allergic sensitization is a possible mechanism of action involved, and the induction of airway inflammation is suggested to be of importance in PM-induced adjuvant activity.

Objective Because differently sized PM have different toxic potentials, we studied the role of particle size in the induction of airway inflammation and allergic sensitization. This was done using fine (0.250 and 0.260 µm) and ultrafine (0.029 and 0.014 µm) titanium dioxide (TiO₂) and carbon black particles (CBP) with known differences in airway toxicity.

Methods Mice were intranasally exposed to ovalbumin (OVA) alone or in combination with one of the different particles. The induction of airway inflammation and the immune adjuvant activity were studied in the lungs and lung-draining peribronchial lymph nodes (PBLN) at day 8. OVA-specific antibodies were measured at day 21, and the development of allergic airway inflammation was studied after OVA challenges (day 28).

Results When administered at the same total particle mass (200 µg), exposure to ultrafine TiO₂ and CBP-induced airway inflammation, and had immune adjuvant activity. The latter was shown by increasing both the PBLN cell numbers and the production of OVA-specific T-helper type 2 (Th2) cytokines (IL-4, IL-5, IL-10 and IL-13). Whereas OVA-specific IgE and IgG1 levels in serum were only increased in animals exposed to the ultrafine TiO₂, allergic airway inflammation could be detected in both ultrafine TiO₂- and CBP-treated groups after challenges with OVA.

Conclusion Our data show that only the ultrafine particles, with a small diameter and a large total surface area/mass, cause airway inflammation and have immune adjuvant activity in the current model supporting the hypothesis that particle toxicity is site-dependent and related to adjuvant activity.

Keywords adjuvant, airway, allergic, inflammation, intranasal, mouse, particulate matter, sensitization, ultrafine particles

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Introduction

Epidemiological studies have provided evidence that exposure to ambient particulate matter is associated with adverse health effects [1]. These adverse health effects consist of pulmonary [2] as well as cardio-vascular diseases [3, 4], and lead to increased morbidity [5] and mortality [6, 7]. Diesel exhaust particles (DEP) are among the most important and well-studied examples of ambient

particulate matter (PM) and these, but also other ambient PM, usually consist of a mixture of particles with varying physicochemical properties like: size, surface area, surface charge and chemical composition. Which of these characteristics are most important in PM-induced health effects, and the underlying mechanisms are still not clear.

Epidemiological and laboratory studies have shown that pulmonary health effects resulting from PM exposure include airway inflammation [8, 9], increased allergic sensitization [10–12] and exacerbation of asthmatic symptoms [13, 14]. For instance, it is shown that DEP are able to increase sensitization against antigens in human

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subjects [11], whereas we [15] and others [16–18] have demonstrated that various particles are able to induce allergic sensitization against co-administered ovalbumin (OVA). Importantly, allergic sensitization occurred only at doses that also induced airway inflammation [15], which is in line with experimental data that indicate that the induction of oxidative stress and/or inflammation is involved in this particle adjuvant activity [18, 19]. In particular, induction of local airway damage and inflammation, and concomitant oxidative stress would provide the danger signals [20] required to fully activate and sensitize the immune system.

In animal models using airway exposure, particles themselves [15, 21] but also attached chemicals such as polycyclic aromatic hydrocarbons [22], transition metals [19, 23], and biologics [24] have been shown to cause airway inflammation. Others have shown that the proinflammatory effect of particles was size dependent and that compared with fine particles, ultrafine particles, because of their high collective surface area, cause more oxidative stress [25, 26]. Previous airway instillation studies have shown that the ultrafine TiO₂ particles and carbon black particles (CBP) were more toxic than the fine particles of the same type [27] and that the ultrafine particles had a higher potency to damage airway epithelium, impair macrophage functions and induce recruitment of neutrophils [21]. As a corollary, we suggest that particles have size-dependent adjuvant activity on airway-induced immune responses. To test this hypothesis, we have compared fine with ultrafine TiO₂ particles and CBP in a previously developed intranasal adjuvant model [15]. Briefly, because previous data have shown that 200 µg ultrafine CBP caused allergic airway sensitization, mice were intranasally exposed to 200 µg fine or ultrafine CBP and TiO₂ particles in combination with OVA antigen. Airway toxicity, inflammation and allergic immune sensitization were assessed on day 8 after first exposure, and allergic sensitization was further studied after intranasally challenging the animals with OVA.

Methods

Mice

Specific pathogen-free female BALB/cANNCrl mice (6–8 weeks old) were obtained from Charles River (Sulzfeld, Germany) and randomly assigned to specific treatment. Mice were maintained under barrier conditions in filter-topped Macrolon cages with wood chips bedding, at a mean temperature of 23 ± 2 °C, a relative humidity of 50–55% and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were conducted according to the guidelines of the animal experiments committee of the Veterinary Faculty of Utrecht University.

Particle preparation and protein binding

Fine and ultrafine TiO₂ particles and CBP were kindly provided by the group of Ken Donaldson, who have also previously analysed these particles [27]; details are shown in Table 1. The particles were suspended in phosphate-buffered saline (PBS) at a concentration of 3.3 mg/mL, and sonicated using a Branson 1510 Ultrasonic bath (Branson, Danbury, CT, USA) for 2 h.

Ovalbumin (Sigma-Aldrich, Zwijndrecht, the Netherlands) was prepared in PBS and endotoxin was removed using Pierce detoxigel (Perbio Science, Etten-Leur, the Netherlands). Endotoxin levels were 0.6 ng/mg OVA, and no endotoxin could be detected in any of the particles used as determined by an LAL assay (LAL Kinetic-QCL™ Kit, Bio-Whittaker, Walkersville, MD, USA). OVA was then added to the particle suspensions to a final concentration of 0.5 mg/mL. The amount of OVA that was bound to the particles was detected in the supernatant of the particle suspensions using the Pierce BCA kit (Perbio Science), after spinning down the particles for (15 min at 8000 g) (Table 1).

