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Original Article

Characterization of surgical models of postoperative tumor recurrence for preclinical adjuvant therapy assessment

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Abstract: Purpose: Nearly 30% of cancer patients undergoing curative surgery succumb to distant recurrent disease. Despite large implications and known differences between primary and recurrent tumors, preclinical adjuvant therapy evaluation frequently occurs only in primary tumors and not recurrent tumors. We hypothesized that well characterized and reproducible models of postoperative systemic recurrences should be used for preclinical evaluation of adjuvant approaches. Experimental Design: We examined traditional animal models of cancer surgery that generate systemic cancer recurrences. We also investigated models of systemic cancer recurrences that incorporate spontaneously metastatic cell lines and surgical resection. For each model, we critiqued feasibility, reproducibility and similarity to human recurrence biology. Using our novel model, we then tested the adjuvant use of a novel systemic inhibitor of TGF-β, 1D11. Results: Traditional surgical models are confounded by immunologic factors including concomitant immunity and perioperative immunosuppression. A superior preclinical model of postoperative systemic recurrences incorporates spontaneously metastatic cell lines and primary tumor excision. This approach is biologically relevant and readily feasible. Using this model, we discovered that "perioperative" TGF-β blockade has strong anti-tumor effects in the setting of advanced disease that would not be appreciated in primary tumor cell lines or other surgical models. Conclusions: There are multiple immunologic effects that rendered previous models of postoperative cancer recurrences inadequate. Use of spontaneously metastatic cell lines followed by surgical resection eliminates these confounders, and best resembles the clinical scenario. This preclinical model provides more reliable preclinical information when evaluating new adjuvant therapies.

Keywords: Surgery, recurrence, models, surgical oncology, concomitant immunity, perioperative immunosuppression, TGF-β

Introduction

Systemic cancer recurrences occurring after "curative" surgical resection account for more than 300,000 deaths each year [1]. These recurrences often result from the proliferation of micrometastatic disease that was undetected at time of surgery [2]. These metastatic foci are established during primary tumor growth and are the result of hematogenous and/or lymphatic spread to distant locations [3].

In attempts to prevent morbidity and mortality from systemic cancer recurrences, surgical candidates often receive adjuvant protocols incorporating chemotherapy, targeted biologics, or immunotherapy. Despite common use in an adjuvant setting, few of these modalities have been critically analyzed using preclinical models of recurrent diseases. This is at least partially driven by the paucity of such recurrence models readily available to the translational researcher. Instead, preclinical analysis has been carried out in models of primary cancer then assumed to be applicable to recurrent disease scenarios.

Such an assumption is concerning given well-documented immunologic changes that occur

after surgery and differences between primary and recurrent disease [4]. With this in mind, our group has become interested in investigating adjuvant therapies in preclinical models incorporating surgery and systemic recurrence development. More specifically, we sought to critically evaluate and develop murine models of systemic cancer recurrences that occur following surgery. Using a biologically relevant and reliable model of systemic recurrence, we examined the impact of immunotherapy (systemic TGF-β) on postoperative recurrences. This novel model revealed an impressive synergy between surgery and immunotherapy that would have been overlooked in traditional preclinical tumor models that fail to incorporate surgical resection.

Materials and methods

Animals

Female C57Bl/6, BALB/c, and B6x129/J1 hybrid (6-8 weeks old) mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All mice were maintained in a pathogen-free animal facility for one week prior to experimentation. The Animal Care and Use Committees of the Wistar Institute and University of Pennsylvania approved all animal study protocols described in this publication, and experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

Cell lines

The murine malignant mesothelioma cell line, AB12, was derived from an asbestos-induced tumor and has been previously described in detail [5]. The murine esophageal carcinoma cell line, AKR, was derived from mouse esophageal squamous epithelia with cyclin D1 over expression via Epstein-Barr virus ED-L2 promoter in p53 deficient genetic backgrounds [6]. The murine lung cancer cell line, TC1, was derived from mouse lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene [7]. The spontaneously metastatic murine lung cancer line, LKR, was derived from an explanted pulmonary tumor from an activated KrasG12D mutant mouse that had been induced in an F1 hybrid of 129Sv.J and C57BL/6 [8]. The metastatic NSCLC cell line, murine Lewis lung carcinoma (LLC), was obtained from American Type Culture Collection (Manassas, VA).

AB12, LKR, AKR, and LLC cell lines were cultured and maintained in high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS; Georgia Biotechnology, Atlanta, GA), 5 Ag/mL penicillin/streptomycin, and 2 mmol/L glutamine. The TC1 cell line was grown in vitro in RPMI, 10% FBS, 5 Ag/mL penicillin/streptomycin, and 2 mmol/L glutamine. Cell lines were regularly tested and maintained negative for *Mycoplasma spp.*

Animal models

Mice were injected subcutaneously on the flank with $5x10^5$ AKR tumor cells (C57Bl/6 mice), $1x10^6$ AB12 cells (BALB/c mice), $1.2x10^6$ TC1 cells (C57Bl/6 mice), $2x10^6$ LLC cells (C57Bl/6 mice), or $2x10^6$ LKR cells (Bl/6x129/J1) unless otherwise noted. Tumor cells for subcutaneous injections were suspended in 100μL PBS. Tumor volume was calculated using the formula (3.14 x long-axis x short-axis^2)/6. Lungs were harvested at various time points and fixed in formalin to assess for pulmonary tumor burden in LKR and LLC models.

Intraperitoneal (i.p.) and intravenous (i.v.) tumor cell inoculations utilized the same quantity of tumor cells as in flank inoculations unless otherwise noted Intraperitoneal cell inoculants were suspended in 500μL PBS, and injected into the peritoneal cavity. Cells for i.v. injections were suspended in 300μL PBS. Pulmonary involvement was determined by the percentage of total lung cross-sectional area with tumor involvement.

