Methods

Reagents for Ace-DEX synthesis and Ace-DEX PAMP MP fabrication

The materials used for polymer synthesis and microparticle production were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. 2-ethoxy propene was purchased from Matrix Scientific (Elgin, SC). Imiquimod, 3'3'-cGAMP, Murabutide, and Poly(I:C) were purchased from Invivogen (San Diego, CA).

Ace-DEX synthesis

Ace-DEX was synthesized and characterized according to Kauffman et al. using dextran with a 70 kDa average molecular weight. To obtain Ace-DEX with 40% cyclic acetal coverage (CAC), dextran was reacted with 2-ethoxypropene for 33.5 minutes. After synthesis, Ace-DEX was hydrolyzed in deuterium oxide with the addition of 10% v/v deuterium chloride and analyzed by ^{1}H NMR spectroscopy (Inova 400 MHz spectrometer; Varian Medical Systems, Palo Alto, CA). The relative cyclic acetal coverage was determined to be 40 ± 3%.

Fabrication and Quantification of PAMP Electrosprayed Micropartcles

Ace-DEX MPs loaded with cGAMP, Imiquimod, Murabutide, or Poly(I:C) were fabricated by using a electrohydrodynamic spraying method. A stainless steel plate was sterilized for 1 hour by UV-treatment and incubated at 265° C for 1 hour. Ace-DEX was dissolved in ethanol at either 20 mg/mL or 30 mg/mL. cGAMP, Imiquimod, Murabutide, or Poly(I:C) were dissolved in pure molecular grade water. Ace-DEX (20 mg/mL) and cGAMP or Poly(I:C) (0.1% w/w) mixtures were generated at a 90:10 ethanol:water ratio. Ace-DEX (30 mg/mL) and Imiquimod and Murabutide (0.1% w/w) mixtures were generated at a 95:5 ethanol:water ratio. Ace-DEX and

PAMP mixtures were sprayed in 2.5 mL batches with a flow rate of 0.2 mL/hour. The needle and steel plate were charged at -5 kV and +2.5 kV, respectively using high voltage power sources (Gamma High Voltage Research, Inc. Ormond Beach, FL). Aerolized MPs were collected on the plate for 12.5 hours. Blank Microparticles were fabricated by the same process using pure molecular grade water (20 mg/mL Ace-DEX, 90:10 Ethanol:Water).

PAMP Microparticle	Concentration of Ace-DEX (mg/mL)	Ethanol:Water ratio
Blank	20	90:10
cGAMP	20	90:10
Imiquimod	30	95:5
Murabutide	30	95:5
Poly(I:C)	20	90:10

cGAMP loading was quantified by high performance liquid chromatography (HPLC, Agilent 1100 series, Santan Clara, CA) using a gradient method on an Aquasil C18 column (150 mm length, 4.6 mm inner diameter, 5µm pore size, Thermo Fisher Scientific, Waltham, MA). cGAMP MPs were dissolved in a 75:25 water:acetonitrile mixture with 0.1% v/v trifluoroacetic acid (TFA). Particles were sonicated and vortexed vigorously. cGAMP was detected at a UV-absorbance wavelength of 256 nm. A standard curve using soluble cGAMP was also analyzed by HPLC. Blank MPs were dissolved and analyzed by HPLC to determine background levels and subtracted from cGAMP MP samples.

Time (minutes)	H_2O, 0.1% TFA (%)	Acetonitrile, 0.1% TFA (%)	Flow (mL/min)
0.0	75	25	1
5.0	45	55	1

5.1	20	80	1
7.0	20	80	1
7.10	75	25	1
13.0	75	25	1

Imiquimod was quantified by dissolving MPs in DMSO. A standard curve of Imiquimod was generated in DMSO. Blank MPs were dissolved in DMSO to determine background levels and subtracted from Imiquimod MP samples. Imiquimod was analyzed by using a fluorescent plate reader (excitation: 325 nm, emission: 365 nm, SpectraMax M2, Molecular Devices, San Jose, CA). Murabutide was quantified by dissolving MPs in DMSO. A standard curve of Murabutide was generated in DMSO. Blank MPs were dissolved in DMSO to determine background levels and subtracted from Murabutide MP samples. Fluorescamine (50μL, 3 mg/mL acetone) was added to Murabutide MPs, Blank MPs, and the standard curve and incubated briefly. These solutions were analyzed by using a fluorescent plate reader (excitation: 390 nm, emission: 460 nm, SpectraMax M2, Molecular Devices, San Jose, CA).