Intranasal sensitization and challenge

Mice were anaesthetized by an intramuscular injection of 40 µL ketamine (25 mg/mL)/xylazine (5 mg/mL), and exposed to 20 µL of OVA (0.5 mg/mL) or particle + OVA (3.3 and 0.5 mg/mL, respectively) in PBS by intranasal droplet application on days 0, 1 and 2 (total dose of 200 µg particles and 30 µg OVA). Mice were killed on day 8 by an overdose of pentobarbital, or challenged to study asthma-like allergic airway inflammation.

Mice were anaesthetized by an intramuscular injection of 40 µL ketamine (25 mg/mL)/xylazine (5 mg/mL) and challenged by intranasal droplet application of 20 µL OVA (0.5 mg/mL) in PBS on days 25, 26 and 27. Mice were killed on day 28 by an overdose of pentobarbital.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by flushing the lungs three times with 1 mL of sterile PBS 24 h after

Table 1. Particle characteristics

Particles	Size (nm)	Surface		Manufacturer	Reference
		Size (nm)	% OVA bound		
ufTiO ₂	29.0	49.8	9	Degussa	[27]
fTiO ₂	250.0	6.6	6	Tioxide td.	[27]
ufCB	14.3	253.9	100	Degussa (printex 90)	[27, 28]
fCB	260.2	7.9	14	H. Haefner & Co. Ltd (Huber 990)	[27, 28]

TiO₂, titanium dioxide; CB, carbon black; uf, ultrafine; f, fine.

the last intranasal exposure. The first lavage was kept separate from the other two. BAL was centrifuged at 900 *g* for 5 min, and the BAL fluid (BALF) from the first lavage was stored at -80°C until further analysis. The cells from all lavages were pooled, total BAL cell numbers were determined using a Coulter counter (Coulter Electronics, Luton, UK) and differential cell count was performed using FACS staining.

Lactate dehydrogenase (LDH), total protein and TNF- α levels were determined in BALF using the TOX-7 kit (Sigma-Aldrich), the Pierce BCA kit (Perbio Science), and the TNF- α ELISA kit (Biosource Europe, Fleurus, Belgium), respectively, according to the manufacturer's protocols.

Isolation and culture of lymph node cells

Peribronchial lymph nodes (PBLN) were isolated and minced using frosted objective slides. Single-cell suspensions were taken up in complete RPMI 1640 with Glutamax-I (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin.

Cell suspensions were plated in round bottom 96-well plates (2×10^{-6} cells/mL) and restimulated with 100 μg OVA for 4 days. Levels of IL-4, IL-5, IL-10 and IFN- γ in culture supernatants were measured by ELISA (BD Pharmingen, Hamburg, Germany) according to the manufacturer's protocol.

Flow cytometry

Cells from BAL were stained with fluorescein isothiocyanate (FITC)-labelled anti-GR-1, PE-labelled anti-B220 and APC-labelled anti-CD11c. Cells from PBLN were stained with FITC-labelled anti-CD4, PE-labelled anti-CD8, PerCP-labelled anti-CD3 and APC-labelled CD19. Cells were incubated with Fc block (2.4G2 Ab) for the reduction of non-specific Ab binding, and stainings were performed at 4°C . Antibodies used were from BD Pharmingen and cells were measured using a FACScanTM and analysed using CellQuestTM software (BD Bioscience, Hamburg, Germany). Dead cells and debris were excluded from BAL samples using propidium iodide staining (Sigma-Aldrich) measured in FL-3.

Serum collection

Blood was drawn from mice on days 21 and 28 using orbita puncture, and collected serum was stored at -20°C until measurement of OVA-specific antibodies.

ImmunoglobulinG1 and immunoglobulinG2a enzyme-linked immunosorbant assay

Briefly, plates (highbond 3950; Costar, Cambridge, MA, USA) were coated (overnight [on], 4°C) with 10 $\mu\text{g}/\text{mL}$ OVA in 0.05 M bicarbonate buffer (pH 9.6), and blocked

with PBS containing 0.05% Tween 20 (PBS-T) and 3% milk powder (ELK, Campina Melkunie, Eindhoven, The Netherlands) (1 h, 37°C). Serum was serially diluted in PBS-T containing 1% bovine serum albumin (PBS-T 1%BSA) and incubated for 1 h at 37°C . This was followed by a 1-h incubation with alkaline phosphatase-coupled goat anti-mouse IgG1 or IgG2a (SouthernBiotech, Birmingham, UK). Next, wells were incubated with p-nitrophenyl phosphate (1 mg/mL in diethanolamine buffer) for 30 min at RT. The colouring reaction was stopped by adding 10% EDTA; the absorbance was measured at 405 nm and compared between treatment groups.

ImmunoglobulinE sandwich enzyme-linked immunosorbant assay

Briefly, plates (highbond 3950; Costar) were coated (overnight [on], 4°C) with 2 $\mu\text{g}/\text{mL}$ rat anti-mouse IgE antibody (BD Pharmingen) in 0.05 M bicarbonate buffer (pH 9.6), and blocked with PBS-T and 3% milk powder (Campina Melkunie) (1 h, 37°C). Serum was serially diluted in PBS-T 1%BSA and incubated for 1 h at 37°C . This was followed by a 1-h incubation with biotinylated OVA. After incubation with poly-HRP-streptavidin (Sanquin, Amsterdam, the Netherlands) (1 h, RT), the TMB substrate was added. (15 min, RT). The colouring reaction was stopped by adding 2 M H_2SO_4 and absorbance was measured at 450 nm and compared between treatment groups.

Lung histology

Lungs from non-lavaged mice were inflated with 1 mL 1.85% gelatin in PBS, snap-frozen using liquid nitrogen and histological sections (5 mm thick) were stained with haematoxylin and eosin (HE) and examined by light microscopy.

Statistical analysis

Data are presented as mean values $\pm$ standard error of the mean (SEM) of six toeight mice per treatment group. Because our data were not normally distributed, they did not meet the assumptions needed for ANOVA. Therefore, we used the non-parametric Kruskal-Wallis' test for multiple independent samples, followed by a Mann-Whitney U test to compare the different treatment groups. All tests were performed using SPSS software 12.0 for windows. Data are considered significant when *P* values are < 0.05 .

