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## Supplementary<br/>Materialhttp://www.jimmunol.org/content/suppl/2011/12/21/jimmunol.1100156.DC1.html

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## Inert 50-nm Polystyrene Nanoparticles That Modify Pulmonary Dendritic Cell Function and Inhibit Allergic Airway Inflammation

# Charles L. Hardy,<sup>\*,†,‡</sup> Jeanne S. LeMasurier,<sup>\*,†,‡</sup> Gabrielle T. Belz,<sup>§</sup> Karen Scalzo-Inguanti,<sup>\*</sup> Jun Yao,<sup>\*,†,‡</sup> Sue D. Xiang,<sup>\*</sup> Peter Kanellakis,<sup>¶</sup> Alex Bobik,<sup>¶</sup> Deborah H. Strickland,<sup>∥,#</sup> Jennifer M. Rolland,<sup>\*,†,‡</sup> Robyn E. O'Hehir,<sup>\*,†,‡</sup> and Magdalena Plebanski<sup>\*,‡</sup>

Nanoparticles are being developed for diverse biomedical applications, but there is concern about their potential to promote inflammation, particularly in the lung. Although a variety of ambient, anthropogenic and man-made nanoparticles can promote lung inflammation, little is known about the long-term immunomodulatory effects of inert noninflammatory nanoparticles. We previously showed polystyrene 50-nm nanoparticles coated with the neutral amino acid glycine (PS50G nanoparticles) are not inflammatory and are taken up preferentially by dendritic cells (DCs) in the periphery. We tested the effects of such nanoparticles on pulmonary DC function and the development of acute allergic airway inflammation. Surprisingly, exposure to PS50G nanoparticles did not exacerbate but instead inhibited key features of allergic airway inflammation including lung airway and parenchymal inflammation, airway epithelial mucus production, and serum allergen-specific IgE and allergen-specific Th2 cytokines in the lung-draining lymph node (LN) after allergen challenge 1 mo later. PS50G nanoparticles themselves did not induce lung oxidative stress or cardiac or lung inflammation. Mechanistically, PS50G nanoparticles did not impair peripheral allergen sensitization but exerted their effect at the lung allergen challenge phase by inhibiting expansion of CD11c<sup>+</sup>MHCII<sup>hi</sup> DCs in the lung and draining LN and allergen-laden CD11b<sup>hi</sup>MHCII<sup>hi</sup> DCs in the lung after allergen challenge. PS50G nanoparticles further suppressed the ability of CD11b<sup>hi</sup> DCs in the draining LN of allergen-challenged mice to induce proliferation of OVA-specific CD4<sup>+</sup> T cells. The discovery that a defined type of nanoparticle can inhibit, rather than promote, lung inflammation via modulation of DC function opens the door to the discovery of other nanoparticle types with exciting beneficial properties. The Journal of Immunology, 2012, 188: 1431-1441.

**B** ngineered nanoparticles are increasingly being developed for use in industries ranging from biomedicine to cosmetics. In view of their potential harmful effects, there is a critical need to understand their impact on human health (1). The particle concentration in ambient air is dominated by ultrafine particulates (nanoparticles of <100 nm), which are postulated to

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Abbreviations used in this article: AAI, allergic airway inflammation; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; DC, dendritic cell; LN, lymph node; PAS, periodic acid–Schiff.

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contribute disproportionately to the morbidity and mortality associated with particle inhalation (1, 2). Exposure to pollution particulates has been associated with asthma exacerbations (2, 3), and the past 20 y have seen a dramatic increase in the prevalence of allergic asthma in Western countries (4), with increased pollution a potential link. Indeed, ambient pollutant particles and diesel exhaust particles can promote lung inflammation and allergic sensitization (5-8). Studies have also shown that carbon black nanoparticles (9-11), titanium dioxide nanoparticles (12), and carbon nanotubes (13, 14) can promote allergic sensitization, allergic airway inflammation (AAI), and/or stimulate dendritic cell (DC) and allergen-specific T cells. However, a significant component of these particle adjuvant effects has been attributed to adsorbed toxic chemicals and metallic impurities and the ability to induce oxidative stress (6, 7, 13, 15-19). Notably, information regarding the long-term effects of inert, nontoxic and noninflammatory nanoparticles delivered into the lung on asthma development and pulmonary DC distribution and function is lacking. As well as being a core question for immunology, this is an important practical question with immediate clinical and regulatory consequences in view of the growing number of particulate, nanosized vaccines and drug delivery systems in development (20, 21).

We and others have shown that DCs in the draining lymph node (LN) preferentially internalize 25- to 50-nm nanoparticles that have been injected intradermally (22, 23). In contrast to the prevailing belief that nanoparticles exacerbate asthma, we report in this study that such inert glycine-coated polystyrene 50-nm nanoparticles (PS50G nanoparticles), which do not promote cardiac inflammation or lung oxidative stress, have the novel property of inhibit-

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ing pulmonary inflammation, airway mucus hypersecretion, and allergen-specific IgE and local allergen-specific Th2 cytokine production after allergen challenge 1 mo following particle instillation. This inhibition of allergen-specific immunity was achieved by suppressing expansion of total and allergen-laden DCs in the lung and draining LN after allergen challenge and by impairing the ability of lung-draining LN DCs to induce proliferation of OVA-specific CD4<sup>+</sup> T cells. These findings show that the long-term effect of PS50G nanoparticles is to inhibit lung inflammation via modulation of DC function, demonstrating that not all nanoparticles necessarily promote lung inflammation. Moreover, our findings offer the possibility that other nanoparticle types may have such novel and unexpected immunomodulatory properties and support the possibility of safe development of nano-sized formulations for treatment of lung inflammatory diseases.

#### **Materials and Methods**

Mice

Female BALB/c, C57BL/6, or DO11.10 mice aged 7–12 wk were obtained from Laboratory Animal Services (Adelaide, Australia) or The Walter and Eliza Hall Institute of Medical Research and housed in the Alfred Medical Research and Education Precinct or The Walter and Eliza Hall Institute of Medical Research animal facilities. BALB/c mice were used in all experiments except those where C57BL/6 mice were used to assess strain-specificity or for the influenza virus infection studies. DO11.10 mice (BALB/c background) were used for the DC–T cell cocultures. All experimental protocols were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee and The Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee.

