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Supplementary Materials for

Generation of Effector Memory T Cell–Based Mucosal and Systemic Immunity with Pulmonary Nanoparticle Vaccination

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Supplementary Materials

Supplementary Materials and Methods

Synthesis of ICMVs

ICMV nanocapsules were synthesized as previously described with slight modifications (*16*, *17*). Briefly, lipid films (1.26 μmol lipids) were dried from chloroform (lipid composition: DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and MPB (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimidophenyl) butyramide) in a 1:1 molar ratio (Avanti Polar Lipids). For samples incorporating MPLA in ICMVs, 2.9 μg MPLA was included in the lipid composition. Lipid films were rehydrated in 20 mM bis-tris propane at pH 7.0 with antigens ovalbumin (OVA; Worthington) at 1.63 mg/ml, SIV-gag AL-11 (AAVKNWMTQTL) at 3.25 mg/ml, or PADRE (AKAVAAWTLKAAAC) at 1 mg/ml. After vigorous vortexing every 10 min for 1 h, the liposomal suspension was then sonicated in alternating power cycles of 6 and 3 W in 30-s intervals for 5 min on ice (Misonix Microson XL probe tip sonicator). DTT and Ca^{2+} were then added at final concentrations of 3 mM and 40 mM, respectively, and incubated for 1 h at 37°C. After the particles were washed twice in deionized water by centrifugation at 14,000 × *g* for 4 min, 10 mg/ml of 2-kDa PEG-thiol (Laysan Bio) was added and incubated for 30 min at 37°C. The final product was washed twice before resuspension in PBS, and the particles were used within 24 h of synthesis.

The amount of protein or peptide encapsulated in ICMVs was determined using LavaPep

kits (Gel Company). Measurements of Alexa647-OVA fluorophore-to-protein ratios for protein recovered from lysed ICMVs vs. soluble (stock OVA) protein showed that the fluorophore labeling of protein entrapped in ICMVs was not statistically different from the starting stock solution. Based on the error of the LavaPep assay readout, doses of antigen administered were accurate within ± 9.3%. For immunizations, 10 μg of low MW polyI:C (average size 0.2-1 kb, Invivogen) per injection dose was mixed into particle suspensions**.**

Intratracheal immunization

Intratracheal instillations were performed as described previously (*40*). Briefly, anesthetized mice were placed on a platform by their front teeth. An Exel Safelet IV catheter was inserted into the trachea, guided with a Fiber-Lite Illuminator shining on the mouse's chest, and 75 μl of vaccine dose (containing 10 μg of OVA or 10 μg AL-11 peptide + 3.3 μg PADRE peptide, 0.3 μg MPLA, and 10 μg polyI:C) was directly administered into lungs. For subcutaneous tail base vaccinations, anesthetized mice were immunized with an equivalent vaccine dose in 100 μl.

Analysis of antigen trafficking

To examine antigen uptake and trafficking, 10 μg of Alexa Fluor 647-conjugated OVA (Invitrogen) was administered in either soluble or ICMV formulations with 0.3 μg MPLA and 10 μg polyI:C. Lungs, mdLNs, and bronchoalveolar lavage (BAL) were collected at various time points, and the amount of OVA in each tissue was measured with a fluorescent microplate reader. Histologic images of lungs and mdLN cryosections were taken with a Zeiss LSM 510

confocal microscope. Lungs were cut into small pieces with scissors and meshed over 70-μm cell strainers. RBCs were removed with ACK lysis buffer (Gibco). Mediastinal LNs were isolated, meshed between two frosted glass slides, and passed through cell strainers. BAL fluid was collected by inserting a catheter down the trachea to inject and retrieve 300 μl of PBS from the lungs. The process was repeated once. After washing cells with 1% BSA in PBS, cells were stained with anti-CD11c and DAPI, and analyzed by flow cytometry to examine antigen uptake among $CD11c^+$ antigen-presenting cells. To ensure consistency between experiments and across different time points, the same settings were used for staining, data acquisition, and analysis on flow cytometry, including the same gating schemes, and non-treated controls were included in all the experiments.

Ex vivo determination of antigen presentation to antigen-specific $CD8^+$ T cells

Lungs, mdLNs, and spleen were harvested from mice 3 days after immunization. Whole tissue homogenates were co-incubated with 50,000 $Thy1.1^+CD8^+$ T cells isolated from OT-I TCR-transgenic mice and labeled with 1 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE). After 3 days, dilution of CFSE in OT-I T cells was analyzed by staining the culture with DAPI, anti-CD8a (BD), anti-α4β7 integrin (eBioscience), and anti-Thy1.1 (BD) followed by flow cytometry analysis.

