Supplemental Material

Nanoparticles targeting an immune checkpoint protein VISTA induces potent antitumor immunity

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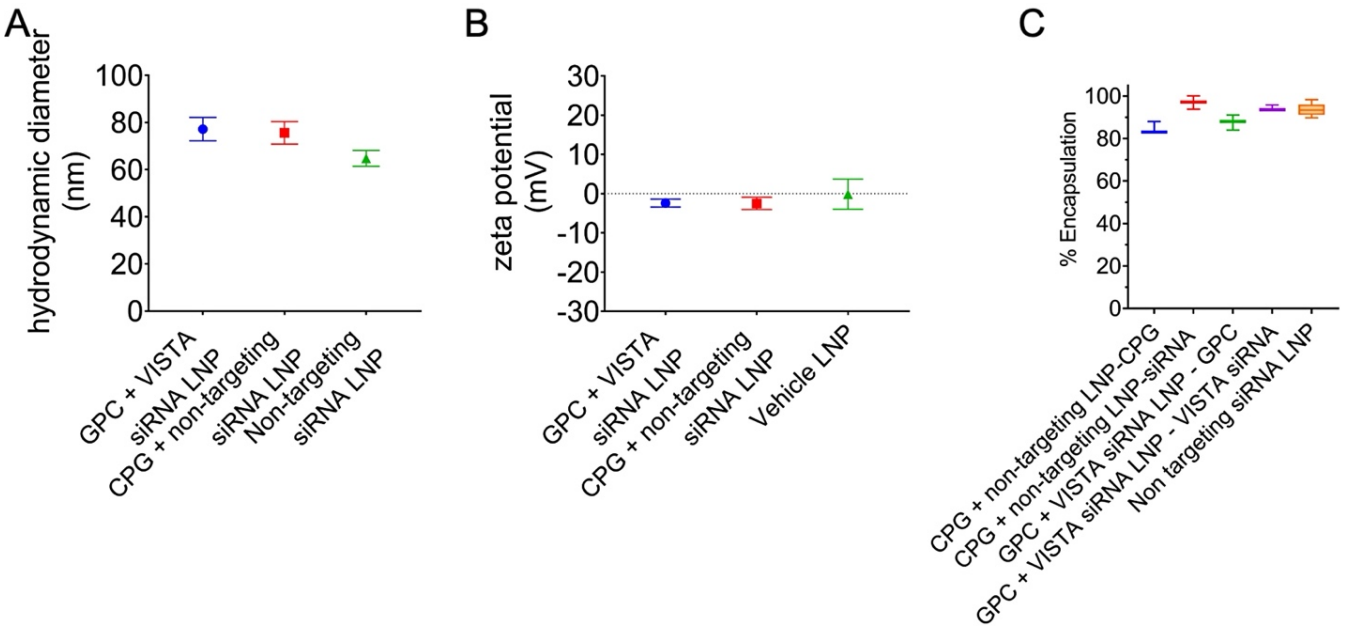
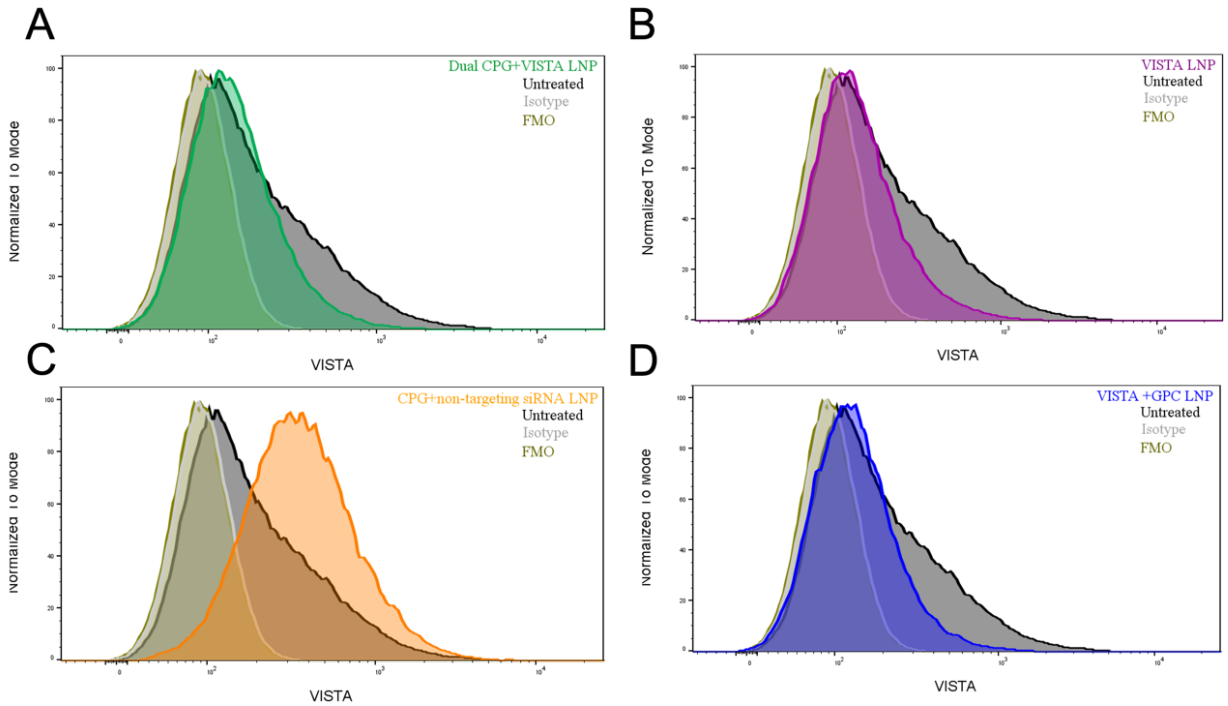
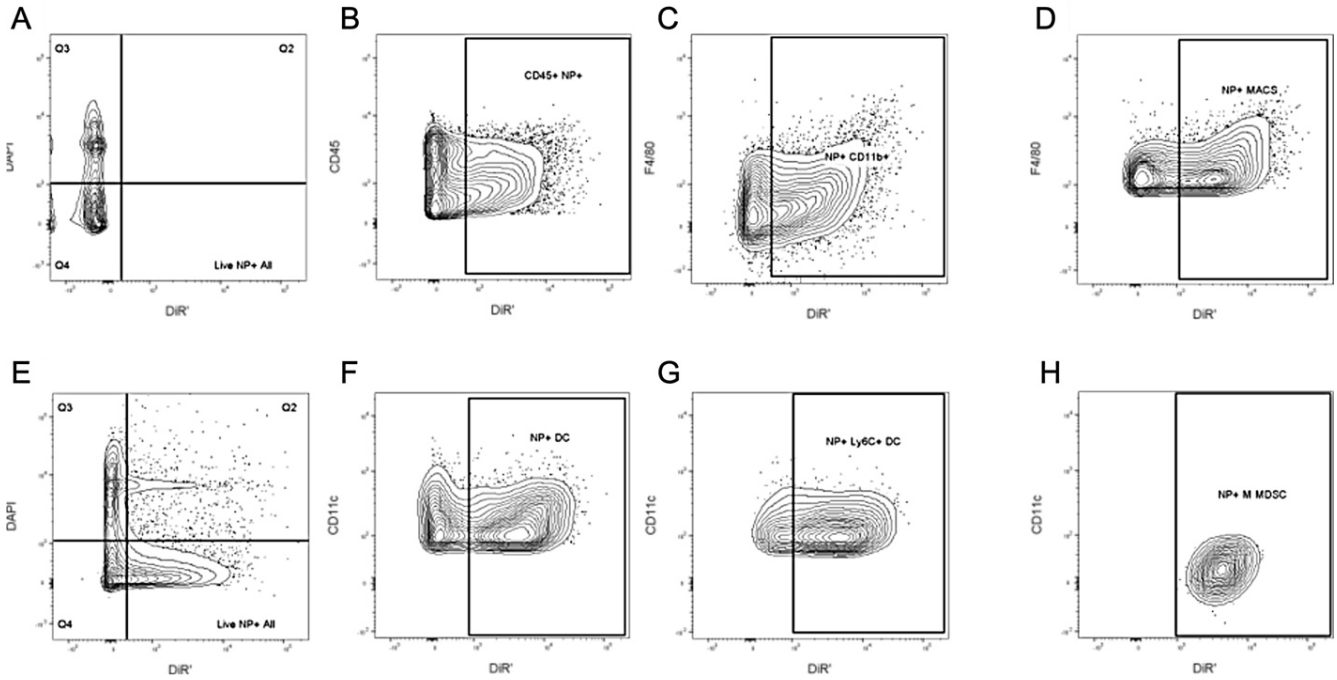