#### Particle preparation and physical characteristics

Polybead carboxylate microspheres (unlabeled, nominally 0.05  $\mu$ m; no. 15913; Polysciences, Warrington, PA) were glycine coated as described (22), hereafter referred to as PS50G nanoparticles. Because of manufacturer batch variation, typical particle sizes ranged from 45 to 49 nm (SD = 1.5 to 7 nm) for the 0.05- $\mu$ m (PS50G) nanoparticles when purchased. The Zetasizer (Malvern Instruments, Worcestershire, U.K.) measurement for the size distribution of glycine-coated particles in sterile 0.9% saline (saline for clinical use; Pfizer) was 39.2  $\pm$  3.5 nm. Surface charge was -37.5  $\pm$  4.6 mV (Supplemental Fig. 1*A*, 1*B*). Importantly, all particles when resuspended in saline for instillation were monodisperse, aggregate free, and carried a substantial negative charge. The manufacturer's measurements on the particles prior to glycine coating (which are performed in water and thus register as slightly larger but also monodisperse non-aggregated solutions by light scattering) were also checked by Zetasizer, which confirmed the manufacturer's claims (data not shown).

#### Particle instillation and immunizations

To determine particle effects on AAI, BALB/c mice received saline or nanoparticles (200 µg/50 µl) intratracheally on day 0 and day 2 (Fig. 1). The trachea was intubated with a soft plastic 20-gauge catheter and guide wire, and saline or particles were delivered via a 50-µl microsyringe (SGE International Pty., Melbourne, Australia) attached to a blunted 20gauge needle (24). Mice were sensitized i.p. with saline or OVA (50  $\mu$ g; Sigma-Aldrich, St. Louis, MO) in aluminum hydroxide on days 12 and 22 and challenged intratracheally with saline or OVA (25 µg) on days 32, 34, 37, and 39 as described (24) (Fig. 1). In pilot experiments to optimize nanoparticle dose, mice received 20, 100, or 200 µg PS50G nanoparticles intratracheally, with the 200-µg dose showing the most consistent (across cytokines) inhibition of Th2 cytokine production in the lung-draining LN (Supplemental Fig. 2), and the 200-µg dose was used for the remainder of our studies. In certain experiments, we used C57BL/ 6 mice instead of BALB/c mice, and in other experiments, mice received FITC-labeled OVA (no. O-23020; Invitrogen) for the final (fourth) challenge.

#### Tissue sampling and cell isolation

Collection and preparation of blood and bronchoalveolar lavage (BAL) were as described (24). Tissue digestion was performed as described (25)

with modifications. The right ventricle was perfused with 5 ml  $Ca^{2+}/Mg^{2+}$ free HBSS (no. 14175095; Invitrogen) with 0.01 M EDTA, pH 7.2. Lung and draining LN were chopped with a tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, U.K.). Tissue fragments were digested in collagenase type III (1 mg/ml; Worthington, Lakewood, NJ) and DNase type I (0.025 mg/ml; no. 1284932; Roche Diagnostics, Sydney, Australia) in a volume of 7 ml at 25°C by manual pipetting for 20 min. The reaction was stopped by adding one 10th volume of 0.01 M EDTA and mixing for 5 min. The cell suspension was filtered through a 70-µm cell strainer (BD Falcon) and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation (350  $\times$  g, 4°C). The cell pellet was resuspended in red cell lysis solution for 3-5 min (no. R7757; Sigma-Aldrich), diluted to 10 ml in RPMI 1640 and 10% FCS, and underlaid with 1 ml 0.01 M EDTA in FCS prior to centrifugation ( $350 \times g$ , 4°C). Cells were resuspended in staining buffer [3% FCS, 3% pooled normal mouse serum, 5 mM EDTA (pH 7.2), and 0.1% Na-azide in Ca2+/Mg2+-free HBSS], and viable cells counted in a hemocytometer. Airway hyperreactivity (AHR) was assessed invasively in separate groups of mice by measurement of lung resistance and dynamic compliance as described (26).

#### Assessment of nanoparticle effects in influenza virus infection

Naive C57BL/6 received saline or PS50G nanoparticles (200  $\mu$ g/50  $\mu$ l) intratracheally on day –1 and day 0 and on day 25 were infected intranasally with 1  $\times$  10<sup>4.5</sup> PFU HKx31 influenza A diluted in 25  $\mu$ l PBS, and body weight was recorded daily. On days 8 and 10 postinfection, BAL cells were stained with Ab to CD8 $\alpha$  and tetramer specific for the H-2D<sup>b</sup>– restricted nucleoprotein peptide of amino acids 366–374 [D<sup>b</sup>NP(366–374)], as described (27).

#### Assessment of cardiac inflammation

Naive BALB/c mice received PS50G nanoparticles on day 0 and were killed for analysis on day 45. The heart was removed and the aortic sinus dissected. The heart was dissected into three transverse slices from the base, mid-region, and apex. Aortic sinus and heart tissues were embedded in either paraffin or OCT compound (28). Five-micrometer-thick sections were used for staining [H&E and Oil Red O (for lipids)] and immunohistochemistry (CD68 for macrophages) as previously described (28).

#### Measurement of oxidized proteins and lipid peroxidation

Naive BALB/c mice received PS50G nanoparticles, LPS (10  $\mu$ g; LPS from *Escherichia coli*, no. K-235; Sigma-Aldrich) or saline intratracheally, and lungs were collected on days 1 and 3 postinstillation. Lungs were homogenized in a tissue homogenizer (Ultra-Turrax T25, IKA Works, Wilmington, NC). Oxidized proteins and lipid peroxidation were measured via spectrophotometric measurement of protein carbonyls and lipid hydroperoxides per the manufacturer's instructions (cat. no. 10005020 and no. 705002, respectively; Cayman Chemical, Ann Arbor, MI).

