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ORGAN TOXICITY AND MECHANISMS

Exposure to multi-walled carbon nanotubes results in aggravation of airway inflammation and remodeling and in increased production of epithelium-derived innate cytokines in a mouse model of asthma

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Abstract With the development of nanotechnologies, the potential adverse effects of nanomaterials such as multi-walled carbon nanotubes (MWCNT) on the respiratory tract of asthmatics are questioned. Furthermore, investigations are necessary to understand how these effects might arise. In the present study, we hypothesized that epithelium-derived innate cytokines that are considered as important promoting factors in allergy may contribute to an aggravating effect of MWCNT on asthma. We investigated in the mouse the effect of MWCNT on systemic immune response and airway inflammation and remodeling induced by the most frequent allergen so far associated with asthma, house dust mite (HDM), and we examined the production of the innate cytokines thymic stromal lymphopoietin (TSLP), IL-25, IL-33, and GM-CSF. Mice exposed to HDM exhibited specific IgG1 in serum and inflammatory cell infiltration, and increased Th2 cytokine production, mucus hyperproduction, and collagen deposition in the airways when compared to naïve animals. Levels of total IgG1 and HDM-specific IgG1, influx of macrophages, eosinophils and neutrophils, production of collagen, TGF-β1, and mucus, as well as levels of IL-13, eotaxin, and TARC, were dose-dependently increased in mice exposed to HDM and MWCNT compared to HDM alone. These effects were associated with an increased production of TSLP, IL-25, IL-33, and GM-CSF in the airways. Our data demonstrate that MWCNT increase in a dose-dependent manner systemic immune response, as well as airway allergic inflammation and remodeling induced by HDM in the mouse. Our data suggest also a role for airway epithelium and innate cytokines in these effects.

Keywords Carbon nanotubes · Asthma · Airway epithelium · Innate immunity · Thymic stromal lymphopoietin · IL-25 · IL-33

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Introduction

With the development of nanotechnology, the use of engineered nanomaterials has increased tremendously in recent years. Carbon nanotubes (CNT) are one of the most promising nanoparticles in nanotechnology and the most predominant nanomaterials in use today (Paradise and Goswami 2007). Indeed, because of their remarkable technological properties, CNT, and particularly multi-walled carbon nanotubes (MWCNT), have potential applications in various industrial fields, including electronics, optics, aeronautics, manufacturing of everyday consumer goods like clothes and sports gears, or medicine (Kostarelos et al. 2009).

Asthma is a chronic airway inflammatory disease characterized by eosinophil infiltration, Th2 cytokine production, mucus hypersecretion, and subepithelial fibrosis (Bousquet et al. 2000). In allergic asthma, these features result from an aberrant immune response to common environmental allergens. Mechanisms of asthma are complex, and there are several risk factors for developing the disease, among which exposure to nonallergenic airborne pollutants (Peden 2002). Epidemiological and experimental studies on air pollution have provided substantial evidence that exposure to particulate matter is associated with a variety of adverse effects on the respiratory tract, including inflammation, airway wall remodeling, allergic sensitization, and

exacerbation of asthma (Dockery and Pope 1994; Ghio and Devlin 2001; Churg et al. 2003; Nygaard et al. 2005). Among environmental particles, fine particles, and in particular diesel exhaust particles (DEP), have a high potency to induce inflammation and display adjuvant activity (Riedl and Diaz-Sanchez 2005; de Haar et al. 2006; Alberg et al. 2009). Thus, with the development of nanotechnology, the potential adverse effects of engineered nanoparticles such as MWCNT on the respiratory tract of asthmatics are questioned.

Several groups including ours have shown that MWCNT can trigger inflammation, as well as fibrotic response in the lung of mice, suggesting that this nanomaterial may promote asthma (Muller et al. 2005; Porter et al. 2010; Ronzani et al. 2012). In agreement with this hypothesis, studies using ovalbumin (OVA) as a model antigen found that MWCNT promote allergic immune response and airway inflammation (Inoue et al. 2009; Nygaard et al. 2009; Inoue et al. 2010) or increase allergen-induced airway fibrosis (Ryman-Rasmussen et al. 2009) in the mouse. However, the mechanisms by which MWCNT may contribute to asthma remain so far poorly understood. *In vitro* studies investigating the ability of MWCNT to modulate the function of immune cells involved in the initiation and driving of asthma, including monocytes, macrophages, T lymphocytes, or dendritic cells gave so far contradictory results (Bottini et al. 2006; Inoue et al. 2009; Fiorito et al. 2009; Thurnherr et al. 2009; Wang et al. 2009; Palomaki et al. 2010; Laverny et al. 2013). Therefore, further investigations are necessary to fully understand how MWCNT could promote allergic asthma.

The airway epithelium is the first structure that inhaled environmental agents encounter upon their entry in the respiratory tract. This first line of defense against toxicants also plays a central role in initiating and driving respiratory diseases, and particularly allergic asthma (Lambrecht and Hammad 2012). Indeed, upon exposure to environmental factors, and in particular airborne particles, airway epithelial cells are prone to release mediators involved in airway allergic inflammation and remodeling (Baulig et al. 2003; Val et al. 2012; Dergham et al. 2012). Among these mediators, innate cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, IL-33, and GM-CSF are currently the subject of great interest and intensive research. Indeed, these cytokines are increasingly regarded as major mediators in the interplay between the airway epithelium and immune cells during asthma (Wang and Liu 2009; Smith 2010; Bartemes and Kita 2012).

While studying the deposition of MWCNT in the respiratory tract of mice, we and others found that this nanomaterial is internalized in airway epithelial cells (Porter et al. 2010; Ronzani et al. 2012). This led us to hypothesize that airway epithelium-derived innate cytokines might mediate the effects evoked by MWCNT on asthma. In the present study, we used the most frequent respiratory allergen so far associated with asthma in humans, house dust mite (HDM) to trigger allergen-specific systemic immune response and airway inflammation and remodeling in the mouse. We investigated the effect of MWCNT on these responses and examined the production of the epithelium-derived innate cytokines TSLP, IL-25, IL-33, and GM-CSF.