Poly(I:C) was quantified by dissolving MPs in DMSO. A standard curve of Poly (I:C) was generated in DMSO. Blank MPs were dissolved in DMSO to determine background levels and subtracted from Poly(I:C) samples. Quant-iT OliGreen kit (Thermo Fisher Scientific, Waltham, MA) was used according to manufacturer's specifications to quantify Poly(I:C). OliGreen reagent (1 mL) was added to Poly(I:C) MPs, Blank MPs, and the standard curve and incubated for 5 minutes in the dark. These solutions were analyzed by using a fluorescent plate reader (excitation: 480 nm, emission: 520 nm, SpectraMax M2, Molecular Devices, San Jose, CA).

Loading capacity and encapsulation effiency of PAMPs in MPs were calculated as shown:

$$Loading\ capacity\ (\%) = \frac{Mass\ PAMP}{Mass\ MPs} \times 100$$

$$Encapsulation\ Efficiency\ (\%) = \frac{Experimental\ PAMP\ loading}{Theoretical\ PAMP\ loading} \times 100$$

Physical Characterization of PAMP Electrosprayed Micropartcles

The hydrodynamic diameter, PDI, and zeta potential of all MPs (n=3) were measured using a Brookhaven NanoBrook 90Plus Zeta Particle Size Analyzer (Holtsville, NY). Each MP formulation was suspended into deionized water (1 mL) and sonicated/vortexed before measurement.

Scanning electron microscopy (SEM) images were taken of all particles to determine morphology using an S-4700 scanning electron microscope (Hitachi High Technologies America, Schaumburg, IL). MPs were resuspended in 100 µL water and 30 µL was pipetted onto the surface of an aluminum pin stub (Ted Pella, Inc., Redding, CA). This was incubated at 100°C for 20 minutes before sputter-coating with 6 nm of AuPd (Sputter Coater 108 Auto/Thickness Monitor MTM-10, Cressington Scientific Instruments, Hertfordshire, United Kingdom).

In vitro BMDC analysis

Murine bone marrow derived dendritic cells (BMDCs) were cultured from C57BL/6 mice as described previously[34]. BMDCs were treated with soluble compounds and encapsulated in MPs described above. The treatments were suspended in supplemented RPMI media (10% inactivated fetal bovine serum [FBS], 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-

mercaptoethanol, 1× non-essential amino acids, and 2 mM L-glutamine). After 24 hours, the supernatant was removed and analyzed on the following ELISAs from BD Biosciences (San Jose, CA): TNF and IL-6. ELISAs were run according to manufacturer's instructions. Viability was measured using an MTT assay described previously[35]. IFN-β was detected by ELISA using murine specific IFN-β antibodies from Santa-Cruz Biotechnology (Dallas, TX) and R&D Systems (Minneapolis, MN), anti-Rabbit IgG HRPO from Cell Signaling Technology (Danvers, MA), and recombinant IFN-β standard from R&D Systems.

Murine tumor experiments

All studies were conducted in accordance with National Institutes of Health's guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) at UNC. Mice were inoculated with tumors between the age of 8-12 weeks. The B16F10 cell line was obtained from ATCC (Manassas, VA cat. CRL-6475) and cultured in DMEM medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Mice were inoculated with 200,000 B16F10 cells in 50% matrigel (Corning, Corning, NY cat. 354234) on the flank. The E0771 cell line was obtained from CH3 biosystems (Buffalo, NY cat. 940001) and cultured in RPMI 1640 medium with 10% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. Mice were inoculated with 750,000 E0771 cells in 50% matrigel into the mammary fat pad. Tumors were allowed to grow for six days before the first treatment. MPs were injected in 1 mg/mL Egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, A) in phosphate-buffered saline (PBS) to help with suspension.

Mice were injected every three days, for a total of three injections (B16F10) or seven injections (E0771). Tumor volume was measured every three days and was calculated using the formula $V=½ L*W^{2.}$ Unless otherwise specified, mice were sacrificed when tumors reached 20 mm in diameter, in accordance with IACUC protocol.