Evaluation of local and systemic toxicity

To assess potential toxicity following pulmonary immunizations, histopathological assessment of

H&E-stained lung tissue sections collected 1 or 7 days post prime and post boost was performed by a pathologist blinded to the study groups (M.A.S). Cytokines and chemokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, MIP-1α, MIP-1β, and MCP-1) were quantified in the lungs, BAL, and sera samples at multiple time points after prime and boost with a multiplex assay kit (Cytometric Bead Array, BD Biosciences). For the lung and BAL fluid samples, supernatants obtained after cell isolation from each tissue as described above were used. Overall body condition was assessed by measuring weights of immunized mice over time.

Isolation of lymphocytes

Spleens, lungs, mdLNs, and Peyer's patches from the small intestines of immunized animals were collected, meshed, and passed through 70 μm cell strainers to isolate lymphocytes. Lymphocytes from vaginal tract tissues were isolated by cutting the tissues into small pieces, followed by digestion in 100 MU/ml collagenase D (Roche) at 37°C for 30 min, and passing cells through 70 μm strainers. To isolate intraepithelial lymphocytes, cells from small intestines were collected by vigorous shaking of tissue sections after incubation with RPMI 1640 supplemented with 5 mM EDTA and 0.145 mg/ml DTT at 37°C. Lymphocytes were then resuspended in 44% Percoll solution (Sigma) and layered over 67% Percoll. Samples were centrifuged at 600 x g for 25-30 mins at RT with gentle deceleration. Intraepithelial lymphocytes were collected at the 67%/44% Percoll interface. Cells were washed with HBSS to remove Percoll.

Assessment of cellular and humoral immune responses

Frequencies of antigen-specific $CD8^+$ T cells and their phenotypes elicited by immunization were determined by staining lymphocytes with DAPI, anti-CD8a (BD), anti-CD44 (BD), anti-CD62L (eBioscience), anti-CD127 (eBioscience), anti-KLRG1 (eBioscience), and AAVKNWMTQTL/H-$2D^b$ (NIH) or SIINFEKL/H-$2K^b$ peptide-MHC tetramers (BD), followed by flow cytometry analysis using a FACSCanto-II flow cytometer and FlowJo software. Absolute cell numbers were enumerated by including AccuCheck Counting Beads (Invitrogen) in the samples. To assess functionality of primed $CD8^+$ T cells, lymphocytes from spleen and lungs were stimulated *ex vivo* with 20-30 μM of OVA peptide SIINFEKL or Gag peptide AAVKNWMTQTL for a total of 5-6 hrs with brefeldin A (eBioscience) added for the last 2 hours of incubation. Cells were then fixed, permeabilized, stained with anti-IFN-γ (BD), anti-TNFα (BD), anti-Granzyme B (BD) and anti-CD8α (BD), and analyzed by flow cytometry.

OVA-specific antibody responses were examined in serum and vaginal washes collected at 10-11 weeks after immunization. Anti-OVA IgG concentrations were determined by including a monoclonal mouse anti-OVA IgG_1 (clone OVA-14, Sigma-Aldrich) as a standard reference during ELISA.