Surgery

Mice with flank tumors were anesthetized using ketamine (80mg/kg) and xyalazine (10mg/kg) then shaved. A 1 cm incision was made adjacent to the tumor. Full resection was achieved by excising 100% of the tumor (including vascular supply) using standard blunt dissection. Mice undergoing sham surgery were anesthetized using ketamine (80mg/kg) and xyalazine (10mg/kg), the flank contralateral flank was shaved, a 1cm incision was made. Sterile silk 4-0 sutures were used to close wounds. Buprenorphine (0.1mg/kg) was administered immediately following surgery and 4 hours after as

Table 1. Summary of tested models of cancer recurrence (* Best approaches)

Model	Advantages	Disadvantages
Systemic Recurrence		
I. Two Nodules		
IA. Concurrent Injection	• Technically simple • Easy to monitor primary and metastatic nodules • Consistent growth within each cell line	• Various growth patterns among different cell lines • Timing does not resemble sequence of events in patients with cancer
IB. Interval Injection	• Technically simple • Easy to monitor both nodules • Good representation of sequence of events that occur in patients with cancer	• Growth of the metastatic tumor is inconsistent and varies between cell lines • Primary and metastatic tumor do not differ in phenotype
IC. Rechallenge and Post-Op Rechallenge	• Easy to monitor both nodules • Consistent growth of metastatic tumor in selected cell lines	• Preoperative immunosuppression is an induced immune response that allows for rechallenge • Rechallenge is only feasible in selected cell lines • Metastases are not established at time of surgery
II. Spontaneously Metastatic Cell Lines*	• Technically simple • Consistent, characterizable patterns of systemic recurrence • Resembles events occurring in human cancer biology	• Monitoring recurrent disease is difficult • Requires larger sample sizes (n=15-20) to allow for periodic monitoring & biological variation • Processing of samples analyzed is time consuming

postoperative analgesia.

Transforming growth factor-β (TGF-β) inhibitor, 1D11

The anti-murine TGF-β mAb, 1D11, neutralizes the three isoforms of TGF-β [9], while the murine IgG1 mAb against Shigella toxin, 13C4, serves as an isotype control. Antibodies were provided by the Genzyme Corporation. Antibodies (1D11 and 13C4) were suspended in 0.2 ml of distilled water and administered intraperitoneally at a dose of 25 mg/kg. Once begun, antibodies were given three times per week for three weeks [10, 11].

Statistical analysis

Differences among two groups were compared by Student t-test or one-way analysis of variance (ANOVA) for multiple comparisons with appropriate post hoc testing. Fisher exact test was used for categorical data. Results are expressed as the mean and the standard error of the mean, unless otherwise noted. Time to disease progression was defined as the time from surgery until the recurring tumor reached a volume of 500mm^3. Best-fit linear regressions for models were generated using time from surgery as the independent variable with mean tumor volume or surface area as dependent variables. A $p<0.05$ was considered a statistically significant result.

Results

Manipulation of non-metastatic cell lines to model systemic recurrences

We performed a literature search in the U.S. National Library of Medicine (PubMed) and identified small animal models of cancer surgery that model postoperative systemic recurrences. A frequent approach involves simultaneously establishing multiple tumor sites in a mouse [12-17]. One tumor is dubbed the “primary tumor” while other site(s) is designated “metastatic”. Surgery is then performed on the primary site, and the other lesions are monitored as systemic recurrences. There are multiple variations of this model documented in the literature: the second nodule is established at the time of the primary tumor, after the primary tumor, at the time of surgery, or following surgery (**Table 1**) [12-16]. The "metastatic foci" may be located in the flank (subcutaneous injection), peritoneum

