Jun 04, 2021

Tertiary Lymphoid Structures in Mouse Models of Lung Cancer: From Induction to Imaging

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In Development Share dx.doi.org/10.17504/protocols.io.bun4nvgw

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ABSTRACT

Tertiary lymphoid structures (TLSs) are a recently-described immune response that occurs when chronic inflammation leads to an organ-like organization of immune cells and specialized blood vessels. In humans, they have been shown to develop in and around many different types of tumors, and often lead to a more promising prognosis when found. In non-small cell lung cancer (NSCLC), they have a highly beneficial effect on patients' survival. What remains to be studied is the heterogeneity of the TLSs and the mechanisms of their development. In order to study these important aspects of the immune response to NSCLC, we developed a protocol to image a time course of TLS development in murine bronchiolo-alveolar adenomas and adenocarcinomas, and further expanded on how to quantify, and therefore characterize, these structures.

DOI

dx.doi.org/10.17504/protocols.io.bun4nvgw

PROTOCOL CITATION

Wendy Memishian, Teresa McGee, Julie Wells, Carol Bult 2021. Tertiary Lymphoid Structures in Mouse Models of Lung Cancer: From Induction to Imaging. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bun4nvgw

KEYWORDS

null, Immune system, immune response, cancer, tumor, immune microenvironment, IHC, immunohistochemistry, imaging, multiplexed, serial section, cancer biology, tumor microenvironment

CREATED

Apr 30, 2021

LAST MODIFIED

Jun 04, 2021

PROTOCOL INTEGER ID

49596

Adenovirus Infection 6w

1 At 6-7 weeks of age, mice are separated by sex and treatment group (i.e. female control, female experimental, etc.). 6w

2 Combine MEM, $CaCl_2$, and Ad5mSPC-Cre (of a known titer) to get 2.5 EE8 PFU. Let the combination sit for 20 minutes. 25m

3 Mice are given an intraperitoneal injection of tribromo-ethanol (Avertin) at a dosage of 0.02 mL/g of body weight, which should render the mouse unable to respond to a toe pinch. 10m

4 Using a pipet, have the mouse breathe in 25 uL of the virus by holding droplets against one nostril slowly. After ~5 min, have the mouse breathe in another 25 uL through the other nostril in droplets. 10m

5 Lay the mouse on its side. Put the pen over a heating pad or under a heat lamp in order to keep the mouse's temperature up while it's anesthetized. Observe to make sure all mice wake up and are able to easily get food and water before putting back on the shelf. 1h 30m

Perfusion and Harvest of Lungs 22m

6 Euthanize mouse using an IACUC-approved method, such as carbon dioxide, until it no longer responds to toe pinches. 5m

7 Weigh mouse before dunking completely in soapy water. 1m

8 Pin down limbs and cut open abdomen from the intestines to the rib cage using surgical scissors. 2m

9 Cut through the diaphragm as a secondary form of euthanasia, being careful not to cut into the liver, lungs, or heart. Continue snipping it all the way across the bottom of the rib cage. Cut completely through the sternum and the rib cage in order to allow complete inflation of the lungs. 1m

10 Find the inferior vena cava and snip it in order to begin perfusion of the lungs 1m

Davenport ML, Sherrill TP, Blackwell TS, Edmonds MD (2020). Perfusion and Inflation of the Mouse Lung for Tumor Histology.. Journal of visualized experiments : JoVE. https://doi.org/10.3791/60605

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11 **5 mL 1XPBS** Using a 5 mL syringe with a 23g needle, find the right ventricle of the heart and slowly inject 1XPBS until lungs inflate completely. 3m

BD Luer-Lok Tip Syringe (5 mL)
Syringe
BD **309646**

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General Use and PrecisionGlide Hypodermic Needles - 23g (0.064 cm x 2.5 cm)
needle
BD **14-826A**

12 5m
5 mL 10% NBF To fix lungs, prepare a new 5 mL syringe filled with 10% neutral buffered formalin and a new 23g needle. Using the surgical scissors, snip soft tissue away from the throat area until the trachea is in view. Holding the needle parallel to the trachea, insert the needle with the bevel up, careful to enter the trachea and not go completely through it. Slowly inject all of the fixative and hold the needle in the trachea for at least 1 minute afterwards to ensure all of the lung gets contact with the 10% NBF.

13 4m
20 mL 10% NBF After removing the needle, grab the trachea gently with forceps and cut above where it is held. Lift the trachea and, holding the scissors parallel to the body, snip away tissue attaching the trachea, thymus, heart, and lungs to the body. Immediately place the organs in a 50 mL conical tube filled with 10% NBF.

Fisherbrand™ Curved Medium Point General Purpose Forceps
Forceps
Fisher Scientific **16-100-110**

14 1d
Overnight **20 mL 1XPBS** Leave the organs in 10% NBF overnight. Move to 1XPBS the following day.

Sectioning and Staining of Lungs 1d 2h 11m

15 8m
Put organs into a dish with some of the 1XPBS and, using a dissecting scope, cut or pull the trachea, thymus, and heart from the lungs. Then separate the 5 lobes of the lungs, removing as much connective tissue as possible without damaging them.

Dissection Microscope
Microscope
Zeiss **Stemi 305**

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PYREX™ Reusable Petri Dishes: Complete Dish

Fisher Scientific 08-747A

16 **100 mL 70% EtOH** Move the lung lobes from the dish into a slotted tissue cassette, making sure to spread the lobes out as much as possible to ensure good staining and imagine. Put the cassettes into 70% ethanol. 3m

Epredia™ Cassette II Slotted Tissue Cassettes in Tube Packs
Cassette

Epredia B851729YW

17 Paraffin embed the tissues. Wait to begin sectioning until all future materials are prepared, as antigens degrade once sectioning begins. 2h

18 Serial section the paraffin block into 5um sections, preferably with one section per slide, but up to three per slide. 1d

19 Stain every fourth slide with hematoxylin and eosin (H&E) in order to find "levels" of interest in the block (ex. stain the first slide with H&E, if it appears interesting, then the first level, consisting of slides 2, 3, and 4, will proceed to the rest of the stains). 12h

19.1 Look for hyperplasia, adenomas, and adenocarcinomas. 15m

19.2 Around the abnormal cells, look for areas of lymphoid aggregates that are peritumoral or intratumoral (lymphoid aggregates near vessels in the lungs are common with aging mice, and are not a result of the tumor). 10m

20 For each level of interest, perform IHC on the second slide with anti-CD3/CD20 dual staining, on the third slide with anti-PNAd, and on the fourth slide with anti-CD11c. 1w

CD3/CD20 Antibody Cocktail **Thermo Fisher Scientific Catalog #MA5-14433**

Peripheral Node Addressin Antibody (MECA-79R) **Novus Biologicals Catalog #NBP2-78792SS**

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Recombinant Anti-CD11c

Antibody **Abcam Catalog #EPR21826**

21 Scan the slides and use software (ImageJ, CellProfiler, etc.) to overlay the images of the different parts of the TLS, showing a bona fide TLS.

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