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Differential Uptake of Nanoparticles and Microparticles by Pulmonary APC Subsets Induces Discrete Immunological Imprints

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There is increasing interest in the use of engineered particles for biomedical applications, although questions exist about their proinflammatory properties and potential adverse health effects. Lung macrophages and dendritic cells (DC) are key regulators of pulmonary immunity, but little is known about their uptake of different sized particles or the nature of the induced immunological imprint. We investigated comparatively the immunological imprints of inert nontoxic polystyrene nanoparticles 50 nm in diameter (PS50G) and 500 nm in diameter (PS500G). Following intratracheal instillation into naive mice, PS50G were preferentially taken up by alveolar and nonalveolar macrophages, B cells, and CD11b+ and CD103+ DC in the lung, but exclusively by DC in the draining lymph node (LN). Negligible particle uptake occurred in the draining LN 2 h postinstillation, indicating that particle translocation does not occur via lymphatic drainage. PS50G but not PS500G significantly increased airway levels of mediators that drive DC migration/maturation and DC costimulatory molecule expression. Both particles decreased frequencies of stimulatory CD11b+ MHC class IIhi allergen-laden DC in the draining LN, with PS50G having the more pronounced effect. These distinctive particle imprints differentially modulated induction of acute allergic airway inflammation, with PS50G but not PS500G significantly inhibiting adaptive allergen-specific immunity. Our data show that nanoparticles are taken up preferentially by lung APC to stimulate cytokine/chemokine production and pulmonary DC maturation and translocate to the lung-draining LN via cell-associated transport. Collectively, these distinctive particle imprints differentially modulate development of subsequent lung immune responses. These findings support the development of lung-specific particulate vaccines, drug delivery systems, and immunomodulators. The Journal of Immunology, 2013, 191: 000–000.

Inhalation of air pollution particulates is epidemiologically linked to reduced lung function, bronchitis, and asthma exacerbations (1). Evidence suggests that ambient ultrafine particles (<100 nm) play a disproportionate role in asthma exacerbations compared with fine-sized microparticles (100–2500 nm) (2–5), suggesting that inhaled particle size influences their relative ability to modulate lung function. Similarly, man-made carbon black and titanium dioxide nanoparticles (6–8) and carbon nanotubes (9, 10) all promote allergic sensitization and/or allergic airway inflammation (AAI). However, counterintuitively, we recently made the discovery that preadministration of inert nontoxic polystyrene nanoparticles 50 nm in diameter (PS50G), which do not induce oxidative stress, can inhibit development of AAI by impairing pulmonary dendritic cell (DC) expansion or stimulatory function (11). It is currently unknown if the novel beneficial immunological imprint of particle exposure in the lung that we identified using PS50G is affected by particle size.

DC and macrophages play critical roles in the induction and regulation of pulmonary immunity and AAI (12–16). However, surprisingly little is known about how different-sized particles are handled by pulmonary APC. Previous studies by our group have shown that 40–50 nm nanoparticles, when conjugated to protein Ag, are taken up by lymph node (LN) DC and potently stimulate immune responses, whereas particles >200 nm fail to do so (17). An early study suggested that 400-nm latex particles were taken up predominantly by F4/80+ (CD11c+ or CD11c+Ihi) cells, consistent with a macrophage phenotype, in the lung up to 48 h after instillation (18). Further, diesel and ambient particulates were shown to promote Th2 cytokine production in the draining LN and activate lung CD11c+ cells (19), although these CD11c+ cells were not segregated into macrophage and DC subsets. Similarly, carbon black nanoparticles coadministered with allergen promote AAI by increasing costimulatory capacity of DC (20). The above would suggest nanoparticles (<100 nm) and fine-sized particles (>100 nm) could be handled differently in the lung and may have different imprinting consequences. Particle-adsorbed toxic chemicals and metallic impurities and the induction of oxidative stress are thought to play significant roles in the adjuvant and/or inflammatory

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Abbreviations used in this article: AAI, allergic airway inflammation; BAL, bronchoalveolar lavage; DC, dendritic cell; FSC, forward light scatter; i.t., intratracheally; LN, lymph node; MHC II, MHC class II; pDC, plasmacytoid DC; PS50G, nontoxic polystyrene nanoparticle 50 nm in diameter; PS500G, nontoxic polystyrene nanoparticle 500 nm in diameter.
effect of ambient and diesel particulates (5, 21). However, no studies have compared the long-term effects of different-sized inert particles, which are devoid of potential toxic contaminants and do not induce oxidative stress, on pulmonary APC distribution and function and development of subsequent lung immune responses.

We investigated comparatively the uptake by different APC subsets of ultrafine PS50G nanoparticles and fine 500-nm micro-particles (PS500G) in the lung and lung-draining LN, as well as the activation status of these APC at both early and late time points after exposure. We also compared the potential downstream effects of PS50G and PS500G particles in preventing the subsequent development of AAI. Use of fluorescent-labeled particles allowed us to determine the rates of particle clearance, track particle migration, and identify the subsets of particle-laden APC over time for PS50G and PS500G. Our results provide new insights into particle effects on pulmonary APC distribution and function and indicate that when engineering particles for topical lung administration, the substantial differences in biological function due to size-dependent effects of particles on APC should be taken into account.

Materials and Methods

Mice

Female BALB/c mice aged 7 to 8 wk were obtained from Laboratory Animal Services (Adelaide, South Australia) and housed in the Alfred Medical Research and Education Precinct animal facility. All experimental protocols were approved by the precinct Animal Ethics Committee.

