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Supplemental Information

DNA-Based Delivery of Checkpoint Inhibitors in Muscle and Tumor Enables Long-Term Responses with Distinct Exposure

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SUPPLEMENTAL FIGURES

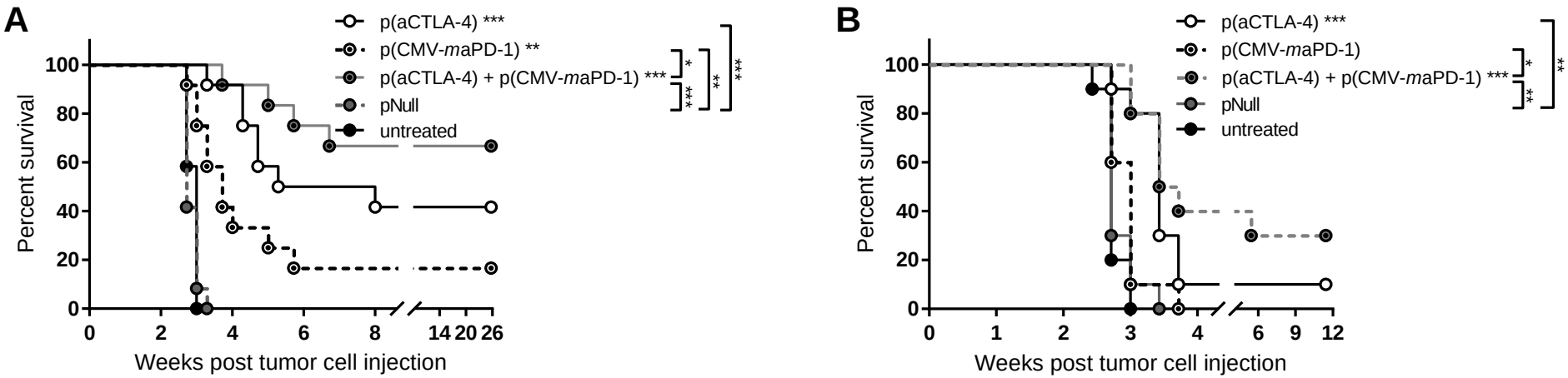

Figure S1. Kaplan-Meier survival curves after intramuscular electrotransfer of DNA-based checkpoint inhibitors. C57BL/6J mice received an intramuscular pDNA electrotransfer in the *tibialis anterior* seven days before (**A**) or three days after (**B**) s.c. MC38 tumor cell injection. One day later, pDNA electrotransfer was repeated in the *gastrocnemius* muscle. Each electrotransfer, the single-treatment groups received 60 µg p(aCTLA-4) or p(CMV-*m*aPD-1) in the left leg, and the combination-treatment group received both DNA-based mAbs in different legs. Control mice got an equimolar amount of pNull in both legs, or were left untreated. Data were analyzed with the log-rank (Mantel-Cox) test with a Holm's test for multiple comparisons. Asterisks in the legend indicate the statistical difference compared with untreated mice (n = 10 or 12 mice per group, *p < 0.05, **p < 0.01, ***p < 0.001).