Materials and methods

Animals

Specific-pathogen-free male BALB/cByJ mice were purchased at the age of 9 weeks from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). They were housed in polycarbonate exhaust ventilated cages (M.I.C.E.® cages, Animal Care Systems) at a rate of 4 mice per cage, with bedding made from spruce wood chips (Safe, Villemoisson, France). Ventilation in the cages was set to 10–12 changes per hour, according to the manufacturer's recommendations. The animal room was maintained under controlled environmental conditions, with a temperature of 20 ± 2 °C, a relative humidity of 50 ± 10 %, and a 12 h/12 h light/dark cycle (lighting 07:00–19:00). Food (standard diet 4RF21, Mucedola) and tap water were available ad libitum. The animals were acclimated for 1 week before the initiation of the study.

MWCNT and allergen

CNT used in this study were multi-walled CNT (MWCNT, Graphistrengh C100, Arkema, Colombes, France). Their specifications and endotoxin and trace metal content were as previously reported (Ronzani et al. 2012). MWCNT were dispersed just before their use in a synthetic lung surfactant, as previously described (Ronzani et al. 2012). A *Dermatophagoides pteronyssinus* (HDM) extract was purchased from GREER® Laboratories Inc., as a lyophilized preparation (Lenoir, NC). This extract was dissolved in saline, and its content in the major allergen Der p 1 (210 µg/mL) and in endotoxin (12.5 ng/mL, i.e., 59 pg endotoxin per µg Der p 1) was determined using a Der p 1 ELISA (Indoor Biotechnologies, Charlottesville, VA) and the Chromo LAL assay (Associates of Cape Cod Inc., East Falmouth, CA), respectively.

Experimental protocol

Animal experiments were performed in accordance with the European Union guidelines for use of laboratory animals and with the approval of the government body that

regulates animal research in France (Agreement number: C67–121). Mice were randomly distributed into 6 groups of 6 animals: a group that received the vehicle alone (Control group), a group that received HDM extract alone (HDM group), two groups that received MWCNT alone (MWCNT groups), and two groups that received HDM and MWCNT (HDM + MWCNT groups). HDM extract (2 μg Der p 1/administration) or vehicle was administered on days 0, 7, 14, and 21 of the protocol. MWCNT (25 μg/administration) were administered on days 0, 7, and 14 (75 μg MWCNT group and HDM + 75 μg MWCNT group) or every other day from day 0 to day 18 (225 μg MWCNT group and HDM + 225 μg MWCNT group). All animals were used on day 23. The experiment was terminated by i.p. injection of an overdose of anesthetic. HDM, MWCNT, or their vehicle were administered by intranasal instillation (25 μL) of a solution containing HDM alone, MWCNT alone, HDM + MWCNT, or saline containing synthetic surfactant (vehicle). Intranasal instillation was used as it is noninvasive, effective in delivering substances into the lung of mice and reproducible. Instillations were carried out under light anesthesia (50 mg/kg ketamine (Imalgen®, Merial, Lyon, France) and 3.33 mg/kg xylazine (Rompun®, Bayer, Puteaux, France) given i.p.). Dose of MWCNT per administration was set to 25 μg, based on previous studies (Porter et al. 2010; Shvedova et al. 2005) and our own data (Ronzani et al. 2012).

Serum, bronchoalveolar lavage fluid, and lung collection

Blood was drawn from mice by vena cava puncture, and collected serum was stored at −80 °C until immunoglobulin measurements. After tracheotomy, lungs were lavaged by 6 instillations of ice-cold saline supplemented with EDTA (saline–EDTA). Bronchoalveolar lavage fluids (BALF) recovered from the first two instillations were centrifuged, and the resulting supernatant was stored at −20 °C until cytokine measurements. Cell pellets recovered from the 6 instillations were resuspended in saline–EDTA and used to determine total and differential cell numbers. After BALF collection, lungs were perfused with ice-cold phosphate-buffered saline (PBS), collected, and either frozen in liquid nitrogen and stored at −80 °C until mediator assays, or fixed in 4 % paraformaldehyde for histology.

Immunoglobulin assay in serum

Serum levels of total and HDM-specific IgG1 were determined by ELISA. Briefly, microtiter plates were coated with an anti-mouse IgG1 antibody (0.2 μg/well in PBS, pH 7.4, BD Biosciences, Le Pont de Claix, France) or with HDM (0.25 μg Der p 1/well in 0.1 M bicarbonate buffer, pH 9.6) and blocked with PBS containing 1 % bovine serum albumin (BSA). Serums diluted in PBS containing 1 % BSA were then incubated overnight at 4 °C. Next, the plates were incubated with a biotinylated anti-mouse IgG1 antibody (BD Biosciences), an extravidin-horseradish peroxidase (Sigma-Aldrich, Saint Quentin Fallavier, France), and the horseradish peroxidase substrate tetramethylbenzidine (TMB, BD Biosciences), successively. Absorbance intensity was measured at 450 nm.

Determination of total and differential cell counts in bronchoalveolar lavage fluids

BALF were centrifuged to pellet cells, and erythrocytes were lysed by hypotonic shock. Cells were then resuspended in ice-cold saline–EDTA, and the total cell counts were determined using a Neubauer's chamber. Differential cell counts were assessed on cytologic preparations obtained by cytocentrifugation (Cytospin 4, Thermo Scientific, France). Slides were stained with Microscopy Hemacolor® (Merck, Germany). Cell determinations were performed by counting at least 400 cells on each preparation. Eosinophil, neutrophil, lymphocyte, and macrophage numbers were expressed as absolute numbers from total cell counts.