Particle instillation and immunization

FITC-labeled carbohydrate-modified microspheres (nominally 0.04 and 0.5 μm; Invitrogen-Molecular Probes, Carlsbad, CA; #F8795 and #F8813, respectively) were glyco-encased as described previously (17). PS50G and PS500G had a narrow size distribution (58.54 ± 0.3 and 488.33 ± 7.87 nm, respectively) and carried a negative surface charge (−14.8 ± 1.65 and −38.87 ± 4.71 mV, respectively) (Supplemental Fig. 1). Mice received saline (control), PS50G, or PS500G particles (200 μg/50 μl) intratracheally (i.t.) (22) on days 0 and 1, 5, 7, and 30/31 postinstillation. In experiments to test particle effects on AAI, mice received PS50G or PS500G (200 μg/50 μl) i.t. on days 0 and 2 prior to i.p. sensitization with OVA (50 mg; Sigma-Aldrich, St. Louis, MO) in aluminum hydroxide on days 12 and 22 and i.n. OVA challenge (25 μg) on days 32, 34, 35, and 37 as described (Fig. 1A) (11). In experiments to test particle effects on allergen uptake, mice received particles i.t. prior to OVA–Alexa Fluor 488 (100 μg/50 μl) (Molecular Probes, Grand Island, NY; #034781) on day 3, with analysis performed on days 4 or 31.

Tissue sampling and cell isolation

Methods were as described previously (22). Blood was collected from the inferior vena cava and serum collected. Bronchoalveolar lavage (BAL) was performed with 0.4 ml 1% FCS in PBS and three further lavages of 0.3 ml. For differentials, BAL cytospots were Giemsa stained (Merck, Kilsyth, VIC, Australia) and ≥200 cells identified by morphological criteria. Tissue digestion was performed as described previously (11). The right ventricle was perfused with 5 ml Ca2+-Mg2+-free HBSS (Invitrogen; #1244901) at 25°C mixing continuously for 45 min (LN) or 1 h (lung). The reaction was stopped by adding one-tenth volume of 0.01 M EDTA (pH 7.2). Lung-draining LN were minced with appropriate isotype control Abs and/or fluorescence-minus-one controls were used. All dilutions were in staining buffer (see above). Cells were protected from light at all times. Acquisition was on an FACScalibur, LSRII, or LSRFortessa (all BD Bioscienes) and analysis performed on FlowJo (Tree Star, Ashland, OR).

Measurement of BAL fluid cytokines and chemokines

IL-5 and IL-13 were detected using IL-5 and IL-13 ELISA kits (#88-7054 and #88-7137; eBioscience). Fifty-microliter standards and samples were tested. Details were otherwise as specified in the manufacturer’s instructions. IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, eotaxin/CCL11, G-CSF, GM-CSF, keratinocyte chemoattractant/CXCL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, and TNF were detected by Bio-Plex (Bio-Rad, Hercules, CA). TGF-β1 was detected using the TGF-β1 ELISA DuoSet following acid activation of latent TGF-β according to the manufacturer’s instructions (#DY1679; R&D Systems).

Statistical analysis

Statistics were analyzed using GraphPad Prism v5.02 software (GraphPad). 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 posttests, as appropriate. Differences were considered statistically significant at p < 0.05. Group sizes are indicated in the figure legends. All values are mean ± SEM.

Results

PS50G nanoparticles cause mild transient airway inflammation and sustained increases in lung and draining LN cell numbers

To understand the broad effects of PS50G and PS500G within the pulmonary compartment, we analyzed their effects on leukocyte numbers in the airways, lung parenchyma, and lung-draining LN. Mice were exposed to an equal mass (200 μg) of PS50G or PS500G, consistent with other studies in the particulate literature (7, 23, 24). Relative to saline controls, PS50G nanoparticles induced a 5-fold expansion of BAL cells at day 3, declining rapidly to near baseline values by day 31 (Fig. 1A). PS500G nanoparticles induced a smaller expansion in BAL cell numbers at day 1, returning to baseline by day 31. To put these particle-induced increases in context, BAL counts in acute AAI (11, 25) are typically 6–10-fold greater (3–5 × 106 cells) than the maximum values seen in PS50G-treated mice. PS50G nanoparticles caused an ∼2-fold increase in lung leukocyte counts relative to controls at all time points (Fig. 1B). PS500G microparticles induced a smaller expansion in BAL cell numbers at day 1, although cell counts returned to control values by day 7. Nanoparticles increased draining LN cell counts 4-fold at day 7 relative to saline controls, and this was maintained at day 31, whereas PS500G did not cause any significant change (Fig. 1C). The peak PS50G-induced increases in lung and LN cell counts were ∼2-fold lower than those seen during acute AAI (11, 25).

