



Iron oxide particles modulate the ovalbumin-induced Th2 immune response in mice

Masarin Ban, Isabelle Langonné, Nelly Huguet, Yves Guichard, Michèle Goutet*

Department of Pollutants and Health, National Institute for Research and Safety 54519 Vandoeuvre-les-Nancy, France

ARTICLE INFO

Article history:

Received 25 July 2012

Received in revised form 31 October 2012

Accepted 2 November 2012

Available online 9 November 2012

Keywords:

Submicron and nano sized iron particles

OVA-induced Th2 response

Mice

Immunomodulation

ABSTRACT

This study was designed to investigate the modulatory effects of submicron and nanosized iron oxide (Fe_2O_3) particles on the ovalbumin (OVA)-induced immune Th2 response in BALB/c mice. Particles were intratracheally administered four times to mice before and during the OVA sensitization period. For each particle type, three different doses, namely 4×100 , 4×250 or $4 \times 500 \mu\text{g}/\text{mouse}$, were used and for each dose, four groups of mice, i.e. group saline solution (1), OVA (2), particles (3), and OVA plus particles (4), were constituted.

Mice exposed to OVA alone exhibited an allergic Th2-dominated response with a consistent increase in inflammatory scores, eosinophil numbers, specific IgE levels and IL-4 production. When the mice were exposed to OVA and to high and intermediate doses of iron oxide submicron- or nanoparticles, the OVA-induced allergic response was significantly inhibited, as evidenced by the decrease in eosinophil cell influx and specific IgE levels. However, the low dose ($4 \times 100 \mu\text{g}$) of submicron particles had no significant effect on the OVA allergic response while the same dose of nanoparticles had an adjuvant effect on the Th2 response to OVA. In conclusion, these data demonstrate that the pulmonary immune response to OVA is a sensitive target for intratracheally instilled particles. Depending on the particle dose and size, the allergic response was suppressed or enhanced.

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1. Introduction

In industrialized countries, the rapid development of new technologies over the past two decades has generated new chemical environmental pollutions, including micro, submicron or nano-sized particles. Exposure to these new materials may have adverse health effects and may be responsible for the development of various lung diseases such as repeated lung infection, respiratory allergy and lung cancer (Nicolai, 1997; Chen et al., 1989; Timonen and Pekkamäen, 1997; Granum and Lovik, 2002; Akgun et al., 2008; Inoue and Takano, 2011). With respect to health concerns and risk management against the widespread presence of particles in the environment and workplaces, numerous experimental studies have been carried out to investigate the harmful effects of these new contaminants. Among the manufactured nanoparticles encountered in workplaces, iron oxide particles, which are mostly used in construction, paints/coating, plastics, cosmetics and nutriment, are part of a list of nanomaterials to be tested (OECD, 2010). Recently, the toxicological effects of nano- and micro-sized iron

oxide particles have been investigated and compared in two *in vitro* studies. Bhattacharya et al. (2012) and Guichard et al. (2012) reported that particles of iron oxide (Fe_2O_3) induced intracellular generation of reactive oxygen species in human bronchial epithelial cells (SV40-transformed BEAS-2B cells) and in Syrian hamster embryo cells. Using *in vivo* experiments, we recently demonstrated that submicron- and nanosized iron oxide particles induced, in mice, not only pulmonary inflammation but also suppression of the adaptive immune response against sheep red blood cells (Ban et al., 2012).

Earlier *in vivo* and *in vitro* studies showed that exposure to environmental toxicants composed of smoke and particles may affect the respiratory epithelium and activate its adjacent immune cells, including the macrophages and dendritic cells, in addition to the pulmonary epithelial cells (Horvath et al., 2011). This cellular activation may result in the production of a variety of inflammatory factors such as IL-1, IL-6, IL-8, TNF- α , GM-CSF and RANTES which modulate the lung immune responses (Bezemer et al., 2011). According to a study by Shoenfelt et al. (2009), pattern recognition receptors (PRRs), expressed by macrophages and dendritic cells, are involved in particle recognition. After recognizing the particles, these PRR-expressing cells are able to release mediators such as a danger signal which determines the inflammatory reaction type. In animal experimentation, the release of inflammatory factors may depend on exposure conditions, including composition,

* Corresponding author at: Département Polluant et Santé, Institut National de Recherche et de Sécurité (INRS) – 1, Rue du Morvan-CS 60027-54519 Vandoeuvre Cedex, France. Fax: +33 383 50 20 96.

E-mail address: michele.goutet@inrs.fr (M. Goutet).

concentration, route and type of toxicant (Wegesser and Last, 2009). Likewise, the mediator concentration and composition in lungs may play a key role in balancing the local adaptive Th1/Th2 immune response. Indeed, Hilgaertner et al. (2011) showed the influence of the hydrocarbon composition and exposure conditions on jet fuel induced immunotoxicity. Previous *in vivo* studies (Takano et al., 2002; Inoue et al., 2006) designed to evaluate the effects of diesel exhaust particles (DEPs) on lung inflammation induced by LPS identified a biphasic phenomenon related to the administration route. Indeed, the authors reported an exacerbation of lung inflammation when DEPs were administered by intratracheal instillation and an inhibition when the same particles were administered by inhalation.

Exposure to environmental particles may contribute to the onset of allergic diseases including asthma. Humans and animals are normally protected against particle exposures by the mucociliary barrier of the respiratory epithelium, which can also modulate immune responses to microbes or allergens (Bauer et al., 2012). Indeed, diesel exhaust particle inhalation in mice enhanced allergen-related eosinophil recruitment, local IL-5 production and airway hyperresponsiveness (Takano et al., 1998), whereas in atopic subjects, diesel exhaust particles had adjuvant effects on IgE synthesis (Diaz-Sanchez, 1997). However, the impact of particle parameters, such as dose and size, on the allergic Th2 response was not much studied. In addition, despite the large-scale production of nanoparticles for industrial, cosmetic and medical use, there is currently a lack of knowledge concerning the potential adjuvant effects of these particles on the pulmonary allergic immune response. Iron compounds, which are encountered either in metallic form or in complex form with pollutant particles such as DEPs or residual oil fly ashes, may contribute to the expression of allergic immune response through the production of reactive oxygen species, creating a microenvironment favorable to allergic immune response development (Nel et al., 1998).