Preparation of lung homogenates

Frozen lungs were homogenized in 2 mL of PBS containing a protease inhibitor cocktail (Complete EDTA-free tablets, Roche, Germany) using an Ultra-Turrax® homogenizer (T25, Ika, Staufen, Germany). Lung homogenates were centrifuged before collagen, cytokine, or mucin assays.

Cytokine assay

IL-13, eotaxin, TARC, TSLP, GM-CSF, IL-25, and IL-33 in lung homogenates and total tumor growth factor (TGF)-β1 in BALF were measured by ELISA according to the manufacturer's instructions (R&D Systems, Lille, France).

Collagen assay

Collagen was assessed in lung homogenates by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions. Data were expressed as total soluble collagen per lung.

Mucin assay

Mucins were assayed in lung homogenates by enzyme-linked lectin assay using *N*-acetylglucosamine specific lectin from *Triticum vulgaris* (Chen et al. 2011). Briefly,

microtiter plates were coated with 100 µL of lung homogenates diluted in PBS and blocked with 200 µL of PBS containing 0.05 % Tween and 1 % BSA. Next, the plates were incubated with 1 µg/mL horseradish peroxidase-conjugated wheat germ agglutinin (Sigma-Aldrich) in PBS containing 0.05 % Tween, followed by TMB. Absorbance intensity was measured at 450 nm.

Histology

Fixed lungs were rinsed in PBS, dehydrated, and embedded in paraffin using standard procedures. Tissue sections (5 µm) were prepared and stained with hematoxylin and eosin (H&E) for routine morphologic assessment, and Masson's trichrome and periodic acid-Schiff (PAS) for collagen deposition and mucus visualization, respectively.

Statistical analysis of the data

Data are presented as mean ± SEM. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's test, using the GraphPad Prism 4.0 software. Data were considered as significantly different when $p < 0.05$.

Results

Effect of MWCNT on serum levels of total and allergen-specific IgG1

Total and HDM-specific IgG1 levels were measured in mouse serum to examine whether MWCNT had an adjuvant effect on allergen-induced systemic immune response. Mice exposed to HDM alone exhibited significant levels of specific IgG1 in serum when compared to control animals ($p < 0.01$), demonstrating sensitization to mite allergens (Fig. 1b). Specific IgG1 levels were significantly increased in HDM + 75 µg MWCNT ($p < 0.01$) and HDM + 225 µg MWCNT ($p < 0.001$) mice when compared to control mice, and in HDM + 225 µg MWCNT ($p < 0.05$) mice when compared to HDM mice (Fig. 1b). Furthermore, total IgG1 levels were significantly increased in the HDM + 225 µg MWCNT group when compared to control ($p < 0.001$) or HDM ($p < 0.01$) group (Fig. 1a).

Effect of MWCNT on allergen-induced airway inflammation

To investigate the effect of MWCNT on allergen-induced airway inflammation, we examined the cell infiltrate in BALF and lung tissue sections of mice. Although not significant, an inflammatory cell infiltrate was observed in BALF of mice that received 75 or 225 µg MWCNT when compared to controls (Fig. 2a). Likewise, a nonsignificant cell infiltrate was observed in mice exposed to HDM alone (Fig. 2a). By contrast, exposure to HDM + MWCNT caused a massive influx of inflammatory cells into mouse airways. Indeed, the total number of cells was significantly greater in BALF from HDM + 75 µg MWCNT ($p < 0.01$) and HDM + 225 µg MWCNT ($p < 0.001$) mice compared to control mice, and in BALF from HDM + 225 µg MWCNT animals ($p < 0.001$) compared to HDM animals. As well, the numbers of eosinophils, neutrophils, and lymphocytes were significantly greater in HDM + 75 µg MWCNT ($p < 0.01$, $p < 0.001$, and $p < 0.01$, respectively) and HDM + 225 µg MWCNT ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively) groups when compared to the control group, and in HDM + 225 µg MWCNT mice ($p < 0.001$, $p < 0.001$, and $p < 0.05$, respectively) when compared to HDM mice. Histological analysis showed peribronchial and perivascular inflammatory infiltrates in the lung of mice exposed to 225 µg MWCNT (Fig. 2c), HDM (Fig. 2d), and HDM + 225 µg MWCNT (Fig. 2e) when compared to control animals (Fig. 2b). These infiltrates were the most important in animals exposed to HDM + MWCNT.

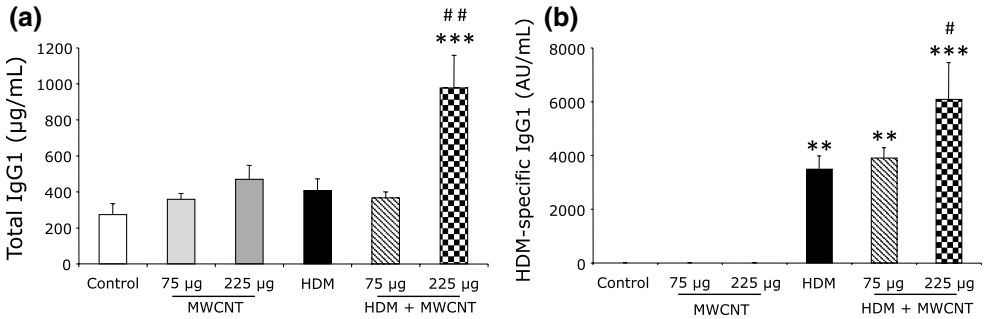

Fig. 1 Effect of MWCNT on total and HDM-specific IgG1 serum levels. Levels of total (**a**) and HDM-specific (**b**) IgG1 in serum from control, MWCNT, HDM, and HDM + MWCNT mice. Data are mean ± SEM of $n = 6$ animals. Statistically significant differences at $p < 0.05$ (*one symbol*), $p < 0.01$ (*two symbols*), and $p < 0.001$ (*three symbols*) when compared to control (*asterisks*) or HDM (*hash*)

