Supporting Information

Fromen et al. 10.1073/pnas.1422923112

SI Materials and Methods

Reagents. Solvents and buffers of reagent grade were obtained from Fisher Scientific. Particles were fabricated by using PRINT molds (Liquidia Technologies) using preparticle reagents of 2-aminoethylmethacrylate (AEM), poly(ethylene glycol)$_{700}$ diacrylate ($PEG_{700}DA$), diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide (TPO) (Sigma-Aldrich), and tetra(ethylene glycol) monoacrylate (HP_4A), synthesized in house via described methods (1). Dyes of cell trace violet and pHrodo red succinimidyl ester were obtained from Invitrogen, and maleimide-Dylight 650 was obtained from Fisher. Coupling reagents for the carboiimide conjugation of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (s-NHS) were obtained from Thermo Fisher. CpG-B 1826 oligonucleotide (5′-TCCATGACGTTCCTGACGTT-3′) and OVA grade V were obtained from Sigma-Aldrich. Endotoxin Free OVA (EndoFit Ovalbumin) and MPLA were (VacciGrade) from Invivogen. RPMI 1640 supplemented with 0.1 mM NEAA, 1 mM sodium pyruvate, 2 mM glutamine, 55 μM 2-ME (Invitrogen), 100 units/ml penicillin-streptomycin and 10% (vol/vol) FCS (Sigma-Aldrich).

Particle Functionalization. For (ζ^+)NP-OVA, OVA was activated with five molar excess of EDC and s-NHS for 30 min in 0.1 M MES buffer at pH 6, reacted with (ζ+)NP in a 1:100 molar ratio (OVA:NP) for 2 h at 0.1 M sodium phosphate buffer at pH 7.5, then washed through centrifugation three times in sterile water. For (ζ^-)NP-OVA, (ζ^+)NPs in DMF were incubated with 100 M excess succinic anhydride for 30 min, washed first with borate buffer at pH 9.5, and then three additional washes in water to achieve (ζ^-)NP. (ζ^-)NP were then activated with five molar excess of EDC and s-NHS for 30 min in 0.1 M MES buffer at pH 6, reacted with OVA a 1:85 molar ratio (OVA:NP) for 2 h at 0.1 M sodium phosphate buffer at pH 7.5, then washed through centrifugation three times in sterile water.

Particle Characterization. Particle morphology was monitored by using scanning EM; samples were sputter-coated with 1–5 nm of Au/Pd (Cressington Scientific Instruments) and imaged by using Hitachi model S-4700. Particle size and zeta potential were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments) and thermogravimetric analysis (TGA) was used to determine particle concentrations. Protein concentration on the NPs was determined by BCA assay (Thermo Fisher, SpectraMax M5 plate-reader), following manufacturer's instructions. Particle samples were assayed at 1 mg/mL, and the amount of bound OVA to NPs was determined by using a standard curve of soluble OVA and unreacted particles of equivalent concentration as the absorbance blank.

Tissue and Cell Preparation. BMDCs used in coculture and NP uptake studies were isolated as described with the following modifications (2). Bone marrow was obtained from C57B/6 mice, and red blood cells were lysed with ACK buffer (Gibco), washed, and seeded in media with recombinant murine GM-CSF (Peprotech) (20 ng/mL). After 6 d, cells were enriched by using $CD11c^+$ MACS beads (Miltenyi Biotec). Single-cell suspensions were generated OT-II spleens by using gentle agitation between frosted glass slides and straining through a 70-μm sieve. RBCs were lysed with ACK buffer and naive $CD4^+$ T cells were enriched by using $CD25^+$ cell depletion and $CD4^+$ cell selection with L3T4 MACS beads (Miltenyi Biotec).