#### Tissue histology and quantitative image analysis

Formalin-fixed, paraffin-embedded, 4-µm-thick lung sections were stained with H&E for histological analysis or periodic acid–Schiff (PAS) reagent for quantitation of mucus-producing cell frequency. For the analysis of airway mucus-producing cells, photomicrographs of every airway in each mouse lung section (~20–50 airways per mouse) were analyzed by Fiji Open Source image analysis software (http://fiji.sc). The mean number of PAS-positive cells per micrometer of airway epithelial basement membrane was calculated for each mouse.

#### Flow cytometry

Nonspecific FcR binding was blocked by incubating cells in CD16/CD32 block (BD Biosciences, San Jose, CA). Cells  $(0.5 \times 10^6 \text{ to } 1 \times 10^6)$  were stained on ice for 20 min with the following Abs (all BD Biosciences unless noted): CD8 $\alpha$ -FITC, CD11b-PE, CD11b-PerCP-Cy5.5, CD11c-allophycocyanin, MHC class II (MHCII)–PE, MHCII-allophycocyanin-eFluor 780 (eBioscience), MHCII-biotin, and streptavidin–PerCP. Appropriate isotype control Abs were used. All dilutions were in staining buffer (see earlier). Data were acquired on a FACSCalibur or LSR II (BD) and analyzed with FlowJo (Tree Star, Ashland, OR).

#### CFSE staining of transgenic T cells

 $CD4^+$  T cells were purified from spleen and mesenteric LN of OVAspecific DO11.10 (H-2<sup>d</sup> haplotype, syngeneic with respect to BALB/c) TCR transgenic mice by incubating with Abs against Mac-1 (M1/70), F4/80, Ter 119, GR-1, MHCII (M5/114), and CD8 (53.6.7 from BD Pharmingen) and removal of Ab-binding cells as described (29). Cell purity was >95%. Cells were labeled with CFSE as described (30).

#### DC stimulation of naive T cells

LNs from BALB/c (H-2<sup>d</sup>) mice that had been treated with saline or nanoparticles prior to OVA sensitization and challenge were digested as described earlier and non-DCs removed by incubating with predetermined concentrations of Abs [anti-CD3 (KT3), anti-Thy1 (T24/31.7), anti-CD19 (ID3), anti-erythrocyte (TER-119)] and removal of Ab-binding cells as described (29). DCs were gated to exclude doublets and dead cells (propidium iodide) and sorted on the basis of CD8a and CD11b expression (27). CD4<sup>+</sup> DO11.10 T cells (5  $\times$  10<sup>4</sup>) were cocultured with sorted DCs (range  $6 \times 10^3$  to  $12 \times 10^3$ ) in duplicate as described (29). For the peptide pulsing experiments, DCs ( $12 \times 10^3$  to  $24 \times 10^3$ ) were isolated from naive mice by enzymatic digestion and sorting as above, pulsed with OVA MHCII-restricted peptide OVA323-339 (25 µg/ml) for 1 h at 37°C, washed, and cocultured with 5  $\times$   $10^4$  purified CFSE-labeled OVA-specific CD4+ T cells. Cultures were analyzed for proliferation at 60 h by staining with CD4-PE and propidium iodide and gating on CD4<sup>+</sup> propidium iodide<sup>-</sup> cells.

## Cytokine ELISPOT assay and measurement of BAL fluid cytokines

IL-4, IL-5, and IL-13 ELISPOT assays were performed as described (31). IFN- $\gamma$  ELISPOT assay was performed using AN18 capture and R4-6A2 biotinylated detection Abs (no. 3321-3-1000 and no. 3321-6-1000; Mabtech, Mossman, Australia) and hydrophobic membrane plates (no. MAIPS4510; Millipore). BAL fluid IL-5 and IL-13 were detected by specific ELISA (no. 88-7054 and no. 88–7137; eBioscience). Fifty-microliter standards and sample were tested as specified in the manufacturer's instructions.

#### OVA-specific IgE ELISA

OVA-specific IgE was detected as described (24).

#### Statistical analysis

Data were analyzed for normality and log-transformed as necessary prior to analysis by independent samples *t* test, ANOVA, or two-way ANOVA with Tukey or Bonferroni post tests, respectively (Graph Pad Prism v5.02). Differences were considered statistically significant at p < 0.05. Group sizes are indicated in the legends to figures that accompany this article. All values are mean  $\pm$  SEM.

#### Results

#### PS50G nanoparticles inhibit acute AAI

In the course of studies to investigate the long-term influence of inert 50-nm nanoparticles on development of allergic inflammation in the lung, we treated mice with ("ultrafine" or nanoparticle-sized particulates) inert PS50G nanoparticles intratracheally 32 d prior to induction of acute allergic asthma (24, 31) (Fig. 1). Unexpectedly, rather than promoting AAI, nanoparticle pretreatment prior to OVA sensitization and challenge (nano/OVA/OVA) dramatically decreased the frequency and number of airway eosinophils (Fig. 2A-C) compared with the positive control group (sal/ OVA/OVA). Nanoparticles instilled into mice without airway inflammation (i.e., which were sensitized with saline; nano/sal/ OVA) caused a negligible increase in the frequency of airway eosinophils compared with the sal/sal/OVA group (Fig. 2A). Mice with AAI had significantly increased concentrations of serum allergen-specific IgE (sal/OVA/OVA), and this was significantly inhibited by nanoparticles (nano/OVA/OVA) (Fig. 2D). However, nanoparticles themselves, in the absence of AAI (nano/sal/OVA), did not increase allergen-specific IgE levels over the sal/sal/OVA group.

