

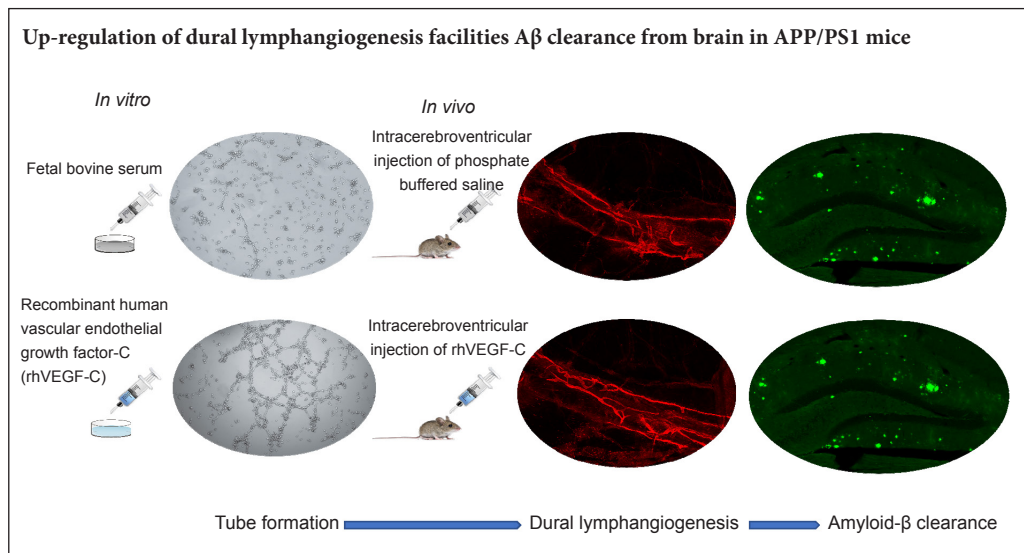
Induced dural lymphangiogenesis facilitates soluble amyloid-beta clearance from brain in a transgenic mouse model of Alzheimer's disease

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Graphical Abstract



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Abstract

Impaired amyloid- β clearance from the brain is a core pathological event in Alzheimer's disease. The therapeutic effect of current pharmacotherapies is unsatisfactory, and some treatments cause severe side effects. The meningeal lymphatic vessels might be a new route for amyloid- β clearance. This study investigated whether promoting dural lymphangiogenesis facilitated the clearance of amyloid- β from the brain. First, human lymphatic endothelial cells were treated with 100 ng/mL recombinant human vascular endothelial growth factor-C (rhVEGF-C) protein. Light microscopy verified that rhVEGF-C, a specific ligand for vascular endothelial growth factor receptor-3 (VEGFR-3), significantly promoted tube formation of human lymphatic endothelial cells *in vitro*. In an *in vivo* study, 200 μ g/mL rhVEGF-C was injected into the cisterna magna of APP/PS1 transgenic mice, once every 2 days, four times in total. Immunofluorescence staining demonstrated high levels of dural lymphangiogenesis in Alzheimer's disease mice. One week after rhVEGF-C administration, enzyme-linked immunosorbent assay results showed that levels of soluble amyloid- β were decreased in cerebrospinal fluid and brain. The Morris water maze test demonstrated that spatial cognition was restored. These results indicate that the upregulation of dural lymphangiogenesis facilitates amyloid- β clearance from the brain of APP/PS1 mice, suggesting the potential of the VEGF-C/VEGFR-3 signaling pathway as a therapeutic target for Alzheimer's disease.

Key Words: nerve regeneration; dura mater; lymphangiogenesis; amyloid- β ; Alzheimer's disease; recombinant human vascular endothelial growth factor-C; lymphatic endothelial cells; lymphatic clearance; neural regeneration

Introduction

Alzheimer's disease (AD), the most common cause of dementia, causes severe impairments to AD patients and heavy financial and social burden to their families and society (Reitz and Mayeux, 2014). Accumulation and deposition of amyloid- β (A β) is regarded as the main causative pathology in AD development, and a variety of strategies to promote

A β clearance have been developed and tested both in experimental and clinical studies (Wisniewski and Drummond, 2016; Bate, 2017; Wang et al., 2017). However, the therapeutic effects of current A β clearance-based pharmacotherapies are unsatisfactory and can cause severe side effects including encephalitis and meningitis (Cash et al., 2014). In general, these therapeutic methods involve active immunization, pas-

sive immunization or other mechanisms to directly increase the clearance of A β from the brain. Nevertheless, there has been no report of a method to promote the lymphatic clearance of A β from the cerebrospinal fluid (CSF). This might be because the structure and function of the lymphatic system of the central nervous system (CNS) were not described in detail until 2015 (Aspelund et al., 2015; Louveau et al., 2015). Before 2015, it was widely thought that no classical lymphatic vessels were present in the CNS, including the brain and meninges. Therefore, few studies argued that the lymphatic clearance of A β from the CNS mattered (Shibata et al., 2000; Abbott, 2004), although several studies reported the presence of A β in cervical lymphatic nodes (Tsai et al., 2003; Pappolla et al., 2014). In 2015, Aspelund et al. (2015) and Louveau et al. (2015) verified that cells and solutes in the CSF drained into the deep cervical lymphatic nodes through classical lymphatic vessels located in the dura. Therefore, the transport of A β from the brain into the CSF indicates another potentially important route for brain-produced A β clearance.

