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A Mouse Model of Incompletely Resected Soft Tissue Sarcoma for Testing Perioperative Treatments to Prevent Local Recurrence

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RE: JoVE submission JoVE60882

16 December 2019

Dear Dr Dsouza,

We thank you and the reviewers for the constructive comments which have helped to improve the quality of the manuscript. Please find attached the revised manuscript and a point-by-point reply to the comments.

Please note that we also added co-author Prof Terrance Johns, who has contributed to the development of the model and has been involved in the analysis and interpretation of the data. Not having Prof Johns as a co-author on this paper in the first version was an oversight from our side. He has read and provided feedback to the manuscript and agrees to be a co-author. All co-authors agree with the addition of Prof. Johns as a co-author.

Sincerely,

Rachael M. Zemek
Willem Joost Lesterhuis
On behalf of all authors

TITLE:

A Mouse Model of Incompletely Resected Soft Tissue Sarcoma for Testing (Neo)adjuvant Therapies

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KEYWORDS:

Soft tissue sarcoma, perioperative, surgical resection, mouse model, debulking surgery.

SUMMARY:

In this protocol, we describe a mouse model of incomplete surgical resection of soft tissue sarcoma for testing (neo)adjuvant therapies.

ABSTRACT:

Surgery is often the first treatment for many solid tumors. However, local relapses frequently occur following primary tumor resection, despite adjuvant or neo-adjuvant therapies. This occurs when surgical margins are insufficiently tumor-free, resulting in residual cancer cells. From a biological and immunological perspective, surgery is not a null event; the wound healing environment is known to induce both pro- and anti-tumorigenic pathways. As a consequence, preclinical models for drug development aimed at preventing local relapse should incorporate surgical resection when testing new (neo)adjuvant therapies, to model the clinical settings in patients treated with surgery.

Here, we describe a mouse model of incomplete surgical resection of WEHI 164 soft tissue sarcoma that allows testing of (neo)adjuvant therapies in the setting of a wound healing response. In this model, 50% or 75% of the tumor is removed, leaving behind some cancer tissue in situ to model gross residual disease after surgery in the clinical setting. This model allows testing therapies in the context of surgery while also considering the wound healing response,

which may affect the efficacy of (neo)adjuvant treatments. The incomplete surgical resection results in reproducible regrowth of the tumor in all mice in the absence of adjuvant therapy. Adjuvant treatment with checkpoint blockade results in reduced tumor regrowth. This model is thus appropriate for testing therapies in the context of debulking surgery and its associated wound healing response and can be extended to other types of solid cancer.

INTRODUCTION:

Surgery remains the main treatment option for many solid tumors[1], including soft tissue sarcoma[2,3]. Despite improvements in cancer surgery techniques, and combinations with (neo)adjuvant therapies, there is still a high risk of cancer relapse and metastasis following primary tumor resection[4,5]. In soft tissue sarcoma, relapses occur particularly locoregionally, at the site of surgery, resulting in increased morbidity and mortality. In the clinical setting, it can be difficult to obtain wide enough margins (e.g., due to anatomical constraints), resulting in incomplete resection and subsequent tumor recurrence[6]. Surgical stress and the subsequent process of wound healing are known to create an immunosuppressive tumor microenvironment favorable for tumor recurrence[7,8]. Therefore, the discovery and development of new therapies for soft tissue sarcoma, particularly immunotherapies, should ideally take the surgical wound healing response into account.

Most preclinical studies for adjuvant therapies are initially carried out using subcutaneous syngeneic or xenotransplant mouse models, without incorporating the surgical stress and wound healing response[9,10]. Therefore, we developed a syngeneic subcutaneous mouse soft tissue sarcoma model incorporating incomplete surgical resection. WEHI 164 fibrosarcoma cells are inoculated subcutaneously, and once tumors are established, we remove 50-75% of the tumor bulk (**Figure 1A-E**). Tumors consistently re-grow from the remaining tumor. This model allows for testing adjuvant therapies while considering the effect of surgical stress and wound healing. Similar surgical models of incomplete resection have been used in a number of studies by several groups and found to be reproducible and effective[11-13]. Here, we provide a detailed description of this protocol.

PROTOCOL:

Animals used in these experiments were obtained from the Animal Resource Centre (Perth, Western Australia). Animals were maintained under standard pathogen-free conditions at the Harry Perkins Institute of Medical Research Bioresources North Facility (Perth, Western Australia). All experiments were carried out following the protocol as approved by the Harry Perkins Institute of Medical Research Animal Ethics Committee. BALB/c mice of 8-12 weeks of age were used in these experiments. The WEHI 164 fibrosarcoma cell line was obtained from CellBank Australia (Westmead, NSW).

1. Inoculation of cells

1.1. Preparation of cells and animals

1.1.1. Ensure that the cell line is maintained in the recommended media. For example, maintain WEHI 164 cell line in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin.

NOTE: Passage cells at least 3 and up to 5 times after being removed from cryogenic storage. To ensure an optimum cell viability, cells should be split when they are between 70-80% confluent. Tumor cell lines should be tested for mycoplasma, as infection can alter the cell growth and influence the immune response in vivo.

1.1.2. One day before inoculation, shave mice on the lower right flank using clippers.

NOTE: Female BALB/c mice, aged between 8-12 weeks, of normal weight (16 -22 grams) were used in this experiment.

1.1.3. On the day of inoculation, harvest WEHI 164 cells when 70-80% confluent by trypsinization.

1.1.3.1. Aspirate the culture medium from the tissue culture flasks and then add sterile phosphate buffered solution (1x PBS), to remove remaining traces of fetal bovine serum (FBS).

