Nasal BCG Exposure Accelerates Dural Lymphatics Development via Macrophages’ Role in Newborn Mice

Junhua Yang (ORCID: 0000-0001-8037-9644)^{1#}, Lifang Yuan(ORCID: 0000-0002-6315-2824)^{2,#}, Linyang Song(ORCID: 0000-0002-2770-9568)¹, Fangfang Qi³, Zejie Zuo³, Jie Xu³, Zhibin Yao(ORCID: 0000-0003-1613-9157)^{3,*}

¹ Department of Anatomy, School of Biosciences & Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou 510006, PR China.

² School of Public Health (Shenzhen), Sun Yat-sen University, Shenzhen 510275, PR China.

³ Department of Anatomy and Neurobiology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, PR China.

[#]Junhua Yang and Lifang Yuan contributed equally to this study and share first authorship.

*Corresponding author. Fax: +862087330709. E-mail address: yao.zb@163.com (Zhibin Yao).

20   **Abstract**

21   The dural lymphatics develop mainly during the first postnatal month. Lymphatics
 22   may be shaped by immune activation when bacterial infection happens. BCG, a strong
 23   immune activator, is widely injected to newborns. Moreover, human beings are
 24   nasally exposed in daily life to bacterial stimuli. These background prompted us to
 25   investigate whether neonatal BCG injection combined with nasally exposure exerts an
 26   influence on dural lymphatics develop. Here, mice received a single dose
 27   intracutaneous (i.c.) BCG injection immediately after birth followed by repeated nasal
 28   BCG challenge once a day (i.c./nasal group). These mice had an accelerated dural
 29   lymphatics growth and increased levels of several cytokines and VEGFR-3.
 30   Furthermore, macrophages were identified as a key mediator of these alterations.
 31   Mice that received mere one dose i.c. BCG injection showed no significant alterations
 32   in these indexes. Additionally, mere repeated nasal BCG challenge induced similar
 33   effects to i.c./nasal challenge but with a slighter extent. Taken together, these findings
 34   show that repeated nasal BCG vaccination accelerates dural lymphatics development
 35   in neonatal mice, especially in the presence of neonatal i.c. BCG injection.

36   **Keywords:** Meningeal lymphatic vessels; Development; Vaccine; Central nervous
 37   system; Mucosal immunity

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41 Introduction

42 Since two publications in 2015 describing the structure and function of the dural
 43 lymphatics (1, 2), these vessels have drawn increasing attention. A series of researches
 44 as well as review articles focused on them (3, 4). In 2017, Salli Antila et al. reported
 45 the development and plasticity of dural lymphatics in mice, revealing that the dural
 46 lymphatics network starts to develop after birth and they were already well developed
 47 at about four weeks old (5). As well known, lymphatics may be shaped by
 48 inflammatory factors produced in immune activation. It has been reported that
 49 immune activation induced by bacterial or virus infection led to lymphangiogenesis
 50 outside the central nervous system (6-8). BCG is a live attenuated vaccine containing
 51 live *Mycobacterium tuberculosis* and is widely used to newborns. Planned BCG
 52 vaccination is a strong and unnatural neonatal immune stimuli. About 90 percent of
 53 infants of the world were administrated neonatally with BCG (9).

54 We here imitated the immune challenge by BCG that happens to human infants, by
 55 means of a single dose i.c. BCG injection immediately after birth. Moreover, we also
 56 imitated the daily nasal exposure to microbiota that exist in the air by repeated nasal
 57 challenge with BCG performed once a day within the first 14, 19 or 21 postnatal days.
 58 For the nasal immune challenge with bacteria, BCG was used for three reasons: 1)
 59 this constitutes a boosting immunization when combined with neonatal i.c. BCG
 60 injection in spite of a different exposure pathway, 2) in the long period of human
 61 evolution and survival, *Mycobacterium tuberculosis*, as a kind of bacteria mainly
 62 transmitted through the air, has been exposed to almost all human individuals (10), 3)

63 despite advances in modern medicine, one third of the world's population is estimated
64 to have contracted the bacteria, with estimates of ten million new infections globally
65 each year (11). Meanwhile, mice receiving PBS, mere one dose i.c. neonatal BCG
66 injection or mere repeated nasal BCG challenge were also prepared and observed. We
67 found that neonatal BCG vaccination accelerates lymphatics development in mice,
68 especially in the presence of neonatal i.c. BCG exposure.

