

**Supplemental Materials for**

**CHITOSAN ENHANCES NANOPARTICLE DELIVERY FROM THE  
REPRODUCTIVE TRACT TO TARGET DRAINING LYMPHOID ORGANS**

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## Supplemental Methods

### *Materials and reagents*

Red fluorescent PS NPs (carboxylate-modified FluoSpheres with excitation/emission 580/605, Molecular Probes, Eugene, OR) in two different diameters (20 and 200 nm) were used to track biodistribution *in vivo* following IVG administration. Ultra-pure chitosan (MW <200 kDa, deacetylation > 75%, NovaMatrix, Sandvika, Norway), Nonoxynol-9 (Options Conceptrol, Madison, NJ), and CpG-ODN (ODN 1826, Invivogen, San Diego, CA) were used to treat the mouse vaginal tract.

### *In vivo IVG administration of materials*

All procedures were approved by the University of Washington Institutional Animal Care and Use Committee. *In vivo* administration of reagents and materials to mice is summarized in Figure 1a. Briefly, female C57BL/6J (8-10 weeks old) mice were subcutaneously administered 2.5 mg of medroxyprogesterone acetate (Depo-Provera, Greenstone LLC, Peapack, NJ) formulated in 1X sterile PBS (100  $\mu$ l) per mouse in order to synchronize the estrous cycle. Six days post-Depo-Provera administration, mice were anesthetized and vaginal tracts were lavaged three times with 80  $\mu$ l of 1X sterile PBS. The vaginal lumen was swabbed using a sterile calginate swab to remove mucus. Mice were intravaginally administered 1% (w/v) chitosan, 1% (w/v) N9, or 0.15% (w/v) CpG-ODN formulated in endotoxin-free water (20  $\mu$ l per mouse). 24 hours later, vaginal tracts of anesthetized mice were lavaged and swabbed as described above, and 10  $\mu$ l of 0.5 % (w/v) fluorescent PS NPs were intravaginally administered. Mice in the negative control group were intravaginally administered 10  $\mu$ l of 1X sterile PBS. Mice were kept anesthetized in dorsal recumbence for 30 minutes following all IVG administrations to minimize

external leakage. To prevent self- and inter-grooming, mice were taped around their abdomen with FisherBrand tape and individually caged from the day of chitosan pre-treatment through the end point (Figure 1a). Mice were euthanized at 6, 24, 48, or 72 hours following NP administration, and organs were isolated and processed for fluorescence imaging, flow cytometry, histology, or cytokine secretion measurement.

#### *Fluorescence imaging and NP biodistribution*

Isolated organs were placed on a black construction paper and imaged together (per organ type per trial) using a Xenogen *in vivo* imaging system (IVIS) (Xenogen Corporation, Alameda, CA) to evaluate biodistribution of fluorescent NPs. Fluorescence was normalized to the negative control group (PBS) to set a minimum threshold for tissue auto-fluorescence for each independent trial (Figure 2a). Following this normalization to the PBS control, frequency of fluorescence-positive LNs was calculated for each ILLN and IGLN per mouse to quantitatively compare NP accumulation in the dLNs (scored 0, 0.5, or 1 for 0, 1, or 2 positive LNs, respectively, out of 2 ILLNs or 2 IGLNs per mouse). In addition, using LivingImage 3.0 software, a region of interest (ROI) encompassing the fluorescent area was defined and the average radiance (photon/second/cm<sup>2</sup>/steradian) of each organ was obtained. The average radiance per organ was normalized to the radiance of organs from the negative control group (PBS) in each independent trial, or to organ mass. This allowed us to directly compare NP biodistribution in various organs across all independent trials. For this fluorescence imaging study, each experimental or control group included 2~4 mice per independent trial and a total of 6 independent trials were conducted.

