# Supporting Information for

## *In situ* genetic engineering of tumors for long-lasting and systemic immunotherapy

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**Classification**

BIOLOGICAL SCIENCES: Applied Biological Sciences

www.pnas.org/cgi/doi/10.1073/pnas.1916039117

## Supporting Materials and Methods

### Detailed poly(beta-amino ester) (PBAE) synthesis

Backbone B4 (1,4-butanediol diacrylate), sidechains S3 (3-amino-1-propanol) and S5 (5-amino-1-pentanol), and end-cap E7 [1-(3-aminopropyl-4-methylpiperazine)] were purchased from Alfa Aesar (Tewksbury, MA). B5 (1,5-pentanediol diacrylate) was purchased from Monomer-Polymer and Dajac Labs (Ambler, PA) and S4 (4-amino-1-butanol) from Fisher Scientific (Hampton, NH). E6 [2-(3-aminopropylamino)ethanol], E27 (4,7,10-trioxa-1,13-tridecanediamine), and E49 (N,N-dimethyldipropylenetriamine) were purchased from Sigma Aldrich (St. Louis, MO), and E60 (pentaethylenehexamine) was purchase from Santa Cruz Biotechnology (Dallas, TX). All other chemicals used were anhydrous and reagent-grade.

Briefly, one backbone (B) monomer was polymerized with one sidechain (S) monomer at a 1.1:1 molar ratio of acrylates to primary amines in a neat solution at 90°C for 24 hr. The resulting diacrylate-terminated base polymer was then reacted with an excess of an end-cap (E) monomer in anhydrous tetrahydrofuran (THF) at room temperature for 1 hr. The end-capped polymer was isolated by precipitation into anhydrous diethyl ether and collected by centrifugation at 3200 rcf for 5 min at 4°C. The supernatant was decanted and the polymer washed twice with ether, using centrifugation after each wash to pellet the polymer. The resulting product was dried under vacuum for 48 hr at room temperature, then dissolved in anhydrous dimethyl sulfoxide (DMSO) and stored as a 100 mg/mL solution at -20°C with desiccant until use.

### *In vitro* transfection of B16-F10 and MC38 cells: screening with reporter gene

B16-F10 or MC38 cells were cultured in complete growth medium consisting of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were maintained at <80% confluency. The day before the transfection, cells were seeded in flat-bottom 96-well plates at $5\times10^4$ cells/well in 100 μL complete growth medium. On the day of transfection, nanoparticles were formed by diluting green fluorescent protein (GFP) plasmid DNA (pEGFP-N1,

purchased from Clontech and amplified by Elim Biopharmaceuticals, Hayward, CA) and an array of PBAE polymers in 25 mM sodium acetate buffer, pH 5 (NaAc) and then mixing the diluted DNA and PBAEs to allow self-assembly. After 10 min, nanoparticles were added to the cells in complete growth medium at a final DNA concentration of 5 μg/mL and final PBAE concentrations ranging from 150-450 μg/mL. The cells were incubated with nanoparticles at 37°C and 5% $CO_2$ for 2 hr, and then the media were replaced with 100 μL fresh complete growth medium per well.

To assess toxicity of the PBAE/DNA nanoparticles, an MTS assay was carried out 24 hr after transfection (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) to measure the metabolic activity of B16-F10 or MC38 cells. Transfection efficacy was assessed by flow cytometry 48 hr after transfection, using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) with a Hypercyt high-throughput attachment (IntelliCyt, Albuquerque, NM) and 1×PBS with 2% FBS as buffer. Transfection was measured as the percentage of total cells per well that were $GFP^+$ as well as by geometric mean GFP fluorescence intensity. For both toxicity and transfection assays, PBAE/DNA nanoparticle-treated cells were compared to untreated cells as a control.

***In vitro* expression of 4-1BBL and IL-12 by B16-F10 cells**

To ensure that exogenous signals 2 and 3 could be expressed by B16-F10 cells after transfection, nanoparticles were formed as described above for *in vitro* transfection using PBAE 5-3-49, the lead polymer for *in vivo* transfections. Following the results of *in vitro* screenings, the polymer was combined with DNA, at a mass ratio of 90 w/w. Cells were seeded in 96-well plates as described above and transfected with nanoparticles carrying DNA encoding fLuc (control), 4-1BBL, IL-12, or a mixture of 4-1BBL and IL-12 at plasmid mass ratios of 1:3, 1:1, and 3:1. The total amount of DNA in each nanoparticle formulation was the same, and 600 ng DNA was added per well for transfection.

To measure secreted IL-12 expression, the B16-F10 culture medium was collected after 24 hr and 48 hr and measured by mouse IL-12 ELISA (ELISA MAX Deluxe kit, BioLegend, San Diego, CA). To measure 4-1BBL surface expression, transfected cells were trypsinized and stained for mouse

4-1BBL [phycoerythrin (PE)-labeled antibody against mouse 4-1BBL, clone TKS-1, BioLegend; 1:80 dilution] or an isotype control (PE-labeled rat $IgG_{2a,\kappa}$ isotype control antibody, BioLegend; 1:80 dilution) in 1×PBS with 2% FBS. The stained cells were washed twice, then analyzed by flow cytometry (Accuri C6 with Hypercyt attachment).

