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Original Research

Comparison of a Minimally Invasive Transthoracic Approach and a Surgical Method for Intrapleural Injection of Tumor Cells in Mice

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Intrapleural injections can be used in mice to deliver therapeutic and diagnostic agents and to model human disease processes (for example, pleural fluid accumulation, malignant pleural disease, and lung cancers). In the context of establishing cancer models, minimally invasive methods of intrapleural injection are desirable because inflammation at the injection site can have a major impact on tumor growth and progression. Common approaches for intrapleural injection include surgical exposure of the thoracic wall or the diaphragm prior to injection; however, these invasive procedures require tissue dissection that triggers an undesirable inflammatory response and increases the risk of pneumothorax. While nonsurgical procedures can minimize this concern, 'blind' injections may lead to off-target inoculation. In this study, we hypothesized that a minimally invasive transthoracic approach (MI-TT) would produce a tumor distribution and burden similar to that of a surgical transabdominal approach (SX-TA). Prior to performing the procedures on live mice, surgeons were trained using cadavers and terminal procedures. Then a total of 14 nude mice (female, 4 to 6 wk old) were injected with 50 µL (5 million) A549-Luc2 human cancer cells either using the MI-TT (*n* = 8) or SX-TA (*n* = 6) approach under carprofen analgesia and isoflurane anesthesia. Our results indicate that with training, a minimally invasive transthoracic approach for intrapleural injection provides more consistent tumor placement and a greater tumor burden than does the surgical method. However, additional studies are necessary to confirm anatomic placement and characterize tumor profiles.

Abbreviations: BLI, bioluminescence imaging; CT, computed tomography; DPBS, Dulbecco's phosphate buffered saline; MI-TT, minimally invasive transthoracic approach; MRI, magnetic resonance imaging; PBS, phosphate buffered saline; SX-TA, surgical transabdominal approach; wk, week

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Introduction

Intrapleural injections target the pleural space (region between the lung and chest wall). Clinically, this space can be accessed during disease treatment and diagnosis. In a research setting, injection into this space can be used to model disease processes (pleural fluid accumulation,[13] malignant pleural effusion/disease,[1,29] and lung cancer[16]), deliver therapeutic agents, or trace neuronal pathways.[28] Most commonly, intrapleural injection of cancer cells can be used to model malignant pleural effusion/disease in humans, which is most frequently caused by primary malignant pleural mesothelioma,[6,22] metastatic lung cancer, breast cancer, and lymphoma.[26]

Current methods of intrapleural injection include 1) minimally invasive transthoracic (MI-TT) injection (no surgical exposure or visualization of the intrapleural space), 2) surgical exposure of the thoracic wall via tissue dissection, or 3) surgical exposure of the diaphragm via abdominal exposure.[4,16,26,28] In addition to logistical benefits such as reduction of the surgical time and supplies, a minimally invasive approach without surgical exposure can be considered an animal welfare refinement[24]) and may improve research reproducibility by reducing stress and pain-related confounds.[23] Furthermore, a minimally invasive injection reduces risk of pneumothorax, which is a concern during surgical exposure of the thoracic wall. The minimally invasive approach is especially desirable for studies involving inflammation and immunology in which surgical stress can significantly affect measured study outcomes. However, because the technique is performed 'blindly' (that is, the injection space is not directly visualized),[28] a potential concern with the minimally invasive method is a greater likelihood of creating an off-target effect in the subcutaneous space or chest wall during injection.

Existing literature[26,28] describes different techniques and associated concerns for intrapleural injection. Although a previous study reported that the intrapleural injection technique does not affect fluorescent tracing of motor neurons,[28] and another study assessed the effect of injection technique on tumor growth in adjacent tissues such as the lung,[16] to our knowledge no study has compared the effect of different intrapleural injection techniques on tumor placement and quantitative tumor burden. In this study, we compared a minimally invasive transthoracic (MI-TT) approach to a surgical approach (SX-TA, exposure of

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the intrapleural space through the diaphragm via the abdomen). We hypothesized that the MI-TT approach would result in similar tumor placement and qualitative burden as a surgical transabdominal approach (SX-TA).