For immunization studies, whole blood was obtained through submandibular bleed or cardiac puncture and collected in heprin-coated tubes; from these samples, plasma was obtained by centrifugation. Draining medistinal LNs were collected, gently agitated between frosted glass slides and passed through a 70-μm sieve to obtain a single-cell suspension (3). BAL was performed by inserting a cannula in an incision in the trachea and flushing the lungs with 1 mL of HBSS, and centrifugation was used to separate BALF cells from supernatant.

Quantitative Real-Time RT-PCR. Total RNA was harvested using TRIzol(Invitrogen) followed by oligo(dT_{20}) primed reverse transcription using M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Quantiative PCR for murine *Cd80*, *Cd86*, *H2-Aa*, *Tnfa*, *Il6*, *Il12*, *Il18*, *Cxcl10*, *Il10*, *Ifnb*, *Il1b*, *Ccl2*, *Tnf*, *Tgfb1*, and *Actb* (used for normalization) was performed by using TaqMan primer/probe sets and master mix (Applied Biosystems).

Antibodies: Flow Cytometry and ELISAs. Single-cell suspensions from either tissue or cell culture were kept on ice and blocked with anti-CD16/32 (Fc block, eBioscience) and stained with the following antibodies to mouse cell surface molecules: CD80-APC, CD86-PE, GL7-eFluor-450, CD62L-APC-eFluor-780, CD62L-PE-Cy7 CD19-PE-Cy7, CD4-Fitc, CD4-PE-Cy7, CD3 FITC (eBioscience); and IA/IE-PacificBlue, CD11b-PE-Cy7, CD11c-APC-Cy7, CD4-BV510, CD44-APC, CD25-PE, Vβ5.1.-APC (BioLegend). Cells were fixed by using 2% (vol/vol) PFA in PBS. All data were collected by using LSRII (BD Biosciences) flow cytometer and analyzed by using FlowJo software (Tree Star).

ELISA kits for IL-6 and IL-12 were purchased from BD Biosciences. For OVA-specific indirect ELISAs, plates were coated with 2 μg/mL OVA, incubated with serum or BALF samples from immunized mice. OVA-specific mouse IgG and IgA were detected by using either goat anti-mouse IgG-HRP or goat anti-mouse IgA-HRP (SouthernBiotech).

1. Guzman J, Iglesias MT, Riande E (1997) Synthesis and polymerization of acrylic monomers with hydrophilic long side groups. *Polymer (Guildf)* 38(20):5227–5232.
2. Lutz MB, et al. (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223(1): 77–92.
3. Van den Broeck W, Derore A, Simoens P (2006) Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. *J Immunol Methods* 312(1-2):12–19.

