Supporting Information

Display of DNA on Nanoparticles for Targeting Antigen Presenting Cells

Nicholas M. Molino[1], Medea Neek[1], Jo Anne Tucker[2], Edward L. Nelson[2,3,4], Szu-Wen Wang[1,3,5]

[1]Department of Chemical Engineering and Materials Science
[2]Department of Medicine
[3]Chao Family Comprehensive Cancer Center
[4]Institute for Immunology
[5]Department of Biomedical Engineering
University of California, Irvine, CA 92697 USA

Materials and Methods

Materials

All buffer reagents were purchased from Fisher Scientific, unless otherwise noted. The oligodeoxynucleotide Toll-like receptor 9 ligand CpG 1826 (5'-tccatgacgttcctgacgtt-3') (CpG) and non-CpG 1982 (5'-tccaggacttctctcaggtt) were synthesized with a phosphorothioated backbone and thiol linker by TriLink Biotechnologies. The methoxy-PEG-N-hydroxysuccinimide (mPEG-NHS; molecular weight 2000 Da) and maleimide-PEG-NHS (mal-PEG-NHS; molecular weight 2000 Da) were purchased from Nanocs. Alexa Fluor 488 C5-maleimide was from Life Technologies. Fluorescently-tagged monoclonal antibodies CD11c (clone N418), CD3 (clone 145-2C11), B220 (clone RA3-6B2), F4/80 (clone BM8), DEC-205 (clone 205yekta), and rat IgG2a,κ (clone eBR2a, isotype control) were purchased from eBioscience. All cell culture media, unless otherwise noted, was comprised of RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Lonza), 100 units/ml penicillin (Hyclone), 100 µg/ml streptomycin (Hyclone), 50 µM 2-mercaptoethanol (Fisher), and 0.1 mM non-essential amino acids (Lonza) (complete RPMI media).

Animals and Cell Lines

All experiments were performed under approved protocol by the IACUC at the University of California, Irvine. Animals were housed and maintained by the ULAR. For generation of

bone marrow-derived dendritic cells and for *in vivo* biodistribution assays, wild-type C57Bl/6 female mice aged 6-12 weeks were used (Jackson Laboratories).

The NIH 3T3 mouse fibroblast cell line was purchased from ATCC and maintained in DMEM + 10% FBS. B3Z, a $CD8^+$ T cell hybridoma, was kindly provided by Prof. Nilabh Shastri (University of California, Berkeley). CH12, a B cell lymphoma line was kindly provided by Prof. Paolo Casali (University of Texas Health Science Center at San Antonio). Murine bone marrow-derived macrophages were kindly provided by Prof. Wendy Liu (University of California, Irvine).[1]

E2 Preparation

The D381C E2 protein (E2) was prepared as previously described.[2] D381C is an E2 mutant with a non-native cysteine introduced to the internal cavity of the nanoparticle for site-directed functionalization. Briefly, proteins were expressed in *E. coli*, cells were lysed, and soluble cell lysates were applied to a HiPrep Q Sepharose anion exchange column (GE Healthcare) followed by a Superose 6 (GE Healthcare) size exclusion column for purification. The purified proteins were analyzed by dynamic light scattering (Zetasizer Nano ZS, Malvern) for size measurements. Electrospray ionization mass spectrometry and SDS-PAGE were performed for molecular weight and purity confirmation. Final protein preparations were stored in 50 mM potassium phosphate at pH 7.4 with 100 mM NaCl (phosphate buffer). Lipopolysaccharide (LPS) was removed, and endotoxin levels were checked as described previously.[3]

Zeta potential measurements

Zeta potential measurements were conducted with a Malvern Zetasizer (Nano ZS) using a diffusion barrier method. This method can measure the zeta potential of low volume samples in physiological ionic strengths, a condition which is needed due to aggregation of our nanoparticles at low salt conditions. Malvern capillary cells were filled with 50 mM potassium phosphate at pH 7.4 with 100 mM NaCl, and 150 µl of nanoparticles (at ~1.3 mg/ml) were gently injected to the bottom of the buffer-filled capillary cells. Since E2 samples are of relatively high conductivity/ionic strength, the monomodal analysis mode within the Malvern software was used.

