

Video Article

A Mouse Tumor Model of Surgical Stress to Explore the Mechanisms of Postoperative Immunosuppression and Evaluate Novel Perioperative Immunotherapies

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Abstract

Surgical resection is an essential treatment for most cancer patients, but surgery induces dysfunction in the immune system and this has been linked to the development of metastatic disease in animal models and in cancer patients. Preclinical work from our group and others has demonstrated a profound suppression of innate immune function, specifically NK cells in the postoperative period and this plays a major role in the enhanced development of metastases following surgery. Relatively few animal studies and clinical trials have focused on characterizing and reversing the detrimental effects of cancer surgery. Using a rigorous animal model of spontaneously metastasizing tumors and surgical stress, the enhancement of cancer surgery on the development of lung metastases was demonstrated. In this model, 4T1 breast cancer cells are implanted in the mouse mammary fat pad. At day 14 post tumor implantation, a complete resection of the primary mammary tumor is performed in all animals. A subset of animals receives additional surgical stress in the form of an abdominal nephrectomy. At day 28, lung tumor nodules are quantified. When immunotherapy was given immediately preoperatively, a profound activation of immune cells which prevented the development of metastases following surgery was detected. While the 4T1 breast tumor surgery model allows for the simulation of the effects of abdominal surgical stress on tumor metastases, its applicability to other tumor types needs to be tested. The current challenge is to identify safe and promising immunotherapies in preclinical mouse models and to translate them into viable perioperative therapies to be given to cancer surgery patients to prevent the recurrence of metastatic disease.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51253/>

Introduction

Surgery is a critical component in the curative cancer treatment of solid tumors, but despite complete resection, many patients develop a metastatic recurrence and ultimately die of their disease. Increasingly, the postoperative period, as a result of the physiologic stress response to surgery, including blood coagulation, release of growth factors, and immune suppression, is recognized as critical time for the development of metastases. Our group^{1,2} and others³⁻⁵ have shown that the immediate postoperative period is a uniquely susceptible time for the formation of metastases.

One of the key mechanisms responsible for the prometastatic effects of surgery is postoperative dysfunction of Natural Killer (NK) cells¹⁻³. NK cells are cytotoxic lymphocytes of the innate immune system involved in the control of tumor growth and metastases⁶. NK cell dysfunction following surgery has been documented in both human patients^{1,7-9} and animal models^{1,10,11}. Postoperative NK cell suppression correlates with increased metastases in animal models of spontaneous and implanted metastases^{1,11-14}, while in human studies, low NK activity during the perioperative period is associated with a higher rate of cancer recurrence and mortality^{15,16}.

Despite this, there are currently no cancer therapies specifically addressing the prometastatic changes that occur immediately following cancer surgery. The perioperative period represents a therapeutic window of opportunity in which to intervene in the metastatic process. While traditional cancer therapies, such as cytotoxic chemotherapy, are considered too toxic to be administered to patients recovering from major surgery¹⁷, immune therapies are ideal candidates for perioperative administration. Perioperative use of recombinant IL-2 and IFN- γ have been explored in

early phase clinical trials demonstrating their potential to prevent postoperative NK cell suppression and improve progression-free survival¹⁸⁻²¹. Unfortunately, further development has been hindered by tolerability of this nonspecific cytokine therapy combined with major surgery¹⁷. Viruses are also potent nonspecific activators of NK cells. Our research has previously demonstrated that preoperative administration of replicating viruses, such as novel anti-cancer Oncolytic Viruses (OV), and nonreplicating viral vaccines, such as influenza vaccine, can inhibit surgery-induced NK cell dysfunction and attenuate metastatic disease^{1,22}.