***In vitro* immune stimulation by transfected MC38 cells**

For studies with colorectal carcinoma, MC38 cells were seeded in 96-well plates and transfected as described above with plasmids encoding fLuc (control), 4-1BBL, or IL-12 or a combination of the 4-1BBL and IL-12 plasmids. The next day, splenocytes were isolated from nine-week-old female C57BL/6J mice, red blood cells were lysed, and splenocytes were resuspended in complete RPMI growth medium as described. To each well of transfected MC38 cells, $10^5$ splenocytes in 50 µL medium were added and co-cultured for 18 hr or 3 days. The secreted IFN-γ was quantified in the supernatant by ELISA, as described for the B16-F10 model.

**Immunohistochemistry on B16-F10 tumors**

B16-F10 tumors were established subcutaneously (s.c.) in the right flank of C57BL/6 mice as described in **Methods**. After 7, 9, and 11 days, mice were treated by intratumoral (i.t.) injection of nanoparticles as described, following the groups used for the survival study. On days 7 and 9, mice were also treated with intraperitoneal (i.p.) injection of anti-PD-1 antibody where specified, as described previously. On t=14 days, mice were euthanized by cervical dislocation, and their tumors were removed, flash-frozen in a bath of dry ice and isopropanol, and stored at -80°C until use. Before cryosectioning, tumors were mounted in optimal cutting temperature (OCT) medium, and 20-µm sections were adhered to Superfrost Plus slides (Thermo Fisher). Sections were allowed to dry at room temperature (RT) for approximately 30 min, then stored at -80°C until staining.

For immunohistochemistry (IHC), sections were fixed for 5 min in cold acetone and allowed to dry at RT. Sections were then rehydrated in 1×PBS at RT for 10 min and blocked for 1 hr at RT in

1×PBS with 3% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% TritonX-100. Slides were then stained either for CD8 or for CD31 and LYVE-1 using the primary antibodies listed in **Supporting Table 3**. Antibodies were diluted in carrier solution (1×PBS with 3% NGS and 0.3% TritonX-100) and incubated with the slides for 2 hr at room temperature in a humidified box. The slides were then washed in 1×PBS four times for 5 min each, then incubated with the secondary antibodies described in **Supporting Table 3**. Slides with anti-CD8 were probed with AF405-labeled anti-rabbit secondary antibodies; slides with anti-CD31 and anti-LYVE-1 were probed with AF405-labeled anti-rat and AF488-labeled anti-rabbit antibodies, respectively. After 1 hr of incubation at room temperature in a humidified box, protected from light, the slides were washed with 1×PBS four times for 5 min each. Coverslips were mounted on the slides in a mixture of 90% glycerol and 10% 1×PBS. Slides were sealed with clear nail polish, stored at 4°C until use, and then imaged by fluorescence microscopy (Axio Observer.Z1, Zeiss).

**Assessment of local immune response: IFN-γ secretion into tumor interstitial fluid**

Mice were inoculated s.c. with $3\times10^5$ B16-F10 cells on the right flank. At t=7 days, mice were treated with nanoparticles and/or anti-PD-1 was described above. At t=14 days, 7 days after initiating treatment and 3 days after the final nanoparticle treatment, n=4 mice per group were euthanized. The tumors were excised and cut into pieces of 2 mm or smaller, weighed, and resuspended in ELISA diluent buffer from the mouse IFN gamma uncoated ELISA kit (Invitrogen/Thermo Fisher). The tissue was incubated at 37°C for 1 hr, then centrifuged at 300 rcf for 5 min, and the supernatant was removed and measured by IFN-γ ELISA according to the manufacturer's instructions. Differences in IFN-γ secretion among groups were detected by one-way ANOVA with Dunnett post-tests against the control (i.t. control nanoparticle administration only). Differences were considered statistically significant for $p<0.05$.

**Assessment of local immune response: qPCR**

Mice were inoculated with B16-F10 s.c. flank tumors and treated as described above. After 10 and 14 days, or 3 days after the start of treatment and 3 days after the final treatment, n=4 mice per group were euthanized. Their tumors were excised, flash frozen in liquid nitrogen, crushed with pestles, and dissolved in TRIzol reagent (Invitrogen/Thermo Fisher). RNA was isolated according to the manufacturer's protocol and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher, Foster City, CA). cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems/Thermo Fisher) and a StepOnePlus Real-Time Polymerase Chain Reaction (RT-PCR) System (Applied Biosystems). Expression levels of the genes listed in **Supporting Table 1** were calculated by the delta delta $C_T$ method using beta-actin (ACTB) as a reference gene. Differences in relative expression levels were detected by one-way ANOVA with Dunnett post-tests against the control (i.t. control nanoparticle administration only). Differences were considered statistically significant for $p<0.05$. Normality of the distributions was confirmed by Shapiro-Wilk tests.