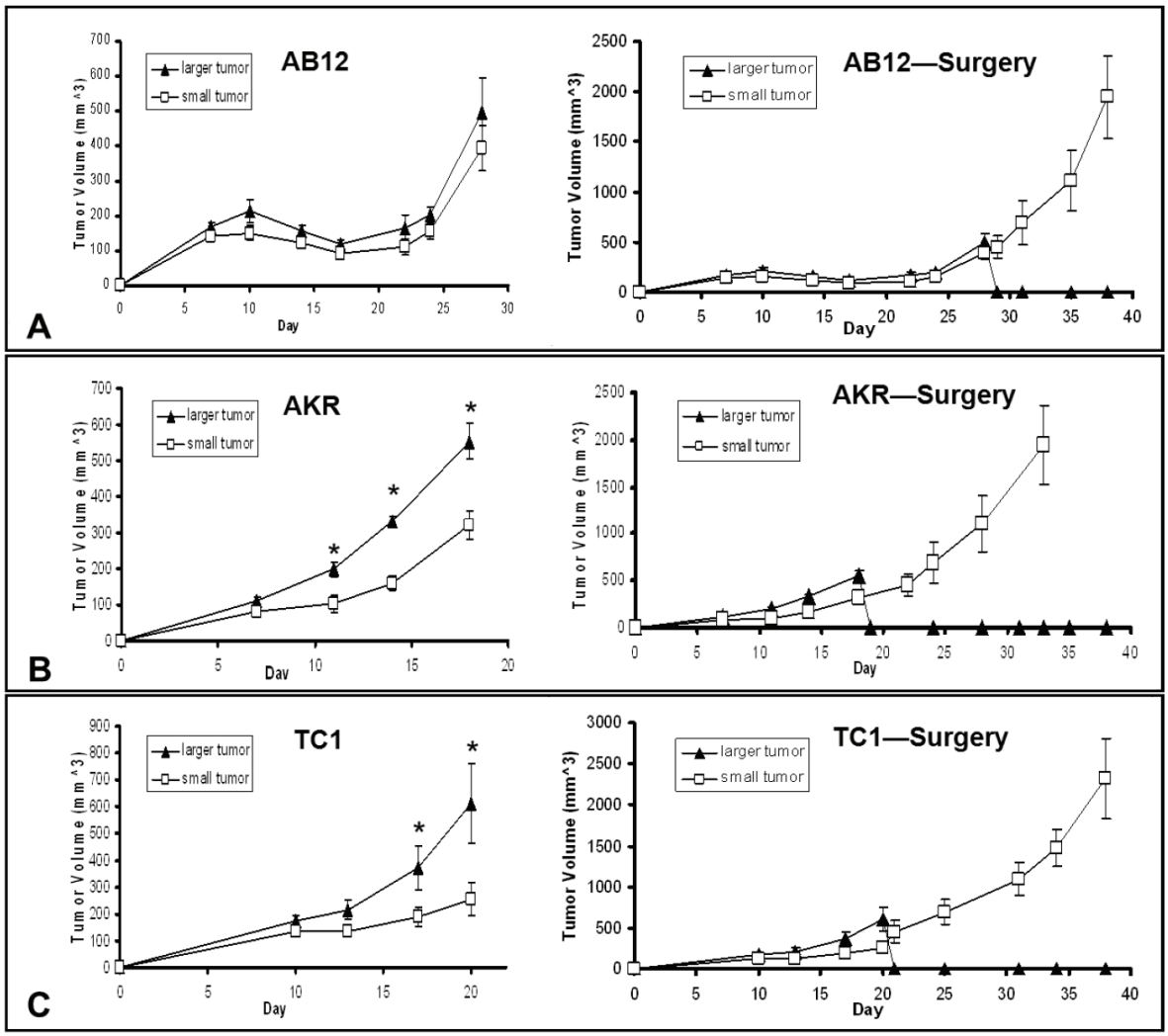

Figure 1. Concurrent injection of two flank tumors as a model of systemic recurrence after surgery. Growth was consistent and predictable for AB12, AKR and TC1 cell lines. Larger tumors were resected at a volume of 500 mm^3. A. Initial growth of AB12 flank tumors is similar between both nodules. Following removal of one nodule, the second nodule rapidly enlarges. B. Initial growth of AKR is different between the tumor nodules. After resection of the dominate nodule, postoperative growth of the remaining, smaller nodule rapidly increases. C. TC1 demonstrates a similar finding to AKR. *denotes statistical difference at time point ($P \leq 0.05$).

(intraperitoneal injection) or the lung (intravenous tail injection). We evaluated each variation in turn.

Simultaneous tumor nodules

The simplest approach incorporates concurrent injection of tumor cells at two locations ("primary" tumor and "metastatic" tumor) followed by resection of the primary tumor at a later date. To evaluate this approach, we injected an equal number of tumor cells simultaneously into subcutaneous locations on both the left and right flanks of mice. We tested this model using three cell lines in separate experiments: TC1-lung cancer, AKR-esophageal cancer, and AB12-mesothelioma. We observed bilateral tumor growth in more than 90% of the mice, and cell lines developed two distinct growth patterns. The first trend, exemplified by the AB12 cell line, demonstrated similar growth patterns in both nodules (similar rates with less than 15% variability in the dimensions between the two nodules) (**Figure 1A**). The second pattern, exemplified by TC1 and AKR cell lines, was characterized by a "dominant" and a "non-

dominant nodule". One nodule often became the dominant nodule and displayed continued growth; the other non-dominant nodule would plateau in size following an initial burst of growth (**Figure 1B** and **1C**). Regardless of the initial growth pattern, we performed surgery on the larger nodule (our primary lesion). In all animals, the remaining nodule displayed continued growth after the primary lesion was excised (**Figure 1A, 1B** and **1C**). All mice were ultimately euthanized due to tumor burden. Although this approach was technically feasible and provided predictable growth within given cancer models, it poorly mimics the sequence of events occurring in patients with cancer. Instead, this approach better mimics the scenario of synchronous primary lesions.

Injection of a second tumor inoculum before surgery

A second frequent approach in the literature includes injecting tumor cells at a distant site after the first flank nodule is established, followed by resection of the first (primary) nodule. This has been argued to be biologically relevant as it better reflects clinical sequences that occur in patients with metastatic disease. To test this method, tumor cells (either TC1, AKR, and AB12) were injected into the flanks of mice. After tumors were fully established and measured 500mm^3 (approximately 2 weeks later), tumor cells were injected into the contralateral flank.

Growth of the second tumor was observed in less than 30% of mice in each cell line (except for non-immunogenic Lewis Lung Cancer, LLC [12]. Tumor cells used in the second injection were confirmed to be viable by simultaneously injecting them into a tumor naïve mouse (data not shown). We repeated these experiments and injected two- to four-fold more tumor cells in the second inoculum. Despite increasing the number of tumor cells by several orders, growth of the second nodule was unsuccessful (data not shown).

To determine if the second flank position was in a suboptimal location due to inadequate blood supply, we experimented with alternate locations. For example, the second inoculum was injected into the peritoneal space rather than a subcutaneous location. Abdomens were then followed for signs of tumor burden (ascites and tenderness) for 3 weeks. After four weeks of observation, no mice displayed signs of abdominal disease. On necropsy, no other evidence of abdominal or systemic disease was found. Again, after increasing cell inoculums by two- and three-fold, no growth was observed (data not shown). We also injected tumor cells intravenously via the tail vein as a second inoculum. Again, systemic tumor involvement was not observed. Even after doubling and tripling the inoculum, additional growth was not observed. Although theoretical benefits exist for the interval injection of flank tumors, the growth of a second nodule is inconsistent once a cell line has established a tumor in a syngeneic immunocompetent mouse.

Evolution of concomitant immunity

Given our above observations, we performed additional experiments to sequence the timing of tumor immunity that develops when using syngeneic models of murine cancer. We injected fifty mice with various tumor cell lines (AB12, AKR, TC1, LLC) into a subcutaneous flank location. At variable time periods following the initial injection (Day 3, Day 6, Day 9, Day 14), ten mice in each group were injected with an equal number of the same initial tumor cell line into the contralateral flank.

In the AB12 and TC1 cell lines, we observed consistent growth (greater than 95% of mice) of the second inoculum when injected within three to six days of the first. Following this period, fewer than 30% of the second tumors would implant (**Figure 2A**). Interestingly, in AKR and LLC, cell lines which have lower levels of immunogenicity, growth of the second inoculum was more common than in the AB12 and TC1 cell lines at all time points (**Figure 2A**). We repeated these experiments and injected two- to four-fold more tumor cells in the second inoculum. Despite increasing the number of tumor cells by several fold, growth of the second nodule remained unsuccessful (data not shown). This propensity of the host immune system to recognize and eradicate the second inoculum likely results from development of concomitant immunity.