### *Isolation of single cell suspensions from necropsied organs*

After fluorescence imaging, organs were immediately digested to prepare single cell suspensions for flow cytometry. To optimize detection of immune cell subset and fluorescent NPs, mouse reproductive tracts (RTs) (including the vagina, cervix, and uterine horns without ovaries), ILLNs, or IGLNs were pooled within each group for isolation of a single cell suspension. All RTs within a group were pooled and the 4 ILLNs or 4 IGLNs showing the highest fluorescence signal by Xenogen imaging were pooled. Then, these organs were digested as previously described<sup>1-3</sup> with minor modifications.

Organs were cut into small pieces and incubated on a rotational shaker at 37°C for 45 minutes (RTs) or 30 minutes (LNs) in complete digestion media consisting of RPMI 1640 supplemented with 1% (v/v) Penicillin-Streptomycin (Life Technologies, Carlsbad, CA), 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies), 1.5 mg/ml Collagenase D (Roche Diagnostics, Indianapolis, IN), and 40 µg/ml DNase I (Roche Diagnostics). Then, to deactivate enzymes, 5 mM EDTA (Sigma) was added to each single cell suspension preparation and incubated for another 5 minutes at room temperature. After digestion, tissues were further mechanically disrupted with a sterile syringe plunger on a cell strainer (70 µm, BD Falcon, San Jose, CA) and washed using RPMI 1640 medium. Single cell suspensions were then washed twice with 1X sterile PBS, counted, and transferred into a flow cytometry tube prior to antibody staining.

### *Antibody staining of single cell suspensions*

Cells were stained for 20 minutes at room temperature with a Live/Dead Fixable stain kit (Life Technologies, Carlsbad, CA) and then resuspended in FACS buffer [1X sterile PBS

supplemented with 1% (v/v) heat-inactivated FBS] to  $10^6$  cells/ml. Cells were blocked with anti-mouse Fc $\gamma$  III/II receptor monoclonal antibody (clone 2.4G2; IgG2b $\kappa$ ), and stained with fluorescently conjugated anti-mouse monoclonal antibodies against CD11c (clone HL3; IgG1 $\lambda$ 2), CD11b (clone M1/70; IgG2b $\kappa$ ), CD45 (clone 30-F11; IgG2b $\kappa$ ) (all from BD Biosciences, San Jose, CA), F4/80 (clone BM8; IgG2a $\kappa$ ), Gr-1 (clone RB6-8C5; IgG2b $\kappa$ ), Ly6C (clone HK1.4; IgG2c $\kappa$ ) (all from BioLegend, San Diego, CA), and MHCII (clone M5/114.15.2; IgG2b $\kappa$ ) (eBiosciences, San Diego, CA) for 30 minutes at 4°C in the dark. Isotype staining was performed to use as negative controls in flow cytometry analysis. After staining, cells were extensively washed and fixed using 2% paraformaldehyde. Cells were examined using flow cytometry – LSRII (BD Biosciences). Data were analyzed with FlowJo Software (Tree Star, Ashland, OR).

Consistent with literature,<sup>1,4,5</sup> live CD45+ immune cells (leukocytes) were subsequently gated and defined as total DCs (MHCII+CD11c+), CD11b–DCs (MHCII+CD11c+CD11b–), CD11b+DCs (MHCII+CD11c+CD11b+), monocytes (CD11b+Ly6C+), macrophages (CD11b+F4/80+), and granulocytes (CD11b+Gr-1+).

Extra mice of negative control groups (PBS) were spiked with fluorescent NPs (administered NPs right before isolation and digestion of RTs) and used to evaluate non-specific and time-sensitive association between NPs and cells during the window of flow cytometry preparation. As seen in Supplemental Figure 4a, when a cluster of very bright cell-free NPs is excluded in gating analysis, the live CD45+NP+ cell frequency of the spike control (PBS + instant NPs) (3.76%) is very close to the CD45+NP+ cell frequency of the PBS control not administered NPs (1.16%). Five independent trials (out of 6 carried out in the Xenogen imaging study) were analyzed in flow cytometry experiments.