We hypothesized that an increase in dural lymphangiogenesis improves the lymphatic clearance of A β from the CSF and thus would have therapeutic effects on AD pathology concerning the accumulation and deposition of A β in the brain. To the best of our knowledge, no reports to date have addressed this issue.

Vascular endothelial growth factor (VEGF)-C plays an important role in lymphangiogenesis and angiogenesis in embryos and tumors (Kukk et al., 1996; Karkkainen et al., 2004; Hirakawa et al., 2007; Gore et al., 2011). Recombinant human VEGF (rhVEGF)-C is a human recombinant protein specific to VEGF receptor-3 (VEGFR-3); these are critical factors for lymphatic growth (Iwami et al., 2015), and VEGF-C/VEGFR-3 signaling is involved in maintaining tissue electrolyte balance (Machnik et al., 2009; Wiig et al., 2013). The current study applied rhVEGF-C to a transgenic mouse model of AD to investigate the effect on A β clearance from the brain and CSF and the underlying mechanism.

Materials and Methods

Human lymphatic endothelial cell (hLEC) culture

hLECs were purchased from ScienCell Research Laboratories (Catalog #2500, Carlsbad, CA, USA) and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Shanghai, China) containing 10% fetal bovine serum (Bio-Ind, Israel) and 1% penicillin/streptomycin solution (Life Technologies, Shanghai, China) and were seeded in culture bottles (Corning, New York City, NY, USA), and then incubated at 37°C in a humidified incubator with an atmosphere of 5% CO₂. hLECs were digested with 0.25% trypsin-ethylenediamine tetraacetic acid (Life Technologies, Burlington, Canada) for 1 minute, and complete medium was added to terminate digestion. hLECs were then treated with rhVEGF-C protein (100 ng/mL) (Cys156Ser, R&D Systems, Minneapolis, MN, USA) at the indicated time points.

hLEC tube formation assay

Tube formation assay was performed to examine the effects

of rhVEGF-C on the tube formation of hLECs *in vitro*. Ten μ L/well liquid Matrigel (Corning, Cat. #356243, Bedford, MA, USA) was coated on the inner walls of 15-well Ibidi μ -slides for angiogenesis (Ibidi, Martinsried, Germany) (Gagliostro et al., 2016), and polymerized for 1 hour at 37°C. hLECs were synchronized by serum starvation for 12 hours prior to treatment with equal amounts of purified factor (100 ng/mL) or 10% fetal bovine serum for 48 hours (Joukov et al., 1998; Chien et al., 2009). hLECs (1×10^4 /well) in 50 μ L media were seeded into the upper well and incubated for 12 hours at 37°C to allow tube formation. Images of tube formation of each chamber were captured using an inverted phase contrast microscope (Leica DMI 4000B, Wetzlar, Germany) at 5 \times magnification. Total lengths of tube-like structures (exceeding 20 μ m in diameter) were measured and quantified using ImageJ (NIH, Bethesda, MD, USA) (Kazenwadel et al., 2012; Shin et al., 2015).

Animals

Nine-month-old female APP^{swe}/PS1^{dE9} (APP/PS1) mice were purchased from the Guangdong Medical Laboratory Animal Center in China (Foshan, China) (No. 44007200040575). All animals were housed in a specific-pathogen-free facility and maintained on a 12 hour-light/12 hour-dark cycle with a standard diet. This study was approved by the Institute Research Ethics Committee of Sun Yat-sen University of China (approval No. 2017-002) and performed in strict accordance with the UK Animals (Scientific Procedures) Act, 1986. The mice were randomly divided into rhVEGF-C and control groups ($n = 16$ per group, including 6 mice for morphological analysis and 10 for behavioral tests).

In vivo VEGFR-3 activation

Mice were anesthetized with an intraperitoneal injection of chloral hydrate (10% solution) and placed into a stereotaxic frame. The neck muscles were bluntly dissected to expose the dura mater overlying the cisterna magna through a small midline incision. Cannulae (outer diameter 0.3 \times inner diameter 0.14 mm, depth 1 mm, custom-made by RWD, Shenzhen, China) were implanted above the cisterna magna and affixed with dental acrylic to maintain their position (de Lange et al., 1997). On day 5 after cannulae implantation, mice were intracerebroventricularly injected (into the cisterna magna) with 5 μ L of rhVEGF-C (Cys156Ser, R&D Systems, 200 μ g/mL) or PBS with a 5 μ L microliter syringe (RWD, Cat. #79004, Shenzhen, China) at a rate of 1 μ L/min over 5 minutes (Kajiya et al., 2009; Heishi et al., 2010; Rangroo Thrane et al., 2013). A total of four injections were performed once every 2 days. Meninges were harvested 7 days after the last injection for immunofluorescence staining. The experimental timeline is shown in **Figure 1**.