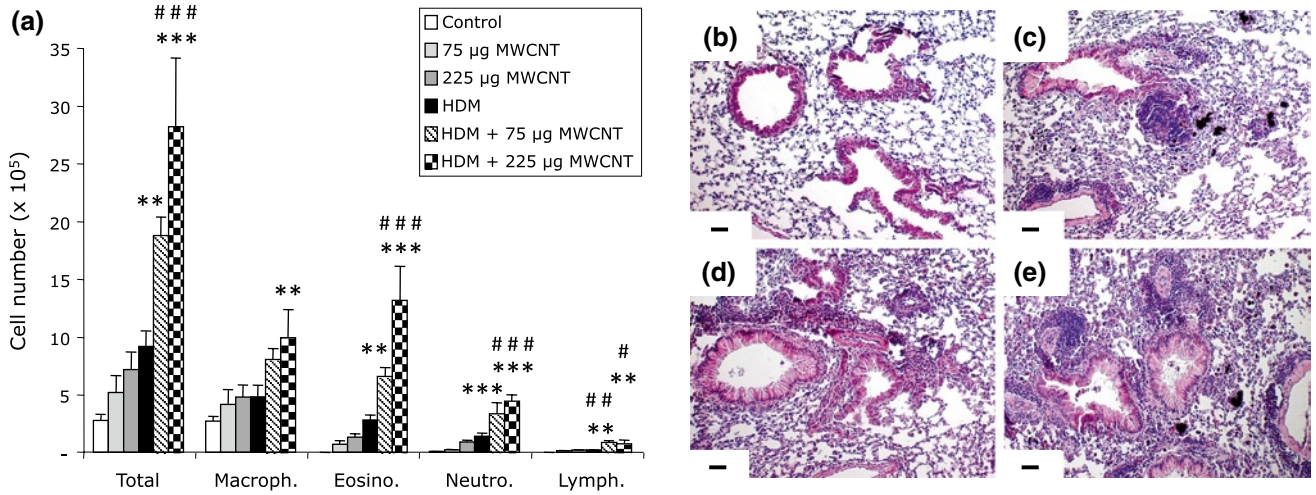

Fig. 2 Effect of MWCNT on allergen-induced airway inflammation. **a** Total and differential cell counts in bronchoalveolar lavage fluids from control, MWCNT, HDM, and HDM + MWCNT mice. Data are mean ± SEM of $n = 6$ animals. Statistically significant differences at $p < 0.05$ (*one symbol*), $p < 0.01$ (*two symbols*), and $p < 0.001$ (*three symbols*) when compared to control (*asterisks*) or HDM (*hash*). **b–e** Histopathology of lung tissues from control (**b**), 225 µg MWCNT (**c**), HDM (**d**), or HDM + 225 µg MWCNT (**e**) mice. Lung sections were stained with hematoxylin and eosin (*scale bar* 50 µm)

Effect of MWCNT on allergen-induced airway remodeling

To evaluate the effect of MWCNT on allergen-induced airway remodeling, we assessed the fibrogenic response by quantifying total soluble collagen in lung homogenates and total TGF-β1 in BALF. Total soluble collagen and TGF-β1 levels were significantly increased in mice exposed to HDM alone ($p < 0.01$ and $p < 0.001$, respectively) or HDM + 75 µg MWCNT ($p < 0.001$) when compared to control mice, and even more in mice exposed to HDM + 225 µg MWCNT compared to control ($p < 0.001$) or HDM ($p < 0.05$) group (Fig. 3a, b). Collagen deposition was also assessed qualitatively on Masson's trichrome–stained lung tissue sections. An increased collagen deposition was seen around bronchioles and vessels of mice exposed to 225 µg MWCNT (Fig. 3d), HDM (Fig. 3e), and HDM + 225 µg MWCNT (Fig. 3f) when compared to control animals (Fig. 3c).

To further characterize airway remodeling, we assessed mucus production in lung homogenates and on tissue sections. Mice exposed to 225 µg MWCNT ($p < 0.05$) or to HDM ($p < 0.001$) alone exhibited mucus hyperproduction when compared to control mice, as evidenced by increased mucin levels in lung homogenates (Fig. 4a). Mucin levels were further increased in lung homogenates of mice exposed to HDM + 75 µg MWCNT or to HDM + 225 µg MWCNT ($p < 0.05$ and $p < 0.001$, respectively) when compared to HDM animals (Fig. 4a). Mucus hyperplasia was observed on lung sections from mice that received 225 µg MWCNT (Fig. 4c), HDM (Fig. 4d), or HDM + 225 µg MWCNT (Fig. 4e) when compared to control animals (Fig. 4b).

Effect of MWCNT on the production of allergic and epithelium-derived innate cytokines

We quantified the allergic cytokines IL-13, eotaxin and TARC (Fig. 5a), and the epithelium-derived innate cytokines TSLP, IL-25, IL-33, and GM-CSF (Fig. 5b) in mouse lung homogenates. In mice exposed to MWCNT alone, levels of allergic cytokines were not different from control mice, but levels of TSLP and IL-25 were significantly increased ($p < 0.01$ and $p < 0.001$, respectively) in the case of animals exposed to 225 µg MWCNT. Eotaxin, TARC, IL-25, and IL-33 were significantly increased in mice exposed to HDM ($p < 0.001$, $p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively) compared to control mice, whereas TSLP and GM-CSF were unchanged. But all allergic and epithelium-derived cytokines were significantly increased in the lung of mice exposed to HDM + 225 µg MWCNT when compared to control ($p < 0.001$ for all cytokines) or to HDM animals ($p < 0.05$ for TARC; $p < 0.01$ for IL-13 and GM-CSF; $p < 0.001$ for eotaxin, TSLP, IL-25, and IL-33).