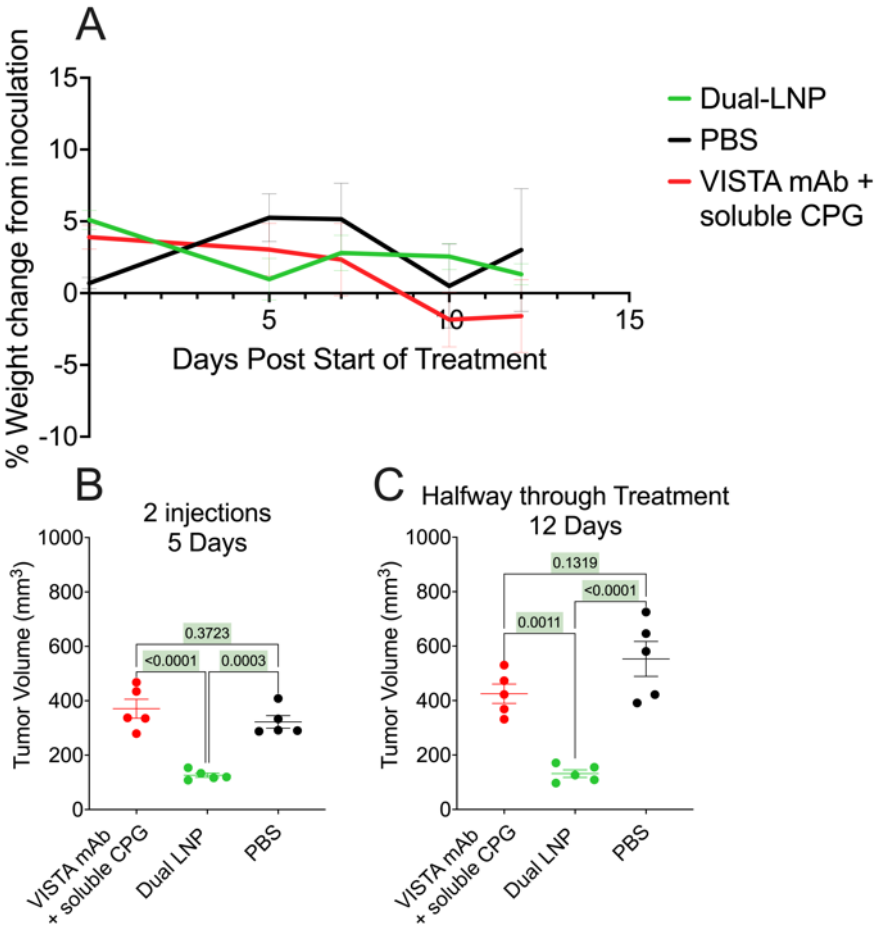
Supplemental figure S1. Characterization of control LNP formulations. (A) Hydrodynamic diameter of control LNPs (GPC + VISTA siRNA, CPG + non-targeting siRNA, and nontargeting LNP only). (B) Zeta potential of control nanoparticles. (C) Percent encapsulation of CPG, non-targeting siRNA, GPC, VISTA siRNA in control nanoparticles.