Results

Ultrafine carbon black particles, but not fine carbon black particles or titanium dioxide particles, bind all OVA

Because binding of particle may be involved in their adjuvant activity, we determined the percentage of OVA that was bound to the particles in our suspensions. The

data were added to Table 1, which also show other particle characteristics. Fine CBP and both ultrafine and fine TiO₂ bind only small and similar percentages of the OVA, whereas the ultrafine CBP bind all the OVA.

Ultrafine, but not fine particles induce lung inflammation

To investigate whether particle-size-related toxicity is linked to particle adjuvant activity, we determined the airway toxicity and immunostimulatory potential of fine and ultrafine TiO₂ and CBP. At day 8, both the ultrafine TiO₂ and CBP induced a local inflammatory response in the airways. The levels of LDH (Fig. 1a) were higher, and the levels of total protein (Fig. 1b) and TNF- α (Fig. 1c) were significantly higher ($P < 0.05$) in BALF from mice exposed to ultrafine particles compared with mice exposed to the fine particles of the same type. Compared with the OVA-only exposed mice, mice exposed to ultra-

fine TiO₂ particles showed higher levels of LDH (Fig. 1a), and significantly higher levels of total protein (Fig. 1b) TNF- α (Fig. 1c; $P < 0.05$), whereas only TNF- α levels were significantly increased ($P = 0.001$) after ultrafine CBP exposure. Exposure to ultrafine TiO₂ or CBP combined with OVA caused an influx of inflammatory cells into the airways. The number of neutrophils, and to a lesser extent eosinophils, were significantly increased ($P < 0.05$) in ultrafine particle-exposed mice compared with those exposed to fine particles of the same type or to OVA alone (Fig. 1d).

In concordance with the inflammatory parameters detected in BAL(F), histological analysis of the airways showed that exposure to ultrafine TiO₂ (Fig. 2b) or ultrafine CBP (Fig. 2d) leads to peribronchial and perivascular inflammatory infiltrates (mostly neutrophils). Exposure to OVA alone (Fig. 2a), or combined with fine TiO₂ (Fig. 2c) and fine CBP (Fig. 2e), had no effects on lung histology.

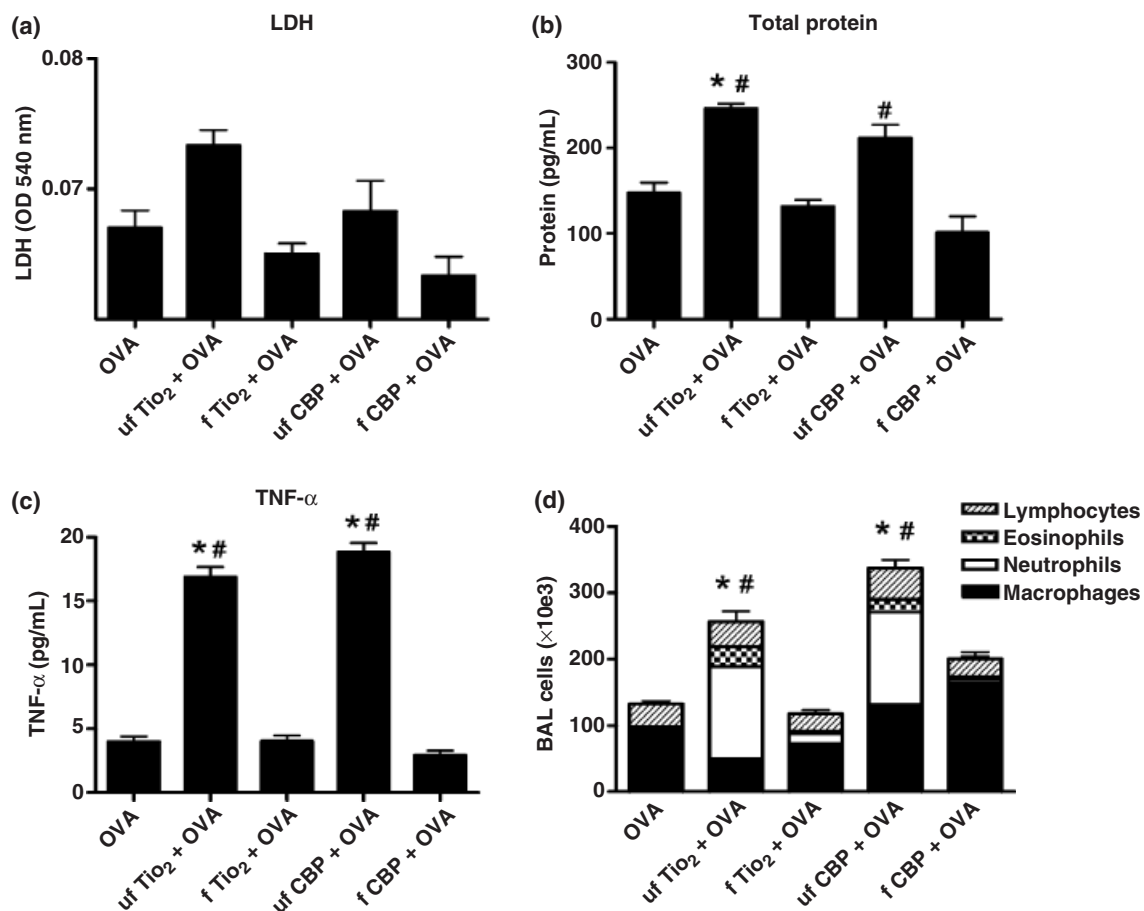


Fig 1. Effects of combined exposure to particles and antigen on bronchoalveolar lavage BAL(F) parameters of airway inflammation. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP), combined with OVA or OVA alone on days 1–3. At day 8, the lungs were lavaged and the induction of airway inflammation was assessed in the BAL(F). Cell damage was detected by lactate dehydrogenase (LDH) measurements in the BALF (a). Levels of protein (b) and TNF- α (c) were also detected in BALF as a measure of inflammation. Flow cytometry was used on BAL cells to assess the influx of inflammatory cells into the airways (d). In all groups $n = 6$, * significantly different from the OVA control ($P < 0.05$), # significantly different from the fine particles of the same kind ($P < 0.05$).

