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Depth of tumor implantation affects response to in situ vaccination in a syngeneic murine melanoma model

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ABSTRACT

An important component of research using animal models is ensuring rigor and reproducibility. This study was prompted after two experimenters performing virtually identical studies obtained different results when syngeneic B78 murine melanoma cells were implanted into the skin overlying the flank and treated with an in situ vaccine (ISV) immunotherapy. Although both experimenters thought they were using identical technique, we determined that one was implanting the tumors intradermally (ID) and the other was implanting them subcutaneously (SC). Though the baseline in vivo immunogenicity of tumors can depend on depth of their implantation, the response to immunotherapy as a function of tumor depth, particularly in immunologically 'cold' tumors, has not been well studied. The goal of this study was to evaluate the difference in growth kinetics and response to immunotherapy between identically sized melanoma tumors following ID versus SC implantation. We injected C57BL/6 mice with syngeneic B78 melanoma cells either ID or SC in the flank. When tumors reached 190–230 mm^3, they were grouped into a 'wave' and treated with our previously published ISV regimen (12 Gy local external beam radiation and intratumoral hu14.18-IL2 immunocytokine). Physical examination demonstrated that ID-implanted tumors were mobile on palpation, while SC-implanted tumors became fixed to the underlying fascia. Histologic examination identified a critical fascial layer, the panniculus carnosus, which separated ID and SC tumors. SC tumors reached the target tumor volume significantly faster compared with ID tumors. Most ID tumors exhibited either partial or complete response to this immunotherapy, whereas most SC tumors did not. Further, the 'mobile' or 'fixed' phenotype of tumors predicted response to therapy, regardless of intended implantation depth. These findings were then extended to additional immunotherapy regimens in four separate tumor models. These data indicate that the physical 'fixed' versus 'mobile' characterization of the tumors may be one simple method of ensuring homogeneity among implanted tumors prior to initiation of treatment. Overall, this short report demonstrates that small differences in depth of tumor implantation can translate to differences in response to immunotherapy, and proposes a simple physical examination technique to ensure consistent tumor depth when conducting implantable tumor immunotherapy experiments.

INTRODUCTION

Scientific investigations conducted in an effective and reproducible manner are the mainstay of basic, translational, and clinical biomedical research. The National Institutes of Health (NIH) has implemented new guidelines in an effort to improve rigor and reproducibility in scientific research, with the goal of enhancing scientific integrity and transparency.[1] In syngeneic implantable models for tumor immunotherapy, care is taken to control confounding factors. Subtle variances within these systems can lead to differing responses, despite holding constant as many factors as possible. Variations in diet, animal housing temperature, and even vendor source can affect the response to immunotherapy.[2–4] These and other potentially unknown factors can result in differences in the tumor-immune microenvironment and may influence response to immunotherapy. Murine tumor immunology experiments can be plagued by high variability, making statistical evaluation difficult and hampering the ability to reliably extend and build on the published results of others.[5 6]

Previous work in vaccine development demonstrated that different cutaneous tissue planes can influence the degree of immune response to an implanted tumor.[7 8] In studying the immunogenic EL4-OVA transfected tumor line, Joncker *et al* demonstrated that an EL4-OVA inoculum injected intradermally (ID) failed to develop, while the same tumor inoculum injected subcutaneously (SC) grew into a progressive and lethal tumor.[9] The difference in response was due to a difference in the kinetics of dendritic cell (DC) migration and tumor-specific T-cell activation at the tumor-draining lymph node between the two tumor implantation depths, but only controlled the tumor in the ID space. In this setting, the stronger immune response

in the ID space was sufficient to prevent growth of the EL4-OVA immunogenic tumor. However, less immunogenic (immunologically 'colder') tumor models may still be able to develop in both the ID and SC space, but retain a difference in baseline antitumor immune response. For these tumor models, immunotherapy is required to drive a sufficient antitumor response. Yet, the effect of depth of tumor implantation in the skin on response to immunotherapy has not been well studied in syngeneic murine models. This manuscript expands on Joncker *et al*'s published work by determining a role of tumor implantation depth in response to immunotherapy.

We have previously demonstrated that local external beam radiation therapy (RT) primes immune responses and synergizes with intratumoral injections of hu14.18-IL2 immunocytokine ((IC), an anti-disialoganglioside (GD2) antibody fused to interleukin 2 (IL2)) to achieve tumor control in an implantable, GD2-positive B78 syngeneic melanoma model that does not respond to immune checkpoint blockade therapy alone.[10] This therapy was mechanistically T-cell dependent, and mice rendered disease free developed immunological memory, demonstrating that this RT+IC immunotherapy acts as an in situ vaccine (ISV). Yet, our group noted substantial inter-experimenter variability in tumor response rates to ISV without a clear distinguishing cause. This study investigates the influence of tumor implantation depth on response to this ISV immunotherapy.

METHODS

Syngeneic tumor cell line

B78-D14 (B78) murine melanoma (provided by Ralph Reisfeld, Scripps Research Institute, La Jolla, California) is a GD2-positive cell line distantly derived from B16-F10 melanoma, cultured as previously described.[11 12] Unlike the B16 line, this tumor grows more slowly, has an encapsulated mass, and does not spontaneously metastasize.[10]

Animals and tumor models

Animals were cared for using a protocol approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. Female, 6–8-week-old C57BL/6 mice from Taconic Biosciences (Rensselaer, New York) were inoculated after ~1 week with 2×10^6 B78 cells in 100 µL phosphate buffered saline (PBS). For tumors intentionally implanted ID, the beveled tip of the needle was inserted face up at a <10° angle and advanced 2–3 mm into the tissue parallel to the skin while lifting the needle to create a tenting effect. This resulted in a well-demarcated weal and could be felt as pressure or resistance during injection. For tumors intentionally implanted SC, the needle was inserted at a ~20° angle and advanced 5–7 mm into the tissue while remaining superficial to the flank musculature. This created a less clearly observable weal, without strong resistance. Tumor volume ((width^2×length)/2) was measured two times per week using calipers. Mice were randomized using a random number generator into treatment groups when tumors reached 190–230 mm^3 and assigned to a 'wave.' One to two mice per wave were randomly assigned to receive PBS control treatment.

Tumor treatments

On day 1 post randomization, tumors received a single 12 Gy fraction of RT as previously described.[10] On days 6–10 post randomization, 50 µg of hu14.18-IL2 IC (Apeiron Biologics, Vienna, Austria) was injected in 100 µL PBS intratumorally (figure 1A).

Tissue harvest, preparation, and histological analysis

Following CO_2 asphyxiation, tumors were dissected en bloc to preserve anatomical relation to surrounding tissues. Specimens were fixed in 10% neutral buffered formalin for 48 hours. Samples were paraffin processed, sectioned into 5 µm slices, and stained with H&E. Sections were visualized under a SCOPE.A1 microscope (Zeiss), and images were captured using an AxioCam HR camera (Zeiss).

Statistical analysis

The data are presented as mean±SEM, except where noted. Time to treatment size and overall survival analyses was performed using the Kaplan-Meier method with comparisons using a log-rank test. Comparisons of tumor volume at treatment day 33 were done using a one-way analysis of variance with multiple comparisons conducted by the Sidak method. Comparison of growth rates between ID and SC tumors treated with ISV was done using linear mixed effects models of log-transformed data to estimate slopes. Statistical analyses were conducted using R and GraphPad Prism software (San Diego, California).

RESULTS

Experimenters conducting the same experiment obtained disparate results associated with different tumor implantation depths

We noticed that the efficacy of ISV varied, especially when comparing results obtained by different experimenters. To test implantation technique as a possible reason for this variability, two cohorts of C57BL/6 mice were implanted with B78 tumor cells, one by experimenter A and another by experimenter B. Both experimenters injected tumors consistent with their understanding of a 'subcutaneous' injection. Once each cohort reached ~150 mm^3 average, they were treated by experimenter A using our ISV regimen.[10] Four out of five tumors implanted by experimenter B became tumor free following treatment, compared with only 1/5 implanted by experimenter A (figure 1B).

Examination of untreated tumors implanted by both experimenters revealed that most tumors implanted by experimenter A had a 'fixed' phenotype, where lateral displacement of the skin over the tumor did not result in tumor displacement. There was no apparent tumor