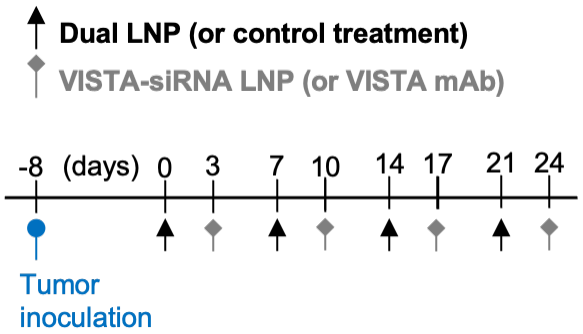
Supplemental figure S2. VISTA expression following in vitro treatment VISTA BV421 staining in VISTA fluorescent minus one control, BV421 Isotype, and untreated RAW 264.7 compared to 24 hours of (A) Dual CPG+VISTA LNP treatment, (B) VISTA LNP treatment, (C) CPG+non-targeting siRNA LNP, and (D) VISTA+GPC non-stimulatory control LNP. All experiments were performed with at least n=3 wells.



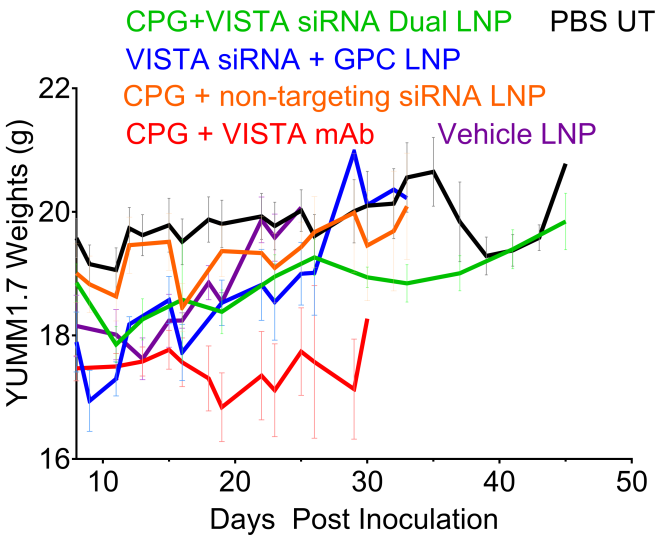
Supplemental figure S3. Representative flow gating strategy for nanoparticle biodistribution. (A) Live stain (DAPI counterstain) in NP absent samples. (B) Live stain (DAPI counterstain) in NP treated samples. (C) CD45+ gate on all LNP+ Cells, (D) CD11b+ gate on all LNP+ Cells, Nanoparticle positive gate in (E) macrophages (F) dendritic cells, (G) Ly6C+ dendritic cells, and (H) M MDSCs. All experiments were performed with at least 5 mice per group.



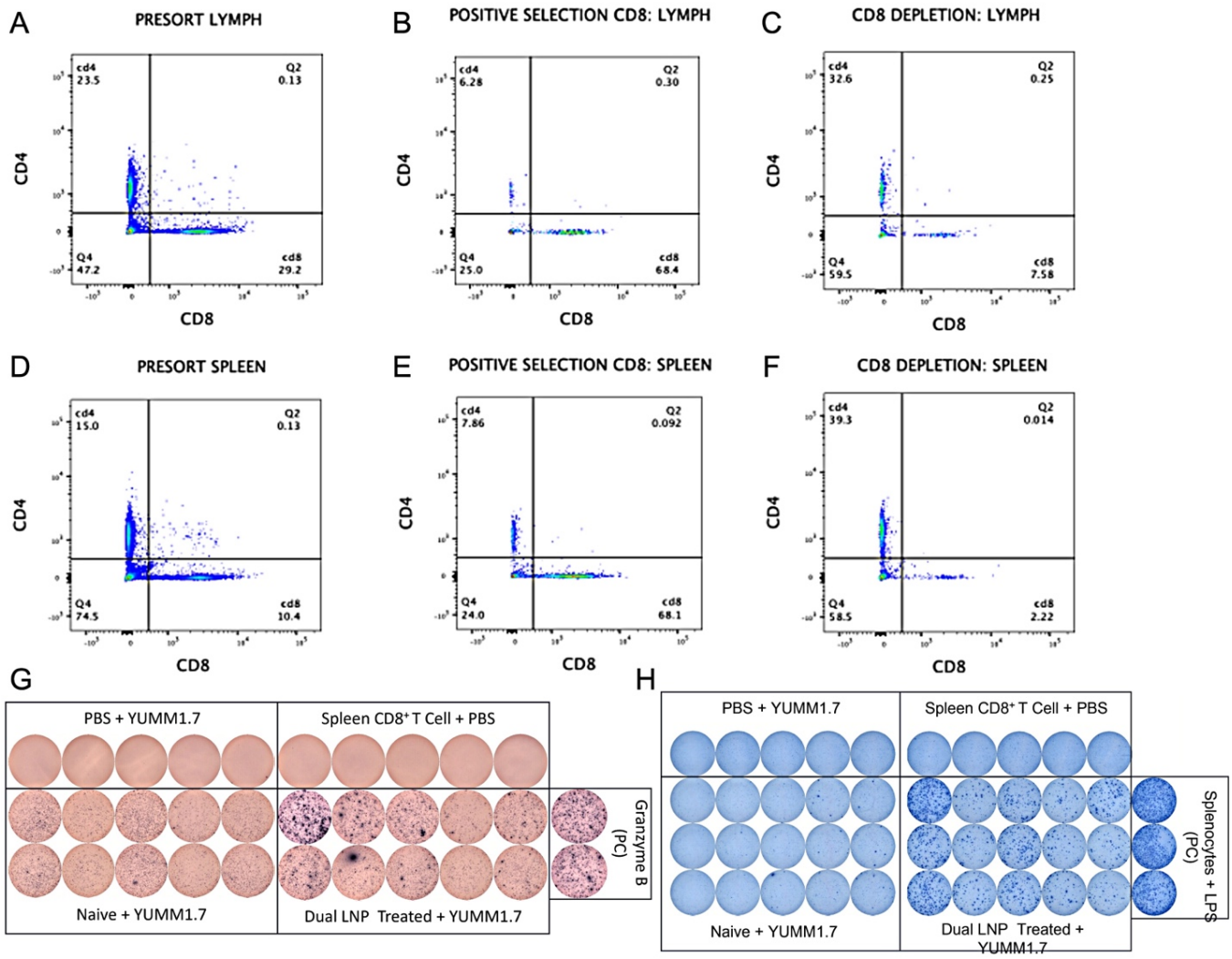
Supplemental figure S4. Safety analysis weights and tumor volume of YUMM1.7 tumor bearing mice. (A) Percent change in weight of mice compared to their starting weights during safety analysis experiment. Tumor volumes following (B) 2 injections (5 days after the start of treatment) of therapy and (C) 4 injections (12 days after the start of treatment).