Lungs from mice with airway inflammation (sal/OVA/OVA) showed a pronounced cellular infiltrate surrounding the airways, composed predominantly of eosinophils and mononuclear cells (Fig. 3*A*). The magnitude of this infiltrate was significantly inhibited by nanoparticle treatment (nano/OVA/OVA). Lungs of



FIGURE 1. Timing of particle instillation in mouse model of acute AAI. Mice received PS50G nanoparticles or saline intratracheally prior to i.p. OVA/alum sensitization and intratracheal saline or OVA challenge at the indicated times. Tissue sampling was performed 24 h after the final lung allergen challenge. In certain experiments, mice received saline/alum immunization instead of OVA/alum or were not sensitized or challenged.

mice that received nanoparticles in the absence of OVA challenge resembled normal healthy lungs, with no cellular infiltrate and normal lung architecture (Fig. 3A). These histological changes were directly reflected in total leukocyte yields obtained from enzymatically digested lungs, with a significant increase in cell numbers seen in the sal/OVA/OVA group compared with the negative control group (sal/sal/OVA), and the magnitude of this infiltrate was significantly inhibited by nanoparticle treatment (nano/OVA/OVA) (Fig. 3B). In agreement with the histology, nanoparticles instilled into mice without AAI (nano/sal/OVA) did not cause lung inflammation (Fig. 3B). Differential analysis of lung leukocytes isolated from mice with AAI showed significantly increased numbers of B cells (FSC<sup>low</sup>CD11c<sup>neg</sup>MHCII<sup>+</sup>) (32), eosinophils (FSC<sup>low</sup>SSC<sup>high</sup>CD11c<sup>low/neg</sup>MHCII<sup>neg</sup>) (32, 33), and DCs (CD11c<sup>+</sup>MHCII<sup>hi</sup>) (34), with no change in macrophage numbers (CD11c<sup>+</sup>MHCII<sup>+</sup>) (34) (DC and macrophage gating as in Fig. 7A). Nanoparticle instillation significantly inhibited this increase in numbers of B cells, eosinophils, and DCs, in agreement with the histological and lung cell count data (Fig. 3C). There was no change in numbers of lung CD4<sup>+</sup> or CD8<sup>+</sup> T cells (data not shown). Once again, nanoparticles themselves did not increase numbers of any of these cell populations (nano/sal/OVA). Quantitative assessment of mucus production, a characteristic of AAI critically regulated by IL-13, showed a dramatic increase in the frequency of mucus-producing cells in the airway inflammationpositive group (sal/OVA/OVA), and this was significantly attenuated by nanoparticle pretreatment (Fig. 3D). Nanoparticles themselves (nano/OVA/sal) did not induce mucus production (the frequency for the nano/OVA/sal group is virtually identical to that seen in naive unmanipulated lungs; data not shown).

## *PS50G nanoparticles inhibit Th2 cytokine production in the lung and draining LN*

We next performed a more detailed examination of PS50G nanoparticle effects on pulmonary allergen-specific Th2 cell responses. Mice with AAI had a marked increase in allergenspecific IL-4, IL-5, and IL-13 production in the draining LN, as expected, and this was significantly inhibited by nanoparticle treatment (Fig. 4A-C). The decreased frequencies of Th2 cytokine-producing cells in nano/OVA/OVA mice translated into 2to 4-fold reductions in the total numbers of IL-4-, IL-5-, and IL-13-producing cells in the draining LN compared with sal/OVA/ OVA mice (Supplemental Fig. 3A-C). Nanoparticles (nano/OVA/ OVA) also inhibited the increase in BAL fluid levels of IL-5 and IL-13 seen in sal/OVA/OVA mice (Fig. 4E, 4F). We observed an equivalent inhibitory effect of PS50G nanoparticles with the clinically relevant Bermuda grass pollen allergen (data not shown). Despite the pronounced nanoparticle inhibition of IL-13 production, nanoparticles did not impair the development of AHR (Supplemental Fig. 3D, 3E). However, this inhibition of local Th2 immunity was not counterregulated by nanoparticle-driven



FIGURE 2. PS50G nanoparticles inhibit eosinophilic airway inflammation and allergen-specific IgE. BALB/c mice received PS50G nanoparticles or saline prior to saline or OVA sensitization and challenge as in Fig. 1. *A*–*C*, Differential analysis of absolute BAL cell numbers (*A*, *C*) and frequencies (*B*). *D*, OVA-specific serum IgE. n = 5-10 mice/group, representative of five to six separate experiments. Mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.001. Significances for *A* and *C* refer to eosinophil numbers.

induction of Th1 cells in the draining LN (35), as the frequency of IFN- $\gamma$ -producing cells in both the nano/OVA/OVA and sal/ OVA/OVA groups was comparable and low, although still clearly elevated relative to the negative control (sal/sal/sal) group (Fig. 4D). Additional studies by us demonstrated that numbers of influenza virus nucleoprotein-specific CD8+ T cells in the BAL of saline- and PS50G nanoparticle-treated mice were equivalent at day 8 and day 10 postinfection (day 8:  $9.8 \times 10^4 \pm 3.0 \times 10^4$ versus  $8.3 \times 10^4 \pm 1.2 \times 10^4$ ; day 10:  $7 \times 10^4 \pm 0.5 \times 10^4$  versus  $10.1 \times 10^4 \pm 3.2 \times 10^4$ , saline versus PS50G, respectively). Furthermore, nanoparticle-treated mice showed similar kinetics of weight loss and recovery compared with saline-treated mice (Supplemental Fig. 3F). Therefore, nanoparticles do not impair bona fide Th1 immunity in an acute lung influenza virus infection model. Thus, nanoparticles had the unexpected property of specifically inhibiting the elicitation of local allergen-specific IL-4, IL-5, and IL-13 production, cytokines that together mediate acute allergic reactions, airway mucus production, and IgE isotype switching. This dampening of Th2 immunity in the lung and draining LN, without impairing Th1-biased immunity, was consistent with a local nanoparticle-induced immune modulatory mechanism.

#### PS50G nanoparticles do not impair systemic allergen priming

It could be speculated that nanoparticle pretreatment affected pulmonary Th2 immunity to allergen by dampening peripheral allergen sensitization prior to lung allergen challenge. We investigated this in separate experiments by comparing the effect of nanoparticles in mice that were sensitized with allergen but not challenged. OVA/alum i.p. in the absence of allergen challenge induced allergic sensitization—production of serum OVA-specific IgE and IL-4, IL-5, and IL-13 in the lung-draining LN (Fig. 5). Nanoparticle instillation prior to this OVA sensitization had no effect on any of these parameters. Thus, nanoparticles do not exert their effects at the systemic priming stage, but rather impair efficient induction of inflammation in the lung at the allergen challenge phase.