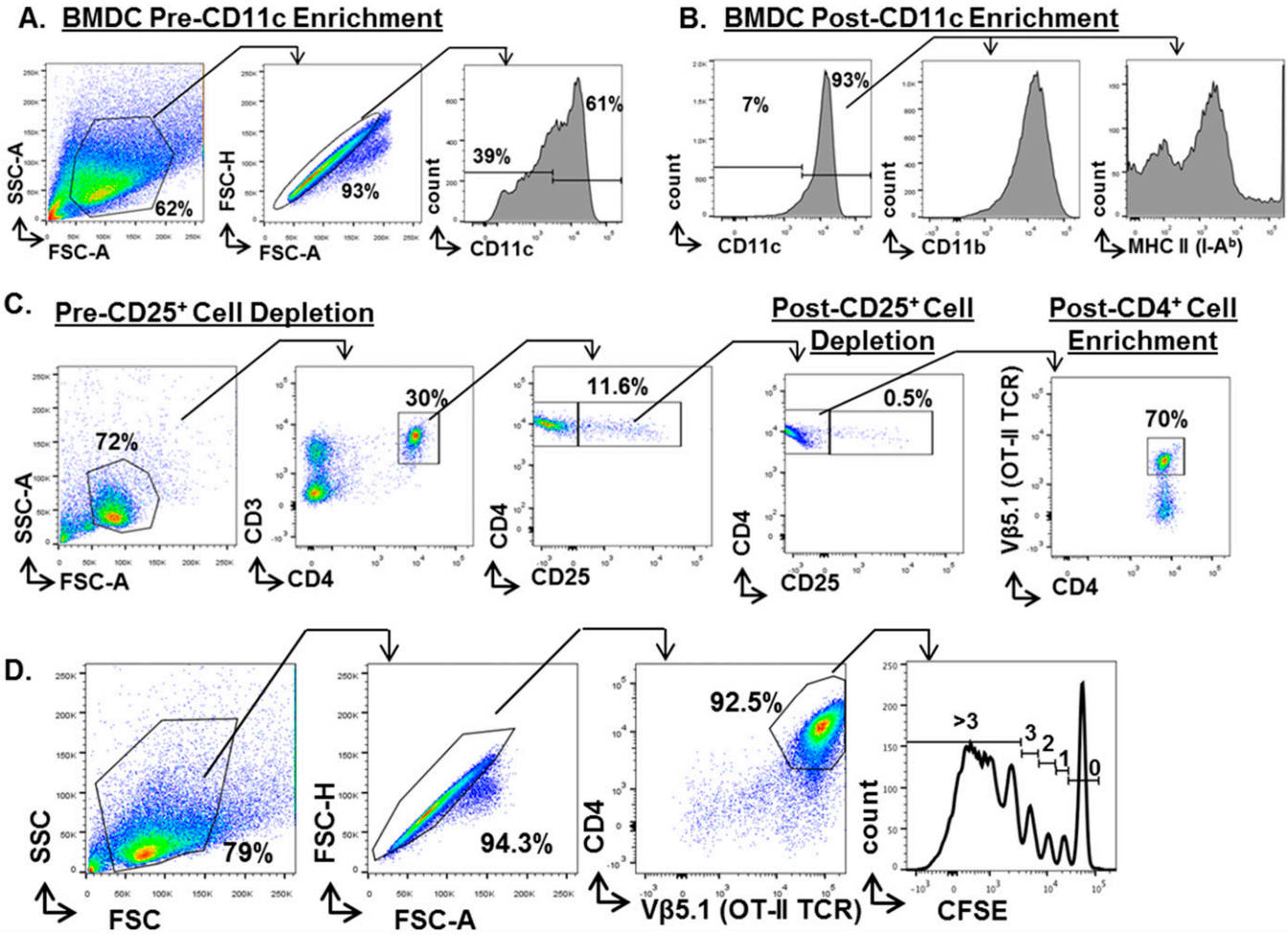


Fig. S1. Representative gating for BMDC and OTII T-cell purification and proliferation. (*A*) Flow cytometric analysis of CD11c$^+$ BMDCs 6 d after culture with GM-CSF before CD11c enrichment. (*B*) Purity and phenotype of CD11c$^+$ BMDC population following magnetic enrichment of CD11c$^+$ cells from *A*. (*C*) Flow cytometric analysis of total OVA-specific OT-II transgenic splenocytes predepletion and postdepletion of CD25$^+$ cells (regulatory and activated T-lineage). The CD25-depleted fraction was then enriched for CD4$^+$ cells and the frequency of OVA-specific T cells was determined by using TCR-Vβ5.1 staining. (*D*) Gating for CFSE studies using enriched naïve OVA-specific CD4$^+$ T-cells cocultured with NP-treated BMDC. CFSE dilution was only analyzed in the OVA-specific TCR-Vβ5.1$^+$CD4$^+$ T-cell population. Number above each peak represents the number of divisions.