1.1.3.2. Aspirate the PBS from the tissue culture flasks. Add 3 mL of 0.05% trypsin (for a T75 flask) and then swirl the flask so that the whole surface of flask with cells is covered by trypsin.

1.1.3.3. Incubate the flask at 37 °C, 5% CO_2 incubator for 3 min. Check cells periodically, by tapping on the sides of the flask to see if cells have dislodged.

1.1.3.4. Remove flasks from cell culture incubator and add 5 mL of media supplemented with FBS to neutralize the trypsin.

NOTE: Do not leave cells in trypsin longer than necessary, as this can damage cells and lead to low cell viability.

1.1.3.5. Pipet suspension multiple times to obtain a single cell suspension. Transfer cell suspension to a conical centrifuge tube.

1.1.3.6. Pellet cells by spinning at 350 x *g* for 3 min.

1.1.4. Wash the cells three times in 1x PBS.

1.1.4.1. Resuspend cells in 50 mL of sterile 1x PBS and wash cells by pipetting cell suspension up and down. Pellet cells by spinning at 350 x *g* for 3 min.

1.1.4.2. Aspirate the supernatant and resuspend cells in 15 mL of sterile 1x PBS. Wash cells by pipetting cell suspension up and down. Pellet cells by spinning at 350 x *g* for 3 min.

1.1.4.3. Aspirate the supernatant and resuspend cells in exactly 10 mL of sterile 1x PBS. Wash cells as in step 1.1.4.2 and transfer a small amount (approximately 100 µL) of cell suspension to an centrifuge tube for counting. Pellet cells by spinning at 350 x *g* for 3 min.

1.1.5. Determine the cell number using the Trypan blue exclusion method by either using a hemocytometer or an automated cell counter. Resuspend cells in sterile 1x PBS at a concentration of 5 x 10^6 cells/mL. Keep cell suspension on ice.

NOTE: The viability of tumor cells should be equal or above 80 % to ensure reproducible tumor growth.

1.2. Subcutaneous inoculation

1.2.1. Mix the cell suspension thoroughly and fill a syringe with a 26 G needle with 100 µL of cell suspension (5 x 10^5 cells) in sterile 1x PBS. Repeat mixing of cells before loading the next syringe.

NOTE: Keep cells on ice throughout the procedure to maintain viability.

1.2.2. Restrain the mouse appropriately, ensuring access to the lower-right flank. Inoculate the mouse subcutaneously on the shaved lower-right flank.

NOTE: Make sure the inoculation is not in the peritoneum by lifting the needle slightly, which should be visible under the skin. A bubble-like lump should form under the skin following inoculation.

1.2.3. Monitor mice as required by the applicable ethics approval and perform surgical resection when the tumours have grown to a size of about 50 mm^2.

2. Partial surgical resection of the tumor

NOTE: This protocol requires TWO researchers; one for surgical procedures (SURGEON), and another for mouse monitoring (ASSISTANT).

2.1. Surgery setup

2.1.1. On day 12 post inoculation, when tumors have reached a size of approximately 50 mm^2, dose mice with 100 µL (0.1 mg/kg) of buprenorphine s.c. in the scruff of the neck, 30 minutes prior to surgery.

2.1.2. Set up the surgical area with a heat pad covered with bench coat and set up a nose cone for anaesthesia. Have the following surgical equipment clean and within easy reach: chlorhexidine, swab, gauze, eye gel, two curved forceps, scissors, clip applicator, clip remover, clip refills (**Figure 2A, 2B**).

2.1.3. Warm the heating chamber to 37 °C and set up another heat pad for recovery (**Figure 2C**).

2.2. Anesthesia

2.2.1. Place the mouse in the induction chamber and anesthetize the mouse with 4% isoflurane (4% in 100% oxygen at a flow rate of 1 L/min) until the breathing rate slows to approximately 60 breaths per minute (1 per second) (this usually takes <1 min).

NOTE: Do not leave the mouse in the chamber for too long as that may lead to asphyxiation and death. Only have one mouse under anesthesia at a time.

2.2.2. Transfer the mouse onto the heat pad on the surgery table, place the mouse with its nose in the nose cone and maintain the anesthetic state with 3-4% isoflurane in 100% oxygen at a flow rate of 0.5 L/min. Monitor the breathing rate to ensure that the depth of anesthesia is maintained.

NOTE: The ASSISTANT must monitor the breathing of the mouse throughout the surgery to ensure the correct level of anesthesia is maintained. Lower the anesthetic concentration if breathing becomes too slow or increase the concentration if the depth of anesthesia is too shallow. If the mouse begins gasping, remove mouse from the nose cone, decrease the anesthetic concentration, and wait until breathing normalises before placing on the nose cone again.

2.2.3. Perform a “pinch test” and “corneal reflex test”[14] to ensure that the mouse is fully anesthetized before commencing surgery.

NOTE: Movement of any part of the mouse is an indication that the mouse is not fully anesthetized. The animal should immediately be given additional anesthetic by increasing the anesthetic concentration.

2.2.4. Cover the mouse’s eyes with a small amount of ophthalmic gel to avoid eye dryness.

2.3. Surgical procedure (SURGEON)

2.3.1. Swab the surgical area with chlorhexidine. Using forceps and a pair of scissors, make a 1 cm straight incision along the dorsal side, 3 mm away from the tumor (**Figure 3A, 3B**).

NOTE: Standardizing the incision to 1 cm in every mouse (using a ruler) allows for even assessment of wound healing between mice. Locating the incision 3 mm away from the tumor allows for subsequent intratumoral adjuvant therapy without leakage from the wound.

2.3.2. Using tweezers, pull away the facia and subcutaneous fatty tissue between the tumor and peritoneum. The subcutaneous tumor is normally attached to the skin-side.