Discussion

In the present study, we found that MWCNT increase in dose-dependent manner systemic immune response and airway inflammation, mucus production, and fibrotic response induced by HDM in the mouse. We showed as well that these effects are associated with an increased production of the epithelium-derived innate cytokines TSLP, IL-33, and IL-25.

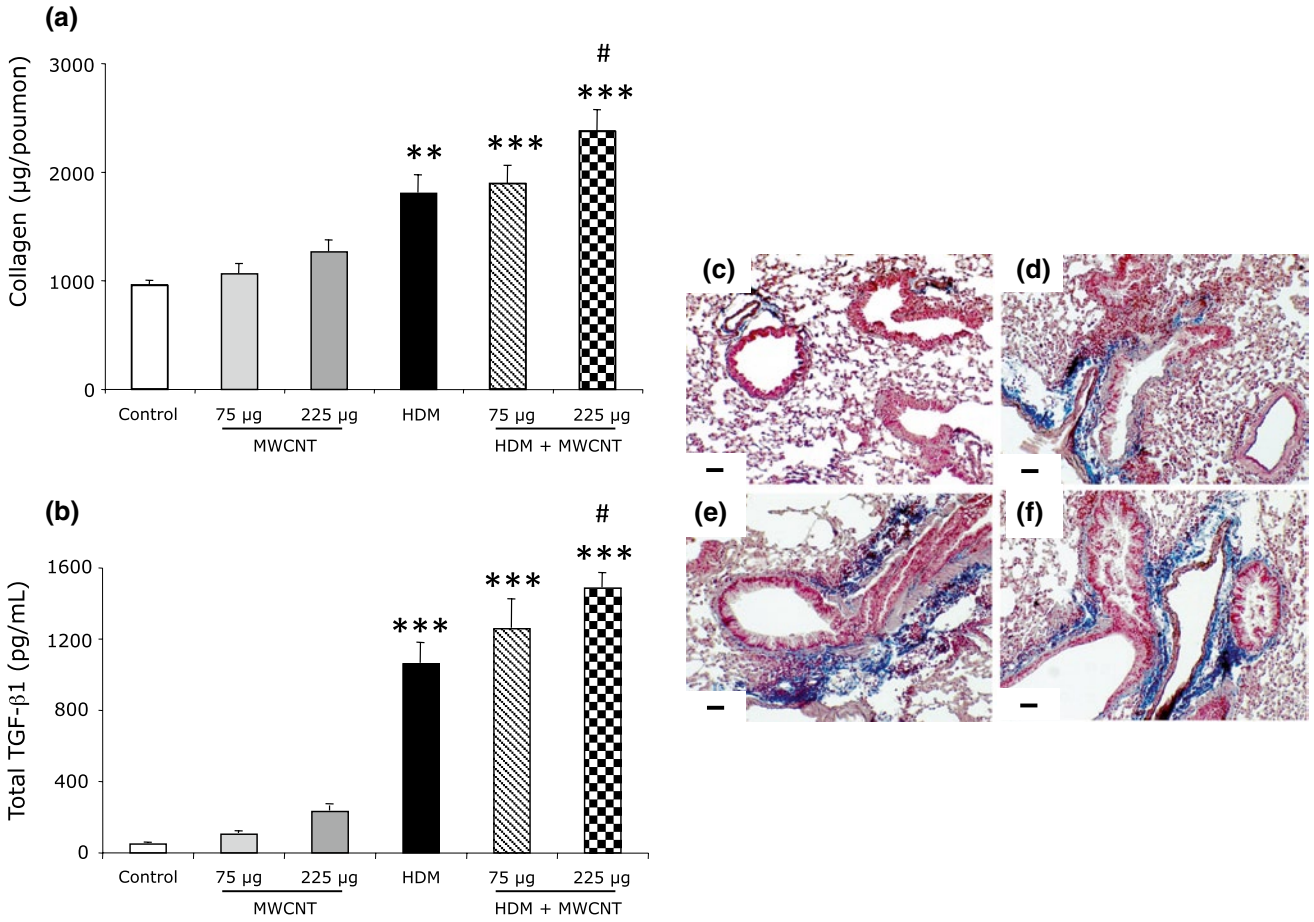

Fig. 3 Effect of MWCNT on allergen-induced airway remodeling. Concentration of total collagen in lung tissue (**a**) and total TGF-β1 in bronchoalveolar lavage fluids (**b**) from control, MWCNT, HDM, and HDM + MWCNT mice. Data are mean ± SEM of $n = 6$ animals. Statistically significant differences at $p < 0.05$ (*one symbol*), $p < 0.01$ (*two symbols*), and $p < 0.001$ (*three symbols*) when compared to control (*asterisks*) or HDM (*hash*). **c–f** Collagen deposition in the lung from control (**c**), 225 µg MWCNT (**d**), HDM (**e**), and HDM + 225 µg of MWCNT (**f**). Lung sections were stained with Masson's trichrome stain (*scale bar* 50 µm)