Immunofluorescence staining

Mice were euthanized with an intraperitoneal injection of chloral hydrate (10% solution) 7 days after the last injection. Approximately 5–10 μ L CSF was obtained from the cisterna

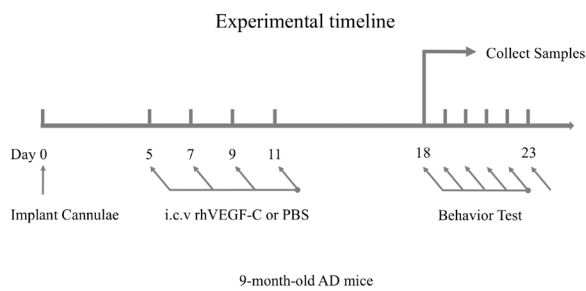


Figure 1 Experimental timeline.

Mice were implanted with a cannula for 4 days and were intracerebroventricularly (i.c.v.) injected with 5 μ L of recombinant human vascular endothelial growth factor-C (rhVEGF-C) (200 μ g/mL) or phosphate-buffered saline (PBS) once every 2 days. Four injections were performed. Immunofluorescence staining, enzyme-linked immunosorbent assay and the behavioral tests were performed 7 days after the last injection.

magna per mouse according to the protocol described previously for enzyme-linked immunosorbent assay (ELISA) (Liu and Duff, 2008). After perfusion with 0.1 M of ice-cold phosphate-buffered saline (PBS) for 5 minutes, surgery was performed as previously described (Louveau et al., 2015). After removal of the skin and muscle from the skull, the skull cap was removed with surgical scissors. Then, the brain was immediately removed and bisected sagittally. The right hemisphere was snap frozen for ELISA, and the left hemisphere for Thioflavine-S staining. Deep cervical lymph nodes were isolated by forceps. Whole-mount meninges, brains and deep cervical lymph nodes were immediately fixed in PBS with 4% paraformaldehyde overnight at 4°C. The meninges (dura mater/arachnoid) were then carefully stripped from the skull cap, washed in PBS three times and processed for staining. Brains and deep cervical lymph nodes were dehydrated in a graded series of sucrose solutions at 4°C. At the level of the hippocampus (bregma -1.58 mm to -2.46 mm), serial coronal sections (30 μ m) were obtained from each brain with a freezing microtome (Leica Microsystems, Inc., Exton, PA, USA), and stored in PBS at 4°C prior to immunostaining.

Dura and deep cervical lymph node sections were washed twice with PBS for 5 minutes each and blocked in PBS containing 1% bovine serum albumin and 0.25% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C and subsequently incubated overnight at 4°C with appropriate dilutions of primary antibodies: goat anti-Lyve-1 (1:600; R&D Systems) and rabbit anti-Prox1 (1:500; Abcam). Specimens were washed three times in PBS followed by incubation with the appropriate secondary antibody at 37°C for 2 hours. The secondary antibodies were: Alexa 555-conjugated donkey anti-goat, Alexa Fluor 488 goat anti-rabbit, (1:400; both from Invitrogen). Subsequently, the samples were washed three times in PBS and mounted on glass slides with fluorescence mounting media. Brain sections were washed twice with PBS for 5 minutes each and stained with 1% thioflavine-S (Li et al., 2017).

Confocal micrographs were obtained using an LSM 780 confocal laser-scanning microscope (Zeiss, Heidelberg,

Germany). For images of the whole mount of the meninges, brain sections and lymphatic nodes, images were acquired with a 10 \times objective using the Tile Scan function. Images of the meninges were acquired at 512 \times 512 pixel resolution and with a z-step of 4 μ m. Lymphatic vessels were stained by Lyve-1 antibody (red), and the nuclei of hLECs were stained by Prox-1 antibody (green). The numbers of Prox-1⁺ nuclei colocalized with Lyve-1⁺ vessel were counted using Stereo Investigator (MicroBrightField, Williston, VT, USA). The superficial area of dural lymphatic vessels was quantified with Imaris 8.4 software (Bitplane AG, Zurich, Switzerland). Prox1-positive cell density was determined by dividing the number of Prox1⁺ nuclei by the superficial area of lymphatic vessels. The length of lymphatic vessels of the meninges was quantified with ImageJ. The meningeal lymphatic vessel diameter of dural lymphatic vessels was determined by dividing the superficial area by the length of lymphatic and π . Relative areas of Lyve-1⁺ lymphatic vessels of the deep cervical lymph nodes were measured by ImageJ. For the quantitative image analysis of A β plaques, six 30 μ m coronal sections (150 μ m apart) were selected per mouse. The hippocampus and cerebral cortex of each sample were chosen for quantitative analysis using ImageJ. Data are reported as the percentage of immunolabeled area captured relative to the total demarcated brain region.