### *Tissue homogenization and cytokine analysis*

Separate trials were also conducted to perform cytokine analysis of treatment groups. Cytokine analysis was performed on RTs from mice intravaginally administered 1% (w/v) chitosan, or 0.15% (w/v) CpG-ODN formulated in endotoxin-free water (20  $\mu$ l per mouse) without NPs. At 6, 12, 24, and 48 hours post-IVG administration of chitosan or CpG-ODN, RTs were isolated and homogenized as previously described<sup>6,7</sup> with minor modifications. Briefly, frozen RTs were massed and placed in 500  $\mu$ l of ice-cold Hank's Balanced Salt Solution (HBSS) containing 0.05% Triton-X 100 (non-ionic, Fisher Scientific, Pittsburgh, PA) and 1  $\mu$ l of a protease inhibitor cocktail (104 mM AEBSF, 80  $\mu$ M Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A) (Sigma, St. Louis, MO). Then, the tissue was homogenized using a tissue-tearor (BioSpec Products, Bartlesville, OK) and centrifuged at 4,000  $\times$ g for 5 minutes to eliminate cellular debris. Supernatants were aliquoted and stored at -20°C until cytokine analysis. Standard ELISA kits (PeproTech, Rocky Hill, NJ) were used to analyze supernatants for TNF- $\alpha$  and IL-1 $\beta$  secretion in RT tissues. For cytokine analysis, a single mouse per time point was analyzed per trial and 4~5 independent trials were conducted per time point.

### *Histology and immunofluorescence imaging*

RTs and dLNs were separately prepared for histology and immunofluorescence analysis. RTs and dLNs were isolated from 3~4 mice per mouse group, and frozen in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA). Frozen tissues were cryo-microtomed into 3~10 sections, each of which was 5  $\mu$ m thick, using a Leica CM1850 cryostat (Leica Biosystems, Buffalo Grove, IL). RTs were cryo-microtomed into transverse sections in the middle of the

frozen tissue block along the length of the tissue. LNs were cryo-microtomed in the middle of the frozen tissue block without a specific orientation. Five different areas per section slide were examined under a microscope for histology and immunofluorescence imaging analysis.

Cryo-microtomed RT and dLN tissue sections were stained with Phalloidin (Life Technologies) or primary anti-mouse monoclonal antibodies against CD11c (clone HL3; IgG1 $\lambda$ 2 or biotinylated) (BD Biosciences) or Gr-1 (clone RB6-8C5; IgG2b $\kappa$  or biotinylated) (BioLegend) and labeled using secondary fluorescent antibodies including anti-hamster IgG (DyLight 488, clone Poly4055; Goat IgG) (BioLegend) or streptavidin (DyLight 488) (BioLegend). Tissue sections were then counter-stained with Fluoromount-G anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) (SouthernBiotech, Birmingham, AL).

Fluorescently stained RT and dLN tissue sections were imaged under a Zeiss Axio Observer Z1 fluorescence microscope with an ApoTome optical sectioning attachment (Zeiss, Thornwood, NY) using 20X, 40X, or 63X (oil immersion) objective. A cryo-microtomed section of each tissue was further 'optically sectioned' on the microscope by collecting x-y plane images (slices) at 10-11 different z-direction altitudes, ensuring that a full thickness of a single tissue section was visualized (x-y slices were collected every  $\Delta z = 1 \mu\text{m}$ ). A 'z-stack' was reconstructed, a single z-direction altitude at the middle of the tissue section thickness was selected, and NPs or cells in various x-y coordinates in this single slice were visualized. Specifically, using z-stack reconstruction, NP co-localization with the cellular cytoskeleton or specific cell markers according to previous studies<sup>8-10</sup> was visually captured.

For histological analysis, RT tissue sections were stained with hematoxylin and eosin (H&E), and then examined under a Nikon Eclipse Ti microscope equipped with a Nikon Digital

Sight DS-Fi2 camera (Nikon, Melville, NY). All H&E stain images were obtained using a 10X objective.