The surgical model described in this paper has facilitated our understanding of the mechanisms involved in the spread and growth of tumor cells after surgery and allowed us to explore novel targeted therapies that can be administered in the perioperative period. To accomplish this goal, an animal model of surgical stress and spontaneous metastasis was developed. The model makes use of mouse breast carcinoma tumors (BALB/c - 4T1) that are able to spontaneously metastasize from the primary mammary gland to multiple distant sites in particular the lungs. At day 0, breast cancer cells are implanted in the mouse breast fat pad. At day 14 post tumor implantation, a complete resection of the primary mammary tumor is performed in all animals. A subset of animals receives additional surgical stress in the form of an abdominal nephrectomy. At day 28, lung tumor nodules in surgically stressed and no surgery control mice compared are isolated and quantified. In this animal model of cancer and surgery, the effects of surgery on cancer metastases are being studied and the efficacy of perioperative administration of innovative immunotherapies, including replicating and nonreplicating virally based immune- stimulants are tested for the first time.

Protocol

1. Maintaining 4T1 Tumor Cells *in vitro*

1. Culture unmodified 4T1 tumor cells in complete DMEM (DMEM, 10% FBS, 1x Penicillin/Streptomycin) in 10 cm tissue culture plates. Incubate in 37 °C, 5% CO₂ tissue culture incubator.
2. Split cultures 2-3 times/week. To maintain optimum viability cells should not exceed 80% confluence. Do not use cells for establishing *in vivo* primary tumors if cultures have been passaged *in vitro* for >1 month as this decreases malignancy and metastatic potential than earlier passaged cells.

2. Harvesting 4T1 Tumor Cells for Injection

1. Aspirate culture medium from the tissue culture plate using a Pasteur pipette.
2. Add 10 ml of 1x sterile PBS containing 2 ml EDTA. Let the PBS solution incubate in the plate at 37 °C, 5% CO₂ tissue culture incubator for 5-7 min.
3. Harvest PBS solution from plate, rinsing the plate 2-3x with solution, transfer to 15 ml conical tube.
4. Centrifuge cells for 5 min at 500 x g, at 4 °C, in a bench-top centrifuge.
5. Aspirate the supernatant and resuspend the cell pellet in serum-free DMEM.
6. Determine cell concentration using a cell counter.
7. Dilute cells with serum-free medium to 1x10⁶ cells/500 µl (1x10⁵ cells/50 µl (for injection and place cells on ice).

3. Injecting Mice with 4T1 Tumor Cells

All animal studies performed were in accordance with institutional guidelines at the Animal Care Veterinary Services of the University of Ottawa recommendations for all animals receiving orthotopic injections or tumor implants.

1. Treat mice subcutaneously with Buprenorphine (0.05 mg/kg) 1 hr before surgery for pain management.
2. Induce and maintain mouse under anesthesia using 2.5% isoflurane during the duration of the injection. Pinch the footpad of the mouse to detect reflex response. If none is detected, effective anesthesia levels are reached. Sterile eye lubricant is then applied to prevent corneal drying.
3. Load a 30 G ½ in "Ultra-fine" Syringe with precisely 50 µl of cells suspension. Ensure that all air bubbles are removed from the syringe column by tapping the side of the syringe to dislodge air bubbles.
4. Place mouse (under anesthesia) ventral side up.
5. Clean the injection site using an alcohol swab and introduce the needle horizontally and directly into the 4th mammary fat pad, slowly dispense the syringe volume.
6. Use a cotton swab to clean any possible leakage.
7. Allow mice to recover from anesthesia.
8. Maintain Buprenorphine (0.05 mg/kg) for pain management administered s.c. every 8 hr for 2 days.

4. Administering Perioperative Treatment in 4T1 Tumor Bearing BALB/c Mice

The treatment regimen and route for influenza vaccine administration is identical to the oncolytic virus preoperative treatment.

1. At 13 days post-tumor cell injection, measure the primary tumor using an external caliper. Measure the greatest longitudinal diameter (length) and the greatest transverse diameter (width) with the caliper. The modified ellipsoidal formula is used to calculate the tumor volume ($Tumor\ volume = 1/2(length \times width^2)$)
2. At 13 days post-tumor cell injection, the primary tumor should measure approximately 1 cm³. When this tumor measurement is attained, prepare perioperative therapy reagent.
3. Oncolytic virus is one innovative therapeutic that one can administer in the perioperative period. Prepare oncolytic virus (1x10⁹ PFU/1 ml) in 1x sterile PBS and place on ice prior to injection.
4. Secure and place mouse into restrainer for intravenous tail vein injection.

