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**Stimulation and inhibition of lymphangiogenesis for tissue
regeneration and disease therapy**

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Running title: Stimulation and inhibition of lymphangiogenesis

Abstract

The lymphatic vessels can be selectively stimulated to grow in adult mice, rats and pigs by application of viral vectors expressing lymphangiogenic factors VEGF-C or VEGF-D and lymphangiogenesis in various pathological settings can be inhibited by the blocking of the VEGF-C/VEGFR3 interaction. Furthermore, the recently discovered plasticity of meningeal and lacteal lymphatic vessels provides novel opportunities for their manipulation in disease. For gene transduction, adeno-associated viral vectors (AAVs), which have a superior safety profile compared to adenoviral vectors, have already been successfully used as tools for establishing long-term gene expression in clinical trials. As an example, we describe here ways to manipulate the meningeal lymphatic vasculature in the adult mice by using AAV-mediated gene delivery. The possibility of stimulation and inhibition of lymphangiogenesis in adult mice has enabled us to explore the role and function of lymphatic vessels in mouse models of disease.

Key words: Lymphangiogenesis, Lymphatic vessel egression, Meningeal Lymphatics, AAV-mediated gene therapy, VEGF-C, VEGFR3

1 Introduction (about 250 words)

The lymphatic system, found exclusively in vertebrates, consists of the lymphatic vessels (capillaries, pre-collectors and collectors) and the lymphoid organs (lymph nodes, spleen, thymus, Peyer's patches, tonsils, appendix and the bone marrow) [1]. Under physiological conditions, the main functions of the lymphatic system include tissue fluid homeostasis by draining the interstitial fluid back to the systemic circulation, immune surveillance and dietary fat uptake from the gut [2]. Aberrant lymphatic vessel growth and lymphatic vessel dysfunction have been associated with pathological conditions such as inflammation, lymphedema and tumor metastasis; therefore, lymphatic vessels have been an attractive target for therapeutic intervention in such conditions [3].

Lymphatic vessels have long been considered to be independent of VEGFR3 signaling in adulthood; however, recent studies demonstrated differential and unique dependence of lymphatic endothelial cells in certain vascular beds such as the intestinal lacteals and meningeal lymphatic vessels on VEGF-C/VEGFR3 signaling [4,5]. The discovery of meningeal lymphatic vessels has raised new questions about their role in diseases of the central nervous system [6]. Answering of these questions requires the manipulation of lymphatic vessels in a variety of already pre-clinical disease models.

Adeno-associated viral vectors (AAVs) have become valuable tools for *in vivo* gene therapy due to their excellent safety profile, high transduction efficacy and ability to target a broad range of tissues [7]. Due to their low immunogenicity, AAVs also provide long-term *in vivo* expression of their genetic cargo, eliminating the need to administer proteins with short half-lives. Here we describe how to manipulate the meningeal lymphatic vasculature in adult mice using AAV-mediated gene delivery. Successful systemic transduction of AAVs that encode secreted growth factors or soluble inhibitors can be confirmed by western blotting of tissue or serum samples (**Fig. 1**), while the stimulation and inhibition of the meningeal lymphatics can be visualized in whole mounts of the skull caps (**Fig. 2**).

2 Materials

2.1 Intraperitoneal administration

1. AAV vector in PBS (see **Note 1**).
2. 1 mL syringe.
3. 25G needle(s).
4. Ice.

2.2 Intracerebroventricular administration of AAVs

2.2.1 Materials for surgery and injection

1. AAV vector in PBS (see **Note 1**).
2. Anesthesia (see **Note 2**).
3. Analgesic (see **Note 3**).
4. Ethanol : 70 % and 95 % in milliQ water.
5. Eye ointment (see **Note 4**).
6. MilliQ water.
7. Ice.Cotton swabs.
8. Eppendorf tubes.
9. Surgical equipment : forceps, scissors and scalpels. Hamilton Cleaning Solution (Hamilton Company).
10. Nanofil syringe 10 μ L and 33G needle (World Precision Instruments).
11. 5-0 Nylon sutures or wound clips (Clay Adams).
12. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates atlas [8] or equivalent.

2.2.2 Apparatus for surgery

1. Timer.
2. Electric shaver.
3. Dental drill.

4. Sterilizer (Fine Science Tools).
5. Stereotaxic apparatus (Stoelting).
6. Electronic grid (Stoelting).
7. Micro syringe pump controller (Stoelting).
8. Heating chamber.