Flow Cytometry

Tumors were harvested from treated mice. These organs were then passed through a 40 μM cell strainer to create a single cell suspension, lysed with ammonium-chloridepotassium (ACK) solution, and washed in Hank's balanced salt solution (HBSS) containing 3% FBS before staining and fixation in 1% paraformaldehyde. Intracellular cytokines were stained using the intracellular staining kit (Biolegend cat. 420201) and restimulated using PMA/ionomycin with brefeldin A (Biolegend, San Diego, California cat. 423303). Immune cell populations were identified by flow cytometry in either LSRII or the LSRFortessa (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (Tree Star, Ashland, OR). Tumors or splenocytes were stained with the following antibodies from Biolegened. anti-mouse CD3-PE-cy7, CD4- PercP cy 5.5, CD8-AF700, CD69-APC-cy7, NK1.1- PE-Dazzle594, CD27- FITC, CD11b-BV510. Granzyme B- AF647, and IFN-γ-BV605

Depletion

Cellular subsets were depleted as described previously[36]. Briefly, mice were injected with 400 μg of antibody twice weekly by intraperitoneal injection (i.p.). The first injected was done one day

before initial tumor inoculation. The following cell types were depleted: NK cells with anti-NK1.1 antibody (BioXcell, West Lebanon, NH, clone PK136, cat. BE0036), CD8 T cells with anti-CD8alpha antibody (BioXcell, clone 2.43 cat. BE0061), and CD4 T cells by anti-CD4 antibody (BioXcell, clone GK1.5 cat. BE0003). Mice were inoculated with 200,000 B16F10 tumor cells on day 0. Six days after tumor inoculation, mice were treated with either PBS or cGAMP MPs. Mice were treated three times, on day 6, 9, and 12. On day 15, the NK cell, CD4 T cell, and CD8 T cell depletion was confirmed by taking 100-200 uL of blood by submandibular blood collection. To confirm depletion, blood was prepared for flow cytometry using a red blood cell lysis/fixation buffer kit (Biolegend, San Diego, California cat. 422401) and stained with the following antibodies from biolegend NK1.1- PE/Dazzle™ 594, CD3-PE-cy7, CD4- APC/Cy-7 (clone RM4-5), CD8-AF700, and CD45- BV421. Tumor volume was monitored every three days and mice were sacrificed when tumors reached 20 mm in a single direction.

Figures

A

Particle Batch	Storage Time (Months)	Initial Diameter (nm)	Initial PDI	Final Diameter (nm)	Final PDI
Batch 1	1	626 ±154	0.061	647 ± 111	0.029
Batch 2	3	607 ± 170	0.078	648 ± 184	0.081
Batch 3	8	671 ± 144	0.046	705 ± 143	0.041
Batch 4	10	726 ± 146	0.040	655 ± 167	0.065
Batch 5	18	668 ± 188	0.079	619 ± 193	0.097

B

Time After Suspension (min)	Effective Diameter (nm)	PDI
0	1079	0.231
5	1147	0.199
15	1208	0.547
30	940	0.450
60	955	0.456

Fig. S1. **Ace-DEX MPs storage and stability.** (A) Diameter and dispersity for cGAMP loaded MPs after various storage times at -20 °C. Diameter is reported as the average and standard deviation of *n*=50 particle measurements taken from SEM micrographs. Dispersity is reported as the square of the ratio of the standard deviation of particle diameter to the mean. (B) Stability of cGAMP-loaded MPs after suspension. MPs were suspended in a 1 mg/mL solution of egg phosphatidylcholine in PBS using the same procedure and concentration (200 μg cGAMP/mL) as used for *in vivo* administration. At each specified time point, 5 μL of the resulting suspension was transferred to a cuvette and diluted with 1 mg/mL egg phosphatidylcholine to a concentration of 0.1 mg MPs/mL, and particle size was measured using dynamic light scattering (DLS) over the span of 90 seconds.