Previously, we showed through inhalation experiments on mice that chemicals such as chloroform, 1,1-dichloroethylene and styrene exacerbated the allergic response to ovalbumin and increase total and specific IgE levels, Th2 cytokines, mucus production and inflammatory cells in the lungs (Ban et al., 2006). Using a similar ovalbumin model without aluminium hydroxide adjuvant, the present study investigated in mice the impact of submicro- and nanosized iron oxide (Fe₂O₃) particles on the ovalbumin-induced pulmonary Th2 response.

2. Materials and methods

2.1. Materials

Iron oxide (Fe₂O₃) particles and ovalbumin (OVA), grade V, were purchased from Sigma-Aldrich, France. The chemical and physical particle characterization is shown in Table 1. The particles were freshly prepared just prior to instillation by resuspension in a sterile saline solution, sonication at 47 kHz for 30 min and vortexing for 10 s just before use.

2.2. Mice

Female, seven-week old BALB/c mice were obtained from Charles River (Italy). All experiments were carried out in respect to the recommendations of the National Ethics Committee. The animals were housed in an environmentally controlled animal facility maintained at 22 °C with a 12 h/12 h light/dark cycle. They were provided with food and water *ad libitum* and used one week after arrival.

2.3. Experimental protocol

Three concentrations of each particle type, 2 mg, 5 mg and 10 mg/mL were prepared and studied in separate experiments (Fig. 1). In each experiment the particles were administered every 4 days to mice by means of 4 repeated intratracheal instillations of 50 µL each. Thus, the total doses administered were 4 × 100 µg, 4 × 250 µg or 4 × 500 µg/mouse. The same experimental procedure was used in each experiment: four groups of 14 mice/treatment received control saline solution in the

vehicle groups, saline solution and OVA in the OVA groups, iron oxide particles in the iron particle groups and iron oxide particles plus OVA in the OVA + iron particle groups. On days 0, 4, 8, and 12, mice in iron particle groups and in OVA + iron particle groups received an intratracheal instillation of iron particles while mice in vehicle groups and in OVA groups received saline solution in the same conditions. In addition, on day 7, the mice in OVA groups and in OVA + iron particle groups received, on both flanks, four intradermal injections (50 µL/mouse) of 0.1% OVA in saline solution, followed, on day 11, by a single intranasal administration (50 µL/mouse) of 0.01% OVA in saline solution. At the same time, the mice in vehicle groups and iron particle groups received intradermal and intranasal injections of saline solution only. On days 19, 26, and 32, mice in OVA groups and in OVA + iron particle groups received challenge intranasal doses of 0.01% OVA in saline solution (50 µL/mouse) while mice of vehicle groups and iron particle groups received saline solution alone. 24 h after the last challenge, 8 mice per group were euthanized by intraperitoneal injection of pentobarbital (60 mg/kg, Sanofi Sante Animale, France), bled and the lung-associated lymph nodes were removed. The remaining mice were sacrificed 48 h after the last challenge. Blood samples were obtained by cardiac puncture for the IgE level assessment. Lungs were washed and excised for cytology and histology examination.

2.4. Lung-associated lymph node cell culture

The lung-associated lymph nodes were removed and pooled to obtain four lymph node pools per animal group. Single cell suspensions were prepared by gently mashing the lymph nodes through a sterile 70 µm cell strainer (Falcon) in cold RPMI-1640 medium (Gibco) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). After washing by centrifugation, the cell number was adjusted to 2 × 10⁶ cells/mL. The cells were seeded on 96-well plastic plates at 5 × 10⁵ cells/well. Concanavalin A (Sigma) was added (3 µg/mL) and the cells were cultured for 48 h at 37 °C in a 5% CO₂ incubator. The culture supernatants were collected and stored at –80 °C before assessing cytokine levels.

2.5. Cytokine levels in culture supernatants

Cytokines, IL-4 and IFN-γ, present in culture supernatants were assayed using ELISA kits (R&D system and Pharmigen respectively), according to the manufacturer's protocols. For each sample, two measurements were taken on the micro-plate reader at an optical density of 450 nm. The results were expressed as the mean ± SEM of four different samples obtained from four lymph node pools in each group.

2.6. Bronchoalveolar lavage (BAL)

After blood sampling, BAL fluid was collected by cannulating the trachea and flushing the lung with 4 × 0.3 mL sterile phosphate buffered solution (PBS). Each mouse yielded approximately 0.95 mL BAL fluid. Total cell numbers were counted and adjusted to 1 × 10⁵ cells/mL. For the differential cell counts, 100 µL of cell suspension was spun onto microscope slides (cytospin). After drying, each slide was immersed successively for 5 s each in Diff-Quik fixative; Diff-Quik stain solution and Diff-Quik counter stain solution. The Diff-Quik stained slides were then rinsed in tap water, dehydrated in absolute alcohol, cleared and mounted. At least 200 cells were differentiated on each slide based on conventional morphologic criteria.

2.7. Lung histology

After washing the lungs, the left lung was removed, frozen in liquid nitrogen and stored at –80 °C until cryostat sectioning. Two slides were prepared for each mouse by sectioning the lung lobe (6 µm) along the main bronchial stem. One slide was stained by the Diff-Quik method described above to highlight the influx of inflammatory cells around the airway and/or vessel, and another slide was stained by periodic acid-Schiff to detect any proliferation of airway goblet cells. Briefly, after 5 min fixing in picric acid-formaldehyde solution, these slides were successively immersed in periodic acid solution for 5 min, in reducing rinse for 5 min and in Schiff's reagent for 30 min. After nuclei staining for 1 min with methyl green solution, they were then dehydrated and mounted. Blind scoring (scores 0–4) for lung injury was carried out by an experienced pathologist based on the histological criteria described in the previous study (Ban et al., 2006). The results were expressed as the means of the numerical scores.