Materials and Methods

All experimental procedures were performed at an AAALAC-accredited institution (Protocol #: PRO00009736), according to the standards established by *the Guide for the Care and Use of Laboratory Animals*[9] and were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Mice. Fourteen female 4- to 6-wk-old nude mice (NU/J, Jackson Laboratory, Bar Harbor, ME) were used for this study. The first cohort had 2 SX-TA mice and 8 MI-TT mice. The second cohort had 4 SX-TA mice. Mice were negative for mouse parvovirus, minute virus of mice, mouse hepatitis virus, mouse norovirus, Theiler murine encephalomyelitis virus, enzootic diarrhea of infant mice virus, Sendai virus, pneumonia virus of mice, reovirus, mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus, ectromelia virus, K virus (murine pneumotropic virus), polyomavirus, and mouse cytomegalovirus. Mice were socially housed in groups of 4 to 5 per individually ventilated cage on corncob bedding (1/8 in. [3.175 mm], Shepherds Cob, Shepherd Specialty Papers, Milford, NJ) in a temperature- and humidity-controlled room (70 to 73 °F [21 to 23 °C], 30% to 70% relative humidity [humidity occasionally around 20% in winter months]) with ad libitum feed (Teklad global 18% protein, irradiated, 2918, Envigo, Indianapolis, IN) and water on a 12:12-hr light:dark cycle. Crinkle paper (EnviroDri, Shepherd Specialty Papers) and a paper cup were provided for enrichment. All supplies were autoclaved prior to contact with mice. Upon arrival, mice were acclimated for 5 to 6 d prior to experimental manipulation.

Surgeon training. Prior to performing each procedure, the 2 surgeons were trained in MI-TT and SX-TA approaches using euthanized mice and terminal surgery. Approximately 2 one-hour sessions were performed for each. Injection of blue dye (Evans blue [Acros Organics, Geel, Belgium] or blue food coloring [Lot# 8236269, Chef-O-van, C.O.V. Extract Company, Rockford, OH]) into the pleural space was visually confirmed during necropsy.

Intrapleural injections. Mice were anesthetized using isoflurane anesthesia (Fluriso, VetOne, Boise, ID or Isoflurane, Phoenix Pharmaceuticals, Burlingame, CA). The area of injection (MI-TT group) or surgery (SX-TA group) was prepared (one alcohol pad swab on the right thoracic wall for MI-TT, 3 alternating chlorhexidine and saline scrub cycles on the ventral abdomen for SX-TA). Because nude mice do not have fur, shaving was not necessary. Mice received an injection of the analgesic carprofen (5 mg/kg, subcutaneous; Rimadyl, Pfizer, New York, NY) immediately before injection or surgery, and once the following day. The techniques followed for intrapleural injection were modified from a previously published paper,[28] using the "transthoracic approach" for MI-TT and the "transdiaphragmatic approach" for SX-TA.

For MI-TT, the mouse was placed in left lateral recumbency, and the landmark for the percutaneous injection was 1 to 2 mm caudal to the right elbow when the right forelimb was flexed against the body. A tuberculin needle (29-gauge, 12.7-mm needle length, EXELINT, Los Angeles, CA) was inserted through the chest wall (approximately half the needle length), and angled laterally toward the chest wall after insertion to avoid piercing the lungs. The needle was removed immediately after injection of tumor cells. No bleeding was noted, so pressure was not applied after the injection.

For SX-TA, the mice were placed in dorsal recumbency, and abdominal midline incisions were made to expose the diaphragm from inside the abdomen. The lungs and pleural space (lateral to the lungs) were clearly visible through the diaphragm. A tuberculin needle containing the cells was inserted through the diaphragm 1 to 2 mm to the right of the ventral midline (approximately half the needle length) and a single injection was given into the pleural space. The abdominal wall was closed with an absorbable suture, and the skin was closed with either the same suture or wound clips. Each mouse was injected with 5 million (50 µL, 1:1 PBS:Matri-gel) A549-Luc2 tumor cells, a human lung carcinoma line (The American Type Culture Collection [ATCC], Manassas, VA).[15]

For both groups, mice were moved to ventral recumbency and monitored until ambulatory. Mice were monitored daily for 1 wk, and then at least once weekly until study endpoint.

For 6 of 6 SX-TA mice and 4 of 8 MI-TT mice, we calculated the length of the procedure by subtracting surgical start time (for SX-TA group) or beginning of surgical preparation time (for the MI-TT group) from the surgical end time.