2.2.3 Materials for blood collection

1. Small piece of tissue paper.
2. 23G needle.
3. 50 mL Falcon tubes with air holes.
4. Blood collection capillaries, non-heparinized, 75 mm - 75 μ L (Hirshmann).
5. MiniCollect gel tubes for serum separation (Greiner Bio-One).
6. Electric shaver.
7. Microfuge.

2.2.3 Materials and equipment for western blotting

1. Regular western blot materials and equipment.
2. Tris-buffered saline (TBS; 10 \times) : 1.5 M NaCl, 0.1 M Tris-HCl, pH 7.4.
3. Blocking solution : 1 % (w/v) bovine serum albumin, 0.05 % (v/v) Tween 20, in TBS.
4. Polyclonal goat-anti-mouse VEGFR3 antibody (R&D, AF743).
5. Rabbit anti-goat-IgG, HRP conjugate.

3 Methods

3.1 Intraperitoneal administration of AAV

1. Restrain the mouse by firm grip behind the neck and turn over so that abdomen is exposed and tail is secured between your pinky or ring finger. Monitor chest movements to make sure that the mouse is breathing normally.
2. Aim your i.p injection to the lower right or left quadrant of the abdomen. Avoid injecting to the bladder, liver, or other internal organs. Perform your injection with steady pressure, remove the needle and release mouse back to into its cage (see **Note 5**).

3.2 Intracerebroventricular administration of AAV

3.2.1 Pre-surgical procedure

1. Sterilize the area and the stereotaxic apparatus with 70% ethanol to create a sterile field for surgery.
2. Sterilize the equipment: place the tips of surgical equipment into the bead sterilizer for 15 seconds. Remove and place in 70 % ethanol. When performing more operations, re-sterilize the tips of the instruments in between animals.
3. Sterilize the Hamilton injection needle in the following order:
 - i.* Hamilton Cleaning Solution.
 - ii.* MilliQ water.
 - iii.* 95% ethanol (see **Note 6**).
4. Weigh the mouse.

5. Anesthetize the mouse based on the weight with i.p. injection using a mixture of Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and monitor that an unconscious state (no withdrawal reflex of paw is observed or another equivalent parameter of anesthesia depth) has been achieved prior to continuing (see **Note 7**).
6. Deliver Carprofen (5 mg/kg) for mouse pre-operative pain relief using a subcutaneous injection between the shoulder blades.
7. Remove hair in the area from the top of the shoulder to the eyes using an electric shaver.
8. Secure the anesthetized mouse in the stereotaxic apparatus (see **Note 8**).
9. Apply eye ointment to each eye (see **Note 4**).
10. For disinfection of the surgical area, wipe the top of the head and neck areas starting from the center of the area and moving outwards using a cotton swab dipped in 95% ethanol.

3.2.2 Surgical procedure

1. Start by making an incision from the base of the neck up to the midline between the eyes with a scalpel or scissors.
2. Clean the skull with sterile cotton swabs and a cotton swab dipped in 95 % ethanol to enhance visualization of bregma. Let the skull dry for better visualization of skull sutures.
3. Find bregma and mark it with surgical marker, preferably of blue or black color for easy visualization.
4. Check that bregma, lambda and both hemispheres are at same level.

5. Attach your drill to holder of stereotaxic apparatus, guide the tip of the drill to the bregma and carefully lower it so that its base will touch the skull. Set this zero to all three-dimensional coordinates. Move the drill to the right coordinates (in A/P and M/L axis's). A/P -0.34mm ; M/L -1.00mm (see **Note 9**). In this protocol, these coordinates are set by using Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates book [8].
6. Drill gently so that the dura is left untouched. As the thickness of the mouse skull is about 2-3 mm, don't drill more than that. Observe constantly the depth of the Burr hole (see **Note 9**).
7. When the drilling has been completed, fill your injection needle with AAV particles and attach it to the stereotaxic apparatus holder and recheck the coordinates.
8. Place the injection needle carefully to the hole so that it will touch the dura (D/V 0,00) and slowly lower the needle to the right coordinates: D/V -2.50 from dura.
9. Wait for 2 minutes for the brain to seal around the needle.
10. Infuse up to $4\ \mu\text{L}$ of AAV vector mixture in PBS at $0.5\ \mu\text{L}/\text{min}$ (see **Note 1**).
11. Wait for 4 minutes before removing the needle.
12. Remove the needle from the hole steadily at an approximate speed of $1\ \text{mm}/\text{second}$.
13. Remove the mouse from the stereotaxic apparatus. Apply $30\ \mu\text{L}$ of lidocaine on the surface of the skull and close the wound with 5-0 nylon sutures or wound clips.
14. Check that the mouse is breathing normally and place it into a heating chamber to recover.