2.3.3. Open the wound by gently holding the skin on the tumor bearing side using tweezers, and "invert" the tumor so that it is visible outside (**Figure 3C, 3D**).

NOTE: The section of tumor to be debulked should be closest to the opening, to have enough skin to close the wound. Be careful not to cut the skin when removing the tumor.

2.3.4. Using a pair of scissors, cut away the tumor capsule from the half to remove, starting from the base of the tumor closest to the opening.

2.3.5. For 50% debulk surgery, cut across the middle of the tumor. Using curved forceps, scoop up the section of the tumor to be removed (50%); scoop up any remnants from the debulked area.

2.3.6. For 75% debulk, perform a 50% tumor debulk as in part 2.3.5 above. Then cut in half the remaining 50% of tumor and scoop up 25% of the tumor, using curved forceps as described above.

2.4. Closing the surgical site

2.4.1. Place the remaining tumor back underneath the skin, and using forceps, pull the skin flaps together and line up the skin along the wound.

2.4.2. Hold the skin together 5 mm from the edge of the wound, and use surgical clips to close the wound, starting on the side closest to the forceps. Apply as many clips as needed to ensure no underlying tissue is exposed. Generally, three to four clips are applied with 2 mm gaps between clips.

NOTE: If any clips is not well applied, remove it using a clip remover and replace with new clips.

2.5. Recovery of mice (ASSISTANT)

2.5.1. Allow the mice to recover by putting them into the warm (37 °C) heating chamber.

2.5.2. Place the mouse's cage on the heat pad. Monitor the mice in the heating chamber until they have recovered from the anesthetic (awake and walking) and then put the mice back into the cage. Leave the cage on the heat pad for a further 10 minutes, until the mice have become more active.

2.5.3. Give the mice wet and soft food. Monitor the mice 1 hour after surgery for recovery and ensure clips remain in place.

2.5.4. Dose mice with 0.1 mg/kg buprenorphine (100 µL subcutaneously in the scruff of the neck), 6-8 hours after surgery (at the end of the day). Monitor mice early the following morning, and dose mice again with 0.1 mg/kg buprenorphine (100 µL subcutaneously in the scruff of the neck).

Give more wet food as needed.

2.5.5. Monitor mice daily for the next seven days. Clips may be removed after seven days using the clip remover.

2.6. Adjuvant or neoadjuvant treatment

2.6.1. Treat mice peri-operatively with (neo)adjuvant therapy at any given time, depending on the treatment of interest.

2.6.2. For example, treat mice with one dose of 100 µg of anti-CTLA-4 intraperitoneally (i.p.) on day 15 after inoculation, or with three doses of 200 µg anti-PD-1 i.p. on day 15, 17 and 19 after inoculation.

2.7. Experimental controls

2.7.1. When using this model to assess the effects of inflammation/wound healing, consider using the following control groups: 1) No-surgery control (treatments can still be administered intratumorally); 2) Sham surgery control: A surgical incision is made in the skin; the tumor is manipulated and exposed, but no tumor tissue is removed; the wound is closed with clips.

REPRESENTATIVE RESULTS:

Tumor growth to a size of 50 mm^2 is an ideal size for partial debulk. The incomplete surgical resection of 50 mm^2 tumors results in 100% (n=5) reproducible regrowth of the tumors in the absence of adjuvant immunotherapy (**Figure 4A**). We next used the model to test adjuvant immunotherapies using antibodies against checkpoint molecules Cytotoxic T Lymphocyte Associated Protein 4 (CTLA-4) and Programmed Death Receptor 1 (PD-1). Treatment of mice with anti-CTLA-4 or anti-PD-1 resulted in a cure rate of 80% and 25% (n=4-5 per group), respectively (**Figure 4B, 4C**). The response with anti-PD-1 provides an opportunity to test novel combinations to improve the response rate further.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of partial surgical resection of the tumor. (**A**) BALB/c mice are inoculated with 5 x 10^5 WEHI-164 cells on the lower right flank. (**B**) When the tumor reaches 50 mm^2, surgery can commence. (**C**) The tumor is partially resected (50 % shown). (**D**) The surgical site is closed with clips. (**E**) Adjuvant therapy can be administered, intravenously, intraperitoneally (shown) or intratumorally in the wound area.

Figure 2: Representative images of the surgery set up. (**A**) A whole image of the surgery set up showing the surgical tools (listed in step 2.1) and the anesthetic machine. (**B**) A snapshot image of the surgical table showing all materials within an easy reach. (**C**) A heating chamber and a heating pad for mouse recovery.

Figure 3: Representative pictures of partial tumor debulk technique. (**A**) A fully anesthetized mouse with a tumor of 50 mm^2 in size before surgery. (**B**) Incision site 3 mm away from the tumor; 1 cm incision. (**C-D**) Opening of the wound by gently holding the skin on the tumor bearing side using tweezers, and "inverting" the tumor so that it is visible outside.

Figure 4: Tumor regrowth following incomplete tumor resection and immunotherapy. (**A**) Tumor regrowth curves of partially resected WEHI-164 tumors in the absence of adjuvant immunotherapy. (**B-C**) Tumor regrowth after surgery and adjuvant treatment with anti-CTLA-4 (**B**) or anti-PD1 (**C**). The dotted line indicates the day of surgery.

DISCUSSION:

We provide a protocol for a mouse model of incomplete surgical resection of soft tissue sarcoma to test peri-operative therapies. We also standardized the surgical incision to allow assessment of wound healing between mice following treatment.

Tumor placement is an important part of this protocol. We have opted for a subcutaneous tumor model to allow easy surgical access to the tumor site and administration of local therapies with minimal burden on the mice. It is also important to ensure that the tumors grow in the subcutaneous space and not within the peritoneum, which can result in unexpected morbidity and mortality.