Measurement of tumor burden. Mice were imaged using bioluminescence imaging (BLI) (IVIS SpectrumCT In Vivo Imaging System, PerkinElmer, Waltham, MA) for qualitative and quantitative analysis. A fresh solution of D-Luciferin in DPBS (15 mg/mL) was prepared before each imaging session. Each mouse was given 150 mg D-Luciferin/kg of body weight via intraperitoneal (IP) injection. After 15 min, each mouse was imaged to monitor tumor burden. The exact imaging schedule was variable depending on group and cohort, but all mice were imaged at endpoint, which was postoperative/procedural day 46 or 48. BLI images were used for both qualitative analysis (characterizing spread of tumor distribution in mouse [chest compared with other parts of the body]), and quantitative analysis (tumor burden as measured with radiance [photons/s/cm^2/sr]). After the study, all mice were necropsied to assess gross tumor burden.

Histopathologic analysis. Hematoxylin and eosin and Ki67 immunohistochemical staining of tumor tissue was only performed on only one SX-TA mouse, as histology was not planned in the original study and tissues from other mice were needed for other research objectives.

Analysis. SX-TA and MI-TT quantitative tumor burden (BLI, radiance [photons/s/cm^2/sr]) were compared at the final time point by using a Wilcoxon Kruskal Wallis Rank Sum test for nonparametric data with JMP Pro 16 (SAS, Cary, NC).

Results

Animals. The interprocedure interval for the SX-TA group ranged from 22 to 33 min. The interprocedure interval for the MI-TT ranged from 6 to 10 min.

All mice recovered from anesthesia uneventfully. One of 8 MI-TT mice was unexpectedly found dead on postoperative day 45. The 7 remaining MI-TT mice and all 6 of the SX-TA mice survived until the end of the study.

Measures of tumor burden.

Qualitative measurements. All 8 MI-TT mice expressed bioluminescence signal in the appropriate location (bilaterally in the thoracic cavity) throughout the study. The mouse that was found dead during the study had also expressed bilateral thoracic signal until it died. In contrast, the 6 SX-TA mice were more variable in their signal. Only 3 of the 6 SX-TA mice expressed consistent signal bilaterally in the thoracic cavity. Among the