## *PS50G nanoparticles do not induce cardiac inflammation or lung oxidative stress*

Previous studies by us in mice and sheep showed that PS50G nanoparticles do not induce inflammatory reactions at sites of peripheral injection or in any organ up to 2 mo after intradermal or s.c. injection (22, 36-38). Polystyrene particles are further reported as unable to induce oxidative stress in vitro (39). However, because ultrafine particle exposure has been linked to the induction of cardiovascular disease and oxidative stress (1, 2), we also confirmed formally that our nanoparticles do not induce cardiovascular inflammation or lung oxidative stress when instilled into the lung. Our results showed no particle-induced cardiac pathology, with normal heart and aortic sinus morphology in mice treated with nanoparticles (Fig. 6A, 6B). Additionally, whereas LPS strongly induced expression of protein carbonyl and lipid hydroperoxides at day 3 postinstillation, nanoparticles did not induce significant oxidative stress in the lung (Fig. 6C, 6D). Furthermore, nanoparticles instilled into naive mice did not increase BAL fluid levels of IFN-y or IL-17 at days 1, 3, 7, or 31 postinstillation (IFN- $\gamma$  and IL-17 concentrations were below the limit of detection).



**FIGURE 3.** PS50G nanoparticles inhibit lung inflammation and airway epithelial mucus production. BALB/c mice received PS50G nanoparticles or saline intratracheally prior to saline or OVA sensitization and challenge as in Fig. 1. *A*, Representative micrographs from H&E-stained lung sections (original magnification ×400). *B* and *C*, Total lung leukocyte counts (*B*) and differential analysis of isolated lung leukocytes (*C*). n = 3/group (each replicate consisting of pools of three to nine mice); data are from two separate experiments. *D*, Quantitative image analysis of frequency of mucus-producing airway epithelial cells for all airways in the mouse lung. n = 6 mice/group. Mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

#### PS50G nanoparticle effects are not strain specific

To confirm that the protective effects induced by nanoparticles were not unique to our OVA-induced model of AAI in the BALB/c  $(H-2^d)$  strain, we tested the effects of nanoparticles in C57BL/6 mice. Consistent with the results in BALB/c mice, pretreatment of C57BL/6  $(H-2^b)$  mice with nanoparticles also markedly inhibited eosinophilic airway inflammation and Th2 cytokine production in the draining LN (Supplemental Fig. 4A-C).

## *PS50G nanoparticles inhibit DC expansion in the lung and draining LN in AAI*

Pulmonary DCs play a critical role in the generation of allergic airway immune responses (40). To investigate the mechanism by which nanoparticles promoted lung homeostasis in the face of allergen challenge, we therefore investigated direct PS50G nanoparticle effects on DC numbers and function. Nanoparticles (nano/ OVA/OVA) significantly inhibited the increased numbers of draining LN migratory CD11c<sup>+</sup>MHCII<sup>hi</sup> DCs (41) seen in mice with AAI (sal/OVA/OVA) (Fig. 7A, 7B), without affecting numbers of nonmigratory resident CD11c<sup>+</sup>MHCII<sup>+</sup> DCs (Supplemental Fig. 4D). As shown above, nanoparticles also inhibited the expansion of CD11c<sup>+</sup>MHCII<sup>hi</sup> DCs (34) in the lung, without affecting macrophage numbers (Fig. 3C, gated as in Fig. 7A). These data indicate that nanoparticles specifically modify the behavior of migratory DCs in the draining LN.

#### *PS50G nanoparticles inhibit expansion of allergen-laden CD11b<sup>hi</sup>MHCII<sup>hi</sup> DCs in the lung*

To investigate further the mechanisms by which nanoparticles influence function of migratory DCs and gain insight into how this could result in impaired immune stimulation during allergen challenge, we tested the capacity of DCs to take up allergen in vivo in the context of nanoparticle treatment. Sensitized mice were



**FIGURE 4.** PS50G nanoparticles inhibit Th2 cytokine production in the lung and lung-draining lymph node. BALB/c mice received PS50G nanoparticles or saline intratracheally prior to saline or OVA sensitization and challenge as in Fig. 1. A-D, Frequency of IL-4– (A), IL-5– (B), IL-13– (C), and IFN- $\gamma$ – (D) producing lung-draining LN cells stimulated with medium or OVA. E and F, BAL fluid concentrations of IL-5 (E) and IL-13 (F). n = 7-12 mice/group, representative of two to five experiments. Mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

challenged with FITC-labeled OVA to allow the identification of allergen-laden DCs (OVA-FITC<sup>+</sup>CD11c<sup>+</sup> cells) (41) (Fig. 7C). Initial experiments showed that lung OVA-FITC<sup>+</sup>CD11c<sup>+</sup> cells consisted almost entirely of cells with DC morphology. The absolute number of OVA-FITC+CD11c+ DCs was increased 2-fold in sensitized/challenged mice ( $0.34 \pm 0.02 \times 10^6$  versus  $0.67 \pm 0.05 \times$  $10^6$ , saline versus OVA, p = 0.003), and this was marginally prevented by nanoparticle instillation  $(0.58 \pm 0.05 \times 10^6)$ . More profoundly, nanoparticle treatment decreased both the proportion and absolute numbers of lung OVA-FITC<sup>+</sup>CD11c<sup>+</sup> cells that coexpressed high levels of MHCII or CD11b by ~40% (nano/ OVA/OVA versus sal/OVA/OVA; Table I and Fig. 7D-F). Notably, the proportion of CD11b<sup>hi</sup> DCs increased 5.2-fold in salinetreated mice (sal/sal/OVA versus sal/OVA/OVA) but increased only 2.1-fold in nanoparticle-treated mice (Table I). OVA+CD11c+ cells in the lungs of control nonsensitized mice (sal/sal/OVA) were MHCII<sup>lo</sup> and CD11b<sup>lo</sup> and were therefore relatively immature, and this was not altered by nanoparticle treatment (nano/sal/OVA; Table I and Fig. 7D-F). Nanoparticles therefore modify stimulatory DC ability to be recruited to or activated in the lung during an allergic inflammatory response.