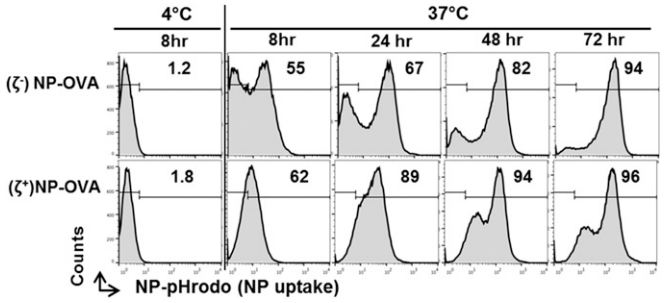


Fig. S2. Internalization of (ζ$^-$) NP-OVA and (ζ$^+$) NP-OVA by BMDCs by using the pH-sensitive dye pHrodo. Cells were incubated with 10 μg/mL (ζ$^-$) NP-OVA (*upper*) or (ζ$^+$) NP-OVA (*lower*) at 4 °C (no internalization) and at 37 °C for the indicated times. Number indicates the frequency of BMDC in the pHrodo$^+$ gate that have internalized NP (gate set based on 4 °C control).

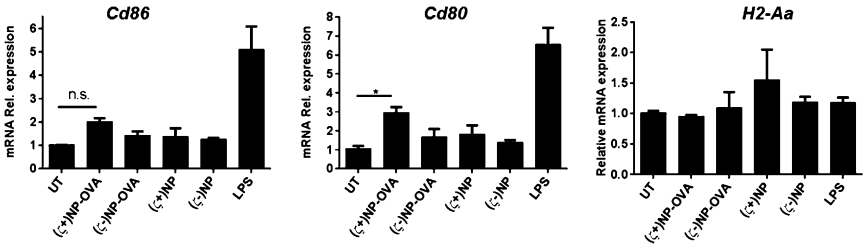
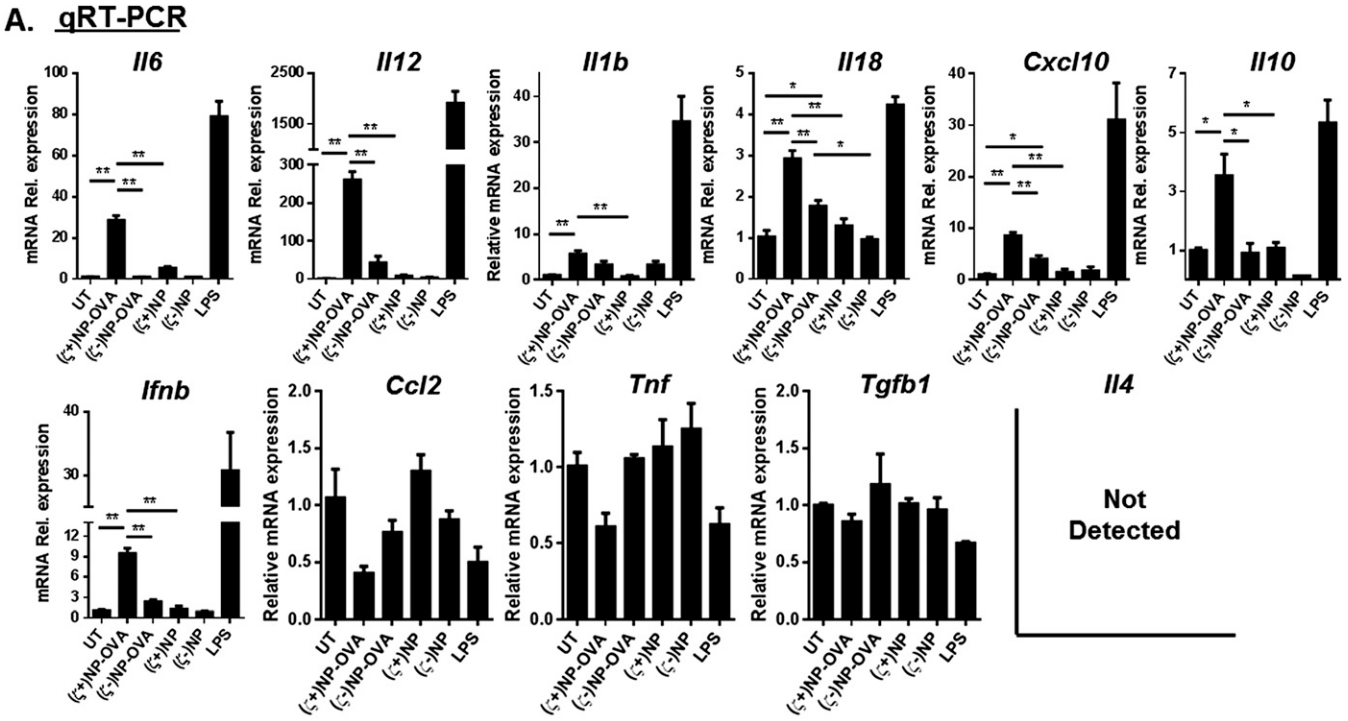