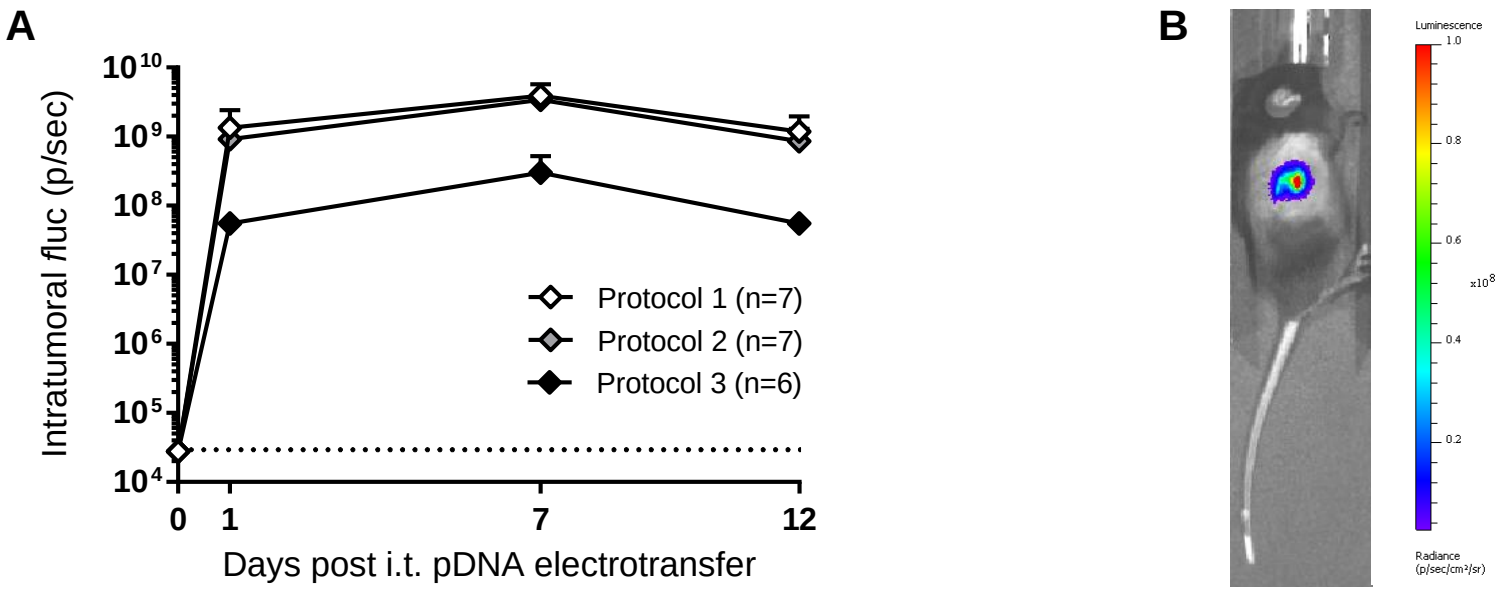

Figure S2. Validation of an intratumoral electroporation protocol. (**A**) Intratumoral *firefly* luciferase (*f*luc) expression following a single intratumoral (i.t.) electrotransfer of 60 µg pFluc, seven days after s.c. MC38 tumor cell injection in C57BL/6J mice. Three electroporation protocols were compared, each consisting of two series of four square-wave pulses applied in perpendicular directions at a frequency of 1 Hz. Protocol 1: 600 V/cm, 5 ms pulse length, skin covered with Eco Ultrasound transmission gel. Protocol 2: 200 V/cm, 20 ms pulse length, skin covered with Signa Electrode Gel. Protocol 3: 400 V/cm, 5 ms pulse length, skin covered with Signa Electrode Gel. The dotted line indicates the average bioluminescence background in untreated mice. Data are represented as mean + SEM (n = 6 or 7 mice per group). The less-conductive Eco Ultrasound Transmission Gel applied in protocol 1 and the lower voltage applied in protocol 2 resulted in lower electrical currents, which correlated with higher *f*luc expression. Since protocol 1 is extensively validated by Cemazar *et al.*,[25, 26] this intratumoral electroporation protocol was applied in all subsequent experiments. (**B**) Representative bioluminescence image of *f*luc expression in an MC38 tumor one day after intratumoral pFluc electrotransfer, using electroporation protocol 1. p/sec: photons per second. p/sec/cm²/sr: photons per second that leave a square centimeter of tissue and radiate into a solid angle of one steradian.