Differential analysis of BAL cells showed that, relative to saline mice, PS50G treatment caused an expansion of macrophages at day 3 (routine differential analysis of BAL cells does not discriminate between macrophages and DC, and these are hereafter referred to as macrophage/DC) (Fig. 1D). There was a small expansion of eosinophils and neutrophils at day 3. These changes had markedly subsided by day 7, although macrophage/DC numbers were still elevated 2-fold in PS50G-treated mice (Fig. 1E). By day 31, macrophage/DC numbers were still slightly elevated in PS50G nanoparticle-treated mice, whereas BAL cell composition in PS50G nanoparticle-treated mice resembled controls (Fig. 1F).
PS50G nanoparticles increase pulmonary DC and macrophage numbers

We predicted that these broad changes in pulmonary leukocyte numbers would translate into marked alterations in pulmonary APC distribution. We performed a detailed analysis of lung APC populations at days 3 and 30 postparticle instillation, as our time-course analysis (Fig. 1A–C) showed that these represented peak and resolution phases of particle-induced inflammation, respectively. We analyzed proportions of lung CD11c+ alveolar macrophages (CD11c+CD11b–F4/80+), CD11c–CD11b–F4/80– B cells using Boolean gating), B cells (CD11c–CD11b–MHC II–CD45R[B220]low forward light scatter [FSC]low), and CD11c+MHC II+CD11b+CD103+ and CD11b+CD103– DC (excluding F4/80–Siglec-F– cells using Boolean gating) (Fig. 2). To validate our macrophage gating strategy, we confirmed that the majority (98 ± 0.15%) of alveolar macrophages identified as CD11c+CD11b–F4/80– expressed Siglec-F–, a marker of alveolar macrophages (26), and we therefore used this phenotype to identify alveolar macrophages across different time points. We also confirmed that <0.5% of nonalveolar/interstitial macrophages identified as CD11c+CD11b–F4/80– (excluding MHC II–CD45R– B cells using Boolean gating) expressed Siglec-F– (data not shown).

Both PS50G and PS500G caused an ∼3-fold decrease in proportions of lung CD11c+ alveolar macrophages at day 3, with this decrease maintained at day 30 in the PS500G group (Supplemental Fig. 2A). In contrast, PS500G caused a small increase in frequency of CD11c–nonalveolar macrophages at day 3, although proportions of this population had returned to baseline by day 30 (Supplemental Fig. 2A). PS50G and PS500G caused a small but significant decrease in proportions of B cells at day 3. Neither particle size caused a significant change in proportions of plasmacytoid DC (pDC) (CD11cintMHC IIlow/intCD11b–CD45R+; the majority confirmed to be pDC Ag-1+ in preliminary experiments; region 3, Supplemental Fig. 2B) (0.4–0.7% of lung leukocytes across all groups; data not shown). PS50G decreased proportions of CD11b–CD103+ lung DC over 2-fold at day 3, with PS500G having a slightly smaller effect, and this pattern was largely maintained at day 30 (Supplemental Fig. 2A). In contrast, PS50G caused a ∼2-fold increase in proportions of CD11b+CD103+ lung DC at both early and late time points, whereas PS500G caused a smaller increase at day 3 only. Additional analysis showed that PS50G nanoparticles caused an ∼7-fold increase in proportions of CD11c+MHC II+ lung DC at day 3 (region 1; Supplemental Fig. 2B) and increased proportions of CD11b+CD11c+MHC II+ DC by 10%, whereas PS500G similarly increased the frequency of CD11b+ DC at days 3 and 7 (Supplemental Fig. 2B).

In the lung-draining LN, PS50G induced a 5-fold expansion of migratory (Ag-transporting) CD11c+MHC IIhi DC (27, 28) (region 1;
fold greater than LN-resident DC numbers, regardless of treatment. There were no marked changes in proportions of lung-derived migratory CD11c<sup>+</sup>MHC II<sup>+</sup> alveolar macrophages (29) (region 3; Supplemental Fig. 2C) (0.41, 0.36, and 0.51%; saline, PS50G, and PS500G, respectively). Further analysis showed that ~60% of this minor CD11c<sup>+</sup>MHC II<sup>+</sup> population were CD11b<sup>+</sup> CD45R<sup>-</sup> pDC in saline controls, and this was not altered by either particle size (data not shown). There were no significant changes in CD11c<sup>+</sup>MHC II<sup>-</sup> cells (>99% small leukocytes by FSC/side scatter, <1% CD11b<sup>+</sup>, region 4; Supplemental Fig. 2C) (74.8, 72.5, and 69.1%; saline, PS50G, and PS500G, respectively) or CD11c<sup>+</sup>MHC II<sup>-</sup> B cells (region 5; Supplemental Fig. 2C) (20.9, 23.2, and 24.4%; saline, PS50G, and PS500G, respectively) among the three groups.

**Nanoparticles and microparticles are taken up and cleared from the lung and lung-draining LN with different kinetics**

To gain insight into how particle size affects cellular uptake and clearance within the pulmonary compartment, we identified cells which internalized particles as fluorescent positive (Fluo<sup>+</sup>) (Fig. 3A). The frequency of PS50G-laden cells in the lung was significantly greater than PS500G-laden cells at day 1 (38%), dropping steadily over the next month (Fig. 3B). In contrast, whereas the frequency of PS50G- and PS500G-laden cells in the draining LN was the same at day 1, the frequency of PS50G-laden cells increased rapidly until day 7 (an 18-fold expansion of cell numbers). There was little change in the frequency of PS500G-laden cells over this time (Fig. 3C). Background frequencies (i.e., mice that received saline instead of particles) were 0.2–0.6% in the lung and lung-draining LN at all times. Thus, the frequency of nanoparticle-laden cells peaked early in the lung, dropping gradually over time, with a delayed increase in particle-laden cells seen in the lung-draining LN, whereas smaller changes were observed for PS500G.