When choosing a tumor cell line for this protocol, we advise that the cells when grown in vivo form a solid mass (e.g., WEHI-164 model), rather than a semi-solid mass (such as the B16 model) as it is technically difficult to partially resect. In addition, if the tumor begins to grow through the skin (usually seen in tumors larger than 100 mm^2), debulking is not recommended as the skin may become necrotic and not heal well after surgery. We have overcome this problem by debulking tumors once they reach 50 mm^2 in size.

As our model can be used to assess the effect of wound healing on therapy, we propose a control/sham group as a comparison. The control may be unaltered tumor, or sham surgery which would only have the skin incision, exposure of the tumor, and wound closure without partial tumor debulk. This sham control group could be used when discerning the effect of surgery-induced inflammation and wound healing from the partial debulk on the treatment outcome.

For successful partial debulking surgery, some technical points need to be considered. An important aspect is the correct implantation and growth of the tumor. Tumors need to be implanted on the lower-right flank, away from the hind leg. Tumors that are implanted too close to the hind leg can interfere with their ability to walk and can result in extra force on the clips causing them to come loose. In addition, consistency in tumor size is critical in order to avoid variability in the relative percentage of debulking. We chose to perform surgery with tumors that have a size of 50 mm^2, to make surgery technically straightforward, although we envisage that partial resection on smaller tumors is feasible. To prevent inconsistency in tumor size, the used cell line needs to be passaged following the appropriate standard cell culture techniques, and the

researcher needs to be adequately trained in the proper tumor inoculation technique. When extending this protocol to other subcutaneous tumor models, the physical characteristics of the tumor are of importance. For example, we found that cell lines that give rise to soft, gelatinous tumors (e.g., M3-9-M rhabdomyosarcoma, and B16 melanoma[15]) are technically challenging to debulk.

There are also technical points that need to be considered during surgery. Mice need to be adequately anesthetized to prevent movement during the procedure. Apart from the impost that inadequately anesthetized mice will endure, any movement of mice during the procedure can make the surgical resection difficult, resulting in variability in the size of tumor removed between mice. In addition, mouse respiratory rate should be carefully monitored during surgery Isoflurane concentration should be adjusted to maintain the appropriate depth of anesthesia. Therefore, an assistant is always needed during the surgical procedure to monitor the breathing rate during surgery, and to ensure an adequate level of anesthesia. The size of the incision needs to be consistent in order to avoid variability in the wound healing response. We found that a 1-1.5 cm incision is sufficient for tumor debulking, with a minimal chance of wound dehiscence.

Our model of partial resection mimics residual disease remaining after surgery as seen in the clinical setting of many solid tumors and offers advantages over traditional syngeneic mouse models by taking into account the effect of surgical wound healing. In addition, existing traditional models of surgery have used complete tumor resection, which does not always result in tumor recurrence[16]. Other researchers have successfully used partial resection models using other cancer cell lines [11-13], underscoring the robustness of this method. Furthermore, it has been demonstrated that partial resection, but not complete resection, resulted in protective anti-tumor immune memory when adjuvant therapy is given[12] which was attributed to the persistence of antigens from the residual tumor.

This model is designed to study the effect of inflammation and wound healing on therapy. Our debulking approach clinically resembles clinical situations where gross residual disease is left behind after surgery (R2 resection), rather than macroscopically complete resection with microscopic residual disease (R1 resection). For example, surgical resection in invasive soft tissue sarcoma can result in positive margins when the tumor is located next to critical structures such as nerves, arteries or adjacent organs, precluding complete resection with wide margins[17]. Surgery models for resection resulting in microscopic positive margins have been published[13]; our protocol can be used to study the effect of the wound healing response on therapy when macroscopic residual disease is present.

A limitation of our model is that it does not give rise to distant relapse and micrometastasis, which is common after surgery in solid tumors such as breast cancer or pancreatic cancer. Other surgery models, such as the murine breast cancer model 4T1[18-20] or murine models of de novo mammary cancer metastasis[21] are better suited to investigate systemic relapse after local resection. Another limitation is that this protocol is for subcutaneous models and thus does not allow assessment of tissue-specific pathology. For this purpose, orthotopic tumor mouse models are appropriate[7,22,23]. However, orthotopic models are more challenging and usually involve

greater impost to mice, and are more laborious and costly[22]. Subcutaneous models are well suited to assess the effects of (neo-)adjuvant therapies, either systemically or locally, on local cancer relapse, in a cost-effective and relatively high-throughput manner with minimal impost to the animals.

The incomplete partial resection as outlined in this protocol is useful for testing adjuvant therapies while incorporating surgical wound healing as a factor, a variable which is often overlooked.

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DISCLOSURES:

No disclosures

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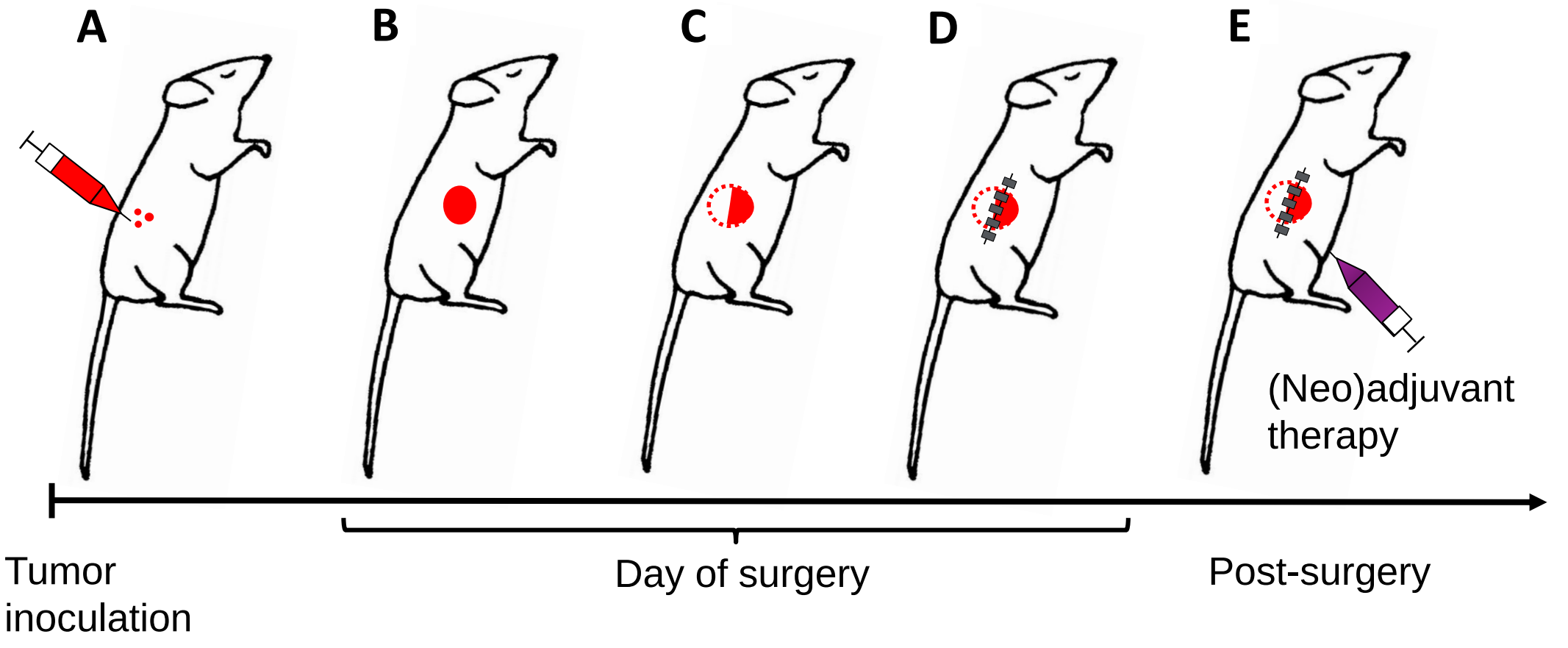

Figure 1

Figure

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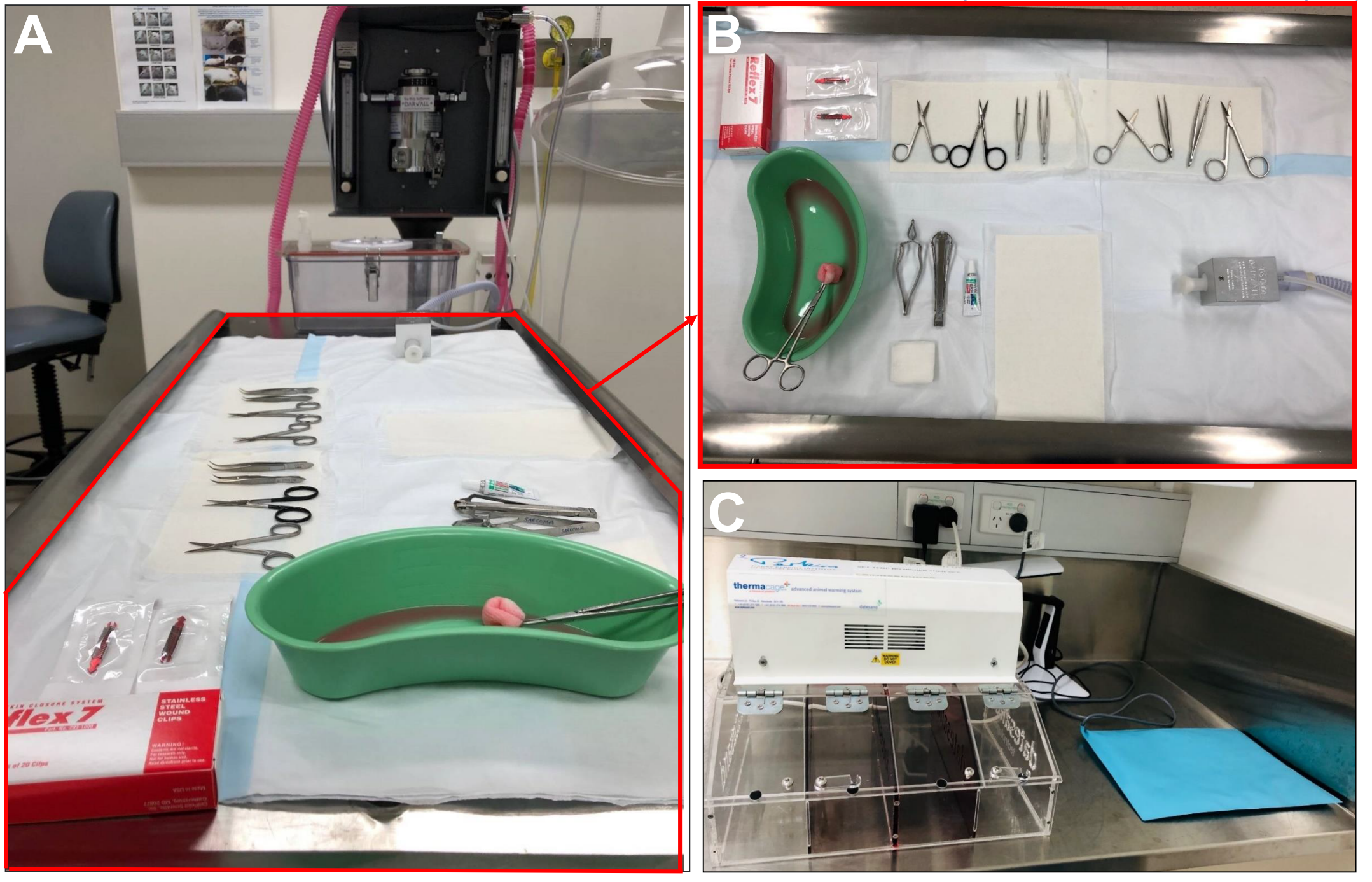