15. On the following day, administer Carprofen (5 mg/kg) by subcutaneous injection between shoulder blades and examine the behavior and appearance of the mouse (see **Note 10**).

3.3 Validation of AAV transduction

3.3.1 Blood collection

1. In this protocol, we use the saphenous vein for peripheral blood sampling (see **Note 11**).
2. Restrain the conscious mouse in an uncapped 50 mL Falcon tube, which has air holes in the bottom end, with the tail and the hind legs of the mouse exposed at the open end of the Falcon tube (see **Note 12**).
3. Extend the left hind leg and fix firmly by applying pressure on the thigh muscle at a point close to the hip joint. This will also help making the saphenous vein protrude.
4. Remove hair from outer surface of the hind leg by using the electric shaver and reveal the saphenous vein.
5. Using a 23G needle, make a puncture to the saphenous vein and gently reduce the pressure on the leg. A drop of blood should appear on the surface of the skin.
6. Hold the capillary at a 45° angle and fill with blood approximately one third of its length (approximately 25 mm/25 µL) by capillary force. Put the capillary aside.
7. Apply compression to the puncture site until the bleeding stops (a few seconds). Return the mouse to its cage.
8. Dispense the blood into the gel tube for serum separation by pipetting air into the capillary from the other side of the capillary.

9. Let the blood sit for 20 minutes at room temperature, then centrifuge the gel tube in a microfuge for serum separation at 2300 g for 5 minutes.
10. Pipet the separated serum from the upper partition into a clean Eppendorf tube. For a single Western blot sample, only 0.5 μ L of serum will be needed.

3.3.2 Western blotting for mVEGFR3₁₋₄-Ig and mVEGFR3₄₋₇-Ig

1. Perform western blotting with common protocols using the following specific modifications:
 - a. Use 0.35-0.5 μ L of serum collected from mice injected with AAV-mVEGFR3₁₋₄-Ig or AAV-mVEGFR3₄₋₇-Ig.
 - b. Use an 8 % gel to separate the proteins.
 - c. For detection of both mVEGFR3₁₋₄-Ig and mVEGFR3₄₋₇-Ig, use polyclonal goat-anti-mouse VEGFR3 antibody at 1:1000 dilution (see **Note 13**).
 - d. In the resultant blot, both mVEGFR3₁₋₄-Ig and mVEGFR3₄₋₇-Ig should be detected around 95 kDa (see **Fig. 1**).

[Insert Fig. 1 near here]

3.3.3 Whole mount tissue preparation

1. Follow the descriptions in Antila *et al.* [5] for the required materials and detailed tissue preparation protocol. While the whole mount of a normal mouse skull cap looks like in **Fig. 2a**, stimulation of lymphangiogenesis with AAV-VEGF-C presents with multiple sprouts of the meningeal lymphatics, which is shown in **Fig. 2b**. The i.p. injected control AAV (AAV-mVEGFR3₄₋₇-Ig) does not cause significant alteration of the meningeal lymphatics, whereas the AAV-mVEGFR3₁₋₄-Ig results in regression of the pre-existing lymphatic vessels in the meninges (**Fig. 2c and d**).

[Insert Fig. 2 near here]