Fig. S3. MHCII and T-cell coreceptor expression by BMDC following 24-h NP-OVA treatment. qRT-PCR for mRNA expression of *Cd80 Cd86* and *H2-Aa* (MHCII) by cells treated for 24 h. Data are normalized to β-actin (*Actb*) mRNA and graphed as fold change over UT. Equivalent OVA dose [1 μg/mL] corresponds to [10 μg/mL] NP dose and LPS treatment for 24 h at 10 ng/mL. $*P < 0.05$, $**P < 0.001$; one-way ANOVA with Tukey's multiple comparisons test. LPS serves as a positive control for the assay and was excluded from statistical analysis. Representative of three independent experiments. Each experiment used independently synthesized NP and NP-OVA batches. Bar represents mean ± SEM.



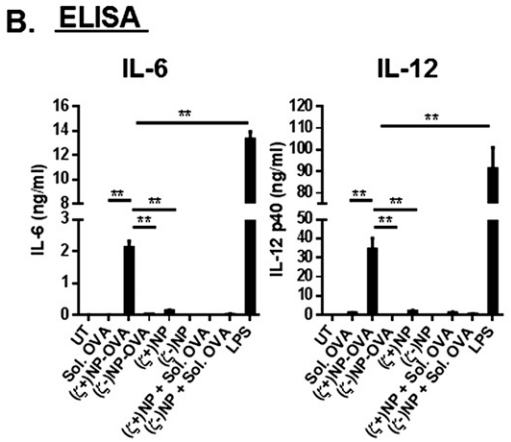


Fig. S4. Cytokine and chemokine expression by NP-OVA–treated BMDC. (*A*) qRT-PCR for mRNA expression of indicated cytokines by NP-treated BMDC at 24 h (*Ifnb*, 48 h). Data are normalized to β-actin (*Actb*) mRNA and graphed as fold change over untreated control. (*B*) ELISA for IL-6 and IL-12 protein secretion by BMDC 24 h after NP treatment. Equivalent OVA dose is 1 μg/mL; LPS dose is 10 ng/mL. $*P < 0.05$, $**P < 0.001$; one-way ANOVA with Tukey's multiple comparisons test. LPS serves as a positive control for the assay and was excluded from statistical analysis. Representative of two independent experiments by using independently synthesized NP and NP-OVA batches. Graph represents mean ± SEM.