**Assessment of local immune response: flow cytometry**

Mice were inoculated with B16-F10 s.c. flank tumors and treated as described above. After 14 or 18 days, or 3 or 7 days after the final treatment, n=4 mice per group were euthanized. Tumors were excised, cut into 2-mm pieces, and digested with collagenase D (Sigma Aldrich) for 1 hr at 37°C. The digested tissue was pressed through a 70-µm cell strainer with a pestle and washed with cold 1×PBS. The cells were pelleted by centrifugation at 300 rcf for 5 min at 4°C and the supernatant removed. The cells were then resuspended in 1 mL ACK lysing buffer for 1 min at room temperature, then diluted in 10 mL cold 1×PBS, passed through a 100-µm cell strainer, and centrifuged at 300 rcf for 5 min at 4°C.

The supernatant was aspirated, and the cell pellet was resuspended in FACS buffer (1×PBS with 2% FBS) and separated into three aliquots for staining. All samples were centrifuged again to pellet the

cells, and the supernatant was removed and replaced with a cocktail of antibodies to stain for (1) CD3ε and CD8a, (2) CD3ε and CD49b, or (3) CD4 and Foxp3. Details of all antibodies used are described in **Supporting Table 2**. The cells were resuspended in the antibody cocktail and incubated on ice and protected from light for 20 min, then washed three times in FACS buffer by centrifugation. Samples stained for intracellular Foxp3 were first stained for CD4 as described here, then fixed, permeabilized, and stained for Foxp3, and washed using the anti-mouse/rat Foxp3 APC staining set (eBioscience, Thermo Fisher, Carlsbad, CA) according to the manufacturer's instructions. All samples were finally resuspended in FACS buffer for analysis by flow cytometry using the Accuri C6 with Hypercyt attachment. The gating strategy used is shown in **Supporting Figure 5**. Differences in cell populations were detected by one-way ANOVA with Dunnett post-tests against the control (i.t. control nanoparticle administration only). Differences were considered statistically significant for $p<0.05$. Normality of the distributions was confirmed by Shapiro-Wilk tests.

***In vivo* anti-tumor efficacy of tAPC reprogramming nanoparticles in MC38 model**

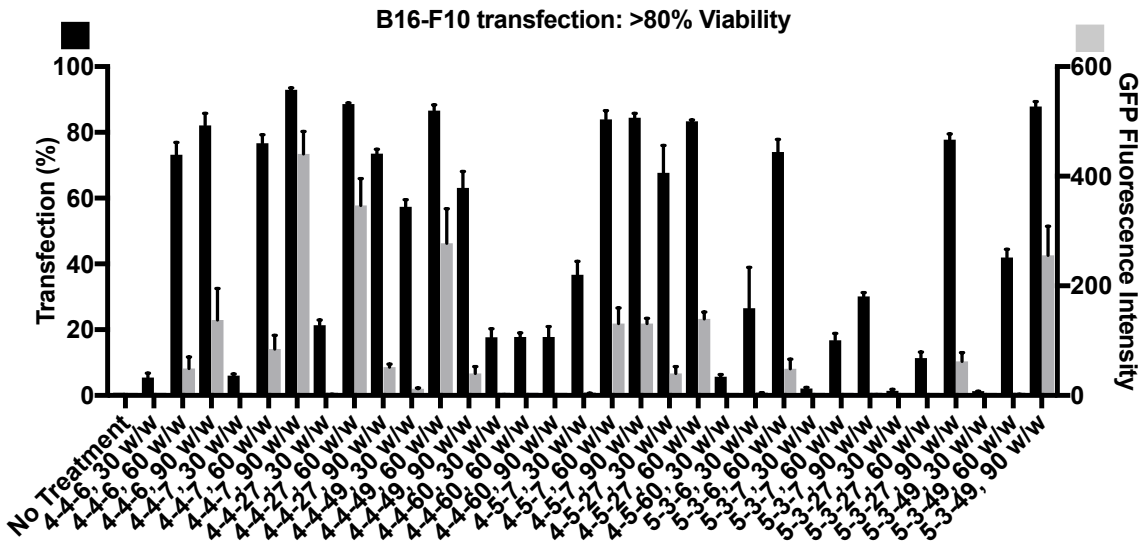
For MC38 anti-tumor efficacy studies, female nine-week-old C57BL/6J mice were inoculated s.c. with $5\times10^5$ cells on the right flank as described above. The study was carried out according to the procedure and schedule described for the B16-F10 model. For the MC38 model, only the lead treatment nanoparticle group (4-1BBL/IL-12-encoding DNA nanoparticles i.t.) and a control (fLuc-encoding DNA nanoparticles i.t.) were tested, both with or without anti-PD-1 antibody administered i.p. N=8 mice were assigned to each group. The tumor re-challenge was carried out on long-term surviving mice by inoculating mice on the left flank s.c. with $5\times10^5$ cells/mouse and following procedures described above for the B16-F10 model. Differences in tumor size detected by two-way repeated-measures ANOVA with post hoc Tukey tests. Differences in survival curves were detected by Mantel-Cox log-rank tests, with a Bonferroni correction for multiple comparisons.