5. Gently heat mouse tail in warm tap water to visualize lateral tail veins.
6. Load a 27 G $\frac{1}{2}$ in "Insulin Syringe" with precisely 100 μ l of oncolytic virus therapy. Ensure that all air bubbles are removed from syringe column.
7. Inject the mouse with 1×10^8 PFU/100 μ l/mouse to one of the lateral tail veins. If the needle is appropriately inserted into a lateral vein, no resistance should be felt when depressing the syringe.

5. Complete Resection of Primary Tumor and Abdominal Left Nephrectomy

1. At 14 days post-tumor cell injection, initiate routine perioperative care following University of Ottawa Animal Care and Veterinary Service approved protocols. Treat mice subcutaneously with Buprenorphine (0.05 mg/kg) 1 hr before surgery for pain management. Induce and maintain anesthesia using 2.5% isoflurane during the surgery. Under sterilized conditions and using sterile surgical instruments, surgical site is shaved and scrubbed. Animals are administered subcutaneous fluids and eye lubricant prior to surgery.
2. Make a small incision (1-2 cm in length) to gently and completely remove the primary 4T1 tumor from the mammary fat pad.
3. Close the incision with 2-3 9 mm staples.
4. Expose the abdomen by cutting through the skin and subcutaneous layer along the ventral midline of the mouse.
5. Make an incision at the linea alba (3-4 cm) to access mouse peritoneum.
6. Expose the left interior side of the abdomen by moving the overlying bowels to the side. Make sure the intestines are kept moist with a saline soaked sterile gauze.
7. Using a blunt pair of surgical forceps, grasp the left kidney gently.
8. Using a 3-0 wax coated braided silk suture tied into a loop, ligate the hilum of the left kidney and secure with 3 surgical knots. Remove the left kidney with surgical scissors.
9. Inspect the suture tie carefully to ensure that adequate hemostasis is achieved.
10. Close the subcutaneous layer with a continuous loop suture using 5-0 braided absorbable, then staple skin layer using 9 mm staples. Steps 5.2 to 5.10 should require 10 min/animal.
11. Maintain Buprenorphine (0.05 mg/kg) for pain management administered s.c. every 8 hr for 2 days.

6. Euthanizing Mice and Processing and Quantification of Lung Tumor Burden

1. At 28 days post 4T1 tumor injection, euthanize mice according to Animal Care and Veterinary Services protocol at The University of Ottawa.
2. Spray down mouse with 70% ethanol.
3. Make initial incision with scissors just below the rib cage.
4. Expose the thorax by cutting through the skin and subcutaneous layer along the ventral midline of the chest cavity of the mouse.
5. Make lateral incisions through skin and tissue on each side up to the neck of the mouse.
6. Dissect out the lungs by gently grasping the lung while snipping away the connective tissue above and below the lungs.
7. Presoak extracted lungs in cold PBS to remove residual blood, then place in 10% Buffered Formalin.
8. Photograph each lung for tumor metastases assessment. Weigh lungs for tumor burden quantification.

Representative Results

A reproducible mouse model of surgical stress that results in the dramatic enhancement of pulmonary metastases has been developed. At day 28 post 4T1 tumor inoculation (and 14 days post tumor resection +/- abdominal nephrectomy), lungs were harvested and visualized for metastases. Surgery clearly increases the amount of pulmonary metastases compared to untreated mice as demonstrated by lung photographs (**Figure 1A**), enumeration of lung nodules (**Figure 1B**) and lung weight (**Figure 1C**). Preoperative administration of replicating oncolytic virus and inactivated influenza vaccine significantly rescues the prometastatic effects of cancer surgery (**Figures 1A-C**).

