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Orthotopic transplantation of syngeneic lung adenocarcinoma cells to study PD-L1 expression

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TITLE:

Orthotopic Transplantation of Syngeneic Lung Adenocarcinoma Cells to Study PD-L1 Expression

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Summary:

Here we describe a minimally invasive syngeneic orthotopic transplantation model of mouse lung adenocarcinoma cells as a time- and cost-reducing model to study non-small cell lung cancer.

Abstract:

The use of mouse models is indispensable for studying the pathophysiology of various diseases. With respect to lung cancer, several models are available, including genetically engineered models as well as transplantation models. However, genetically engineered mouse models are time-consuming and expensive, whereas some orthotopic transplantation models are difficult to reproduce. Here, a non-invasive intratracheal delivery method of lung tumor cells as an alternative orthotopic transplantation model is described. The use of mouse lung adenocarcinoma cells and syngeneic graft recipients allows studying tumorigenesis under the presence of a fully active immune system. Furthermore, genetic manipulations of tumor cells before transplantation makes this model an attractive time-saving approach to study the impact of genetic factors on tumor growth and tumor cell gene expression profiles under physiological conditions. Using this model, we show that lung adenocarcinoma cells express increased levels of the T-cell suppressor programmed death-ligand 1 (PD-L1) when grown in their natural environment as compared to cultivation *in vitro*.

KEYWORDS:

Non-small cell lung cancer, orthotopic transplantation, minimally invasive, syngeneic, PD-L1, intratracheal delivery

INTRODUCTION:

Lung cancer is still by far the biggest cancer-related killer in both men and women[1]. Indeed, according to the American Cancer Society, every year more people die of lung cancer than of breast, prostate, and colon cancer together[1]. Until recently, the majority of patients suffering from non-small cell lung cancer (NSCLC), which is the most abundant subtype of lung cancer, were treated with platinum-based chemotherapy in a first-line setting, mostly with the addition of angiogenesis inhibitors[2]. Only a subset of patients harbors oncogenic mutations in the epidermal growth factor receptor (EGFR), in anaplastic lymphoma kinase (ALK), or in ROS1, and can be treated with available targeting drugs[3,4]. With the advent of immune checkpoint inhibitors, new hope for lung cancer patients has arisen, although until now, only 20% - 40% of patients respond to immune therapy[5]. Hence, further research is required to improve this outcome by fine-tuning immune checkpoint therapy and investigating combinatory treatment options.

To study lung cancer, a vast array of preclinical models are available, including spontaneous models triggered by chemicals and carcinogens and genetically engineered mouse models (GEMM) where autochthonous tumors arise following the conditional activation of oncogenes and/or the inactivation of tumor suppressor genes[6-8]. These models are of particular value to investigate fundamental processes in lung tumor development, but they also require extensive mice breeding, and experiments are time-consuming. Therefore, many studies evaluating potential inhibitors take advantage of subcutaneous (patient-derived) xenograft models where human lung cancer cell lines are subcutaneously injected into immunodeficient mice[9].

In these models, the micromilieu of tumors is not represented accordingly; hence, researchers also use orthotopic transplantation models, where tumor cells are injected intravenously, intrabronchially, or directly into the lung parenchyma[10-20]. Some of these methods are technically challenging, difficult to be reproduced, and require intensive training of the researchers.[21] Here we adapted a non-invasive orthotopic, intratracheal transplantation method in immunocompetent mice, where tumors develop within 3–5 weeks and exhibit significant similarities to human tumors, to induce the expression of the T-cell suppressor Programmed death-ligand 1 (PD-L1) on tumor cells.[11,12,20] The use of mouse tumor cells derived from GEMM models and syngeneic recipient mice allows proper studying of the tumor microenvironment including immune cells. Furthermore, gene editing tools like CRISPR/Cas9 technology[22] can be used *in vitro* before transplantation which facilitates the investigation of the impact of genetic factors in lung tumorigenesis.

PROTOCOL:

All experimental protocols as outlined below follow ethical guidelines and were approved by the Austrian Federal Ministry of Science, Research and Economy.