**PS50G and PS500G are taken up by distinct cell populations in the lung and lung-draining LN**

Previous data from our group showed size-differential uptake of PS50G and PS500G by DC and macrophages, respectively, in the draining LN following footpad injection (17), and we speculated that a similar phenomenon would operate in the lung. We analyzed particle uptake by lung CD11c<sup>+</sup> alveolar macrophages (CD11c<sup>+</sup> CD11b<sup>+</sup> F4/80<sup>-</sup>Siglec-F<sup>-</sup>), CD11c<sup>-</sup> nonalveolar (interstitial) macrophages (CD11c<sup>-</sup> CD11b<sup>-</sup> F4/80<sup>-</sup>CD45R<sup>-</sup>MHCII<sup>-</sup>), B cells (CD11c<sup>+</sup> CD11b<sup>-</sup> F4/80<sup>-</sup> MHC II<sup>+</sup>CD45R<sup>-</sup>), and CD11b<sup>-</sup>CD103<sup>+</sup> and CD11b<sup>-</sup>CD103<sup>-</sup> DC (CD11c<sup>+</sup>MHC II<sup>-</sup>F4/80<sup>-</sup>Siglec-F<sup>-</sup>) as gated in Fig. 2. PS50G were preferentially taken up by CD11c<sup>-</sup> alveolar macrophages at day 3, whereas the proportion of PS500G-laden CD11c<sup>-</sup> macrophages more than doubled by day 30 (Fig. 3D). Similarly, nonalveolar CD11c<sup>-</sup> macrophages preferentially took up PS50G nanoparticles, exceeding 30% of PS50G-laden cells at day 3 and dropping to 18% at day 30. The proportion of PS500G-laden CD11c<sup>-</sup> macrophages was steady at ~5% at both time points. Approximately 10% of B cells took up PS50G at day 3, dropping to < 6% at day 30, whereas uptake of PS50G was ~3-fold lower. Approximately 60% of CD11b<sup>+</sup> CD103<sup>+</sup> and CD11b<sup>-</sup> CD103<sup>-</sup> DC took up PS50G at day 3, whereas uptake of PS500G microparticles was ~4-fold lower. The proportion of PS500G-laden CD103<sup>-</sup> DC had decreased ~2-fold (to 30%) at day 30, whereas the proportion of PS500G-laden CD11b<sup>-</sup> DC remained static. The frequency of PS500G-laden CD103<sup>-</sup> and CD11b<sup>-</sup> DC remained virtually unchanged from day 3 to 30.

In the draining LN, we determined particle uptake by CD11c<sup>+</sup> MHC II<sup>-</sup> DC examining CD103<sup>+</sup>CD11b subsets (gated on total CD11c<sup>+</sup>MHC II<sup>-</sup> and CD11c<sup>+</sup>MHC II<sup>-</sup> cells; Fig. 5A, Supplemental Fig. 2C), lung-derived migratory CD11c<sup>+</sup>MHC II<sup>-</sup> al-
veolar macrophages (29) (region 3; Supplemental Fig. 2C), CD11c<sup>+</sup>MHC II<sup>+</sup> cells (region 4; Supplemental Fig. 2C), and CD11c<sup>+</sup>MHC II<sup>+</sup>B cells (region 5, Supplemental Fig. 2C). We observed strong preferential size-dependent uptake of PS50G by CD11b<sup>+</sup>CD103<sup>+</sup>, CD11b<sup>+</sup>CD103<sup>-</sup>, and CD11b<sup>+</sup>CD103<sup>+</sup> DC subsets (90–95% of these three DC subsets lacked expression of the monocytic marker Ly6C; data not shown). The proportion of PS50G-laden CD11b<sup>+</sup>CD103<sup>+</sup> DC was ~10% at day 3, increasing to 30% by day 30 (Fig. 5B). The proportion of CD11b<sup>+</sup>CD103<sup>-</sup> DC that took up PS50G remained relatively stable at ~30% across both time points. The strongest particle uptake occurred in CD11b<sup>+</sup>CD103<sup>-</sup> DC, with ~45% of these cells containing PS50G at both time points. Overall, uptake of PS50G nanoparticles was ~3-fold greater than for PS500G microparticles for all three draining LN DC subsets (Fig. 5B). In contrast, the proportion of B cells that took up PS50G or PS500G at day 3 was 0.1–0.5% and not examined further (data not shown). Similarly, the proportion of particle-laden CD11c<sup>+</sup>MHC II<sup>+</sup> lymphocytes was 0.01–0.03%, whereas particle uptake by CD11c<sup>+</sup>MHC II<sup>+</sup> migratory alveolar macrophages and CD11c<sup>+</sup>MHC II<sup>+</sup>CD45R<sup>+</sup>pDC was <1% (data not shown).

**Particle trafficking to the lung-draining LN is cell associated**

The above data clearly show that particles instilled into the lung subsequently appear within DC in the draining LN. However, it was unclear whether these particles were transported from the lung via migration of particle-laden DC or direct lymphatic drainage. Data from several studies suggest that migration of Ag-laden DC occurs within 6 h postinstillation of Ag to the lung, peaking at 12–24 h (27, 30–32). However, soluble tracers appear within the draining LN within minutes postinjection due to lymphatic drainage (33, 34). Therefore, to distinguish between these two particle-transport possibilities, we instilled fluorescent particles into naive mice and examined proportions of particle-laden cells 2 h later. Our data show that the frequency of particle-laden cells in the draining LN was extremely low (~0.02%) at 2 h postinstillation for both the PS50G and PS500G groups and not significantly different from background levels in saline-treated mice (Fig. 6A). Accumulation of PS50G and PS500G within migratory CD11c<sup>+</sup>MHC II<sup>+</sup> draining LN DC (27, 28) was also very low and not significantly different from saline controls (0.07 ± 0.05, 0.16 ± 0.04, and 0.4 ± 0.14%; saline, PS50G, and PS500G, respectively). In contrast, the proportion of particle-laden total lung cells (isolated from the same mice) was already >20% for both particle sizes by 2 h (Fig. 6B), confirming that particles had been instilled correctly.