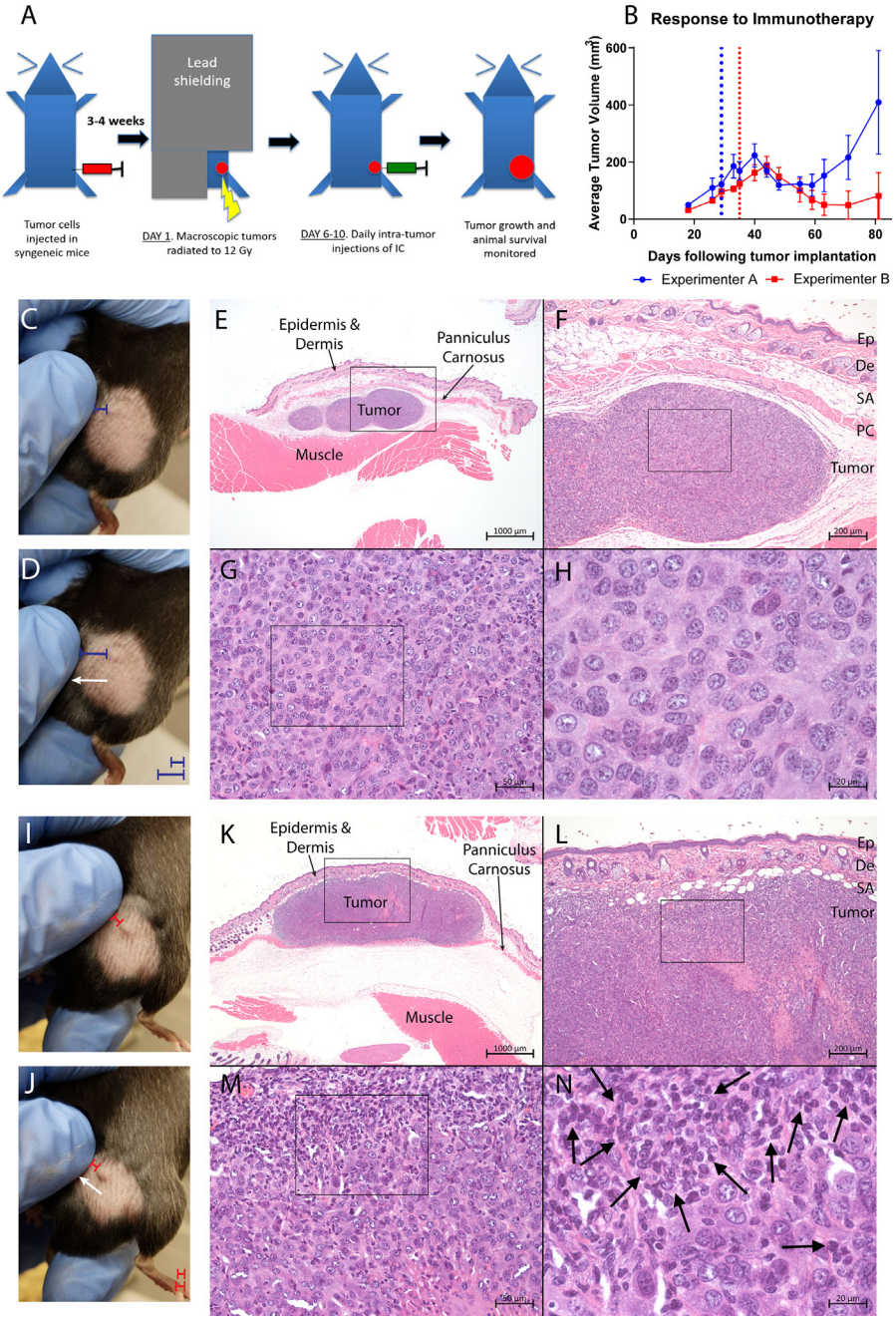

Figure 1 Different results obtained from two different researchers conducting the same experiment. (A) Schema of ISV treatment approach, as described in the 'Methods' section. (B) Average±SEM tumor volume of n=5 mice implanted with tumors by either experimenter A (blue) or experimenter B (red) and treated using the ISV described in (A). Both groups were treated and measured identically by experimenter A. Vertical dotted lines represent the day on which treatment began (when tumors reached ~150 mm^3) for the mice implanted by experimenter A (blue) or by experimenter B (red). (C,D) Physical examination of tumors implanted by experimenter A reveals a distinct 'fixed' phenotype of the tumors in response to lateral displacement of the overlying skin. Note in (C) the short distance (blue bar) between the experimenters finger and the left margin of the tumor, in contrast note in (D) how the lateral leftward movement (white arrow) of the overlying skin increases the distance from the finger to the left margin of the tumor (blue bar), indicating that the tumor is not attached to the skin. (E–H) Histologic examination by H&E staining of 'fixed' tumors demonstrates the tumors reside deep to the cutaneous proper, which is delineated by the PC. (I,J) Physical examination of tumors implanted by experimenter B reveals a distinct 'mobile' phenotype of the tumors in response to lateral displacement of the overlying skin. Note in (I) the short distance (red bar) between the experimenters finger and the left margin of the tumor does not increase in (J) with lateral leftward movement (white arrow shown in (J)) of the skin that is attached to the 'mobile' tumor. (K–N) Histologic examination shows that 'mobile' tumors are either superficial to or invading the PC (seen in (K)) and reside in the true skin compartment. Further, qualitative assessment identifies distinct mononuclear cells, likely infiltrating lymphocytes, infiltrating the 'mobile' tumor ((N), marked by arrows). Online supplemental videos 1 and 2 demonstrate the 'fixed' and 'mobile' phenotypes shown in (C,D) and (I,J) in greater detail. De, dermis; Ep, epidermis; ISV, in situ vaccine; PC, panniculus carnosus; SA, subcutaneous adipose.

connection to the skin, as it would move over the tumor (figure 1C,D). In contrast, most tumors implanted by experimenter B had a 'mobile' phenotype, where lateral displacement of the skin resulted in displacement of the tumor. These tumors appeared connected to, and moved freely with, the skin (figure 1I,J and online supplemental videos 1 and 2).

Histologic analysis confirmed that these 'fixed' and 'mobile' tumors occupied different tissue planes within the skin compartment. 'Fixed' tumors were deep to the thin layer of dermal striated muscle called the panniculus carnosus (PC), which is present in most mammals and separates the true skin compartment from underlying fascial layers (figure 1E,F).[13 14] In contrast, 'mobile' tumors were either completely superficial to or invading/involved with the PC; they could be seen invading true skin compartment structures including the dermal white adipose tissue, dermis, and lymphatics (figure 1K,L). Together, these observations demonstrate that different experimenters with substantial mouse-handling experience can implant tumors at different depths in the skin, which may impact treatment outcome and contribute to increased variability.

Response rates to ISV are greater in ID versus SC tumors

The starting size of a tumor influences treatment response to ISV.[15] To explore the effect of tumor implantation depth on response to ISV while controlling for tumor size, a staggered treatment start experiment was conducted. A cohort of mice was injected with B78 cells to intentionally be ID/mobile, while a second cohort was injected to be intentionally SC/fixed. Whenever tumors reached 190–230 mm^3, they were grouped into a treatment 'wave' and treated with ISV (figure 1A), with 1–2 untreated control mice per wave.

In total, there were 10 distinct treatment 'waves' (figure 2). SC-implanted tumors grew faster, reaching treatment size in a median of 22 days post implantation, while ID-implanted tumors took a median of 36 days to reach treatment size (figure 2A). Consistent with figure 1B, ID-implanted tumors responded to ISV better than SC-implanted tumors (figure 2B, table 1). Median survival post treatment for ID-implanted tumors was significantly longer than SC-implanted tumors (figure 2B and table 1). Both SC-implanted and ID-implanted tumors had a prolonged survival following treatment compared with untreated controls (figure 2B, table 1). Overall, 11 out of 22 (50%) ID-implanted mice demonstrated a complete response to ISV and remained disease free, compared with 0/24 SC-implanted mice (figure 2C and D). Direct comparison of tumor volumes on post treatment day 33 demonstrated significant differences between all treated and untreated groups, with significantly lower average tumor volumes in the ID-treated compared with the SC-treated groups (figure 2E). In addition, linear mixed effects modeling predicted a growth factor of 0.42 (0.37–0.48) every 30 days for ID-implanted/treated tumors, while SC-implanted/treated tumors grew by a factor of 3.07 (2.64–3.53) every 30 days ($p<0.0001$). Lastly, response to ISV as measured by overall survival did not depend on treatment 'wave' for both ID-implanted ($p=0.877$) and SC-implanted ($p=0.340$) tumors (figure 2F and online supplemental figures 1 and 2).

'Mobile' versus 'fixed' tumor status is associated with treatment outcome

As a means of non-invasively confirming tumor implantation depth, the 'fixed' or 'mobile' status of each tumor was evaluated in a blind fashion. At each time point during this experiment, tumors were designated as entirely 'fixed,' entirely 'mobile,' or 'intermediate' if they had qualities of both, such as a bilobed or partially mobile phenotype (figure 3). Observed tumor status was largely consistent with the intended injection depth; the majority of ID-implanted mice developed 'mobile' tumors, and the majority of SC-implanted mice developed as 'fixed' tumors. A heat map was generated to track physical-examination status of the tumors over time. After ranking all mice based on the tumor volume at treatment day 60 (blind to intended implantation depth), a clear clustering phenomenon was observed (figure 3). All mice rendered disease free had a predominantly 'mobile' phenotype; all mice that died from tumor burden had predominantly 'fixed' or 'intermediate' phenotype. In general, mice with the smallest day 60 tumor volumes were 'mobile,' and those with the largest tumor volumes were 'fixed.' In addition, those tumors intended to be ID, but with 'fixed' phenotypes, behaved similarly to the other 'fixed' tumors. This implies a strong association between the response to ISV and depth of tumor implantation; the physical 'fixed' or 'mobile' status appeared more closely associated with treatment outcome than the original 'intended' treatment group.

Following these observations, four additional syngeneic tumor models and five immunotherapy regimens were investigated to determine the degree of applicability of these findings (online supplemental figures 3-6 and online supplemental tables 1-4). 'Mobile' and 'fixed' phenotypes were observed in each of these four additional tumor models. In murine models of CT26 colon adenocarcinoma, B16-GD2 melanoma, and MOC2 head and neck squamous cell carcinoma, SC-implanted tumors grew faster than ID-implanted tumors. The degree of response to multiple immunotherapies (detailed in online supplemental figures 3-6 and online supplemental tables 1-4) was substantially different between ID and SC tumors in the CT26, B16-GD2, and Panc02 pancreatic adenocarcinoma models as well.

DISCUSSION

Our findings highlight the need for detailed documentation of experimental methods and expand published studies indicating that tumor depth can influence antitumor immune response.[8 9] B78 melanoma tumors grow at different rates in the ID versus SC space and show