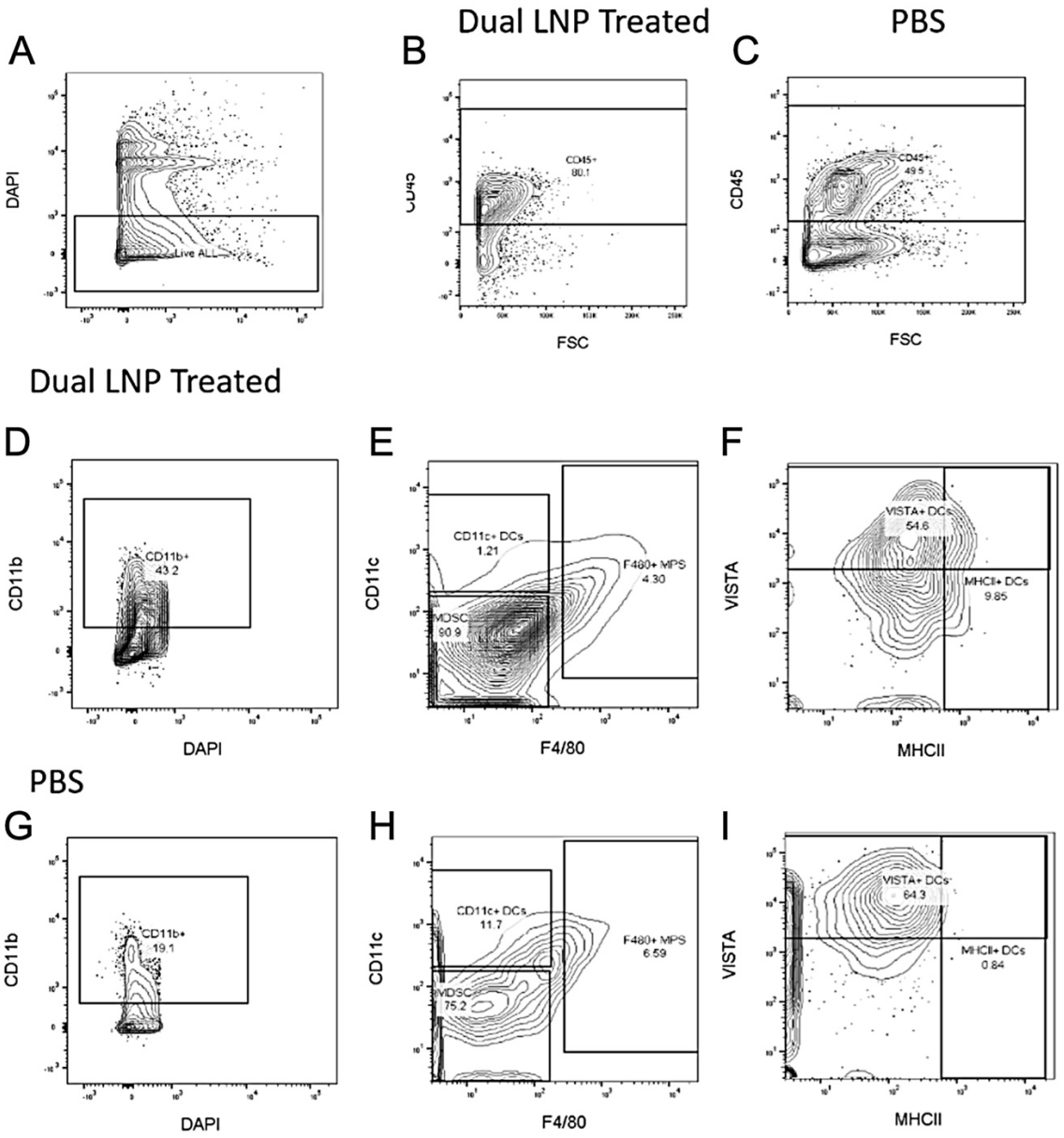
Supplemental figure S5. Treatment schedule in the YUMM1.7 model. Treatment began 8 days after inoculation, when the tumors reached a size of ~60 mm^3. The Dual LNPs were intratumorally injected weekly for a total of 4 weeks at a dose containing 4 µg of CPG and 10 µg of VISTA siRNA. Mice were also injected with LNPs containing only VISTA-siRNA but devoid of CpG 3 days after every Dual LNP treatment. Control treatments followed the same schedule.