Second tumor inoculum (rechallenge) at the time of surgery-perioperative immunosuppression

Previous reports on surgical models have described growth of a second tumor cell inoculum

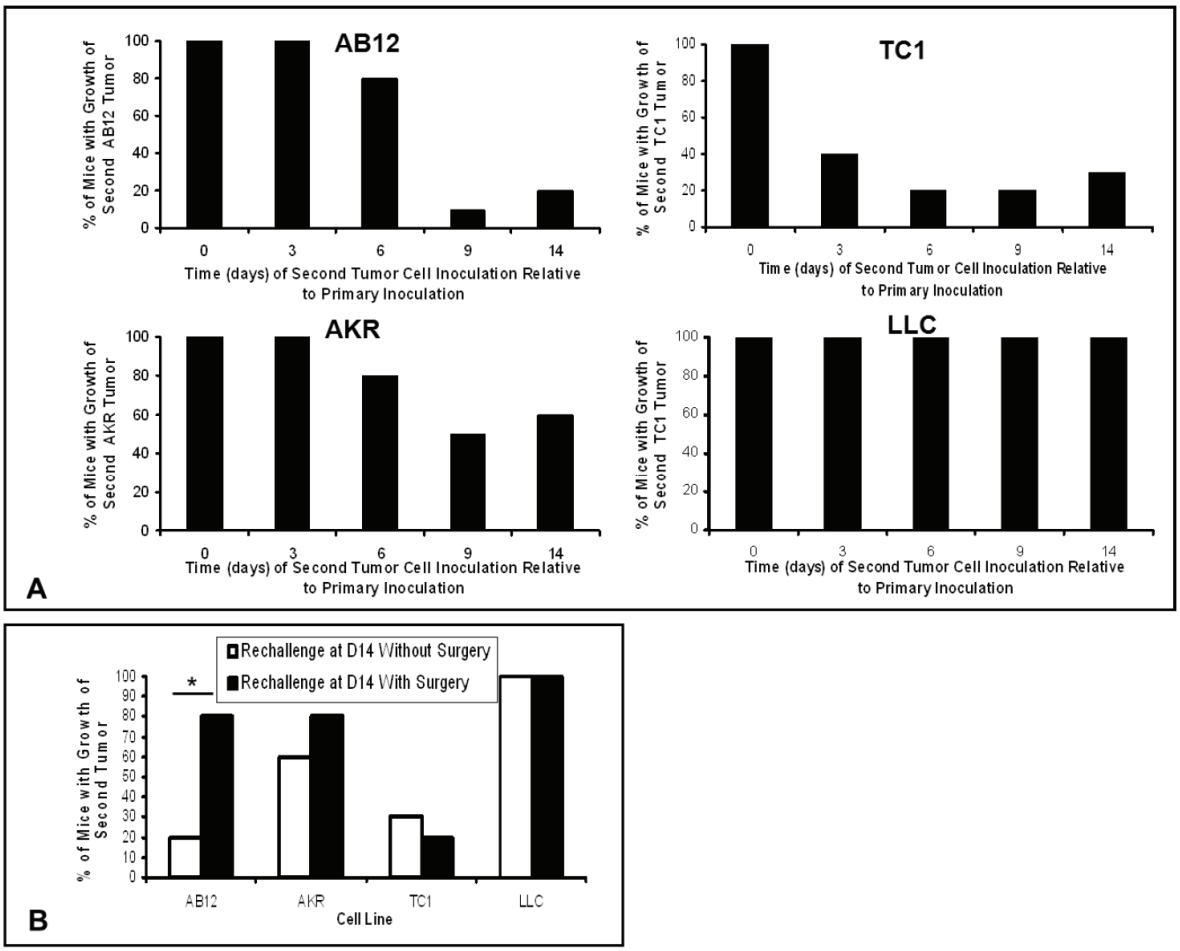

Figure 2. Interval injection of a second "metastatic" tumor focus as a model for systemic recurrence after surgery. A. When performing surgery on Day 14, second tumor implantation was observed more frequently in the AB12 and AKR cell lines. The rate of second inoculum establishment did not change in the TC1 and LLC cell lines. B. Interval injection of flank tumors occurred at the time of primary tumor inoculation (Day 0) or at various times following this (Day 3, 6, 9, 14). The percentage of second tumors which established decreased with time; however, this trend varied between the AB12, TC1, AKR and LLC cell lines. *signifies a statistically significant increase in the second tumor implantation ($P \leq 0.05$).

when injected immediately after excision of the first nodule [16]. We thus examined whether surgical resection of the primary tumor allowed growth of a second inoculum of tumor cells. Mice were injected with syngeneic tumor cells (either AB12, AKR, LLC, and TC1) into a subcutaneous location in the right flank. At Day 14, a time when we found establishment of a secondary to be unreliable, we fully excised the primary tumor using sterile surgical technique. While under anesthesia, fresh tumor cells were injected into the contralateral flank.

For the AB12, AKR, and LLC cell lines, over 80% of the tumor re-challenges grew consistently if injected at the time of primary flank tumor surgery (**Figure 2B**). However, if the second inoculum was injected more than 3 days following surgery, less than 20% of the tumor rechallenges implanted (data not shown). All secondary tumors that grew were detected within 7 days of surgery. Growth rate of the secondary tumor was similar to that of the primary tumor (data not shown). For the TC1 cell line, injection of the second inoculum of cells immediately after surgery resulted in no tumor growth. Of note, previous studies have identified TC1 as a very immunogenic cell line which likely explains this finding [12]. These findings likely the phenomena of perioperative immunosuppression