Bone Marrow-Derived Dendritic Cells (BMDCs)

Murine bone marrow-derived dendritic cells (BMDCs) were prepared as described previously,[3] and involved plating of 2 million red blood cell-depleted bone marrow cells in 10 mL of complete RPMI containing 20 ng/mL murine GM-CSF for 8 days.

Internal Conjugation with Alexa Fluor 488 Fluorescent Marker and External Conjugation of Poly(ethylene glycol) and CpG

The thiol-reactive Alexa Fluor 488 C5-maleimide (Thermo Fisher) was added to a solution of E2 (D381C mutant[2]) in phosphate buffer at a 3-fold molar excess to E2 monomer. Unreacted dye was removed by the 40 kDa molecular weight cutoff Zeba Spin Desalting Columns (Thermo Fisher).

The mPEG-NHS or mal-PEG-NHS linkers were conjugated to the external lysines on E2 by incubating at a 25-fold molar excess of PEG to E2 monomer for 2 hr at room temperature, with excess linker removed by the Zeba Spin desalting column. To the mal-PEG-NHS-functionalized E2 nanoparticle, TCEP-reduced CpG-SH or (non-CpG)-SH was added at a 10-fold molar excess to E2 monomer and incubated for 2 hr at room temperature followed by an overnight incubation at 4°C, and the reaction was quenched with L-cysteine (Fisher) at a 20-fold excess to E2 monomer. Unreacted CpG-SH and L-cysteine were removed by the Zeba Spin desalting columns. All nanoparticles (E2, PEGylated E2 [mPEG-E2], and E2 with CpG and non-CpG ligands on surface [CpG-PEG-E2]) were characterized by BCA (to measure concentration), SDS-PAGE, zeta potential, and DLS (to measure particle size) as previously described.[2] In order to determine the number of conjugated CpG molecules per E2 nanoparticle, relative band intensities on SDS-PAGE were evaluated using the NIH ImageJ software as previously described.[3] Endotoxin levels of final conjugated nanoparticles were checked to confirm acceptable limits as previously discussed.[3]

In Vitro Uptake Assays

Cells were prepared at 1 million cells/mL in complete RPMI for BMDCs, BMDMs, CH12 B cells, and B3Z T cells or DMEM + 10% FBS for NIH 3T3 cells. E2, mPEG-E2, CpG-PEG-E2, or (non-CpG)-PEG-E2 was added to the cells at either 5 μg/mL or 1 μg/mL for 1 hr at 37°C. Cells were harvested and prepared in PBS + 1% BSA (Fisher) and 0.02% sodium azide (FACS

buffer) for flow cytometry analysis on the BD Accuri C6 flow cytometer. Mean fluorescence intensity (MFI) is reported as relative to cells only (background fluorescence) and represents the mean ± standard deviation of at least 3 experiments. For discrimination of surface bound versus internalized fluorescent particles, cells were incubated with 0.5% trypsin for 30 min at 37°C to remove surface-bound proteins.

For inhibition of cellular uptake, well-established pathway-specific inhibitors and conditions were used.[4] Cells were incubated for 1 hr at 37°C with 1 μM cytochalasin D (macropinocytosis and phagocytosis; variably for receptor-mediated endocytosis[5]), 30 min with 5 mM amiloride HCl (macropinocytosis), 20 min with 10 μg/mL chlorpromazine (clathrin-mediated endocytosis), or 20 min with 25 μg/mL nystatin (lipid raft-mediated endocytosis). E2 or CpG-PEG-E2 (1 μg/mL) was added for an additional 1 hr at 37°C and cells were harvested for analysis.

Biodistribution and In Vivo Cell Interaction

E2, mPEG-E2, and CpG-PEG-E2 protein nanoparticles (50 μg in PBS) were administered subcutaneously in the left hock region of 6-12 week old female C57BL/6 wild-type mice, which has a well-described unilateral drainage pattern.[6] Following 6 hr or 48 hr, secondary lymphoid tissues were isolated from euthanized animals and crushed through a 70 μM cell strainer with 1 mL PBS. Cells from the draining lymph nodes were prepared for flow cytometry analysis in FACS buffer and labelled with fluorescently-tagged monoclonal antibodies.

