The role of the meningeal lymphatic system in local inflammation and trigeminal nociception implicated in migraine pain

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Abstract

Background: A system of lymphatic vessels has been recently characterized in the meninges, with a postulated role in 'cleaning' of the brain via cerebro-spinal fluid drainage. As meninges are an important tissue involved in the origin of migraine pain, we hypothesized that malfunctioning of a 'cleaning' lymphatic system should affect the functional properties of meningeal nociception. To test this hypothesis, we studied migraine-related nociceptive and inflammatory mechanisms in the meninges, in a mouse model of primary lymphedema (K14-VEGFR3-Ig mice) characterized by the lack of functional meningeal lymphatic system.

Methods: To study the migraine-related nociceptive and inflammatory mechanisms we recorded spiking activity from meningeal fibers of the trigeminal nerve, estimated the local mast cells infiltration, calcitonin gene-related peptide (CGRP) and cytokine levels (basal and under stimulating conditions), as well as the dura mater innervation in freshly-isolated hemiskull preparations from K14-VEGFR3-Ig (K14) or wild type C57BL/6 mice (WT).

Results: We found that the level of CGRP and the production of TNFα, which both are implicated in migraine, were reduced in meninges of K14 mice. There was a trend of having a larger number of dural mast cells, consistent with the increased level of the mast cell activator MCP-1 found in these animals. In addition, we found an increased spontaneous and ATP-induced nociceptive firing in the meningeal afferents of mice lacking meningeal lymphatic system. However, the patterns of trigeminal innervation in meninges remained unchanged.

Conclusions: In summary, the lack of meningeal lymphatic system leads to a new balance between pro- and anti-inflammatory mechanisms implicated in peripheral nociception, affecting key cellular and humoral factors implicated in migraine.

Keywords

Meningeal lymphatic, neuroinflammation, migraine, pain

Abbreviations:

WT – wild type mice

K14 – K14-VEGFR3-Ig mice

mLVs – meningeal lymphatic vessels

1. Introduction

Migraine is a complex neurological disorder, which originates from the meninges (Bolay et al., 2002; Olesen et al., 2009). Meninges are densely innervated by somatic trigeminal (Ramachandran, 2018; Zakharov et al., 2015) and parasympathetic innervation (Delépine and Aubineau, 1997), which runs along and in contact with meningeal blood vessels (Ebersberger et al., 2006). It has been proposed that so-called sterile neurogenic inflammation (Moskowitz, 1993, 1984; Moskowitz et al., 1979) in the meninges is an important initiator of the pathophysiology of migraine. The initial step in neurogenic inflammation is the activation of the trigeminal nociceptive system in meninges (Moskowitz, 1993). Indeed, trigeminal neurons release neuropeptide calcitonin gene-related peptide (CGRP), which activates dural mast cells (Ottosson and Edvinsson, 1997), promotes sensitization of trigeminal afferents (Cady et al., 2011; Giniatullin et al., 2008), and induces local vasodilation (Goadsby et al., 1988). Vasodilation, in turn, activates mechanosensitive receptors expressed in trigeminal nerves in meninges (Mikhailov et al., 2019). Trigeminal neurons can also be activated by mast cells-secreted serotonin (Kilinc et al., 2017), and by extracellular ATP, which is released from endothelial and mast cells, as well as from neurons, upon stimulation of the migraine mediator CGRP (Koroleva et al., 2019; Suleimanova et al., 2020; Yegutkin et al., 2016). In addition, endothelial (Loberg et al., 2006) and mast cells (Mukai et al., 2018) release various pro-inflammatory cytokines as potential contributors to local inflammation. Moreover, our previous data show that acetylcholine (ACh, known to be released from parasympathetic fibers) can activate trigeminal afferents and induce meningeal mast cell degranulation (Shelukhina et al., 2017).

The presence of these diverse endogenous agents in the meninges suggests the existence of regulatory mechanisms modulating the local levels of these, mostly pro-inflammatory compounds in meninges

in order to prevent potential inflammation and minimize the activity of nociceptive fibers. In analogy with the suggested role of the recently characterized meningeal lymphatic system (Antila et al., 2017; Aspelund et al., 2015; Louveau et al., 2015) **(Figure 1)** in the clearance of the CNS from toxic molecules in neurodegenerative diseases such as Alzheimer's disease, epilepsy, stroke and TBI (Kwon et al., 2019; Noé and Marchi, 2019; Patel et al., 2019; Rasmussen et al., 2018; Tamura et al., 2019), we hypothesized that the meningeal lymphatic system plays a similar protective/cleaning role also in migraine.