Supplementary Figures

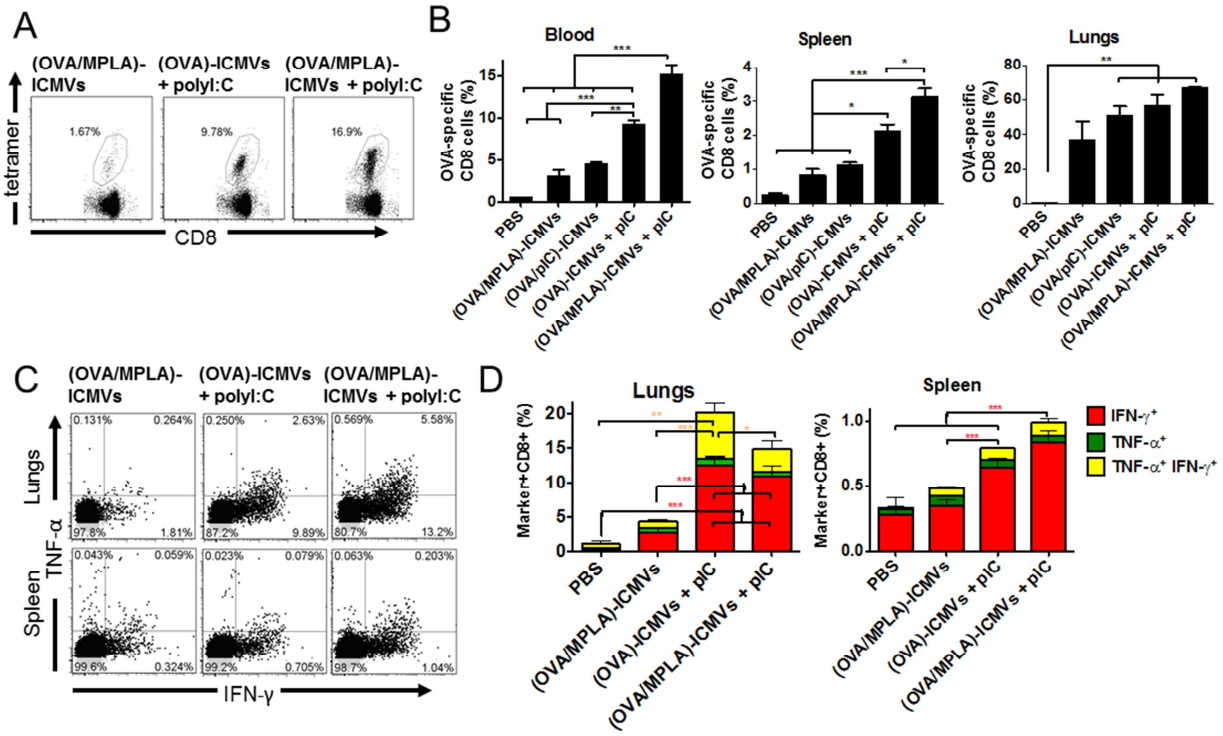

Fig. S1. ICMV nanoparticles adjuvanted with TLR agonists elicit CD8$^+$ T cell responses after pulmonary vaccination. C57Bl/6 mice (*n*=3-4/group) were immunized with 10 µg OVA in ICMVs formulated with either 0.3 µg MPLA (co-entrapped with OVA in ICMVs), 10 µg polyI:C (added externally to ICMVs or co-entrapped with OVA within ICMVs), or the combination of the two via intratrachael administration on days 0 and 42. (**A** and **B**) Frequencies of OVA-specific CD8$^+$ T cells were analyzed on day 7 after boost (blood, spleen, and lungs) by SIINFEKL-MHC-I tetramer staining. Shown are representative flow cytometry dot plots gated on CD8$^+$ T cells (A) and mean frequencies of tetramer$^+$ CD8$^+$ T cells (B). (**C** and **D**) Functionality of OVA-specific CD8$^+$ T cells was assayed on day 7 post-boost after *ex vivo* restimulation with SIINFEKL and intracellular staining for IFN-γ and/or TNF-α. Shown are representative flow cytometry dot plots gated on CD8$^+$ T cells (C) and mean frequencies of cytokine$^+$ CD8$^+$ T cells (D). Data are means ± SEM of two or three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, by one-way ANOVA (B) or two-way ANOVA (D).