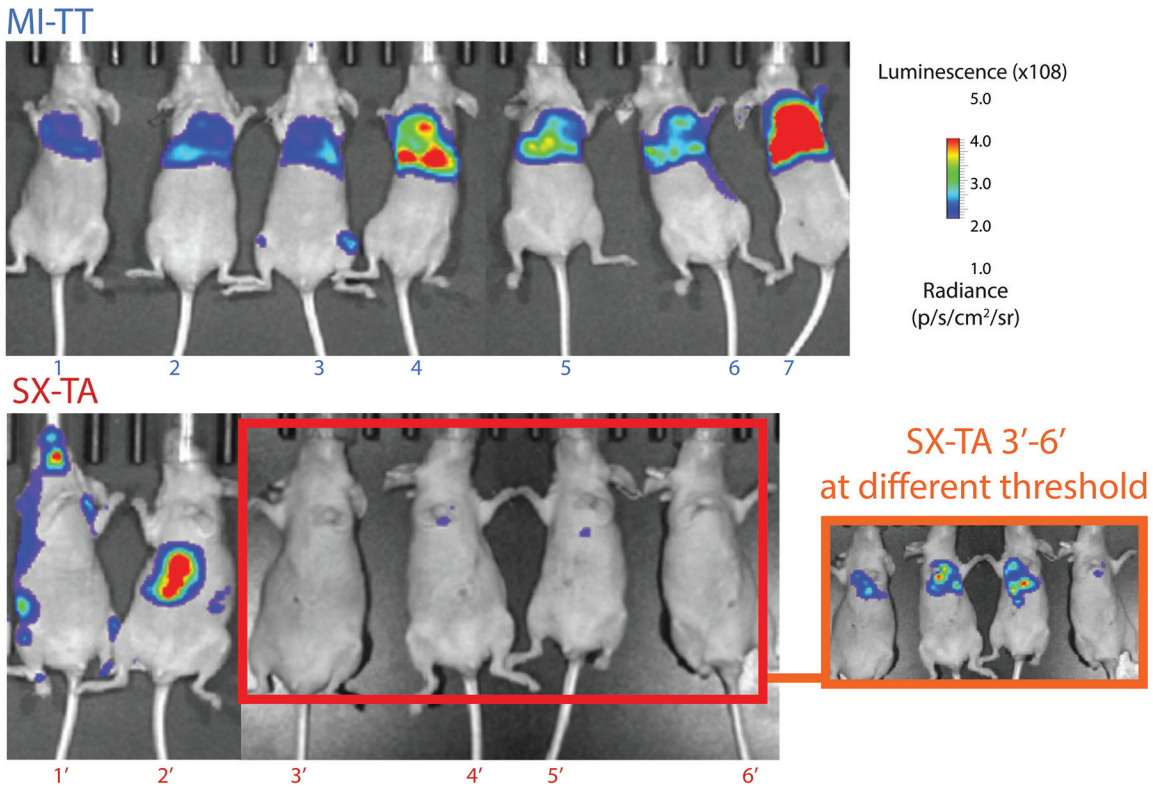

Figure 1. Bioluminescent images from MI-TT and SX-TA groups at endpoint. Top panel, (MI-TT group, mice 1-7) and lower left panel (SX-TA group, mice 1′-6′) are processed at the same luminescence threshold. Red box (lower left panel), and orange box (lower right panel) both refer to mice SX-TA mice 3′-6′; the red box at the default luminescence threshold, and the orange box at a different processing threshold to focus the signal in the chest. The MI-TT group expressed consistent signal on both sides of the chest for all mice. The SX-TA mice had more variable expression, and three mice did not express consistent bilateral signal in the chest. SX-TA mouse 1′ had signal on the face, flank, and stifle, mouse 2′ had signal in the abdomen, and mouse 6′ had very small signal in the chest. Several mice in both groups (for example, mouse 3 and 1′ at endpoint) had small BLI signal in the flank and stifle regions. However, this signal was not consistently present over time in the same mice.

other 3 SX-TA mice, at endpoint, one had signal in the face, flank, and stifle (mouse 1′), one had signal in the abdomen (mouse 2′), and one had very small signal in the chest (mouse 6′) (Figure 1). Several mice in both groups (for example mice 3 and 1′ at endpoint) had a small BLI signal that was occasionally and intermittently seen in the flank and stifle regions. However, this signal was not present consistently over time in the same mouse (Figure 1).

Quantitative measurements. Tumor burden as measured by BLI was generally higher in the MI-TT group compared with the SX-TA group during the study (Figure 2). At the study endpoint (day 46 to 48), after excluding the MI-TT mouse that was found dead before endpoint and the SX-TA mouse (mouse 2′) that had tumor in the abdomen, the MI-TT group had significantly greater tumor burden than the SX-TA group ($P = 0.0058$) (Figure 3). At necropsy, obvious tumor was seen in the MI-TT group, but not the SX-TA group (Figure 4).

Histopathologic analysis. Despite lack of obvious gross pathology, the single SX-TA lung submitted revealed Ki67 positive tumor tissue associated with the surface of the lung, but not infiltrating or associated with lymph nodes (Figure 5).

Discussion

This study investigated a minimally invasive method of intrapleural tumor injection in mice and demonstrated its utility in seeding tumor cells within the pleural cavity compared to an invasive surgical method. We demonstrated that despite concerns of off-target effects in the subcutaneous space or chest wall,[28] the minimally invasive "blind" approach (MI-TT) was more consistent in establishing tumor burden in the chest cavity and increased tumor burden compared to the invasive surgical (SX-TA) method in which the pleural space was clearly visualized.

In contrast to our predictions of no difference in research outcomes (as demonstrated in a neuronal tracer study showing a similar number of labeled motor neurons between the 2 techniques[28]), or that visualization of the diaphragm and intrapleural space with the SX-TA method would provide more accurate injection,[28] tumors developed more consistently in the lungs with the MI-TT method. While all MI-TT mice developed tumors bilaterally in the thoracic cavity, 3 SX-TA mice (mice 1′, 2′, and 6′) did not develop consistent BLI signal bilaterally in the chest. While physical differences between the 2 techniques could attribute to the discrepancy in tumor location (for example the tumor cells "spilled" out of the chest through the hole created by the injection in the diaphragm, and grew in the abdominal cavity), the systemic inflammation caused by an invasive surgical approach could also alter the tumor's microenvironment and ability to grow. In humans, postsurgical systemic inflammation results in proinflammatory cytokine release, microcirculatory disturbance, and cell-mediated immune dysfunction, and is followed by a compensatory anti-inflammatory response syndrome.[18]