**Assessment of systemic B16-F10 tumor-specific immune response**

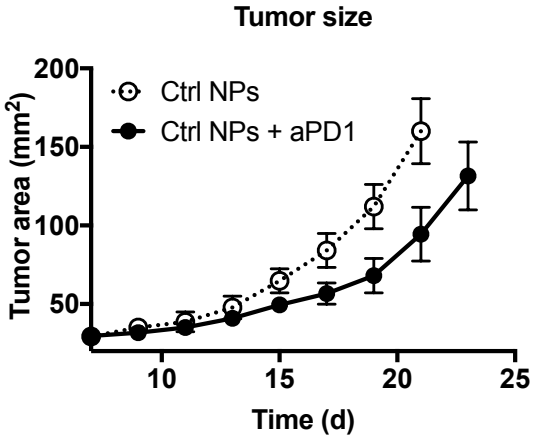
Mice were inoculated with B16-F10 s.c. flank tumors and treated as described above, with n=4

per group. At t=12 days post-tumor inoculation, B16-F10 or MC38 cells were seeded *in vitro* into 96-well plates, then transfected with 4-1BBL and IL-12 in combination on t=13 days as described above. On t=14 days, mice were euthanized by $CO_2$ asphyxiation, and their spleens were excised and pressed through 70-μm cell strainers using pestles. The red blood cells were lysed using ACK lysing buffer as described above. The $CD8^+$ T cells in each spleen were isolated using MACS negative isolation kits and columns as described above. To each well of B16-F10 or MC38 tAPCs, $10^5$ $CD8^+$ T cells were added in 50 μL complete RPMI growth medium, with a final volume of 150 μL per well, and the co-culture was incubated at 37°C with 5% $CO_2$. After 18 hr of incubation, the media from the co-cultures were analyzed by IFN-γ ELISA as described above.

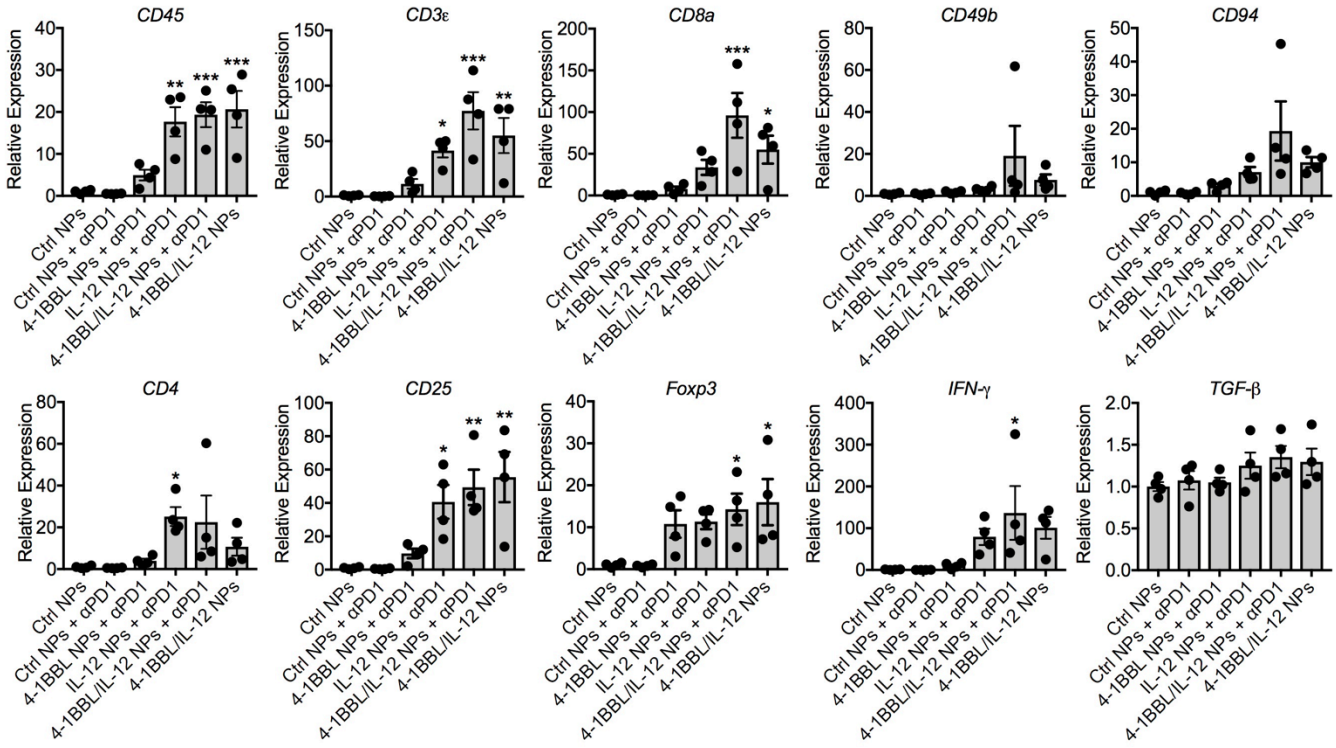
The isolated $CD8^+$ T cells from the spleens of treated and control mice were also stained with a phycoerythrin (PE)-labeled gp100-loaded MHC I tetramer (gp100-Tet; MBL International Corporation, Sunnyvale, CA) to quantify the proportion of gp100-specific $CD8^+$ T cells. Following the manufacturer's instructions, $4\times10^5$ $CD8^+$ T cells per sample were stained in in 60 μL volume, consisting of FACS buffer with 0.1% sodium azide and 1 μg TruStain FcX anti-CD16/32 antibody (Biolegend) along with 10 μL gp100-Tet. Cells were incubated on ice in the dark for 60 min, then washed twice with FACS buffer and resuspended in 200 μL PBS with 0.5% formaldehyde. Stained cells were incubated on ice in the dark for an additional 1 hr, then analyzed by flow cytometry (Attune NxT).



