Controlled analysis of nanoparticle charge on mucosal and systemic antibody responses following pulmonary immunization

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Pulmonary immunization enhances local humoral and cell-mediated mucosal protection, which are critical for vaccination against lung-specific pathogens such as influenza or tuberculosis. A variety of nanoparticle (NP) formulations have been tested preclinically for pulmonary vaccine development, yet the role of NP surface charge on downstream immune responses remains poorly understood. We used the Particle Replication in Non-Wetting Templates (PRINT) process to synthesize hydrogel NPs that varied only in surface charge and otherwise maintained constant size, shape, and antigen loading. Pulmonary immunization with ovalbumin (OVA)-conjugated cationic NPs led to enhanced systemic and lung antibody titers compared with anionic NPs. Increased antibody production correlated with robust germinal center B-cell expansion and increased activated CD4+ T-cell populations in lung draining lymph nodes. Ex vivo treatment of dendritic cells (DCs) with OVA-conjugated cationic NPs induced robust antigen-specific T-cell proliferation with ~100-fold more potency than soluble OVA alone. Enhanced T-cell expansion correlated with increased expression of surface MHCI, T-cell coactivating receptors, and key cytokines/chemokine expression by DCs treated with cationic NPs, which were not observed with anionic NPs or soluble OVA. Together, these studies highlight the importance of NP surface charge when designing pulmonary vaccines, and our findings support the notion that cationic NP platforms engender potent humoral and mucosal immune responses.

vaccine | cationic | nanoparticle | pulmonary | mucosal

The lung is a primary site of pathogen entry and is therefore a critical target for mucosal vaccination. Conventionally administered vaccines (e.g., s.c. or intramuscular injection) provide strong humoral protection, but often fail to generate mucosal immunity, especially in the form of IgA (1). Mucosal vaccines not only provide local protection, but also confer systemic immunity, including distal mucosal sites (1–3). Mucosal and systemic antibody responses require complex cross-talk between innate and adaptive immune cells. Antigens are first encountered by professional antigen presenting cells (APCs), such as dendritic cells (DCs), at the site of infection or injury. Activated DCs migrate to the draining lymph node (dLN), where they present antigenic peptides to MHCI, which allows for activation of antigen-specific CD4+ T cells. Activated T cells instruct antigen-specific B cells to form germinal centers (GC) in the dLN where B cells expand, undergo affinity maturation, and Ig class switch recombination, resulting in production of highly specific antibodies with specialized functions (4). All three cell types (DCs, CD4+ T cells, and B cells) are required for GC formation, because T-cell–deficient mice fail to form GCs and DC-depleted mice exhibit reduced antibody production (5–7).

Nanoparticle (NP) formulations are being engineered to improve subunit vaccine approaches that induce pathogen mimicry while still maintaining the safety of subunit vaccines (8, 9). In the lung, NP formulations offer potential solutions to overcome biological barriers and target APCs (2, 10–13). Cationic NP formulations have been shown to increase mucosal antibody production following pulmonary or intranasal administration (14, 15), whereas independent studies using anionic NP approaches show only minimal improvement in antibody production compared with immunization with soluble protein alone (16, 17). To date, no direct evaluation of NP surface charge on mucosal immune responses has been performed, likely due to limitations in NP formulation that prevent such a comparison without dramatically changing NP composition. Our studies used the unique Particle Replication in Non-Wetting Template (PRINT) process to exquisitely control all other nanoparticle characteristics and specifically investigate the role of NP charge on vaccine responses.

Significance

To our knowledge, no other nano-based vaccine delivery platform has directly assessed the effects of nanoparticle charge on pulmonary vaccination without affecting other physio/chemical particle characteristics and/or antigen loading. The Particle Replication in Non-Wetting Templates nanoparticle fabrication process is unique in that it allows for isolation of charge as the sole variable in these studies while maintaining all other physical and chemical parameters constant. We find that positively charged nanoparticles induce robust mucosal and systemic antibody responses following pulmonary administration, whereas negatively charged nanoparticles fail to do so. Therefore, our studies underscore the importance of considering nanoparticle charge as a critical design parameter when generating pulmonary-based vaccines and may have implications for particulate vaccination through other routes of administration.