69

70 **Materials and methods**

71 **Animals and study design**

72 Newborn litters of C57Bl/6 mice were obtained from the SYSU Laboratory Animal
73 Center (Guangzhou, China) and were housed in a specific pathogen-free facility. This
74 study consisted of different tests, each of which used four groups of mice, namely
75 con-group, intracutaneous(i.c.)-group, nasal-group and i.c./nasal-group. All
76 experiments in the study were started on postnatal day 0 (P0).

77 For each test, six newborn litters of C57Bl/6 mice were used. Within each litter, four
78 male pups were used and randomly divided into four groups, receiving i.c. PBS
79 injection combined with nasal PBS administration (con-group), i.c. BCG injection
80 combined with nasal PBS administration (i.c.-group), i.c. PBS injection combined
81 with nasal BCG vaccination (nasal-group) or i.c. BCG injection combined with nasal
82 BCG vaccination (i.c./nasal-group). The pups that were sacrificed after P21 were
83 weaned on P21. The study was approved by the SYSU Institute Research Ethics

84 Committee and all animal experiments were conducted in compliance with the
85 relevant laws and institutional guidelines.

86 **Immunization procedures**

87 A single dose of BCG was administered (i.c.) to the mice in i.c.-group and
88 i.c./nasal-group in the back on P0. For nasal-group and i.c./nasal-group, each mouse
89 received nasal administration of BCG once a day from P0 to P14, P19 or P21.
90 Freeze-dried living BCG (D2-BP302 strain, Biological Institute of Shanghai, China)
91 was suspended in PBS. The dose both of i.c. injection and nasal administration is
92 about 10^5 CFU.

93 **Immunofluorescence staining and quantification**

94 To test the proliferating cells in dural lymphatics, the mice received five
95 intraperitoneal injections of BrdU (Sigma-Aldrich, 50 mg/kg) from P14 once a day to
96 label the dividing cells. At P19, the animals were deeply anesthetized with chloral
97 hydrate (10% solution) and then transcardially perfused with PBS. After removing the
98 mandibles and the skull rostral to the maxillae, the skull cap and the attached
99 dura/arachnoid containing superior sagittal sinus (SSS) and two transverse sinus (TS)
100 areas were removed with surgical scissors. The dura mater tissue used for all the other
101 tests were obtained from mice at P14 or P21 that were not treated with BrdU. Whole
102 mounted meninges that were still attached to the skull cap were placed in 4%
103 paraformaldehyde (PFA) in PBS and postfixed for 24 h at 4°C. The dura/arachnoid
104 were then carefully and gently separated from the skull cap using tweezers with a thin
105 head. Actually, the adhesion between dura mater and calvaria is relatively loose in

106 young mice, and therefore it is easy to keep them intact during peeling from calvaria.

107 The dura were then washed with PBS three times and processed for staining. The dura

108 were blocked in PBS containing 1% bovine serum albumin and 0.25% Triton X-100

109 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C before being incubated with

110 primary antibodies diluted in the same blocking solution overnight at 4°C. The

111 primary antibodies were as follows: goat anti-Lyve-1 (R&D Systems, Minneapolis,

112 MN, USA; 1:600), rabbit anti-Prox-1 (Abcam, Cambridge, MA, USA; 1:500), rat

113 anti-BrdU (Santa Cruz, CA, USA; 1:500), goat anti-VEGFR-3 (R&D Systems,

114 Minneapolis, MN, USA; 1:50) and rat anti-F4/80 (Abcam, Cambridge, MA, USA;

115 1:200). Specimens were washed three times with PBS before being incubated with the

116 following secondary antibodies for 2 h at 37°C: Alexa Fluor 555-conjugated donkey

117 anti-goat, Alexa Fluor 647-conjugated donkey anti-rat and Alexa Fluor

118 488-conjugated donkey anti-rabbit antibodies. All secondary antibodies (Invitrogen

119 Molecular Probes®, Eugene, Oreg., USA) were diluted 1:400. Each of the dura

120 samples was then placed on a glass slide with the skull face attached on the slide. This

121 process was performed in PBS to ensure that the SSS area and two TS areas stretched

122 without folding.