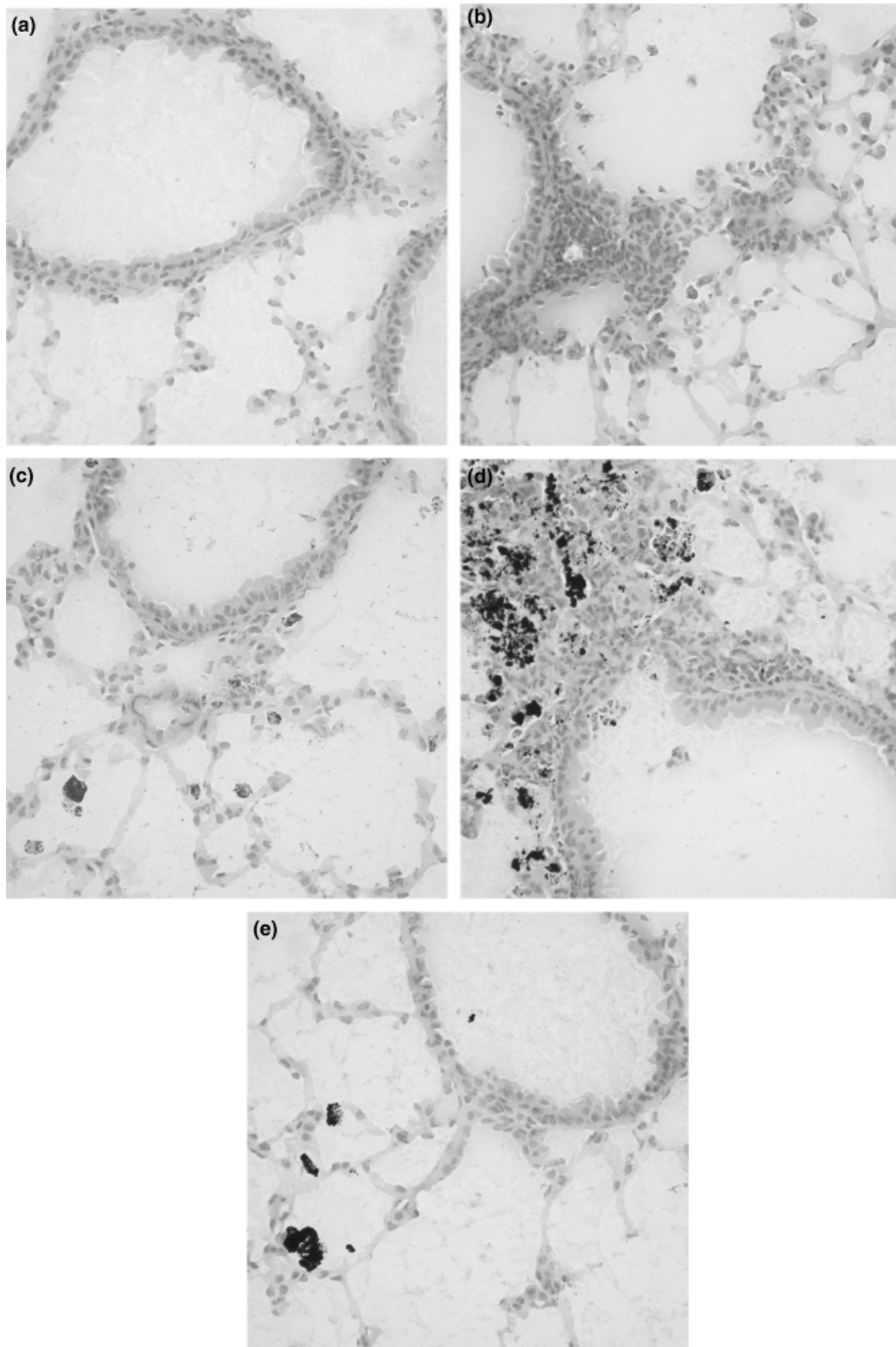


Fig 2. Effects of combined exposure to particles and antigen on lung histology. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP), combined with OVA or OVA alone on days 1–3. At day 8, the lungs were inflated using and snap-frozen in liquid nitrogen. Histological sections were stained with haematoxylin and eosin (HE) and photographed at x100 magnification. Mice exposed to OVA (a), fine TiO₂ (c) and fine CBP (e) do not show signs of inflammation, but some particle-containing macrophages are detected in c and e. Exposure to ultrafine TiO₂ (b) and ultrafine CBP (d) induced airway inflammation with clear neutrophilic infiltrates around the bronchi. Particle-containing macrophages are also present in these slides.