The heart, kidney, and liver were diced and submerged in 1 mL PBS containing Liberase TM (0.25 U/mL for heart and liver and 0.5 U/mL for kidney), incubated at 37°C for 30 min (heart and liver) or 45 min (kidney). The lung and skin from the injection site (and contralateral hock, for comparison) were diced and submerged in 1 mL PBS containing 0.5 mg/mL Collagenase D (Roche) for 30 min at 37°C. Digested tissues were subsequently crushed through a 70 μM cell strainer. Cells from tissues were prepared for flow cytometry analysis in FACS buffer and labelled with fluorescently-tagged monoclonal antibodies.

Blood was collected and centrifuged at 300 × g for 5 min to separate cells from plasma. Blood plasma was centrifuged at 18,000 × g for 5 min to remove debris and analyzed for

fluorescence by spectrophotometry (96-well plate format) at excitation/emission wavelengths of 490/525 nm (SpectraMax M2).

Statistical Analysis

Statistical analyses were carried out using Microsoft Excel and GraphPad Prism. Data is reported as mean ± standard deviation for particle characterization and DEC-205 expression, and mean ± standard error of the mean (S.E.M.) for all else, of at least three independent experiments unless otherwise noted. For *in vitro* assays, each datum is the result of duplicate measurements (unless otherwise noted). Statistical significance was determined by one-way ANOVA using post-hoc Tukey's test (comparing all means) for *in vitro* and *in vivo* cellular uptake assays, to determine differences between the nanoparticle formulations, and t-test for *in vivo* biodistribution experiments (pairwise comparison of experimental groups to background fluorescent control).

References

1. Kim, Y. K.; Que, R.; Wang, S. W.; Liu, W. F., Modification of biomaterials with a self-protein inhibits the macrophage response. *Adv Healthc Mater* **2014,** *3* (7), 989-94. DOI: 10.1002/adhm.201300532.
2. Dalmau, M.; Lim, S.; Chen, H. C.; Ruiz, C.; Wang, S. W., Thermostability and molecular encapsulation within an engineered caged protein scaffold. *Biotechnol Bioeng* **2008,** *101* (4), 654-64. DOI: 10.1002/bit.21988.
3. Molino, N. M.; Anderson, A. K.; Nelson, E. L.; Wang, S. W., Biomimetic protein nanoparticles facilitate enhanced dendritic cell activation and cross-presentation. *ACS Nano* **2013,** *7* (11), 9743-52. DOI: 10.1021/nn403085w.
4. Ivanov, A. I., Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? *Methods Mol Biol* **2008,** *440*, 15-33. DOI: 10.1007/978-1-59745-178-9_2.
5. Fujimoto, L. M.; Roth, R.; Heuser, J. E.; Schmid, S. L., Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic* **2000,** *1* (2), 161-71.
6. Kamala, T., Hock immunization: a humane alternative to mouse footpad injections. *Journal of Immunological Methods* **2007,** *328* (1-2), 204-14. DOI: 10.1016/j.jim.2007.08.004.
7. Destito, G.; Yeh, R.; Rae, C. S.; Finn, M. G.; Manchester, M., Folic acid-mediated targeting of cowpea mosaic virus particles to tumor cells. *Chemistry & Biology* **2007,** *14* (10), 1152-62. DOI: 10.1016/j.chembiol.2007.08.015.
8. Zheng, C.; Ma, G.; Su, Z., Native PAGE eliminates the problem of PEG-SDS interaction in SDS-PAGE and provides an alternative to HPLC in characterization of protein PEGylation. *Electrophoresis* **2007,** *28* (16), 2801-7. DOI: 10.1002/elps.200600807.
9. Ren, D.; Kratz, F.; Wang, S. W., Engineered drug-protein nanoparticle complexes for folate receptor targeting. *Biochemical Engineering Journal* **2014,** *89*, 33-41. DOI: 10.1016/j.bej.2013.09.008.