ELISA

Briefly, brain samples were weighed and radioimmune precipitation assay buffer (Beyotime, Shanghai, China) containing 1% phenylmethyl sulfonylfluoride (Beyotime) was added and then sonicated with a homogenizer (Qiagen, Hilden, Germany) for 20 seconds on ice. Samples were then centrifuged for 5 minutes at 12,000 \times g at 4°C, and supernatants were transferred into a new tube and stored at -80°C. The total protein concentration of each sample was adjusted to 4.5 mg/mL using the bovine serum albumin protein assay kit (Beyotime). The levels of human soluble A β 1-40 and A β 1-42 in brain, CSF and deep cervical lymph nodes were analyzed by ELISA kits (Cusabio, Wuhan, China). Brains were also assayed for oligomeric A β using ELISA kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Morris water maze test

The Morris water maze test was performed to assess the hippocampus-dependent spatial learning and memory of transgenic mice 7 days after the last injection (Vorhees and Williams, 2006). Mice were trained to find the escape platform in white non-toxic colored water at 23°C in a 0.8-m diameter pool (Yang et al., 2016). The behavioral test was administered for 6 consecutive days. The acquisition phase was from the first to the fifth day, and the spatial probe occurred on day 6. In each trial, mice were given 60 seconds to find the hidden platform. The escape latency is the mean time required to find the platform of four trials starting from different quadrants, recorded up to 60 seconds. On day 6, the platform was removed from the pool and each mouse was tested by a probe trial for 60 seconds (Counts and Lahiri, 2014). Data were re-

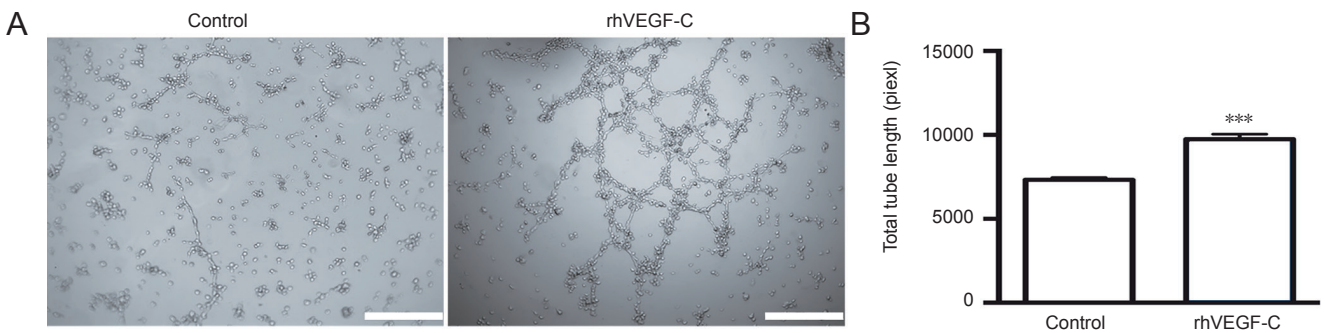


Figure 2 Tube formation of hLECs following rhVEGF-C treatment.

(A) hLECs (1×10^4 cells) treated with rhVEGF-C (100 ng/mL) or 10% fetal bovine serum for 48 hours were cultured on Matrigel in 50 μ L media and 12 hours later images were captured. Scale bars: 500 μ m. (B) The total tube length was measured and shown as pixel length per well. Data are presented as the mean \pm SEM, and each group was tested in triplicate in three independent experiments ($n = 4$), *** $P < 0.001$, vs. control group. (two-tailed Student's t -test) hLECs: Human lymphatic endothelial cells; rhVEGF-C: recombinant human vascular endothelial growth factor-C.