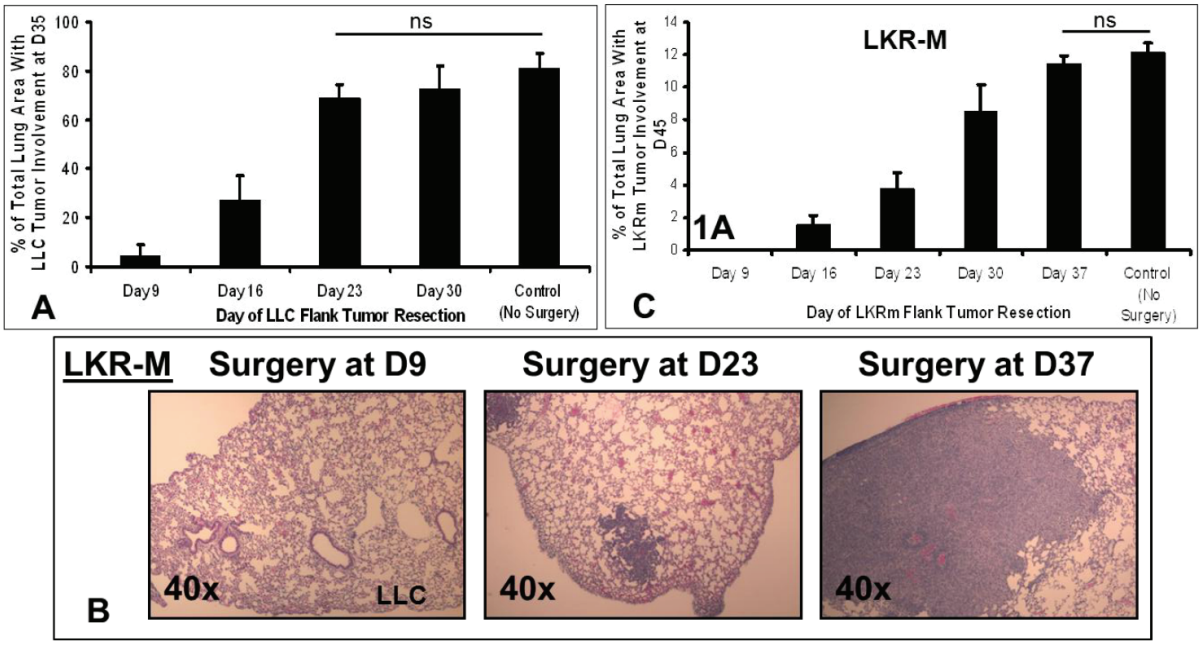

Figure 3. Spontaneously metastatic murine NSCLC cell lines (LLC and LKR) as models of systemic recurrence following surgery. A. Even when fully resecting LLC flank tumors as early as 9 days, lung metastases are seen. Later flank tumor resection was associated with increased metastatic involvement at Day 35. This trend was observed until flank resection at Day 23, after which point lung burden at Day 35 remained consistent and similar to controls. B. After 45 days of LKR flank tumor growth, a significant amount of metastatic burden is found in the lungs. C. Even after resecting LKR flank tumors as early as 16 days, lung metastases are seen. Later flank tumor resection was associated with increased metastatic involvement. There was no difference in lung involvement at Day 45 when compared to mice undergoing flank tumor resection at Day 37. ns signifies lack of statistical difference in percentage of lung surface area with tumor involvement ($P > 0.05$).

which describes a short window of immune system dysfunction that follows a surgical intervention [18, Markovic, 1993 #546, Akiyoshi, 1985 #550, Ogawa, 2000 #373].

These results suggest that previous models that incorporating "metastatic focus" establishment at the time of "primary focus" excision are dependent on complicated immunologic principles, which confound preclinical durg evaluation. Further, second inoculum establishment is only feasible in certain tumor lines and poorly mimics the order of events observed in human cancer biology (when metastatic foci are present prior to surgical excision).

Spontaneously metastatic tumor cell lines create rigorous systemic recurrence models

Given the described immunologic confounders associated with previous models of postoperative systemic cancer recurrences, we evaluated recurrence models utilizing cell lines that spontaneously metastasize [19, 20]. We characterized systemic recurrences after surgery in LLC cells and a new lung cancer cell line developed in our lab, LKR [8].

To characterize systemic recurrences (pulmonary metastases) following surgery, we began by injecting LLC or LKR tumor cells into the right flanks of C57Bl/6 and Bl6x129/J1 fifty mice, respectively. At day 9, 16, 23, and 30, ten mice underwent complete resection of the flank tumor. A control group underwent sham surgery at Day 9. Forty days after the injection, mice were sacrificed, lungs were harvested, and pulmonary tumor burden was quantified. After day 9, despite flank tumor resection, all mice undergoing complete resection of LLC flank tumors were had evidence of pulmonary disease (**Figure 3A**). Similarly, all mice undergoing LKR flank tumor resection after day 16 were found to have evidence of pulmonary involvement (**Figure 3B** and **3C**). In both models, it was also noted that delayed excision of flank tumors cor-

related with increased pulmonary tumor burden (Figure 3A and 3C).

In attempts to monitor for pulmonary metastasis with non-invasive imaging, we transduced LKR cells with several bioluminescent gene products include luciferase, Katushka, and Tomato Red prior to injecting them into mice [21, 22]. The flank tumors initially grew as large as 150mm^3 but eventually fully regressed (data not shown). On necropsy, no evidence of pulmonary involvement was found. When analyzing residual tumors, bioluminescent genes were no longer present, presumably due to an immune response against tumors cells containing foreign gene products.

In conclusion, flank tumors that develop spontaneous metastases despite complete surgical resection are practical and much more closely mimic the systemic recurrence chronology observed in cancer patients. Unfortunately, optimal quantification of systemic disease requires animal sacrifice and imaging probes have the potential to alter the natural history of this disease.

Surgical models of systemic recurrence allow for accurate evaluation of adjuvant immunotherapy (TGF-β inhibitor, 1D11)

Multiple preclinical studies have demonstrated that systemic TGF-β blockade impedes tumor progression in murine models of mesothelioma [23, 24], gliomas [11], NSCLC [25] and renal cell carcinomas [26]. Additional studies have suggested that TGF-β blockade, when combined with tumor vaccination, generates augmented anti-tumor responses [10, 11]. Given these potent effects, a role for TGF-β inhibition as a surgical adjuvant has been proposed despite only being evaluated in non-surgical models. Using spontaneously metastatic models along with surgical resection, we sought to determine the efficacy of adjuvant TGF-β inhibition with the soluble anti-murine TGF-β mAb, 1D11.

We began by determining the role of 1D11 as monotherapy without a surgical intervention in treating the spontaneously metastatic lung cancer model, LKR. To do this we injected 2x10^6 LKR tumor cells into the flank of Bl/6x129/J1 mice. Once tumors were established (approximately 300 mm^3), mice (n=20) were randomized to 1D11 or control antibody protocols which consisted of intraperitoneal injections at twice weekly for 3 weeks. After 40 days of tumor growth, mice were sacrificed and lungs were analyzed for tumor invasion. Mice randomized to 1D11 therapy were found to have a modest reduction in metastatic lung involvement as compared to mice randomized to control antibodies: 15.8% of lung surface area was found burdened by cancer in mice receiving control antibody versus 13.7% in mice receiving 1D11; p=0.83 (Figure 4A).