Supplementary Figures

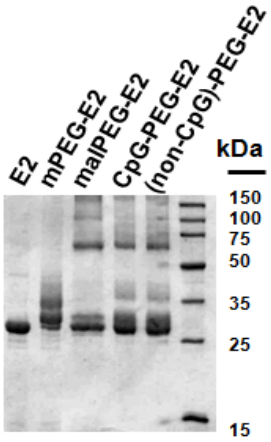
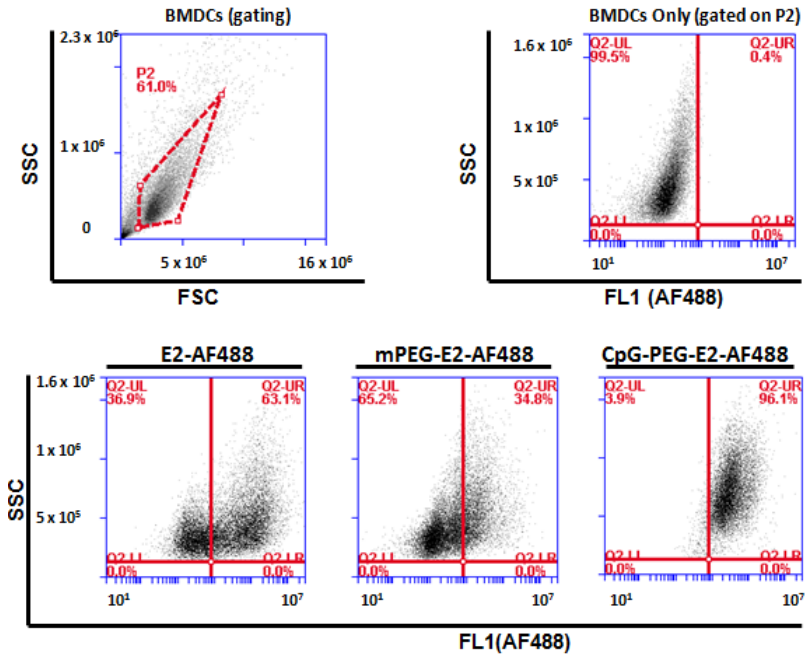


Figure S1. SDS-PAGE. The lane for mPEG-E2 indicates heterogeneous numbers of PEG polymers attached to E2 subunits. CpG-PEG-E2 and (non-CpG)-PEG-E2 nanoparticles comprise of individual subunits with no attachment of PEG or CpG/(non-CpG), attachment of PEG, and attachment of both PEG and CpG/(non-CpG). Bands >60 kDa are indicative of the attachment of bi-functional PEG linker (see lane "malPEG-E2"). This observation for PEGylated protein is consistent with other studies and has been hypothesized to be due to the complex interaction between PEG chains and SDS micelles. [7-9]

A



B

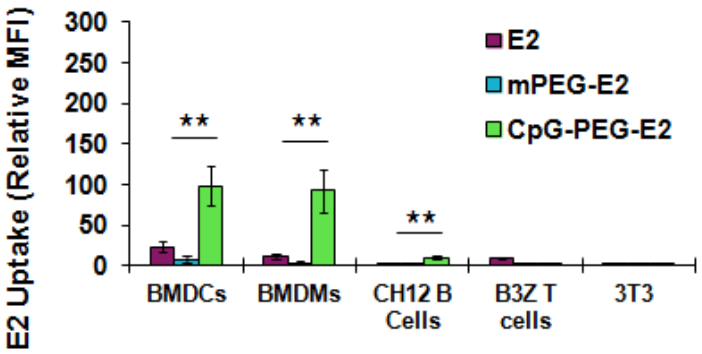


Figure S2. A. A representative BMDC uptake experiment analyzed by flow cytometry following 1 hour incubation with 5 ug/ml of AlexaFluor 488-tagged E2, mPEG-E2, CpG-PEG-E2, or (non-CpG)-PEG-E2. Cells were gated with a standard gating strategy, using a live gate based on FSC/SSC**.** Events to the right of the red bar are considered positive for the specific marker (AF488). **B.** Antigen presenting cells show increased association with CpG-PEG-E2 nanoparticles *in vitro*, compared to their interactions with E2 and mPEG-E2. Cellular association was measured by mean fluorescence intensity (MFI) of cells incubated with 1 μg/mL E2 nanoparticles for 1 hr at 37°C. Data is reported as average ± S.E.M. relative to cellular background fluorescence (PBS) of 3 independent experiments, and statistical significance was determined with a one-way ANOVA using a post-hoc Tukey's test (** $p < 0.01$).