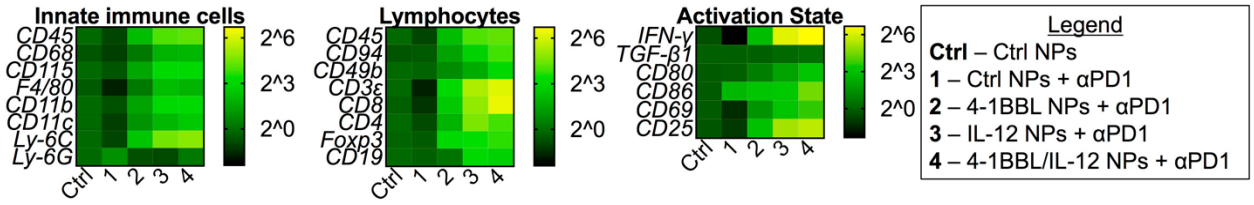

**Supporting Figure S1.** After an initial screen (**Fig. 1**), the PBAEs considered for further study were those that caused <20% toxicity (>80% viability) in B16-F10 cells. The formulations that met this criterion were then ranked by percent of cells transfected and geometric mean GFP fluorescence intensity. Four (n=4) replicates were tested per group. Mean ± standard error are shown.



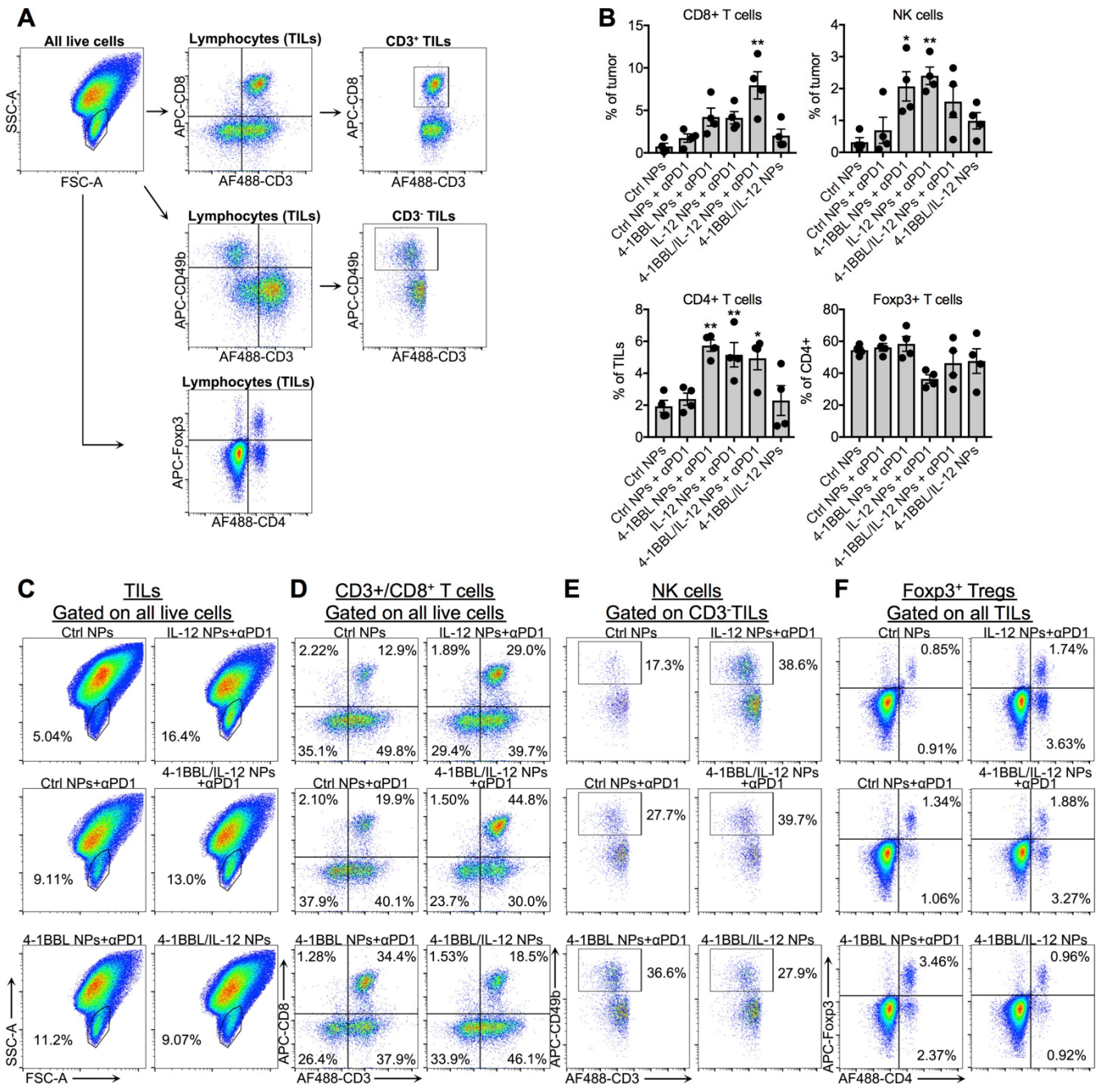

**Supporting Figure S2.** B16-F10 melanoma-bearing mice were treated with either control (fLuc) nanoparticles alone or control nanoparticles along with anti-PD-1 checkpoint blockade. Anti-PD-1 slows tumor growth slightly, but differences are not statistically significant. Significance was measured by a two-way repeated measures t-test with Sidak-Bonferroni correction for multiple comparisons. Results were considered significant when $p<0.05$. Mean ± standard error is shown for each point, and n=7 mice were measured per group.



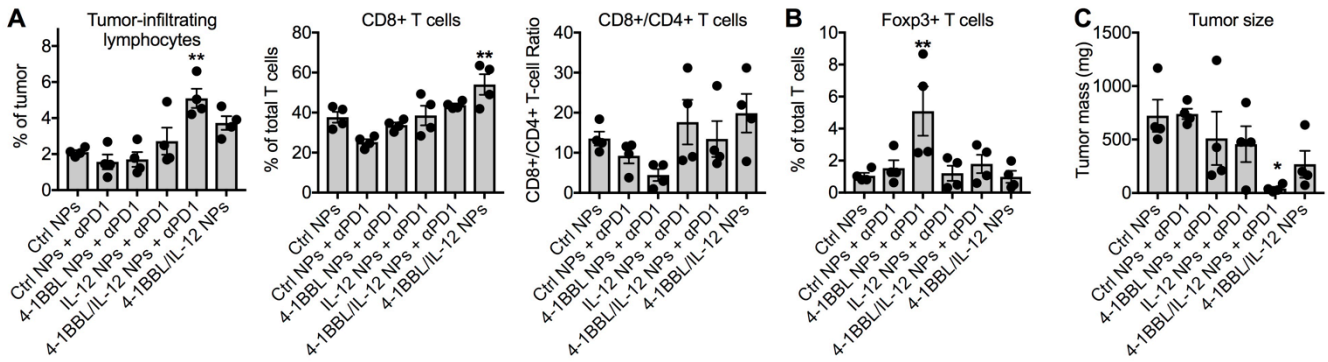

**Supporting Figure S3**. qPCR performed 14 days after tumor inoculation shows strong trends in the expression of various immune cell markers. In general, tAPC-reprogrammed tumors showed higher expression of leukocyte (CD45) and lymphocyte (CD3ε) markers, and there was an increase in the mRNA of genes expressed by cytotoxic lymphocytes, such as $CD8^+$ T cells and NK cells (CD49b, CD94). The slight increase in CD4 expression indicates that $CD4^+$ T cells, important for T-cell help as well as immune regulation, are also stimulated by the tAPC reprogramming strategy, either via direct signaling by transfected cells or via downstream signaling by cells in a tumor microenvironment altered by NP-based gene delivery. All groups measure n=4 animals. Graphs show mean ± standard error. *$p<0.05$; **$p<0.01$; ***$p<0.001$; statistically significant differences were measured by one-way ANOVA with Dunnett post-tests comparing to the control (Ctrl NPs).