Note: The protocol here describes an orthotopic transplantation model of mouse lung adenocarcinoma cells into syngeneic recipients. Cells may be isolated from tumor-bearing lungs of $Kras^{LSL\text{-}G12D}$:$p53^{fl/fl}$ (KP) mice[7,18], if available in-house, and transplanted into mice of the same background and sex. If cells were provided from other research groups and the exact background remains unknown, we recommend the use of the F1 generation of a cross between C57BL/6 and 129S mice as transplant recipients to guarantee maximal tolerance.

1. Cell Preparation

1.1. Seed KP cells 24 h before transplantation at approximately 50% confluency in RPMI supplemented with 10% fetal calf serum (FCS), glutamine, and 100 U/mL penicillin and 100 µg/mL streptomycin (hereafter referred to as standard culture medium). Incubate the cell cultures at 37° C, 5% CO_2, and around 95% relative humidity.

1.2. On the next day, harvest cells using 1 mL of trypsin-EDTA (0.05% in phosphate-buffered saline [PBS]) for 5 min per 10-cm plate and, subsequently, resuspend detached cells with 9 mL of standard culture medium.

1.3. Count the cells in a hemocytometer and transfer the number of cells needed for the experiments in a 50-mL conical centrifuge tube.

Note: We recommend transplanting between 2.5 x 10^5 and 1 x 10^6 KP cells per mouse, but this might be adapted based on the researcher's needs.

1.4. Subsequently, centrifuge the cells for 5 min at 300 x *g*, aspirate the supernatant, and, using a pipette, resuspend the cells at a density of 2 x 10^7/mL (for the inhalation of 1 x 10^6 KP cells per mouse) in serum and antibiotic-free RPMI, supplemented with 0.01 M ethylenediaminetetraacetic acid (EDTA).

1.5. Keep the cells on ice until transplantation.

2. Orthotopic Transplantation *via* Intratracheal Delivery

2.1. Sedate a mouse (8 - 12 weeks of age) by a subcutaneous injection of a mixture of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight).

2.2. While the anesthesia sets in, prepare the catheter for intubation. Therefore, blunt the needle of a catheter by simply cutting the end with scissors. Afterward, push the catheter completely over the end of the needle.

2.2. Confirm the appropriate level of anesthesia by pedal reflex *via* firm toe pinching and apply ophthalmic ointment to the eyes.

2.3. Fix the mouse on the intubation platform (**Figure 1A**) by hooking its upper incisors over a suture and confirm that the chest is vertical underneath the suture.

2.4. Place a fiber optic cable in between the front legs to illuminate the chest (**Figure 1B**).

2.5. Carefully open the mouth of the mouse and pull out the tongue using disinfected flat forceps. Look for the emission of white light to locate the larynx and visualize the epiglottis and arytenoid cartilages (**Figure 1C**).

2.6. Once the opening of the trachea is clearly visible, gently slide the catheter into the trachea (**Figure 1D**). The length of the catheter to be inserted depends on the age and size of the animal, since it should not go below the bifurcation to guarantee an even distribution of the lung adenocarcinoma cells within the lung. Quickly remove the needle from the catheter.

2.7. The proper placement of the catheter in the trachea is indicated by the white light shining through the catheter (**Figure 1E**). In order to confirm the placement of the catheter in the trachea, attach a 1-mL syringe containing water to the catheter. The water in the syringe will rapidly move up and down in accordance with the breathing (**Figure 1F**).

Note: This step can be omitted by experienced researchers.

2.8. Warm up the cell suspension by holding the tube in hand and, subsequently, pipette 50 µL of the suspension containing 1 x 10^6 cells (the number of cells may be variable) into the center of the catheter hub. The suspension will be aspirated immediately. Subsequently, attach a 1-mL syringe and dispense 300 µL of air to assure a consistent distribution within the lungs.

2.9. Gently remove the catheter, remove the mouse from the intubation platform, and put it on a heat pad until it recovers from the anesthesia.

3. Lung Preparation for Flow Cytometry

3.1. At the desired experimental endpoint, sedate the mouse by a subcutaneous injection of a mixture of ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight) and euthanize it by cervical dislocation.

3.2. Soak the carcass in 70% ethanol and secure the mouse on a dissection board using tape.

3.3. Make a ventral midline incision and gently invert the skin to expose the thoracic wall muscles and the abdominal organs. Puncture the diaphragm and cut the ribs with scissors to expose the thoracic cavity.