4 Notes

1. AAV batches vary by their viral particle (vp) numbers. Calculate carefully prior to the injections how much vector you need for your experiment. Keep AAVs on ice prior to administration. In this protocol, we use AAV9-pseudotyped AAVs. For induction of lymphangiogenesis we use AAVs encoding murine VEGF-C (AAV-mVEGF-C; [9]), delivered i.c.v. ($10^{7.5}$ - 10^{10} vp / mouse). Please note that AAV-mVEGF-C cannot be injected intraperitoneally. For inhibition of lymphangiogenesis or induction of lymphatic vessel regression, we use AAVs encoding the ligand binding domains 1-4 of murine VEGFR-3 fused to the Fc part of murine IgG1 (AAV-mVEGFR3₁₋₄-Ig; [10]). Control mice receive AAVs encoding the domains 4–7 of murine VEGFR3 (that do not bind VEGF-C or VEGF-D) fused to the Fc part of murine IgG1 (AAV-mVEGFR3₄₋₇-Ig; [11]). These can be delivered via i.p. (10^{11} - 10^{12} vp / mouse) or i.c.v (10^9 - 10^{10} vp / mouse) administration routes. Please note that i.p. injection technique requires larger vp amount.
2. Induction of anesthesia in mice can be achieved by using injectable anesthetics or inhalation delivery method (isoflurane or equivalent). In this protocol, we use a combination of Ketamine (100 mg/kg) and Xylazine (10 mg/kg), which results in deep and reliable anesthesia over the course of the surgical operation. For inhalation anesthesia, additional equipment is required for induction and maintenance. Validate most suitable and convenient method for your own experimental setup and animal experiment license.
3. Surgical operations require analgesic administration. In this protocol, Carprofen (5 mg/kg) is used prior the operation and 24 hours after surgery. Check your own institutional regulations and guidelines for the analgesic administration. Based on our

experience, 48 hours after the operation, the operated mice are indistinguishable from non-operated mice based on their behavior or their general appearance, indicating that a longer analgesic administration is not required.

4. Eye ointment prevents drying of the mouse eyes during and after the surgery while the mice are still under anesthesia. Gel formulas generally provide longer lasting protection than the aqueous ones.
5. Systemic AAV transduction for adult mice can be achieved by administration of a single dose of viral particles (10^{11} - 10^{12} viral particles in PBS). If the concentration of your AAV prep is low, check the maximum i.p. administration volume allowed by your institutional authorities.
6. Fill and empty the Hamilton needle in the order described in the methods section. Rinse with the liquids up and down 3-5 times before changing to the next one. Be careful not to touch the tip of the needle to the wall of the Eppendorf tube as this may cause breaking of the needle.
7. The combination of anesthetics we use contains 1/5 parts Ketamine (50 mg/mL), 1/20 parts Xylazine (20 mg/mL) and 3/4 parts of 0.9% NaCl. Dosing is based on the weight of the mouse (10 μ L/g).
8. Hook the front teeth on to hole in the frame's bite grip. After this, gently position the ear bars in the ear canal and secure the holding screws. At this point make sure that skull is leveled. Then position the nose clamp down and secure it.
9. You can also attach the injection needle to the stereotaxic apparatus holder and use that for coordinate navigation. With this method, the thinning of the skull is none by hand only. This saves time so that you don't need to de-attach the drill and needle, but it requires practice so that the dura will stay untouched when drilling is done with the free-hand method.

10. On the first day after operation, mice usually behave relatively normally with small differences in appearance. For instance, their fur might not appear as clean as prior to the operation. However, on the second day they are not distinguishable from unoperated mice.
11. Make sure that your experimental permit allows the collection of the required amount of blood. It may be helpful to have assistance by a second person during this procedure.
12. After the i.p. administration, allow at least 48-72 hours for the AAV transduction and protein production to take place.
13. This polyclonal antibody is raised against domains 1-7 of the murine VEGFR-3; therefore, it recognizes both mVEGFR3₁₋₄-Ig and mVEGFR3₄₋₇-Ig.

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Figures and figure legends

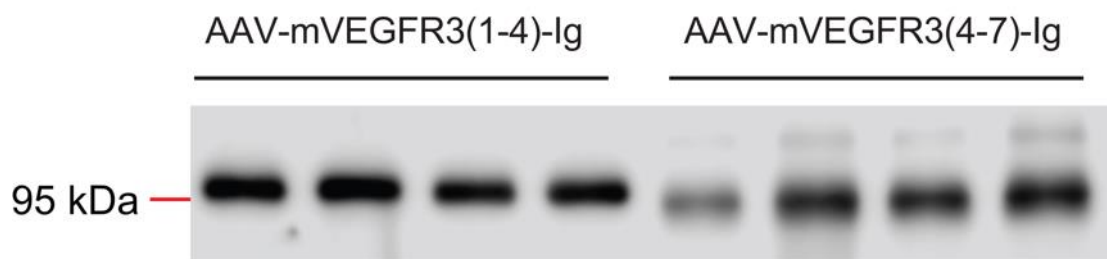


Fig. 1 Validation of AAV-transduction after i.p. administration. Western blot showing mVEGFR3-Ig protein in serum 8 weeks after AAV injection.