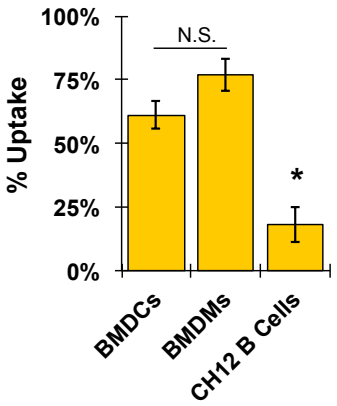
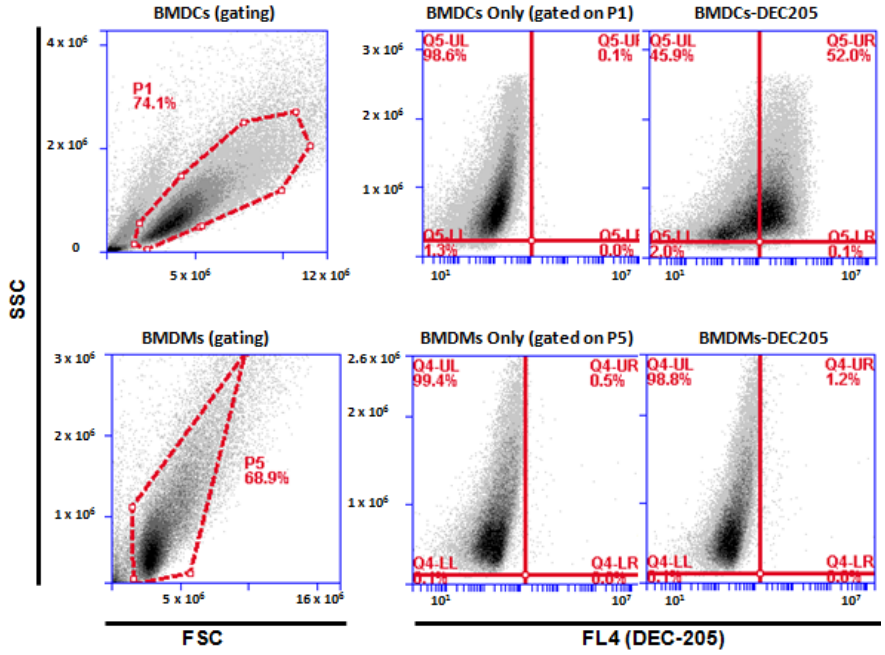


Figure S3. The majority of E2 is internalized within BMDCs and BMDMs, but not B cells. Cells were incubated with 5 μg/mL of the CpG-PEG-E2 nanoparticle and subsequently treated with 0.5% trypsin to remove surface bound proteins. The percentage of fluorescence remaining following treatment with trypsin is shown. Data is reported as average MFI ± S.E.M. of 3 independent experiments. Statistical significance was determined with a one-way ANOVA followed by a post hoc Tukey's test (* $p < 0.05$). Significance for CH12 B cells was compared to both BMDCs and BMDMs.

A



B

Cell Type	DEC-205 Expression% ± SEM
BMDCs	46.4% ± 4.2
BMDMs	2.2% ± 1.6
CH12 B Cells	0.80% ± 0.4
B3Z T cells	0.20% ± 0.1
3T3	0.13% ± 0.03

Figure S4. Representative samples of BMDCs and BMDMs were labeled with fluorescently-tagged DEC-205 antibody and analyzed by flow cytometry for DEC-205 expression. A. Representative data quantifying DEC-205 expression by flow cytometry. The events to the right of the red bar are considered positive for DEC-205 expression. B. Average percent of cells residing within the gate for DEC-205 expression for the different cell types examined *in vitro*. Data are presented as average percent ± standard deviation for at least three independent experiments.