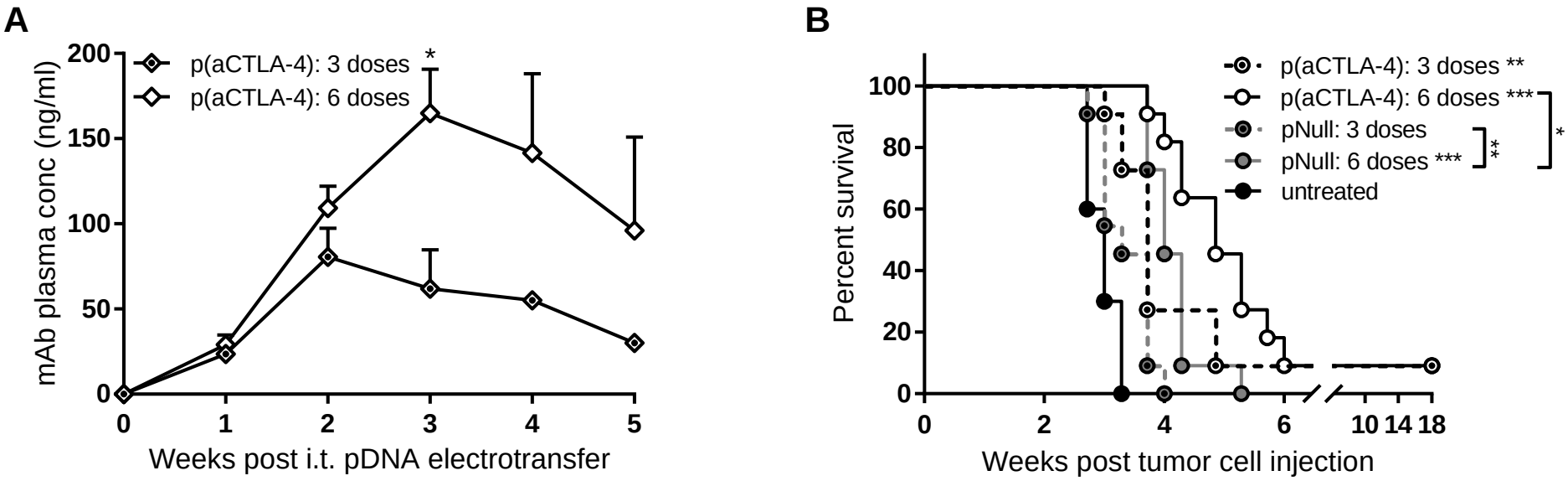

Figure S3. Dose comparison for intratumoral p(aCTLA-4) or pNull electrotransfer. MC38-bearing C57BL/6J mice received three or six intratumoral (i.t.) electroporations of 60 µg p(aCTLA-4) or an equimolar amount of pNull. Control mice were left untreated. (**A**) Anti-CTLA-4 mAb plasma concentrations over time, represented as mean + SEM. Peak levels and levels at each time point were compared between both dose regimens with an unpaired t test. (**B**) Kaplan-Meier survival curves. Data were analyzed with the log-rank (Mantel-Cox) test with a Holm's test for multiple comparisons. Asterisks in the legend indicate the statistical difference compared with untreated mice (n = 10 or 11 mice per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

