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Blocking Lymph Flow by Suturing Afferent Lymphatic Vessels in Mice

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TITLE:

Blocking Lymph Flow by Suturing Afferent Lymphatic Vessels in Mice

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SUMMARY:

A protocol to block lymph flow by surgical suturing of afferent lymphatic vessels is presented.

ABSTRACT:

Lymphatic vessels are critical in maintaining tissue fluid balance and optimizing immune protection by transporting antigens, cytokines, and cells to draining lymph nodes (LNs). Interruption of lymph flow is an important method when studying the function of lymphatic vessels. The afferent lymphatic vessels from the murine footpad to the popliteal lymph nodes (pLNs) are well-defined as the only routes for lymph drainage into the pLNs. Suturing these afferent lymphatic vessels can selectively prevent lymph flow to the pLNs. This method allows for interference in lymph flow with minimal damage to the lymphatic endothelial cells in the draining pLN, the afferent lymphatic vessels, as well as other lymphatic vessels around the area. This method has been used to study how lymph impacts high endothelial venules (HEV) and chemokine expression in the LN, and how lymph flows through the adipose tissue surrounding the LN in the absence of functional lymphatic vessels. With the growing recognition of the importance of lymphatic function, this method will have broader applications to further unravel the function of lymphatic vessels in regulating the LN microenvironment and immune responses.

INTRODUCTION:

The spatial organization of the lymphatic system provides structural and functional support to efficiently remove extracellular fluid and transport antigens and antigen-presenting cells (APCs) to the draining LNs. The initial lymphatic vessels (also named lymphatic capillaries) are highly permeable due to their discontinuous intercellular junctions, which facilitate the effective collection of fluids, cells, and other materials from surrounding extracellular spaces[1]. The initial lymphatic vessels merge into collecting lymphatic vessels, which have tight intercellular junctions, a continuous

basement membrane, and lymphatic muscle coverage. Collecting lymphatic vessels are responsible for transporting collected lymph to the draining LNs and eventually returning lymph to the circulation[2,3]. The collecting lymphatic vessels that propel lymph into the draining LN are the afferent lymphatic vessels[4-7]. Obstruction of afferent lymphatic vessels can block lymph flow into the LNs, which is a useful technique when studying the function of lymph flow.

Previous studies have shown that lymph flow plays a significant role in transporting antigens and APCs, as well as maintaining LN homeostasis. It is well understood that tissue-derived APCs, typically activated migrating dendritic cells (DCs), travel through the afferent lymphatic vessels to the LN to activate T cells[8]. The idea that free-form antigens, such as microbes or soluble antigens, passively flow with lymph to the LN to activate LN-resident APCs has been gaining acceptance in the past decade[9-12]. Free-form antigens traveling with lymph take minutes after the infection to travel to the LN, and the LN-resident cell activation may occur within 20 min after the stimulation. This is much faster than the activation of migrating DCs, which takes more than 8 h to enter the draining LN[9]. Besides transporting antigens to initiate immune protection, lymph also carries cytokines and DCs to the LN to maintain its microenvironment, and to support immune cell homeostasis[13,14]. Previously, blocking lymph flow by suturing the afferent lymphatic vessels demonstrated that lymph is required to maintain the HEV phenotype required for supporting homeostatic T cell and B cell homing to the LN[15-17]. CCL21 is a critical chemokine that directs DC and T cell positioning in the LN[8,18]. Blocking lymph flow interrupts CCL21 expression in the LN and potentially interrupts DC and T cell positioning and/or interaction in the LN[19]. Thus, blocking lymph flow can directly or indirectly abrogate antigen/DC access to the draining LN by disrupting the LN microenvironment that regulates immune responses in the LN. To better investigate the function of lymph flow, an experimental protocol is presented (**Figure 1**) to block lymph flow in mice by suturing the afferent lymphatic vessels from the footpad to the pLN. This method can be an important technique for future studies on lymphatic function in healthy and diseased conditions.

PROTOCOL:

All animal work needs to be approved by institutional and governmental ethics and animal handling committee.