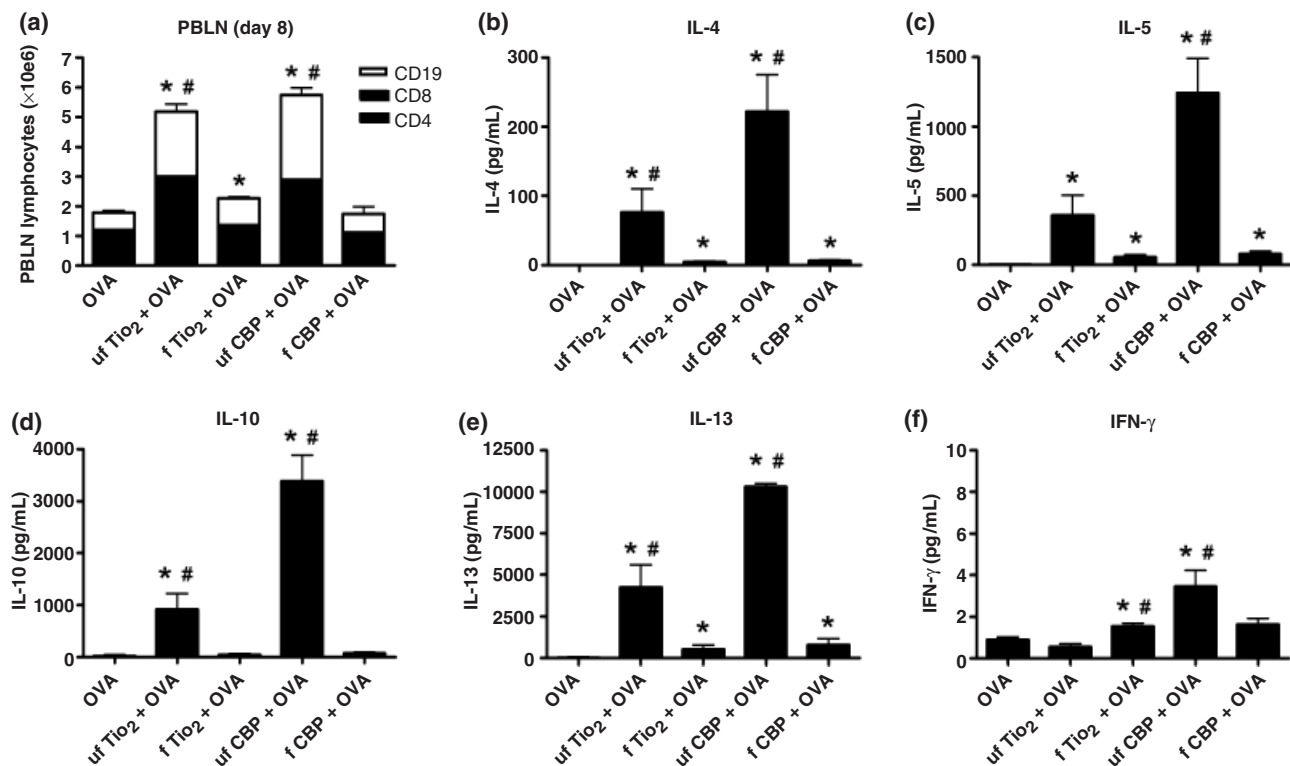


Fig 3. Effects of combined exposure to particles and antigen on PBLN cells and cytokine production. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP), combined with OVA or OVA alone on days 1–3. At day 8, the PBLN cells were counted and lymphocyte subtypes were analysed using flow cytometry (a). PBLN cells were also re-stimulated *ex vivo* with OVA for 96 h, and the production of IL-4 (b), IL-5 (c), IL-10 (d), IL-13 (e) and IFN-γ (f) is displayed. In all groups $n = 6$, * significantly different from the OVA control ($P < 0.03$), # significantly different from the fine particles of the same kind ($P < 0.01$).

Ultrafine, but not fine particles stimulate local immune responses

Both ultrafine CBP and TiO₂ particles stimulated the local immune response against the co-administered OVA antigen. The cell numbers of PBLN from ultrafine particle-exposed animals were significantly increased ($P < 0.01$) compared with numbers in mice exposed to OVA only or to fine particles. Fine TiO₂ particles induced a very low but significant ($P = 0.015$) increase in PBLN cell number (Fig 3a). Phenotypical analysis of the PBLN cells revealed that although CD4 and CD8 populations had doubled in number, the major increase in PBLN cellularity was caused by increased numbers of B cells (five to six times). This preferential increase in B cell number was also obvious for the fine TiO₂ particles.

The levels of Th2-associated cytokines IL-4, IL-5, IL-10 and IL-13 produced by PBLN lymphocytes after *ex vivo* re-stimulation with OVA were significantly higher ($P < 0.02$) in all mice exposed to particles combined with OVA than in control mice (Figs. 3b, c and e). Whereas both types of ultrafine particles elicited much higher levels of these cytokines compared with the fine particle, ultrafine CBP on their turn were more potent in stimulating cytokine-

producing cells than ultrafine TiO₂ particles, in particular with regard to IL-5, IL-10 and IL-13. The production of the Th1-associated cytokine IFN-γ was low compared with the levels of Th2-associated cytokines and only significantly higher ($P < 0.05$) in animals exposed to ultrafine CBP or fine TiO₂ compared with the OVA only or other particles of the same kind.

Together, the PBLN parameters show that, at the concentration used, only the ultrafine particles show clear adjuvant activity, defined by high increases in cell numbers, increased number of T and in particular B cells and production of high levels of type 2 cytokines.

Ultrafine titanium dioxide particles increase ovalbumin-specific immunoglobulin E and immunoglobulin G1 levels

Systemic sensitization and the response to an airway challenge were studied to determine the relevance of the day 8 sensitization data. In order to establish whether the differences between ultrafine and fine particles with respect to local airway effects are reflected in systemic sensitization, the levels of OVA-specific antibodies (IgE, IgG1 and IgG2a) were examined at day 21 after first intranasal exposure (Fig. 4).