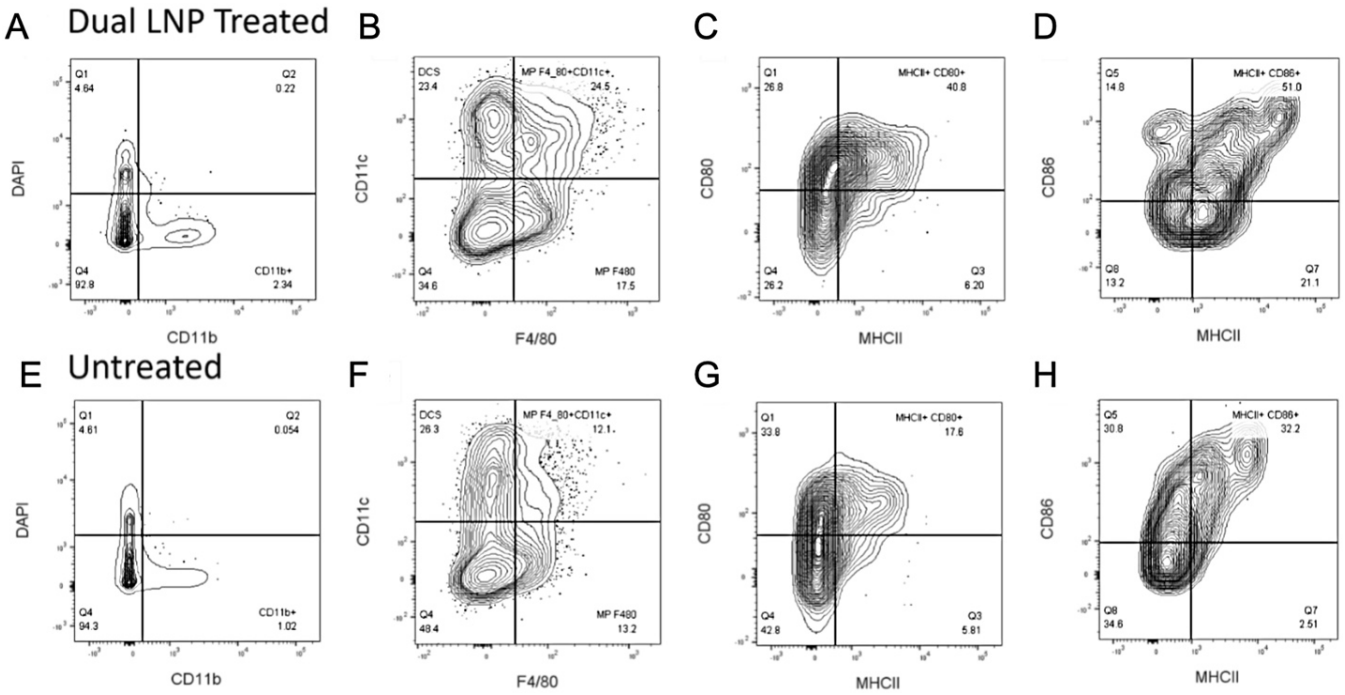
Supplemental figure S6. YUMM1.7 melanoma survival weights. Mice bearing a YUMM1.7 tumor were treated starting 8 days after inoculation, when the tumors were ~60 mm3. The Dual LNPs were injected intratumorally (IT) weekly for a total of 4 weeks at a dose containing 4 µg of CPG and 10 µg of VISTA siRNA. Mice were IT injected with VISTA-siRNA-only LNPs at a dose of a 10 µg siRNA 3 days following every Dual LNP treatment. Similar schedule and dose were used for the control treatments. Treatment included Dual LNP, GPC LNP +VISTA siRNA, CPG + non-targeting siRNA LNP, vehicle LNP, CPG + VISTA mAb, and PBS treatments.



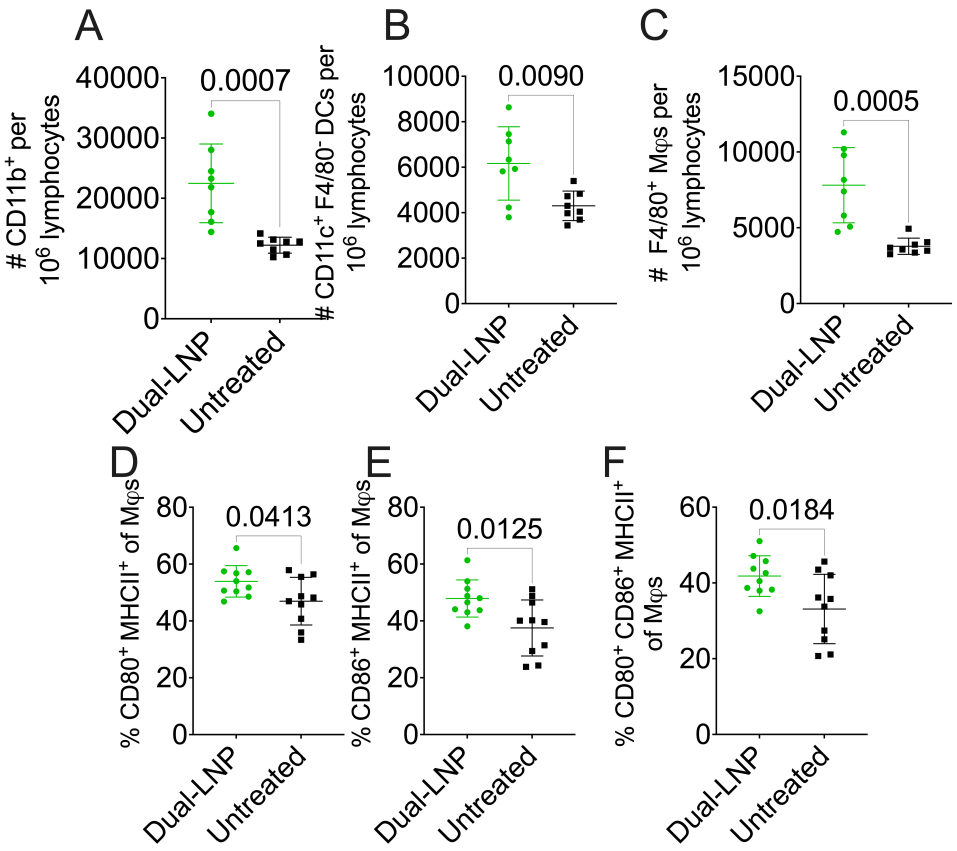
Supplemental figure S7. ELISPOT Validation. Lymph Node CD4 vs CD8 distribution in (A) presorted, (B) positively selected CD8+ samples, and (C) CD8+ depleted samples. Spleen CD4 vs CD8 distribution in (D) presorted, (E) positively selected CD8+ samples, and (F) CD8+ depleted samples. (G) Well images of Granzyme B ELISPOT. (H) Well images of IFNy ELISPOT. All experiments were performed with at least 5 mice per group.