Figure 2

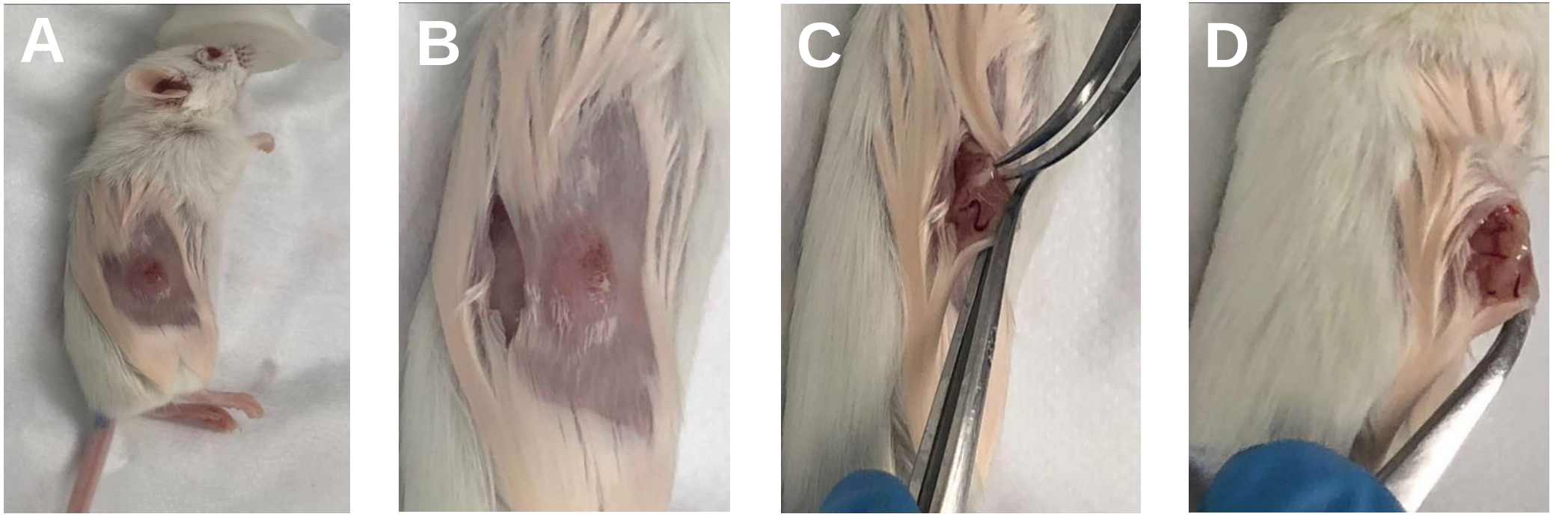

Figure 3

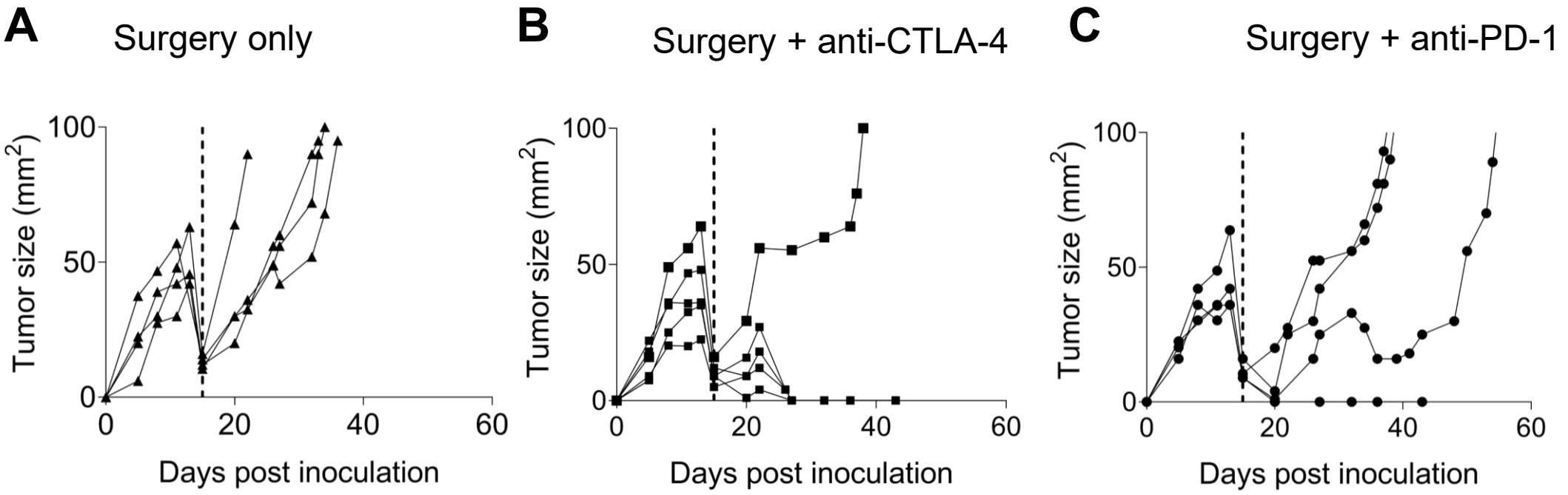

Figure 4

Name of Material/Equipment	Company	Catalog Number	Comments/Description
26 gauge 0.5 mL insulin syringe	Becton Dickinson, Australia	326769	None
2-Mercaptoethanol	Life Technologies Australia Pty Ltd	21985023	None
Anaestetic gas machine	Darvall Vet, Australia	SKU: 2848	None
Anti-CTLA-4	BioXcell, USA	BE0164	None
Anti-PD-1	BioXcell, USA	BP0273	None
Buprenorphine Hydrochloride Injection, 0.3mg/mL	RB healthcare UK Limited, UK	55175	Prescription order
Chlorhexidine Surgical Scrub 4%	Perigo Australia, Australia	CHL01449F(scrub	None
Fetal Bovine serum	CellSera, Australia	AU-FBS-PG	None
Forceps Fine 10.5 cm	Surgical house, Western Australia	CC74110	None
Forceps Fine 12 cm Serrated	Surgical house, Western Australia	CC74212	None
Forceps Halsted 14 cm	Surgical house, Western Australia	CD01114	None
Heating chamber	Datesand Ltd, UK	Mini-Thermacage	None
HEPES (1M)	Life Technologies Australia Pty Ltd	15630080	None
Isoflurane	Henry Schein Animal Health, Australia	SKU: 29405	Prescription order
Lubricating Eye Ointment	Alcon	n/a	None
Penicillin/streptomycin 1000X	Life Technologies Australia Pty Ltd	15140122	None
Phosphate Buffered Solution 10x	Life Technologies Australia Pty Ltd	70013-032	None
Reflex 7mm Clips	Able scientific, Australia	AS59038	None
Reflex 7mm Wound Clip Applicator	Able scientific, Australia	AS59036	None
Reflex Wound Clip Remover	Able scientific, Australia	AS59037	None
Rodent Qube Anesthesia Breathing Circuit	Darvall Vet, Australia	#7885	None
Roswell Park Memorial Institute (RPMI) 1640 Medium + L-glutamine	Life Technologies Australia Pty Ltd	21870092	None

Scissors Iris STR 11 cm	Surgical house, Western Australia	KF3211	None
Scissors Iris STR 9 cm	Surgical house, Western Australia	JH4209	None
Small Induction Chamber	Darvall Vet, Australia	SKU: 9630	None
TrypLE express 1x	Life Technologies Australia Pty Ltd	12604-021	None

Addressing editorial and reviewer's comments: JoVE submission JoVE60882

A. Editorial comments

Proofreading: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have proofread the manuscript for any potential misspelling or grammatical errors.

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 1.1.1: mention examples of cell line, culture media and culture conditions.
   We added details of the culture media and culture conditions of cell line WEHI-164 to page 3.

2) 1.1.2: Mention animal strain, age, sex, weight.
   We mention animal strain, age, sex, and weight on page 4." Female BALB/c mice, aged between 8-12 weeks, of normal weight (16 -22 grams) were used in this experiment."