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DCs Treated with Particulate OVA Induce Robust OT-II CD4\(^+\) T-Cell Proliferation ex Vivo. To determine how NP charge affects cellular uptake, antigen processing, and presentation by MHCIi, we treated bone marrow-derived dendritic cells (BMDCs) (gating specifically and was undetectable with soluble OVA (Fig. 1D). At the 0.01 μg/mL dose, the (ζ\(^+\))NP-OVA–treated DCs declined precipitously and was undetectable with soluble OVA (Fig. 1D). The ability of DCs treated with (ζ\(^+\))NP-OVA to induce robust T-cell proliferation was highly reproducible, because similar results were observed with multiple batches of independently synthesized NP-OVA and cell preparations (Fig. 1E). From these data, we conclude that DCs treated with (ζ\(^+\))NP-OVA induce more robust T-cell proliferation than (ζ\(^−\))NP-OVA and soluble OVA alone.

(ζ\(^+\))NP-OVA Up-Regulates BMDC Costimulatory Receptors, Cytokines, and Chemokines. The ability of BMDCs treated with cationic NPs to induce strong OTIII T-cell proliferation with ~100-fold lower concentrations of soluble OVA could be explained by multiple mechanisms. One explanation is that cationic NPs bind more readily to the BMDC surface, resulting in increased antigen uptake and presentation compared with anionic NP or soluble OVA. We tested this possibility by incorporating a pH-sensitive dye (pHrodo) during NP fabrication that fluoresces upon internalization in the endosome. The largest observed difference in NP uptake occurred at 24 h with 20% fewer pHrodo\(^+\) cells in (ζ\(^+\))NP-OVA–treated BMDC cultures compared with (ζ\(^−\))NP-OVA treatment (Fig. S2). By 72 h, >94% of DCs were pHrodo\(^+\) regardless of NP charge (Fig. S2), suggesting that internalization at longer time points is not grossly different between (ζ\(^+\))NP-OVA and (ζ\(^−\))NP-OVA.

We also hypothesized that (ζ\(^+\))NP-OVA–treated DCs induce stronger T-cell proliferation due to increased MHCIi and CD80/CD86 expression on the DC surface, which would allow for stronger T-cell proliferation due to increased MHCII and CD80/CD86 expression on the DC surface, which would allow for stronger T-cell receptor engagement and more robust T-cell activation. Flow cytometric analysis indicated that surface MHCIi, CD80, and CD86 were up-regulated at 72 h after (ζ\(^+\))NP-OVA treatment, which were similar to the expression...
levels observed on LPS-treated DCs after 24 h (Fig. 2A). In contrast, (ζ+)NP-OVA only induced a modest increase in MHCII and coreceptor expression compared with untreated controls (Fig. 2A). Maximal levels of MHCII and coreceptor expression induced by (ζ+)NP-OVA depended on direct OVA conjugation to the NP surface, because blank (ζ+)NP alone, or (ζ+)NP administered with soluble OVA [(ζ+)NP + sol. OVA], were unable to induce strong surface expression of these molecules (Fig. 2A). The increase in coreceptor expression was not due to general up-regulation of cell surface molecules, because CD11b expression remained constant among all treatment groups (Fig. 2A). Similarly, results were observed at the mRNA level by using quantitative RT-PCR (qRT-PCR) for CD50 and CD56 at 24 and 48 h after NP treatment (Fig. S3 and Fig. 2B, respectively); however, the mRNA levels of H2-Aa (MHCII encoding) remained unchanged, suggesting that up-regulation of surface MHCII occurs posttranscriptionally.

We further tested whether BMDC cytokine profiles changed following NP treatment and whether NP charge was a contributing factor. We found significant increases in IL-6 and IL-12 mRNA expression and protein secretion by DCs treated with (ζ+)NP-OVA compared with (ζ+)NP-OVA treatment and untreated controls (Fig. S4A and B). Increases in key cytokines/chemokines, including Il1b, Il18, Cxcl10, 110, and Ifnb, were detected after DC treatment with (ζ+)NP-OVA, compared with untreated or (ζ+)NP-OVA-treated DCs (Fig. S4A). Significant increases in Il18 and Cxcl10 were observed in DCs treated with (ζ+)NP-OVA compared with untreated controls; however, at a lower level than (ζ+)NP-OVA (Fig. S4A). Although Il1b mRNA expression was elevated in (ζ+)NP-OVA-treated DCs compared with untreated controls (Fig. S4A), we failed to detect IL-1β protein in the supernatant, which is consistent with previous findings that this formulation of NPs does not induce inflammatory activation (18). We also assessed the mRNA expression of several other cytokines (Ccl2, Tnf, Tgfb1, Ifna4) and found that they were either not expressed or were different from untreated cells (Fig. S4A). Similar to T-cell coreceptor expression, the increased cytokine expression/secretion required direct OVA conjugation to the NP, because blank NPs and blank NPs + soluble OVA did not induce strong cytokine responses (Fig. S4A and B). These data suggest that cationic NPs induce an activated DC phenotype that requires direct conjugation of protein antigen to the NP surface.