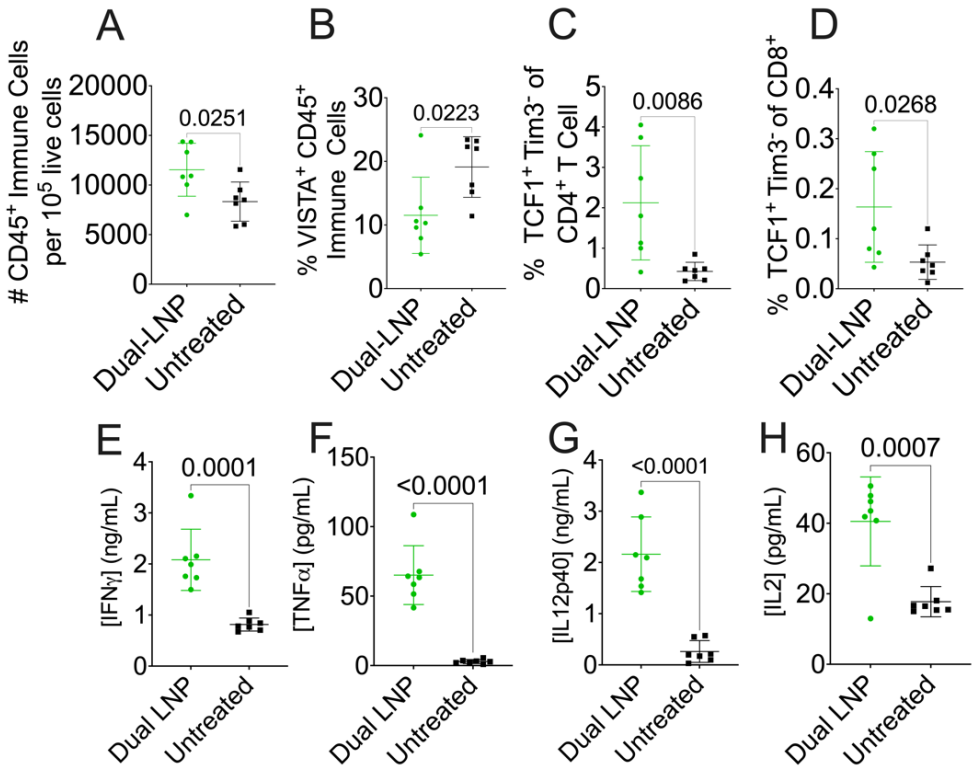
Supplemental figure S8. Representative flow plots for myeloid immune cell population identification in the tumor. (A) Live stain (DAPI counterstain). CD45+ plots for (B) Dual-LNP treated and (C) PBS treated samples. Representative flow plots of immune cells in the Dual-LNP treated mice of (D) CD11b+ cells, (E) CD11c+ vs F4/80+ cells, (F) VISTA+ vs MHCII+ in DCs. Representative flow plots of immune cells in the PBS treated mice of (G) CD11b+ cells, (H) CD11c+ vs F4/80+ cells, (I) VISTA+ vs MHCII+ in DCs. All experiments were performed with at least 5 mice per group.