3.4. Perfuse the lungs 3x with 6 - 8 mL of ice-cold PBS through the right ventricle using a 27-G needle after cutting a small opening in the left ventricle to allow blood to leave. The lungs should be cleared of blood and turn completely white.

3.5. Take out the lungs and mince the lobes into small pieces using scissors. Transfer the lung pieces to a 2-mL microcentrifuge tube and incubate it in 1.5 mL of lung digestion buffer (RPMI, 5% FCS, 150 U/mL collagenase I, and 50 U/mL DNase I).

3.6. Incubate the lung pieces 30 - 60 min at 37 °C and with constant shaking.

3.7. Transfer the lung cell suspension through a 70-µm cell strainer into a 50-mL tube. Clear the strainer with the back of a sterile 10-mL syringe and rinse the strainer with 15 mL of PBS with 2% FCS.

3.8. Centrifuge the cells at 300 x *g* for 5 min at 4 °C and aspirate the supernatant. Resuspend the cells in 1 mL of ammonium-chloride-potassium (ACK) lysing buffer and incubate them for 5 min at room temperature for the lysis of residual erythrocytes.

3.9. Centrifuge the cells at 300 x *g* for 5 min at 4 °C and resuspend the cells in 1 mL of PBS with 2% FCS and proceed with the desired staining protocol for flow cytometry[23].

Note: Alternatively, the cells may be resuspended in RPMI containing 30% FCS and 10% DMSO and frozen using a freezing container for later analysis.

REPRESENTATIVE RESULTS:

We used the orthotopic transplantation model *via* intratracheal tumor cell delivery to test whether the tumor microenvironment stimulates PD-L1 expression. Therefore, we isolated mouse lung AC cells from the autochthonous KP model (KP cells), 10 weeks following tumor induction *via* Cre-recombinase-expressing adenovirus (Ad.Cre) delivery[24]. Subsequently, we labeled the lung AC cells using a green fluorescent protein (GFP)-expressing lentivirus[25] and orthotopically engrafted them into immunocompetent, syngeneic mice *via* intratracheal delivery. To validate the model, we transplanted different amounts of tumor cells and performed survival analysis. As expected, the survival of recipient mice was correlated to the number of engrafted cells, and the survival time was between 2 weeks for recipients of 2 x 10^6 cells and around 10 weeks for recipients of 2.5 x 10^5 cells (**Figure 2A**). When the lungs were dissected following the death of the mice used for survival analysis, we noticed an even distribution of tumor nodules throughout all lobes of the lungs (**Figure 2B**). Regarding the morphology of the tumors, we compared transplanted tumors with autochthonous $KRAS^{G12D}$-driven tumors[7] and did not notice any obvious difference (**Figure 2C**).

To study the PD-L1 expression of transplanted tumor cells, we euthanized recipient mice 3 weeks after the transplantation of 1 x 10^6 cells and prepared the lungs for flow cytometric analysis. Probing for PD-L1 expression and gating for GFP^+ cells, we identified a significant shift in $PD\text{-}L1^+$ positive cells as compared to cells cultured *in vitro* (**Figure 2D**). Hence, we validated this model as a time-saving model to test for gene expression alterations in tumor cells under physiologic conditions, which, for instance, can be used to investigate the effects of genetic alterations or pharmacological treatments on the PD-L1 expression in lung AC cells.

FIGURE LEGENDS:

Figure 1: Intratracheal lung tumor cell transplantation. (**A**) This panel shows the home-made intubation platform using a polystyrene lid, two 15-mL tubes, and a 6.0 silk suture. (**B**) The fiber optic wire is directed to the chest of the mouse and (**C**) after gently pulling out the tongue, white light emitted from the opening of the trachea can be seen. (**D** and **E**) Proper placement of the mouse is indicated by light shining through the catheter and can be verified (**F**) by the up-and-down movement of water placed in a 1-mL syringe.