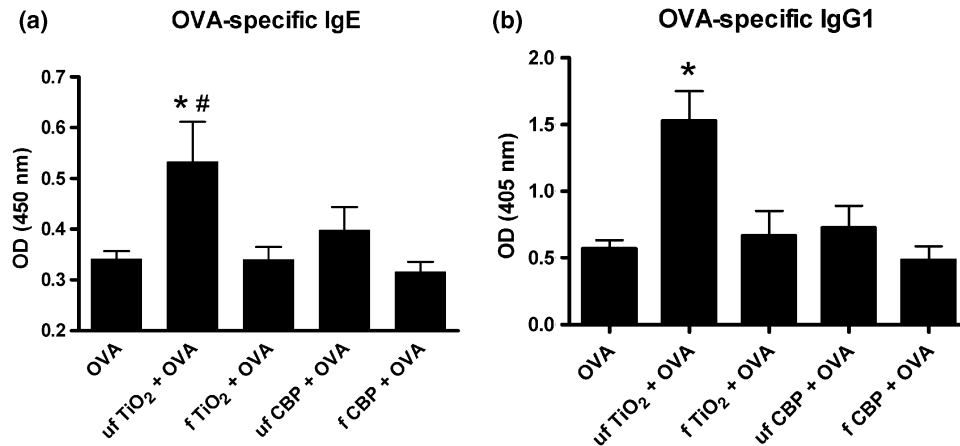


Fig 4. Effects of combined exposure to particles and antigen on serum levels of OVA-specific IgE and IgG1 antibodies. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP), combined with OVA or OVA alone on days 1–3. Serum was collected at day 21 and antibodies were detected using ELISA. The OD values of a 1 : 8 dilution for IgE (A) and a 1 : 32 for IgG1 (b) are displayed. In all groups $n = 8$, *significantly different from the OVA control ($P < 0.02$) #significantly different from the fine particles of the same kind ($P = 0.032$).

Levels of OVA-specific IgE, represented by the OD value obtained from the linear part of the serum dilution curves (Fig. 4a) and IgG1 (Fig. 4b), are significantly increased in animals exposed to ultrafine TiO₂ combined with OVA, compared with the fine TiO₂ particles (IgE only $P = 0.032$) or the OVA-only control ($P < 0.02$). The average IgE level in mice exposed to ultrafine CBP combined with OVA was increased but not significantly. OVA-specific IgG2a could not be detected in any of the samples (data not shown).

Ultrafine particles stimulate allergic airway sensitization against ovalbumin

At day 28, after challenge with OVA at days 25, 26 and 27, the PBLN cell numbers were significantly higher in both ultrafine in combination with OVA (Fig. 5). The increase in different subtypes was comparable with that seen at day 8, with a two- or threefold increase in both CD4 and CD8 cells, and a five- or sixfold increase in CD19 cells.

The production of OVA-specific IL-4, IL-5, IL-10 and IL-13 by PBLN cells was significantly increased in both ultrafine TiO₂- and CBP-exposed groups as compared with groups exposed to fine particles or OVA-only exposed. Although levels of IFN- γ were significantly increased in ultrafine CBP+OVA-treated animals compared with animals exposed to the fine particles of the same kind or to OVA only, they were again low compared with those of the Th2 cytokines. Fine TiO₂ showed low but significantly increased levels of IL-4 and IFN- γ compared to OVA only. However, because there were no or hardly any signs of systemic sensitization (Figs. 4 and 5) or allergic airway inflammation (Fig. 6) in these animals, these levels are probably not sufficient to cause profound immunological or pathological effects. In addition, influxes of eosino-

phils, neutrophils and lymphocytes were only found in both groups exposed to ultrafine PM (Fig 6), and allergic airway inflammation (perivascular and peribronchial infiltrates, and goblet cell hyperplasia) was only seen in the ultrafine particle-treated groups (Fig 6). All particle-treated groups still showed some macrophages containing particles, indicating that not all particulates were removed from the airways at day 28.

Discussion

In the current study, we show that ultrafine (below 30 nm in diameter) but not fine (over 200 nm in diameter) CBP and TiO₂ particles induce airway inflammation and display adjuvant activity. The latter is evidenced by the induction of immune sensitization to co-administered OVA and demonstrated by high levels of cytokines reminiscent of a Th2-skewed immune response, increases in serum OVA-specific antibodies and by the occurrence of allergic airway inflammation, after an intranasal OVA challenge.

The cause of the inflammatory and adjuvant potential of the ultrafine particles is not known. Whereas TiO₂ are chemically inert, some CBP are known to contain transition metals [28] and PAHs [29], but it is unlikely that these chemicals are responsible for the adjuvant effect found in the present study with the CBP we have used. Notably, Brown et al. [28], who provided us with their CBP, showed that airway inflammation induced in rats by these ultrafine CBP could not be inhibited by the metal chelator desferrioxamine. Moreover, Borm et al. [30] also used the same CBP and have recently shown that PAHs that adhered to these CBP are not bio-available, suggesting that this factor is also not involved in the effects described here.