1. Preparation of materials

1.1. Prepare 100 mL of 70% ethanol by mixing 70 mL of 100% ethanol with 30 mL of sterile water. Autoclave all surgical tools before surgery and keep the tools in 70% ethanol before and during the surgery to maintain sterilization.

1.2. Prepare an injection apparatus.

1.2.1. Cut ~30 cm of polyethylene tubing (0.28 mm in diameter). Connect the tip of a 30 G x ½ needle (needle A) to one end of polyethylene tubing. Carefully dislodge another 30 G x ½ needle (needle B) and connect the broken side to the other end of polyethylene tubing.

1.2.2. Attach needle A to a 1 mL tuberculin syringe.

NOTE: For this polyethylene tubing, 1.6 cm of fluid in the tubing corresponds to 1 µL[20].

1.3. Prepare a 10:1 ketamine/xylazine mixture (10 mg/mL ketamine and 1 mg/mL xylazine) in saline (bacteriostatic 0.9% [w/v] sodium chloride). Prepare the solution freshly before use.

2. Preparation of the animal for surgery

NOTE: Use mice aged 6−10 weeks. Both female and male mice can be used. In this study, 6−10-week-old, C57BL/6 female mice were used. This method can be adapted for other strains of mice.

2.1. Anesthetize the mouse by injecting 250 µL of the ketamine/xylazine mixture intraperitoneally. Wait until the mouse is completely asleep. Ensure mouse does not react to a toe pinch to detect full anesthetization.

2.2. Shave fur around the legs with hair clippers.

2.3. Apply the depilatory cream around the leg and wait for 5 min. Wipe off the residual fur and the depilatory cream using a moist tissue and clean the leg with sterile water. Spray 70% ethanol around the leg to sterilize the operating area.

3. Surgical suturing of afferent lymphatic vessels

NOTE: The right leg is sutured, and the left leg is used as the sham control. The lymphatic suture protocol (steps 3.1−3.8) takes 20−30 min.

3.1. Keep mouse at a prone position and fix it with surgical tape to expose the operation area on the right leg.

3.2. Intradermally inject 5 µL of 1% Evans blue dye or 9 cm of the fluid of the injection apparatus tubing into the footpad. Gently massage the footpad to help Evans blue enter the lymphatic vessels.

NOTE: The insulin syringe is not easy to control for small volume injection. The volume can be controlled more accurately using the injection apparatus. Lymphatic vessels are visualized by blue dye under the skin. With extensive training, both afferent lymphatic vessels can be seen with the naked eye as transparent vessels in the adipose tissue, parallel to the Saphenous artery. With extensive training, it is possible to suture the vessels without injecting Evans Blue dye in cases where there are concerns of potential disturbances from the dye.

3.3. Under a dissecting microscope, choose an incision site 5 mm from the bottom edge of the popliteal fossa. Make a small incision (~5 mm) in the skin with scissors. Using fine operation forceps, stretch the incision, and expose the collecting lymphatic vessels (**Figure 1A**).

NOTE: If necessary, a small skin fragment can be removed to expose the lymphatic vessels.

3.4. Identify both afferent lymphatic vessels leading to the pLNs under the dissecting microscope (**Figure 1B**).

NOTE: There are two afferent lymphatic vessels from the footpad to the pLN. Both need to be sutured to block lymph flow completely.

3.5. Using a needle holder, cautiously insert the suture needle (0.7 metric or smaller) between the afferent lymphatic vessel and the Saphenous artery and pull the needle gently out around the afferent lymphatic vessel. Gently pull the suture string and leave about 2 cm of the suture string behind. Use the needle holder to help tie the string tightly to suture one lymphatic vessel with a surgeon's knot (**Figure 1C**).

NOTE: The tissue underneath the incision may dry out with prolonged exposure to air. Making the incision as small as possible and performing the suture quickly (i.e., within 5 min) will prevent the tissue from drying out. Maintain the tissue moisture by applying a small volume of saline with a cotton swab.