Outside of body cavities directly exposed in surgery (thoracic and abdominal), mice in both groups had BLI signal in distant regions of the body, such as the stifle (mice 3 and 1′ at endpoint) or head (mouse 1′ at endpoint). Distant tumor metastasis is a possibility; to our knowledge, metastasis secondary to intra-

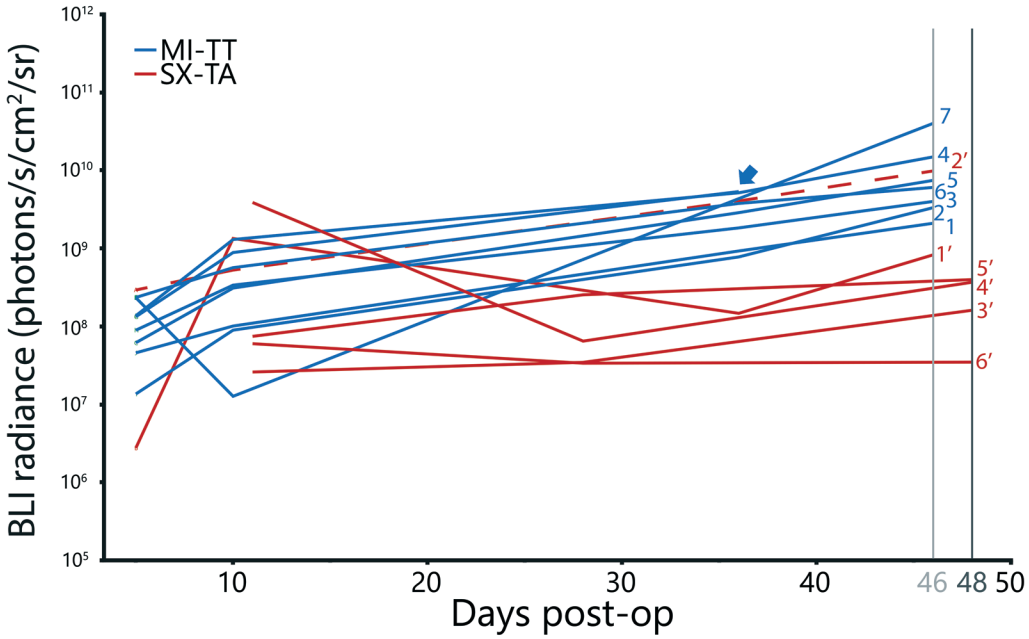

Figure 2. Bioluminescence radiance (photons/s/cm^2/sr) of each individual mouse measured over the study. Lines connect sample readings. Blue arrow indicates the last measurement of a MI-TT mouse before it was lost from study (found dead). Red dashed line indicates a SX-TA mouse whose tumor expressed in the abdominal instead of the thoracic cavity. Blue lines show mice 1-7 from the MI-TT group, red lines show mice 1′-6′ from the SX-TA group. Light gray and dark gray lines denote endpoint days 46 and 48 of the respective cohorts.