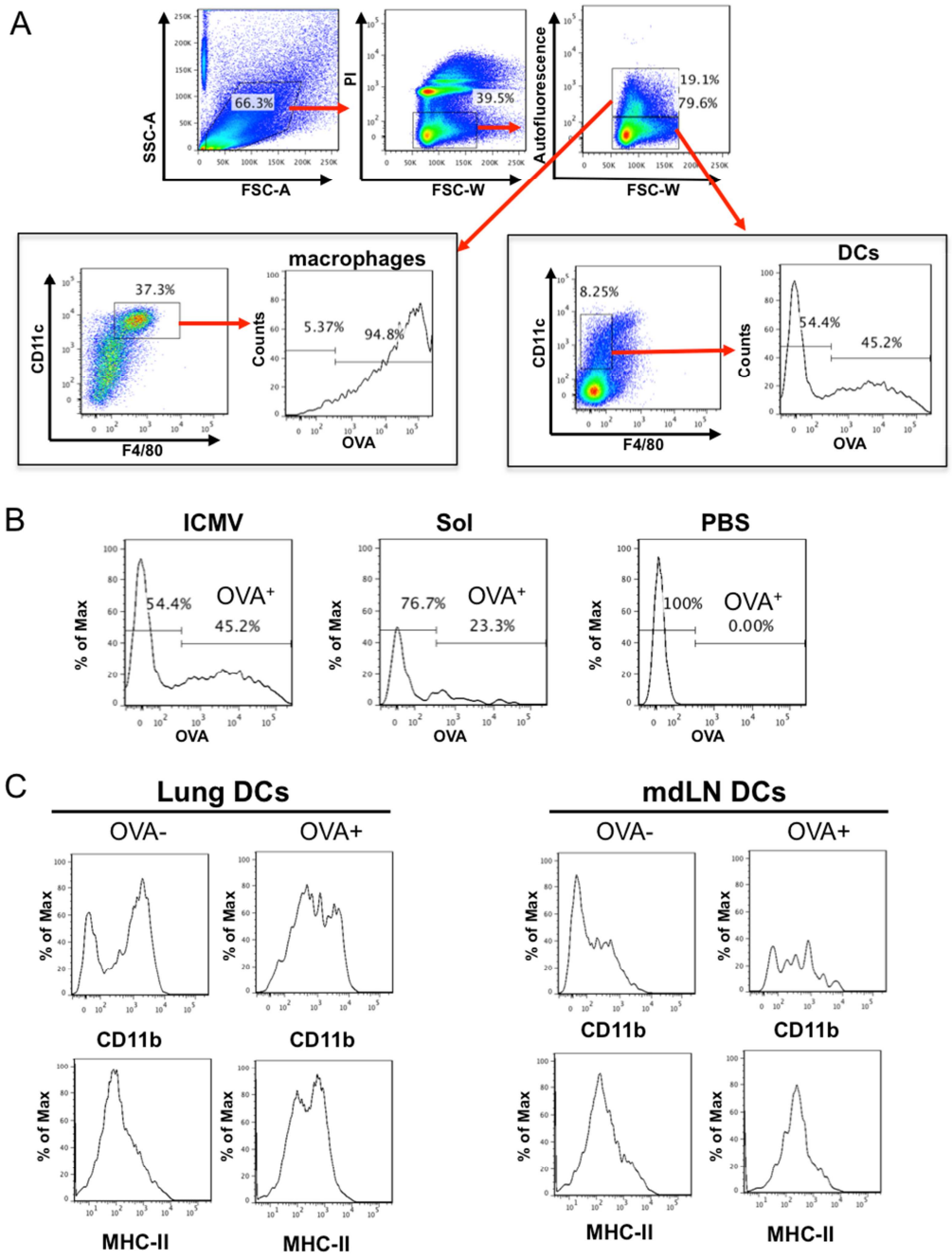

Fig. S2. Phenotype of OVA$^+$ APCs in lungs and mdLNs. C57Bl/6 mice were immunized with ICMVs encapsulating fluorescent Alexa647-OVA (with MPLA and polyI:C as in Fig. 1), equivalent doses as soluble vaccines, or PBS. On day 2, lung tissues were harvested and analyzed for OVA$^+$ DCs and macrophages. (**A**) Gating strategy for identification of macrophages (autofluorescent, CD11c$^+$F4/80$^+$MHC-II$^-$ cells) and lung DCs (non-autofluorescent, CD11c$^+$F4/80$^-$CD11b$^+$MHC-II$^+$ cells). (**B**) Example flow cytometry plots illustrating gating for OVA$^+$ cells. (**C**) Flow cytometry characterization of CD11b and MHCII expression by OVA$^+$ and OVA$^-$ DCs from the lungs and draining mdLNs.

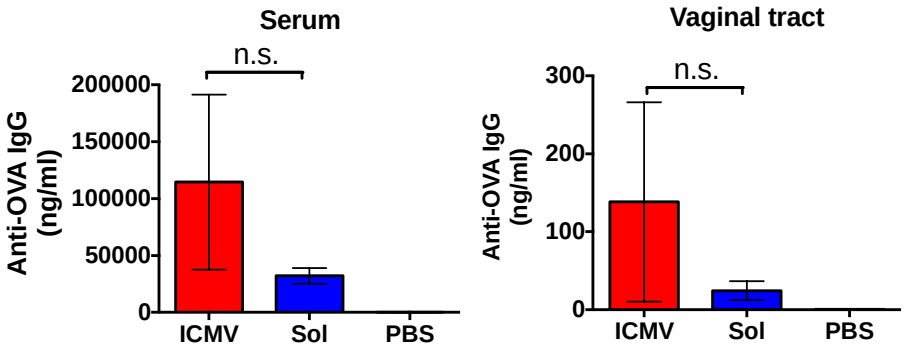

Fig. S3. Pulmonary vaccination elicits humoral immune responses in the blood and vaginal tract. C57Bl/6 mice (*n*=3-12/group) were immunized as in Fig. 1. Sera and vaginal washes were collected and analyzed 7 weeks after priming and concentrations of OVA-specific IgG were measured with ELISA. Data are means ± SEM. n.s., not statistically significant by one-way ANOVA.