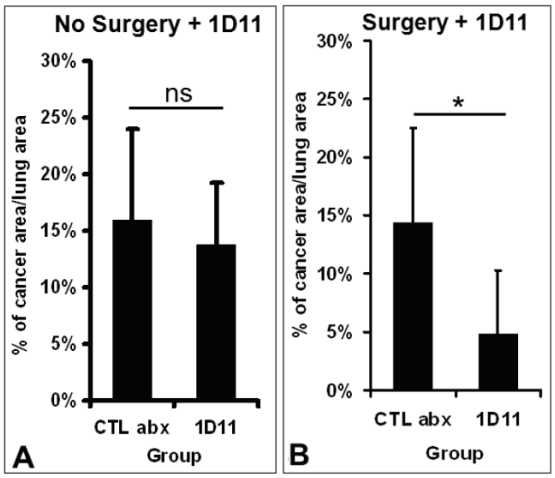

Figure 4. Efficacy of TGF-β inhibition in preventing systemic metastases in non-small cell lung cancer. A. 2x10^6 LKR tumor cells were injected into the flank of Bl/6x129/J1 mice (Day 0). On Day 16, animals were randomized to 1D11 or control antibody protocols which consisted of intraperitoneal injections at 2 times per week for 3 weeks. After 40 days of tumor growth, mice were sacrificed and lungs were analyzed for tumor invasion. Mice randomized to 1D11 therapy were found to have a negligible differences in metastatic lung involvement compared to mice randomized to control antibodies, 15.8% of lung surface area was found burdened by cancer in mice receiving control antibody versus 13.7% in mice receiving 1D11 (p=0.83). B. Experiments were repeated and flank tumors were completely excised on Day 16. Three days after excision of the primary subcuatenous focus, mice were randomized to 1D11 (n=6) or its control antibody, 13C4, (n=6). At 40 days following flank tumor inoculation, mice were sacrificed and lungs were assessed for tumor burden. The use of postoperative 1D11 provided more than a 50% reduction in lung surface area involved with tumor; 14.4% in mice randomized to surgery with control antibody versus 4.89% in mice receiving adjuvant 1D11 with surgery (p=0.03).

We next focused attention to evaluate 1D11 as

surgical adjuvant using the systemic recurrence cancer model. Mice were inoculated with LKR flank tumors, and flank tumors were completely excised once the tumors reached a size of 300 mm^3. Three days after excision of the primary subcutaneous focus, mice were randomized to 1D11 (n=6) or its control antibody, 13C4, (n=6). At 40 days following flank tumor inoculation, mice were sacrificed and lungs were assessed for tumor burden. The use of postoperative 1D11 provided more than a 50% reduction in lung surface area involved with tumor: 14.4% in mice randomized to surgery with control antibody versus 4.89% in mice receiving adjuvant 1D11 with surgery; p=0.03 (**Figure 4B**). Of note, no mice randomized experienced wound breakdown.

Using systemic recurrence cancer models (spontaneously metastatic cell lines with complete flank tumor resection), we determined a large decrease in metastatic lung lesions in mice randomized to surgery with adjuvant 1D11 versus surgery alone. It is important to appreciate that this significant advantage would have been underestimated if traditional non-surgical models of systemic disease had been utilized as described in previous reports. These results demonstrate the necessity for accurate surgical models to evaluate adjuvant treatments in preclinical studies.

Discussion

In this study, we examined a series of animal models with the goal of simulating a common, important, but understudied clinical scenario: systemic cancer recurrences after surgery. Using our optimal models of local and systemic recurrences, we evaluated the efficacy of an adjuvant systemic TGF-β inhibitor (1D11) in eliminating postoperative recurrences.

Each model displayed distinct advantages and disadvantages (summarized in **Table 1**). Although previous approaches are reproducible and feasible, they fail to account for several important themes which render them of limited utility.

First, as previously described, metastatic foci are established during primary tumor growth [3]. Unfortunately, multiple flank tumor models previous models poorly recapitulate these important steps in disease progression. For example, simultaneous injection of two flank nodules, a technique used widely in the literature, poorly mimics the time course of systemic metastasis developing, rather, it more closely resembles the circumstances of synchronous primary tumors (given that lesions are injected simultaneously). Similarly, the “rechallenge” approach fails to consider the biology of metastatic foci which exist prior to surgical resection.

A second consideration that is overlooked in traditional models is concomitant immunity. The concept of "concomitant immunity" was first reported by North and colleagues in 1984, and describes the acquired ability of a host with a progressive tumor to reject a second inoculum of the same tumor at a distant site [27]. The mechanism of this immunity against tumors is primarily due to a protective T-cell memory that develops during the initial tumor exposure [28, 29]. Although this immune response is not powerful enough to eliminate the established tumor, it is able to eliminate a second inoculum. This immunity variably disappears (depending on the tumor and mouse strain), due to induction of suppressor cells, most notably T-regulatory cells [29]. Our experiments support the early work described by both North and Bursuker and the more recent work conducted by Turk and her colleagues [27, Bursuker, 1986 #33, Turk, 2004 #45]. Due to concomitant immunity, we found it was difficult to inject multiple flank sites with cancer cells and have a reproducible model of metastasis. Further such findings likely confound data acquired using such approaches.

A third consideration involving previous models of postoperative cancer recurrences is perioperative immunosuppression. In our experiments, resecting the primary tumor indeed did provide a temporary window in which tumor growth occurred. Many studies suggest that surgery generates a transient immunosuppression that allows increased tumor growth [4, 30-32]. This window is thought to result from inflammatory, neural, and hormonal factors. One arm stems from general anesthesia, which has a role in decreasing natural killer cell activity [33]. The other arm, surgery itself, impairs production of IL-2 [34] and generates immune suppressor cells [35]. These immunologic forces confound the already complicated task of developing clinically relevant animal models to study post-surgery recurrences.

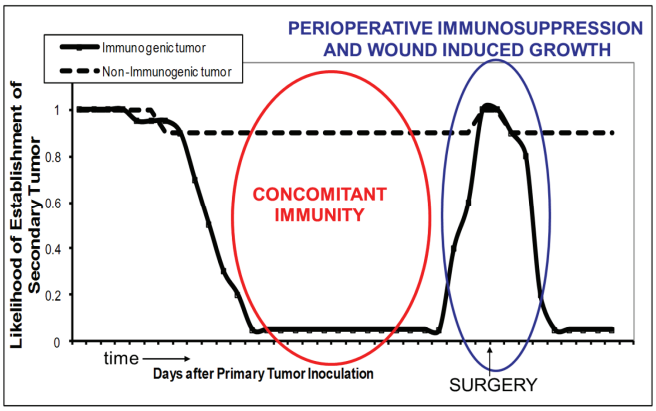

Figure 5. Representation of immunologic forces that influence animal models involving tumor resection. During a short period following initial tumor cell inoculation, a second inoculation will result in consistent growth. Following this period, growth of a second inoculum is inhibited by concomitant immunity in immunogenic cell lines. For non-immunogenic cell lines, concomitant immunity is weak and growth of second inoculum remains feasible. The perioperative effects of surgery temporarily overcome the anti-tumor effects associated with concomitant immunity, and allow for successful establishment of a second tumor in some immunogenic cell lines.