2.8. Total serum IgE levels

The total serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocols (Pharmigen). Briefly, 96-well microimmunoplates (NUNC) were incubated with 2 µg/mL of rat monoclonal anti-mouse IgE antibody overnight at 4 °C, washed three times with PBS containing 0.05% Tween-20, and incubated successively with (1) 200 µL per well of PBS containing 10% foetal calf serum (blocking buffer), (2) 100 µL per well of mouse serum diluted 1:70 in PBS/foetal calf serum or mouse IgE standard, and (3) 100 µL per well of rat anti-mouse IgE antibody labeled with peroxidase. After incubation which was carried out at room temperature, the plates were washed five times. To highlight

Table 1
Chemical and physical particle characterization.

Iron oxides	Description ^a	Particle size ^b (nm)	Specific surface area ^c (m ² /g)	Particle size distribution ^d			
				Number		Volume	
				NMD ^e (μm)	RSF ^f	VMD ^g (μm)	RSF
Nano-sized particles	Iron (III) oxide, nanopowder Sigma 544884	35 ± 14	39	0.894	2.83	4.430	1.26
Submicron-sized particles	Iron (III) oxide, Powder Sigma 310050	147 ± 48	6	0.664	3.92	4.415	1.43

^a According to the supplier.

^b Values represent the mean ± SD of particle diameter measured by TEM in 100 particles.

^c Specific surface area was evaluated by BET method.

^d Particle size distribution in saline solution is measured by the technique of laser diffraction Mastersizer 3000).

^e Number median diameter.

^f Relative span factor is a dimensionless parameter indicative of the uniformity of particle size distribution.

^g Volume median diameter.

peroxidase activity, a substrate buffer containing tetramethyl benzidine and H₂O₂ was added to each well and the reaction was terminated 30 min later by adding H₂SO₄. Absorbance was measured at 450 nm by a microplate reader (Lab System, Multiskan, MS). The total serum IgE level (ng/mL) was expressed as the mean ± SEM.

2.9. OVA-specific serum IgE levels

The technique to assess OVA-specific serum IgE was similar to that employed for total IgE, but the mouse anti-IgE was replaced by 5% OVA solution and the OVA-specific IgE levels were expressed as optical density values.

3. Data analysis

Data were analyzed using the “Statgraphics” software. One-way analysis of variance (ANOVA) followed by *post hoc* Fisher LSD multiple comparisons were carried out. When the variance was heterogeneous, the data were log-transformed. Significance was set at ($p < 0.05$).

4. Results

To determine the ability of the submicron and nanosized iron oxide particles to modulate the OVA-induced Th2 immune response in BALB/c mice, animals were exposed either to OVA alone or to OVA and different doses of iron particles as described in Section 2. We examined next the impact of particles, in relation to their type and doses, on the expression of different parameters of the lung immune responses.

4.1. Mouse OVA-sensitization model

The mouse OVA-sensitization model of allergic inflammation (OVA groups) used in this study showed an allergic Th2 response

characterized in each experiment by increases in the expression of Th2 markers. In comparison to the vehicle control groups, the OVA groups demonstrated all along the study significant increases in OVA-specific IgE levels, IL-4 production, BAL fluid eosinophil numbers, inflammatory and hyperplasia scores (Figs. 2B, 3A and B, 4A and 5A). The expression of other parameters such as total IgE levels, neutrophil and lymphocyte numbers in BAL fluids and IFN-γ production also increased in OVA-sensitized mice but the differences observed, between control and OVA-treated mice, were sometimes not statistically significant as in Figs. 2A (right), 2C (right), 4B (right) and 5B (left and right).

4.2. Effect of iron particles on inflammatory cell influx into alveolar spaces

Changes in the BAL fluid cellular composition were dependent on particle types and concentrations

4.2.1. Lymphocyte influx

The comparison between the OVA groups and the OVA/iron particle groups underlines significant differences in lymphocyte influx into the alveolar spaces (Fig. 2A). The percentages of lymphocytes in BAL fluids from mice exposed to the 4 × 100 μg or 4 × 250 μg of submicron iron particles and from mice exposed to 4 × 500 μg of nano-iron particles decreased significantly.

4.2.2. Eosinophil influx

The same decrease that was seen with lymphocytes was observed with eosinophil influx (Fig. 2B). Significant decreases in the percentages of eosinophils were observed in mice exposed to 4 × 250 μg or 4 × 500 μg of submicron iron particles. In

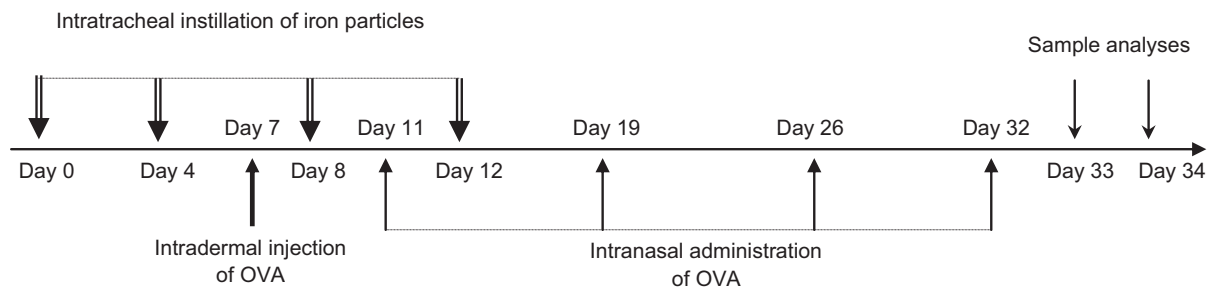


Fig. 1. Scheme of the exposure to iron particles and OVA. Three concentrations of each particle type, 2 mg, 5 mg, and 10 mg/mL, were tested. A 50 μL-aliquot of these concentrations was intratracheally instilled four times to mice (4 × 50 μL/mouse) before and during the period where the mice were sensitized with ovalbumin (on days 0, 4, 8, and 12). The total particle doses administered were 4 × 100 μg, 4 × 250 μg or 4 × 500 μg/mouse.