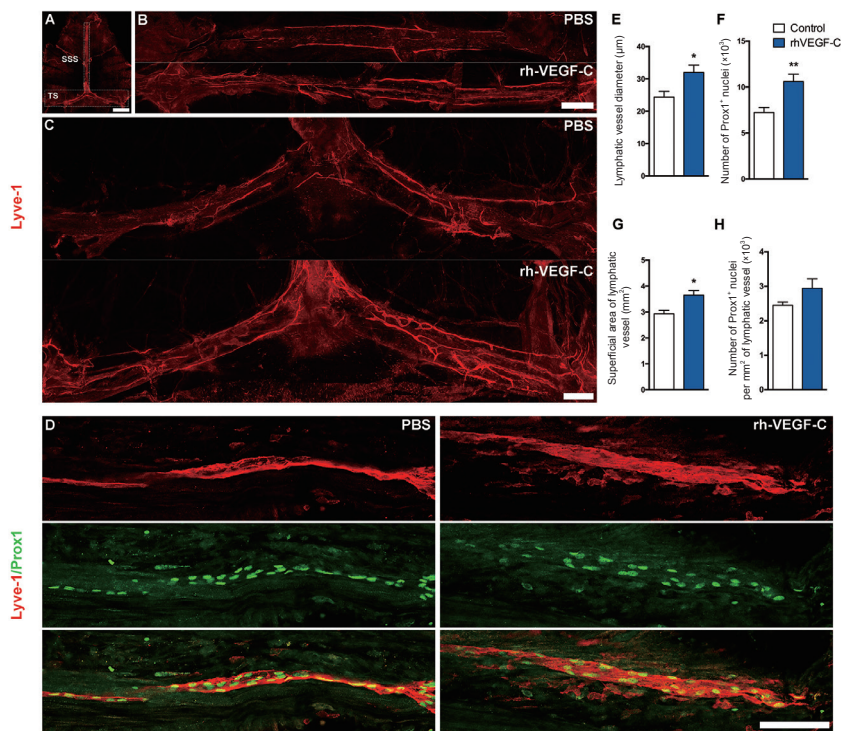


Figure 3 Elevated dural lymphangiogenesis in transgenic mice injected with rhVEGF-C.

(A–H) Analysis of dura mater lymphatic vasculature in transgenic and experimental mice. (A) The dLVs along the SSS and TS, and the location of the images presented in (B) and (C). (D) Representative images of Lyve-1 and Prox1 labeling of meninges on day 7 after the last injection. Scale bars: 1000 μ m (A), 500 μ m (B, C) or 100 μ m (D). (E–H) Quantification of the meningeal lymphatic vessel diameter (E), number of Prox1⁺ nuclei located in dLVs (F), total superficial area of dLVs (G), and number of Prox1⁺ nuclei per mm^2 of lymphatic vessel. Data are presented as the mean \pm SEM ($n = 6$; two-tailed Student's t -test). * $P < 0.05$, ** $P < 0.01$, vs. control group. rhVEGF-C: Recombinant human vascular endothelial growth factor-C; SSS: superior sagittal sinus; TS: transverse sinus; dLVs: dural lymphatic vessels.

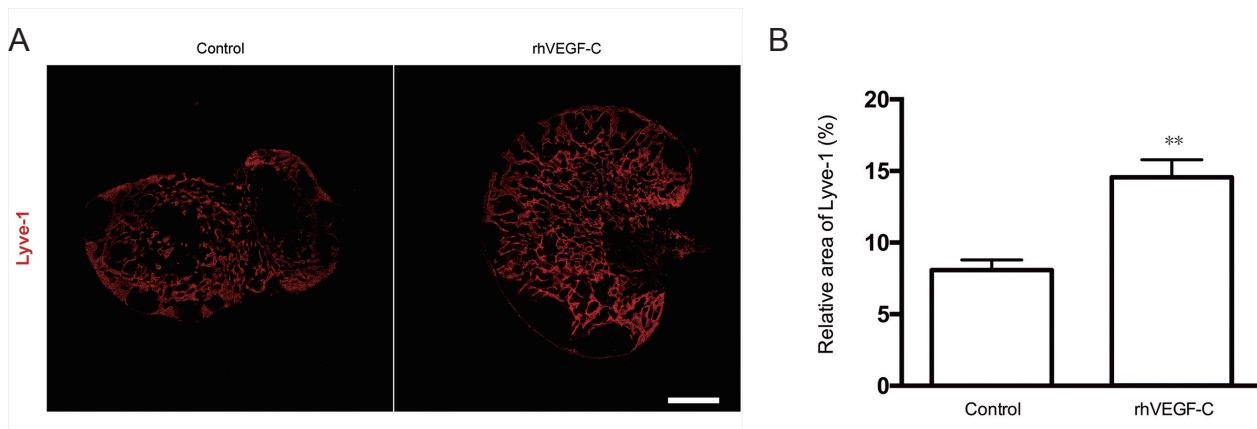


Figure 4 Elevated lymphangiogenesis in dCLNs in APP/PS1 mice treated with rhVEGF-C.

(A) Elevated lymphangiogenesis in dCLNs of 9-month-old APP/PS1 mice treated with rhVEGF-C. Scale bar: 500 μ m. (B) Relative area of the dCLNs of the two groups of mice. Lymphatic vessels were stained by Lyve-1 antibody (red), and the relative area of Lyve-1⁺ lymphatic vessels was measured by ImageJ. Data are presented as the mean \pm SEM ($n = 6$; two-tailed Student's t -test). ** $P < 0.01$, vs. control group. rhVEGF-C: Recombinant human vascular endothelial growth factor-C; dCLNs: deep cervical lymph nodes.