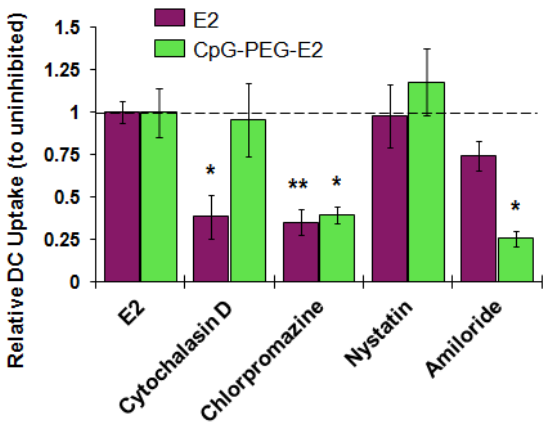


Figure S5. Incubation of BMDCs with various uptake inhibitors shows decreased uptake CpG-PEG-E2 in the presence of chlorpromazine and amiloride, indicating macropinocytic (amiloride) and clathrin-mediated (chlorpromazine) uptake mechanisms, but not lipid-raft mediated (nystatin) or actin-mediated (cytochalasin D) endocytosis. In contrast, BMDCs showed decrease uptake of E2 in the presence of Cytochaslasin D, indicating actin-mediated uptake mechanisms of the bare nanoparticle. Data points were compared to the mean of BMDC uptake for untreated controls (E2). Conditions were chosen that have been well-established for these uptake inhibitors. Data is presented as average MFI ± S.E.M. of at least 3 individual experiments, where statistical significance was determined by t-test, comparing means to untreated nanoparticle (E2; * $p < 0.05$, ** $p < 0.01$).

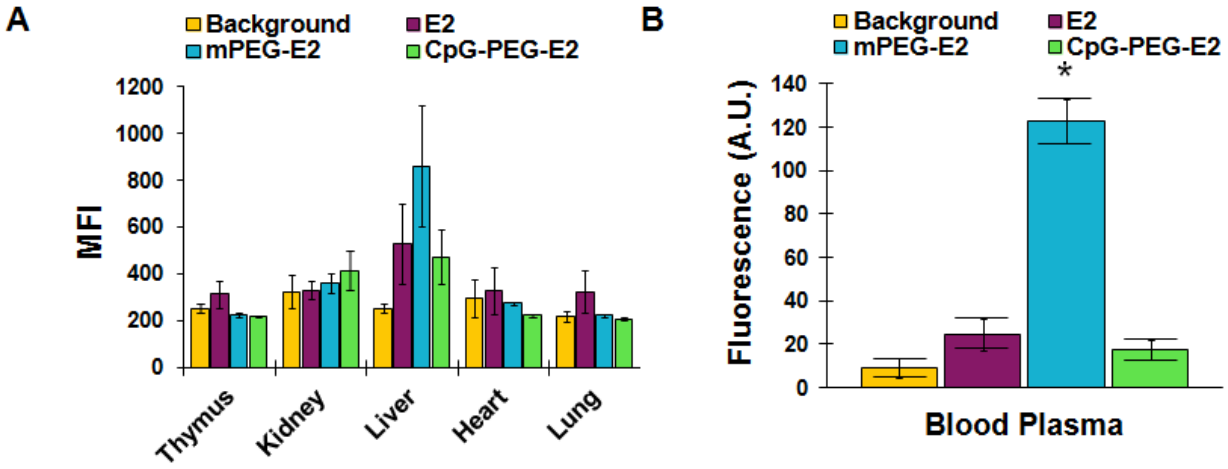


Figure S6. Fluorescence in the thymus, blood draining organs, and blood plasma following subcutaneous administration of the various E2 nanoparticles. A. Tissue MFI was measured by flow cytometry of cells isolated from the thymus and blood draining organs. B. Blood fluorescence was measured by a spectrofluorometer. Data is presented as average MFI ± S.E.M. of 3 independent experiments. For determining statistical significance, each mean was compared to tissue background fluorescence using a t-test (* $p < 0.05$).