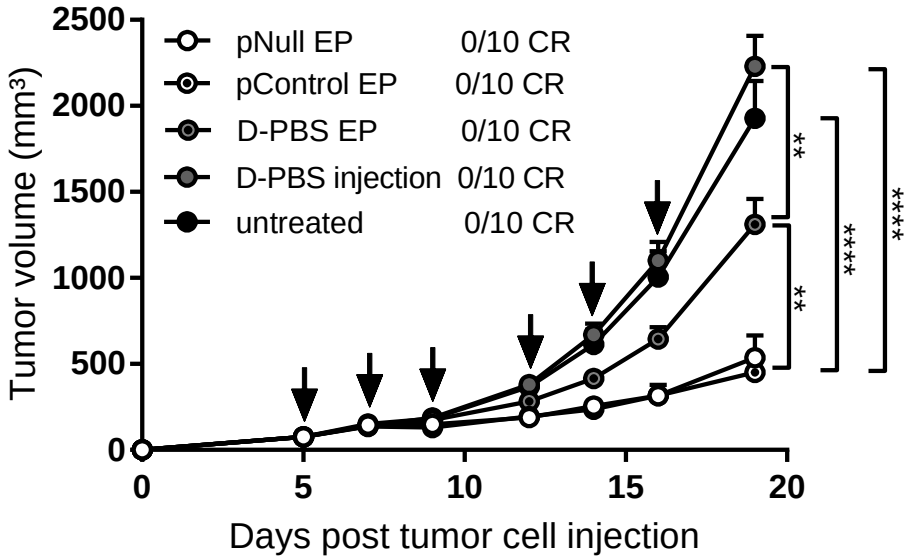

Figure S4. Anti-tumor effect of intratumoral pDNA electroporation. MC38-bearing C57BL/6J mice received six intratumoral electroporations (indicated by arrows). Equimolar amounts of an empty plasmid (26.1 µg pNull or 30.2 µg pControl) or only buffer (30 µL D-PBS) were injected intratumorally, followed by local application of electrical pulses. Control mice received D-PBS injection without electrical pulses or were left untreated. The number of complete responders (CR) is indicated for all treatment groups. Tumor volumes, represented as mean + SEM, were compared with one-way ANOVA on day 19 after tumor cell injection, when the first mice had to be sacrificed (n = 10 mice per group, **$p < 0.01$, ****$p < 0.0001$).

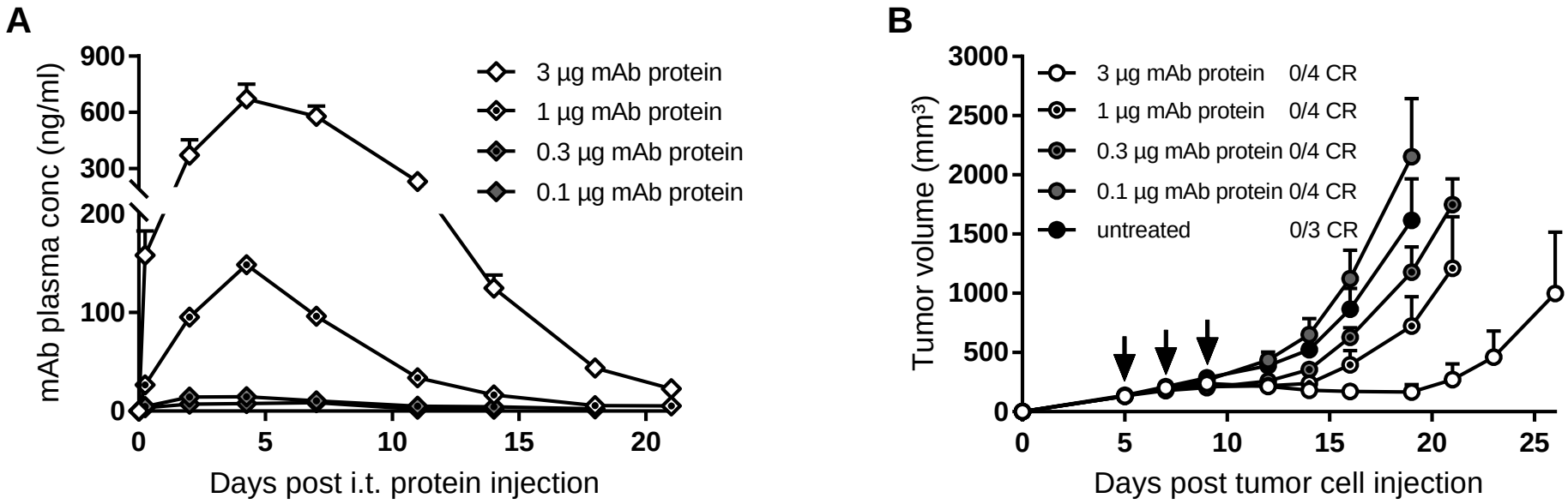

Figure S5. Intratumoral administration of anti-CTLA-4 mAb proteins. MC38-bearing C57BL/6J mice received three intratumoral (i.t.) injections (indicated by arrows) of either 3 µg, 1 µg, 0.3 µg or 0.1 µg anti-CTLA-4 mAb protein, produced and purified in house, in 30 µL D-PBS. Control mice were left untreated. (**A**) Resulting anti-CTLA-4 mAb concentrations in plasma. (**B**) Tumor growth and number of complete responders (CR). Tumor volumes are shown until the time point that the first mouse of the corresponding treatment group had to be sacrificed. Comparison of the tumor volumes on day 19 after tumor cell injection with one-way ANOVA indicated a significant difference between 3 µg mAb protein and 0.1 µg mAb protein ($p < 0.05$), and untreated mice ($p < 0.01$). One µg mAb protein resulted in a significant tumor growth delay compared with untreated mice ($p < 0.05$). All data are represented as mean + SEM (n = 3 or 4 mice per group).