LYVE1

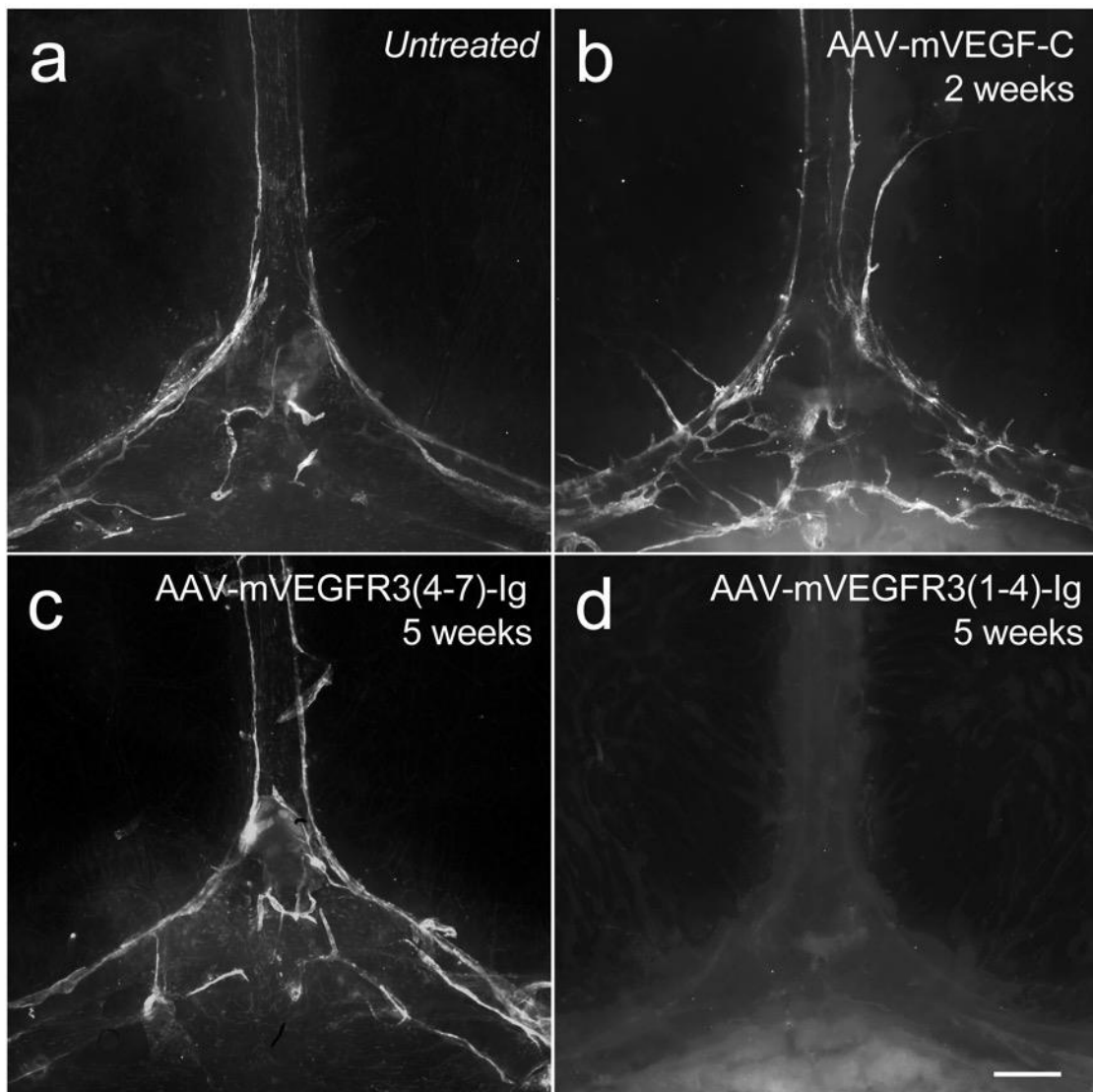


Fig. 2 Meningeal lymphatic vessels in response to the manipulation of VEGF-C/VEGFR3 signaling. **(a–d)** LYVE1 staining (gray) of meningeal LVs in **(a)** untreated mice, **(b)** mice injected i.c.v. with AAV-mVEGF-C, or mice injected i.p. either with **(c)** AAV-mVEGFR3₄₋₇-Ig or **(d)** AAV-mVEGFR3₁₋₄-Ig. Scale bar = 500 μ m.