In the draining LN, the absolute number of OVA-FITC<sup>+</sup>CD11c<sup>+</sup> DCs was increased >3-fold in OVA-sensitized mice (0.035  $\pm$  0.01  $\times$  10<sup>6</sup> versus 0.115  $\pm$  0.02 10<sup>6</sup>; sal/sal/OVA versus sal/OVA/ OVA, respectively), and this was largely inhibited by nanoparticle instillation (0.057  $\pm$  0.03  $\times$  10<sup>6</sup>, nano/OVA/OVA). However, in contrast to the lung, neither nanoparticles nor inflammatory status altered proportions of OVA-FITC<sup>+</sup>CD11c<sup>+</sup> DCs that expressed high levels of CD11b or MHCII in the draining LN. Therefore, nanoparticles substantially decrease numbers of a major stimulatory DC subset (33) among allergen-laden (OVA-FITC<sup>+</sup>) DC in the lung, but not the draining LN, providing a mechanistic explanation for the decreased allergic inflammation locally in the lung. However, the impaired Th2 cytokine production observed in the lung-draining LN suggests that additional mechanisms operate in parallel at this site.

## PS50G nanoparticles inhibit T cell-stimulating function of draining LN CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> DCs

To gain insight into other potential mechanisms by which nanoparticles impair allergen-specific inflammation by acting on DCs, we examined the effect of nanoparticles on the T cell stimulatory capacity of lung-draining LN DCs from mice with AAI. We tested the stimulatory function of the major DC populations in the lung draining LN (27):  $CD8\alpha^{-}CD11b^{-}$ ,  $CD8\alpha^{+}CD11b^{-}$ , and  $CD8\alpha^{-}$ CD11b<sup>+</sup> DCs. Whereas  $CD8\alpha^{-}CD11b^{+}$  DCs from mice that received saline prior to OVA sensitization and challenge (sal/OVA/ OVA) induced strong proliferation of naive OVA-specific CD4<sup>+</sup>



**FIGURE 5.** PS50G nanoparticles do not impair peripheral allergen sensitization in mice that have not been allergen challenged. BALB/c mice received saline or PS50G nanoparticles intratracheally on days 0 and 2, prior to OVA sensitization on day 12. Analysis was performed on day 10 postsensitization. *A*, Serum OVA-specific IgE. *B–D*, Frequency of IL-4– (*B*), IL-5– (*C*), and IL-13– (*D*) producing cells in the spleen stimulated with medium or OVA. *n* = 4 per group. Mean  $\pm$  SEM. \**p* < 0.05.

T cells, pretreatment with nanoparticles (nano/OVA/OVA) reduced proliferative responses to levels seen in saline controls (Fig. 8*A*, 8*B*). T cell-stimulating capability was absent in both the sorted  $CD11b^{-}$  DC subsets (data not shown). Thus, nanoparticles markedly blunt the ability of  $CD11b^{+}$  DCs in the lung-draining LN,

which have captured and processed OVA in vivo, to stimulate OVA-specific T cells. The fact that these three DC subsets were simultaneously sorted by gating on viable (propidium iodide<sup>-</sup>) cells from the same starting population suggested that the lack of proliferative capacity of the CD8 $\alpha$ <sup>-</sup>CD11b<sup>-</sup> and CD8 $\alpha$ <sup>+</sup>CD11b<sup>-</sup>



**FIGURE 6.** PS50G nanoparticles do not cause heart or aortic inflammation or oxidative stress in the lung. Naive BALB/c mice received saline, PS50G nanoparticles, or LPS intratracheally on day 0. *A* and *B*, Analysis of heart (*A*) and aortic sinus (*B*) was performed on day 45 postinstillation. Sections were stained with H&E, Oil Red O (for lipid), and CD68 for macrophages as described (28). Scale bar, 100  $\mu$ m for all images. *C* and *D*, Lung homogenates were assayed for protein carbonyls (*C*) and lipid hydroperoxides (*D*) at days 1 and 3 postinstillation. *n* = 3 to 4/group/time point. Mean ± SEM. \**p* < 0.05, \*\*\**p* < 0.001.

expansion of conventional DCs in the LN and allergen-laden CD11bhiMHCIIhi lung DCs. BALB/c mice received PS50G nanoparticles or saline intratracheally prior to saline or OVA sensitization and OVA challenge as in Fig. 1. Gating strategy for DCs and macrophages in the lung and migratory and resident DCs in the draining LN (A) and numbers of migratory CD11c+MHCII<sup>hi</sup> DCs in the lung-draining LN (B). Gating strategy for allergen-laden DCs (OVA-FITC<sup>+</sup>CD11c<sup>+</sup>) in the lung (C) and expression of CD11b and MHCII by OVA-FITC<sup>+</sup>CD11c<sup>+</sup> cells (D). Numbers of OVA-FITC<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>hi</sup> (E) and OVA-FITC<sup>+</sup>  $\text{CD11c}^+\text{CD11b}^{\text{hi}}(F)$  cells in the lung. n = 3/group (each replicate consisting of pools of three to nine mice); data are from two separate experiments. Mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

FIGURE 7. PS50G nanoparticles inhibit



subsets was not due to a lack of cell viability. However, we formally demonstrated that sorted  $CD8\alpha^{-}CD11b^{-}$ ,  $CD8\alpha^{+}CD11b^{-}$ , and  $CD8\alpha^{-}CD11b^{+}$  DCs that had been loaded with MHCIIrestricted OVA<sub>323-339</sub> epitope ex vivo were fully capable of stimulating proliferation of CFSE-labeled OVA-specific CD4<sup>+</sup> T cells and were thus functionally viable (Fig. 8*C*).