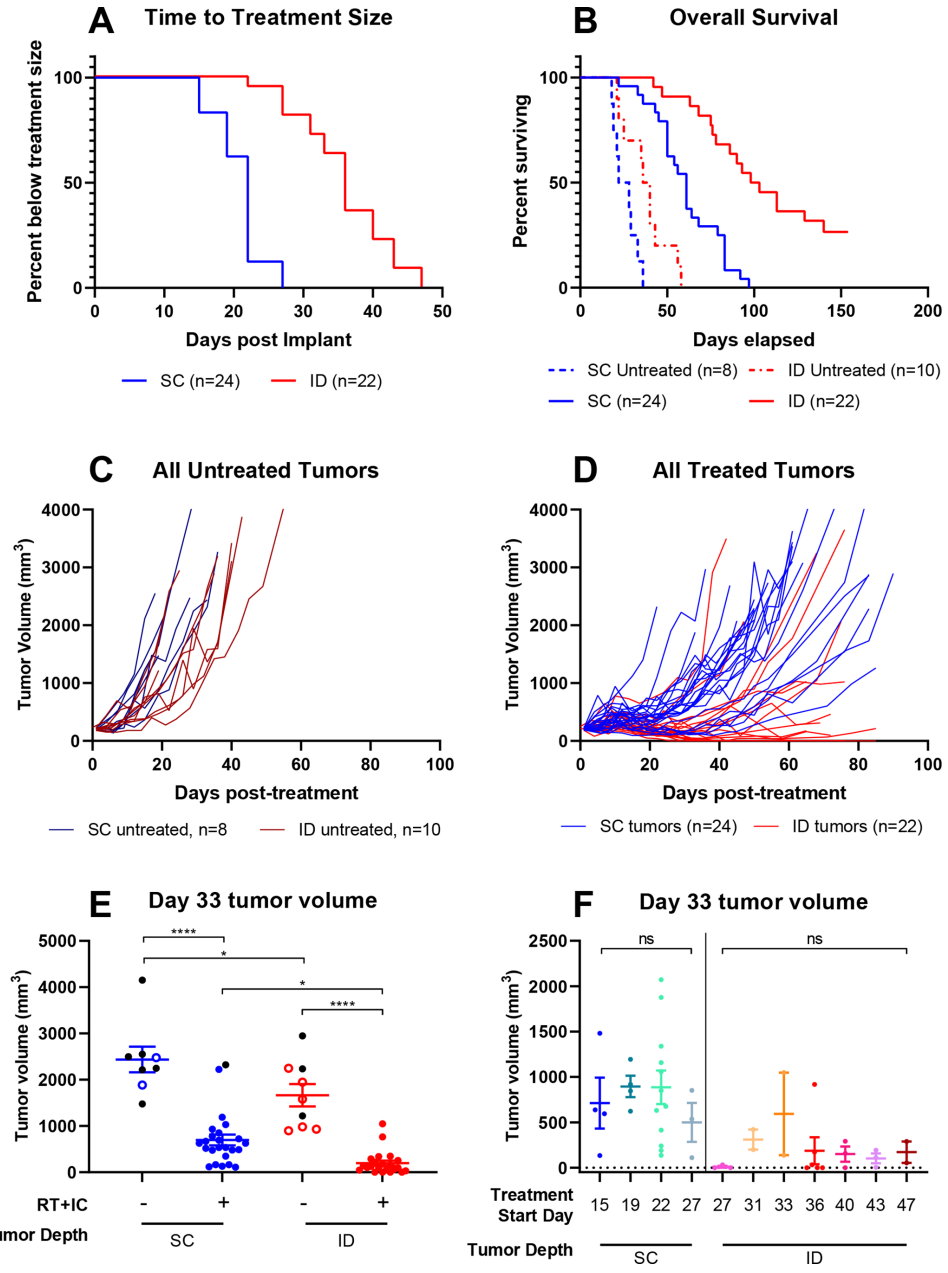

Figure 2 Intradermal tumors are more likely to respond to immunotherapy than subcutaneous tumors. Female, C57BL/6 mice were injected on the same day in the right flank intentionally aiming for either intradermal (ID, red) or subcutaneous (SC, blue) placement of the tumor. Mice were monitored weekly, and when tumors reached treatment size (190–230 mm^3), they were collected into a 'treatment wave' and treated with either PBS control or our previously published in situ vaccine as described in the 'Methods' section. (A) Time to event analysis representing the time from tumor implantation to reaching treatment size. (B) Survival analysis representing the time from initiation of treatment (either PBS control or RT+IC ISV) to death or tumor meeting size criteria for sacrifice. Statistical comparisons for (A) and (B) were conducted using log-rank comparisons, with resulting p values presented in table 1. (C) Tumor volumes (mm^3) were measured two times per week for all untreated tumors. (D) Tumor measurements for all treated tumors were also measured weekly. (E) Tumor volume at day 33 following treatment initiation for both treated and control mice. Data are presented as points representing individual tumor volumes and horizontal bars representing the mean±SEM tumor volume for each treatment group. Black dots represent tumors on mice that died before treatment day 33, with their last value carried forward and shown here. Statistical analysis was conducted by one-way analysis of variance with multiple comparisons using the Sidak method. (F) Tumor volume at day 33 of treatment for mice bearing ID or SC tumors, divided into treatment waves (defined by the day post tumor implant of treatment initiation). Ten different waves are included: waves at days 15, 19, and 22 for the SC tumors, a wave at day 27 for both SC and ID tumors, and waves at days 31, 33, 36, 40, 43, and 47 for the ID tumors. Shown here are only those waves that have two or more mice. A single additional wave (not shown here but included in online supplemental figures 1 and 2) included a single ID mouse starting treatment on day 22. Data are presented as points representing individual tumor volumes, with horizontal bars representing the mean±SEM tumor volume for each treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. IC, immunocytokine; ISV, in situ vaccine; ns, not significant; PBS, phosphate buffered saline; RT, radiation therapy.

Table 1 Time-to-event comparisons corresponding to figure 2

Comparator A	Parameter	Comparator B	Reference figure	A median (days)	B median (days)	P value
ID	Time to treatment	SC	Figure 2A	36	22	<0.0001
ID (treated)	Time to death/sacrifice	SC (treated)	Figure 2B	100.5	61	<0.0001
ID (treated)	Time to death/sacrifice	ID (untreated)	Figure 2B	100.5	38	<0.0001
SC (treated)	Time to death/sacrifice	SC (untreated)	Figure 2B	61	25	<0.0001
ID (untreated)	Time to death/sacrifice	SC (untreated)	Figure 2B	38	25	0.0104

ID, intradermally; SC, subcutaneously.

significant differences in response to ISV immunotherapy (figure 2). This phenomenon appears to extend to additional tumor models and other immunotherapy regimens as well (online supplemental figures 3-6). As reported by Joncker *et al*, OVA-antigen-laden DCs were detected in the draining lymph node of EL4-OVA tumors as early as 2 days post inoculation for ID-implanted tumors, but took 8 days for detection in SC-implanted tumors.[9] Joncker *et al* used immunologically 'hotter' tumors: ID implantation failed to result in tumor growth. Our results here expand on this past work by including the immunologically 'colder' B78 melanoma model and others, which grow readily and avoid immune destruction even in the ID space. Further, we extend the difference in functional

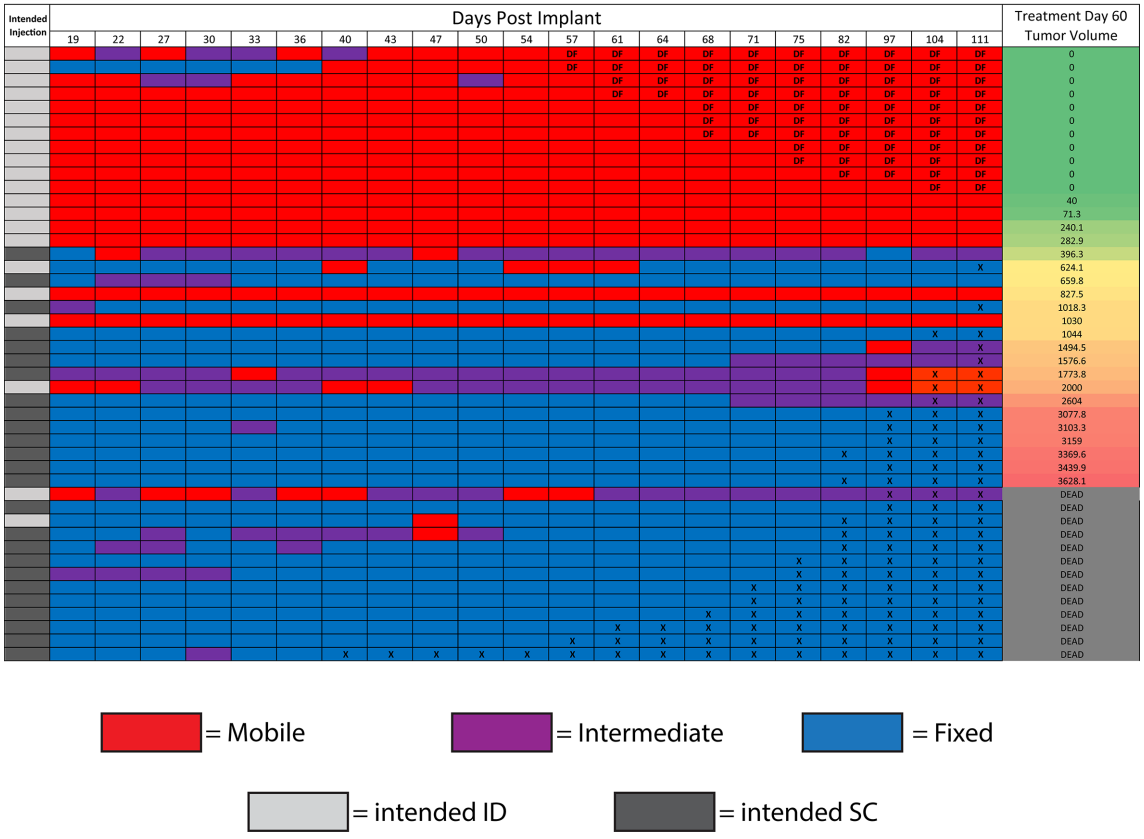

Figure 3 Mobile/fixed status predicts response to RT+IC in situ vaccine. Female C57BL/6 mice used in the experiment described in figure 2 were evaluated by physical examination at each measurement time point. Each row in the figure above represents an individual mouse, and each column represents the measurement time point in days post tumor implantation. Mice with 'mobile' tumors (as described in figure 1, online supplemental video 1, and in the 'Results' section) were coded red, and mice with 'fixed' tumors (figure 1, online supplemental video 2, and the 'Results' section) were coded blue. Mice with mixed or intermediate physical examination findings (as described in the 'Results' section) were coded purple. Mice that were sacrificed due to tumor burden have an 'X' in their cells starting at the time of sacrifice, and mice that were rendered Disease Free (DF) by treatment have a 'DF' in their cell, starting at the time tumor was no longer detected. Mice were then ranked in the far-right column based on their tumor volume at treatment day 60 and listed as dead if they had died or been sacrificed prior to day 60. The 'intended injection' column on the left indicates which depth of tumor was intended on implantation (light gray for ID, dark gray for SC). IC, immunocytokine; ID, intradermal; SC, subcutaneous RT, radiation therapy.

immunogenicity between the ID and SC space to include response to multiple distinct immunotherapy regimens. In this B78 model system, potent RT+ICISV can cure some mice of ID tumors, but can only slow the growth of SC tumors (figure 2B and D). To our knowledge, this is the first explicit demonstration of implantation depth affecting not only tumor growth rate but also response to direct intratumoral immunotherapy.

We observed that mobile, ID-implanted tumors grew either in marginal association with or above the PC while deeper, fixed, SC-implanted tumors were deeper into or below the PC. The ID space is known to contain a higher density of DCs, with specialized blind-ended lymphatics connected to a superficial lymphatic plexus, whereas the SC space contains mostly monocytes and macrophages, with lymphatic vessels connecting to a deeper lymphatic plexus.[16 17] This difference in lymphatic architecture may support Joncker *et al*'s observed differential kinetics of DC mobilization on tumor challenge in these two spaces.[9] Clinically, tumors that have greater immune infiltrates at diagnosis seem to have a higher rate of response to immunotherapy and are considered immunologically 'hotter.'[18] This is likely consistent with both our and Joncker *et al*'s observations, given the greater potential tumor immune cell infiltrate in some ID versus SC tumors (figure 1N).[9] Using this difference, future studies may be able to tune the immunologic 'coldness' of a given tumor line in mouse models through intentional differences in implantation depth. A more complete characterization of the mechanism of antigen recognition, effector immune cell recruitment and infiltration, tumor vascularization, and effector response to immunotherapy may be considered, though is beyond the scope of this report.