3.6. Gently massage the footpad to ensure no Evans Blue dye passes the suture site and then cut the excess string with scissors.

3.7. Perform the same suture steps (i.e., steps 3.5 and 3.6) on the other afferent lymphatic vessel (**Figure 1D**). Close the skin incision with the same suture that was used to suture the vessels in step 3.5 (**Figure 1E**).

3.8. For the sham control, intradermally inject 5 µL of 1% Evans blue dye at the left footpad and massage the footpad to visualize the lymphatic vessels. Open the skin with an excision and then close the wound without suturing the vessel (**Figure 1F**).

3.9. Optionally, monitor the operated mice for 2−4 h. The suture side of the leg should show edema with Evans Blue spread to the thigh, while the control leg will show restricted Evans blue dye in the footpad.

4. Tracking of the lymph flow

4.1. Immediately after the surgery, intradermally inject 10 µL of 2% fluorescein isothiocyanate (FITC) in the footpad of both the control and the lymphatic sutured leg.

4.2. Euthanize the mice with 400 µL of ketamine/xylazine mixture and perform cervical dislocation when the mice are fully anesthetized.

4.3. Collect pLNs from the popliteal fossa and carefully remove the perinodal adipose tissue around the pLNs under the dissection microscope at 2, 6, and 12 h after FITC injection.

4.4. Embed the pLNs with the medullary sinus area facing to the side of the cryomold in optimal cutting temperature (OCT) compound (**Figure 1G,H**).

4.5. Prepare 20 µm frozen sections using a cryotome.

4.6. Image the cryosections under a confocal microscope to determine FITC distribution.

REPRESENTATIVE RESULTS:

Lymphatic vessel suture has been used in previous studies[15-17,19], where it served as an important tool to study the function of lymph flow before the molecular biology of lymphatic vessels was better understood. Blocking lymph flow interrupts LN homeostasis, which leads to HEVs losing the critical gene expression needed for optimal lymphocyte homing to the LN[15-17]. Since then, it took another two decades to demonstrate that DCs traveling with lymph are crucial in maintaining the HEV gene expression profile and lymphocytes homing to the LN[13]. The shear stress provided by the lymph flow is critical to stimulate chemokine expression in the LN. Blocking lymph flow interrupts chemokine CCL21 expression in the LN[19], which is critical in directing DC and T cell positioning in the LN. Therefore, interrupted flow may compromise DCs and T cells positioning in the LN[8,18].

Immediately after the surgery, a small molecular weight fluorescent tracer, FITC, was used to track lymph flow. FITC (10 µL of 2% FITC) was injected intradermally in the footpad of the sham control and the lymphatic sutured leg. The draining pLNs were collected 2, 6, and 12 h later. The draining pLNs were embedded in OCT, and 20 µm frozen sections were prepared. Confocal images showed substantially reduced FITC accumulation in the pLNs after suture. The residual FITC in the pLNs was preferentially accumulated in the LN sinuses (**Figure 2**).

How the lymph flows through the adipose tissue surrounding the LN was investigated using lymphatic suture. The afferent lymphatic vessels leading to the pLNs were sutured to block lymph flow, and it was determined that the perinodal adipose tissue could support a small amount of lymph flow when lymphatic vessels were blocked[21]. The lymph flow through the adipose tissue to the capsule of the LN was mapped; it appeared to feed into the LN sinuses. Small amounts of lymph may have flowed into the LN sinuses over time (**Figure 3**).

FIGURE LEGENDS:

Figure 1: Steps of popliteal LN (pLN) afferent lymphatic vessel suture. Briefly, after mice were anesthetized with a ketamine and xylazine mixture, their legs were shaved, and the residual fur was removed by a depilatory cream. The right leg was used for suture and the left leg was the sham control. The right side of the footpad was intradermally injected with 5 µL of 1% Evans Blue dye prepared in PBS. (**A**) By gently massaging the footpad, Evans Blue dye filled the afferent lymphatic vessels. (**B**) A small skin cut was performed 5 mm away from the pLN to expose the lymphatic vessels, which are indicated by the two white arrows. (**C**,**D**) Both afferent lymphatic vessels were sutured. (**E**) The skin excision was closed by sutures. (**F**) The control leg received Evans blue injection, skin excision, and suture closure without suturing the lymphatic vessels. (**G**) The success of the lymph flow blockage was indicated by Evans blue dye, which entered the pLN of the control leg but not the

sutured leg. (**H,I**) The collected pLNs were embedded in OCT compound with subcapsular sinus (SCS) and medullary sinus (MS) facing the side of the cryomold before snap freezing in liquid nitrogen.