**PS50G nanoparticles selectively increase expression of cytokines and chemokines in BAL fluid**

The above data show that PS50G and PS500G are differentially taken up by pulmonary APC, suggesting that particle size could influence cytokine/chemokine production in the lung. Indeed, instillation of PS50G nanoparticles caused a rapid induction of IL-6, G-CSF, and GM-CSF at day 1 after instillation. Distinct from these selective PS50G effects, both PS50G and PS500G caused broadly similar increases in BAL fluid levels of IL-1α, IL-1β, IL-12p70, CCL3 (MIP-1α), and TNF (Supplemental Fig. 3). PS500G caused a modest increase in TGF-β1 levels at day 3. Neither particle size significantly increased CXCL1 (keratinocyte chemoattractant) or CCL4 (MIP-1β) over saline control values (Supplemental Fig. 3). Overall, these data show that instillation of PS50G and PS500G into the lung rapidly increases production of selected cytokines and chemokines, with evidence for induction of both distinct (particle size–dependent), and overlapping (particle size–independent) cytokine/chemokine profiles.
PS50G nanoparticles increase costimulatory molecule expression on lung DC

The above findings show that particles induced production of chemokines involved in DC maturation, suggesting possible effects on DC costimulatory molecule expression. Flow cytometry confirmed that PS50G nanoparticles caused a marked increase in expression of CD40, CD80, and CD86 on CD11c⁺ MHC II⁺ DC in the lung at day 3 after instillation, primarily in cells containing the highest number of particles (Fig. 8A, 8B). Further analysis confirmed that CD86 expression was similarly upregulated on PS50G-laden macrophages, B cells, and CD11b⁺ and CD103⁺ DC in the lung, with the degree of CD86 upregulation positively correlated with the level of particle uptake (FL1 fluorescence; data not shown). PS50G-induced costimulatory molecule expression decreased at day 7, but nevertheless

**FIGURE 4.** PS50G are preferentially taken up by macrophages, B cells, and DC in the lung. Naive mice received fluorescent-labeled PS50G or PS500G i.t. on day 0 or saline (Sal) as control. Particle-laden (FL1-positive) cells were gated as per Fig. 2. Proportions of bead-positive cells for each population are shown at days 3 and 30. Mean ± SEM, n = 6 mice/group/time point. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
remained significantly elevated over saline and PS50G levels until day 31 after instillation. PS50G had a negligible effect on costimulatory molecule expression by lung DC. In contrast to the lung, neither particle type affected CD40 expression by DC in the lung-draining LN, although CD80 expression was decreased at day 31 (Supplemental Fig. 4B, 4C). Both PS50G and PS500G increased CD86 expression at days 1 and 3 (Supplemental Fig. 4A, 4D). Overall, these data show that PS50G cause sustained increases in costimulatory molecule expression in the lung, whereas both PS50G and PS500G caused a transient increase in CD86 expression in the draining LN.

**FIGURE 5.** PS50G are preferentially taken up by DC in the lung-draining LN. Naive mice received fluorescent-labeled PS50G or PS500G i.t. on day 0 or saline (Sal) as control. (A) CD11c+ MHC II+ cells were gated for identification of CD11b+ CD103+, CD11b+ CD103+, and CD11b+CD103− cells in the lung-draining LN. (B) Proportions of bead-positive cells for the indicated populations are shown at days 3 and 30. Mean ± SEM, n = 6 mice/group/time point. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
pathway by instilling PS50G or PS500G into the lungs of naive, healthy, otherwise unmanipulated mice prior to instillation of fluorescent-labeled (Alexa Fluor 488) OVA and analyzed allergen-laden cells using established methods (27, 35). Both PS50G and PS500G increased the frequency of allergen-laden (Alexa Fluor 488+) lung-derived DC in the draining LN by 70% (Fig. 9A). Despite this increase in total allergen laden DC, PS50G decreased the frequency of CD11b+MHC II+ DC among allergen-laden CD11c+ cells in the draining LN by 70% at days 3 and 31 post-instillation, whereas PS500G caused a slightly smaller decrease (Fig. 9B).