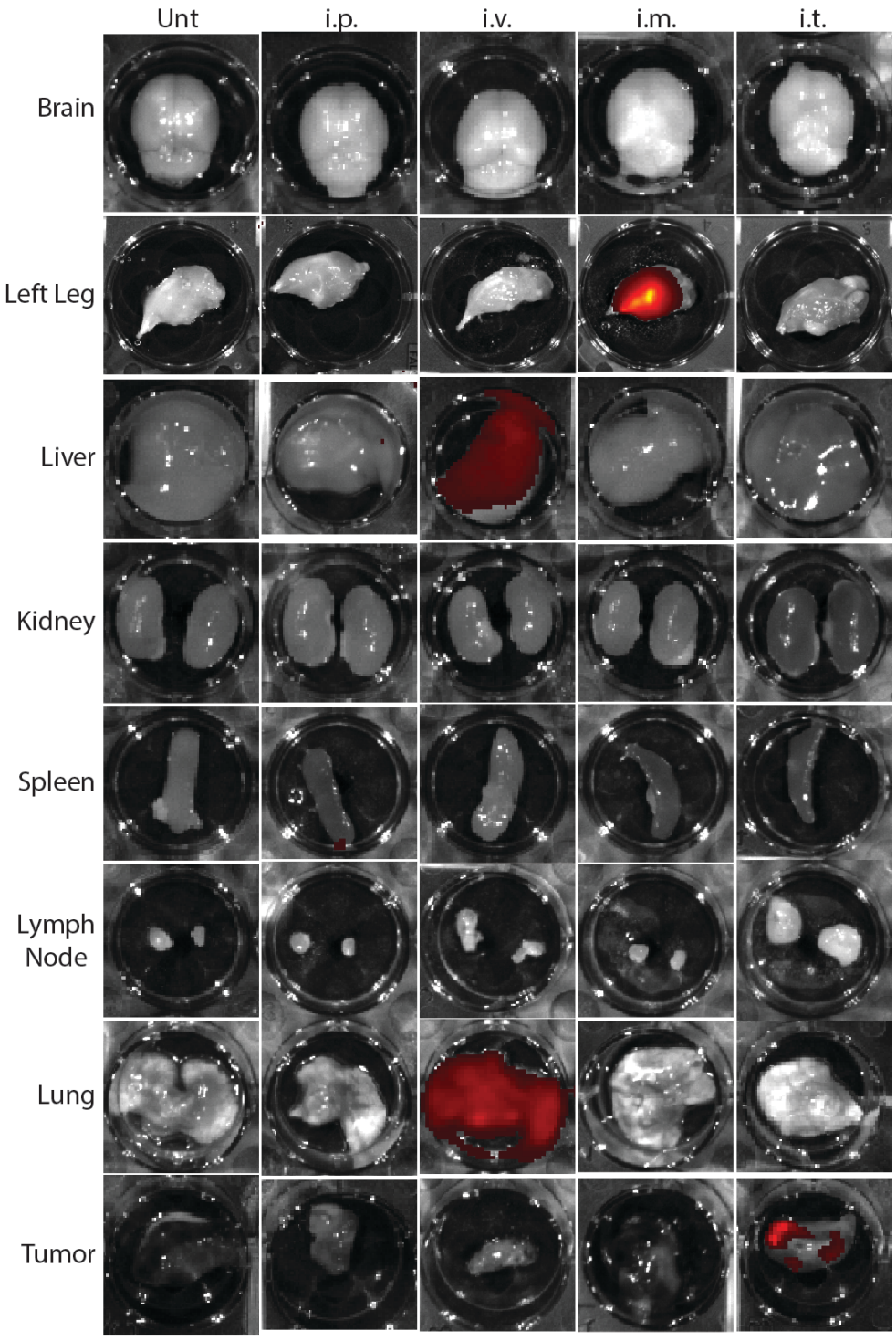

Figure S2. **Biodistribution of cGAMP MPs.** Mice were inoculated with B16F10 cells on day 0. On day 10, mice were injected by four routes of administration with 1 mg of Texas-red labeled cGAMP Ace-DEX MPs. Twenty-four hours later, mice were sacrificed and brain, liver, kidneys, spleen, inguinal lymph nodes, lung, and tumors were removed. Fluorescence was detected by the IVIS Kinetic at an excitation wavelength of 570nm.