Figure 2: FITC distribution in the draining pLNs of the sham or sutured leg. Confocal images of pLNs collected 2, 6, and 12 h after FITC injection showed substantially reduced FITC accumulation in the pLNs after suture. The residual FITC in the pLNs was preferentially found in the LN sinuses.

Figure 3: FITC distribution in the perinodal adipose tissue (PAT), around the draining pLNs of the sham or sutured leg. Confocal images of the PAT and the LN showed that FITC enters the PAT and the LN sinuses but was not effectively distributed throughout the LN when lymphatic vessels were blocked.

DISCUSSION:

Blocking lymph flow will have broad applications in manipulating antigen delivery to the LN in healthy and diseased conditions. It is possible to use this method to control the timing of antigen delivery in order to study how continuous lymph flow regulates immune response in draining LNs. This method of lymph flow interruption can also be used to study how lymph impacts cell compartmentalization, cell activation, cell migration, and cell-cell interactions in the LN.

Mice specifically expressing human diphtheria toxin receptor (DTR) in their lymphatic endothelial cells (*Flt4-cre-dtr*) have been developed; these can be used to specifically deplete lymphatic vessels to study lymphatic function[22]. Administration of DT kills lymphatic endothelial cells along the lymphatic vessels and in the LN. The depletion of lymphatic endothelial cells can completely abrogate lymph flow regionally or systemically to study lymphatic function. This method causes significant fluid accumulation in tissue and serves as a great model to study lymphedema and lymphatic function.

Compared to the lymphatic endothelial cell-specific DTR expression model, the advantage of the lymphatic suture method is that it interrupts lymph flow with minimal damage to lymphatic endothelial cells or any other lymphatic vessel around the area. The intervention does not directly impact cells in the draining LN, so the resulting impact on the LN microenvironment or immune cell communication is a consequence of the lymph flow blockade rather than potential cell death induced by DT. Another benefit of this method is that lymph flow is instantly blocked after the surgery, so the timing of the lymph flow blockade can be better controlled.

The limitation of this method is that it can only be used to study regional intervention of lymph flow in afferent lymphatic vessels from the footpad to the pLN. This method needs identification of the exact location of the collecting lymphatic vessels. Collecting lymphatic vessels are difficult to identify in some anatomical locations, and thus this technique requires extensive anatomical and surgical training before successful identification of afferent lymphatic vessels to block lymph flow. Another limitation is that this method cannot directly block lymph entering the initial lymphatic vessels. After the suture, the blocked lymph flow may increase the interstitial fluid pressure and change the lymph flow direction in the initial lymphatic vessels. Thus, the intact lymphatic vessels around the area may

compensate for the function of the interrupted lymphatic vessels and change the direction of lymph flow.

Moreover, the injection of Evans Blue dye increases the interstitial fluid pressure, which may interfere with the subsequent tracer or antigen injection. The autofluorescence of Evans Blue dye may interfere with other fluorescent tracers or other fluorophores used for immunofluorescent staining. To avoid any interaction between Evans Blue dye and potential antigens, other tracers, or potential molecular mechanisms of lymphatic function regulation, it is possible to identify the collecting lymphatic vessels without Evans Blue dye with the naked eye. This can be achieved with extensive training to identify the vessels. Other dyes, such as isosulfan blue dye can also be used to replace the Evans Blue dye.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