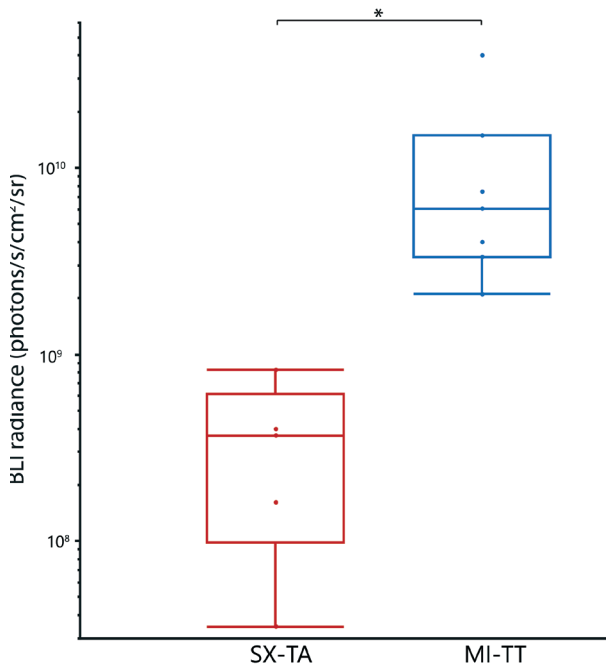

Figure 3. Box plots of BLI for each group at endpoint (day 46-48) after exclusion of 2 mice (MI-TT mouse found dead on day 45, SX-TA mouse with signal in abdomen). Points represent individual samples. Boxes represent first to third quartile range (interquartile range [IQR]), with the line in the middle of each box representing the median. Tips of whiskers extending below and above boxes represent first quartile – 1.5*IQR and third quartile + 1.5*IQR respectively. * $P < 0.05$.

pleural injection has not been documented in A549 cells, but has been documented in other lung cancer cell lines.[30] However, these signals could also be due to artifact.

In addition to more consistent placement in the thoracic cavity, the MI-TT method was associated with greater tumor burden (quantified by a greater BLI signal). Even when BLI showed tumor burden in the thorax of mice in the SX-TA group, the signal strength was lower than that of the MI-TT group. This difference can be visualized qualitatively in SX-TA mice as compared with the MI-TT mice in Figure 1, and is even more apparent in terms of the quantitative measures (Figure 2).

In cancer literature, surgical resection of established tumors promotes tumor progression and metastasis in both humans and animals.[2,20,21,27] In addition to inducing local and systemic immunosuppression that impairs antitumor immunity, surgical manipulation of the tumor can allow the release and dissemination of cancer cells into circulation.[2] However, literature is sparse on how surgery at the time of injection with tumor cells affects tumor growth and metastasis. To our knowledge, only one study[14] has reported that surgery associated with injection of tumor cells accelerates tumor growth and metastasis. Emerging tumors may respond differently to surgery, compared with established tumors, which could explain our unexpected results. In addition, the physiologic response to surgical trauma varies over time, starting with an initial proinflammatory phase, followed by a compensatory anti-inflammatory or immunosuppressive phase;[3] the stage at which tumors are exposed to these different states could affect their growth and development. Finally, cell line and tumor-type specific differences also exist. Alternatively, the low procedural time of the MI-TT method may result in greater cell viability, as cells are injected into the animal more quickly after being collected from culture. However, we view this possibility as unlikely as unpublished data from our lab indicates that maintenance of cells on ice for several hours does not significantly reduce their viability.

An alternative surgical approach (surgical exposure to visualize the pleural space through the thoracic wall[26], a combination of the SX-TA and MI-TT methods) is a slightly less invasive option than the SX-TA method. However, due to the success of the MI-TT method, this third method of intrapleural injection was not investigated in this study, but could be tested in future studies. Other potential experimental modifications of the SX-TA