(ζ+)NP-OVA Have in Vivo Adjuvant Effects Following Pulmonary Immunization. It is well established that effective antibody responses to protein antigens require helper CD4+ T cells. In the absence of T-cell help, the processes of affinity maturation (enhancement of antibody specificity) and Ig isotype switch (IgM to other effector isotypes; IgG, IgA, or IgE) are severely hindered (5). Affinity maturation and isotype switch occur within GCs of dLNs and are comprised of proliferating B cells that up-regulate the surface marker GL7. We used a model of orotracheal NP lung instillation to assess primary and secondary CD4+ T-cell–dependent immune responses, including GC formation and Ig isotype switch by using the immunization schedule described in Fig. S5A. We tested whether particulate OVA induces GC formation in the local lung draining mediastinal LNs following primary and secondary lung instillations and found significantly higher induction of CD19+GL7+ GC B cells in (ζ+)NP-OVA–treated mice compared with (ζ+)NP-OVA or soluble OVA alone (Fig. 3A and B, full gating in Fig. S5B). Increased GC B-cell populations in (ζ+)NP-OVA-treated mice were similar to those treated with soluble CpG/OVA (Fig. 3A and B) and were confined to the draining mediastinal LN and not observed in the spleen (Fig. S5C), suggesting that induction of GC B cells was due to localized adjuvant activity in the lung and not due to systemic increases in GC B-cell populations in these mice. We also investigated the activation status of CD4+ T cells in the mediastinal lymph node after the secondary instillation and found a significant increase in the frequency of antigen-experienced CD4+CD44hiCD62L− T cells in (ζ+)NP-OVA and soluble OVA/CpG-instilled mice compared with mice-treated soluble OVA alone (Fig. 3C, gating in Fig. S6A). In contrast, the antigen-experienced CD4+ T-cell population was not increased in (ζ+)NP-OVA–treated mice compared with soluble OVA alone (Fig. 3C) and we found no difference in the CD4+CD44hiCD62L− T-cell populations in spleen (Fig. S6B), suggesting that the increase in T effector memory cells is specific to the dLN.

Consistent with increased GC formation and antigen-experienced CD4+ T-cell populations, we readily detected OVA-specific IgG in the plasma and bronchoalveolar lavage fluid (BALF) of (ζ+)NP-OVA–treated mice following primary and secondary immunization, with titers comparable to those treated with soluble OVA/CpG (Fig. 4A and B). Three of six mice treated with (ζ+)NP-OVA had very low OVA-specific plasma and BALF IgG antibody titers that were not statistically different from mice treated with soluble OVA alone (Fig. 4A and B). Three of six mice treated with (ζ+)NP-OVA had detectable levels of OVA-specific IgA in BALF following primary immunization, whereas no antigen-specific IgA was detected in any other group, including soluble OVA/CpG (Fig. 4C, Left). OVA-specific BALF IgA was only detectable in (ζ+)NP-OVA and soluble OVA/CpG–treated mice following secondary immunization (Fig. 4C, Right), indicating that (ζ+)NP-OVA has an adjuvant effect capable of inducing systemic and mucosal antibody responses similar to the TLR ligand CpG, with no IgA produced in response to (ζ+)NP-OVA. To determine whether the adjuvant effect(s) of (ζ+)NP-OVA work through a common mechanism to CpG, we delivered soluble CpG with (ζ+)NP-OVA to test for additive effects on OVA-specific antibody titers. We observed that delivery of soluble CpG with (ζ+)NP-OVA significantly increased levels of OVA-specific BALF IgG and IgA compared with soluble OVA/CpG (Fig. 4D), suggesting that (ζ+)NP-OVA and CpG work in an additive fashion to induce more robust antibody responses, especially for mucosal IgA (Fig. 4D, Right). We also observed that codelivery of soluble CpG rescued the ability of (ζ+)NP-OVA to induce BALF IgG and IgA responses (Fig. 4D), suggesting that anionic NPs can still serve as a platform for antigen delivery, but antibody responses require coupling to TLR
agonists. From these data, we conclude that cationic NPs contain inherent adjuvant activity that can further synergize with TLR-stimulating adjuvants, such as CpG, when delivered to the lung.