We identified that slight differences in depth of tumor implantation (~300 µm) can create substantial differences in response to immunotherapy. The NIH has asked researchers to be more transparent and explicit with their methodologic descriptions.[19] Based on our experience, it is likely that in mouse experiments 'subcutaneous' is sometimes used as a common term to describe either SC or ID implantation; most published studies do not detail procedures for confirming the implantation depth of tumors used in their experiments. This study documents the importance of accurately and robustly describing the method and location of tumor implantation, as well as the means of confirming implantation depth. Using histologic and physical observations, we delineate between deeper, 'fixed' tumors and more superficial, 'mobile' tumors and propose incorporating this physical examination finding as an additional criterion for conducting consistent, reproducible implantable tumor immunotherapy experiments.

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Contributors PMC and MM were responsible for experimental design, execution, and analysis of data. PMC created final versions of all figures and drafted the manuscript. MR and CS collected and analyzed experimental data. VS collected tissue for histologic analysis and captured histology images. JB conducted and confirmed statistical analysis of experimental data. RBP, JH, ALR, and ZM contributed to experimental design, thorough edits, and review of the manuscript. AE contributed to experimental design, data collection, mouse colony maintenance, and literature research. PMS provided experimental design and review of data. All authors provided thorough review and editing of the manuscript draft.

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Patient consent for publication Not required.

Ethics approval The research contained in this manuscript does not use patient data in any form. Animal studies were conducted according to a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

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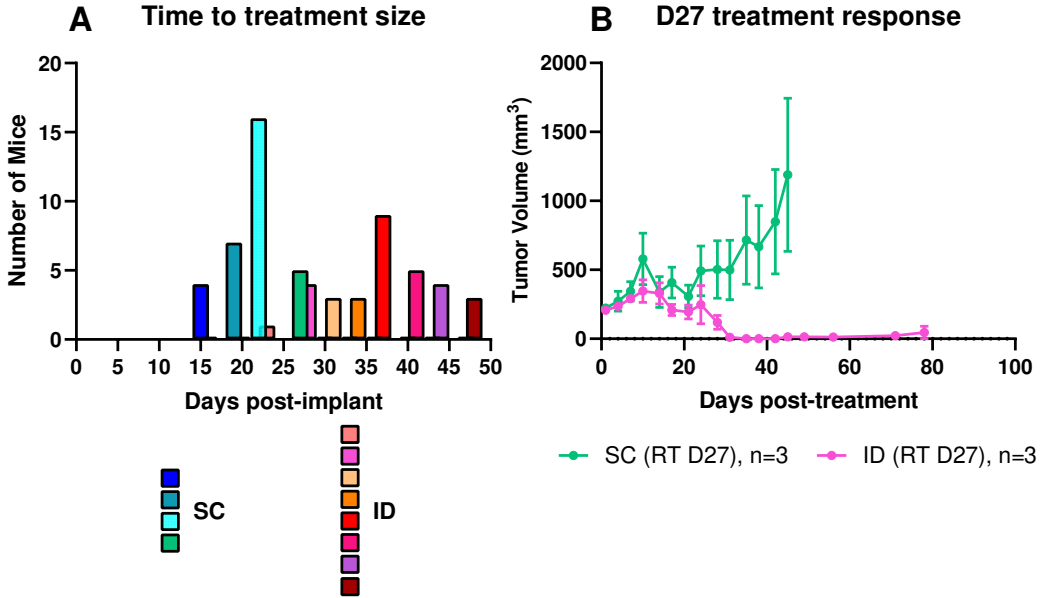
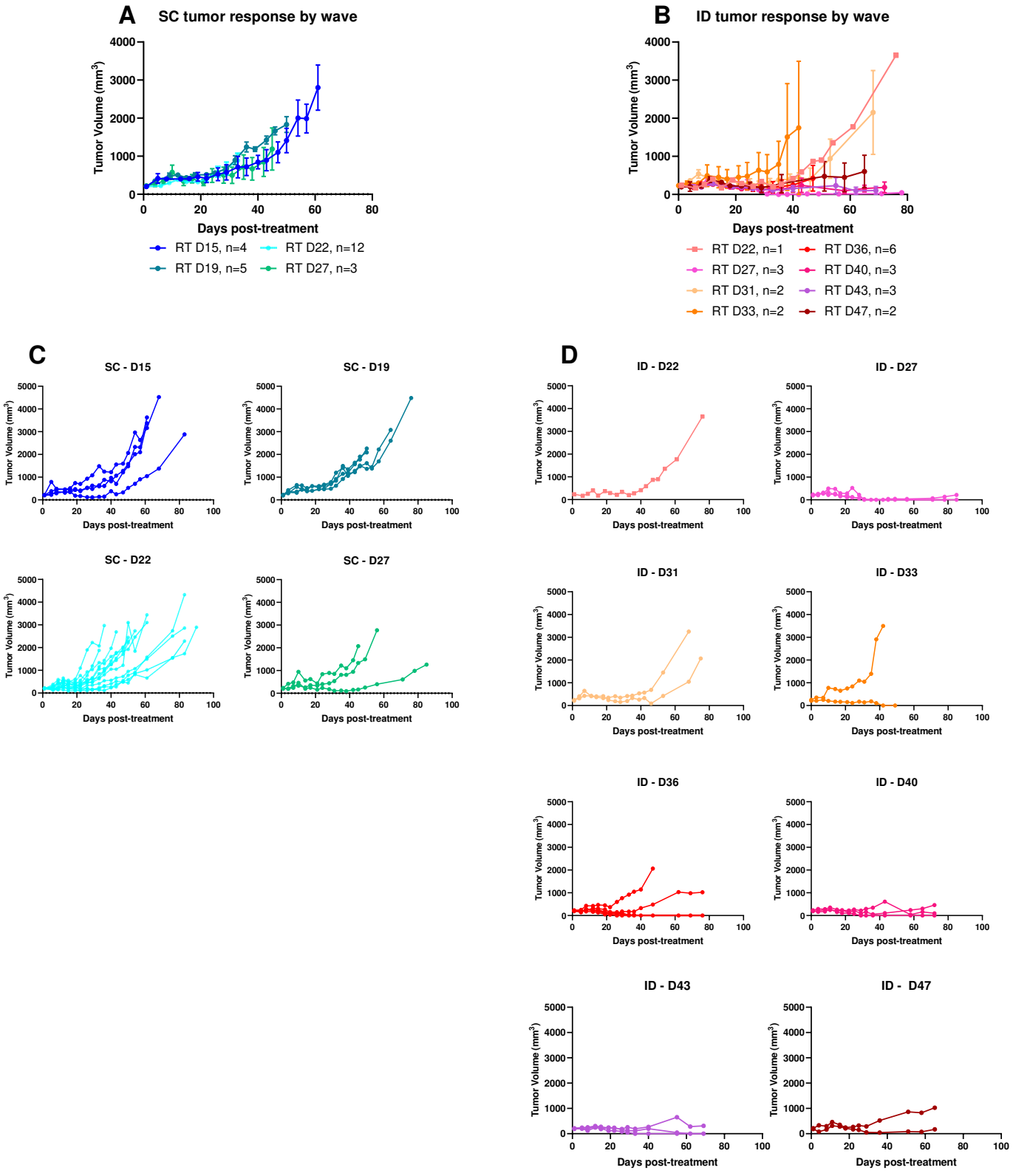
Supplemental Figure 1



Supplemental Figure 2



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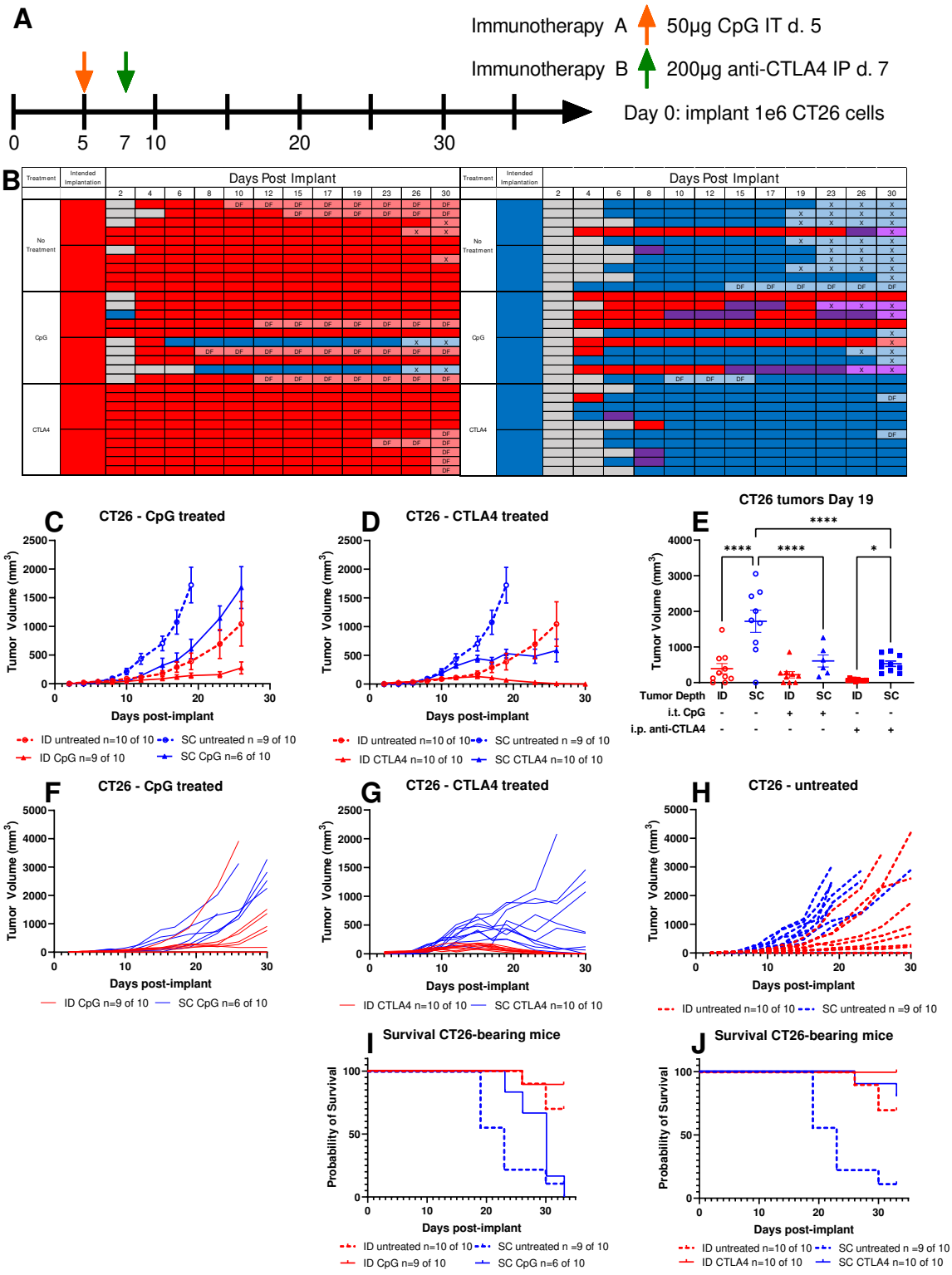