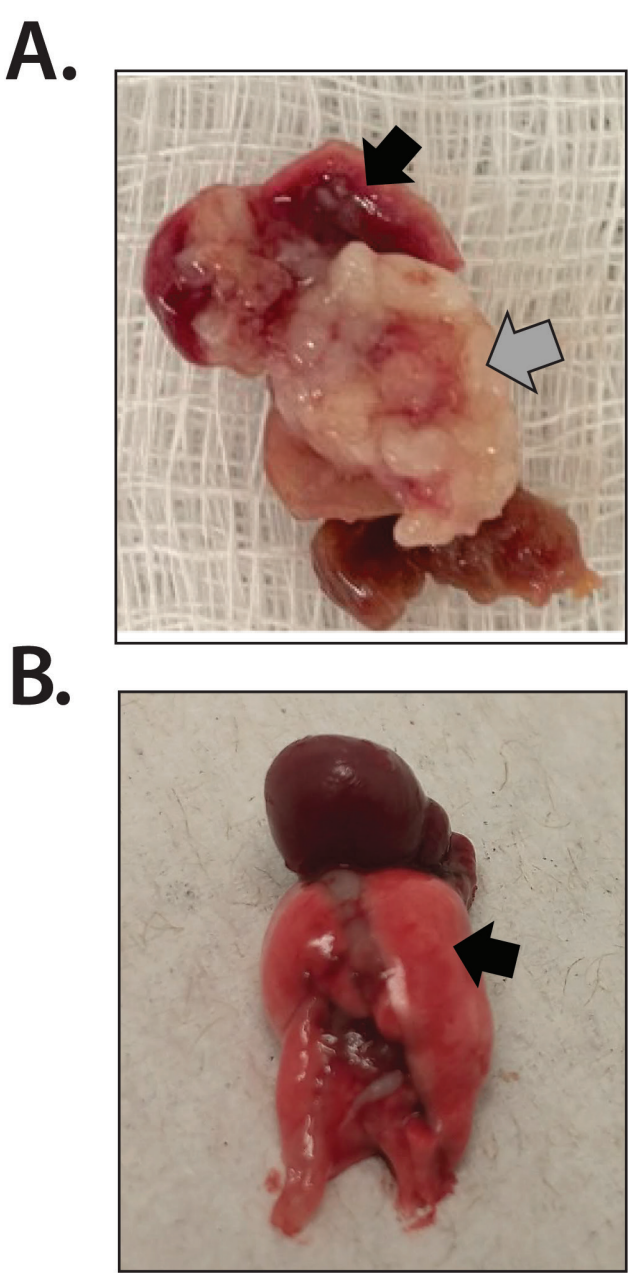

Figure 4. (A) Gross pathology of MI-TT mouse 4 with obvious tumor surrounding the lung. Black arrow points to lung, gray arrow points to tumor. (B) Gross pathology of SX-TA cohort 2 (representative mouse from mice 3′-6′, lungs grossly similar with no obvious visible signs of tumor), tumor not grossly evident. Black arrow points to lung.

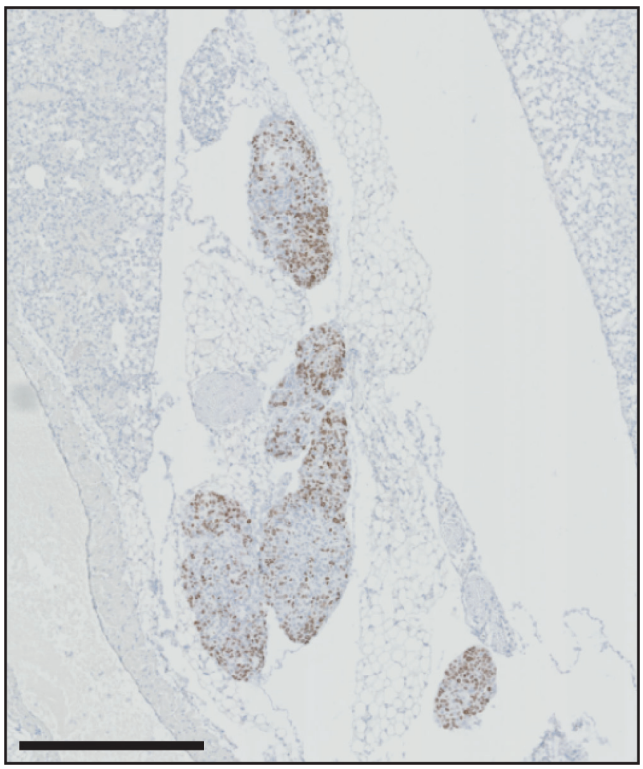

Figure 5. Ki-67 marking of proliferation and neoplasia in lung tissue from SX-TA method, cohort 2 (mice 3′-6′). Focal tumor structures were identified mostly adjacent to and occasionally within the lung (brown stain). Black scale bar on lower left of image is 500 µm.

method include injecting cells multiple times in different parts of the diaphragm,[28] or giving additional injections on the left side to improve distribution and uptake. However, these additional manipulations would increase the risk of pneumothorax and were not thought to be necessary due to communication of the left and right pleural cavities.[26]

In our study, the cell suspension was mixed with Matrigel to promote cell growth and differentiation.[11] Matrigel is liquid when chilled at 4 °C, but becomes gel-like at room temperature, and solid after 30 min at body temperature (37 °C).[8] A concern that the cell suspension will only infiltrate one side of the chest pleural cavity before solidifying. However, consistent with previous studies of MI-TT intrapleural injection using Matrigel, the tumor cells had spread bilaterally across the chest at the time of imaging.[10,17] We hypothesize that either 1) 30 min was adequate for the cell suspension to spread bilaterally before solidifying, as murine pleural spaces communicate between the left and right sides, or 2) the initial injection only seeded the right side of the chest, and the subsequent left side metastasized after injection. Repeating this study without Matrigel may allow for better spread through the body, as an extracellular matrix environment-derived product such as Matrigel may limit tumor growth and dissemination.[25]