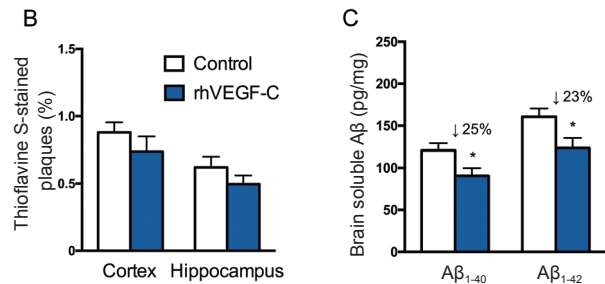
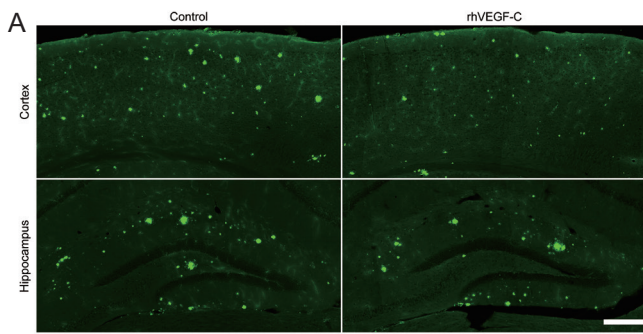


Figure 5 Reduction of cerebral Aβ pathology in APP/PS1 mice treated with rhVEGF-C 7 days after the last injection.

(A) Confocal micrographs of the cortex and hippocampus were obtained to determine the Aβ pathology in brains of APP/PS1 mice treated with phosphate-buffered saline (left) or rhVEGF-C (right). Mouse brain coronal sections were stained with Thioflavine S. Scale bar: 250 μm. (B) Percentages of Thioflavine S-stained plaques (plaque area/total area) in each brain region were quantified by ImageJ. (C–E) Soluble Aβ₁₋₄₀ and Aβ₁₋₄₂ peptides in brain homogenates, CSF and lymph nodes of APP/PS1 mice measured separately by Aβ enzyme-linked immunosorbent assay kits. Compared with the control group, the Aβ levels of brain samples and CSF were decreased in the rhVEGF-C group (C, D), and increased in lymph nodes (E). Data are presented as the mean ± SEM (*n* = 4 mice in B, *n* = 6 mice in C–E; two-tailed Student's *t*-test). **P* < 0.05, vs. control group. rhVEGF-C: Recombinant human vascular endothelial growth factor-C; Aβ: amyloid-β; CSF: cerebrospinal fluid.

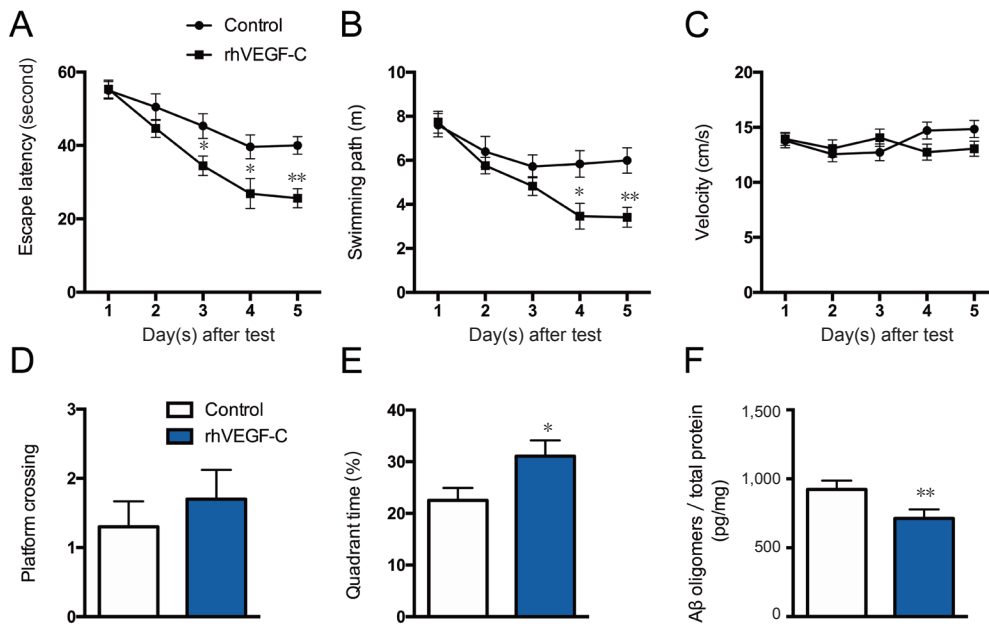
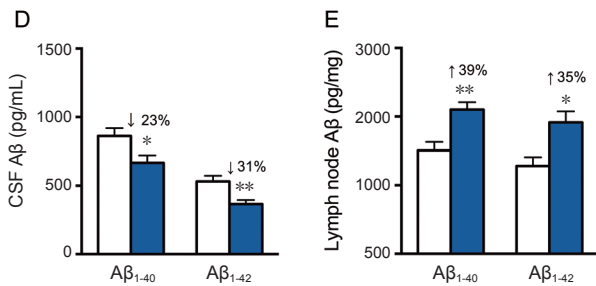


Figure 6 rhVEGF-C improved memory deficits in APP/PS1 transgenic mice.