123 Confocal micrographs of immunofluorescence staining were obtained using a LSM

124 780 confocal laser-scanning microscope (Zeiss, Heidelberg, Germany). The total

125 numbers of each kind of labeled cells showed in Results section within the selected

126 areas were quantified using a Stereo Investigator (MicroBrightField, Williston, USA)

127 stereology system by an investigator who was blinded to the groups.

128 **Multiplex assay**

129 Mice at P21 were anesthetized with chloral hydrate (10% solution). Then, 5-7 μ l of
 130 cerebrospinal fluid (CSF) were obtained from the cisterna magna of each mouse,
 131 according to a previously described protocol (12). The mouse cytokine/chemokine
 132 panel (MCYTOMAG-70K-04; Millipore, Billerica, MA, USA) and bead-based
 133 Luminex system were used to detect the concentrations of IFN- γ , IL-4, TNF- α , IL-7,
 134 IL-17, IL-1 β , IL-6, IL-10 and monocyte chemotactic protein-1 (MCP-1) in the CSF.
 135 We first added 25 μ l of prepared CSF samples that had previously been diluted 1:5 in
 136 assay buffer to each well. Then, 25 μ l of the magnetic beads coated with specific
 137 antibodies were added, and the mixture was incubated overnight at 4°C. Then, the
 138 beads were washed and incubated with 25 μ l of the biotinylated detection antibody for
 139 1 h at room temperature (RT). Afterwards, 25 μ l of the streptavidin-phycoerythrin
 140 conjugate were added, and the reaction was incubated for 30 min at RT. The beads
 141 were then washed with 150 μ l of sheath fluid for 5 min at RT. Data were collected on
 142 a Bio-Plex-200 system (Bio-Rad, Hercules, CA, USA) and analyzed using
 143 professional software (Bio-Plex Manager; Bio-Rad, CA, USA).

144 **ELISA**

145 Mice at P21 were anesthetized with chloral hydrate (10% solution). Then, 5-7 μ l of
 146 CSF were obtained from the cisterna magna of each mouse, according to a previously
 147 described protocol (12). VEGF-C levels were assayed in the CSF of mice using an
 148 ELISA kit (VEGF-C, Cusabio, China), according to the manufacturer's instruction.

149   **Western blot**

150   At P21, levels of the VEGFR-3 proteins in the dura were examined by western blot
151   analyses. After the animals were anesthetized and perfused with PBS, the skull cap
152   and the attached dura/arachnoid were obtained. Before and after the dura/arachnoid
153   were separated from the skull cap, the tissues were washed three times with ice-cold
154   PBS to remove the CSF and intercellular fluid that may have adhered to the meninges.
155   Then, the dura/arachnoid were homogenized in ice-cold RIPA buffer. The homogenate
156   was centrifuged at 9,000 g for 15 minutes at 4°C, after which the supernatant was
157   analyzed by western blotting. Protein concentrations were quantified using the
158   bicinchoninic acid (BCA) protein assay kit (Biotime, China). Membrane homogenates
159   (50 µg) were separated on 6% SDS-PAGE gels and transferred onto presoaked PVDF
160   membranes (Bio-Rad, Pleasanton, CA, USA). Blots were then blocked with 10%
161   non-fat milk in PBST (100 mM phosphate buffer, pH 7.5, containing 150 mM NaCl
162   and 0.1% Tween-20) for 2 h at RT. The the membranes were incubated overnight at
163   4°C with rabbit anti-VEGFR-3 antibodies (Abcam, Cambridge, MA, USA; 1:50).
164   Horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibodies
165   (Sigma-Aldrich Co., USA) were used at a dilution of 1:4000. Protein bands were
166   visualized using a chemiluminescence detection system (Pierce, Milan, Italy). Optical
167   densities were measured by dividing the intensity of the bands by the intensity of the
168   housekeeping protein β -actin (Cell Signaling Technology, MA, USA, 1:1000). Data
169   from control samples were assigned a relative value of 100%.