A limitation of this study is that mice were not consistently examined beyond BLI and gross necropsy to confirm placement into the pleural space as opposed to the subcutaneous tissue or chest wall. However, injections outside of the pleural cavity (subcutaneous, chest wall, or intrapulmonary) would likely be unilateral. In addition, during training, the MI-TT technique consistently delivered colored dye to the pleural space. Finally, assessment of tumors from the MI-TT group on gross necropsy showed them to be in the pleural cavity. Future studies could use MRI, CT or histopathology to confirm tumor placement.

Another limitation was that we did not perform histological comparisons of tumors in the SX-TA and MI-TT groups. This limitation constrained our interpretation of the study. For example, gross necropsy in the SX-TA group showed little evidence of tumor development, but histopathologic evaluation in one mouse showed evidence of tumor cells associated with the intrapleural space, consistent with its small BLI signal. Beyond quantification of tumor location, histopathologic evaluation could have quantified other aspects of the intrapleural tumor microenvironment such as potential pleural eosinophilia (associated with intrapleural injection of saline or air at higher volumes than the 0.1 mL used in this study),[7] or ability for pleural spreading (previously been demonstrated to be af-

fected by different induction methods[16]). These parameters are important for evaluation of the research impact of these injection techniques.

Another limitation was our failure to assess the one mouse in the MI-TT group that was found dead on postoperative day 45. Although counter-intuitive, we cannot rule out that the MI-TT approach has a higher postprocedural complication rate as compared with the SX-TA approach. We did not observe any pleural effusions in necropsies performed at the end of the study. Unlikely possibilities secondary to a space-occupying substance (either tumor or fluid) in the pleural cavity could include congestive heart failure or renal failure.[5] We recommend additional studies with larger cohorts and careful assessment of any mice found dead.

Another limitation of our study was that animal welfare indicators such as weight were not measured. As suggested by previous literature,[28] the MI-TT method may be a refinement[24] as compared with the SX-TA method by causing less pain and stress.[23] The MI-TT method, which requires a smaller incision and less tissue dissection and exposure of body cavities, is less invasive than the SX-TA method and has a shorter procedural time in our experience. We anecdotally noted that SX-TA mice had postoperative weight loss after surgery, which we would not expect in the MI-TT technique, based on our experience with other minimally invasive techniques (for example, percutaneous lung tumor injection). However, we did not collect this information consistently for the 2 cohorts; thus, our data was inadequate for analysis and publication. Information on indicators of animal welfare could support the value of the MI-TT technique as a refinement of the SX-TA technique and should be thoroughly documented in future studies. In addition to collecting weight data, future studies could expand and better characterize the mouse experience by measuring evoked pain (for example, von Frey) and signs of spontaneous pain (for example, burrowing, nesting, locomotion, facial grimace,[12] nest consolidation, and grooming).[12,17]

Finally, our study evaluated only female mice. Future studies should include both sexes.[4]

Our data on how tumor placement and burden vary with different intrapleural injection methods is probably of greatest value to researchers who use this procedure to study malignant pleural disease. However, the intrapleural injection method could also affect study of other diseases (for example, pleural fluid accumulation,[13] lung cancer[16]), as intrapleural injection methods may affect their study outcomes. Beyond tumor distribution and burden, outcomes such as quality and quantity of pleural effusion,[1,13] quantification of tumor through histopathologic interpretation,[6,29] and systemic indicators of animal wellbeing (for example weight loss, mobility, survival)[1] can all be affected by method of injection. Further research is necessary to fully characterize the effects of different methods of intrapleural injection on research and clinical outcomes. In general, literature describing the effects of surgery on newly developing tumors, as compared with established tumors, is sparse, and similar studies would be valuable in other types of surgically-induced tumor models.

In conclusion, we have shown here that a minimally invasive transthoracic approach for intrapleural injection improves tumor placement and increases tumor burden compared with a surgical method. However, additional studies are necessary to confirm anatomic placement and characterize tumor profiles.

Acknowledgments

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