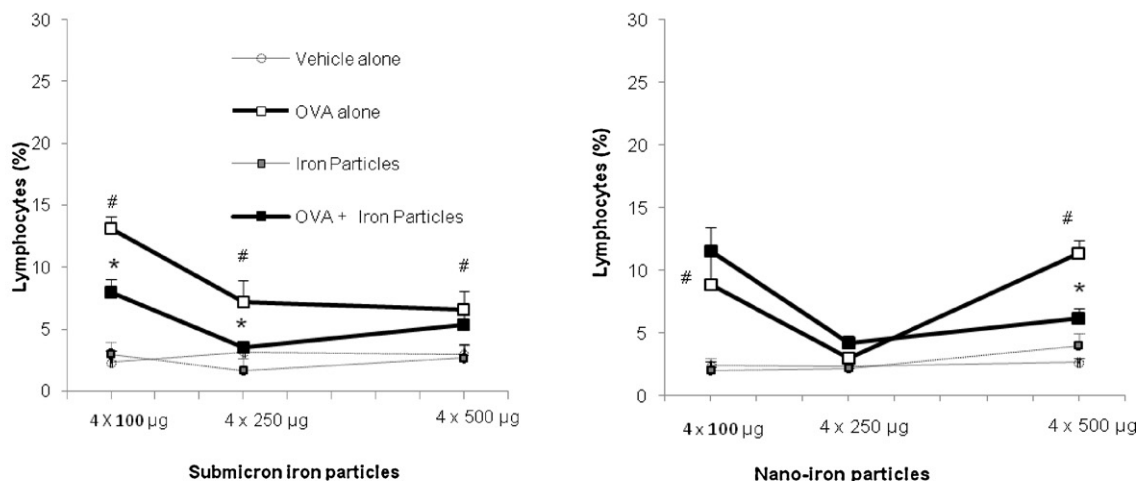
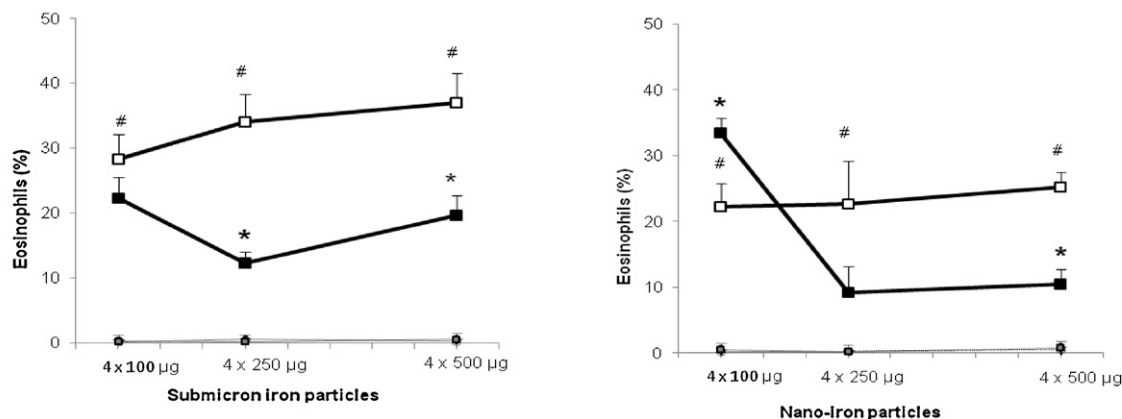
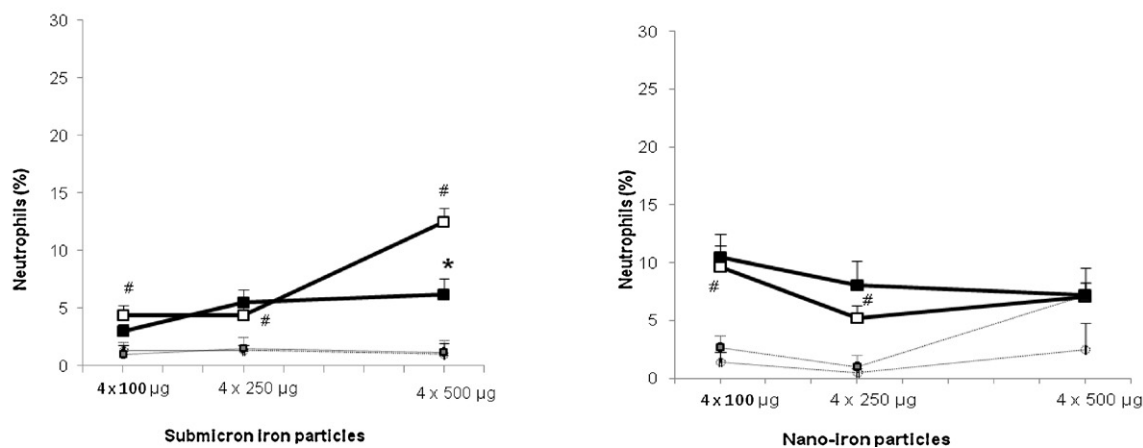
A: Lymphocytes**B : Eosinophils****C : Neutrophils**