The mean escape latency (A), swimming path (B) and mean swimming velocity of APP/PS1 transgenic mice. Two-way repeated measures analysis of variance followed by the Bonferroni *post-hoc* test; **P* < 0.05, ***P* < 0.01, vs. control group. Data show the mean number of platform area crossings (D) and time spent in the target quadrant (E) in the probe trial on day 6. Levels of Aβ oligomers were quantified by enzyme-linked immunosorbent assay kits. Data are presented as the mean ± SEM (*n* = 10 mice; two-tailed Student's *t*-test). **P* < 0.05, ***P* < 0.01, vs. control group. rhVEGF-C: Recombinant human vascular endothelial growth factor-C; Aβ: amyloid-β.

recorded using a MT-200 Morris image motion system (Chengdu Technology & Market Corp., Chengdu, China). Mice were dried and warmed after every trial.

Statistical analysis

All data, expressed as the mean ± SEM, were statistically analyzed by SPSS 21.0 software (IBM Company, Armonk, NY, USA), according to the principle of random and double-blind testing. Data from the Morris water maze test were analyzed by two-way repeated measures analysis of variance

followed by the Bonferroni *post-hoc* test. The remaining data were analyzed by two-tailed Student's *t*-test. The statistically significant level was set at α = 0.05.

Results

rhVEGF-C significantly promoted tube formation of hLECs *in vitro*

Injection of rhVEGF-C, a VEGFR3-specific recombinant protein, into the cisterna magna of C57BL/6 mice increased the mean diameter of lymphatic vessels in the dura mater

(Louveau et al., 2015). This alteration continued for no more than 2 weeks but suggested the promoting role of rhVEGF-C on dural lymphangiogenesis. We examined the effects of rhVEGF-C on tube formation by hLECs *in vitro*. Tube formation was observed 12 hours after hLEC planting on Matrigel-coated Ibidi μ -slides under normal growth conditions. The addition of rhVEGF-C significantly promoted tube formation by hLECs (Figure 2).

Elevated dural lymphangiogenesis in transgenic mice treated with rhVEGF-C

Dura from 9-month-old transgenic mice were labeled immunofluorescently to identify the dural lymphatic vessels (Lyve-1⁺/Prox-1⁺ vessels). Transgenic mice treated with rhVEGF-C showed elevated lymphangiogenesis in the dural lymphatic vessels compared with the control group. The mean diameter of the dural lymphatic vessels (Figure 3A-E), total numbers of Prox-1⁺ nuclei, total superficial area of dural lymphatic vessels and Prox-1⁺ nuclei density were significantly elevated in the rhVEGF-C group compared with controls (Figure 3A-D, F-H). These results suggest that rhVEGF-C induces lymphatic vessel remodeling in the dura mater of transgenic mice. The proportion of Lyve-1⁺ lymphatic vessels in deep cervical lymph nodes was higher in the rhVEGF-C group compared with the control group (Figure 4).

rhVEGF-C reduced A β deposition in transgenic mice

Treatment of rhVEGF-C on APP/PS1 transgenic mice was started at 9 months of age, at which point A β was overexpressed causing severe A β deposition in the CNS. The effect of treatment on plaque burden was quantified by representative confocal micrographs of brain sections (Figure 5A). However, no significant decline was detected regarding the level of A β plaque burden in the rhVEGF-C group compared with the control group (Figure 5B). Soluble A β_{1-40} and A β_{1-42} levels in the brain and CSF were quantified by ELISA and significant alterations in APP/PS1 mice treated with rhVEGF-C were observed. Importantly, levels of A β_{1-40} and A β_{1-42} in brain homogenates were 121 \pm 8 pg/mg and 161 \pm 10 pg/mg in control group, and levels were 91 \pm 9 pg/mg and 124 \pm 12 pg/mg in the injected group at 7 days after the final injection. More specifically, soluble A β_{1-40} and A β_{1-42} levels were reduced by 25% and 23%, respectively (Figure 5C). Similar results were observed in the CSF of rhVEGF-C treated mice, and soluble A β_{1-40} and A β_{1-42} levels were reduced by 23% and 31%, respectively (Figure 5D). To determine whether the decrease of A β_{1-40} and A β_{1-42} in the CNS was associated with cerebral lymphatic drainage from the brain parenchyma to deep cervical lymph nodes, the concentration of A β in deep cervical lymph nodes was measured by ELISA. Notably, A β levels in deep cervical lymph nodes were significantly increased in the rhVEGF-C group compared with the control group: soluble A β_{1-40} and A β_{1-42} levels were increased by 39% and 35% in the rhVEGF-C group, respectively (Figure 5E).

Application of rhVEGF-C reduced spatial cognition deficits in transgenic mice

The Morris water maze test was used to assess the spatial learning and memory of mice. The test consists of 5 days of hidden platform tests, followed by a probe trial 24 hours after the last test. In the hidden platform test, transgenic mice injected with rhVEGF-C showed significant improvement compared with controls. From days 3 to 5, the escape latency of rhVEGF-C treated mice declined compared with control mice, and the treated mice took less time to find the platform compared with untreated mice (Figure 6). The time spent in the third quadrant was higher in the rhVEGF-C group compared with the control group on day 6 of the test (Figure 6E).