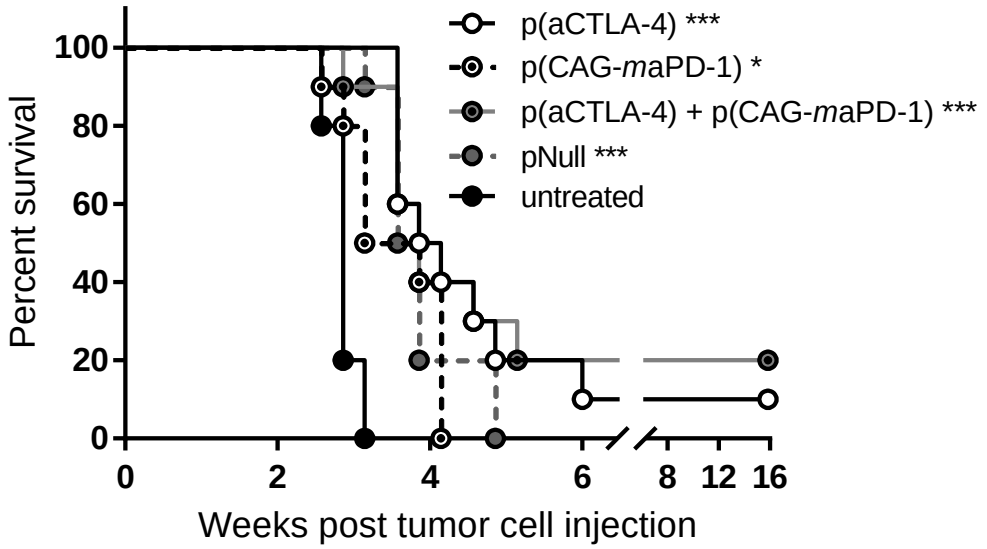

Figure S6. Kaplan-Meier survival curves after combined intratumoral electrotransfer of DNA-based checkpoint inhibitors. MC38-bearing C57BL/6J mice received three intratumoral electroporations of 60 µg p(aCTLA-4) or p(CAG-*m*aPD-1) in 25 µL D-PBS, or the combination of p(aCTLA-4) and p(CAG-*m*aPD-1) or an equimolar amount of pNull in 50 µL D-PBS. One group received no treatment. Data were analyzed with the log-rank (Mantel-Cox) test with a Holm's test for multiple comparisons. Asterisks in the legend indicate the statistical difference compared with untreated mice (n = 10 mice per group, *$p < 0.05$, ***$p < 0.001$).