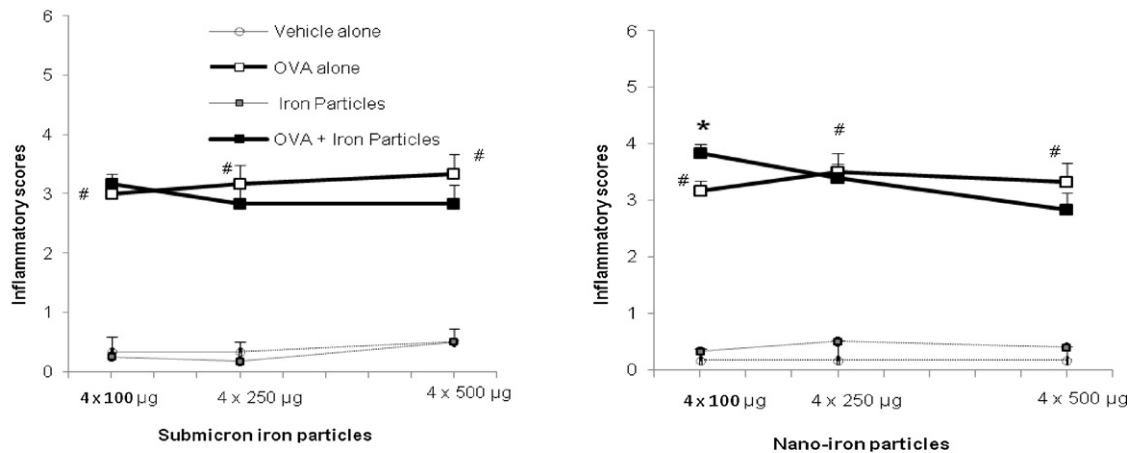
Fig. 2. Percentages of lymphocytes (A), eosinophils (B), and neutrophils (C) in the BAL fluids collected from mice exposed to different doses of submicron iron particles (left side) or nano-iron particles (right side). The cell counts were obtained from Diff-Quick stained cytospin slides. At least 200 cells were differentiated on each slide based on conventional morphologic criteria. Results are shown in cell percentages and represent the mean $\pm$ SEM, $n = 5$ or 6 mice per group. #Values significantly different from those of vehicle control mice. *Values significantly different from those of ovalbumin alone-treated mice.

mice exposed to nano-iron particles, significant decrease was only observed with the $4 \times 500 \mu\text{g}$ dose. However, a significant increase in eosinophil percentages was observed after exposure to $4 \times 100 \mu\text{g}$ of nano-iron particles.

4.2.3. Neutrophil influx

The comparison between the OVA groups and the OVA/iron particle groups shows that only the $4 \times 500 \mu\text{g}$ dose of submicron particles caused a significant decrease in neutrophil percentages

A: Inflammatory scores



B: Hyperplasia scores

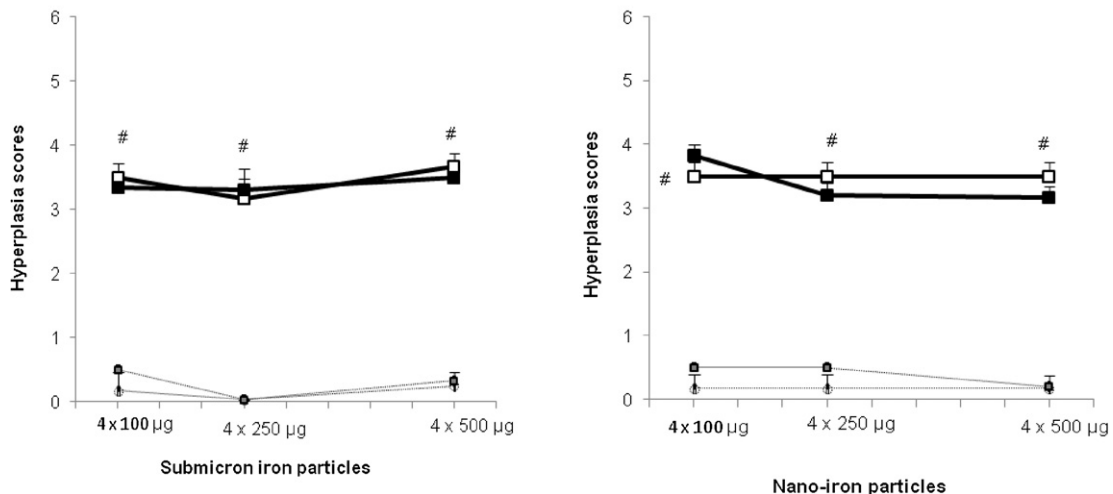


Fig. 3. Inflammatory scores (A) and hyperplasia scores (B) were measured on histological sections of lung tissues removed from mice exposed to different doses of submicron iron particles (left side) or nano-iron particles (right side). Blind scoring (scores 0–4) was based on histological criteria. The different inflammatory scores were determined in proportion to the cell influx around the airways and vessels: score 0 in absence inflammatory cells, score 1 with few inflammatory cells, score 3 with scattered aggregates of inflammatory cells, score 3 with thin layer of inflammatory cells surrounding the airways and vessels and score 4 with a thick layer inflammatory cells surrounding the airways and vessels. The hyperplasia scores were based on goblet cell proliferation: score 0 with positive goblet cells $\leq 5\%$, score 1 with positive goblet cells $\leq 20\%$, score 2 with positive goblet cells $\leq 35\%$, score 3 with positive goblet cells $\leq 50\%$ and score 4 with positive goblet cells $> 50\%$. The data (numerical scores) represent the mean $\pm$ SEM, $n = 5$ or 6 mice per group. #Values significantly different from those of vehicle control mice. *Values significantly different from those of ovalbumin alone-treated mice.

induced by OVA. None of the three doses of nanoparticles changed the percentages of neutrophils caused by OVA treatment (Fig. 2C).

4.3. Effect of iron particles on lung inflammation

Compared to the vehicle control mice and to the iron particle exposed mice, the OVA exposed mice showed a strong lung inflammation as demonstrated by the high scores of inflammation and hyperplasia.

4.3.1. Inflammatory scores

Exposure to iron particles had no effects on the inflammatory scores generated by sensitization to OVA, except the lowest dose ($4 \times 100 \mu\text{g}$) of nano-iron particles which increased significantly the inflammatory scores (Fig. 3A).

4.3.2. Hyperplasia scores

Whatever the doses, none of both types of iron particles change the hyperplasia scores induced by OVA treatment (Fig. 3B).