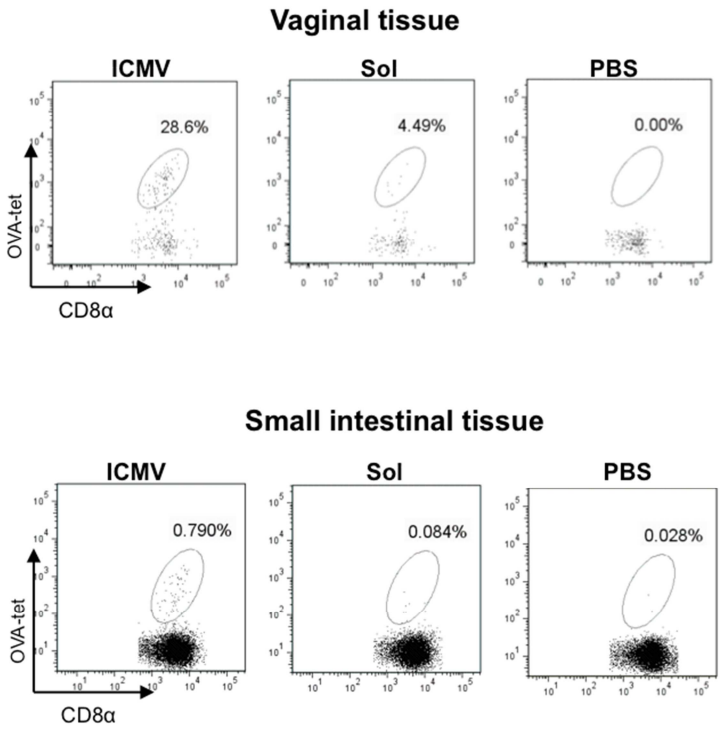

Fig. S4. OVA-tetramer$^+$ T cells from reproductive tract and gut after pulmonary immunization. Shown are representative OVA-tetramer staining plots from individual animals (n = 3 animals/group) from the vaginal tract and intraepithelial lymphocytes corresponding to summary graphs of Fig. 3F.