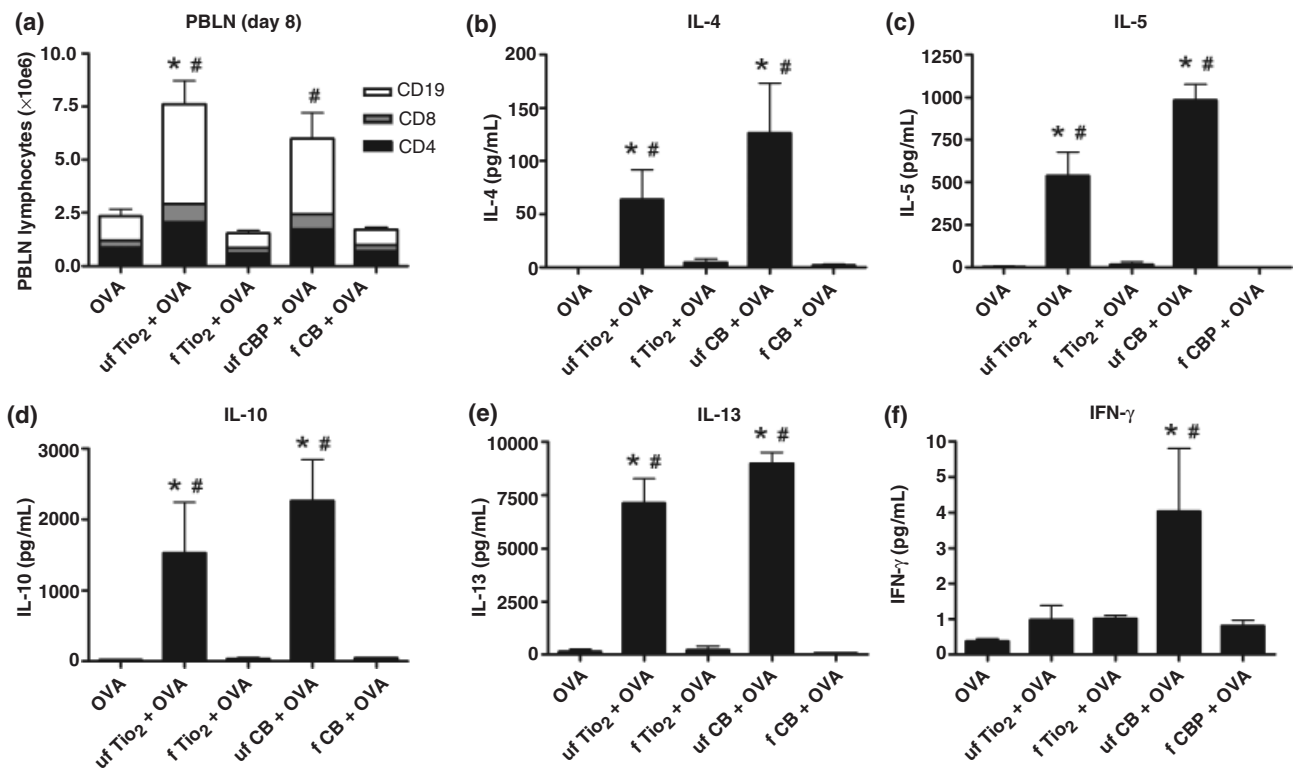


Fig 5. Effects of an intranasal antigen challenge on the PBLN in mice preexposed to different particles combined with antigen. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP) combined with OVA or OVA alone on days 1–3. All mice were intranasally challenged with OVA on days 25, 26 and 27. At day 28, the PBLN cells were counted and lymphocyte subtypes were analysed using flow cytometry (a). PBLN cells were also re-stimulated *ex vivo* with OVA for 96 h, and the production of IL-4 (b), IL-5 (c), IL-10 (d), IL-13 (e) and IFN-γ (f) is displayed. In all groups $n = 6$, *significantly different from the OVA control ($P < 0.03$), #significantly different from the fine particles of the same kind ($P < 0.03$). \$ significantly different from the ultrafine TiO₂ particles ($P < 0.05$).

In the current experiments, we have used identical doses based on milligram/animal for all particles. The number of ultrafine particles per milligram is far higher than those of fine particles; this means that we have administered far higher numbers of ultrafine than fine particles per animal. Because of the relatively large surface area of ultrafine particles compared with fine particles, we suggest that a combination of size and overall surface may be of crucial importance in the induction of airway inflammation and adjuvant activity. Small particle size and/or large surface area are factors able to induce oxidative stress, tissue damage and airway inflammation [25]. Furthermore, ultrafine particles have been demonstrated to have oxidative potential in cell-free systems, but they can also generate oxidative stress when they are taken up by cells [25, 31]. Under normal conditions, the epithelial lining fluid of the airways will be able to prevent damage from particles with oxidative potential by the presence of various antioxidants [32, 33]. However, when this system is overwhelmed by prolonged or high exposures to particles, which cannot be corrected by up-regulation of antioxidant genes, these particles may easily damage the airways and cause inflammation.

In the current study, we show that the ultrafine CBP bind 100% of the OVA, whereas the other particles, including ultrafine TiO₂ particles, bind only around 10% of the OVA. This difference in protein binding may explain some of the differences in response against ultrafine CBP or TiO₂. Ultrafine CBP + OVA caused the highest local cytokine response, but no detectable systemic antibody responses, whereas the ultrafine TiO₂ induced lower local cytokine responses but detectable systemic IgG1 and IgE production. One explanation may be that due to strong binding to CBP, OVA was retained in the lungs for a longer period than in the case of TiO₂. As a result, CBP exposure may cause a higher local concentration of OVA, whereas TiO₂ exposure may result in higher systemic OVA concentrations. Particles with bound antigen may also serve as a slow-releasing antigen depot, as an antigen carrier/concentrator or as uptake stimulators. All these processes have been extensively studied for their role in particle adjuvant activity [34–39]. Importantly, these differences in local and systemic responses did cause differences in the airway inflammation seen after OVA challenge. But overall, it appears from our data that the inflammatory