The aggravation of HDM-induced responses by MWCNT that we observed in the present study is in line with results obtained by other groups using OVA as a model allergen. Indeed, Nygaard et al. (2009) reported that repeated exposure to MWCNT increased the levels of specific IgG1 and IgE in serum and the number of eosinophils in BALF of mice sensitized with OVA (Nygaard et al. 2009). Similarly, Inoue et al. (2009) showed that MWCNT given repeatedly augmented allergen-specific IgG1 and IgE production and inflammatory cellular infiltrate, but also goblet cell hyperplasia in OVA-sensitized mice (Inoue et al. 2009). On their hand, Ryman-Rasmussen et al. (2009) found that acute inhalation of MWCNT increased airway fibrosis in mice with a pre-existing OVA-induced airway inflammation, compared to animals that received OVA or MWCNT alone (Ryman-Rasmussen et al. 2009). Our data add to these previous studies by showing an aggravating effect of MWCNT on the responses evoked by a disease-relevant aeroallergen, HDM. Our work describes also exacerbation of allergen-induced systemic immune response, airway inflammation, and tissue remodeling by MWCNT within the same model. Importantly, it demonstrates as well that these effects are dose-dependent. Indeed, the dose of 225 µg MWCNT caused a more important increase in HDM-induced airway inflammation and mucus production than the dose of 75 µg. Furthermore, only 225 µg MWCNT had an adjuvant effect on the systemic immune response or provoked an increase in the fibrotic response evoked by HDM. In the present study, the dose of MWCNT per administration was set to 25 µg, based on previous studies (Porter et al. 2010; Shvedova et al. 2005) and our own data (Ronzani et al. 2012) in naïve mice. Although the cumulative doses of 75 and 225 µg MWCNT may seem relatively high, these doses are below or within dose ranges for which an aggravating effect of MWCNT on OVA-induced allergic responses was reported so far in

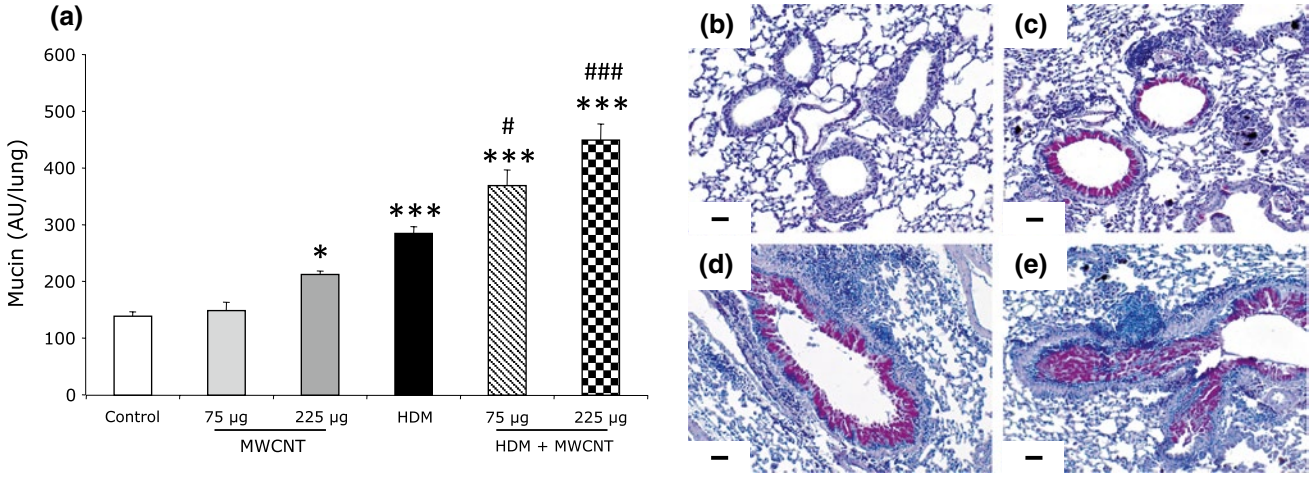

Fig. 4 Effect of MWCNT on allergen-induced mucus production. **a** Mucin content in lung homogenates from control, MWCNT, HDM, and HDM + MWCNT mice. Data are mean ± SEM of $n = 6$ animals. Statistically significant differences at $p < 0.05$ (*one symbol*), $p < 0.01$ (*two symbols*), and $p < 0.001$ (*three symbols*) when compared to control (*asterisks*) or HDM (*hash*). **b–e** Mucus production in the lung from control (**b**), 225 µg MWCNT (**c**), HDM (**d**), or HDM + 225 µg MWCNT mice (**e**). Lung sections were stained with periodic acid-Schiff stain (*scale bar* 50 µm)

the literature, namely 390, 300, and 240 µg (Nygaard et al. 2009; Inoue et al. 2009; Ryman-Rasmussen et al. 2009). Although very few human exposure data are available so far, several authors tried to put in relation the CNT doses they used in naïve mice with human occupational exposure in order to evaluate the relevance of their findings. These attempts gave, however, very disparate estimates. Indeed, Mercer et al. (2008) made the calculation that workers would receive a lung burden equivalent to 10 µg of single-walled CNT (SWCNT) in the mouse in ~200 workdays. On their hand, Shvedova et al. (2005) came to the conclusion that a dose of 20 µg SWCNT given to a mouse is approximately the same as that estimated for a worker exposed to the OSHA PEL for graphite over a period of 20 workdays. At last, Porter et al. (2010) found that a 10 µg MWCNT exposure in the mouse would approximate human deposition for a person performing light work for approximately 9 months to 7.5 years. Considering these estimates and the very few human exposure data available so far, it is very difficult to determine the exact relevance of the CNT doses resulting in exacerbation of allergic responses in the present study and in similar studies published in the literature (Nygaard et al. 2009; Inoue et al. 2009; Ryman-Rasmussen et al. 2009). However, these doses are most likely relevant to long-term occupational exposures. The development of nanotechnology has generated a large variety of engineered nanoparticles. Aggravation of HDM-induced responses by MWCNT that we observed in the present study is also in line with results obtained by other groups on other kind of nanomaterials. Indeed, nanoparticles made of titanium dioxide or gold were shown to promote also allergen-induced responses in mouse models of asthma (Larsen et al. 2010; Hussain et al. 2011). Thus, our study provides further evidence that exposure to nanomaterials may be at risk for asthmatics.