3) 1.1.3: Describe trypsinization, and how trypsin is neutralized.
   We added a description of the trypsinization step, and how trypsin is neutralized on page 4.

4) 1.1.4: mention counting method.
   We added the counting method on page 5 as follow: "Determine the cell number using trypan blue exclusion method by either using a haemocytometer or an automated cell counter."

5) 2.1: How long after inoculation is this?
   We now mention the following on page 6. "On day 12 post inoculation, when tumors have reached a size of about 50 mm^2."

6) 2.3.1: Is the fur shaved?

Yes, we now mention this on page 4. "One day before inoculation, shave mice on the lower right flank using clippers."

7) Ensure that the manuscript title best represents the highlighted portion of the protocol.

The title has been slightly changed to best represent the protocol.

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have rearranged the discussion section to cover the following details in this sequence: 1) modifications and troubleshooting, 2) critical steps within the protocol, 3) limitations of the technique, 4) significance with respect to existing methods, and 5) future applications.

Figures:

1) Fig 3: Use the American spelling for Tumor.

We have used the American spelling for Tumor on this figure which is now Figure 4. A new figure has been included.

2) If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment.

All figures and tables are original.

B. Comments from Peer-Reviewers:

Comments from Reviewer #1

Major Concerns

Reviewer #1 has no major concerns with the manuscript.

Minor Concerns:

1) Section 1.1: The viability of the tumor cells prior to injection should be noted.

   We added the following to page 5. "NOTE: The viability of tumor cells should be equal or above 80 % to ensure tumor growth."

2) Section 1.2: The media for the tumor cell injection should be included. Is it serum free or sterile 1X PBS?

   The media used for inoculation of tumor cells is sterile 1X PBS. We now mention this on page 5. "Mix the cell suspension thoroughly and fill a syringe with a 26-gauge needle with 100 µl cell suspension (5 x 10^5 cells) in sterile 1X PBS."

3) Section 2.1: A picture of the surgical set up would be appreciated.

   We have included a new figure showing visual images of the surgical set up (Figure 2A-C).

Comments from Reviewer #2

Major Concerns:

Overall, the manuscript is well written and provides detailed instructions on carrying out the method described. These instructions are likely to be sufficient for readers to reproduce the method and expected results. All of the critical steps are highlighted.