To determine whether NK cells play a mediating role in preventing metastases post-vaccine treatment, NK cells were pharmacologically depleted using anti-asialo-GM-1 in the tumor metastasis model. In the absence of NK cells, we observed an abrogation of the therapeutic effect of perioperative immunotherapies (**Figures 2A** and **2B**). This data suggests that tumor metastases removal in our surgical stress model is mainly mediated through oncolytic virus and influenza vaccine activation of NK cells and subsequent NK mediated tumor lysis. To further characterize NK cell function following perioperative administration of oncolytic virus and influenza vaccine, *ex vivo* NK cell killing was assessed. Briefly, pooled and sorted DX5+ NK cells were isolated from splenocytes of surgically stressed and control mice. They were cocultured for 4 hr with chromium labeled YAC-1 target cells, at different Effector to Target ratios, followed by measurement of supernatant chromium release with a gamma counter. A significant surgery induced defect in NK cell cytotoxicity along with a significant recovery of NK killing following perioperative administration of oncolytic virus and influenza vaccine compared to surgery alone (**Figures 2C** and **2D**) was observed. Taken together, these results demonstrate that perioperative NK cell suppression can be successfully treated and metastatic disease reduced with novel immunostimulatory therapies.

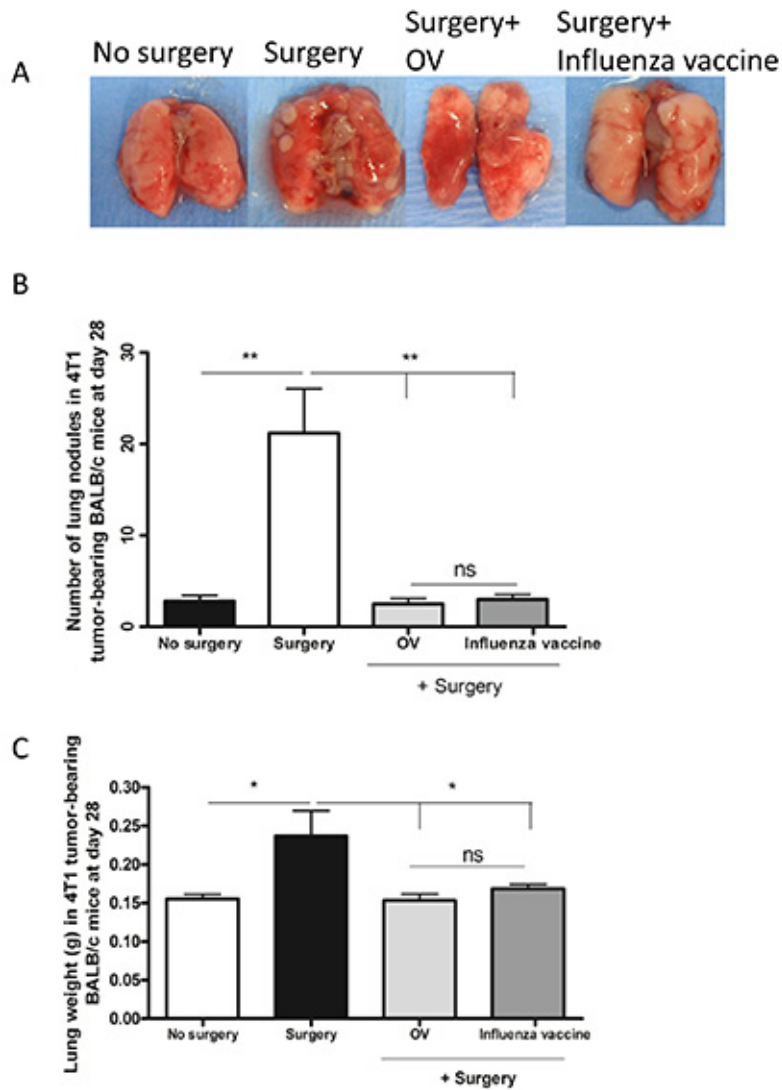


Figure 1. Novel anti-cancer oncolytic virus and influenza vaccine as perioperative therapy against surgery-induced enhancement of lung metastases. Assessment of 4T1 lung tumor metastases at day 28 of indicated treatment groups by **(A)** photographs of representative lungs, **(B)** enumeration of lung tumor nodules and **(C)** lung weights. Data are representative of 3 similar experiments with n=5-10/group (*, p =0.01;**, p <0.0001; n.s., not significant). [Please click here to view a larger version of this figure.](#)