Particle-mediated inhibition of AAI is particle size dependent

Our previous studies showed that PS50G impair the induction of AAI (11). Given the above data showing dramatically different effects of PS50G versus PS500G on pulmonary APC distribution and maturation, we predicted that PS500G would have altered ability to impair AAI relative to PS50G. We investigated this by instilling PS50G or PS500G particles into the lungs of naive mice prior to OVA sensitization and OVA challenge (Fig. 10A). As expected, PS50G inhibited BAL eosinophilia and lung tissue inflammation (Fig. 10B, 10C). PS500G also partially prevented the development of lung airway and parenchymal inflammation, although this was less pronounced than for the PS50G. Of note, PS50G, but not PS500G, additionally inhibited the production of serum OVA-specific IgE (Fig. 10D), a hallmark of allergic disease. PS50G also significantly decreased BAL fluid IL-5 and IL-13 (Fig. 10E, 10F), whereas PS500G had a blunted effect, consistent with their reduced effect on BAL eosinophilia. ELISPOT analysis was performed to further confirm a differential effect of PS50G and PS500G pretreatment on production of IL-13, a key cytokine that regulates mucus production. Consistent with the BAL ELISA data, PS50G but not PS500G significantly decreased the frequency of

**FIGURE 6.** PS50G or PS500G do not accumulate in lung-draining LN cells 2 h postinstillation. Naive mice received fluorescent-labeled PS50G or PS500G i.t. on day 0 or saline (Sal) as control. Frequency of particle-laden cells among total draining LN (A) and lung cells (B) at 2 h post–particle instillation. Mean ± SEM; n = 6 mice/group/time point. ****p < 0.0001.

**FIGURE 7.** PS50G and PS500G induce distinct patterns of BAL fluid cytokines and chemokines. Naive mice received PS50G or PS500G (nano or micro, respectively) i.t. on day 0 or saline as control; groups of mice were killed for analysis at the indicated times. Data show concentrations of IL-6, IL-12p40, G-CSF, GM-CSF, CCL2, and CCL5. Representative of two separate experiments. Mean ± SEM, n = 3–7 mice/group/time point. *p < 0.05, **p < 0.01, ***p < 0.001 (versus saline).
Ambient, pollutant, and man-made ultrafine/nanoparticles are known to promote allergic sensitization and AAI. However, our recent studies counter intuitively showed that inert nontoxic PS50G nanoparticles can inhibit AAI via modulation of pulmonary DC function, demonstrating that nanoparticles in the lung do not inherently promote pathology. However, relatively little is known of the effect of particle size on kinetics of particle uptake by APC in the pulmonary compartment and potential effects on APC distribution, maturation, and inflammatory mediator production. Our data show that PS50G were taken up in much greater proportions than PS500G by all lung APC including alveolar (CD11c+) and nonalveolar (CD11c−) macrophages, B cells, and CD11b+CD103− and CD11b+CD103+ DC, with alveolar macrophages and DC showing the strongest uptake. In contrast, we only detected significant PS50G uptake by DC in the lung-draining LN. Particle uptake by total lung-draining LN cells or migratory CD11c+MHC II+ DC was not detected at 2 h postinstillation, suggesting that particles do not translocate to the draining LN via lymphatic flow, as drainage to LN via the lymphatics occurs within minutes. PS50G instillation was associated with markedly increased costimulatory molecule expression on lung DC and a distinct pattern of cytokine and chemokine production in the lung airways. Although both PS50G and PS500G decreased proportions of stimulatory allergen-laden DC in the draining LN, the effect was most pronounced for PS50G at the day 4 time point. Thus, PS50G and PS500G leave distinct long-lasting immunological imprints in the lung. Based on these data, we predicted that PS500G microparticles would have impaired ability to inhibit AAI relative to PS50G nanoparticles. Our studies confirmed this prediction, showing that...
PS50G significantly inhibited the allergen-specific adaptive immune response, whereas PS500G failed to do so.

It has been convincingly shown that particles are taken up by cells in a size-dependent manner. Foged et al. (36) found that in vitro, DC preferentially take up polystyrene particles of 40 nm, with particle uptake decreasing for particles >100 nm in diameter. In vivo, we previously showed that 40-nm polystyrene particles injected into the footpad preferentially localize to DC in the draining LN (17). Similarly, i.v. infused 25-nm nanoparticles are rapidly transported to the draining LN via the lymphatic system, where they are taken up by DC (37). Several other studies showed that microparticles (1000 nm) are preferentially phagocytosed by macrophages in the LN or lung airways (17, 38), whereas nanoparticles (titanium dioxide, gold, and iridium) are inefficiently and nonspecifically taken up by macrophages in the lung (39–41). Our results using fluorescent particles extend these findings by showing that PS50G were taken up preferentially by alveolar macrophages and both CD11b+CD103− and CD11b+CD103+ DC in the lung, with lower uptake by nonalveolar macrophages and B cells. Uptake of larger PS500G was 2- to 3-fold lower in all lung APC at day 3. Interestingly, the proportion of alveolar CD11c+ macrophages that took up PS500G increased from 33 to 74% from days 3 to 30, indicating ongoing accumulation of PS500G microparticles within these cells. Preliminary data showed that uptake of PS50G by the small population of lung pDC (∼0.4–0.7%) was also significantly higher than PS500G at day 3 (26.3 ± 2.7 versus 6.1 ± 0.7%; n = 6/group; p < 0.001). The increased uptake of PS50G by lung pDC is unlikely to be functionally significant; however, future studies could explore potential changes in frequency of regulatory T cells. Despite uptake of both particle sizes by all lung APC, particle uptake in the lung-draining LN was restricted to CD11b+CD103−, CD11b+CD103+, and CD11b−
CD11b+ DC, with negligible uptake by other cells (alveolar macrophages, pDC, B cells, and CD11c+CD11b+MHC II+ lymphocytes). Our data show that the proportion of PS50G-laden CD11b+CD103+ DC in the draining LN increased from 12 to 32% from days 3 to 30, despite the fact that the proportion of PS50G-laden CD11b+CD103+ DC in the lung had dropped from 60 to 30% over this time, suggesting preferential migration and/or retention of PS50G-laden CD11b+CD103+ DC. We found significant PS50G uptake by CD11b+CD103+ draining LN DC, a minor population of lung-draining LN DC (e.g., Ref. 42). CD11b+CD103+ DC in the small intestine lamina propria were recently shown to play an important role in driving Th17 responses (43), although the function of these cells in the lung-draining LN has not been described. In contrast, CD11b+CD103+ DC in the lung-draining LN have been shown by us and others (11, 28, 44, 45) to play an important role in stimulating CD4+ T cells and driving Th2-biased inflammation, whereas CD11b+CD103+ appear to selectively stimulate cross presentation of apoptotic Ag to CD8+ T cells and drive antiviral immunity (44, 46, 47). Our data raise the possibility that nanoparticle-mediated delivery of appropriate Ag to diverse draining LN DC subsets could be harnessed to selectively promote Th1, Th2, or Th17 immunity.