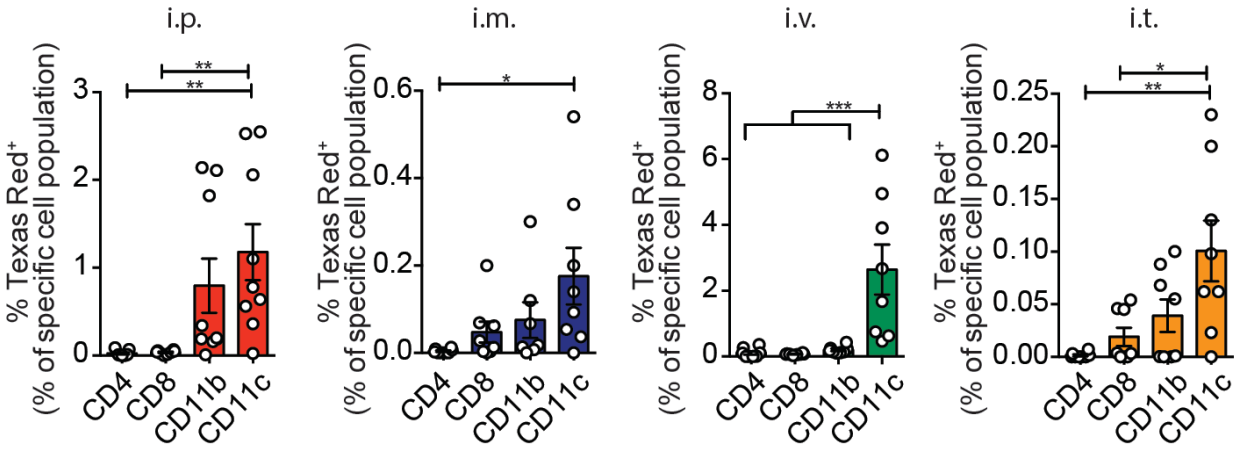

Figure S3. **Cellular uptake of Texas-red labeled cGAMP MPs in the spleen.** Mice were inoculated with B16F10 cells on day 0. (A) On day 10, mice were injected by four routes of administration with 1 mg of Texas-red labeled cGAMP Ace-DEX MPs. Twenty-four hours later, mice were sacrificed and spleen was processed and stained. Cells were analyzed using flow cytometry (n=8 mice ± SEM over 2 experiments, One-way ANOVA *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