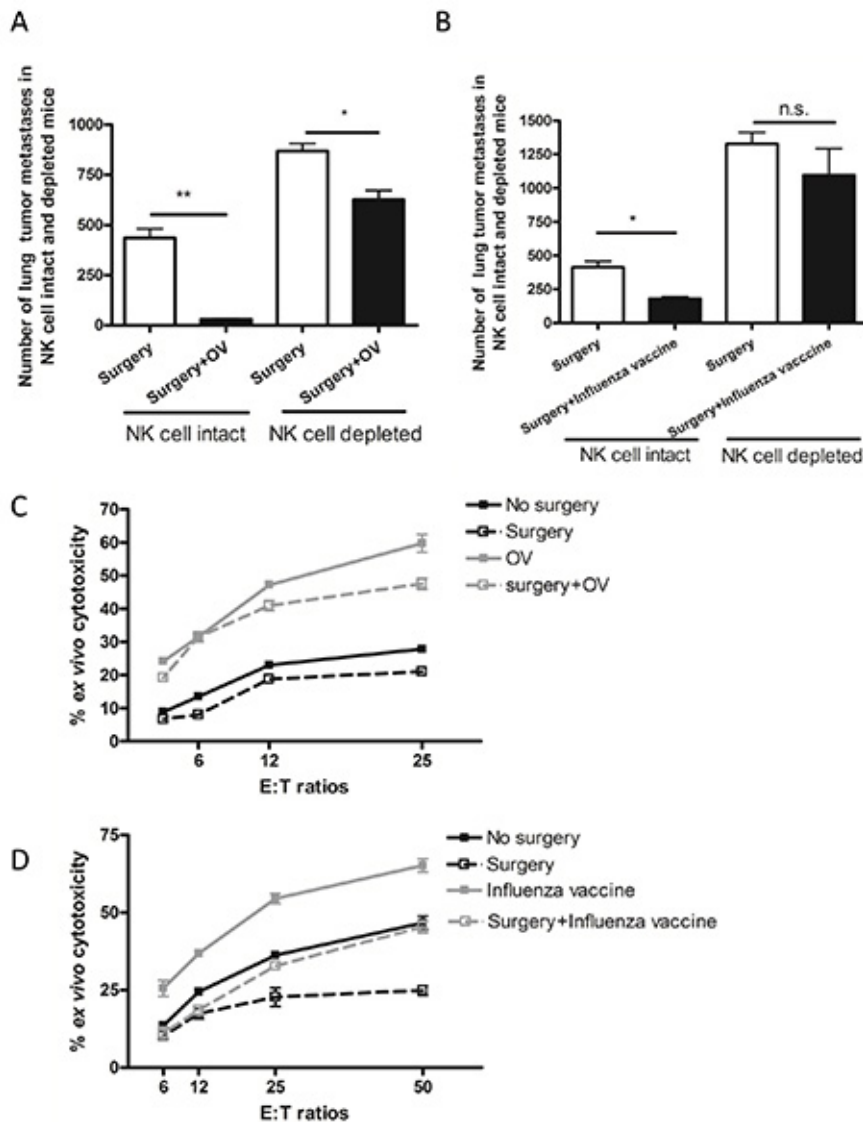


Figure 2. Surgical stress increases lung tumor metastases by impairing NK cells. (A,B) Quantification of lung tumor metastases in surgically stressed mice treated with novel perioperative therapy. (C,D) The ability of purified DX5+ NK cells from surgically stressed and untreated controls to kill tumor cells. The data are displayed as the mean percent (+/- SD) of chromium release from triplicate wells for the indicated E:T ratios. Data are representative of 3 similar experiments where n=4-5/group (*, p =0.01;** p <0.005; n.s., not significant). [Please click here to view a larger version of this figure.](#)

Discussion

Surgical resection is the mainstay of therapy for patients with localized solid malignancies. Even with complete resection, many patients develop a metastatic recurrence and ultimately die of their disease. The immediate postoperative period provides an ideal environment for the formation of cancer metastases, modulated, in large part, by postoperative NK cell suppression. Despite this, it remains a therapeutic window that is largely ignored. There are currently no standard perioperative anti-cancer therapies aimed at preventing postoperative metastases. The current challenge is to identify safe and promising therapies that will activate NK cells in the perioperative period thereby preventing the establishment of micrometastatic disease. These therapies must be rigorously characterized for safety and efficacy in preclinical animal models and then translated into thoughtfully designed clinical trials.