170

171 Statistical analyses

172 All data were statistically analyzed using the Statistical Package for the Social
173 Sciences 25.0. The data were analyzed using randomly completely block design
174 ANOVA followed by Bonferroni's *post hoc* test or paired-samples *t* test. The
175 statistically significant level was set at $\alpha = 0.05$. The normality and homogeneity of
176 variance were conducted using the Shapiro-Wilk test and Levene's test, respectively.

177

178 Results

179 Accelerated dural lymphatics development in nasal-group and i.c./nasal-group in

180 TS zone at P14

181 At P14, lymphatics distribution around the TS was observed by
182 immunofluorescencely staining for Lyve-1. Lyve-1⁺ vessels covered about 59%, 63%,
183 85% and 100% of the length of ipsilateral TS in con-group, i.c.-group, nasal-group
184 and i.c./nasal-group, respectively (Fig.1). Compared with con-group, both nasal-group
185 and i.c./nasal-group had a significant increase in the length of TS covered by Lyve-1⁺
186 vessels, Lyve-1⁺ vessels area, numbers of sprouting from Lyve-1⁺ vessels and
187 numbers of cross points of Lyve-1⁺ vessels (Fig.1). In all these four indexes, the
188 i.c./nasal-group possessed a significant larger extent of increase than the nasal-group
189 (Fig.1). Moreover, the i.c./nasal-group showed obvious sprouting and extending of
190 lymphatics from the TS zone toward both rostral (upward) and caudal side (downward)
191 in the dura mater (Fig.1D). All these findings indicated accelerated dural lymphatics

192 development by neonatal nasal exposure to BCG.

193 **Accelerated dural lymphatics development with more macrophage-derived**
 194 **lymphatic endothelial cell progenitors (M-LECPs) in SSS zone in nasal-group**
 195 **and i.c./nasal-group at P21**

196 Macrophages (F4/80⁺) play a vital role in lymphangiogenesis induced by immune
 197 stimuli because of their capacity to transform into lymphatic endothelial progenitors
 198 (13, 14). F4/80⁺/Lyve-1⁺ cells, F4/80⁺/Prox-1⁺ cells and F4/80⁺/Lyve-1⁺/Prox-1⁺ cells
 199 have previously been reported as the macrophage-derived LEC progenitors (13, 14).

200 In the current study, lymphatics distribution and macrophages presence around the
 201 SSS was observed after the dura mater was immunofluorescently triple-stained for
 202 Lyve-1/Prox-1/F4/80 at P21. Lyve-1⁺ vessels covered about 30%, 50%, 70% and 100%
 203 of the length of SSS in con-group, i.c.-group, nasal-group and i.c./nasal-group,
 204 respectively (Fig.2). Compared with con-group, both nasal-group and i.c./nasal-group
 205 had a significant increase in Lyve-1⁺ vessels area%, numbers of sprouting from
 206 Lyve-1⁺ vessels and Prox-1⁺ nucleus numbers (Fig.2). In all these three indexes, the
 207 i.c./nasal-group possessed a significant larger extent of increase than the nasal-group
 208 (Fig.2). These indexes also indicated accelerated dural lymphatics development by
 209 BCG, especially considering the fact that lymphatics begin to cover 100% of the
 210 length of SSS at P28 under normal condition (5).

211 Compared with con-group, both nasal-group and i.c./nasal-group had a significant
 212 increase in the number of total F4/80⁺ cells (Fig.2H), F4/80⁺/Prox-1⁺ cells and

213 F4/80⁺/Prox-1⁺/Lyve-1⁺ cells (Fig.3M-O). In all these three indexes, the
214 i.c./nasal-group possessed a significant larger extent of increase than the nasal-group
215 (Fig.2H) (Fig.3M-O). In addition, M-LECPs in different stages were found, indicating
216 by different features of phenotype and location to lymphatics (See detailed description
217 in Fig.4 and the corresponding legend).