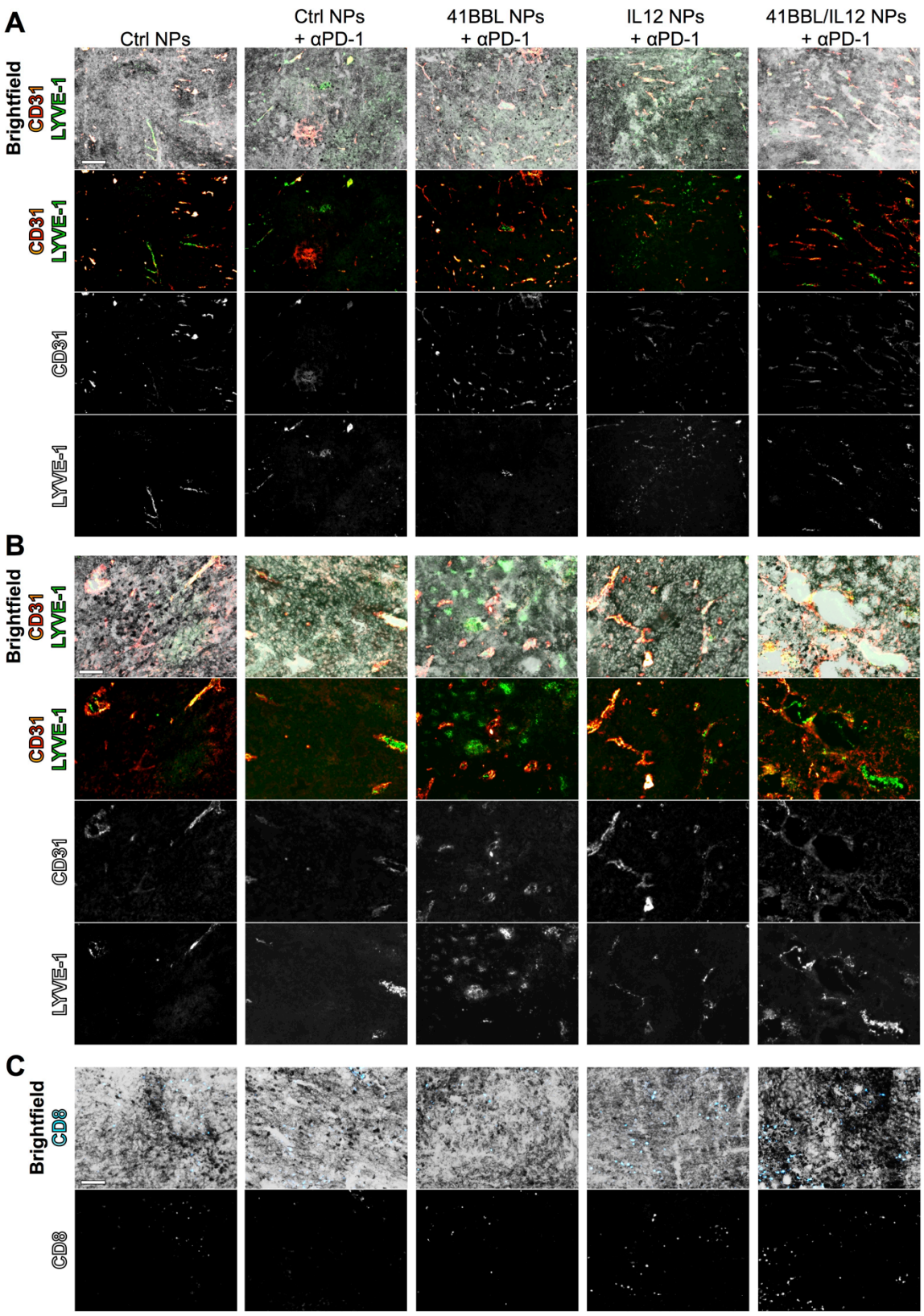

**Supporting Figure S4**. B16-F10 subcutaneous (s.c.) tumors were established in mice and treated as described in **Methods**, then excised and analyzed for mRNA expression of markers indicative of the presence and/or activation state of innate immune cells as well as lymphocytes. Some monocyte, DC, macrophage, and neutrophil markers are elevated in the 4-1BBL-only group, but less so than many of the lymphocyte markers tested. The general trend seen for lymphocytes was also observable for many of the innate immunity and general infiltrating leukocyte markers, with slight upregulation in tumors treated with 4-1BBL, greater upregulation in tumors treated with IL-12, and the greatest effect in tumors treated with both. However, the difference among groups was most striking for lymphocyte markers, particularly CD3 (T cells) and CD8 ($CD8^+$ T cells) and IFN-γ as an activation marker. While this is not definitive proof of our proposed mechanism of action, it does suggest that one of--if not the--major effect of our treatment is on the recruitment, activation, and/or expansion of cytotoxic T lymphocytes, as our technology was originally designed to do.




**Supporting Figure S5**. Flow cytometry on excised tumors 14 days after inoculation showed trends in immune cell populations corresponding to a cytotoxic or Th1 response in tAPC-treated groups. (A) Gating strategy used for all populations gated on all live cells. Separate samples were used for various stains in order to simplify the gating strategy. (B) Proportion of cells found in the tumor or in TILs. All groups measure n=4 animals. Graphs show mean ± standard error. *p<0.05; **p<0.01; statistically significant differences were measured by one-way ANOVA with Dunnett post-tests comparing to the control (Ctrl NPs). (C-F) Flow cytometry pseudocolor plots showing differences among samples. Each plot shows the concatenation of all replicates in order to show the most representative image.




**Supporting Figure S6**. (A) After 18 days, mice treated with reprogramming nanoparticles had more TILs and greater population of cytotoxic lymphocytes at the tumors site than the controls, measured by flow cytometry. (B) The Foxp3$^{+}$ Treg population remained highest in the 4-1BBL nanoparticle-treated group after 18 days. (C) Dramatic differences in tumor size were seen 18 days after inoculation, with those in the 4-1BBL/IL-12 nanoparticles and anti-PD-1 group being significant smaller than controls. *p<0.05; **p<0.01; ***p<0.001; statistically significant differences were measured by one-way ANOVA with Dunnett post-tests comparing to the control (Ctrl NPs). For all bar graphs, mean ± standard error of four (n=4) replicates is shown.