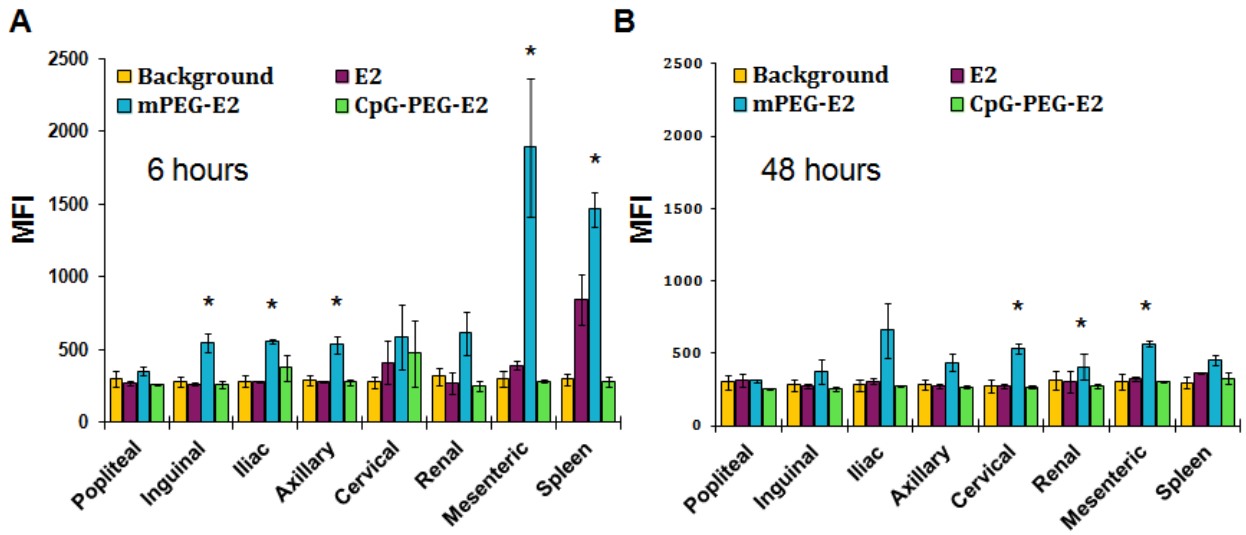


Figure S7. Fluorescence in lymph nodes (contralateral to injection site) and in the spleen, following subcutaneous administration the various E2 nanoparticles. MFI was measured by flow cytometry of cells isolated from the tissues A) 6 hr and B) 48 hr following subcutaneous administration. Data is presented as average MFI ± S.E.M. of 3 independent experiments. For determining statistical significance, each mean was compared to tissue background fluorescence using a t-test (* $p < 0.05$).

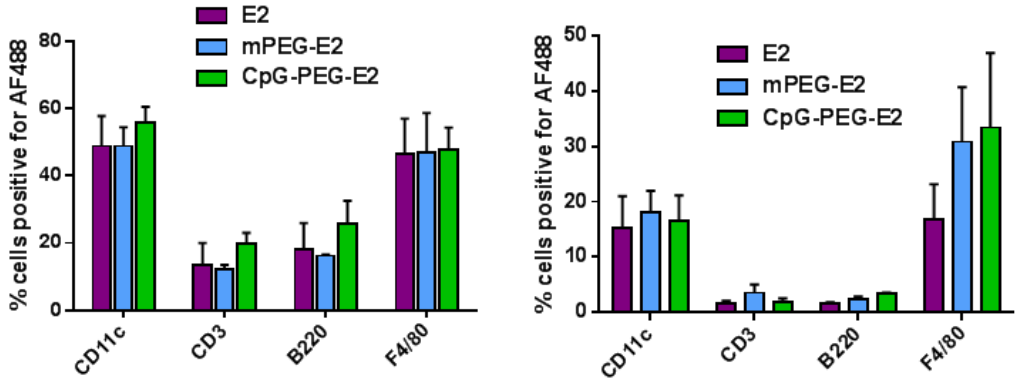


Figure S8. The percentage of the population of a particular cell type within the draining lymph nodes that were associated with E2 nanoparticles was determined by flow cytometry A) 6 hr or B) 48 hr following subcutaneous administration. Relevant cell markers were CD11c (dendritic cells), CD3 (T cells), B220 (B cells), and F4/80 (macrophages). Data is reported as average percentage ± S.E.M. of cells positive for Alexa Fluor 488 (AF488) of 3 independent experiments.