218 **Elevated real time lymphangiogenesis level in SSS zone in nasal-group and** 219 **i.c./nasal-group at P19**

220 The real time lymphangiogenesis level was also evaluated by
221 immunofluorescencely co-staining for Lyve-1/Prox-1/BrdU at P19 and the SSS zone
222 of dura mater was observed. Fig.S1A-D shows whole length graph of SSS area from
223 which representative higher scopes were presented in Fig.5. As expected, both
224 nasal-group and i.c./nasal-group had a significant increase in BrdU⁺ cells (Fig. S1F),
225 BrdU⁺/Prox-1⁺ (Fig.5M) and BrdU⁺/Prox-1⁺/Lyve-1⁺ (Fig.5N) cells within the entire
226 area of SSS compared with con-group besides the increased indexes that reflecting the
227 accelerated dural lymphatics development (Fig. S1E, G-H). In both indexes, the
228 i.c./nasal-group possessed a significant larger extent of increase than the nasal-group.

229 **Pro-lymphangiogenesis and macrophage-recruiting profile of cytokine expression** 230 **in the CSF from mice in nasal-group and i.c./nasal-group at P21**

231 Lymphangiogenesis is a dynamic process that is regulated also by other cytokines,
232 such as vascular endothelial growth factor-C (VEGF-C), monocyte chemotactic
233 protein-1 (MCP-1), interleukin-1 β (IL-1 β), IL-4, IL-6, IL-7, IL-10, IL-17, interferon- γ

(IFN- γ), and transforming growth factor- β (TGF- β) (6, 8, 15-20). Therefore, we determined their concentrations in the CSF and serum of each group of mice. In the CSF, both nasal-group and i.c./nasal-group had a significant increase in the level of VEGF-C, MCP-1, IFN- γ and TGF- β compared with con-group (Fig.6). The i.c./nasal-group possessed a significant larger extent of increase than the nasal-group (Fig.6). The other tested cytokines in the CSF were of no significant differences among groups or undetectable in all groups. In serum, both nasal-group and i.c./nasal-group had a significant increase in the level of MCP-1, IFN- γ , TGF- β , IL-4 and IL-10 compared with con-group (Fig.7). The i.c./nasal-group possessed a significant larger extent of increase than the nasal-group (Fig.7). The other tested cytokines in serum were of no significant differences among groups or undetectable in all groups.

Besides, the level of VEGFR-3, the receptor for VEGF-C and mainly expressed by lymphatics, was also detected in dura mater because of its vital role in lymphangiogenesis (21, 22). WB analysis showed both nasal-group and i.c./nasal-group had a significant increase in VEGFR-3 expression (Fig.8). In both indexes, the i.c./nasal-group possessed a significant larger extent of increase than the nasal-group (Fig.8).

Macrophage identified as a mediator of BCG-induced promotion in dural lymphatics development.

VEGF-C, mainly secreted by macrophages, is the most important cytokine for promoting lymphangiogenesis (16, 17, 23). MCP-1 is the most potential chemokine

for recruiting macrophages (24). Therefore, the increased levels of them in the CSF in nasal-group and i.c./nasal-group may explain the larger number of macrophages counting in nasal-group and i.c./nasal-group as shown in Fig.2H, Fig.3 and Fig. S1.

To further confirm the mediating role of macrophages in BCG-induced promotion in dural lymphatics development, each group of mice were intraperitoneally injected with a macrophage-depleting agent clodronate liposomes (110 mg/kg, once every three days). At P28, dura mater were stained for Lyve-1⁺ vessels. The mean Lyve-1⁺ vessels area in SSS zone in CL/con-group (Fig.S2D) is less than that of con-group at P21 (Fig.2A, Fig. S1A). Moreover, there is no longer any significant difference among four groups in either Lyve-1⁺ vessels area in SSS zone or in the level of VEGF-C in CSF after macrophage-depletion (Fig.S2). What's more, rare Lyve-1⁺ cells out of Lyve-1⁺ vessels could be observed in dura mater when macrophages were depleted.