4.4. Effect of iron particles on antigen-specific and total IgE levels

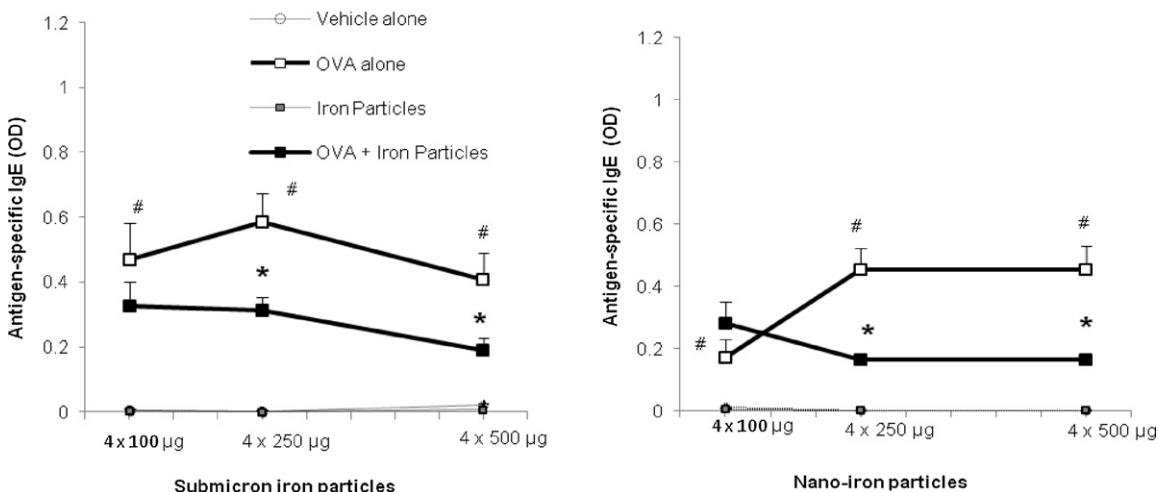
4.4.1. Antigen-specific IgE

The comparison of the OVA groups with the OVA/iron particle groups shows that the two doses, $4 \times 500 \mu\text{g}$ and $4 \times 250 \mu\text{g}/\text{mouse}$, of both types of particles induced a significant inhibition of OVA specific IgE production (Fig. 4A). However, the lowest dose, $4 \times 100 \mu\text{g}/\text{mouse}$, of nano-iron particles tended to increase the antigen-specific IgE levels.

4.4.2. Total IgE

Total IgE production induced by OVA was significantly lowered by only one dose of submicron iron particles, $4 \times 250 \mu\text{g}/\text{mouse}$

A: Antigen-specific IgE levels



B: Total IgE levels

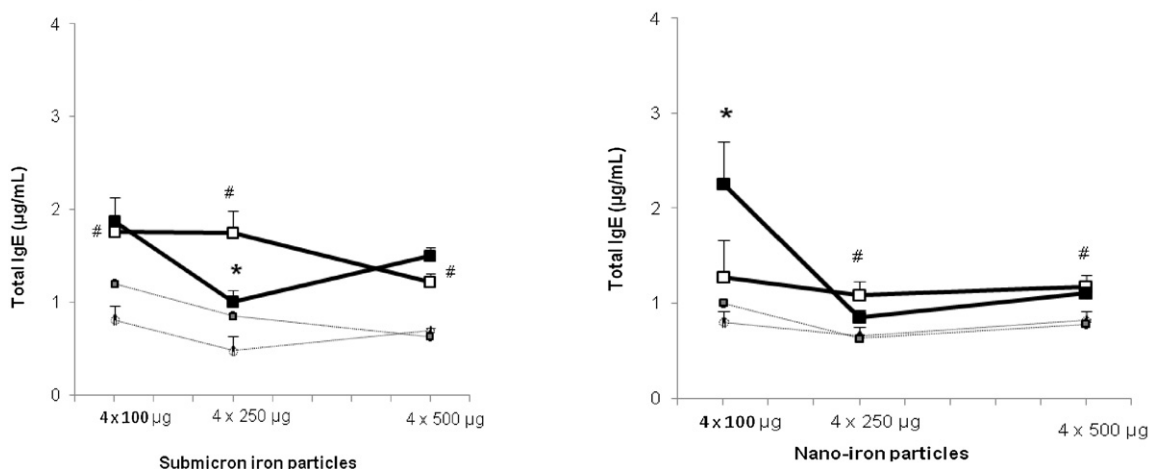


Fig. 4. Levels of antigen (OVA)-specific (A) and total IgE (B) were measured in sera by enzyme-linked immunosorbent assay (ELISA) in sera of mice exposed to different doses of submicron iron particles (left side) or nano-iron particles (right side). Levels of the antigen-specific IgE are expressed as optical density values and represent the mean $\pm$ SEM, $n = 5$ or 6 mice per group. Levels of total IgE are expressed in $\mu\text{g/mL}$ and represent the mean $\pm$ SEM, $n = 5$ or 6 mice per group. #Values significantly different from those of vehicle control mice. *Values significantly different from those of ovalbumin alone-treated mice.

(Fig. 4B). However, the lowest dose of nano-iron particles ($4 \times 100 \mu\text{g}/\text{mouse}$) induced a significant enhancement of the total IgE levels in mice exposed to OVA (Fig. 4B).

4.5. Effect of iron particles on cytokine production in cultures of lung-associated lymph node cells

4.5.1. IL-4 production

Except the highest dose of nano-iron particles ($4 \times 500 \mu\text{g}/\text{mouse}$) which caused a significant decrease in the IL-4 levels (Fig. 5A), the other doses of submicron and nanoparticles did not affect the IL-4 production induced by OVA treatment

4.5.2. IFN- γ production

The doses $4 \times 100 \mu\text{g}$ and $4 \times 250 \mu\text{g}/\text{mouse}$ of submicron and nanoparticles did not affect the IFN- γ production induced by OVA sensitization. However, the highest dose $4 \times 500 \mu\text{g}/\text{mouse}$ of both type of particles decreased significantly the IFN- γ production (Fig. 5B).