### *Statistical analysis*

To observe significant differences between control and treatment groups, various statistical analysis methods were used. A curve fit by a general linear regression, Wilcoxon matched-pairs signed rank test (non-parametric), one-way ANOVA followed by a Tukey's multiple comparison test (parametric), or one-way ANOVA followed by a Kruskal-Wallis test and Dunn's multiple comparison test (non-parametric) was used depending on experimental data sets. For all statistical methods, GraphPad PRISM (Version 5.04, La Jolla, CA) was used. Unless otherwise indicated, a  $p$ -value  $\leq 0.05$  was considered to be significant.

## **Supplemental Results**

### *Chitosan pre-treatment and prevention of oral ingestion of NPs facilitate NP retention in the RT and transport to dLNs*

In small pilot studies (n=1 mouse per group), we tested the effect of different dosing regimens of chitosan in the reproductive tract (RT) for promoting submucosal and dLN accumulation of 20 nm fluorescent NPs administered intravaginally. NPs were either co-administered with chitosan or administered following chitosan pre-treatment, and biodistribution was measured by whole-organ fluorescence imaging. For all our *in vivo* studies, we prevented mice from self- and inter-grooming prior to and during NP administration by applying collars of adhesive tape around the mouse abdomen and by using individual caging.<sup>11</sup> When mice were caged together without taped abdomens, we observed that intravaginally administered NPs



accumulated fluorescence in the liver (Supplemental Figure 1). This observation indicated that NPs were likely ingested orally and accumulated in the liver by first-pass metabolism.<sup>12, 13</sup> Oral ingestion of NPs after vaginal delivery has not been completely investigated and has not been controlled in previous studies of NP administration via the mouse vaginal route, which can confound the data interpretation.<sup>14, 15</sup> However, when mice were individually caged and abdominally taped, we did not observe NP transport to the liver or any other examined organs except for the ILLNs and IGLNs (Supplemental Figure 1).

To compare the biodistribution outcomes for 20NP associated with the different treatments, we measured mean tissue fluorescence and calculated the frequency of positive LNs per mouse. PBS treatment groups were used to set the minimum threshold for sample autofluorescence since they showed similar signal to the chitosan-only treatment groups (Supplemental Figure 2a). We observed that co-administration of chitosan and 20NPs resulted in retention of fluorescence in the RT up to 48 hours post-vaginal administration and detection of fluorescence-positive ILLNs but not IGLNs (Supplemental Figure 2a). In contrast, when 20NPs were intravaginally administered following IVG pre-treatment with chitosan, fluorescence in the RT was extended out to 72 hours post-administration (Supplemental Figure 2b). Chitosan pre-treatment also led to 20NP fluorescence in both dLNs, and a higher frequency of fluorescence-positive IGLN detected as early as 24 hours and persisting to later time points (Supplemental Figure 2b and 2c). We observed that the maximum frequency of positive fluorescent ILLNs and IGLNs occurred at 24 hours. We also measured a time course of the weighted average tissue radiance, which showed a significant decrease for the RT but no change for either dLNs (Supplemental Figure 2d). Based on these data, we demonstrate that mice vaginally administered

20NPs following chitosan pre-treatment maximally retained NPs in the RT and draining ILLNs and IGLNs.

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## Supplemental Figure Legends

Supplemental Figure 1.

**Oral uptake (via self- and inter-grooming) of NPs intravaginally administered was prevented by taping of mouse abdomen and individual caging.** Mice of PBS, 20NP, and CH20NP groups were treated as depicted in Figure 1a. While 5 mice per group were caged in a single cage without taping their abdomen following NP administration, another mouse per group were taped and individually caged. After 24 or 48 hours, organs were excised and imaged under the Xenogen IVIS. Images were obtained from a single mouse per group. ‘No tape + Group caging’ mice are shown only with livers, whereas ‘Tape + Individual caging’ mice are shown with various organs. Images are shown only for 24-hour time point and images observed at 48 hours post-administration of NPs did not show any difference (data not shown). Mouse group identifications for PBS, 20NP, and CH20NP in all organ images are in the same order as ‘No tape + Group caging’ shown at the top-left.