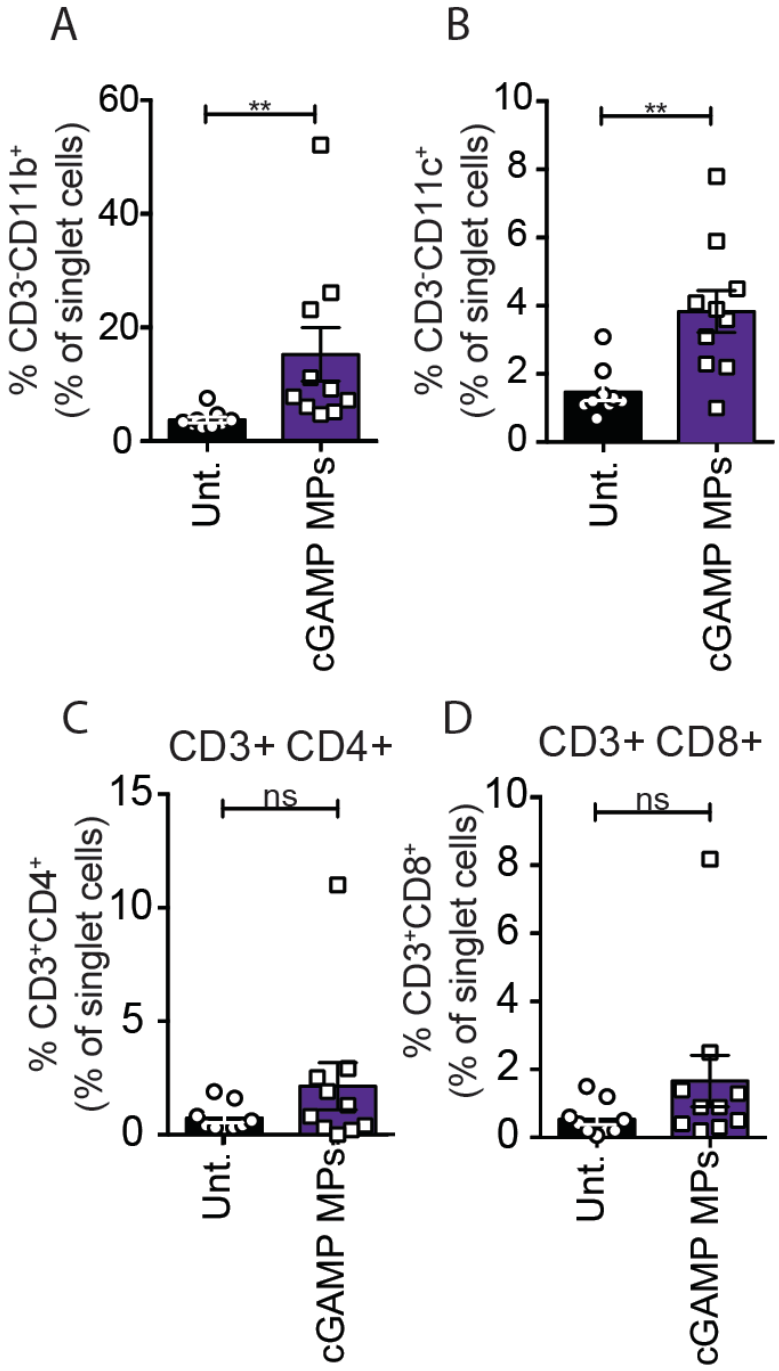

Figure S4. **APCs, but not T cells, increase in the tumor after cGAMP MPs treatment.** (A-B) C57BL/6J mice were inoculated with B16F10 cells on day 0. (A-D) On day 6, 9, and 12 mice were treated with either PBS or 10 μg cGAMP encapsulated in Ace-DEX MPs by i.t. administration. On day 15, mice were sacrificed and tumor were processed and stained for the presence of tumor infiltrating leukocytes by flow cytometry (n=9-10 mice ± SEM over 2 experiments). One-way ANOVA **$p < 0.01$