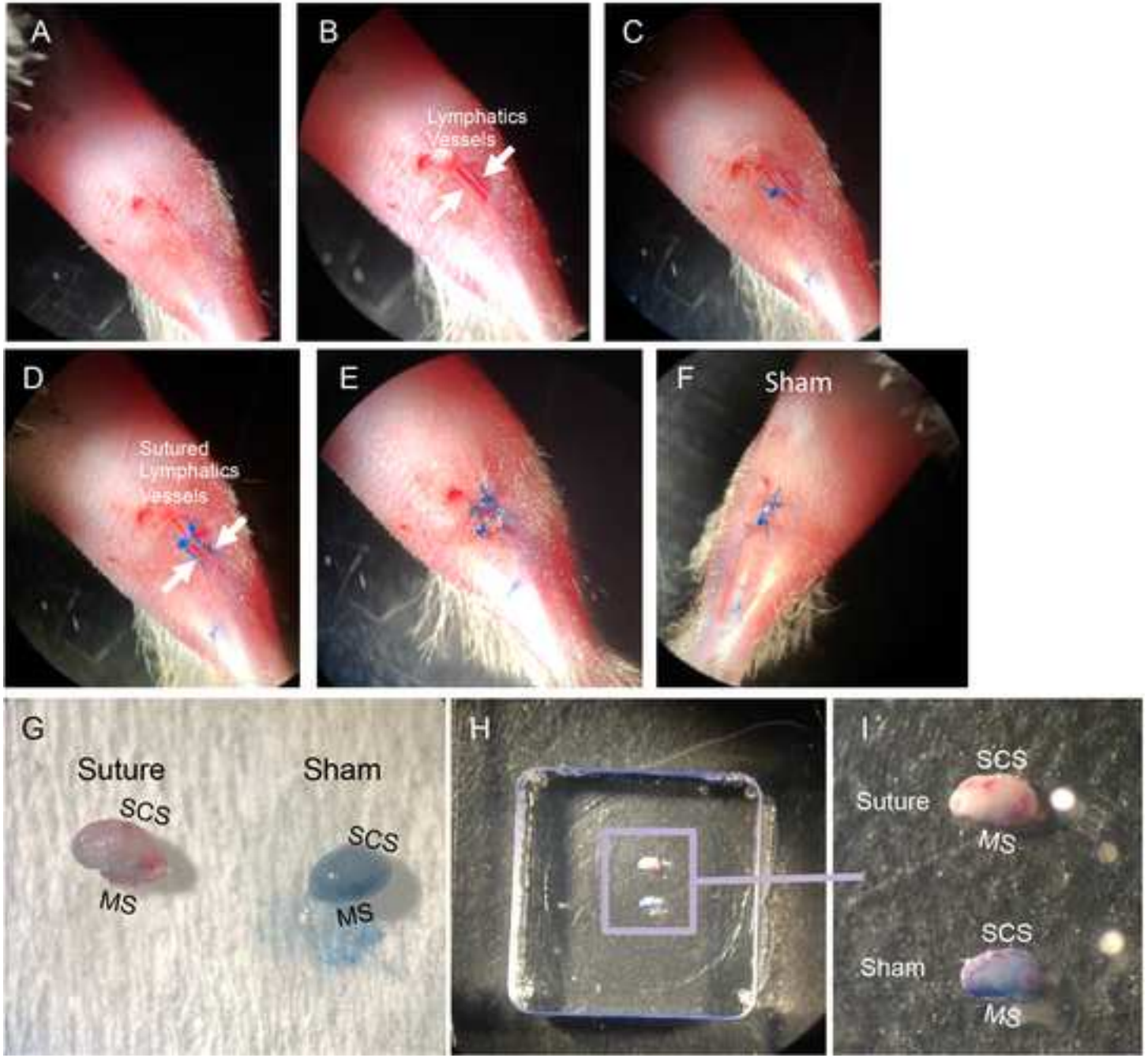

Figure 2

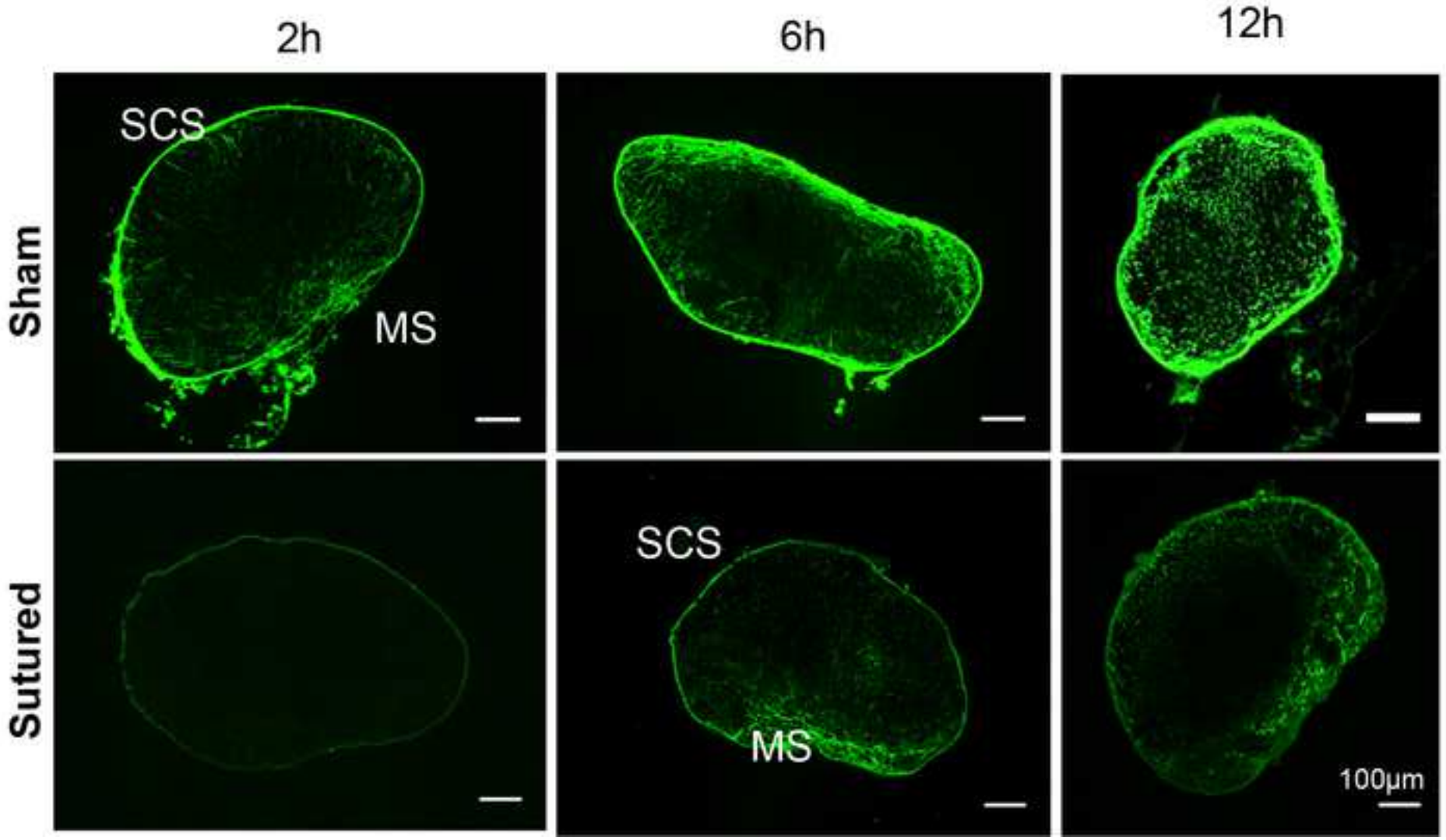

Figure 3

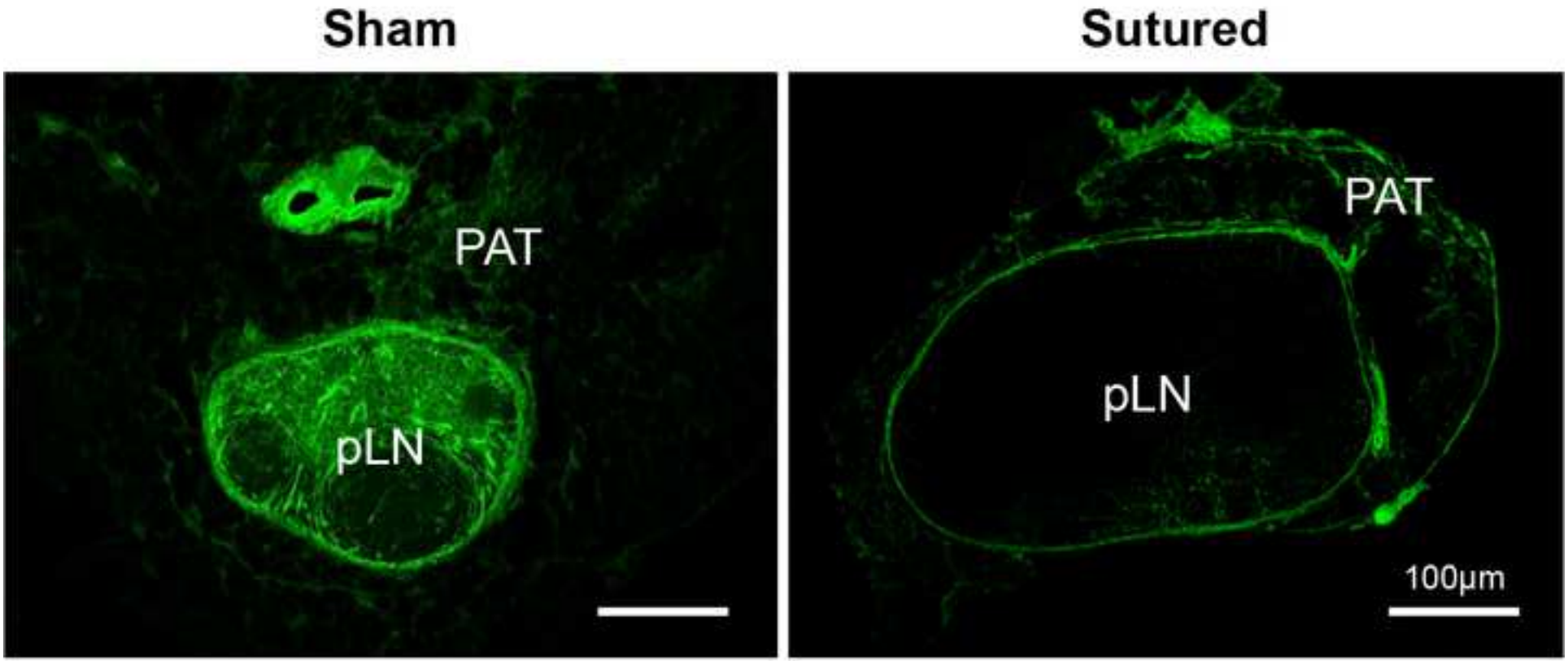

Table of Materials

Click here to access/download;Table of Materials;JoVE_revised_Table_of_Materials.xlsx

Name of Material	Company	Catalog Number
0.9% Sodium Chloride Saline	Baxter	JB1323
100% ethanol	Greenfield Global	
Depilatory cream	Nair	
Evans Blue dye	Sigma Life Science	E2129-10G
Fluorescein isothiocyanate isomer I (FITC)	Sigma Life Science	F7250-1G
Forceps Dumont #3	WPI	500337
Forceps Dumont #5	WPI	500233
Injection apparatus		
Insulin syringe	Becton Dickinson and Company (BD)	329461
IRIS Forcep straight	WPI	15914
IRIS scissors	WPI	14218-G
Ketamine	Narketan	DIN 02374994
Needles (26Gx3/8)	Becton Dickinson and Company (BD)	305110
Needles (30Gx1/2)	Becton Dickinson and Company (BD)	305106
Paton Needle Holder	ROBOZ	RS6403
Phosphate-Buffered Saline (PBS)	Sigma Life Science	P4417-100TAB
Polyethylene tubing	Becton Dickinson and Company (BD)	427401