Mechanisms by which CNT might promote asthma are still unclear. Inoue et al. proposed that MWCNT increase allergic airway inflammation in mice by amplifying the maturation and activation of antigen-presenting cells, including dendritic cells (Inoue et al. 2009). In an OVA-induced mouse model of asthma, the same authors showed that the aggravating effect of SWCNT was associated with an increase in allergen-induced oxidative stress (Inoue et al. 2010). NOD-like receptor pyrin containing 3 inflammasome, a signaling pathway recently implicated in the pathogenesis of asthma, might also mediate CNT effects in the airways, as several types of nanomaterials, including CNT, were shown to activate this pathway in human macrophages or mouse airways (Yazdi et al. 2010; Meunier et al. 2011; Palomaki et al. 2011; Sager et al. 2013). In the present study, we hypothesized that epithelium-derived innate cytokines might play a role in the promoting effect of CNT on airway allergic responses. Our data provided evidence for an increase in TSLP and IL-25 in mice exposed to MWCNT alone compared to control mice, as well as an increase in TSLP, IL-25, and IL-33 in animals exposed to HDM and MWCNT compared to animals exposed to HDM alone. TSLP is considered as a key initiator of allergic inflammation (Wang and Liu 2009). This innate cytokine was found at increased levels in the epithelium and the lamina propria of patients with severe asthma (Shikotra et al. 2012). Lung-specific

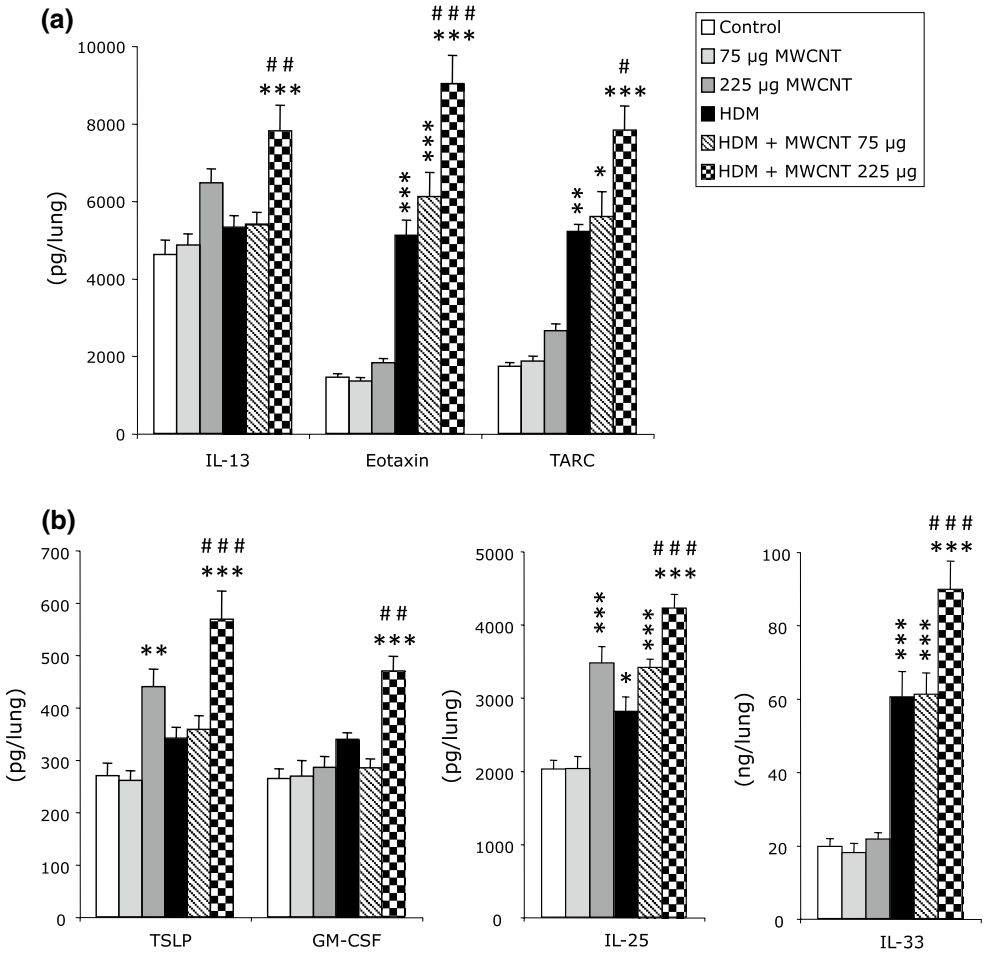

Fig. 5 Effect of MWCNT on the production of allergic and innate epithelial-derived cytokines. Concentrations of the allergic cytokines IL-13, eotaxin and TARC (**a**) and the innate epithelium-derived cytokines TSLP, GM-CSF, IL-25, and IL-33 (**b**) in lung homogenates from control, MWCNT, HDM, and HDM + MWCNT mice. Data are mean ± SEM of n = 6 animals. Statistically significant differences at $p < 0.05$ (*one symbol*), $p < 0.01$ (*two symbols*), and $p < 0.001$ (*three symbols*) when compared to control (*asterisks*) or HDM (*hash*)

over-expression of TSLP resulted in airway inflammation characterized by Th2 cytokine elevation, eosinophil infiltration, goblet cell hyperplasia, and subepithelial fibrosis in the mouse (Zhou et al. 2005). TSLP production by activated structural cells, among which epithelial cells, is believed to contribute to the maturation of dendritic cells, which in turn would favor the Th2 differentiation of naïve T cells. Furthermore, TSLP was proposed as a growth and survival factor for Th2 effector cells. Similarly, IL-33 and IL-25 are recognized as important promoting factors of allergic inflammation (Smith 2010). IL-33 administration triggered airway eosinophilia and increased production of Th2 cytokines and mucus in mouse lung (Schmitz et al. 2005). Over-expression of IL-25 resulted in elevated Th2 cytokine expression in addition to eosinophilia, increased mucus production, and epithelial cell hyperplasia/hypertrophy in the mouse (Fort et al. 2001). In addition to modulating allergic inflammation, IL-25 plays a role in orchestrating allergic airway remodeling (Gregory et al. 2013). Thus, overproduction of TSLP, IL-33, and IL-25 in the respiratory tract of MWCNT-exposed mice might contribute to exacerbate HDM-induced systemic immune response and airway Th2 inflammation, mucus production, and fibrosis.