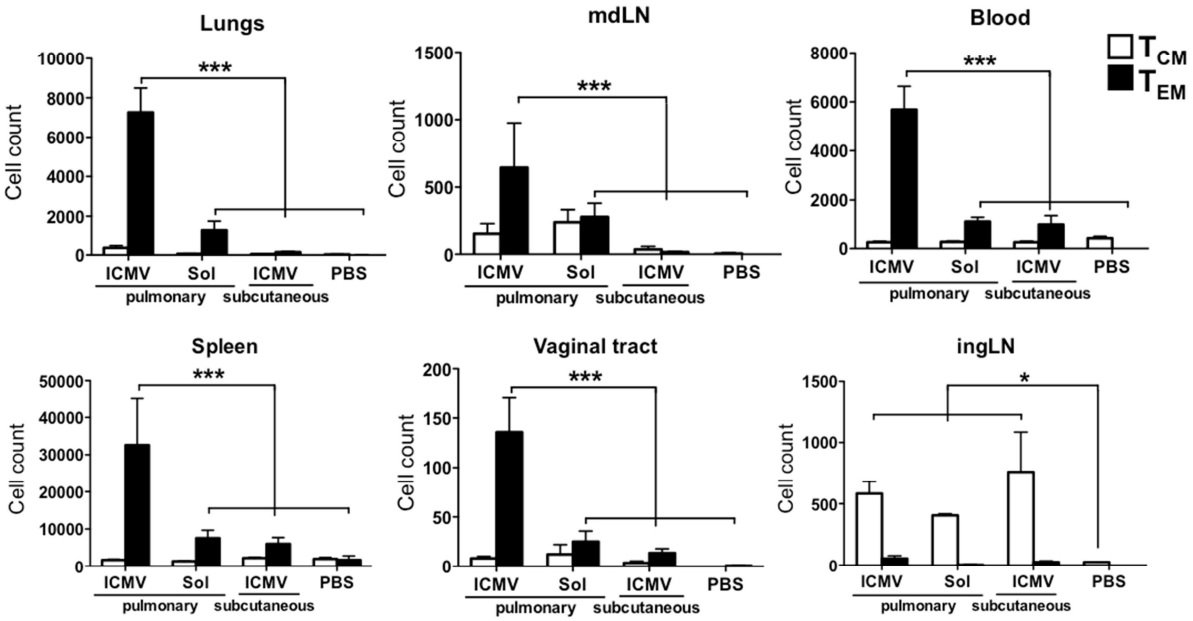

Fig. S5. Pulmonary nanocapsule vaccination promotes T_{EM} cell response in systemic and mucosal compartments. C57Bl/6 mice (n = 3-4) were immunized on days 0 and 28 with either ICMV or soluble OVA vaccines as in Fig. 1. Absolute numbers of OVA-specific effector memory ($CD44^{hi}CD62L^{lo}$) and central memory ($CD44^{hi}CD62L^{hi}$) $CD8^{+}$ T cells were stained and quantified in multiple compartments on day 77. Data are means ± SEM of 2-3 independent experiments. *P < 0.5, ***P < 0.001, by two-way ANOVA.

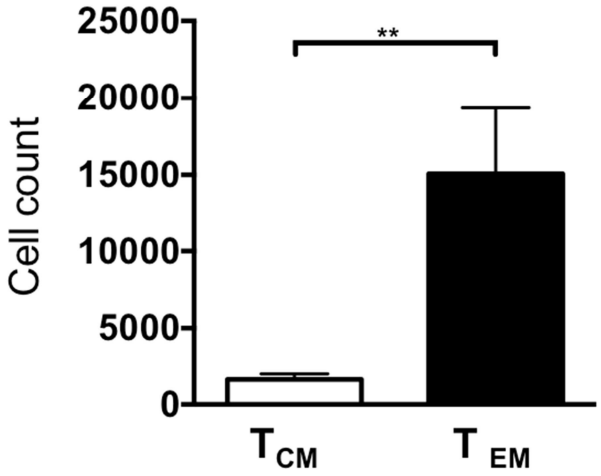

Fig. S6. Immunization with AL11/PADRE peptide ICMVs induces effector memory–biased T cell responses. C57Bl/6 mice (*n*=3-4/group) were immunized as in Fig. 5 with AL11 and PADRE encapsulated in ICMVs, and the frequencies of central memory ($CD44^{hi}CD62L^{hi}$) and effector memory ($CD44^{hi}CD62L^{lo}$) AL11 $tetramer^{+}$ T cells were determined by flow cytometry in the blood on day 35. Data are means ± SEM. $^{**}P < 0.01$, by one-tailed *t*-test.