To summarize, the balance between these complex factors results in "windows of opportunity" for the injected tumor cells to grow (**Figure 5**), which are somewhat cell type dependent.

Based on our experiences and the considerations previously described, we still believe that an optimal model for post-operative systemic recurrences includes spontaneously metastatic flank tumors and surgical resection of the primary (flank) tumor focus. Accurate results can be obtained with diligently planned experiments and data collection techniques. This model is technically simple and parallels the sequence of events observed in human metastatic cancers. In several cell lines (LLC and LRK), we have characterized the metastatic (pulmonary) involvement which is observed following flank tumor resection. The most challenging aspect to this model pertains to monitoring systemic disease because metastatic lung lesions do not produce symptoms until the tumor burden is far advanced. This makes accurate monitoring of recurrent lung disease difficult, requiring periodic imaging (i.e. micro-CT scans) or timed sampling of experimental groups with necropsy to examine tumor infiltrating into the lung. In addition, we found that experiments which require sacrificing animals to measure the quantity of disease in the lung may necessitate 15 to 20 animals per treatment group to obtain statistically significant results. These issues add to the time needed and expense of the model. Despite hurdles, spontaneously metastatic models are predictable and resemble human cancer biology, making them potentially highly useful for studying systemic post-operative recurrence biology (**Table 1**).

Although encouraging, it is essential to understand our findings in the context of recent advances in cancer biology. Spontaneously arising orthotopic tumors in transgenic models have proven effective for studying tumor microenvironment, growth kinetics, and immune system changes. However, with the exception of breast and skin cancers, surgical resection of orthotopic lesions cannot be reliably or safely performed. Furthermore, these models often require prolonged periods for tumor development, which results in low throughput systems.

In order to validate our approach of studying systemic cancer recurrences, we investigated that the role of systemic TGF-β inhibition in preventing systemic recurrences. TGF-β is a cytokine with multiple immunological effects including powerful immunosuppressive features [36]. It directly suppresses activation and maturation of innate and adaptive immune cells including CD3+ T cells, natural killer cells and antigen presenting cells [37]. Tumors and suppressor myeloid cells have been implicated in the production of TGF-β [38], and TGF-β inhibition has been proposed in patients as an adjuvant therapy to other chemotherapeutic and immunological approaches [39]. This cytokine therapy has been thought to be safe, non-toxic and can be administered for long periods of time without side effects [40].

We have over a decade long experience with TGF-β therapy and recently started a clinical trial in TGF-β inhibition of malignant mesothelioma. Therefore, we chose this agent because we were familiar with this approach, and it would inform a clinical trial combining cytore-

ductive surgery for malignant pleural mesothelioma with immunotherapy. Interestingly, in non-surgical models we found no observable decreases in systemic disease associated with TGF-β inhibition. However, when coupling TGF-β blockade with surgical resection we appreciated dramatic decreases in systemic tumor burden. Potential benefits of this approach may have been overlooked if not evaluated in a proper surgical model aimed at studying postoperative recurrences.

Incorporation of accurate systemic recurrence models following cancer surgery has been a challenge primarily due to the lack of systematically evaluated models. After assessing both current and newly proposed techniques, we conclude that the optimal model of systemic recurrence incorporates spontaneously metastatic cell lines followed by complete flank (primary) tumor excision. Once learned, these techniques offer realistic pre-clinical vehicles to study adjuvant therapies.

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References

[1] Aliperti LA, Predina JD, Vachani A and Singhal S. Local and Systemic Recurrence is the Achilles Heel of Cancer Surgery. Ann Surg Oncol 2011; 18: 603-607.

[2] Maruyama R, Sugio K, Mitsudomi T, Saitoh G, Ishida T and Sugimachi K. Relationship between early recurrence and micrometastases in the lymph nodes of patients with stage I non-small-cell lung cancer. J Thorac Cardiovasc Surg 1997; 114: 535-543.

[3] Fisher B. Biological research in the evolution of cancer surgery: a personal perspective. Cancer Res 2008; 68: 10007-10020.

[4] Shakhar G, Ben-Eliyahu S. Potential prophylactic measures against postoperative immunosuppression: could they reduce recurrence rates in oncological patients? Ann Surg Oncol 2003; 10: 972-992.

[5] Davis MR, Manning LS, Whitaker D, Garlepp MJ and Robinson BW. Establishment of a murine model of malignant mesothelioma. Int J Cancer 1992; 52: 881-886.

[6] Opitz OG, Harada H, Suliman Y, Rhoades B, Sharpless NE, Kent R, Kopelovich L, Nakagawa H and Rustgi AK. A mouse model of human oral-esophageal cancer. J Clin Invest 2002; 110: 761-769.

[7] Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM and Wu TC. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996; 56: 21-26.

[8] Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS and Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 2009; 16: 183-194.

[9] Dasch JR, Pace DR, Waegell W, Inenaga D and Ellingsworth L. Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. J Immunol 1989; 142: 1536-1541.

[10] Terabe M, Ambrosino E, Takaku S, O'Konek JJ, Venzon D, Lonning S, McPherson JM and Berzofsky JA. Synergistic enhancement of CD8+ T cell-mediated tumor vaccine efficacy by an anti-transforming growth factor-beta monoclonal antibody. Clin Cancer Res 2009; 15: 6560-6569.

[11] Ueda R, Fujita M, Zhu X, Sasaki K, Kastenhuber ER, Kohanbash G, McDonald HA, Harper J, Lonning S and Okada H. Systemic inhibition of transforming growth factor-beta in glioma-bearing mice improves the therapeutic efficacy of glioma-associated antigen peptide vaccines. Clin Cancer Res 2009; 15: 6551-6559.

[12] Tanaka T, Delong PA, Amin K, Henry A, Kruklitis R, Kapoor V, Kaiser LR and Albelda SM. Treatment of lung cancer using clinically relevant oral doses of the cyclooxygenase-2 inhibitor rofecoxib: potential value as adjuvant therapy after surgery. Ann Surg 2005; 241: 168-178.