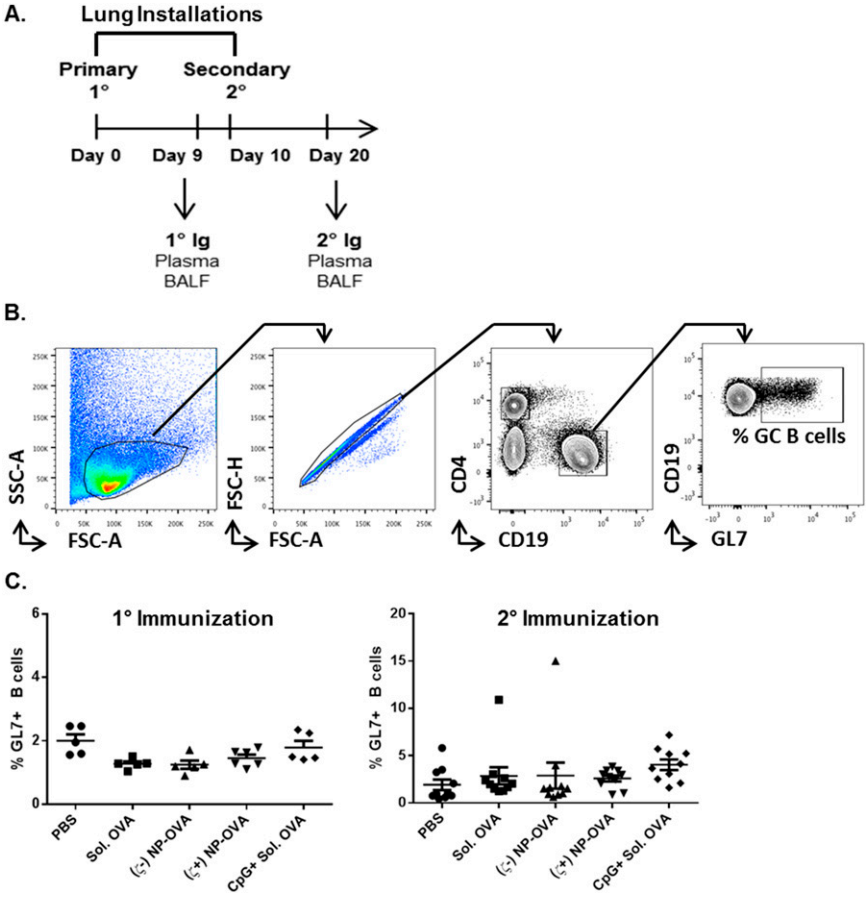


Fig. S5. Pulmonary Immunization schedule and flow cytometric analysis of GC B-cell populations in the spleen. (*A*) Immunization schedule: Primary (1°) orotracheal instillations of 10 μg of OVA conjugated to NP (100 μg of NP), and soluble controls were performed on day 0 with secondary (2°) immunizations occurring on day 10. Mice were bled or euthanized on day 9 (1°) or day 20 (2°) to obtain plasma and BALF. (*B*) Gating scheme used to define the GC B-cell population ($CD19^{+}GL7^{+}$) in spleen and LN of immunized mice. (*C*) Data for frequency of GC B-cell populations in spleen following primary ($n \geq 5$ per group) and secondary ($n \geq 10$) lung immunizations. Data for primary immunization are representative of two independent experiments. Data for secondary immunization are combined from two independent experiments. NP dose = 100 μg per instillation (10 μg of OVA); CpG dose = 2.5 μg per instillation. Line represents mean ± SEM.