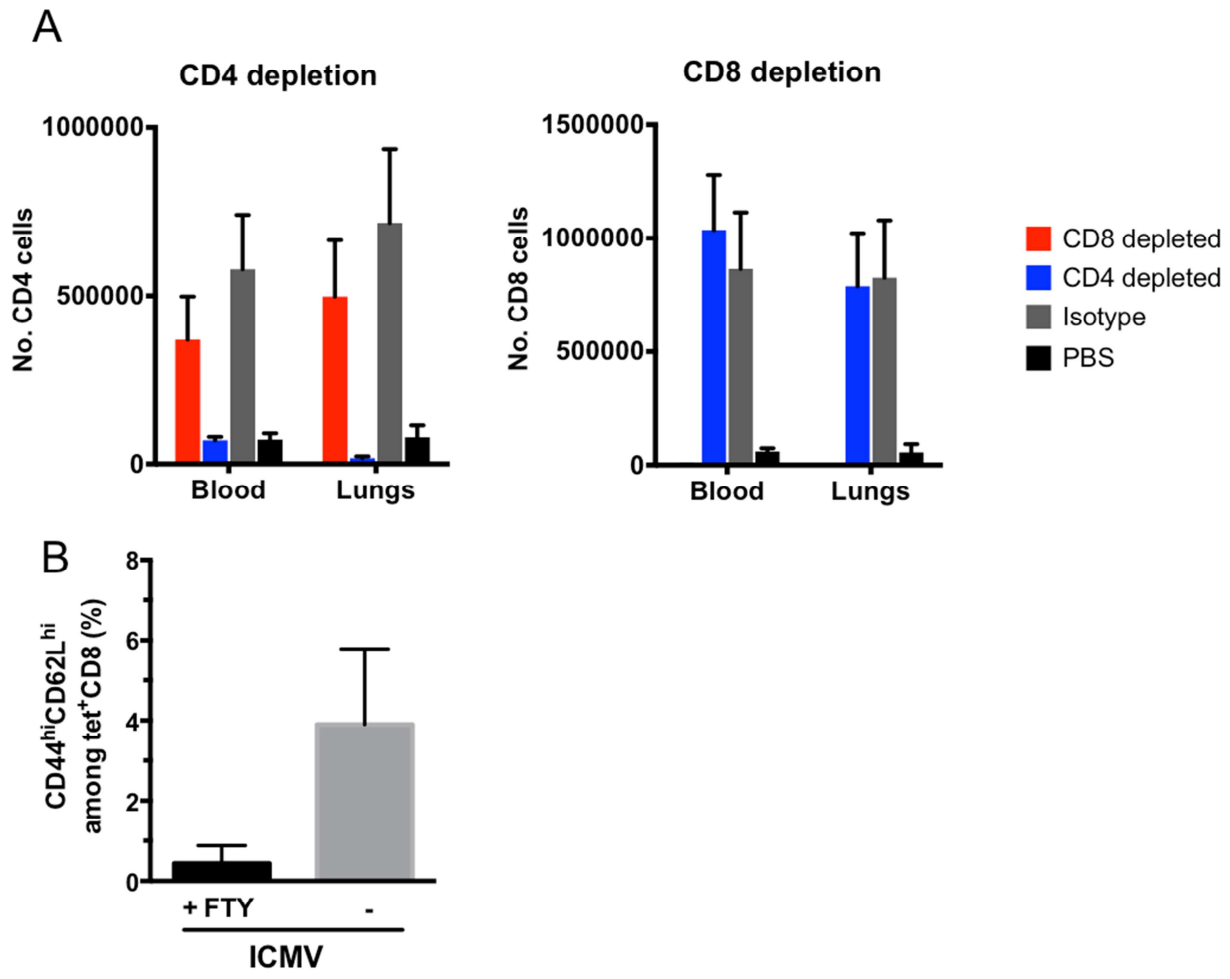

Fig. S7. The role of T_{EM} and T_{CM} cells in ICMV immunization. Groups of C57Bl/6 mice were immunized as described in Fig. 4B. (**A**) On days 40, 42, 44, and 46, depleting antibodies against CD4, CD8, or isotype control IgGs were administered. Total CD4 cells and CD8 cells were detected in blood and lungs on day 48 using flow cytometry. (**B**) FTY720 treatment started 4 days before vaccinia challenge in vaccinated mice depleted AL11-specific $CD8^+$ T_{CM} cells from the blood. Data are means ± SEM (*n*=5-7/group).

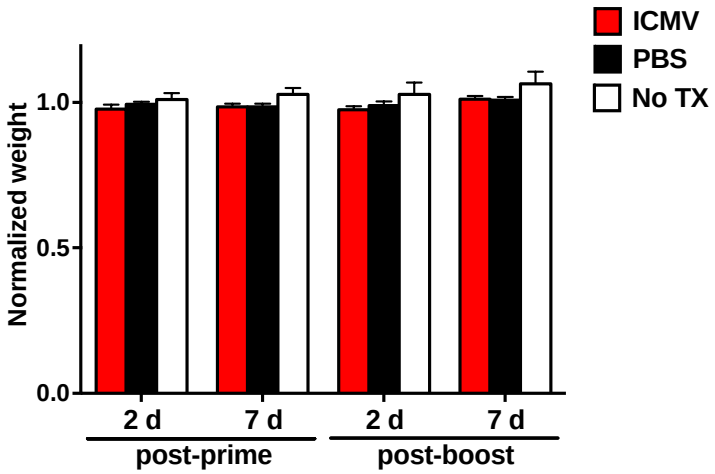

Fig. S8. Pulmonary nanocapsule vaccination does not induce weight loss in healthy animals after vaccination. C57Bl/6 mice were immunized with ICMV OVA vaccines (with MPLA and polyI:C as in Fig. 1) or PBS on days 0 and 28, and the weight of each animal was monitored over time. Untreated mice were also monitored as a control group. The weight of each group was normalized to the weight recorded on the day of prime or boost administration. Data are means ± SEM (n = 8 animals per treatment). Differences between groups were not statistically significant by two-way ANOVA.