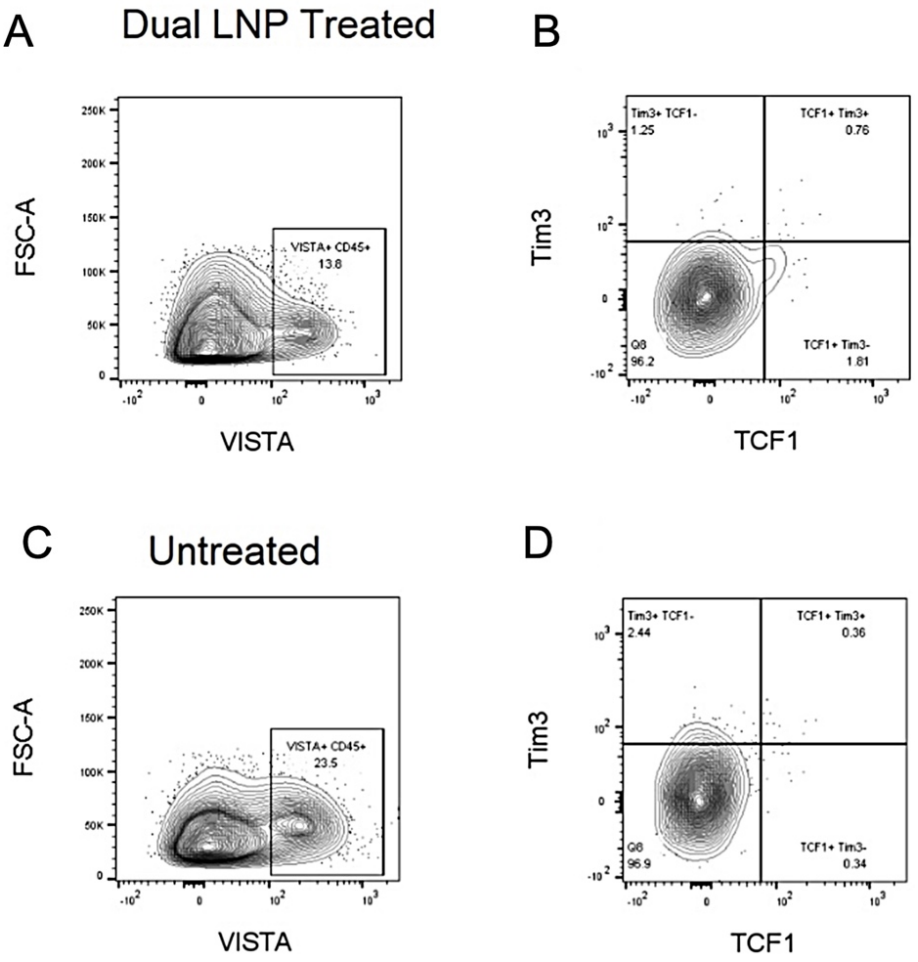
Supplemental figure S9. Representative flow plots for myeloid immune cell population identification in the tumor draining lymph node. Representative flow plots of tumor draining lymph nodes in Dual LNP treated mice (A) CD11b+ vs DAPI counterstain gating, (B) CD11c vs F4/80 gated on CD11b+ live cells, (C) CD80 vs MHCII in macrophages, and (D) CD86 vs MHCII in macrophages. Representative flow plots of tumor draining lymph nodes in untreated mice (E) CD11b+ vs DAPI counterstain gating, (F) CD11c vs F4/80 gated on CD11b+ live cells, (G) CD80 vs MHCII in macrophages, and (H) CD86 vs MHCII in macrophages. All experiments were performed with at least 5 mice per group.



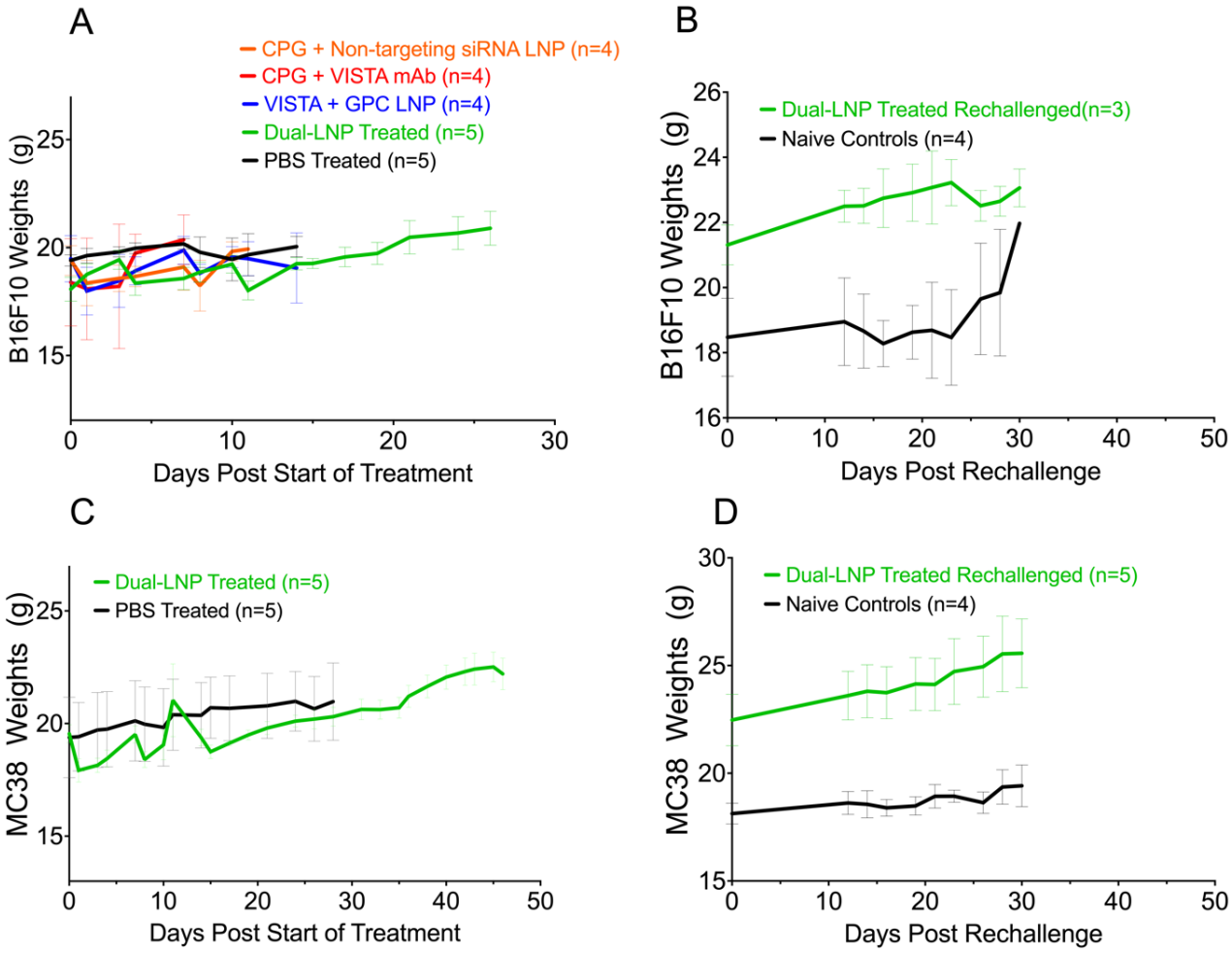
Supplemental figure S10. Myeloid immune cell profile of the tumor draining lymph node 12 days after the initiation of the dual-LNP treatment. Flow cytometry analysis 12 days after the initiation of the dual-LNP treatment. (A) The number of $CD11b^+$ myeloid immune cells per 10^6 lymphocytes. (B) The number of DCs per 10^6 lymphocytes. (C) The number of Mϕ's per 10^6 lymphocytes. (D) The percentage of Mϕ's that are $CD80^+$ and $MHCII^+$. (E) The percentage of Mϕ's that are $CD86^+$ and $MHCII^+$. (F) The percentage of Mϕ's that are $CD80^+$, $CD86^+$, and $MHCII^+$. All experiments were performed with 5 mice per group. Statistics were analyzed by Student's t test.