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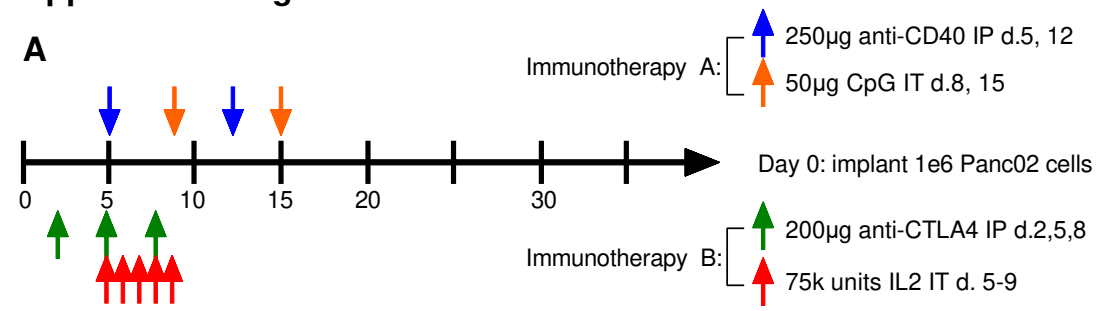
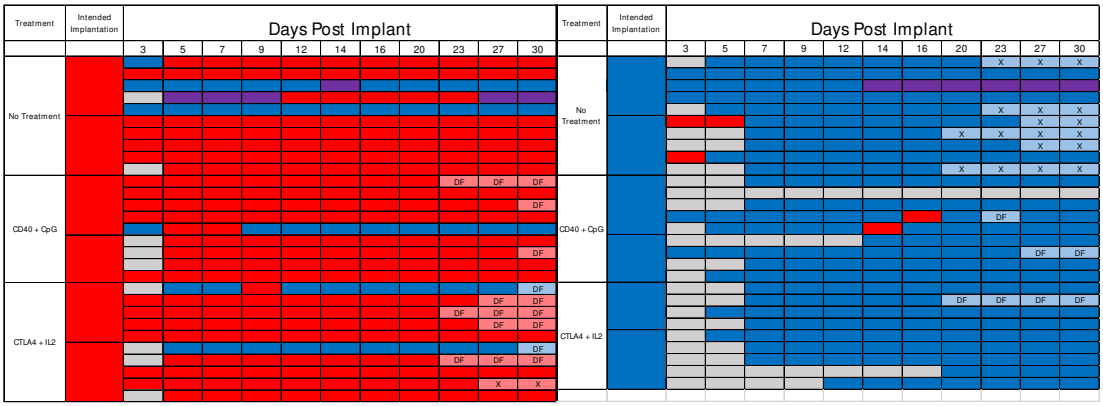
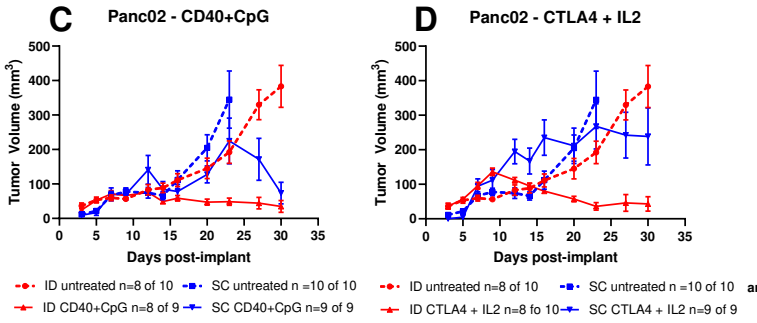
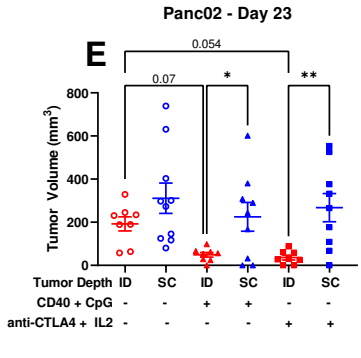
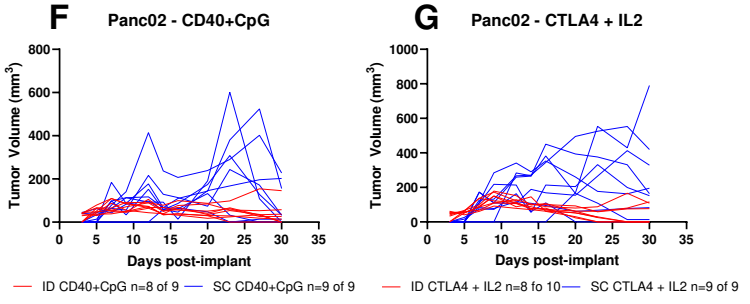
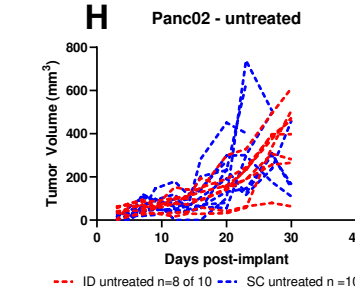