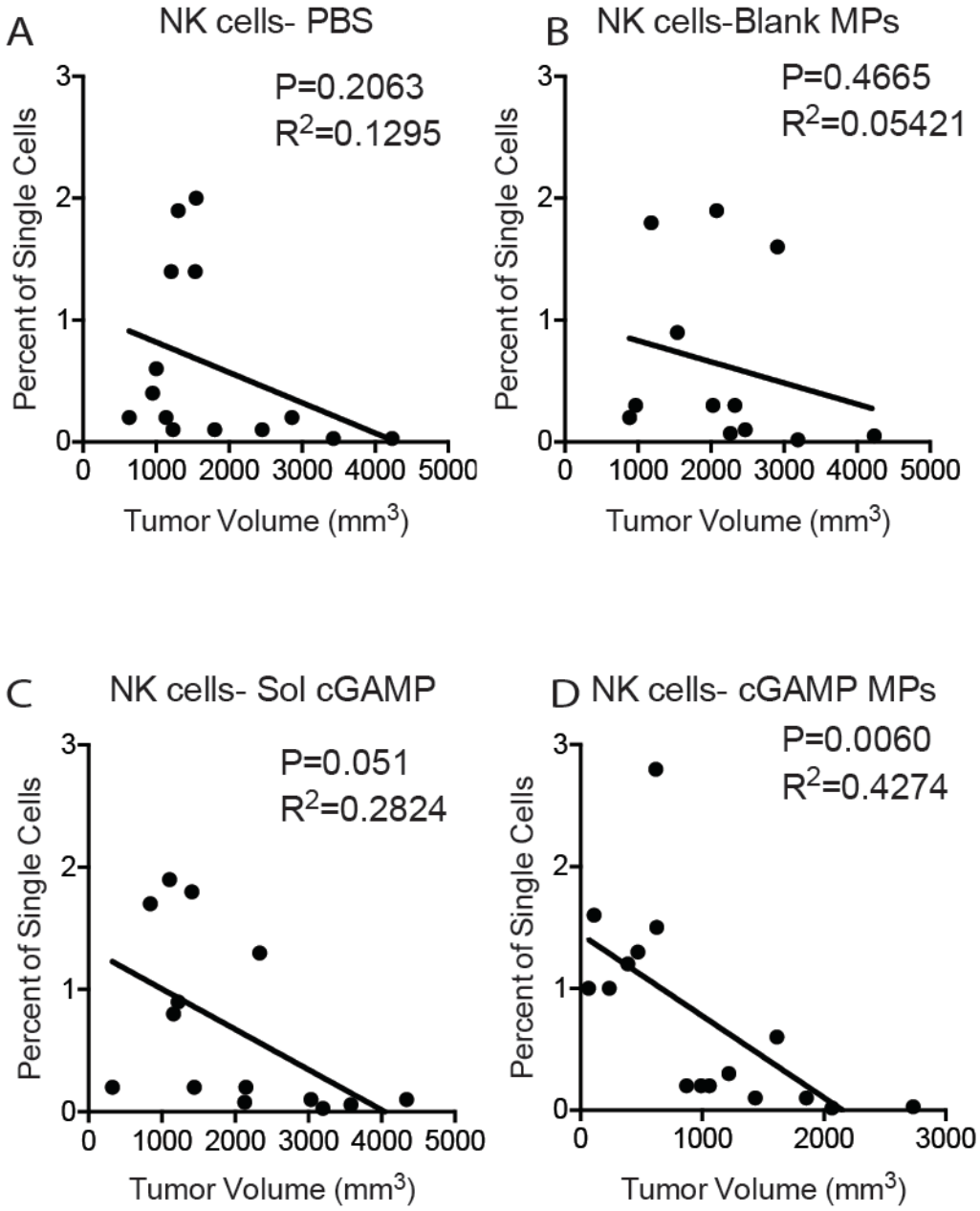

Figure S5. **NK cells number correlates with tumor volume only after cGAMP MP treatment.** Mice were inoculated with B16F10 cells on day 0. On day 6, 9, and 12 mice were treated with 0.1 μg of encapsulated cGAMP. Tumor volume was measured every 3 days. On day 15, mice were sacrificed and tumor infiltrating leukocytes were assessed by flow cytometry. Correlations between day 15 tumor volume and NK cell numbers were assessed by treatment type. (n=14-16 mice ± SEM, Linear regression analysis)