5. Discussion

Due to their extensive use in industrial applications and the need for information about their risk for human health, iron oxide particles have been placed on a list alongside 14 nanoparticle materials by OECD with a view to raising scientists' awareness to them. *In vivo* and *in vitro* studies suggest a low toxic potential for Fe_3O_4 nanoparticles (Hussain et al., 2005; Karlsson et al., 2009) while Fe_2O_3 nanoparticles have been shown to induce lung injury in mice (Zhu et al., 2008) and cytotoxic effects *in vitro* (Soto et al., 2007). The present work builds on the findings of a previous study (Ban et al., 2012) which reported pulmonary immunosuppression in mice intratracheally exposed to iron oxide (Fe_2O_3) particles. The current study shows in mice that exposure to submicro- and nanosized iron oxide particles modulate the OVA-induced allergic response according to particle size and dose. Especially in this study, the particles were given before and during sensitization and not just before or during the challenge phase as it is mostly done in an asthma model.

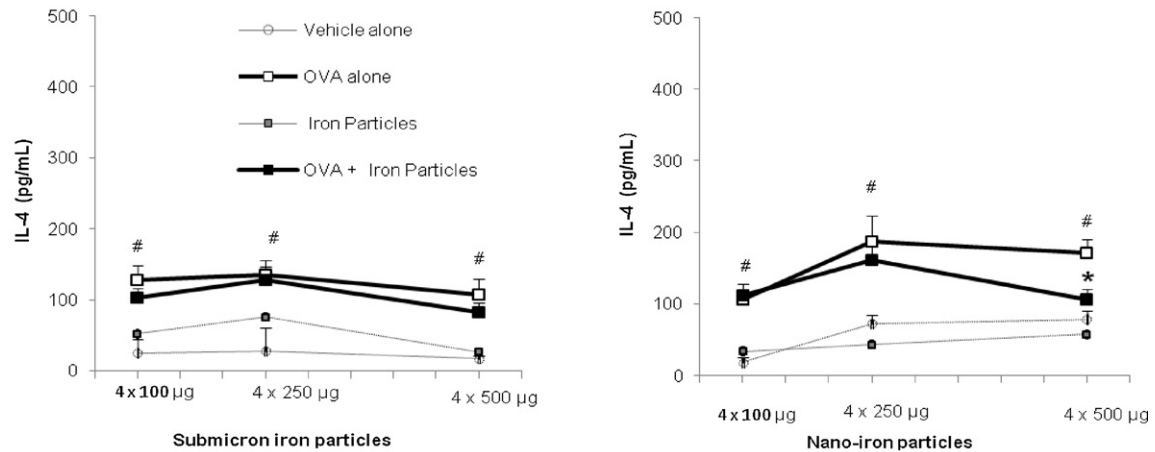
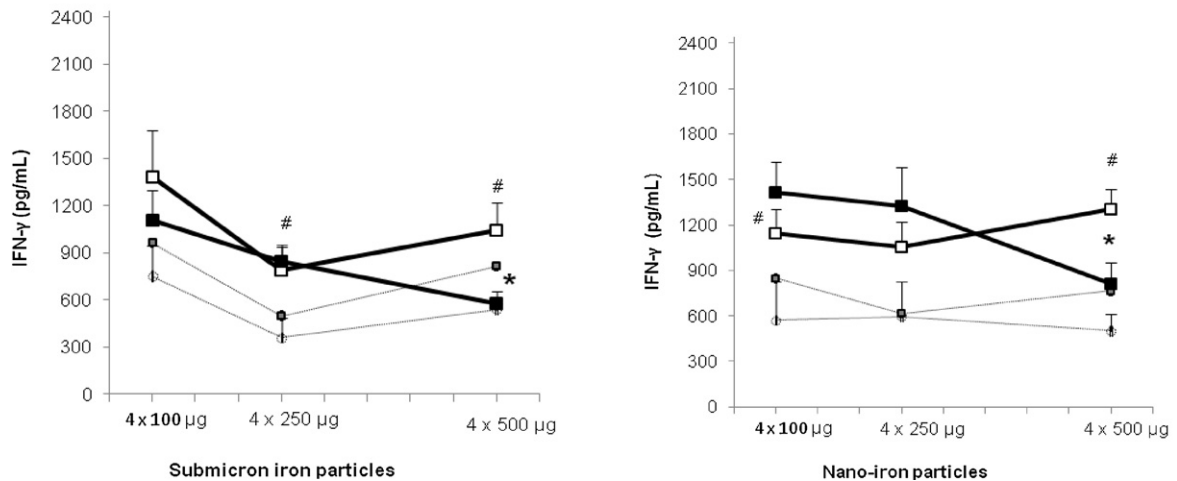
A: IL-4 levels**B: IFN- γ levels**

Fig. 5. Protein levels of IL-4 (A) and IFN- γ (B) in cultures of lung-associated lymph node cells extracted from mice exposed to different doses of submicron iron particles (left side) or nano-iron particles (right side). The associated lymph nodes were pooled in order to obtain four lymph node pools per animal group. The lymph node cells were cultured in presence of Concanavalin A (3 μ g/mL) at 37 °C in a 5% CO₂ incubator levels were assessed in supernatants of lymph node cells cultured for 48 h. The IL-4 and IFN- γ levels obtained by ELISA are expressed in μ g/mL and represent the mean $\pm$ SEM, n = 4 lymph node pools per group. #Values significantly different from those of vehicle control mice. *Values significantly different from those of ovalbumin alone-treated mice.