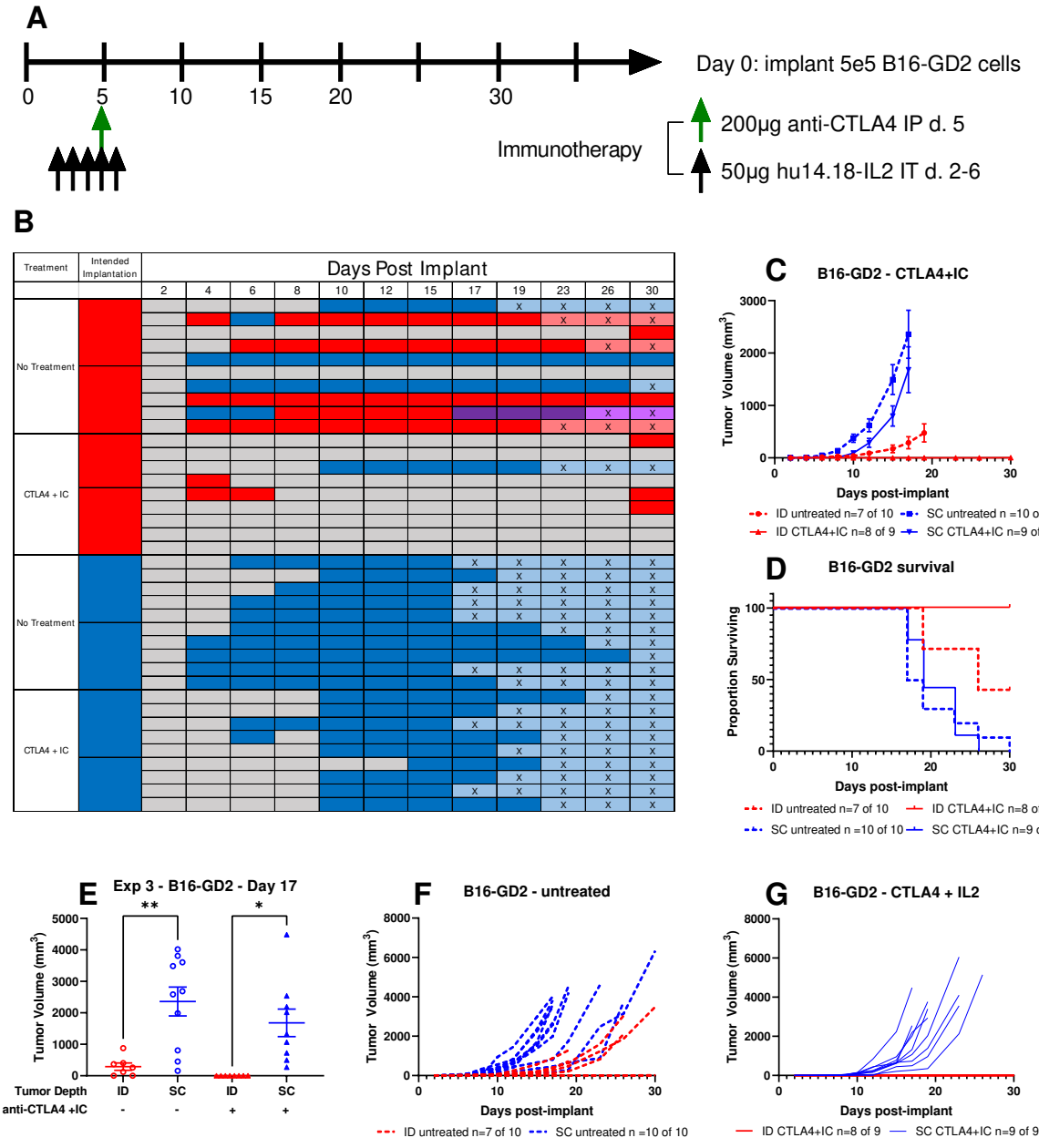




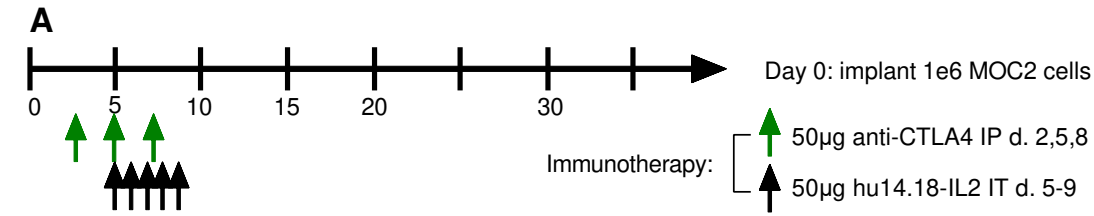


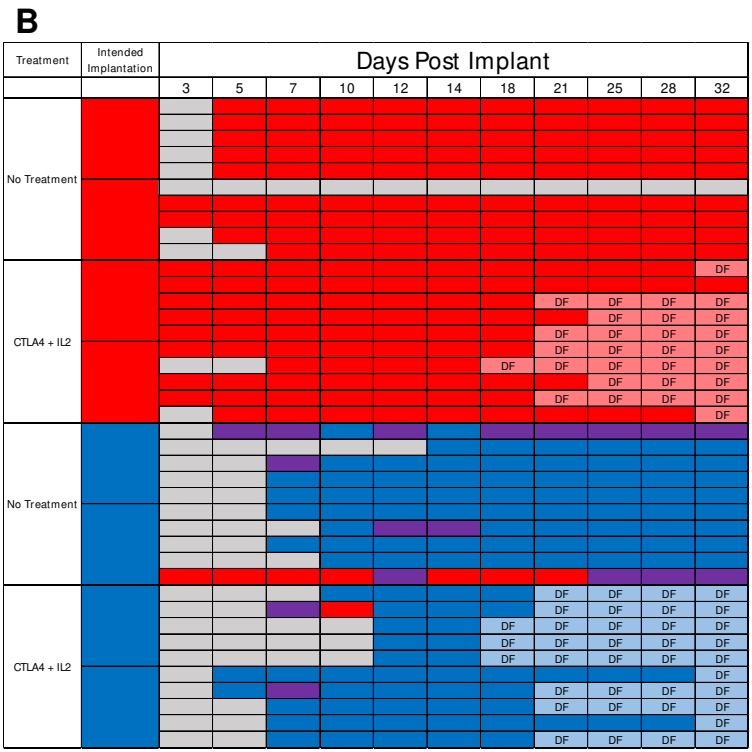


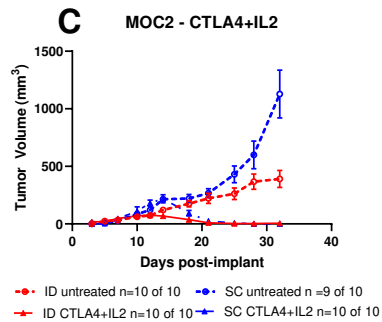
Supplemental Figure 5

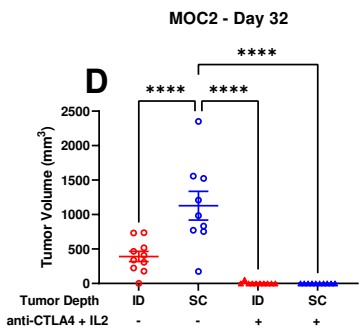


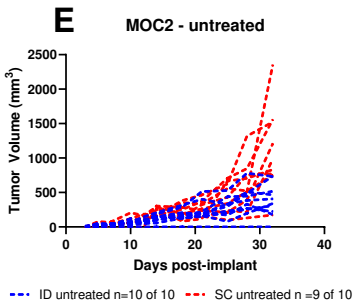
Supplemental Figure 6

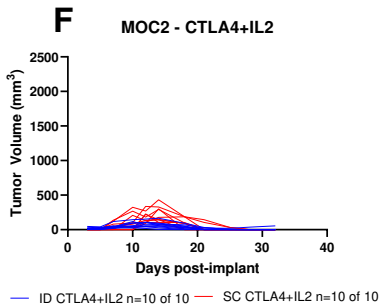












Supplemental Figure 1: Controlling for growth rate, identically sized ID tumors are more likely to respond to RT + IC in situ vaccination. Female, C57BL/6 mice were injected on the same day intentionally aiming for either Intradermal (ID) or Subcutaneous (SC) placement of the tumor. Mice were monitored weekly, and whenever tumors reached treatment size (190-230mm^3), they were collected into a treatment group and treated with either PBS control, or our previously published in situ vaccine (12Gy external beam radiation on treatment day 1 followed by intratumoral hu14.18-IL2 immunocytokine on treatment days 6-10). **A)** Frequency histogram of the number of mice reaching treatment size for each of the 'treatment waves' reached during the course of the experiment. Blue-green bars correspond to the SC waves and red-purple-brown bars correspond to ID waves. **B)** Overlap in time to treatment size between ID and SC implanted tumors occurred for multiple SC and ID mice only on post-implant day 27, in which n=3 mice of both ID-implanted (pink) and SC-implanted (green) groups reached the 190-230mm^3 treatment window. Shown is the average +/- SEM tumor volume following ISV treatment starting on post-implant day 27, to enable comparison of treatment outcome for ID and SC tumors when all treated tumors have the same tumor size and growth rate at the time treatment is initiated.

Supplemental Figure 2: Controlling for starting size, ID tumors are more likely to respond to RT + IC in situ vaccination. Female, C57BL/6 mice were injected on the same day intentionally aiming for either Intradermal (ID) or Subcutaneous (SC) placement of the tumor. Mice were monitored weekly, and whenever tumors reached treatment size (190-230mm^3), they were collected into a treatment group and treated with either PBS control or our previously published in situ vaccine (12Gy external beam radiation on treatment day 1 followed by intratumoral hu14.18-IL2 immunocytokine on treatment days 6-10). For **A** and **B**, the Average +/- SEM tumor volume is plotted for each individual wave, for the SC tumors (in **A,** shades of blue) and the ID tumors (in **B,** shades of red) to enable comparison of treatment

outcome for 190-230mm^3 tumors. Although numbers are small, there are no significant differences in overall survival between the 4 waves shown in **A**, and there are no significant differences between any of the 8 waves plotted in **B** as determined by Cox proportional hazards modeling, for both ID (p=0.877) and SC (p=0.340) implanted tumors. This indicates that in this setting, the initial growth rate of the tumor is not influencing response to treatment. **C)** Tumor volumes for each individual mouse in the 4 waves of treatment for SC mice. **D)** Tumor volumes for each individual mouse in the 8 waves of treatment for ID mice.

Supplemental Figure 3: Depth of implantation impacts growth rate and degree of response to multiple immunotherapies in the CT26 cancer model. A) Treatment Schema. Female, 6 week old Balb/c mice were implanted intentionally either ID or SC with 1x10^6 CT26 colon adenocarcinoma cells [20] as described in the methods section. Mice were then randomized to receive either no treatment, a single IT injection of 50µg CpG1826 (TriLink Biotechnologies) on day 5 post-implantation (orange arrow), or a single IP injection of 200µg anti-CTLA4 (IgG2c, Bristol-Myers Squibb) antibody on day 7 post-implantation (green arrow) [21]. **B)** For all mice, tumors were measured in a blinded fashion, and the mobile or fixed physical status of the tumor was evaluated and recorded. The table in B documents the physical exam status for every mouse in the experiment on each measurement day. Mobile tumors were coded red, fixed tumors were coded blue, and tumors with both properties or intermediate findings were coded purple. Tumors that were not yet palpable were coded grey. Mice that had a tumor and were rendered disease free have a 'DF' in the cell in this graph starting on the measurement day tumor was no longer palpable. Mice that were euthanized due to tumor burden or found dead from metastases have an 'X' in the cell in this graph starting on the measurement day they were euthanized or found dead. Results demonstrate that, like the B78 melanoma tumor model in C57Bl/6 mice, the CT26 colon adenocarcinoma in Balb/c mice can develop in both the ID and SC space, and that the vast majority of intentionally ID-implanted

tumors exhibit the “mobile” physical exam finding, and the vast majority of intentionally SC-implanted tumors exhibit the “fixed” physical exam finding. For all experiments, only ID-implanted mice with ‘mobile’ tumors and SC-implanted mice with ‘fixed’ tumors for greater than half of all measurement days were included in the analysis of tumor growth or survival graphs (C-J, below); (the number of mice included in analyses of tumor growth or survival per group, n, out of the 10 initial mice implanted in each group, is indicated below each graph). **C and D)** average tumor volume for ID (red) and SC (blue) tumors in response to no treatment (dotted lines), CpG immunotherapy (solid lines in **C**), or anti-CTLA4 immunotherapy (solid lines in **D**). Note that when one mouse in the group dies, the average can no longer be graphed. **E)** Individual and mean +/- SEM tumor volumes for each of the treatment groups represented in C and D on day 19 post implantation, the last day for which all mice were alive. Statistical analysis was conducted using a one-way ANOVA. No corrections were made for multiple comparisons. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. **F, G, and H)** Tumor volumes for each individual mouse in each treatment group are plotted over the course of the experiment to demonstrate the range and variability seen. ID tumors are depicted in red, and SC tumors are depicted in blue. **F** shows all mice treated with IT CpG, **G** shows all mice treated with anti-CTLA4, and **H** shows all untreated tumors (dotted lines). **I)** Survival analysis of ID (red) and SC (blue) tumor-bearing untreated mice (dotted lines), and mice treated with CpG (solid lines). **J)** Survival analysis of ID (red) and SC (blue) tumor-bearing untreated mice (dotted lines), and mice treated with anti-CTLA4 (solid lines). Survival analysis in **I** and **J** was conducted using the Kaplan-Meier method, with comparisons between groups conducted using log-rank tests. Results demonstrate that on day 19 post implantation, untreated SC tumors were significantly larger than untreated ID tumors ($p<0.0001$), and untreated ID-implanted mice had a significantly longer overall survival ($p < 0.01$) compared to untreated SC-implanted mice. Untreated SC-implanted mice also had significantly larger tumors than SC-implanted mice treated with CpG ($p<0.0001$) or anti-CTLA4 ($p<0.0001$) on day 19 post implantation. SC-implanted mice treated

with anti-CTLA4 also had significantly larger tumors than ID-implanted mice treated with anti-CTLA4 by day 19 ($p<0.05$). Due to a mouse in the SC-implanted untreated group dying on day 19 post implantation, group comparisons beyond this point for mice with SC-implanted tumors were not technically possible. Nevertheless, comparison of remaining groups on day 26 post implantation by one-way ANOVA demonstrated that ID-implanted untreated mice had significantly larger tumors than ID-implanted mice treated with CpG ($p<0.05$) as well as ID-implanted mice treated with anti-CTLA4 ($p<0.01$). Compared to SC-implanted untreated mice, a greater overall survival was detected in SC-implanted mice treated with anti-CTLA4 ($p<0.001$), but not for mice treated with CpG. Compared to ID-implanted untreated mice, a trend toward greater overall survival was detected in ID-implanted mice treated with anti-CTLA4 ($p=0.064$). No difference in survival was detected among ID-implanted mice treated with CpG vs. untreated ID implanted mice. However ID-implanted mice treated with CpG did have a greater overall survival compared to SC-implanted mice treated with CpG ($p<0.01$). Together, these data suggest that in the CT26 tumor model implanted in balb/c mice, ID- and SC-implanted tumors may behave differently in both the untreated and immunotherapy-treated settings. These CT26 tumors display the same fixed/mobile physical properties associated with SC vs. ID implantation that would allow for more homogeneous selection of study populations when selected for either fixed or mobile features. See Supplemental Table 1 for a summary of all p values referenced for the combinations of tumor depths and immunotherapies studied in this CT26 model.