Figure 2: Morphology of mouse lungs following the syngeneic, intratracheal transplantation of lung AC cells. (**A**) This panel shows a Kaplan Meier analysis of the recipient mice following the orthotopic transplantation of different amounts of tumor cells. The amounts of tumor cells used for intratracheal delivery are indicated in the legend. (**B**) This is a representative picture of a lung of a tumor-cell recipient mouse. Shown is the lung of a mouse that received 5 x 10^5 cells and deceased 43 days following the transplantation. (**C**) This panel shows a hematoxylin and eosin staining of the lung section of autochthonous tumors 10 weeks following Ad.Cre delivery (left panel) and 6 weeks following the orthotopic transplantation of 5 x 10^5 tumor cells (right panel), including a higher magnification of the indicated areas (bottom). (**D**) The PD-L1 expression was measured by flow cytometry in GFP^+ KP cells following cultivation *in vitro* under standard conditions (before transplantation, red) and after orthotopic transplantation and isolation from mouse lungs 3 weeks following engraftment (after transplantation, blue). Rat IgG2a PE-Cyanine7 was used as an isotype control.

DISCUSSION:

To study lung physiologic and pathologic events in the lung, invasive and non-invasive intratracheal intubation methods for the instillation of various reagents are widely used[26-32]. In the cancer field, researchers use the intratracheal (and intranasal) instillation of Cre-recombinase-expressing viruses to introduce somatic mutations in lung epithelial cells. The administration of an Ad.Cre or lentivirus allows the conditional activation of oncogenic K-ras in KRAS-LSL-G12D mice, concomitantly with the knockout of p53 in transduced cells, when mice are bred with p53-floxed mice[7]. The possibility to study lung tumorigenesis from the earliest stage until the death of the animal, as well as a high similarity between mouse tumors and human tumors, makes these models extremely popular. However, from a practical point of view, this model requires extensive mouse breeding to study different genotypes, and in some genotypes, experiments may take up to a year from tumor induction until the experimental endpoint. This requires increased mouse space and, hence, costs for mouse housing.

The possibility to easily manipulate tumor cells *in vitro* by using CRISPR-Cas9 technology[22] makes orthotopic transplantation models a quick alternative to study the impact of selected genes on tumor growth and tumor expression profiles. The tagging of the tumor cells may be used for the real-time monitoring of tumor growth using live cell imagers or to sort tumor cells according to their tags. This also allows for an easy quantification of tumor cells (*i.e.*, tumor burden) according to their labels. Once established, this method of tumor cell delivery is highly reproducible. As

compared to orthotopic transplantation *via* tail vein delivery, the tumor cells are directly delivered to their natural environment in the lungs, whereas exposure to blood and its components may alter tumor cell properties. Further, the effects of manipulated genes on tumor cell survival in the bloodstream and extravasation to the lungs are unclear and may result in genotype-dependent alterations in the quantity of the cells delivered to the lungs.

In the model described here, tumors spread symmetrically throughout the lung. This allows the separate harvesting and analysis of different lesions, for instance, one lobe can be subjected to flow cytometry analysis as described above, while another lobe can be used for immunohistochemical analysis, lung lysate preparation, *etc.* Growing tumors result in the death of the recipient mouse within 3 - 10 weeks following intratracheal delivery, dependent on the number of cells used. This allows the researcher to adapt the number of transplanted cells to individual needs, and smaller cell numbers allow longer tumor growth and tumor cell exposure to the microenvironment. On the other hand, a higher cell number may be desired for pharmacologic studies to shorten the period of drug delivery.