[13] Krewet JA, Ren W, Huang XF, Chen SY and Shah MR. Anti-tumor immune responses following neoadjuvant immunotherapy with a recombinant adenovirus expressing HSP72 to rodent tumors. Cancer Immunol Immunother 2005; 54: 988-998.

[14] Mukherjee S, Nelson D, Loh S, van Bruggen I, Palmer LJ, Leong C, Garlepp MJ and Robinson BW. The immune anti-tumor effects of GM-CSF and B7-1 gene transfection are enhanced by surgical debulking of tumor. Cancer Gene Ther 2001; 8: 580-588.

[15] Jarnagin WR, Zager JS, Klimstra D, Delman KA, Malhotra S, Ebright M, Little S, DeRubertis B, Stanziale SF, Hezel M, Federoff H and Fong Y. Neoadjuvant treatment of hepatic malignancy:

an oncolytic herpes simplex virus expressing IL-12 effectively treats the parent tumor and protects against recurrence-after resection. Cancer Gene Ther 2003; 10: 215-223.
[16] Kruklitis RJ, Singhal S, Delong P, Kapoor V, Sterman DH, Kaiser LR and Albelda SM. Immunogene therapy with interferon-beta before surgical debulking delays recurrence and improves survival in a murine model of malignant mesothelioma. J Thorac Cardiovasc Surg 2004; 127: 123-130.
[17] Ohashi K, Kobayashi G, Fang S, Zhu X, Antonia SJ, Krieg AM and Sandler AD. Surgical excision combined with autologous whole tumor cell vaccination is an effective therapy for murine neuroblastoma. J Pediatr Surg 2006; 41: 1361-1368.
[18] Kutza J, Gratz I, Afshar M and Murasko DM. The effects of general anesthesia and surgery on basal and interferon stimulated natural killer cell activity of humans. Anesth Analg 1997; 85: 918-923.
[19] Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. Curr Protoc Immunol 2001; Chapter 20: Unit 20 22.
[20] Hill HC, Conway TF Jr, Sabel MS, Jong YS, Mathiowitz E, Bankert RB and Egilmez NK. Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. Cancer Res 2002; 62: 7254-7263.
[21] Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer in vivo. Nat Rev Cancer 2005; 5: 796-806.
[22] Kishimoto H, Zhao M, Hayashi K, Urata Y, Tanaka N, Fujiwara T, Penman S and Hoffman RM. In vivo internal tumor illumination by telomerase-dependent adenoviral GFP for precise surgical navigation. Proc Natl Acad Sci USA 2009; 106: 14514-14517.
[23] Suzuki E, Kapoor V, Cheung HK, Ling LE, DeLong PA, Kaiser LR and Albelda SM. Soluble type II transforming growth factor-beta receptor inhibits established murine malignant mesothelioma tumor growth by augmenting host antitumor immunity. Clin Cancer Res 2004; 10: 5907-5918.
[24] Suzuki E, Kim S, Cheung HK, Corbley MJ, Zhang X, Sun L, Shan F, Singh J, Lee WC, Albelda SM and Ling LE. A novel small-molecule inhibitor of transforming growth factor beta type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection. Cancer Res 2007; 67: 2351-2359.
[25] Kim S, Buchlis G, Fridlender ZG, Sun J, Kapoor V, Cheng G, Haas A, Cheung HK, Zhang X, Corbley M, Kaiser LR, Ling L and Albelda SM. Systemic blockade of transforming growth factor-beta signaling augments the efficacy of immunogene therapy. Cancer Res 2008; 68: 10247-10256.
[26] Lee GT, Hong JH, Kwak C, Woo J, Liu V, Lee C and Kim IY. Effect of dominant negative transforming growth factor-beta receptor type II on cytotoxic activity of RAW 264.7, a murine macrophage cell line. Cancer Res 2007; 67: 6717-6724.
[27] North RJ, Bursuker I. Generation and decay of the immune response to a progressive fibrosarcoma. I. Ly-1+2- suppressor T cells down-regulate the generation of Ly-1-2+ effector T cells. J Exp Med 1984; 159: 1295-1311.
[28] Bursuker I, North RJ. Immunological consequences of tumor excision: from active immunity to immunological memory. Int J Cancer 1986; 37: 275-281.
[29] Cote AL, Usherwood EJ and Turk MJ. Tumor-specific T-cell memory: clearing the regulatory T-cell hurdle. Cancer Res 2008; 68: 1614-1617.
[30] Lennard TW, Shenton BK, Borzotta A, Donnelly PK, White M, Gerrie LM, Proud G and Taylor RM. The influence of surgical operations on components of the human immune system. Br J Surg 1985; 72: 771-776.
[31] Park SK, Brody JI, Wallace HA and Blakemore WS. Immunosuppressive effect of surgery. Lancet 1971; 1: 53-55.
[32] Slade MS, Simmons RL, Yunis E and Greenberg LJ. Immunodepression after major surgery in normal patients. Surgery 1975; 78: 363-372.
[33] Markovic SN, Knight PR and Murasko DM. Inhibition of interferon stimulation of natural killer cell activity in mice anesthetized with halothane or isoflurane. Anesthesiology 1993; 78: 700-706.
[34] Akiyoshi T, Koba F, Arinaga S, Miyazaki S, Wada T and Tsuji H. Impaired production of interleukin-2 after surgery. Clin Exp Immunol 1985; 59: 45-49.
[35] Ogawa K, Hirai M, Katsube T, Murayama M, Hamaguchi K, Shimakawa T, Naritake Y, Hosokawa T and Kajiwara T. Suppression of cellular immunity by surgical stress. Surgery 2000; 127: 329-336.
[36] Teicher BA. Transforming growth factor-beta and the immune response to malignant disease. Clin Cancer Res 2007; 13: 6247-6251.
[37] Yang L, Pang Y and Moses HL. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol 2010; 31: 220-227.
[38] Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, Chen W, Wahl SM, Ledbetter S, Pratt B, Letterio JJ, Paul WE and Berzofsky JA. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. J Exp Med 2003; 198: 1741-1752.
[39] Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression by TGF-beta inhibitors. Invest New Drugs 2003; 21: 21-32.

[40] Ruzek MC, Hawes M, Pratt B, McPherson J, Ledbetter S, Richards SM and Garman RD. Minimal effects on immune parameters following chronic anti-TGF-beta monoclonal antibody administration to normal mice. Immunopharmacol Immunotoxicol 2003; 25: 235-257.