#### Discussion

Host defense pathways naturally engaged in handling environmental infectious particulate pathogens may also be triggered by man-made pollutant particulates or engineered nanoparticles. The rules of engagement are still being defined. In this study, we demonstrate that PS50G nanoparticles have the surprising novel property of generating a lung state resistant to AAI. These nanoparticles inhibited lung airway and tissue inflammation and mucus hypersecretion and additionally prevented the elicitation of allergen-specific Th2 immunity. Mechanistically, this was mediated by decreased numbers of CD11c<sup>+</sup>MHCII<sup>hi</sup> DCs in the lung and draining LN, and of allergen-laden CD11c<sup>+</sup>CD11b<sup>hi</sup>MHCII<sup>hi</sup> DCs in the lung. PS50G nanoparticles also markedly suppressed the capacity of CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> DCs in the lung-draining LN to stimulate allergen-specific CD4<sup>+</sup> T cells. Although contentious given the prevailing studies showing that a wide range of nanoparticles/ultrafine particles promote AAI and asthma, our

Table I. Proportions of OVA-FITC<sup>+</sup>CD11c<sup>+</sup> lung cells that are MHCII<sup>hi</sup> or CD11b<sup>hi</sup>

Parameter (%)	Treatment Group			
	Sal/Sal/OVA	Sal/OVA/OVA	Nano/Sal/OVA	Nano/OVA/OVA
MHCII <sup>hi</sup> CD11b <sup>hi</sup>	$18.9 \pm 3.5$ $10.4 \pm 2$	$49.3 \pm 2.7$ $53.7 \pm 0.7$	$14.0 \pm 4.3$ $17.4 \pm 3.2$	$30.5 \pm 5.6^{a}$ $35.8 \pm 3.8^{b}$

All values are mean  $\pm$  SEM.

 ${}^{a}p = 0.06$  versus sal/OVA/OVA.  ${}^{b}p < 0.02$  versus sal/OVA/OVA.

studies bring important information to the field by identifying a defined type of noninflammatory nanoparticle that can inhibit AAI by modulating pulmonary DC function. We speculate that inhibition of local allergic inflammation and Th2 immunity by



FIGURE 8. PS50G nanoparticles impair the ability of CD11b<sup>+</sup> lungdraining LN DCs from mice with AAI to stimulate Ag-specific T cells. Draining LN leukocytes were isolated by collagenase/DNase digestion, and after excluding doublets and gating on viable CD11c<sup>+</sup> cells simultaneously sorted into CD8a<sup>-</sup>CD11b<sup>+</sup>, CD8a<sup>-</sup>CD11b<sup>-</sup>, and CD8a<sup>+</sup>CD11b<sup>-</sup> DC subsets. A and B, BALB/c mice received PS50G nanoparticles intratracheally prior to OVA sensitization and challenge; controls received saline instead of particles or OVA. Sorted viable CD8a<sup>-</sup>CD11b<sup>+</sup> DCs were cocultured with CFSE-labeled OVA-specific syngeneic (DO11.10) CD4<sup>+</sup> T cells. Proliferation was measured after 60 h. Representative plots and pooled data, respectively. n = 3 to 4/group (each replicate consisting of pools of three mice); data are from two separate experiments. \*\*p < 0.01, \*\*\*p < 0.001. C, Sorted viable CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup>, CD8 $\alpha$ <sup>-</sup>CD11b<sup>-</sup>, and CD8a<sup>+</sup>CD11b<sup>-</sup> DCs isolated from the lung-draining LN of naive mice were pulsed with OVA323-339 peptide and cocultured with OVA-specific DO11.10 CD4<sup>+</sup> T cells as above. Proliferation was measured after 60 h. Representative plots from pools of n = 4 mice/group, n = 2 replicates per DC subset. Mean  $\pm$  SEM.

nanoparticle exposure reflects a natural evolutionary mechanism designed to limit the elicitation of inappropriate responses to viralsized particulates in the lung.

As noted above, various types of nanoparticle/ultrafine particles have been shown to promote AAI or asthma. For example, carbon black ultrafine particles, titanium dioxide nanoparticles, carbon nanotubes, and diesel exhaust particles have all been shown to promote allergic sensitization and AAI (5, 6, 8-14). However, there are some important differences between these studies and ours. Carbon black ultrafine particles and carbon nanotubes (9, 13, 14) contain significant concentrations of organic or metallic impurities that can themselves promote pulmonary inflammation (15, 16). Diesel exhaust particles consist of a range of particle sizes from the nanoscale up to 10 µm and contain aromatic hydrocarbon contaminants that can directly promote lung inflammation (6, 7, 17). Titanium dioxide nanoparticles and carbon nanotubes can additionally promote lung inflammation by inducing pulmonary oxidative stress in vivo (13, 18). In contrast, the polystyrene nanoparticles used by us do not contain impurities and did not cause lung oxidative stress or lung inflammation themselves. Furthermore, they did not increase BAL fluid IFN-y or IL-17 levels when instilled into naive mice. Altogether, differences in particle composition, adsorbed toxic chemicals, and potential to induce oxidative stress could explain why inert PS50G nanoparticles can inhibit AAI, rather than promote AAI as previously described for other nanoparticles.

IL-4 and IL-13 play critical roles in IgE class switching and mucus secretion (42-45). Our finding of decreased frequency and numbers of IL-4- and IL-13-producing draining LN cells in PS50G nanoparticle-treated mice is therefore consistent with the inhibition of serum allergen-specific IgE levels and airway epithelial mucus secretion we observed. IL-5, together with eotaxin, plays a key role in eosinophil maturation and recruitment to the lung (46-49), and the decreased frequency and numbers of IL-5producing draining LN cells in nanoparticle-treated mice is thus consistent with the decreased airway and lung tissue eosinophilia in these mice. Consistent with the key role for lung DCs in driving the local allergen-induced Th2-type inflammatory cascade, the inefficient allergen-specific Th2 cytokine production in nanoparticle-treated mice is explained by the impaired recruitment/ expansion of total and allergen-laden DCs in the lung and draining LN and the impaired T cell stimulatory capacity of lungdraining LN DCs. Our finding that PS50G nanoparticles did not affect allergen sensitization indicates that they manifested their effects in the lung after allergen challenge. This finding is important as it illustrates that some nanoparticles, such as PS50G nanoparticles, could help sustain lung homeostasis even in allergyprone individuals and further shows that the nanoparticle effect can be "targeted" to the lung. The observation that nanoparticles exerted their immunomodulatory effects locally in the pulmonary compartment is consistent with the fact that the nanoparticles were delivered directly into the lung and that fluorescent PS50G nanoparticles were detectable in the lung and draining LN up to 31 d postinstillation (data not shown). Although nanoparticles markedly suppressed Th2-biased lung inflammation, they did not significantly impair AHR. However, AHR is currently effectively treated with  $\beta$ -agonists and preventative topical corticosteroids, but there are no current therapies to prevent airway inflammation and remodeling. There is exciting potential to explore the coadministration of nanoparticles with bronchodilatory agents or even novel antifibrotic drugs as an alternative option for treatment of asthma. Notably, PS50G nanoparticles did not exert a "blanket" dampening of inflammation, as they did not inhibit allergenspecific IFN- $\gamma$  production in the lung-draining LNs in the mice with AAI. Furthermore, PS50G nanoparticles did not inhibit bona fide Th1 anti-viral immunity in an acute lung influenza virus infection model.