Supplemental Figure 2

**Sequential administration of chitosan and NPs extends NP retention in the RT as well as facilitates NP accumulation in both ILLNs and IGLNs.** Mice of PBS, 20NP, CH20NP, and/or CH (administered chitosan without NP) groups were (a) co-administered a mixture of chitosan and NPs or (b) sequentially administered as depicted in Figure 1a. After 6, 24, 48, and 72 hours, organs were excised and imaged under the Xenogen IVIS. Images were obtained from a single mouse per group per time point. (c) Fluorescence-positive LNs of CH20NP group are scored based on the ILLN and IGLN images of (a) and (b) (see the scoring criteria in the Methods). (d) Based on the tissue fluorescence images of (b) above, tissue average radiances of organs of 20NP and CH20NP groups are plotted in time course. While a RT curve shows a single radiance per mouse per time point, each of LN curves shows a sum of the radiances from a pair of ILLNs or IGLNs per mouse per time point. To compare radiances across mice along the time points, the radiance is normalized to weight (mg) of respective organs.

Supplemental Figure 3

**NP retention in RTs correlates with NP accumulation in dLNs.** (a) RTs of PBS, 20NP, CH20NP, 200NP, and CH200NP groups were excised and examined under the Xenogen IVIS at 24 hours-post administration of NPs. Then, representative tissue fluorescence images are shown (a single trial selected from a total of 6 independent trials). (b) Average tissue fluorescence radiances of all RTs, ILLNs, and IGLNs are plotted to investigate correlation between NP retention in RT and NP accumulation in dLNs. Each dot represents the radiance of individual dLNs (a total of 4 LNs of ILLNs and IGLNs) versus that of a RT of a single mouse, which shows a total of 4 dots in the plot. All of 20NP, 200NP, CH20NP, and CH200NP groups of all 6 independent trials are pooled together (a total of 340 dots from 85 mice). A curve fit (linear regression) shows R square of 0.08750 and is significantly deviated from zero (non-zero slope) by  $p \leq 0.0001$ .

Supplemental Figure 4

**Chitosan modulates NP association per immune cell but not frequency of immune cells.** (a)(i) Representative flow cytometry plots are shown with gates of CD45+NP+ populations in RT tissues excised at 24 hours post-administration of NPs. An additional control (PBS + Instant NPs) was prepared to check if there is non-specific and instant association between NPs and cells during tissue cell preparation for the flow cytometry analysis (see the Methods for details). (ii) Frequencies of CD45+NP+ populations (out of total live CD45+ cells) are plotted. (iii) Representative NP+ histograms within the CD45+ cell population are shown by an overlay of 20NP and CH20NP or 200NP and CH200NP group. From these NP+ histograms, (iv) mean fluorescence intensities (MFIs) of NP fluorescence are shown to quantitatively compare NPs associated with a single CD45+ cell. (b) CD45+ (leukocytes) or CD45- (epithelial cells) frequency (out of total live RT tissue cells) and CD45-NP+ frequency (out of total CD45- cells) are shown. Data obtained from the flow cytometry are shown with mean±SD, n=5 independent trials (2~4 mice combined per treatment per trial). (c) Fluorescent images of NPs (red) are merged with IFHC-stained cell nucleus (DAPI, blue) and/or CD11c+ or Gr-1+ (green) markers from uterine horn tissue sections excised at 24 hours following treatment with 20NP or CH200NP. Scale bars indicate 50 or 20  $\mu\text{m}$  for 20 $\times$  or 40 $\times$  objective lens, respectively.