Very few studies investigated so far a possible role of epithelium-derived innate cytokines in the pro-inflammatory and adjuvant effects of CNT, or other engineered nanomaterials in the respiratory tract. In agreement with our data, Beamer et al. reported that acute administration of MWCNT evoked the production of IL-33 by lung epithelial cells that contributed to the recruitment of lymphocytes and eosinophils in naïve mice (Beamer et al. 2012). Wang et al. (2011) found an induction of IL-33 mRNA and protein in the lung tissue and BALF from mice exposed to MWCNT (Wang et al. 2011). As well, Inoue et al. reported an exacerbation of allergic inflammation by SWCNT and MWCNT

that was associated with an increased production of IL-33 in a OVA murine model of asthma (Inoue et al. 2009; Inoue et al. 2010). Although the cytokine TSLP was proposed to contribute to the aggravating effects of DEP or cigarette smoke on asthma (Wang and Liu 2009; Bleck et al. 2008; Nakamura et al. 2008), no studies investigated so far its role in the effects evoked by CNT or other engineered nanoparticles in the lung. Interestingly, however, it was recently reported that silica nanoparticles aggravate skin lesions in a mouse model of HDM-induced dermatitis and that these effects were associated with the induction of TSLP (Hirai et al. 2012). Like for TSLP, no data are so far available on a possible implication of IL-25 in the effects triggered by CNT in the lung. Thus, our work is the first one to suggest a role for TSLP and IL-25 together with IL-33 in the responses triggered by MWCNT in the airways of mice in the context of allergic inflammation and remodeling.

Mechanisms by which CNT or other nanomaterials might trigger the release of innate cytokines by airway epithelium are presently unclear (Beamer et al. 2012). One pathway by which ambient particulate matter or nanoparticles are believed to induce or increase airway inflammation is the generation of reactive oxygen species (ROS), which are key messengers in intracellular signaling cascades leading to activation of the pro-inflammatory transcription factor NF-kB and asthma (Marano et al. 2011; Dergham et al. 2012; Li et al. 2008). Oxidative stress was reported in human airway epithelial cells in response to MWCNT (Thurnherr et al. 2011; Snyder-Talkington et al. 2013). Furthermore, ROS were suggested to play a role in the aggravation of allergic inflammation evoked by MWCNT in a mouse asthma model (Inoue et al. 2010). Besides, DEP were proposed to induce TSLP expression in epithelial cells through an oxidative stress-dependent manner (Bleck et al. 2008). Thus, MWCNT might trigger the production of innate cytokines by airway epithelium through the generation of oxidative stress.

A possible suppressive effect of CNT or other engineered nanoparticles on asthma was proposed in the literature. When investigating the immunomodulatory activity of CNT in peripheral blood mononuclear cells from mite-allergic patients, we found that MWCNT inhibit HDM-induced secretion of IL-5, a major player in the immunological and pathological features of allergic asthma (Laverny et al. 2013). Besides, an inhibitory effect of nano-sized titanium dioxide or silver particles was observed in OVA-sensitized mice (Park et al. 2010; Rossi et al. 2010). Reasons for these opposite data are presently unclear. Beside the type of models (in vitro vs in vivo) and the nature of the nanomaterial used in these studies, the dose of particle might raise the difference. Indeed, while investigating the modulatory effects of nano iron on OVA-induced Th2 immune response in mice, Ban et al. (2013) found that according to the particle dose, the allergic response was either enhanced (low dose) or suppressed (high dose). Mechanisms by which CNT or other nanomaterials might inhibit allergen-induced responses have been proposed in the literature. Inhaled MWCNT were shown to suppress systemic immune function in naïve mice by triggering the release of TGF-β from the lung (Mitchell et al. 2009). This cytokine in turn activated the cyclooxygenase pathway in the spleen leading to prostaglandin and IL-10 release ultimately causing T-cell dysfunction. In an in vitro study on human monocyte-derived dendritic cells, our team hypothesized that MWCNT may suppress allergen-induced response by targeting antigen-presenting cells or blunting their differentiation (Laverny et al. 2013). Another work on mouse bone marrow-derived dendritic cells proposed as well that SWCNT may suppress T-cell activation by acting on dendritic cells (Tkach et al. 2011). In their study on nano iron, Ban et al. (2013) assumed that the slight airway inflammation triggered by low doses of the nanomaterial may enhance the antigen-presenting function of dendritic cells leading to stronger T-cell activation, whereas the strong and persistent inflammation induced by higher doses may have a suppressive effect. Similarly, Rossi et al. (2010) hypothesized that the Th2 immune response caused by allergen sensitization in mice may be suppressed by the competing pro-inflammatory response elicited by exposure to titanium dioxide nanoparticles.

In conclusion, using a clinical relevant allergen to model asthma in mice, our data provide evidence that exposure to MWCNT aggravates allergen-induced systemic immune response, as well as airway inflammation and remodeling in a dose-dependent manner. Our data suggest also a role for airway epithelium and the innate cytokines TSLP, IL-33, and IL-25 in these effects. Contribution of epithelium-derived cytokines in the promoting effect of other engineered nanoparticles on airway allergic responses remains to be elucidated. As well, further investigations are necessary to fully understand the impact of CNT and other nanomaterials on asthma.

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