The in Vivo Adjuvant Effect(s) of (ζ+)-NP-OVA Are Independent of Endotoxin Contamination. Certain sources of purified OVA contain endotoxin contaminants that can confound the results of immunization studies. The OVA used thus far in this manuscript is grade V from Sigma, which has the highest protein purity, but has also been found in the past (and reported here; Fig. S7), to contain detectable amounts of endotoxin (19). To test whether the adjuvant effect(s) we observe in vivo are independent of OVA-associated endotoxin, we repeated the in vivo immunization studies by using NPs conjugated to vaccine grade endotoxin-free (ef) OVA (Ovalbumin EndoFitt; Invivogen, endotoxin levels <10 per group). (ζ+)NP-OVA, (ζ−)NP-OVA, and efOVA were formulated and administered to mice at a dose of 100 μg per instillation (10 μg of OVA); CpG dose = 2.5 μg per instillation. Line represents mean ± SEM.

Discussion
NP design features, such as size, shape, modulus, and surface chemistry, are well established for long circulating i.v. delivery; however, these parameters are mainly unverified for other routes of administration, especially pulmonary delivery. Our studies used the PRINT fabrication process to isolate surface charge as the sole variable in NP-based pulmonary vaccines, while maintaining identical particle composition, size, shape, and antigen loading. Our results indicate that cationic hydrogel NPs have adjuvant-like effects, yielding potent mucosal and systemic antibody responses following pulmonary delivery, whereas anionic NPs fail to do so. These responses appear T-cell–dependent, because they correlate with increased GC formation and Ig class switch recombination, which are known to require T-cell help (20, 21).

Possible Mechanisms for Enhanced Mucosal and Systemic Antibody Responses by Cationic NPs. The precise mechanism(s) for how antigen-conjugated cationic NPs induce mucosal and systemic antibody responses require further investigation. Previous vaccine-related studies using cationic liposomes showed increased antibody responses over soluble controls; however, this response was solely attributed to enhanced NP binding to the APC surface and no differences in costimulatory molecule or cytokine expression were reported (22–24). We observed that cationic NPs bound more readily to DCs and macrophages in vitro compared with anionic NPs; however, the degree of NP internalization was not grossly different between these groups, especially at longer time points. Building on previous efforts from our laboratory, which established that pulmonary delivery of PRINT NPs do not trigger any lung inflammatory responses by cytokine release or histopathology (18), neither cationic nor anionic NPs triggered local lung or systemic increases in the inflammatory cytokines IL-6, TNF-α, or IL-1β compared with PBS controls. Rather, our finding that T-cell–activating coreceptors and cytokines are induced with cationic NPs, but not with anionic NPs, suggests a clear role of NP charge in DC maturation and effector function. Similar studies using positively charged chitosan-based NPs have also shown enhanced immune responses over whole protein controls (15, 25, 26), and recent cationic NP studies using the macrophage cell line RAW 264.7 have also demonstrated up-regulation of T-cell costimulatory molecules and cytokines (27). Interestingly, our studies suggest that the positive charge alone is insufficient for DC maturation, because this process required protein conjugation to the NP surface. Therefore, the protein antigen in combination with select NP characteristics (e.g., positive charge) may be required for microbial mimicry effects that trigger DC maturation.

The expansion of GC B cells and antigen-experienced CD4+ T cells following cationic NP delivery were confined to the lung dLN, suggesting that these responses use lung resident immune populations. Previous studies established that similar-sized NP (~200 nm) required lung resident DC transport to the dLN for induction of potent CD4+ T-cell responses (28, 29). Based on these studies and our data reported here, we hypothesize that enhanced antibody responses emanate from cationic NP interaction with lung resident DCs that then migrate to the dLN to induce T-cell–dependent GC formation and antibody production. Treatment of DCs with (ζ+)NP-OVA ex vivo-induced Th11 skewing cytokine production (IL-12, IL-18), but not Th12 skewing cytokines (IL-4), suggesting that cationic NPs may instruct Th11 cell responses following pulmonary delivery, whereas anionic NPs fail to do so. These responses appear T-cell–dependent, because they correlate with increased GC formation and Ig class switch recombination, which are known to require T-cell help (20, 21).