Once established, the intratracheal administration of tumor cells is highly reproducible. However, some critical points have to be considered when performing this procedure. First, caution should be taken to avoid tissue damage when displacing the tongue with the forceps and, in particular, when the catheter is inserted. For the placement of the catheter, it is essential that the researcher can clearly see the white light to locate the opening of the trachea. Nevertheless, by mistake, the catheter can be easily inserted into the juxtaposed esophagus. Therefore, we recommend always checking for the correct placement of the catheter in the trachea as described above. It is also essential to avoid placing the catheter to deep (*i.e.*, the catheter must not be placed below the bronchial bifurcation). This guarantees an even distribution of lung cells and, hence, tumors throughout the lungs.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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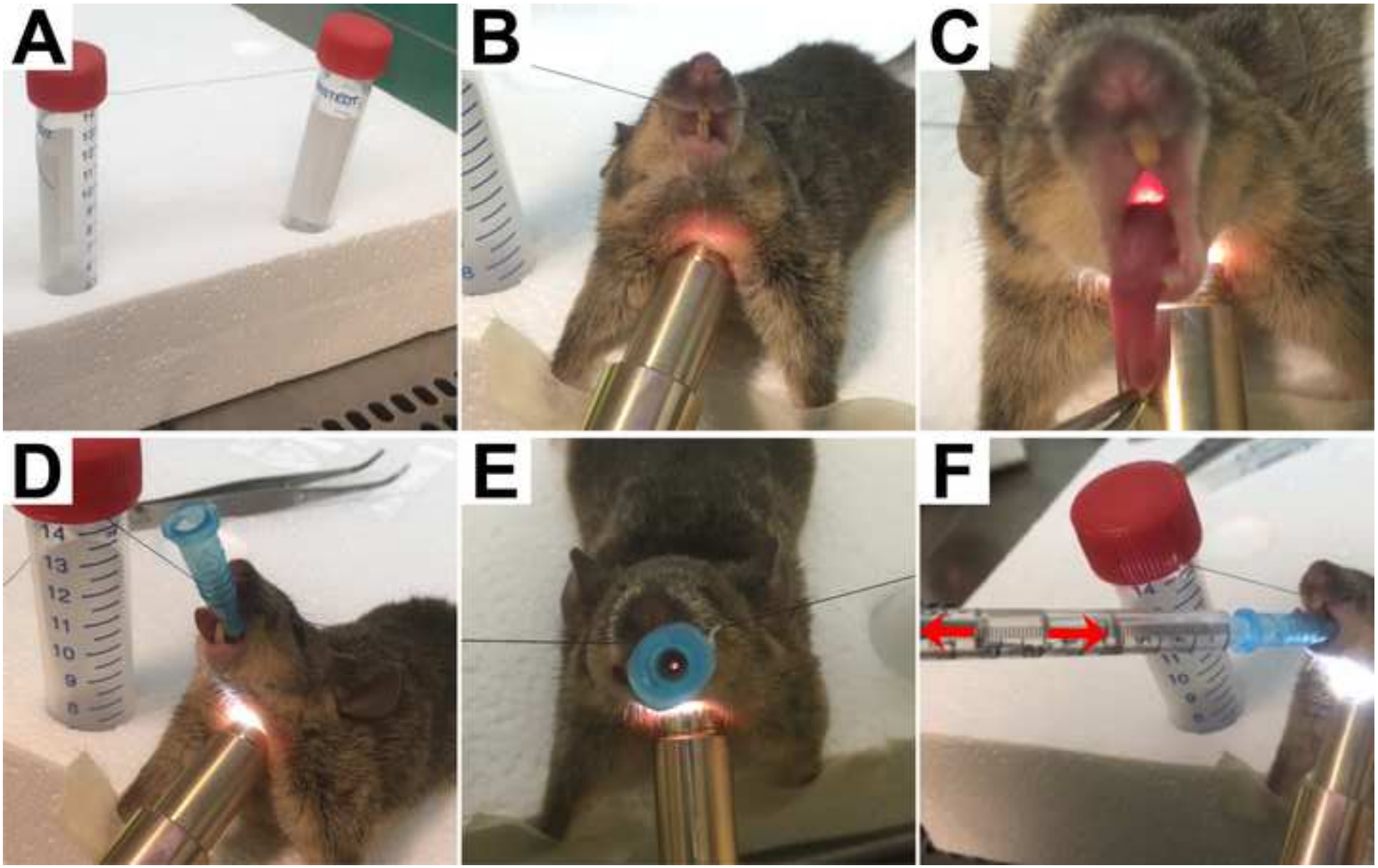