269

270 Discussion

This study for the first time revealed that repeated neonatal BCG vaccination, either combined with or without i.c. injection of BCG, accelerated dural lymphatics development in mice. Furthermore, macrophages were identified as the mediator of BCG-induced dural lymphatics development acceleration.

A notable phenomenon found in this study is that a single dose of BCG i.c. injection resulted in no significant influence in dural lymphatics development at P21.

277 This may be because of the lack of boosting immunization. The possible role of
278 boosting immunization was also supported by the fact that the i.c./nasal-group
279 consistently possessed a significant larger extent of alteration than the nasal-group in
280 nearly all indexes. The present study aimed mainly to explore the potential influence
281 of the immune activation pattern ‘neonatal i.c. injection/nasal immunization of
282 *mycobacterium tuberculosis*’ that happens to people worldwide. Therefore, boosting
283 immunization by repeating i.c. injection of BCG was not attempted in this study.

284 BCG immunization could not only induce Th1 cytokines production such as IFN- γ
285 (25) but also activate macrophages (26). This was confirmed by the results of
286 cytokines analysis in this study. Macrophages is the major source of VEGF-C protein
287 (16). In the nasal-group and i.c./nasal-group, the elevated VEGF-C levels in the CSF
288 (Fig.6A) may be explained by the increased numbers of macrophages in the dura
289 mater. In turn, the larger number of recruited macrophages in the dura mater may be
290 explained by the higher CSF level of MCP-1 that is a very potential chemokine for
291 monocyte/macrophages (27).

292 It is reported that BCG could induce systemic release of MCP-1 (28). However,
293 although the base level of MCP-1 both in serum and CSF indicated by the data from
294 con-group (Fig.6B and Fig.7A), the CSF MCP-1 level was about twice of its serum
295 level in nasal-group and i.c./nasal-group. This suggests that the MCP-1 in the CSF
296 may not derive from circulating blood, but rather produced by local cells. Activated
297 microglia was verified to serve as a possible producer of MCP-1 (29). Neonatal BCG
298 immunization has been shown to activate microglia (25, 30). This deduction provides

299 with an interest cue for future research to investigate the exactly mechanism under the
300 role of BCG in increasing the CSF level of MCP-1.

301 According to the previous report, the frontiers of the growth point of dural
302 lymphatics reach the scalp of skull such as the lateral parts of TS area at about two
303 weeks old (5). Given that the nasal BCG vaccination lasted for two weeks or more,
304 the dura mater of TS and SSS area, not skull base, was chosen for dural lymphatics
305 analysis. Moreover, the complex anatomical structure of the base of skull and the tight
306 attaching of dura mater there would make it difficult to analyze the lymphatics
307 precisely. It is similar with the part of dura mater overlapping attached to the frontal
308 bone overlying the olfactory bulb. In addition, the part of dura mater attached to the
309 scalp of skull is easy to be isolated from bone.

310 The dural lymphatics begin to cover the whole length area along the SSS but are
311 often discontinuous even in adult mice as reported previously (5). These vessels in
312 mice at P21 were seen in about 1/4 to 1/3 of the whole SSS length in con-group mice
313 (Fig. 2A). At P21, mere nasal BCG vaccination mildly increased the dural lymphatics
314 whereas mere neonatal i.c. BCG injection brought no significant effects. Both the
315 nasal-group and i.c./nasal-group showed an advanced development of the dural
316 lymphatics at P21, with their dural lymphatics distribution reaching the level
317 comparable with those in normal adult mice. This advanced development during the
318 critical period for CNS development is very likely to allow an advanced and more
319 effective drainage of CSF and thereby to produce an affect on brain development.