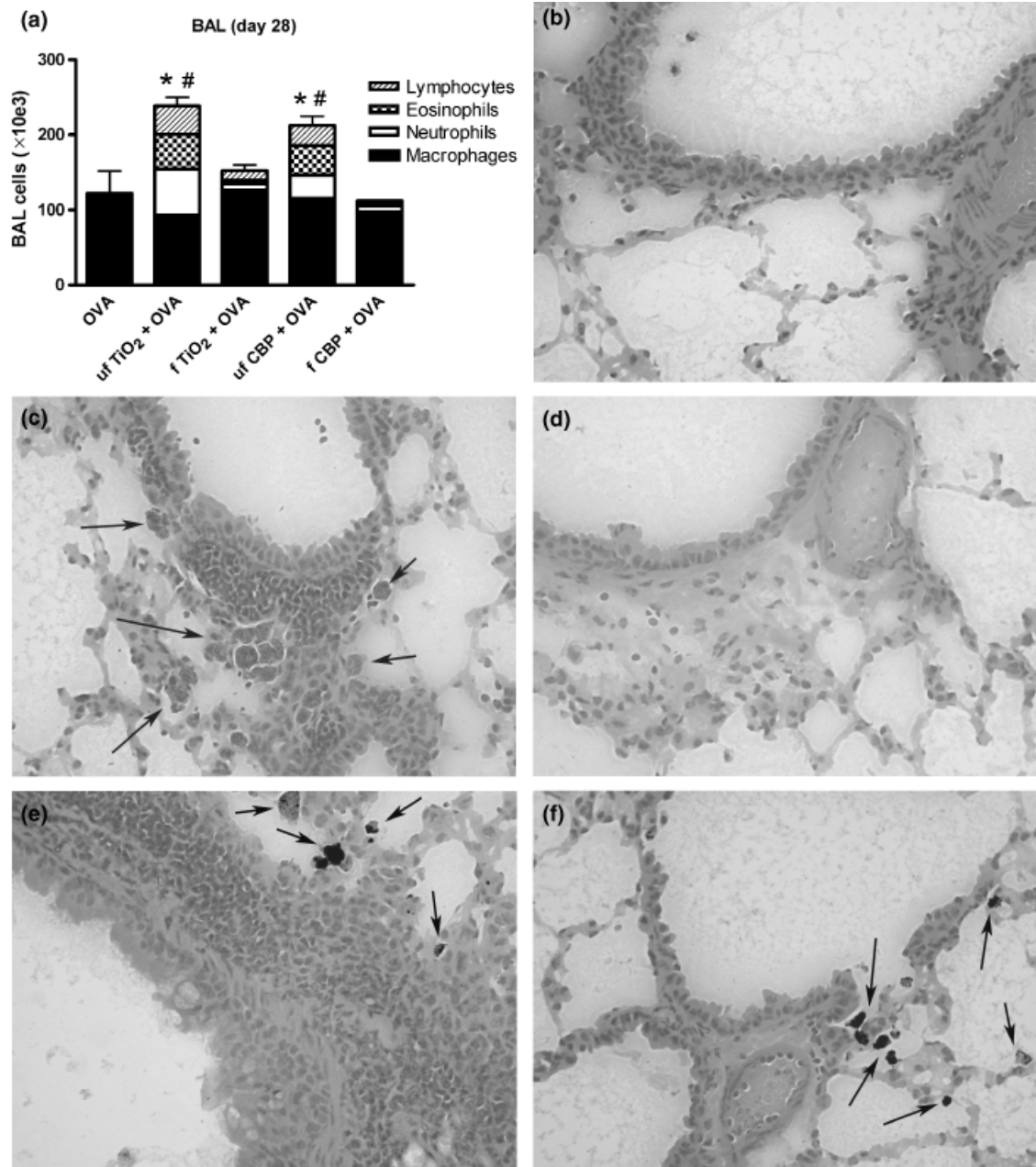


Fig 6. Induction of airway inflammation after an intranasal antigen challenge on in mice pre-exposed to different particles combined with antigen. Mice were exposed to fine or ultrafine particles (TiO₂ or CBP) combined with OVA or OVA alone on days 1–3. At day 8, the lungs of six animals were lavaged, and flow cytometry was used on BAL cells to assess the influx of inflammatory cells into the airways (a). Some lungs were inflated and snap-frozen in liquid nitrogen. Histological sections were stained with haematoxylin and eosin (HE) and photographed at x100 magnification. Mice pre exposed to OVA (b), fine TiO₂ (d) and fine CBP (f) appear normal, with some particle-containing macrophages in C, E and F (arrows). Preexposure to ultrafine TiO₂ (c) and ultrafine CBP (e) induced airway inflammation with perivascular and peribronchial infiltrates, and goblet cell hyperplasia infiltrates around the bronchi. Particle-containing macrophages are also present in these slides.

potential of particles is more important than the protein-binding potential.

In most experimental models studying the effects of PM on allergic sensitization, IgE is measured as an important marker of allergic sensitization, along with the production of IgG1 antibodies and the cytokines IL-4 and IFN- γ [19,

40, 41]. In the present study, we only observed significant increases in OVA-specific IgE and IgG1 serum levels in the animals exposed to ultrafine TiO₂ combined with OVA, but we found clear allergic airway inflammation as well as a stimulation of local immune responses (changes in cellular composition of BAL and PBLN, and production of a

number type 2 cytokines in PBLN) in both ultrafine TiO₂- and CBP-treated groups after intranasal challenges with antigen. Previously, we observed a higher induction of IgE with CBP [15], but in that study we used CBP from a different source. The present low IgE induction, combined with the clear airway allergic response, is also reminiscent of the knowledge that although IgE antibodies are well known for their role in type 1 allergy, these antibodies are not obligatory for the development of allergic airway disease [42]. For instance, allergic airway inflammation has also been observed after anti-IgE treatment [43], as well as in B-cell-deficient mice [44]. However, in these papers, animals were sensitized systemically, and according to Tumas *et al.* [45] the role of IgE in allergic airway disease depends on the site of sensitization and the adjuvant used. In their studies, depletion of IgE greatly reduced the airway inflammation in mice that were sensitized via the airways using a respiratory syncytial virus as an adjuvant, whereas depletion of IgE had no effect when intraperitoneal sensitization with alum as an adjuvant was used. Because we used local sensitization in the current study, IgE may be important in our intranasal model. Furthermore, the fact that we do not detect IgE in serum does not necessarily mean that no IgE is produced. IgE may be produced only locally in the lungs or lung-draining lymph nodes [46], or it may be present bound to mast cells or basophiles [47]. Altogether, we cannot conclude whether or not IgE is involved in allergic airway inflammation in the current experiments. Whereas the presence of antigen-specific IgE may be sufficient to detect allergic sensitization, the absence of antigen-specific IgE does not prove that no allergic airway sensitization has taken place.

Local production of Th2 cytokines, like IL-5 and IL-13, is known to be important in the induction of allergic airway inflammation [48–50] and, in agreement with this, only those animals with a high production of the Th2-associated cytokines IL-4, IL-5, IL-10 and IL-13 after sensitization and challenge showed detectable airway inflammation after challenge.

In summary, our current data show that ultrafine particles are more potent than fine particles in inducing airway inflammation and stimulating immune responses to bystander antigens when used at the same mass dose. These data provide an experimental explanation for the observed correlation between increased allergic sensitization and exposure to traffic-related air pollution, most of which is of the ultrafine size range [10–12]. But the stimulation of the immune response may also explain the exacerbation of airway disease in allergic individuals, which has been reported in various epidemiological studies [13, 14]. The present findings indicate that mere determination of size may be very helpful in determining the potential health effects of particles and particularly warrant careful safety evaluation of pulmon-

ary health effects of ultrafine, including nano-sized, particles.

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