Throughout this study, the OVA-sensitized mice expressed an allergic Th2 dominated response characterized by significant increases in eosinophil numbers, OVA-specific IgE levels, inflammatory scores and IL-4 production by cultured lung-associated lymph node cells. However, when the mice were simultaneously exposed to OVA and to the highest and intermediate doses of submicro- and nanosized iron particles, the OVA-induced allergic response was significantly inhibited, as evidenced by decreases in inflammatory cell influx, specific and total IgE levels and cytokine production. These results, relating to the OVA allergic response, are in line with the findings of our previous work (Ban et al., 2012) which reported, in mice, a suppression of the adaptive pulmonary immune response against sheep red blood cells by the same submicro- and nanosized particles. On the contrary, the exacerbation of allergic response by environmental pollutants has often been reported in the literature (Takano et al., 1998; Leung et al., 2012). These divergent manifestations may result from the use of different particle types and concentrations. As previously reported (Ban et al., 2012), high concentrations of iron oxide particles caused airway inflammation which can interfere with the subsequent adaptive

immune response to particular and soluble antigens. In fact, several mechanisms may be involved in the suppression of the allergic Th2 responses by iron particles, including (i) intensification of pulmonary macrophage activation and of proinflammatory cytokine production which may interfere with the maturation status of dendritic cells at the time of antigen encounter, inhibiting the subsequent immune response (Hubert et al., 2007), (ii) administration of particles which may boost the plasmacytoid dendritic population recognized to have “anti-allergic” properties (Gill, 2012), (iii) particle migration into the lung draining lymph nodes which may interfere with the allergic immune process (Vermaelen and Pauwels, 2005).

Concerning the immunomodulation of the immune response to OVA induced by iron particles, our results showed that this was dependent on the particle type and dose used. Indeed, contrary to high doses, the lower doses of submicron and nanosized iron particles had either no effect or enhanced the OVA-induced allergic response. Environmental pollutants, especially diesel particles, are known for their adjuvant effects on allergic responses (Provoost et al., 2010, 2012), but there is very little information

about the potential of the iron oxide particles to enhance or reduce the immune response in relation to the experimental conditions. The biphasic effects of particles, observed in this study, may be explained by the inflammation level induced by the particles. Contrary to high doses of iron nanoparticles, the low doses may generate conditions favorable to a Th2 cell response via the particle-phagocytised macrophages. Indeed, the slight inflammation of the airways induced by low doses of particles (unlike the strong and persistent inflammation induced by higher doses) may enhance the antigen presenting function of dendritic cells by a subpopulation of macrophages, namely “alternatively activated macrophages” (Raes et al., 2002; Murray and Wynn, 2010), leading to stronger T-cell activation. Anthony et al. (2006) showed in subjects infected with helminthic parasites that the presence of this macrophage subpopulation associated with a higher number of eosinophils may help parasite fighting and tissue repair.

The composition and the size of particles are important factors of toxicity. The difference between micro- and nanoparticles has been investigated in experimental animal and cultured cell systems, but there is, so far, no consensus on the results. According to the conclusions of some of these studies, nanoparticles are more toxic than their larger counterparts because of their greater surface area and their reactivity with living organisms. Using *in vitro* tests to compare the toxicological effects of micro- and nanoscale Fe³⁺-containing particles (hematite), Bhattacharya et al. (2012) reported that nanoparticles were slightly more potent in inducing cyto- and genotoxicity than their microscale equivalent. In an *in vivo* study (Ban et al., 2012), we also showed that in mice, the same dose of iron oxide nanoparticles produced higher levels of inflammation and immunosuppression than their micro-sized counterparts. Although the aggregation state of particles strongly influences their distribution in and excretion from the body (Cho et al., 2009), we did not take this into account in the present study when assessing the particle effects on immune responses. The comparison of three different doses of each particle size revealed that submicron and nanosized particles influenced the immune response to OVA differently. For example, the highest dose of nanoparticles inhibited OVA-induced IL-4 production and lymphocyte influx in the lungs while the equivalent dose of submicron particles inhibited OVA-induced lung neutrophil influx. At the same time, the lowest dose of submicron particles had no effect on OVA-induced allergic response while the lowest dose of nanoparticles produced an adjuvant effect on this response, as evidenced by increases in the number of eosinophils the inflammatory scores and the total IgE levels. Hussain et al. (2012) also reported that TiO₂ nanoparticles were more potent than their pigment counterpart in modulating DNCB sensitization to a Th2 response. In this study, however, it should be noted that the high and intermediate doses may cause pulmonary overload and may not be physiologically relevant. Indeed, Pauluhn (2011) noted the difficulty of data interpretation in the case of pulmonary particle overload.

Modulation of the pulmonary allergic response by a variety of environmental and occupational factors might result from imbalanced cytokine production in the lung microenvironment. Humoral and cellular immune responses develop according to different mechanisms that depend mainly on the specific activation and expansion of CD4⁺ T helper (Th) cells into different subsets such as Th1, Th2, Th17 and regulatory T cells (Treg) with their own cytokine signatures (Adkiss, 2008). Although all of these T cell populations have the potential to influence hypersensitivity responses, this paper focuses on the dichotomy of Th1 and Th2. Previous research works (Mosmann and Sad, 1996; Dai et al., 2009) has demonstrated that the levels of Th1 and Th2 cytokines, including IFN- γ and IL-4, can balance the immune response. Previously, we reported that mice intratracheally exposed to platinum salts developed a Th2 immune response associated with a decrease in

IFN- γ production after repeated challenges (Ban et al., 2010). Zhu et al. (2012a, 2012b) reported that iron oxide nanoparticles initiated a Th1-type immune activation. However, in the present study, the suppression of the OVA immune response by iron oxide micron and nanoparticles did not appear to result from cross-regulation between Th1 and Th2 cytokines since it was not associated with IFN- γ production increases. On the contrary, OVA allergic response suppression was associated with a reduction in IFN- γ production.

In conclusion, the present data has demonstrated that iron particles administered in mice in the form of four successive intratracheal instillations may modulate the pulmonary immune response to OVA. Depending on their dose and size, iron oxide particles are able to suppress or enhance the allergic response. However, future research is needed to clarify the mechanisms of the modulating responses observed in this study. Especially, the role of dendritic cells and T cell subsets will have to be investigated.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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