320 In natural condition, the microbiota to which human beings nasally expose are
321 certainly different from nasal BCG vaccination used in this study both in kind and in
322 dosage. Admittedly, it is not proper to estimate the status of dural lymphatics
323 development in human beings straightly according to the findings in this study
324 especially given that it is not clear whether the dural lymphatics development in
325 perinatal period differs in human beings from that in mice. What is vital and novel in
326 this study is that nasal BCG vaccination does promote dural lymphatics development.
327 Even if this promotion means only a matter of an advanced development or an earlier
328 coming of matured dural lymphatics, rather than a long-lasting elevation of
329 lymphatics structure and function, it is still important for brain development since it
330 occurs during the the early life period when is critical for CNS development
331 programming. In other words, even a transient promoted/advanced dural lymphatics
332 development is likely to exert a long-lasting effects on brain development and
333 function. In addition, the protocol used in this work to immunize mice may serve as
334 an animal model for experiments studying dural lymphatics-related issues.

335

336 **Authors' contributions**

337 Study design: Junhua Yang and Zhibin Yao. Performing the experiments: Junhua
338 Yang (primarily morphological observations and ELISA), Lifang Yuan (primarily
339 animal treatments and grouping, morphological observations and ELISA), Linyang
340 Song (primarily multiplex assays and western blotting). Data analyses (blinded to

341 groups): Junhua Yang. Writing the paper: Junhua Yang. Funding acquisition: Junhua
 342 Yang, Zejie Zuo and Zhibin Yao. Fangfang Qi and Jie Xu participated in discussions,
 343 assisted with data analysis, technical instructions and manuscript editing.
 344 Conceptualization, Supervision All authors read, commented and approved the final
 345 manuscript.

346

347 **Conflict of interest**

348 The authors declare that they have no conflict of interest.

349

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437 **Figure legends:**

438 **Fig.1 Accelerated dural lymphatics development in nasal-group and**

439 **i.c./nasal-group mice at P14.** (A-D) Representative graphs show the differences in

440 dLVs within the corresponding TS areas of the dura in each group of mice. Scale bar,

441 200 μm . (E-H) Bars represent the length of TS covered by Lyve-1⁺ vessels, Lyve-1⁺

442 vessels area%, numbers of sprouting from Lyve-1⁺ vessels and numbers of cross

443 points of Lyve-1⁺ vessels dLVs within the unilateral TS area. $n = 6$ mice/group. *: $p <$

444 0.05; **: $p < 0.01$; ***: $p < 0.001$. Randomly completely block design ANOVA

445 followed by Bonferroni's *post hoc* test. Data are presented as the mean $\pm$ SEM.

446 **Fig.2 Accelerated dural lymphatics development with more M-LECPs in SSS**

447 **zone in nasal-group and i.c./nasal-group at P21.** (A-D) Representative graphs show

448 the differences in dLVs distribution and macrophages number within the

449 corresponding SSS areas of the dura in each group of mice. Scale bar, 400 μm . (E-H)

450 Bars represent Lyve-1⁺ vessels area, numbers of sprouting from Lyve-1⁺ vessels,

451 Prox-1⁺ nucleus numbers and the number of total F4/80⁺ cells. $n = 6$ mice/group. *: p

452 < 0.05 ; **: $p < 0.01$; ***: $p < 0.001$. Randomly completely block design ANOVA

453 followed by Bonferroni's *post hoc* test. Data are presented as the mean $\pm$ SEM.

454 **Fig.3 Selected representative scopes from graphs in Fig.2A-D, showing dural**

455 **lymphatics and M-LECPs presence in SSS zone in nasal-group and**

456 **i.c./nasal-group at P21.** (A-D) Selected representative scopes that were next to the

457 sinus confluence (the left end) of each SSS graph in Fig.2A-D. Scale bar, 100 μm .

(E-H) Selected representative scopes that were located at the middle of each SSS graph in Fig.2A-D. Scale bar, 100 μ m. (I-L) Representative high-magnified scopes showing co-labelled Lyve-1⁺ (white), Prox-1⁺ (green) and F4/80⁺ (red) signals. (M-N) Bars represent the numbers of F4/80⁺/Prox-1⁺ cells and F4/80⁺/Prox-1⁺/Lyve-1⁺ cells within the whole SSS area of each group. (O) The ratio of F4/80⁺/Prox-1⁺/Lyve-1⁺ cells numbers to F4/80⁺/Prox-1⁺ cells numbers within the whole SSS area of each group. $n = 6$ mice/group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Randomly completely block design ANOVA followed by Bonferroni's *post hoc* test. Data are presented as the mean $\pm$ SEM.