Transport of soluble Ag and particles from lung to the lung-draining LN is mediated by active cellular transport, as evidenced by the lack of allergen accumulation in the draining LN in CCR7-deficient mice (28) and the profound inhibition of allergen and latex particle accumulation in mice treated with pertussis toxin, an inhibitor of chemokine signaling (32). The appearance of PS50G in macrophages, B cells, and DC in the lung, but only in DC in the draining LN, suggested onward migration of DC from the lung to the draining LN. The uptake of particles by lung-draining LN DC that we observed could be due to particle drainage via the lymphatics and uptake within the draining LN or by DC uptake in the lung and subsequent migration. Accumulation of allergen or particle-laden DC in the lung-draining LN is apparent within 6 h, peaking at 12–24 h after instillation (27, 30–32). However, soluble tracers can be transported to the draining LN from within minutes postinjection into peripheral sites (33, 34). Therefore, we examined particle uptake in the draining LN at 2 h postinstillation, a time preceding the known dynamics of DC accumulation. Particle accumulation within draining LN cells was undetectable at this time, suggesting that the particle uptake by DC that we observe at day 3 is due to cell-mediated transport, in agreement with studies showing DC-mediated transport of 500-nm particles to the lung-draining LN (18, 32, 47). Our studies advance these findings, showing that nano-sized particles accumulate to a greater degree than microparticles. In contrast, another study found that nanoparticles (20 nm) appear within the subcapsular region of the popliteal LN within 2 h following footpad injection and are taken up by DC (defined as CD11c+) as well as macrophages, B cells, and pDC (F4/80+, B220+, and pDC Ag-1+, respectively) at this early time (48). These findings are consistent with the idea of drainage via the lymphatics and uptake by cells within the popliteal LN, as macrophages and pDC are not considered to be migratory cell populations (28, 32). More surprisingly, this study also showed that a major portion of the 20-nm particle uptake was by macrophages in the draining LN, in contrast to our findings showing uptake exclusively by DC. The differences between this study and ours could be due to differences in the anatomical site (leg versus lung), the route of administration (footpad injection versus i.t.), or the difference in particle size.

DC migration is significantly increased by inflammatory stimuli (30, 32, 49). It is therefore likely that the minor transient inflammation induced by i.t. particle instillation helps drive DC uptake and migration from the lung to the draining LN. The ongoing, albeit gradually decreasing, presence of PS50G in the lung (Fig. 3) would provide a sustained source of particle-laden DC. Our data also indicate that the frequency of PS50G-laden cells in the lung steadily declines to approximately one-third of the initial value, indicating ongoing clearance of PS50G from the lung. Presumably, this clearance would be via a combination of: 1) DC-mediated transport; 2) drainage of particles via the lymphatic system; and 3) clearance of PS50G-laden macrophages via the mucociliary escalator.

PS50G instillation increased BAL fluid levels of mediators involved in recruitment and/or maturation of monocytes and DC, specifically CCL2, G-CSF, GM-CSF, and RANTES/CCL5 (13, 50, 51), explaining the increase in lung DC at day 3. PS50G also increased BAL fluid IL-6 levels, explaining the transient airway neutrophilia we observed. Numerous cell types have been linked to the production of these cytokines, including lung epithelium (IL-6, G-CSF, and GM-CSF) and activated DC (IL-6, IL-12) (13, 51). PS50G instillation was also associated with increased costimulatory molecule expression by lung CD11c+MHC II+ DC, primarily within the particle-laden DC population. Notably, maximum levels of these BAL fluid cytokines/chemokines (day 1 or 3) immediately preceded or coincided with the increased costimulatory molecule expression by lung DC (day 3). This increased costimulatory molecule expression was likely due to inflammatory mediator induced DC maturation, direct effects of PS50G uptake on these cells, or a combination of these factors.

It is now understood that a primary immune response in the lung can modify the nature and/or severity of a subsequent immune response. For example, influenza virus infection protects against infection from the unrelated respiratory syncytial virus (52), and heat-labile Escherichia coli toxin enhances protection to subsequent influenza or respiratory syncytial virus infection (53). Furthermore, in humans, exposure to high levels of LPS inhibits allergies and allergic asthma (54), whereas exposure of mouse lungs to LPS prior to or concomitant with allergen sensitization inhibits development of AAi following allergen challenge (55, 56). This process of innate imprinting is thought to operate by various mechanisms including impairment of pulmonary APC function (53) or induction of regulatory myeloid-derived suppressor cells (56). Our previous study (11) demonstrated an analogous inhibition of AAi with PS50G nanoparticles mediated via modification of pulmonary DC function. Our new findings comparing the same mass of PS50G and PS500G show that PS50G, and to a lesser extent PS500G, decreased proportions of CD11b+ DC allergen-laden in the draining LN, a subset we and others (11, 44) have shown to be responsible for stimulation of allergen-specific CD4+ T cell responses. However, PS50G and PS500G leave distinctive immunological imprints, with PS50G but not PS500G markedly inhibiting the allergen-specific adaptive component of AAi (allergen-specific Th2 cytokines and serum IgE).