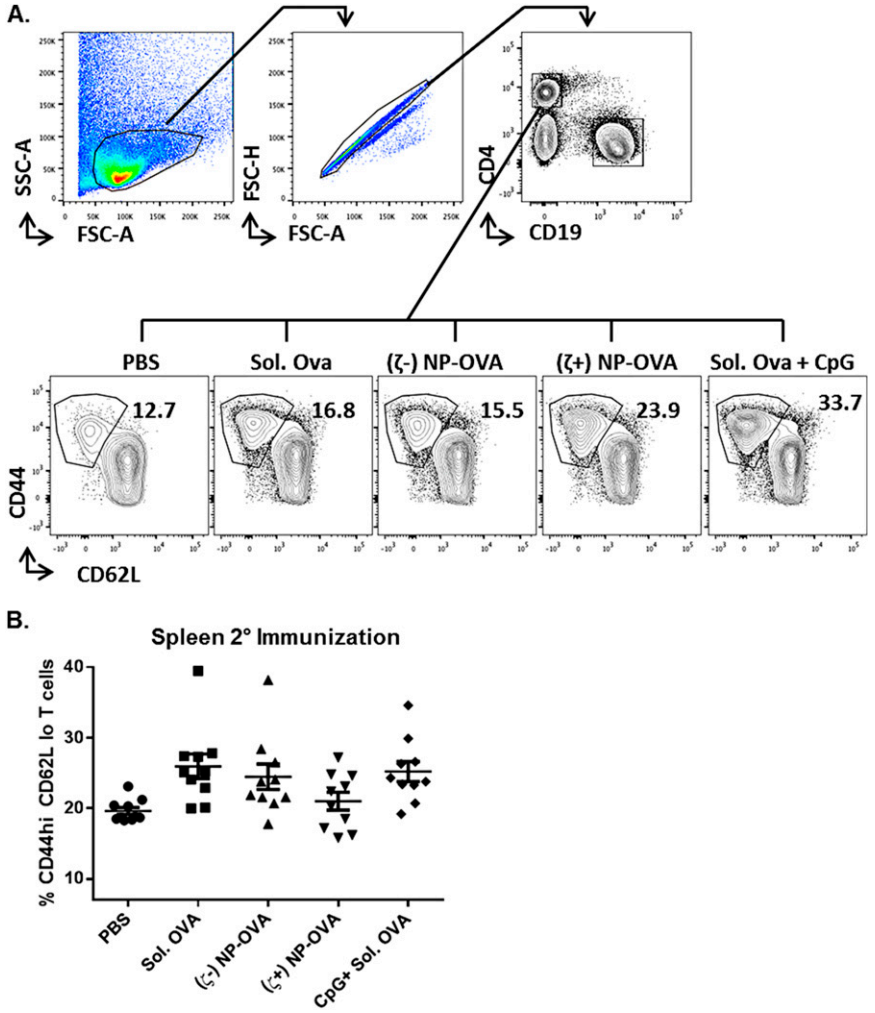


Fig. S6. Representative flow cytometric analysis of antigen-experienced $CD4^+$ T cells with combined data from the spleen. (*A*) Gating scheme used to define the antigen-experienced $CD4^+CD62L^{lo}CD44^{hi}$ T cells in spleen and LN of immunized mice. (*B*) Combined data for frequency of $CD4^+CD62L^{lo}CD44^{hi}$ T cells in spleen following secondary ($n \geq 10$) lung immunizations. Data are combined from two independent experiments. Line represents mean ± SEM.

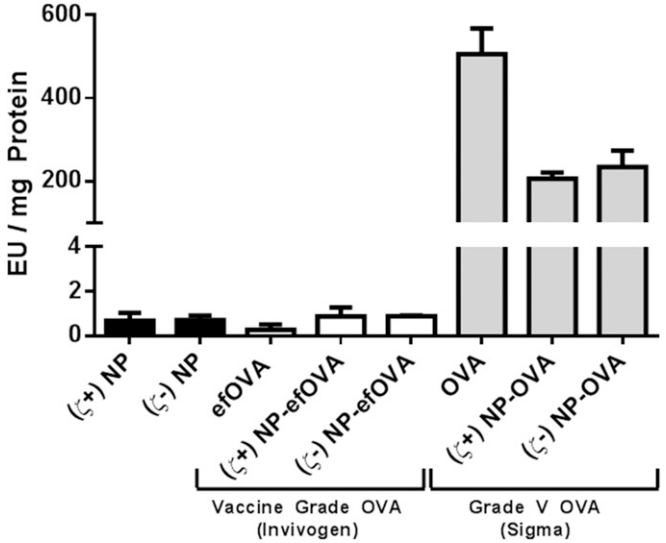


Fig. S7. Endotoxin levels in commercial OVA and NP-OVA formulations. Endotoxin levels determined via LAL Chromogenic Endotoxin Quantitation Kit expressed in entodtoxin units (EU) per mg of protein. Blank particles were dosed at the same particle concentration as OVA-conjugated NP. Graph represents mean ± SD, $n \geq 2$.