**Supporting Figure S7.** (A-B) CD31 expression and LYVE-1 expression show the presence of blood vessels and lymphatic vessels throughout the tumor. (C) CD8 expression shows the presence of cytotoxic T cells throughout the tumor. These results qualitatively support our data from flow cytometry and qPCR, which suggest that cytotoxic immune cells are recruited to the tumor site after treatment with tAPC reprogramming NPs. Scale bar: 200 μm (A) or 100 μm (B-C).

**Supporting Table S1**. Primers used for qRT-PCR

| Gene | Accession Number | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|---|
| β-actin | NM_007393 | GGCTGTATTCCCCTCCATCG | CCAGTTGGTAACAATGCCATGT |
| CD3ε | NM_007648 | ATCACTCTGGGCTTGCTGAT | TGGGCTCATAGTCTGGGTTG |
| CD4 | NM_013488 | AAGAGGAGGTGGAGTTGTGG | GTTTGCACTCTGTCAAGGGG |
| CD8a | NM_009857 | AGCCCACCTTCGTTGTCTAT | AGCCTTCGTTTTCCTTGCTG |
| CD25 | NM_008367 | ACACCACCGATTTCTGGCTA | AGTCTGTGGTGGTTATGGGG |
| Foxp3 | NM_001199347 | ACCTATGCCACCCTTATCCG | GCGAACATGCGAGTAAACCA |
| CD49b | NM_008396 | AGCCCTGTCAGACATCAACA | TGGGAGTACTTGGTGCGAAT |
| CD94 | NM_010654 | GAATGCTGTGTTTGCCTGGA | TCTGGATTGGGGCTGAAGAA |
| IFN-γ | NM_008337 | CATGGCTGTTTCTGGCTGTT | TCCTTTTGCCAGTTCCTCCA |
| TGF-β | NM_011577 | ACGGAATACAGGGCTTTCGA | CCGGTTCATGTCATGGATGG |
| CD45 | NM_001111316 | CCCGGGATGAGACAGTTGAT | ATTCTGCGCACTTGTTCCTG |
| CD68 | NM_001291058 | CCACAGTTTCTCCCACCACA | GTGTAGTTCCCAAGAGCCCC |
| CD115 | NM_001037859 | GGTTGTAGAGCCGGGTGAAA | TCTTGTGGTCAGGGTGCTTC |
| F4/80 | NM_010130 | ACCTGTAAACGAGGCTTCCTG | CTGAGTTAGGACCACAAGGTGAG |
| CD11b | NM_008401 | AGTGCTGGGAGACGTGAATG | GCACTGAGGCTGGCTATTGA |
| CD11c | NM_021334 | GCGTGGAGAACTTTGATGCTTT | TACTGCTGCTTGGTGTCTCTG |
| Ly-6C | NM_010741 | CTTCTTGTGGCCCTACTGTGT | TTGGCACTCCATAGCACTCG |
| Ly-6G | NM_001310438 | CCTGAGACTTCCTGCAACACA | TTGTCCAGAGTAGTGGGGCA |
| CD19 | NM_009844 | TGGTGGAGGTAGAAGAGGGA | AGGAAGGGTGTTGACTGGTT |
| CD80 | NM_009855 | AGCTGACTTCTCTACCCCCAA | TCCAACCAAGAGAAGCGAGG |
| CD86 | NM_019388 | TCTGCCGTGCCCATTTACAA | TGTGCCCAAATAGTGCTCGT |
| CD69 | NM_001033122 | GGAGAGAGGGCAGAAGGACCA | TGAGGACCACTATTAACACAGCC |

**Supporting Table S2**. Antibodies used for flow cytometry

| Protein target | Conjugate | Clone | Dilution | Supplier | Catalog number |
|---|---|---|---|---|---|
| CD3ε | Alexa Fluor 488 (AF488) | 17A2 | 1:50 | BioLegend | 100210 |
| CD8a | Allophycocyanin (APC) | 53-6.7 | 1:100 | BioLegend | 100712 |
| CD49b | Allophycocyanin (APC) | DX5 | 1:100 | BioLegend | 108909 |
| CD4 | Alexa Fluor 488 (AF488) | RM4-5 | 1:80 | BioLegend | 100529 |
| Foxp3 | Allophycocyanin (APC) | FJK-16s | 1:100 | eBioscience | 17-5773-82 |

**Supporting Table S3**. Antibodies used for immunohistochemistry

| Protein target | Conjugate | Host species | Clone | Dilution | Supplier | Catalog number |
|---|---|---|---|---|---|---|
| CD8a | None | Rabbit | Polyclonal | 1:100 | Thermo | PA5-79011 |
| LYVE-1 | None | Rabbit | Polyclonal | 1:200 | Thermo | PA5-19620 |
| CD31 | None | Rat | 390 | 1:50 | BioLegend | 102402 |
| Rabbit IgG | Alexa Fluor 488 (AF488) | Goat | Polyclonal | 1:500 | abcam | ab150077 |
| Rat IgG | Alexa Fluor 405 (AF405) | Goat | Polyclonal | 1:200 | abcam | ab175671 |
| Rabbit IgG | Alexa Fluor 405 (AF405) | Goat | Polyclonal | 1:200 | abcam | ab175652 |