Comparator A	Comparator B	Parameter	P-value
SC-untreated	ID-untreated	Day 19 tumor volume	<0.0001
SC-untreated	SC-CpG	Day 19 tumor volume	<0.0001
SC-untreated	SC-anti-CTLA4	Day 19 tumor volume	<0.0001
SC-anti-CTLA4	ID-anti-CTLA4	Day 19 tumor volume	0.0359
SC-CpG	ID-CpG	Day 19 tumor volume	0.1290
ID-untreated	ID-CpG	Day 26 tumor volume	0.0358
ID-untreated	ID-anti-CTLA4	Day 26 tumor volume	0.0055
ID-CpG	SC-CpG	Day 26 tumor volume	0.0018
ID-untreated	SC-untreated	Overall survival	0.0014
ID-CpG	ID-untreated	Overall survival	0.3589
SC-CpG	SC-untreated	Overall survival	0.2685
ID-CpG	SC-CpG	Overall survival	0.0010
ID-CTLA4	ID-untreated	Overall survival	0.0675
SC-CTLA4	SC-untreated	Overall survival	0.0005
ID-CTLA4	SC-CTLA4	Overall survival	0.1464

Supplemental Table 1: Summary p-values for the analyses conducted for the CT26 colon adenocarcinoma model, corresponding with Supplemental Figure 3. Day 19 and Day 26 analyses were conducted using a one-way ANOVA, with multiple comparisons conducted using the Fisher's Least Square Difference test. Survival comparisons were conducted using the log-rank test of Kaplan-Meier estimates. P-values were not corrected for multiple comparisons.

Supplemental Figure 4: Depth of implantation impacts growth rate and degree of response to multiple immunotherapies in the Panc02 cancer model. A) Treatment Schema. Female, 6 week old C57Bl/6 mice were implanted either intentionally ID or SC with $1x10^6$ Panc02 pancreatic adenocarcinoma cells [22] as described in the methods section. Mice were then randomized to receive either no treatment, a combination of 250 µg/dose agonist CD40 antibody (blue arrow, derived from nude mice ascites following FGK 45.5 hybridoma cell injection and ammonium sulfate precipitation as described previously [23]) intraperitoneally on days 5 and 12 post implantation plus intratumoral injections of 50 µg/dose CpG (orange arrow, TriLink Biotechnologies) on days 8 and 15 post implantation, or a combination of intraperitoneal injections of 200 µg/dose anti-CTLA4 antibody (green arrow, IgG2c, Bristol-Myers Squibb) on

days 2, 5, and 8 post implantation plus intratumoral injections of 75,000 biological units of IL2 (red arrow, BRB Preclinical Biologics Repository) on days 5-9 post implantation. **B)** For all mice, tumors were measured in a blinded fashion, and the mobile or fixed physical status of the tumor was evaluated and recorded. The table in **B** documents the physical exam status for every mouse in the experiment on each measurement day. Mobile tumors were coded red, fixed tumors were coded blue, and tumors with both properties or intermediate findings were coded purple. Tumors that were not yet palpable were coded grey. Mice that had a tumor and were rendered disease free have a 'DF' in the cell in this graph starting on the measurement day tumor was no longer palpable. Mice that were euthanized due to tumor burden or found dead from metastases have an 'X' in the cell in this graph starting on the measurement day they were euthanized or found dead. Results demonstrate that, like the B78 melanoma tumor model in C57Bl/6 mice, Panc02 pancreatic adenocarcinoma in C57Bl/6 mice can develop in both the ID and SC space, and that the vast majority of intentionally ID-implanted tumors exhibit the "mobile" physical exam finding, and the vast majority of intentionally SC-implanted tumors exhibit the "fixed" physical exam finding. For all experiments, only ID-implanted mice with 'mobile' tumors and SC-implanted mice with 'fixed tumors for greater than half of all measurement days were included in the analysis\ of tumor growth graphs (C-J, below); (the number of mice included in analyses of tumor growth per group, n, out of the 9 or 10 initial mice implanted in each group, is indicated below each graph). **C and D)** average tumor volume for ID (red) and SC (blue) tumors in response to no treatment (dotted lines), CD40 + CpG immunotherapy (solid lines in **C**), or anti-CTLA4 + IL2 immunotherapy (solid lines in **D**). Note that when one mouse in the group dies, the average can no longer be graphed. **E)** Individual and mean +/- SEM tumor volumes for each of the treatment groups represented in **C** and **D** on day 23 post implantation, the last day for which all mice were alive. Statistical analysis was conducted using a one-way ANOVA with multiple comparisons performed using uncorrected Fisher's Least Square Difference. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. **F,**

G, and H) Individual tumor volumes for each individual mouse in each treatment group are plotted over the course of the experiment to demonstrate the range and variability seen. Results show that untreated ID-implanted and SC-implanted tumors grew at approximately the same rate with no statistical difference detected in day 23 tumor volume. However, due to invasion of the leg musculature and innervation, SC-implanted mice were more likely to meet criteria for euthanasia per our animal protocol, which did translate to a statistically significantly greater overall survival for ID-implanted mice compared to SC-implanted mice (log rank analysis, p =0.01); these data are evident by the indication of dead or euthanized mice in the untreated groups in **B**. ID-implanted mice treated with both CD40 + CpG and anti-CTLA4 + IL2 immunotherapies had a trending lower tumor volume at day 23 post implantation compared to ID-implanted untreated mice (p=0.07 and 0.054, respectively) (**E**). On day 23 post implantation, ID-implanted mice treated with CD40 + CpG had a lower tumor volume than SC-implanted mice ($p<0.05$, **E**). Similarly, ID-implanted mice treated with anti-CTLA4 + IL2 had a lower tumor volume than SC-implanted mice ($p<0.01$, **E**). Due to a mouse in the SC-implanted untreated group dying on day 23 post implantation, group comparisons beyond this point were not technically possible. Nevertheless, comparison of tumor size in the remaining groups on day 30 post implantation (in **C** and **D**) by one-way ANOVA demonstrated that ID-implanted untreated mice had significantly larger tumors than ID-implanted mice treated with either CD40 + CpG ($p < 0.001$) or with anti-CTLA4 + IL2 ($p < 0.001$). It should be noted, however, that a difference between ID-implanted and SC-implanted tumors treated with CD40+CpG could no longer be detected at day 30 post implantation. Together, these data suggest that the Panc02 tumor line in C57Bl/6 mice displays the same mobile/fixed physical exam findings associated with either ID or SC localization of the tumor, and may exhibit a difference in response to either CD40+CpG or anti-CTLA4 + IL2 immunotherapy based on implanted tumor depth. See Supplemental Table 2 for a summary of all p values referenced for the combinations of tumor depths and immunotherapies studied in this Panc02 model.

Comparator A	Comparator B	Parameter	P-value
SC-untreated	ID-untreated	Day 23 tumor volume	0.1197
ID-untreated	ID-CD40+CpG	Day 23 tumor volume	0.0758
ID-untreated	ID-CTLA4 + IL2	Day 23 tumor volume	0.0543
ID-CD40+CpG	SC-CD40+CpG	Day 23 tumor volume	0.0265
ID-CTLA4 + IL2	SC-CTLA4 + IL2	Day 23 tumor volume	0.0042
ID-untreated	ID-CD40+CpG	Day 30 tumor volume	<0.0001
ID-untreated	ID-CTLA4 + IL2	Day 30 tumor volume	<0.0001
ID-CD40+CpG	SC-CD40+CpG	Day 30 tumor volume	0.5925
ID-CTLA4 + IL2	SC-CTLA4 + IL2	Day 30 tumor volume	0.00091

Supplemental Table 2: Summary p-values for the analyses conducted for the pancreatic adenocarcinoma model, corresponding with Supplemental Figure 4. Day 23 and Day 30 analyses were conducted using a one-way ANOVA, with multiple comparisons conducted using the Fisher's Least Square Difference test. P-values were not corrected for multiple comparisons.

Supplemental Figure 5: Depth of implantation impacts growth rate, overall survival, and degree of response to combination anti-CTLA4 and immunocytokine immunotherapy in the B16-GD2 melanoma model. A) Treatment Schema. Female, 6 week old C57Bl/6 mice were implanted either intentionally ID or SC with $5x10^5$ B16-GD2 murine melanoma cells (B16-F10 melanoma cells transfected to express GD2 as described previously [23]) as described in the methods section. Mice were then randomized to receive either no treatment or a combination of 200μg intraperitoneal anti-CTLA4 antibody (green arrow, IgG2c, Bristol-Myers Squibb) on day 5 post implantation plus intratumoral injections of 50 g/dose hu14.18-IL2 immunocytokine (IC) as described in the Methods section (black arrows) on days 2-6 post implantation. **B)** For all mice, tumors were measured in a blinded fashion, and the mobile or fixed physical status of the tumor was evaluated and recorded. The table in B documents the physical exam status for every mouse in the experiment on each measurement day. Mobile tumors were coded red, fixed tumors were coded blue, and tumors with both properties or