Supplementary figure S11. Adaptive immune response profile in the tumor microenvironment 12 days after initiation of the dual-LNP treatment. Flow cytometry analysis for (A) the number of CD45+ immune cells per 10^5 live TME cells, and (B) the percentage of $CD45^+$ immune cells that are $VISTA^+$. (C) The % of $CD4^+$ T cells that are $TCF1^+$ $Tim3^-$ progenitor exhausted. (D) The % of $CD8^+$ T cells that are $TCF1^+$ $Tim3^-$ progenitor exhausted. ELISA results for the concentration of (E) IFNγ, (F) TNFα, (G) IL12p40, and (H) IL2 in homogenized tumor. All experiments were performed with 5 mice per group. Statistics were analyzed by Student's t test.



Supplemental figure S12. Representative flow plots VISTA expression and T cell phenotyping 12 days following the start of treatment. Representative flow plots of Dual LNP treated tumors. (A) VISTA+ gating on CD45+ immune cells and (B) TCF1 vs Tim3 expression (same gate for CD4 or CD8 T cells). Representative flow plots of untreated tumors (C) VISTA+ gating on CD45+ immune cells and (D) TCF1 vs Tim3 expression (same gate for CD4 or CD8 T cells). All experiments were performed with at least 5 mice per group.



Supplemental figure S13. B16F10 and MC38 survival weights. Mice bearing a B16F10 or MC38 tumors were treated starting 15 days or 8 days, respectively after inoculation, when the tumors were ~60 mm3. The Dual LNPs were injected intratumorally (IT) weekly for a total of 4 weeks at a dose containing 4 µg of CPG and 10 µg of VISTA siRNA. Mice were IT injected with VISTA-siRNA-only LNPs at a dose of a 10 µg siRNA 3 days following every Dual LNP treatment. Similar schedule and dose were used for the saline treated controls. (A) Weights during primary treatment of Dual LNP and PBS treatments in B16F10 tumor bearing mice. (B) Weights during rechallenge of Dual-LNP treated and naïve B16F10 tumor controls. All experiments were performed with at least 5 mice per group. (C) Weights during primary treatment of Dual LNP and PBS treatments in B16F10 tumor bearing mice. (D) Weights during rechallenge of Dual-LNP treated and naïve B16F10 tumor controls. All experiments were performed with at least 5 mice per group.

Methods

Animal Studies

B16F10 cells were purchased from ATCC and cultured in DMEM media (Gibco). Primary tumor models were established in 7-week-old female C57BL/6J mice with intradermal injections of 50,000 cells in 20uL. Dual-LNP treatment was administered on days 15, 22, 29, and 36 post primary inoculation. Dual-LNP mice also received VISTA siRNA-LNP on days 18, 25, 32, and 39 post primary inoculation. PBS treated mice received equivalent volumes of PBS on all treatment days. Monitoring was performed as described previously. MC38 cells were purchase from ATCC and cultured in DMEM media (Gibco). Primary tumor models were established in 7-week-old female C57BL/6J mice with intradermal injections of 100,000 cells in 30 µL. Dual-LNP treatment was administered on days 8, 15, 22, and 29 post primary inoculation. Dual-LNP treated mice also received VISTA siRNA-LNP days 11, 18, 25, and 32 post primary inoculation. PBS treated mice received equivalent volumes of PBS on all treatment days. Monitoring was performed as described previously. Local rechallenge studies were conducted by injecting 50,000 B16F10 or MC38 cells in 30 µL incomplete DMEM media intradermally to the right flank in mice that fully cleared their B16F10 or MC38 original primary tumors, respectively. Naïve C57BL/6J mice served as the control for tumor growth. Mice were monitored as previously described.

Flow Cytometry Processing

Liver, spleen, and tumor draining lymph nodes were processed through 70 µm nylon mesh cell strainers (Fisherbrand) via agitation with the flat end of 3mL or 5mL syringes (BD Syringe). Liver, spleen, and tumor draining lymph node cell pellets were resuspended in DMEM media +1% FBS. Red blood cells in spleen and blood cell pellets via rounds of ACK lysing buffer (Gibco), and then resuspended in DMEM media. Tumor were first cut up and then digested with liberase (0.4 mg/mL, Roche) and DNAse (0.2 mg/mL, Roche) for 20 minutes at 37°C. They were then passed through 70 µm nylon mesh cell strainers and resuspended in DMEM media. If the tumors were used for stimulation studies, they were resuspended in sterile, complete RPMI (Gibco) supplemented with 10% FBS, 1% PS, and 5mM 2-β-mercaptoethanol (Gibco). Single cell suspensions were plated in 96 U bottom well plates (Grenier Bio-one). Cells were washed with buffer (PBS + 0.5% FBS + EDTA). Cells were blocked with CD16/CD32 Fc Block (Biolegend or BD Bioscience) and then stained with specific fluorescent antibodies. Cells undergoing intracellular staining or nuclear staining were fixed in fixation buffer from intracellular staining or FOXP3 staining kits from Thermofisher. They were permeabilized and blocked with 2% normal rat serum (Biolegend). Cells were then stained with antibodies specific for cytokines or transcription factors of interest. Following staining, all cells were resuspended in buffer. All cells were read with the BDFortessa. All samples were analyzed with FlowJo software.