The primary rationale of developing a mouse tumor model of surgical stress is to explore mechanisms of immune suppression and metastatic spread following surgery and to evaluate innovative immunotherapies with potential for future use in cancer patients undergoing surgery to remove the primary tumor. To accomplish this goal, a 4T1 murine mammary carcinoma model coupled with surgical stress was developed. While the 4T1 cell line is a murine "mammary carcinoma", the reason to use this cell line is the reproducibility of spontaneous metastases that allows us to evaluate the effects of surgical stress in a realistic cancer model. In this context, the actual origin of the malignancy is less important than the metastatic potential and tumor biology. The second crucial component of our model is the development of an animal surgical procedure to closely resemble human cancer surgery. In this animal surgical stress model, the primary 4T1 breast tumor is excised after it reaches 1 cm³. Because cancer surgeries in human patients involve significant immune suppression, an open-abdomen nephrectomy in "surgical stress"

treatment groups is additionally performed. As it compares with more conventional tumor resection in humans, the invasive nature of a full left nephrectomy is very comparable to numerous types of surgical treatments for solid malignancies, including surgery for colorectal cancer, ovarian cancer, kidney cancer, pancreatic cancer, lung and esophageal cancer. In addition, we would argue that the profound physiological changes that occur perioperatively, due to laparotomy+nephrectomy, adequately reproduces the overwhelming physiological changes that occur following invasive surgery for most solid malignancies. To control for perioperative factors that could lead to excessive surgical stress and mortality, the duration of anesthesia and surgery and maintain body temperature during the surgery is precisely defined. All these parameters are precisely executed and defined in our surgery protocol to mirror routine perioperative care in human cancer patients.

The timing of the perioperative treatments is an additional critical component to the perioperative rescue model. 4T1 tumor bearing mice have been previously treated with 3 regimens of influenza vaccine: neoadjuvant (given 5 days prior to surgery), perioperative (given on the same day of surgery) and perioperative + multidose (given on the day of surgery, followed by 2 additional doses given 5 days apart). Remarkably, all 3 modes of vaccine treatment significantly decreased lung metastases²². However, influenza vaccine administered perioperatively as a single dose reduced metastases most effectively. Collectively, these experiments highlight the importance of the immediate perioperative period as a narrow therapeutic window to intervene in the metastatic process.

Perioperative use of innovative immunotherapies such as oncolytic virus and vaccines has exclusively been limited to our research group. We demonstrated for the first time that perioperative administration of novel oncolytic ORF and vaccinia viruses can reverse NK cell suppression following surgery in animal models¹. More importantly, this rescue of immune function correlates with a reduction in the postoperative formation of metastases. In human studies, postoperative cancer surgery patients had reduced NK cell cytotoxicity and perioperative OV markedly increases NK cell activity in cancer patients¹. Using commercially available prophylactic vaccines, we demonstrated that perioperative influenza vaccine administration significantly reduced tumor metastases and improved NK cell cytotoxicity in preclinical tumor models. In human studies, influenza vaccine significantly enhanced NK cell activity in healthy human donors and cancer surgery patients²².

Many approaches are used to reduce cancer recurrence, including chemotherapy, and radiation, but these therapies are usually administered weeks to months before (neoadjuvant) or after (adjuvant) surgery. Research in this field shows that the immediate postoperative period is critical in determining long-term tumor recurrence rates. Therefore, clinical interventions in the form of immunotherapies during this critical period may have substantial long-term benefits. Our studies using a mouse model of spontaneous lung metastasis and surgical stress provide a unique opportunity to explore novel therapies and determine their potential to prevent perioperative immunosuppression in cancer surgery patients and thereby also reduce cancer recurrence rates.

Disclosures

The authors declare no competing financial interests.

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