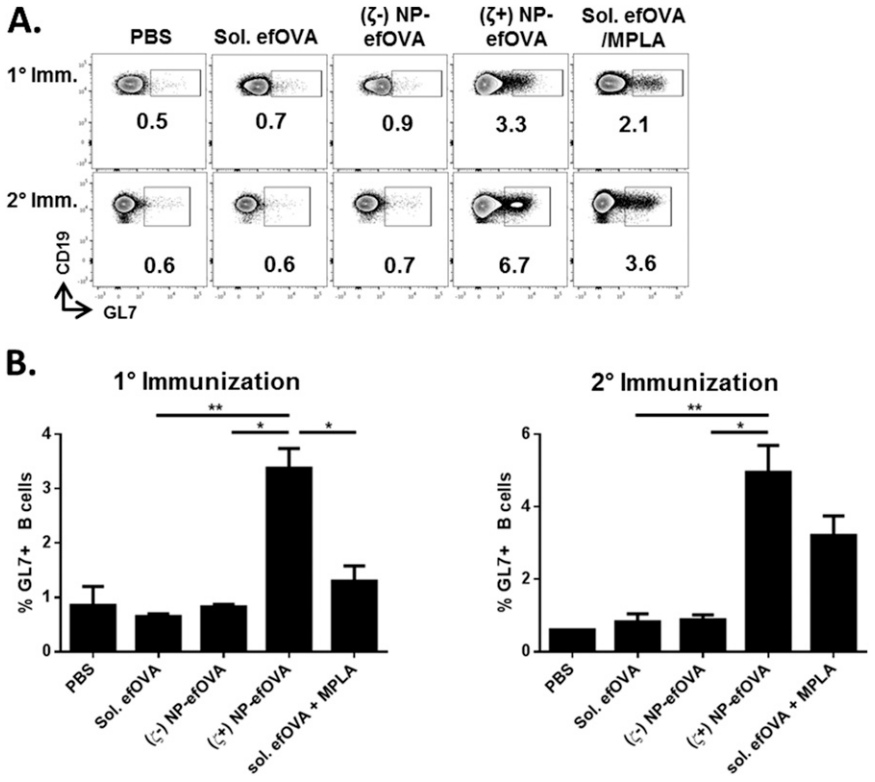


Fig. S8. GC B-cell formation in mediastinal LNs following pulmonary immunization with endotoxin-free OVA-conjugated NP (NP-efOVA). (*A*) Representative flow plots for frequency of $CD19^{+}GL7^{+}$ GC B cells in the mediastinal LN following 1° and 2° immunization. (*B*) Combined data from *A*; 1° immunization, ($n = 4$) per group; 2° immunization, ($n = 4$) per group. $*P < 0.05$, $**P < 0.001$; one-way ANOVA with Tukey's multiple comparisons test. NP dose = 100 μg per instillation (10 μg of OVA); MPLA dose = 0.3 μg per instillation. Line represents mean ± SEM. Graph represents mean ± SEM.

Table S1. Combined DLS results for NPs before and after functionalization

Particle name	Z_{AVG}, nm	PDI	ζ, mV
(ζ^{+}) NP	273 ± 10	0.06 ± 0.02	45 ± 3
(ζ^{+}) NP-OVA	285 ± 4	0.06 ± 0.01	37 ± 3
(ζ^{-}) NP	248 ± 8	0.05 ± 0.02	−38 ± 2
(ζ^{-}) NP-OVA	264 ± 7	0.05 ± 0.01	−38 ± 3

Mean ± SD, $n \geq 5$ of repeated particle batches. PDI, polydispersity index; Z_{AVG}, size; ζ, zeta potential (particle charge).