In summary, our data show that 50- and 500-nm particles leave different immunological imprints in the pulmonary compartment. PS50G were taken up by macrophages, B cells, and DC in the lung, but only by DC in the draining LN, suggesting onward migration of particle-laden DC to the draining LN. Our finding that uptake of particles (of either size) was undetectable in the lung-draining LN at 2 h postinstillation suggests that they do not translocate to the draining LN via simple lymphatic drainage, but are actively transported within DC. PS50G induced DC maturation in the lung and induced a distinct subset of cytokines and chemokines involved in DC recruitment and/or maturation. PS500G were taken up less efficiently than PS50G in the lung and draining LN, with maximal uptake seen at day 30 by lung CD11c+ macrophages, and induced
a more restricted subset of cytokines and chemokines in the lung. The outcome was that pretreatment with PS50G, but not PS500G, significantly inhibited the development of AA. It is tempting to speculate that the impaired Ag-specific costimulatory capacity of CD11b+ DC we observed in the draining LN of PS50G-treated mice (11) is due to a state of PS50G-induced DC refractoriness, possibly occurring due to induction of DC maturation in the absence of specific Ag uptake. An analogous situation occurs during endotoxin tolerance in which refractory or exhausted DC are induced following LPS stimulation, resulting in suppression of AA (57, 58). Overall, these data increase our understanding of how differently sized inert nontoxic particles differentially modulate pulmonary APC function and lung immune homeostasis. These findings provide new insights into particle effects on lung immunobiology and may support the development of lung-specific particulate vaccines, drug delivery systems, and immunomodulators.

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Disclosures

The authors have no financial conflicts of interest.

References


Particulate Size–Dependent Immunological Impacts in the Lung


Fig. S1

(a) Intensity (%) vs. Size (d. nm)

(b) Intensity (kcps) vs. Zeta potential (mV)

(c) Intensity (%) vs. Size (d. nm)

(d) Intensity (kcps) vs. Zeta potential (mV)
Fig. S3
Supplementary Figure legends

Figure S1. Size distribution and surface charge of PS50G and PS500G nanoparticles and microparticles. PS50G (a, b) and PS500G (c, d) were sonicated and resuspended in saline prior to lung instillation and analysed in parallel on a Zetasizer. Size distribution (a, c) and surface charge (b, d). Representative of 2–3 separate analyses.

Figure S2. PS50G and PS500G instillation alters frequencies of macrophages, B cells and DC in the lung and draining LN. Naïve mice received fluorescent-labeled PS50G or PS500G (nano or micro, respectively) i.t. on d0, or saline as control. (a) Frequencies of macrophages, B cells and DC in the lung at d3 and d30 post-particle instillation. (b) MHCII and CD11c expression by DC (region 1), CD11c+ and CD11c− macrophages (regions 2 and 4, respectively), pDC (region 3), and B cells (region 5) in the lung, and frequencies of CD11c−MHCII+ DC and CD11bhi among CD11c−MHCII+ DC following particle instillation. (c) MHCII and CD11c expression by CD11c−MHCIIhi ‘migratory’ and CD11c−MHCII+ ‘resident’ DC (regions 1 and 2, respectively), MHCIIintCD11cint ‘migratory’ alveolar macrophages (region 3), CD11c−MHCII− lymphocytes (region 4) and B cells (region 5) in the lung draining LN, and frequencies of CD11c−MHCIIhi, CD11c−MHCII+ (migratory and resident DC, respectively) and CD11bhi among CD11c−MHCIIhi DC following particle instillation. Mean ± SEM, n=6–9 mice per group per time-point. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure S3. PS50G and PS500G induce overlapping patterns of BAL fluid cytokines and chemokines. Naïve mice received PS50G or PS500G (nano or micro, respectively) i.t. on d0, or saline as control; groups of mice were killed for analysis at the indicated times. Data shows concentrations of IL-1α, IL-1β, IL-12p70, CXCL1, CCL3, CCL4, TGF-β1 and TNF. Representative of 2 separate experiments. Mean ± SEM, n=3–7 mice per group per time-point. *p < 0.05, **p < 0.01, ***p < 0.001 (versus saline).

Figure S4. PS50G and PS500G transiently increase CD86 expression on draining LN DC. Naïve mice received fluorescent-labeled PS50G or PS500G (nano or micro,
respectively) i.t. on d0, or saline as control. (a) Bead uptake (Fluo) versus CD86 expression by draining LN CD11c^+MHCII^{hi} DC (gated as per region 1, Fig. S2c) on d3 post-particles. (b, c, d) CD40, CD80 and CD86 expression by draining LN CD11c^+MHCII^{hi} DC. n=3/group (each replicate consisting of pools of two to three mice). Mean ± SEM, *p < 0.05, ***p < 0.001 (versus saline).