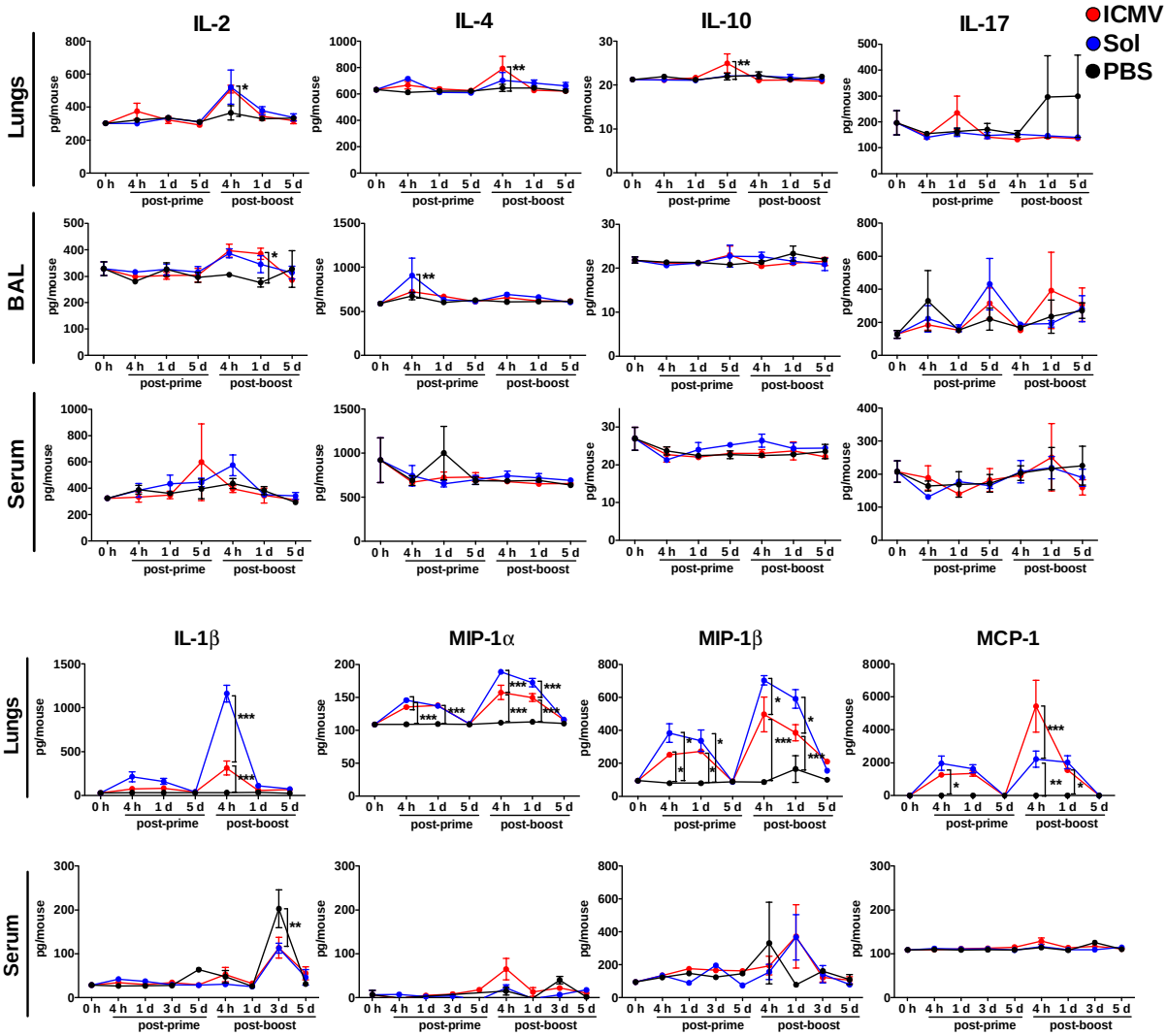

Fig. S9. Chemokine and cytokine levels in lungs, serum, and BAL after pulmonary nanocapsule vaccination. C57Bl/6 mice (n = 3-4/group) were immunized with ICMV or soluble OVA vaccines as in Fig. 1. Supernatants from lungs homogenates, BAL, and sera were collected over time and analyzed for cytokine and chemokine levels by cytometric bead arrays. Data are means ± SEM of two or three independent experiments (one sample of lung tissue, BAL, and serum per mouse was analyzed in each experiment). *P < 0.05, **P < 0.01, ***P < 0.001, by two-way ANOVA. Insufficient BAL samples were collected to analyze the complete panel of cytokines/chemokines; hence, a subset of analyses were carried out on the lavage fluids.