DCs play a critical role in the induction and maintenance of AAI (40). Relative to control mice with noninflamed lungs, DC numbers in the lung increased 5-fold after OVA sensitization and challenge, as expected during acute lung inflammation (50, 51), and this expansion was significantly inhibited by PS50G nanoparticles. CD11b expression reciprocally identifies major DC populations in the lung, being CD11c+CD11bhiCD103- and CD11c<sup>+</sup>CD11b<sup>lo/-</sup>CD103<sup>+</sup>. The CD11b<sup>hi</sup> subset is preferentially expanded during AAI, is the major chemokine-producing population, and induces stronger T cell proliferation than the CD11b<sup>lo</sup> (CD103<sup>+</sup>) subset (33, 52). The selective analysis of allergenladen DCs (OVA-FITC+CD11c+) revealed nanoparticle effects on CD11b expression not detected using a broad analysis of CD11c<sup>+</sup> MHCII<sup>hi</sup> DCs and revealed that nanoparticles inhibit expansion of MHCII<sup>hi</sup> and CD11b<sup>hi</sup> DCs during inflammation in the lung. In agreement with previous studies, OVA-FITC+CD11c+ (allergenladen) cells in the lungs of nonsensitized animals had a relatively immature phenotype as judged by low MHCII (34, 53, 54) and CD11b expression, and this was not changed by nanoparticle treatment. This is consistent with our finding that PS50G nanoparticles themselves did not induce pulmonary inflammation.

DC migration from the lung to draining LN is negligible in homeostasis (55) but increases markedly during inflammation (50, 55). Lung-derived Ag-carrying DCs in the draining LN can be identified within the CD11c+MHCIIhi subset, whereas nonmigratory LN-resident DCs are CD11c<sup>+</sup>MHCII<sup>+</sup> (41). Relative to controls, numbers of migratory CD11c+MHCII<sup>hi</sup> DCs in the draining LN increased ~3-fold in OVA-sensitized/challenged mice, and this was almost totally prevented by PS50G nanoparticle treatment. This inhibition of DC migration from the lung to the draining LN presumably reflects the above-mentioned impaired increase in lung DC numbers. Importantly, nanoparticles significantly inhibited the capacity of CD8α<sup>-</sup>CD11b<sup>+</sup> DCs from the lung-draining LN to stimulate OVA-specific CD4<sup>+</sup> T cells. Stimulatory capacity was not observed in the other draining LN DC subsets tested (CD8a<sup>+</sup>CD11b<sup>-</sup>, CD8a<sup>-</sup>CD11b<sup>-</sup>), despite the fact that they were functional as reflected in their ability to induce OVA-specific CD4<sup>+</sup> T cell proliferation when loaded with OVA peptide ex vivo. Our observation that T cell stimulatory capacity is restricted to the CD11b<sup>+</sup> DC subset is consistent with the finding that CD11c<sup>+</sup>CD103<sup>-</sup> (CD11b<sup>+</sup>) DCs in lung-draining LN are specialized at presenting Ag (e.g., allergen) to CD4<sup>+</sup> T cells (56). In contrast, during lung viral infection, CD11b<sup>-</sup> DCs (both CD8 $\alpha^+$ and  $CD8\alpha^{-}$ ) are the dominant Ag-presenting subset in the draining LN, whereas  $CD8\alpha^{-}CD11b^{+}$  DCs do not play a role (27, 29), indicating that different Ag forms (e.g., noninfectious versus infectious) are presented by distinct DC subsets. Collectively, our data illustrate novel mechanisms by which nanoparticles can inhibit AAI including decreasing numbers of allergen-laden CD11b<sup>hi</sup> lung DCs, together with "conventional" (CD11c<sup>+</sup>MHCII<sup>hi</sup>) DCs in the lung and LN, and strongly suppressing the T cell stimulatory capacity of DCs in the draining LN.

Our findings expand current knowledge on the effects of nanoparticles in the lung in several ways. First, we show that, in contrast to various ambient, anthropogenic and man-made nanoparticles, PS50G nanoparticles can inhibit the development of AAI, demonstrating that not all nanoparticles necessarily promote/ exacerbate AAI. The nanoparticle protective activity occurred similarly in genetically different mouse strains. Second, we show that such nanoparticles can inhibit the generation of allergic lung inflammation and allergen-specific Th2-biased immunity. Third, our findings suggest that pulmonary DC expansion and T cell stimulatory function in mice with AAI can be selectively affected by agents such as PS50G nanoparticles. These changes in DC distribution and function may further contribute to explain natural lung homeostasis in the face of particulate exposure. Importantly, we show that PS50G nanoparticles do not exert their effects at the allergen sensitization phase but rather at the level of local (lung) allergen challenge, showing that pulmonary immunity can be specifically and independently targeted for immunotherapy. The finding that it is possible selectively to inhibit Th2 immunity in the lung without affecting local Th1 immunity or peripheral responses may further open a window to the development of other creative approaches to develop targeted treatments for multiple lung inflammatory diseases.

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#### Disclosures

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