Fig.4 Selected representative scopes showing the various stages of M-LECPs in their transformation into dural lymphatics in SSS zone. (A-B) The same representative scope showing co-labelled Lyve-1⁺ (white), Prox-1⁺ (green) and F4/80⁺ (red) signals. (C) Another representative scope showing co-labelled Lyve-1⁺ (white), Prox-1⁺ (green) and F4/80⁺ (red) signals. (D) Another representative scope showing co-labelled Lyve-1⁺ (purple), Prox-1⁺ (green) and F4/80⁺ (red) signals.

Fig.5 Selected representative scopes from graphs in Fig. S1A-D, showing the elevated real time lymphangiogenesis level in SSS zone in nasal-group and i.c./nasal-group. (A-D) Selected representative scopes that were next to the sinus confluence of each SSS graph in Fig. S1A-D. Scale bar, 50 μ m. (E-H) Selected representative scopes that were located at the middle of each SSS graph in Fig. S1A-D. Scale bar, 50 μ m. (I-L) Representative high-magnified scopes showing co-labelled Lyve-1⁺ (white), Prox-1⁺ (green) and BrdU⁺ (red) signals. (M-N) Bars

480 represent the numbers of BrdU⁺/Prox-1⁺ cells and BrdU⁺/Prox-1⁺/Lyve-1⁺ cells within
481 the whole SSS area of each group. $n = 6$ mice/group. (O) The ratio of
482 BrdU⁺/Prox-1⁺/Lyve-1⁺ cells numbers to BrdU⁺/Prox-1⁺ cells numbers within the
483 whole SSS area of each group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Randomly
484 completely block design ANOVA followed by Bonferroni's *post hoc* test. Data are
485 presented as the mean $\pm$ SEM.

486 **Fig.6 Elevated cytokine levels in CSF in nasal-group and i.c./nasal-group at P21**

487 (A-B) Bars represent cytokines levels. $n = 6$ mice/group. **: $p < 0.01$; ***: $p < 0.001$.
488 Randomly completely block design ANOVA followed by Bonferroni's *post hoc* test.
489 (C-D) Representative bars represent cytokines levels. $n = 6$ mice/group. nd indicates
490 undetectable levels of one cytokine in certain group, such as con-group; ***: $p <$
491 0.001 . Paired-samples t test between nasal-group and i.c./nasal-group. Data are
492 presented as the mean $\pm$ SEM.

493 **Fig.7 Elevated cytokine levels in serum in nasal-group and i.c./nasal-group at**

494 **P21** (A-B) Bars represent cytokines levels. $n = 6$ mice/group. *: $p < 0.05$; **: $p < 0.01$;
495 ***: $p < 0.001$. Randomly completely block design ANOVA followed by Bonferroni's
496 *post hoc* test. Data are presented as the mean $\pm$ SEM.

497 **Fig.8 Increased VEGFR-3 protein level in dura mater of the mice in nasal-group**

498 **and i.c./nasal-group.** (A) Representative western blots showing VEGFR-3 protein
499 level in dura mater of each group. (B) Quantitative data showing alteration in the
500 relative levels of the VEGFR-3 protein in the dura mater. $n = 6$ mice/group. *: $p <$

501 0.05; **: $p < 0.01$; ***: $p < 0.001$. Randomly completely block design ANOVA
502 followed by Bonferroni's *post hoc* test. Data are presented as the mean $\pm$ SEM. (C)
503 Representative graphs show VEGFR-3 expression in dLVs. Scale bar, 50 μm .

Prox-1/F4/80/Lyve-1

● con ● i.c. BCG ● nasal BCG ● i.c./nasal BCG

● con ● i.c. BCG ● nasal BCG ● i.c./nasal BCG Prox-1/F4/80/Lyve-1

● con ● i.c. BCG ● nasal BCG ● i.c./nasal BCG

A

B

C

D

A **B** **C**