1) Including a control group

Given that the authors emphasize the effects of wound healing on influencing the outcome of treatment with adjuvant therapies (in this case systemic therapies), they should suggest to readers including a control group in which all steps of the protocol are carried out except for the actual tumor removal. In other words, the skin incision, exposure of the tumor, and would closure should all occur in this control group. This control will help to differentiate between the role of the partial resection and the inflammation in treatment response

We have included a new step (2.7 Experimental controls) describing experimental controls on page 10. "2.7 Experimental Controls

When using this model to assess the effects of inflammation/wound healing, the following control groups may be considered:

1) *Non-surgery control (treatments can still be administered intratumorally),*
2) *Sham surgery control – A surgical incision is made in the skin; the tumor is manipulated and exposed, but no tumor tissue is removed; the wound is closed with clips."*

The rationale of this control group is discussed on page 12; modification and troubleshooting section.

"As our model can be used to assess the effect of wound healing on therapy, we propose a control/sham group as a comparison. The control may be un-altered tumor, or sham surgery which would only have the skin incision, exposure of the tumor, and wound closure without partial tumor debulk. This sham control group could be used when discerning the effect of surgery-induced inflammation and wound healing from the partial debulk on the treatment outcome."

2) Discussing the difference between an R1 resection and R2 resection

This manuscript would also benefit from a discussion of the differences between an R1 resection (microscopic residual disease) and an R2 resection (gross residual disease) given that this a model of R2 and not R1 resections. In that sense, the value of this model is mostly in studying the context of inflammation and wound healing on therapy and less so of the clinically most common context in which adjuvant therapies applied (i.e. R0 or R1 resections). If up to 50 % of the tumor is retained, then the major difference between this model and a xenograft model without resection is that a wound introduced. More emphasis should be placed on this point and on the contexts when an R2 resection may be clinically indicated (i.e. involvement of vital structures, etc…). This will help readers who are considering applying this method to ensure that it is the most appropriate model for the experimental question they are addressing.

We have included a new paragraph discussing differences between R1 and R2 resections and also addressing the resemblance of our model to the clinical settings of R2 resection on pages 14.

"This model is designed to study the effect of inflammation and wound healing on therapy. Our debulking approach clinically resembles clinical situations where gross residual disease is left behind after surgery (R2 resection), rather than macroscopically complete resection with microscopic

residual disease (R1 resection). For example, surgical resection in invasive soft tissue sarcoma can result in positive margins when the tumor is located next to critical structures such as nerves, arteries or adjacent organs, precluding complete resection with wide margins[17]. Surgery models for resection resulting in microscopic positive margins have been published[13;] our protocol can be used to study the effect of the wound healing response on therapy when macroscopic residual disease is present."

3) Adapting the title to the protocol

Because the model is that of gross residual disease, the title "A mouse model of incompletely resected soft tissue sarcoma for testing perioperative treatments to prevent local recurrence" is somewhat misleading. The term 'local recurrence' carries the implication that the original tumor completely resected, which is not the case in this model and not the goal of the surgery that is performed. It would be more appropriate to change the title to reflect that this is a model for testing treatments in the setting of debulking surgery with gross residual disease. Again, the interesting aspect of this model is that, as the authors appropriately point out, the variable of wound healing is introduced.

Indeed, we perform debulking surgery, not complete surgical resection in our model. The rationale of our model is to introduce a surgery-induced wound healing response in the context where incomplete resections are performed; for example, in soft tissue sarcoma. Throughout the document, we have now emphasized that there will be gross residual disease after surgery in our model, and we changed the title of the manuscript.

"A Mouse Model of Incompletely Resected Soft Tissue Sarcoma for Testing (Neo)adjuvant Therapies"

Minor Concerns:

1) Section 1.1: The role for mycoplasma testing of cell lines prior to injection should be stressed. Is the trypsin quenched with excess media?

   We added the following to page 3: "NOTE: Tumor cell lines should be tested for mycoplasma regularly, as infection can alter the cell growth and influence the immune response in vivo."

2) Section 2.2: Should heart rate also be a monitoring parameter for depth of anesthesia?

We do not monitor heart rate for depth of anesthesia. The mice are under anesthesia for only 5-6 minutes. We have added more details on monitoring breath rate to section 2.2 Anesthesia, step 2: Maintenance of anesthesia, on page 6.

> *"NOTE: The ASSISTANT must monitor the breathing of the mouse throughout the surgery to ensure the correct level of anesthesia is maintained. Lower the anesthetic concentration if breathing becomes too slow, or increase the concentration if the depth of anesthesia is too shallow. If the mouse begins gasping, remove mouse from the nose cone, decrease the anesthetic concentration, and wait until breathing normalises before placing on the nose cone again."*

3) Section 2.5: What is the dose of buprenorphine on post-op day 1?

We now mention this on page 9. Section 2.5, step 4: "…, and dose mice again with 0.1 mg/kg buprenorphine in 100 µl s.c."

4) Section 2.6: Neoadjuvant should also be included in the section heading

We have added "Neoadjuvant" in the section heading on page 9. "2.6 Adjuvant or Neoadjuvant treatment"

5) Figure 1 legend, panel D: Should be staples not clips (or the main text should state clips).

We have changed the main text from staples to clips.

6) Figure 2: Should show an image of the transected tumor.

A representation of this is shown in figure 1. The transection of the tumor will be shown in the video protocol.

7) Table 1: Consider adding the source for the eye ointment.

We have added eye ointment source to table 1.

C. Additional comment

We apologise for having omitted co-author Prof. Terrance Johns in the previous version. Prof. Johns has been involved in the development of this model from the beginning and has been involved in the analysis and interpretation of the data. He has read and provided feedback to the manuscript and agrees to be a co-author. All co-authors agree with the addition of Prof. Johns as a co-author.