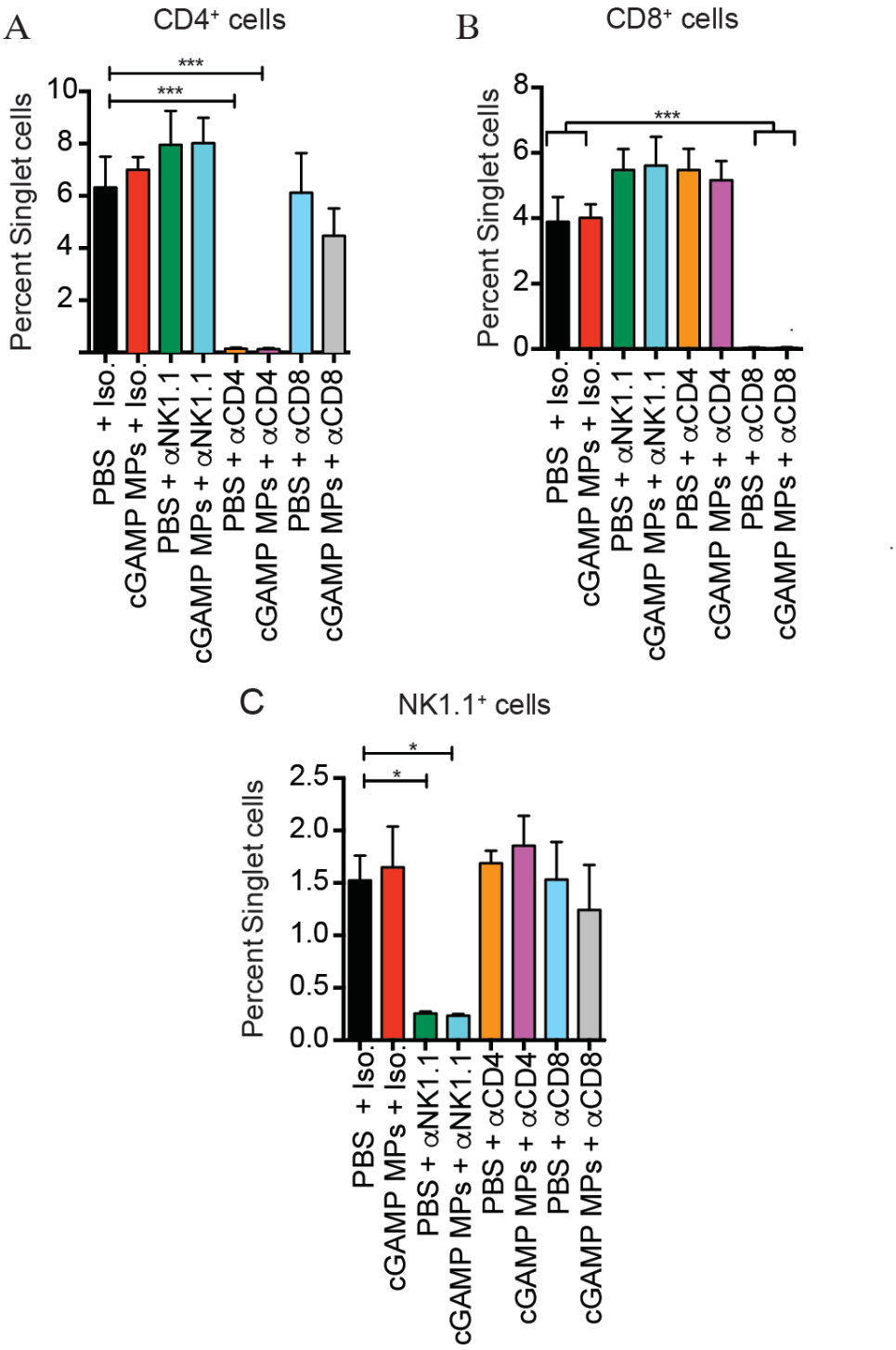

Figure S6. **Cell specific depletions verified in blood.** Mice were inoculated with B16F10 cells on day 0. On day 5, mice received their first i.p. injection of 400 μg of either an isotype control, αCD4, αCD8, αNK1.1 depletion antibody. Mice continued to receive this dose twice a week for the reminder of the study. On day 6, 9, and 12 mice were treated with either PBS or 10 μg cGAMP encapsulated in Ace-DEX MPs. On day 15 of the depletion experiment, < 200 μL of blood was collected from each mouse. Blood was prepared using red blood cell staining kit from Biolegend. Flow cytometry was performed (n=8-10 mice ± SEM One-way ANOVA *$p < 0.05$, ***$p < 0.001$).

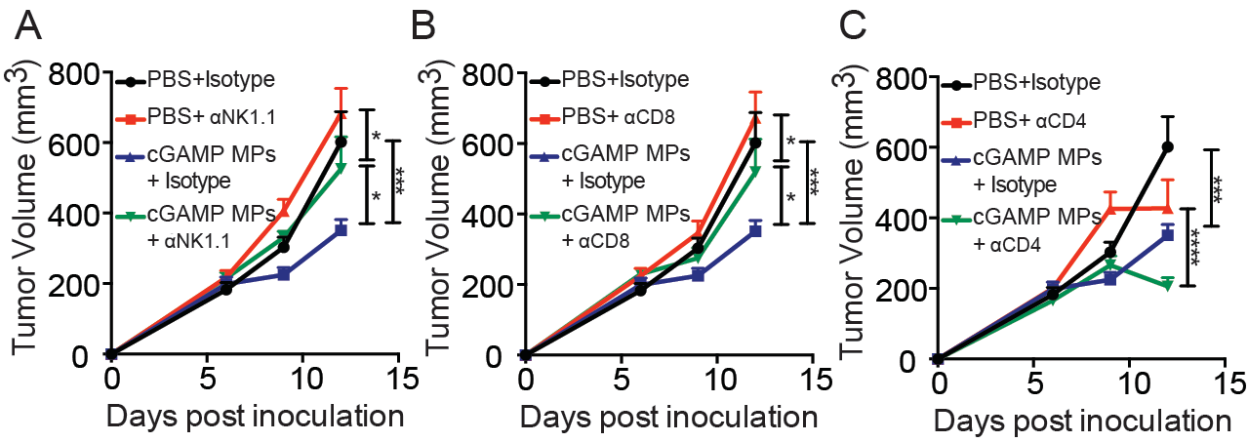

Figure S7. **Tumor volume after cell specific depletions (also seen in Figure 7) graphed over the first 12 days.** Mice were inoculated with E0771 cells on day 0. On day 5 mice received their first i.p. injection of 400 μg of either an isotype control, αCD4, αCD8, αNK1.1 depletion antibody. Mice continued to receive this dose twice a week for the reminder of the study. On day 6, 9, 12, 15, and 18 mice were treated with either PBS or 0.1 μg cGAMP encapsulated in Ace-DEX MPs. Tumor volume was monitored every 3 days for 21 days. To assess early differences, the tumor volumes from the first 12 days were graphed and analyzed. The isotype control groups are repeated in panels C-E. (n=8-10 mice ± SEM over 2 experiments, Two-way ANOVA * $p < 0.05$, ***$p<0.001$, ****$p < 0.0001$).