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T-cell effector populations. Our ex vivo data indicated that DCs treated with NP-efOVA were able to restore antibody responses to soluble OVA, and we expected to see a similar trend in our in vivo studies; however, the response to (ζ+)NP-efOVA was no different from soluble OVA alone. The failure of anionic NPs to induce immune responses was even more apparent when using vaccine grade OVA, which did not induce any detectable OVA-specific antibody. We were able to restore antibody responses to (ζ+)NP-efOVA by coadministering soluble CpG, suggesting that the OVA conjugated to anionic NPs was still intact, but that the negative charge lacks the adjuvant effect associated with cationic NPs.

**NP Charge as a Critical Design Parameter for Pulmonary Therapeutics.** Our study indicates that consideration of NP charge may be of critical importance when designing pulmonary therapeutics. We have demonstrated that cationic NP formulations delivered to the lung enhance both systemic and mucosal antibody responses while also providing an adjuvant effect, whereas anionic NPs do not. However, the use of anionic particles could be advantageous in cases where immune responses are contraindicated. For example, anionic charge with particulate drug depots may afford stealth-like properties allowing for reduced particle clearance and sustained therapeutic delivery. Studies by other groups show that anionic NPs delivered to the lung can induce potent cell-based immunity by activating CD8+ T-cell responses (16, 17). Together, these studies suggest that the ability to control NP
surface charge offers opportunities to tailor the therapeutic application based on desired immunological response.

Continuing to investigate and understand how surface charge and other particle parameters affect cellular interaction/biological responses will be critical for engineering novel NP therapeutics. The degree of control and NP scalability afforded by PRINT allows for intricate vaccine design with the capacity to alter NP dimensions, use cleavable linkers for antigen/adjuvant conjugation, and aerosol formulation to provide a portable pulmonary route of administration (18, 32). Our findings will hopefully contribute to future development of pulmonary-based vaccine platforms that are applicable to a diverse array of pathogens.

Materials and Methods

Particle Fabrication. Amine-containing 80 x 320 nm hydrogel rod-shaped NPs were fabricated on a continuous roll-to-roll PRINT method as described (33). Preparticle solutions contained 1% diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide, 20% (wt/vt) 2-aminoethy methacrylate hydrochloride, 10% (wt/vt) poly(ethylene glycol) diacrylate (Mn ~700), 0–1% functional fluorescent dye, and 69–68% (wt/wt) tetra(ethylene glycol) monacrylate. OVA functionalization was achieved by using carbodiimide chemistry. Characterization of NPs is described in SI Materials and Methods.

OT-II Coculture. Cell preparation and additional analysis are described in SI Materials and Methods. On day 0, BMDCs were seeded in RPMI 1640 with glutamine (Gibco) and 10% (vol/vol) FBS in a round-bottom 96-well plate. NPs or soluble OVA were added on day 1 based on mass of OVA per well. On day 2, splenocytes from OT-II mice were harvested and purified. CD4+ OT-II T cells were labeled by using CellTrace Violet (CFSE analog, referred to in the text as CFSE) following manufacturer’s protocol (Life Technologies). T cells were added to NP-treated BMDCs. Cells were harvested 72 h after T-cell addition.

Pulmonary Delivery and Immunizations. All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee at the University of North Carolina (UNC). All animals were maintained in pathogen-free facilities at UNC and were between 8 and 15 wk of age. C57BL/6 mice were obtained from Jackson Laboratories and OT-II transgenic mice were bred in-house. NP and control formulations were delivered to the lungs of anesthetized mice through an orotracheal instillation in a 50-μL volume. NP doses were 100 μg of NP per instillation, corresponding to 10 μg of OVA per instillation, which was used as the control soluble OVA dose; in studies with adjuvant, 2.5 μg of Cpg per instillation or 0.3 μg of MPLA per instillation was also delivered. For single-dose immunization studies, a dose was given on day 0 and euthanasia performed on day 9. For prime and boost immunization studies, doses were given on day 0 and 10, with submandibular bleeds performed on day 9 and euthanasia on day 20. Characterization of tissue preparation, antibody responses, GC formation, and T-cell activation is described in SI Materials and Methods.

Statistical Analysis. Statistical analyses were performed with GraphPad Prism version 6. Analysis of groups was performed as indicated in figures. All data points were included in the analyses, and no outliers were excluded in calculations of means or statistical significance.

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