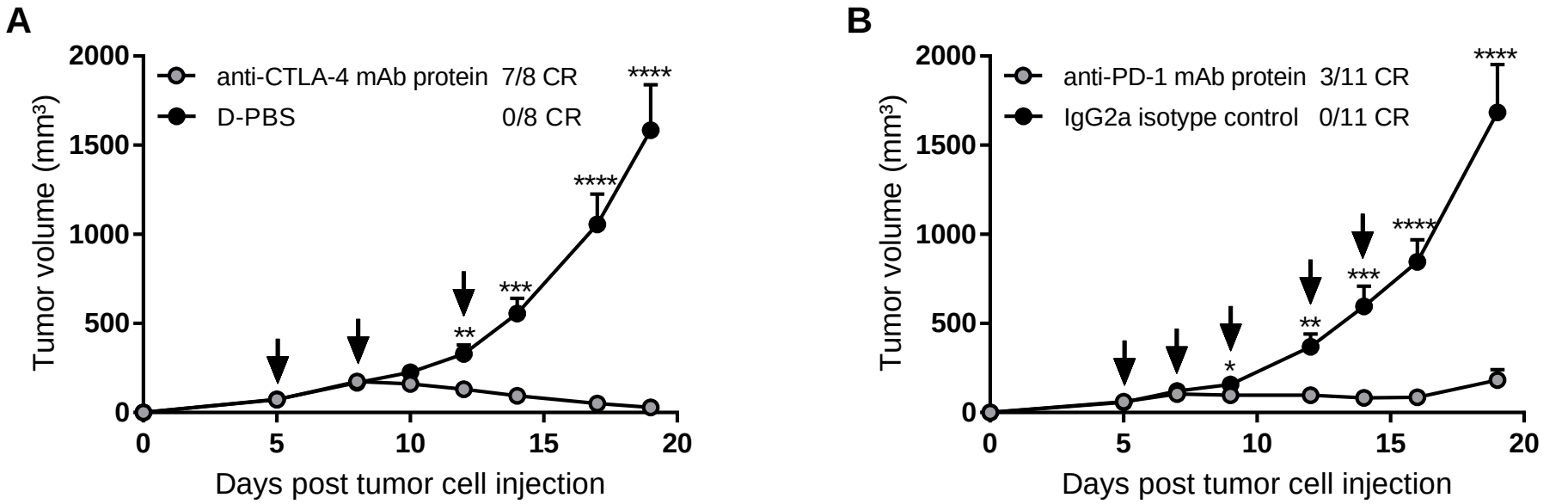

Figure S7. Systemic treatment with checkpoint-inhibiting mAb proteins. (**A**) MC38-bearing C57BL/6J mice received three intraperitoneal injections (indicated by arrows) of 200 µg anti-CTLA-4 mAb protein, produced and purified in house, in 314 µL phosphate buffer (20 mM $Na_2HPO_4.2H_20$, 150 mM NaCl, pH 7.5). Control mice were injected with 314 µL D-PBS. Tumor volumes of both groups were compared with an unpaired t test (n = 8 mice per group). (**B**) MC38-bearing C57BL/6J mice received five intraperitoneal injections (indicated by arrows) of the rat anti-PD-1 mAb RMP1-14 or a rat IgG2a isotype control, at a dose of 10 mg/kg and a concentration of 1.8 mg/mL in D-PBS. Tumor volumes were compared with an unpaired t test (n = 11 mice per group). The number of complete responders (CR) is indicated for all treatment groups. All data are represented as mean + SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

SUPPLEMENTAL MATERIALS AND METHODS

Plasmid DNA constructs and reagents

The *firefly* luciferase 2 (*f*luc) cDNA sequence was cloned into the CAG-driven pDNA backbone (pFluc) by Icosagen (Tartu, Estonia), to be used as reporter pDNA. For the protein study, a rat anti-mouse PD-1 mAb (clone RMP1-14, BE0146) and a rat IgG2a isotype control (anti-trinitrophenol, clone 2A3, BE0089) were purchased from BioXCell (West Lebanon, NH, USA). The variable regions of the mAbs encoded by p(CAG-*c*aPD-1-HC^{2a}+LC), p(CMV-*c*aPD-1-HC^{1}) + p(CMV-*c*aPD-1-LC), p(CMV-*m*aPD-1) and p(CAG-*m*aPD-1) correspond to, or are derived from the variable regions of the RMP1-14 clone.

in vivo bioluminescence imaging

*F*luc expression was quantified by non-invasive bioluminescence imaging (IVIS Spectrum, Perkin Elmer, Waltham, MA, USA) at the Molecular Small Animal Imaging Center (MoSAIC) at KU Leuven. During the whole procedure, mice were anesthetized by isoflurane inhalation. Mice were s.c. injected with 126 mg/kg D-luciferin substrate (E6551, Promega, Madison, WI, USA) at 15 mg/mL in D-PBS, after which bioluminescence intensity was measured every two minutes. Intratumoral *f*luc expression was defined as the maximal total radiance (p/sec) measured in a specified region of interest that included the tumor.