The Morris water maze test was used to estimate hippocampus-dependent spatial learning and memory ability in transgenic mice treated with rhVEGF-C. Increased lymphangiogenesis facilitated the clearance of A β from the CNS to peripheral lymph nodes. A β oligomers are thought to result in the pathogenesis of AD. Here, a decline in the concentration of A β oligomers was detected in the brains of rhVEGF-C treated mice by ELISA (Figure 6F).

Discussion

Meningeal lymphatic vessels were described in detail in 2015 and are regarded as an important pathway for the draining of solutes from the CSF, including A β (Aspelund et al., 2015; Louveau et al., 2015). VEGF-C strongly promotes cytokines to stimulate lymphangiogenesis (Bachmann et al., 2008; Nurmi et al., 2015; Tacconi et al., 2015) and the pro-lymphangiogenesis role of rhVEGF-C was reported *in vitro* (Savetsky et al., 2015). Data from *in vivo* experiments provided direct evidence that rhVEGF-C increases dural lymphangiogenesis. Furthermore, we revealed that large amounts of A β , both soluble and insoluble, were removed from mouse brain by the administration of rhVEGF-C. Furthermore, performances in the Morris water maze were improved significantly by the administration of rhVEGF-C. A notable aspect for the lymphatic clearance of A β is how A β aggregates in the brain were transported into the dural lymphatic vessels. According to previous reports, CNS lymphatic vessels exist only in the dura, but not in the brain (Aspelund et al., 2015; Louveau et al., 2015). Previous studies showed that these tubes drain substances directly from the CSF but not from the brain (Weller et al., 2009; Aspelund et al., 2015; Louveau et al., 2015). Therefore, there is no direct lymphatic pathway for A β produced in the brain to drain into the dural lymphatic vessels. However, the glymphatic system was reported to be a pathway for A β transport from the brain to the CSF (Jessen et al., 2015). Glymphatic CSF influx may serve as a constant pathway carrying soluble A β from the brain into the CSF (Nedergaard, 2013). Indeed, this route has an important role in A β clearance and its failure might worsen AD pathology by increased A β deposition in the brain. Such failure often occurs when para-arterial deposits appear in cerebral amyloid angiopathy (Gupta

and Iadecola, 2015), and this failure always accompanies a marked decrease in the levels of soluble A β in the CSF (Yamada, 2015). In the clinic, reduced levels of soluble A β in the CSF are thought to be a risk factor and a predictor for the emergence of AD (Hoscheidt et al., 2016). In addition, dye injected into brain parenchyma can be detected in the deep cervical lymphatic nodes, indicating substances in the brain are transported through the CNS lymphatic system (Dissing-Olesen et al., 2015). Taken together, the brain-CSF-dural lymphatic vessel pathway is a useful route for the lymphatic clearance of brain-produced A β .

The failure to clear soluble A β and the subsequent deposition of insoluble A β is the core pathological process of AD (Takahashi et al., 2017). In this study, the upregulation of dural lymphangiogenesis by the administration of rh-VEGF-C during the on-setting stage of insoluble A β deposition in the brain significantly reduced the burden of senile plaques in transgenic mice. This suggests that people with a high risk for AD should receive preventive strategies to improve the lymphatic clearance of A β . However, further clinical studies to obtain direct support for this pathway in humans are required.

This study had some limitations. The effects of rhVEGF-C in wild-type animals were not assessed in this study. Moreover, whether a preventive effect of rhVEGF-C on A β -related pathology can be found if it is administered to young APP/PS-1 transgenic mice is unknown. However, data from 9-month-old APP/PS-1 transgenic mice were sufficient to support the conclusion of the present study.

In addition to the drainage of cellular and large molecular components from interstitial fluid or CSF (Murlidharan and Asokan, 2016), lymphatic vessels regulate local immune activity, including immune cell recruitment and activation (Lund et al., 2016). Therefore, the regulation of dural lymphatic vessels might have other potential effects in modifying immune microenvironments in the dura and CSF, which might have complex implications concerning immune activity in the brain. This is a new field of great significance to explore in the future.

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Author contributions: ZBY and YRW designed this study. YRW performed primarily cell culture, animal treatments and immunofluorescence, as well as sample preparation. JHY performed primarily morphological observations and ELISA. XW performed primarily behavioral test. JHY, YRW and XW performed data analyses (blinded to groups). ZBY, YRW and JHY wrote the paper. All authors approved the final version of the paper.

Conflicts of interest: The authors have no conflicts of interest to declare.

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Research ethics: The study was approved by the Institute Research Ethics Committee of Sun Yat-Sen University of China (approval No. 2017-002) and performed in strict accordance with the U.K. Animals (Scientific Procedures) Act, 1986.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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