Surgical tape (1.25cmx9.1m)	Transpore	1527-0
Surgical tape (2.5cmx9.1m)	Transpore	1527-1
Suture	Davis and Geck CYANAMID Canada	Nov-04
Syringe (1ml)	Becton Dickinson and Company (BD)	309659
VANNAS scissors	World Precision Instruments (WPI)	14122-G
Xylazine	Rompun	DIN02169606

Equipment

Dissecting microscope	Olympus
Confocal microscope	Leica

Comments/Description

University of Calgary distribution services UN1170.

Nair Sensitive Formula Hair Removal Crème with Sweet Almond Oil and Baby Oil, 200-ml. Or similar

For 1 ml of Evans blue dye, add 0.1g Evans blue to 10 ml PBS. The Evens Blue solution will be filtered

Connect one end of polyethylene tubing to 30G × ½ needle. Attach a 1ml TB syringe to the needle.

The suppliers of Ketamine and Xylazine are usually under institutional and governmental regulation.

Straight, Without Lock; Serrated

0.7 metric monofilament polypropylene

The suppliers of Ketamine and Xylazine are usually under institutional and governmental regulation.

Olympus S261 (522-STS OH141791) with light source:
Olympus Highlight 3100
SP8

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Feburary 25, 2020

Dear Editor,

We have revised the manuscript as requested. The major changes were highlighted in yellow. The replies to editor are listed below. Please advise if there is anything else we can do.

Sincerely,

Shan Liao

1. Most of these are described in the legend of Figure 1. Please combine this paragraph with Figure 1 legend to avoid repetition.

   This paragraph is now removed and combined with Figure 1.

2. Do you mean “disinfect the tools with 70% ethanol”?
   Yes. I rephrase the sentence.

3. Tuberculin?
   Yes. It is revised.

4. What is the ratio of ketamine/xylazine?
   It is ketamine (10 mg/mL)/xylazine (1mg/mL). It is revised.

5. This is confusing. What is the concentration of xylazine? 1 or 100 mg/mL?
   The stock solution is 100mg/mL. It is revised.

6. Shave with what? Do you both shave and apply depilatory cream?
   Shave with hair clipper and then remove the left over fur with depilatory cream. It is revised.

7. Please specify the operation area.
   It is around the leg.

8. Please reference different panels of Figure 1 in the protocol section to guide the readers.
   It is revised.

9. I added this. Please review for accuracy.
   Correct as revised.

10. Please describe in the imperative tense how to use the injection apparatus prepared in section 1 to inject 5 μL of 1% Evans blue dye.

    Gently inject 9 cm of fluid from the injection apparatus is equivalent to 5 μL. It is revised.

11. 5 mm?
    Yes.

12. I added this. Please review for accuracy.
    Thanks for the revision. It is correct.

13. Please specify.

14. Please specify the steps being repeated for the sham control. The information here is not complete. For instance, Evan blue is also injected to the left leg but this is not mentioned in this sentence.
    It is revised.

15. Under a microscope?
    No, just watch the color of legs with naked eyes.

16. How is this done?
    Euthanize the mice and collect the pLNs. Not sure how to descript this.

17. Can this be removed as Figure 1A does not show this.
    It is revised.

18. Indicated by the white arrow? Otherwise please describe what the arrows point to.
    It is revised. The arrows point to the blue lymphatic vessels.

19. These are already mentioned in protocol and results section. Please delete them to avoid repetition.
    It is deleted.

20. I added these. Please review for accuracy.
    Thanks for the suggestion. It is correct.

21. Please label it in the figure if you are able.

22. Please rephrase this sentence to avoid overlap with previously published text.

    It is revised.

23. Please spell out journal names.
    It is revised.