intermediate findings were coded purple. Tumors that were not yet palpable were coded grey. Tumor-bearing mice that were rendered disease free have a 'DF' in the cell in this graph starting on the measurement day tumor was no longer palpable. Mice that were euthanized due to tumor burden or found dead from metastases have an 'X' in the cell in this graph starting on the measurement day they were euthanized or found dead. Results demonstrate that, like the B78 melanoma tumor model in C57Bl/6 mice, the B16-GD2 model in C57Bl/6 mice can develop in both the ID and SC space, and that the majority of intentionally ID-implanted tumors exhibit the "mobile" physical exam finding, and the majority of intentionally SC-implanted tumors exhibit the "fixed" physical exam finding. Of note, two ID-implanted tumors in the untreated group failed to develop, but almost all of the mice with ID-implanted tumors that were treated with immunotherapy (which began before tumors were palpable) did not develop a tumor. For all experiments, ID-implanted mice with 'fixed' tumors and SC-implanted mice with 'mobile' tumors for greater than half of the measurement days they could be measured were excluded from the analysis (number of mice per group, n, indicated below each graph, of 9 or 10 mice initially implanted in each group). **C)** Average tumor volume for ID (red) and SC (blue) tumors in response to no treatment (dotted lines) or anti-CTLA4 + IC immunotherapy (solid lines). Note that when one mouse in the group dies, the average can no longer be graphed. **D)** Survival curves of ID (red) and SC (blue) B16-GD2 tumor-bearing untreated mice (dotted lines), and mice treated with anti-CTLA4+IC immunotherapy (solid lines). Survival analysis was conducted using the Kaplan-Meier method, with comparisons between groups conducted using log-rank tests. Statistical comparison of these survival times are shown in Supplemental Table 3. **E)** Individual and mean +/- SEM tumor volumes for each of the treatment groups on day 17 post implantation, the last day for which all mice were alive. Statistical analysis was conducted using a one-way ANOVA with multiple comparisons performed uncorrected Fisher's Least Square Difference. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. **F and G)** Individual tumor volumes are plotted over the course of the experiment for all untreated mice (F) and

immunotherapy treated mice (G). Results demonstrate that untreated SC-implanted mice exhibited larger tumors on day 17 post implantation compared to untreated ID-implanted mice ($p < 0.01$) and had a significantly shorter overall survival ($p < 0.05$). No difference (in tumor volume or overall survival) could be detected between SC-implanted mice treated with anti-CTLA4 + IC immunotherapy and untreated SC-implanted mice. By contrast, ID-implanted tumors treated with anti-CTLA4+IC had a smaller average tumor volume on day 17 post implantation compared to SC-implanted treated tumors ($p<0.05$) treated similarly and also had a significantly longer overall survival ($p<0.0001$) than mice with SC-implanted tumors treated similarly. No statistically significant difference could be detected between ID-implanted tumors treated with immunotherapy compared to untreated ID-implanted tumors due to the substantial variance in the experimental group attributable to the SC-implanted untreated group using the one-way ANOVA. However, a student's t test comparing ID-implanted treated and untreated tumors shows they are substantially different ($p = 0.018$). Further, 0 of 8 ID-implanted mice treated with anti-CTLA4 + IC developed a palpable tumor (after excluding the single ID-implanted mouse with a 'fixed' phenotype as indicated in B), whereas all 9 of 9 SC-implanted mice developed tumors ($p < 0.0001$ by fisher's exact test). This does not appear to be a byproduct of only implantation depth, as 5 out of 7 ID-implanted untreated mice developed tumors ($p < 0.001$ by fisher's exact test, when compared to the 0 of 8 mice with ID-implanted tumors that received anti-CTLA4 + IC). Together, these data show that implantation depth may have a profound influence on baseline growth rate, as well as degree of response to anti-CTLA4 + IL2 immunotherapy. Indeed, the B16-GD2 model under these conditions may represent the extreme case where SC-implanted tumors appear to have no response to immunotherapy (no difference from SC-implanted untreated tumors), and ID-implanted tumors have a 'maximum' response to immunotherapy (almost no tumors develop during the experimental period). See Supplemental Table 3 for a summary of all p values referenced for the combinations of tumor depths and immunotherapies studied in this B16-GD2 model.

Comparator A	Comparator B	Parameter	P-value
SC-untreated	ID-untreated	Day 17 tumor volume	0.0004
SC-untreated	SC-anti-CTLA4 + IC	Day 17 tumor volume	0.1706
SC-anti-CTLA4 + IC	ID-anti-CTLA4+IC	Day 17 tumor volume	0.0026
ID-untreated	ID-anti-CTLA4+IC	Day 17 tumor volume (LSD)	0.5985
ID-untreated	ID-anti-CTLA4+IC	Day 17 tumor volume (Students t test)	0.0182
SC-untreated	ID-untreated	Overall Survival	0.0124
SC-untreated	SC-anti-CTLA4 + IC	Overall Survival	0.9096
SC-anti-CTLA4 + IC	ID-anti-CTLA4+IC	Overall Survival	<0.0001
ID-untreated	ID-anti-CTLA4+IC	Overall Survival	0.0152

Supplemental Table 3: Summary p-values for the analyses conducted for the B16-GD2 melanoma model, corresponding with Supplemental Figure 5. Day 17 analyses were conducted using a one-way ANOVA, with multiple comparisons conducted using the Fisher's Least Square Difference test except where otherwise noted. Survival comparisons were conducted using the log-rank test of Kaplan-Meier estimates. P-values were not corrected for multiple comparisons.

Supplemental Figure 6: Depth of implantation affects tumor growth rate in untreated mice, but not response to potent anti-CTLA4 plus IL2 immunotherapy, in the MOC2 tumor model. A) Treatment Schema. Female, 6 week old C57Bl/6 mice were implanted either intentionally ID or SC with $1x10^6$ MOC2 murine squamous cell head and neck cancer cells [24] as described in the methods section. Mice were then randomized to receive either no treatment, or a combination of intraperitoneal injections of anti-CTLA4 antibody (green arrow, IgG2c, Bristol-Myers Squibb) on days 2, 5, and 8 post implantation plus intratumoral injections of 75,000 biological units of IL2 (red arrow, BRB Preclinical Biologics Repository) on days 5-9 post implantation. **B)** For all mice, tumors were measured in a blinded fashion, and the mobile or fixed physical status of the tumor was evaluated and recorded. The table in B documents the physical exam status for every mouse in the experiment on each measurement day. Mobile

tumors were coded red, fixed tumors were coded blue, and tumors with both properties or intermediate findings were coded purple. Tumors that were not yet palpable were coded grey. Tumor-bearing mice that were rendered disease free have a 'DF' in the cell in this graph starting on the measurement day tumor was no longer palpable. Mice that were euthanized due to tumor burden or found dead from metastases have an 'X' in the cell in this graph starting on the measurement day they were euthanized or found dead. Results demonstrate that, like the B78 melanoma tumor model, the MOC2 model in C57Bl/6 mice can develop in both the ID and SC space, and that the majority of intentionally ID-implanted tumors exhibit the "mobile" physical exam finding, and the majority of intentionally SC-implanted tumors exhibit the "fixed" physical exam finding. For all experiments, only ID-implanted mice with 'mobile' tumors and SC-implanted mice with 'fixed tumors for greater than half of all measurement days were included in the analysis (number of mice per group, n, indicated below each graph, out of the 9 or 10 mice initially injected in each group). **C)** Average tumor volume for ID (red) and SC (blue) tumors in response to no treatment (dotted lines) or anti-CTLA4 + IL2 immunotherapy (solid lines). **D)** Individual and mean +/- SEM tumor volumes for each of the treatment groups represented in **C** on day 32 post implantation, the last day of the experiment. Statistical analysis was conducted using a one-way ANOVA with multiple comparisons performed using uncorrected Fisher's Least Square Difference. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. **E and F)** Individual tumor volumes are plotted over the course of the experiment for all untreated mice (**E**) and for all anti-CTLA4 + IL2 immunotherapy treated mice (**F**). Results demonstrate that untreated SC-implanted mice had larger untreated tumors at day 32 post implantation compared to ID-implanted mice ($p < 0.0001$). Untreated SC-implanted mice had significantly larger tumors than anti-CTLA4 + IL2 treated SC-implanted mice ($p<0.0001$). Similarly, untreated ID-implanted mice had tumors that showed a trend toward being larger than immunotherapy treated mice with ID-implanted tumors ($p = 0.054$). However an effect of implantation depth was not detected between SC-implanted and ID-implanted mice treated with anti-CTLA4 + IL2 immunotherapy;

this is because the vast majority of tumors implanted at both depths were rendered disease-free by treatment by day 32. However, although not evaluated as a predetermined study endpoint, the data do appear to suggest that the SC-implanted tumors treated with immunotherapy are larger than the ID-implanted tumors treated with immunotherapy on day 14, before all immunotherapy-treated tumors begin to completely respond. This suggests that the effect of immunotherapy on ID vs. SC implanted tumors may vary depending on the tumor model under investigation. It is certainly conceivable that in the MOC2 model, a less potent immunotherapy (anti-CTLA4 alone or IT IL2 alone, for example) may have elicited a difference in response between ID- and SC-implanted mice. These data suggest that in the MOC2 model, tumors grow at different rates based on tumor implantation depth, though both ID and SC implanted tumors are similarly responsive to potent anti-CTLA4 + IL2 immunotherapy when this regimen is applied under these conditions. See Supplemental Table 4 for a summary of all p values referenced for the combinations of tumor depths and immunotherapies studied in this MOC2 model.

Comparator A	Comparator B	Parameter	P-value
SC-untreated	ID-untreated	Day 32 tumor volume	<0.0001
SC-untreated	SC-anti-CTLA4 + IL2	Day 32 tumor volume	<0.0001
ID-untreated	ID-anti-CTLA4 + IL2	Day 32 tumor volume	0.0524
SC-anti-CTLA4 + IC	ID-anti-CTLA4 + IL2	Day 32 tumor volume	>0.9999

Supplemental Table 4: Summary p-values for the analyses conducted for the MOC2 head and neck cancer model, corresponding with Supplemental Figure 6. Day 32 analyses were conducted using a one-way ANOVA, with multiple comparisons conducted using the Fisher's Least Square Difference test. P-values were not corrected for multiple comparisons.

Supplemental Video 1: Demonstration of “Fixed” phenotype of implanted B78 tumor. The C57BL/6 mouse in the video was implanted SC with B78 syngeneic melanoma cells and allowed to grow for 4 weeks. The tumor exhibited a “fixed” phenotype on physical exam following lateral displacement of the skin. Motion of the finger in the video causes the skin to displace with the finger, sliding over the deeper musculature and tumor. The tumor remains “fixed” to the underlying structures and does not appear to be physically connected to the skin compartment.

Supplemental Video 2: Demonstration of “Mobile” phenotype of implanted B78 tumor. The C57BL/6 mouse in the video was implanted ID with B78 syngeneic melanoma cells and allowed to grow for 4 weeks. The tumor exhibited a “mobile” phenotype on physical exam following lateral displacement of the skin. As skin is displaced by the finger in the video, the tumor displaces as well, moving with the skin. This tumor phenotype is almost fully associated with the true skin compartment and does not appear attached or anchored to any underlying tissue.