**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 women1. Indeed, according to the American Cancer Society, every year more people die of lung cancer than of breast, prostate, and colon cancer together1. 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 inhibitors2. 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 drugs3,4. 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 therapy5. 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 genes6-8. 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 mice9.

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 parenchyma10-20. 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 technology22 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 KrasLSL-G12D:p53fl/fl (KP) mice7,18, 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% CO2, 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 105 and 1 x 106 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 107/mL (for the inhalation of 1 x 106 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.

1. **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 106 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.

1. **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 cytometry23.

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) delivery24. Subsequently, we labeled the lung AC cells using a green fluorescent protein (GFP)-expressing lentivirus25 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 106 cells and around 10 weeks for recipients of 2.5 x 105 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 KRASG12D-driven tumors7 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 106 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-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 thelegend. (**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 105 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 105 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 used26-32. 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 mice7. 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 technology22 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.

**REFERENCES:**

1. Siegel, R. L., Miller, K. D., Jemal, A. Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians.* **68** (1), 7-30 (2018).

2. Zappa, C., Mousa, S. A. Non-small cell lung cancer: current treatment and future advances. *Translational Lung Cancer Research.* **5** (3), 288-300 (2016).

3. Dolly, S. O., Collins, D. C., Sundar, R., Popat, S., Yap, T. A. Advances in the Development of Molecularly Targeted Agents in Non-Small-Cell Lung Cancer. *Drugs.* **77** (8), 813-827 (2017).

4. Stinchcombe, T. E. Targeted Therapies for Lung Cancer. *Cancer Treatment Research.* **170**, 165-182 (2016).

5. Brody, R. *et al.* PD-L1 expression in advanced NSCLC: Insights into risk stratification and treatment selection from a systematic literature review. *Lung Cancer.* **112**, 200-215 (2017).

6. Safari, R., Meuwissen, R. Practical use of advanced mouse models for lung cancer. *Methods in Molecular Biology.* **1267**, 93-124 (2015).

7. DuPage, M., Dooley, A. L., Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nature Protocols.* **4** (7), 1064-1072 (2009).

8. Kwon, M. C., Berns, A. Mouse models for lung cancer. *Molecular Oncology.* **7** (2), 165-177 (2013).

9. Hidalgo, M. *et al.* Patient-derived xenograft models: an emerging platform for translational cancer research. *Cancer Discovery.* **4** (9), 998-1013 (2014).

10. Chen, X. *et al.* An orthotopic model of lung cancer to analyze primary and metastatic NSCLC growth in integrin alpha1-null mice. *Clinical & Experiment Metastasis.* **22** (2), 185-193 (2005).

11. Kang, Y. *et al.* Development of an orthotopic transplantation model in nude mice that simulates the clinical features of human lung cancer. *Cancer Science.* **97** (10), 996-1001 (2006).

12. Kang, Y. *et al.* Proliferation of human lung cancer in an orthotopic transplantation mouse model. *Experimental and Therapeutic Medicine.* **1** (3), 471-475 (2010).

13. Kuo, T. H. *et al.* Orthotopic reconstitution of human small-cell lung carcinoma after intravenous transplantation in SCID mice. *Anticancer Research.* **12** (5), 1407-1410 (1992).

14. Li, B. *et al.* A novel bioluminescence orthotopic mouse model for advanced lung cancer. *Radiation Research.* **176** (4), 486-493 (2011).

15. Mase, K. *et al.* Intrabronchial orthotopic propagation of human lung adenocarcinoma--characterizations of tumorigenicity, invasion and metastasis. *Lung Cancer.* **36** (3), 271-276 (2002).

16. McLemore, T. L. *et al.* Novel intrapulmonary model for orthotopic propagation of human lung cancers in athymic nude mice. *Cancer Research.* **47** (19), 5132-5140 (1987).

17. Tsai, L. H. *et al.* The MZF1/c-MYC axis mediates lung adenocarcinoma progression caused by wild-type lkb1 loss. *Oncogene.* **34** (13), 1641-1649 (2015).

18. Winslow, M. M. *et al.* Suppression of lung adenocarcinoma progression by Nkx2-1. *Nature.* **473** (7345), 101-104 (2011).

19. Zou, Y., Fu, H., Ghosh, S., Farquhar, D., Klostergaard, J. Antitumor activity of hydrophilic Paclitaxel copolymer prodrug using locoregional delivery in human orthotopic non-small cell lung cancer xenograft models. *Clinical Cancer Research.* **10** (21), 7382-7391 (2004).

20. Buckle, T., van Leeuwen, F. W. Validation of intratracheal instillation of lung tumour cells in mice using single photon emission computed tomography/computed tomography imaging. *Lab Animal.* **44** (1), 40-45 (2010).

21. Berry-Pusey, B. N. *et al.* A semi-automated vascular access system for preclinical models. *Physics in Medicine & Biology.* **58** (16), 5351-5362 (2013).

22. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nature Protocols.* **8** (11), 2281-2308 (2013).

23. Singer, B. D. *et al.* Flow-cytometric method for simultaneous analysis of mouse lung epithelial, endothelial, and hematopoietic lineage cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology.* **310** (9), L796-801 (2016).

24. Moll, H. P. *et al.* Afatinib restrains K-RAS-driven lung tumorigenesis. *Science Translational Medicine.* **10** (446) (2018).

25. Campeau, E. *et al.* A versatile viral system for expression and depletion of proteins in mammalian cells. *PLoS One.* **4** (8), e6529 (2009).

26. Gui, L., Qian, H., Rocco, K. A., Grecu, L., Niklason, L. E. Efficient intratracheal delivery of airway epithelial cells in mice and pigs. *American Journal of Physiology - Lung Cellular and Molecular Physiology.* **308** (2), L221-228 (2015).

27. Helms, M. N., Torres-Gonzalez, E., Goodson, P., Rojas, M. Direct tracheal instillation of solutes into mouse lung. *Journal of Visualized Experiments.* (42), e1941 (2010).

28. Lin, Y. W. *et al.* Pharmacokinetics/Pharmacodynamics of Pulmonary Delivery of Colistin against Pseudomonas aeruginosa in a Mouse Lung Infection Model. *Antimicrobial Agents and Chemotherapy.* **61** (3) (2017).

29. Wegesser, T. C., Last, J. A. Lung response to coarse PM: bioassay in mice. *Toxicology and Applied Pharmacology.* **230** (2), 159-166 (2008).

30. Cai, Y., Kimura, S. Noninvasive intratracheal intubation to study the pathology and physiology of mouse lung. *Journal of Visualized Experiments.* (81), e50601 (2013).

31. Lawrenz, M. B., Fodah, R. A., Gutierrez, M. G., Warawa, J. Intubation-mediated intratracheal (IMIT) instillation: a noninvasive, lung-specific delivery system. *Journal of Visualized Experiments.* (93), e52261, (2014).

32. Vandivort, T. C., An, D., Parks, W. C. An Improved Method for Rapid Intubation of the Trachea in Mice. *Journal of Visualized Experiments.* (108), e53771, (2016).