Figure 2

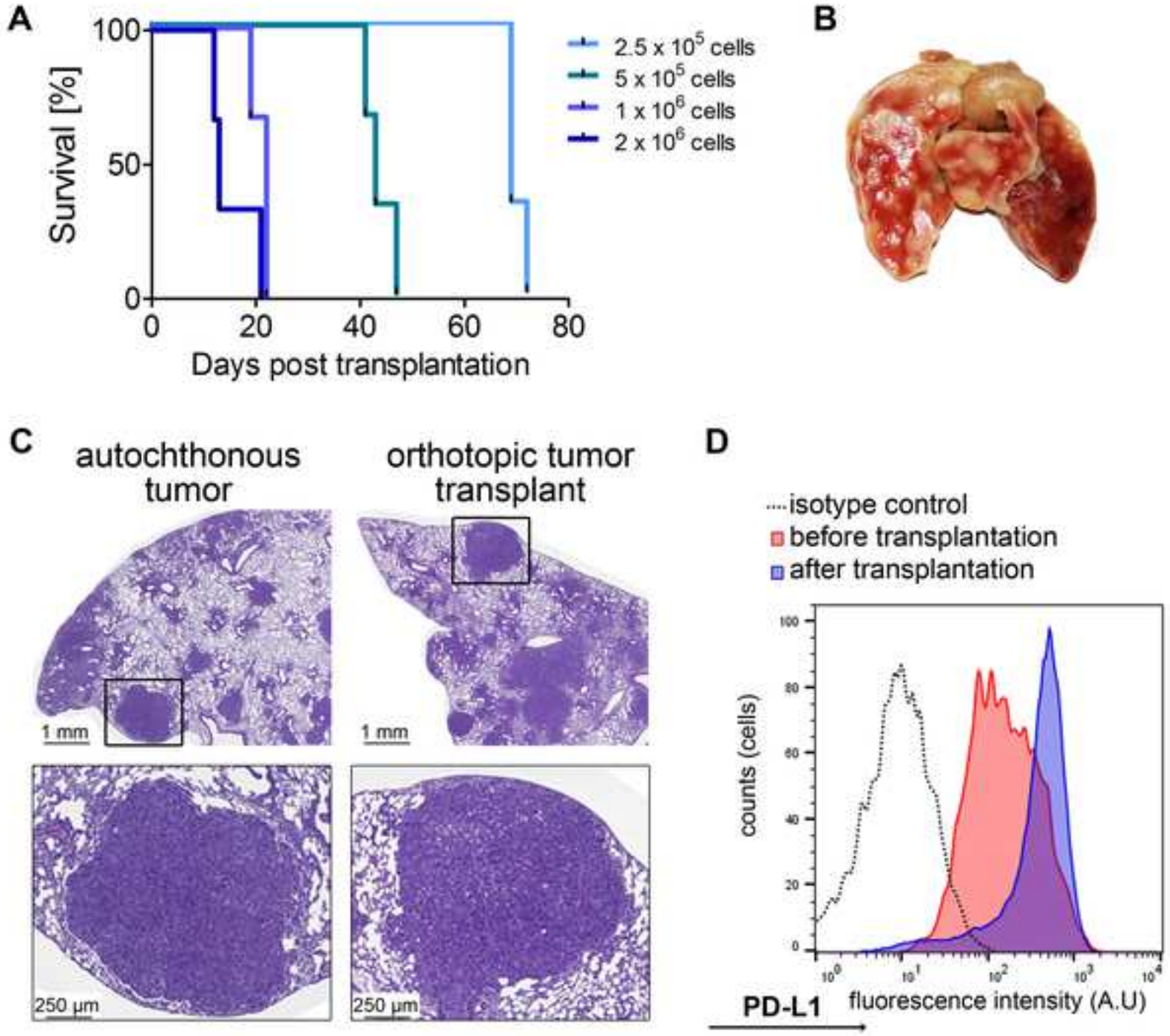

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
mouse lung adenocarcinoma cell line			isolated in house
C57Bl/6 mice 129S mice			F1 of the cross of the two backgrounds may be used (8-12 weeks)
RPMI 1640 Medium	Life Technologies	11544446	
Fetal Calf Serum	Life Technologies	11573397	
Penicillin/Streptomycin Solution	Life Technologies	11548876	
L-Glutamine	Life Technologies	11539876	
Trypsin, 0.25% (1X) with EDTA	Life Technologies	11560626	
UltraPure 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020	
Ketasol (100 mg/ml Ketamine)	Ogris Pharma	8-00173	
Xylasol (20 mg/ml Xylazine)	Ogris Pharma	8-00178	
BD Insyste (22GA 1.00 IN)	BD	381223	
Blunt forceps	Roboz	RS8260	
Leica CLS150 LED	Leica	30250004	Fibre Light Illuminator
Student Iris Scissors	Fine Science Tools	91460-11	
DNase I (RNase-Free)	New England Biolabs	M0303S	
Collagenase Type I	Life Technologies	17100017	
ACK Lysing Buffer	Lonza	10-548E	
CD274 (PD-L1, B7-H1) Monoclonal Antibody (MIH5), PE-Cyanine7	eBioscience	25-5982-82	
Rat IgG2a kappa Isotype Control, PE-Cyanine7	eBioscience	25-4321-82	

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