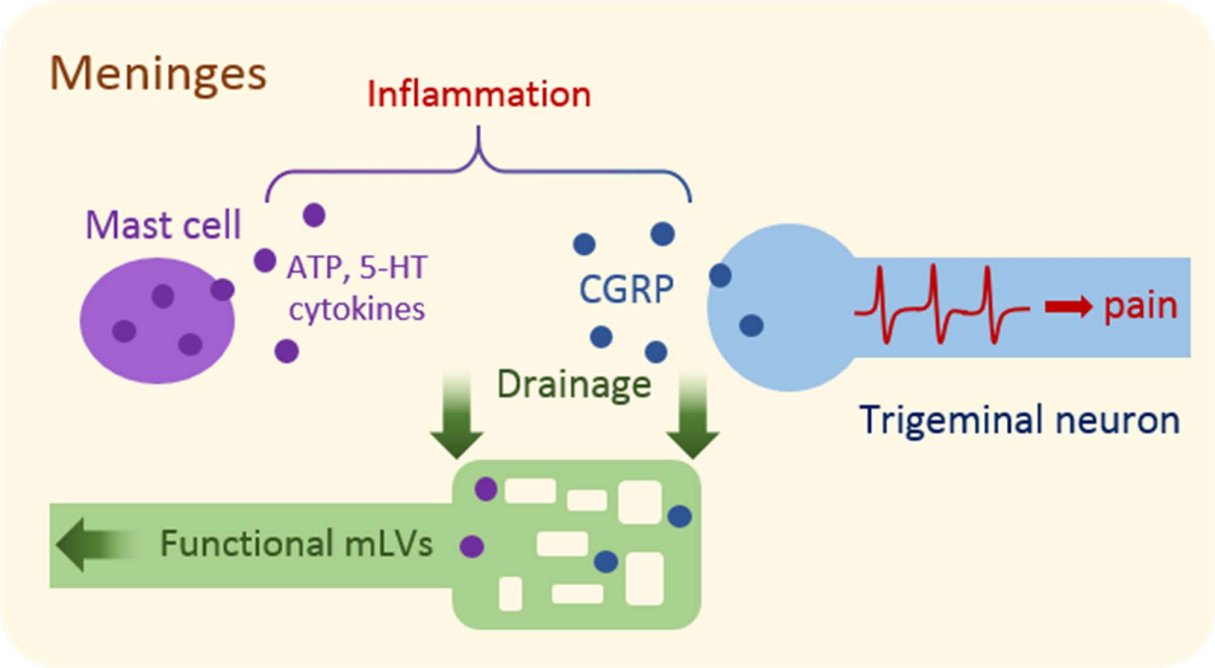

Figure 1. Migraine-related cell types and active compounds presented in meninges. The main migraine mediator CGRP is released from trigeminal neurons, whereas ATP, serotonin (5-HT) and cytokines are released from local immune cells including mast cells. We propose that the local lymphatic system, which is supposed to provide drainage of meninges, prevents the overflooding of these tissues from the pro-inflammatory and pro-nociceptive compounds.

2. Methods and materials

2.1. Animals

We used adult (4 – 20 months old) transgenic K14-VEGFR3-Ig (K14) mice lacking the meningeal lymphatic system (Mäkinen et al., 2001) and wild-type C57BL/6 mice (WT). Total number of animal used for this study was 38 WT and 37 TG animals. Animals were housed at the Lab Animal Center

of the University of Eastern Finland under following housing conditions: 12 hours dark/light cycle, grouped housing, given ad libitum access to food and water, 22°C ambient temperature. All procedures with animals were conducted in accordance with the European Community Council Directive of September 22, 2010 (2010/63/EU). Experimental protocols involving the usage of animals were approved by the Animal Care and Use Committee of the University of Eastern Finland (license EKS-004-2014).

2.2. Hemiskull preparation for ex vivo experiments

For the *in vitro* and *ex vivo* experiments (mast cell staining, CGRP release assay, cytokine release assay, and electrophysiology), we used hemiskull preparations with preserved meninges and meningeal trigeminal innervation (Shatillo et al., 2013; Zakharov et al., 2015). In order to prepare the hemiskulls for subsequent analyses, after decapitation, skin, muscles, and connective tissues, as well as brain were removed with particular care to not touch and damage the meninges. Thereafter, hemiskulls were cut along their sagittal axis (*i.e.*, two hemiskulls were prepared from each animal).

2.3. Mast cell staining

For mast cell staining, WT and K14 animals were transcardially perfused with normal saline (6 minutes at 6 ml/min), followed by 4% paraformaldehyde (PFA) (20 minutes at 3 ml/min). Then hemiskulls were prepared as described above, washed three times for 20 minutes in PBS (phosphate buffered saline), and stained for 60 minutes with 0.1% toluidine blue. Stained meninges were immediately imaged, using a Zeiss Axio Zoom.V16 stereomicroscope (Carl Zeiss AG, Germany), at 30x magnification. We imaged two identical regions-of-interest (ROIs) from each hemiskull: one including the superior sagittal sinus (SSS; at the border of the image) and the next one, which partially overlaps with the previous ROI. In total, four images were acquired from each mouse. From each ROI, the number of stained mast was calculated using ImageJ 1.51v software (National Institute of Health, USA).

2.4. CGRP release assay

The CGRP release assay was conducted as previously described (Mikhailov et al., 2019). Briefly, we used a CGRP enzyme immunoassay kit (EIA kit, SPIbio, France) to measure CGRP release from hemiskull preparations. The levels of CGRP were assessed under control and KCl-treated conditions. First, fresh hemiskull preparations from WT and K14 animals were perfused for 40 minutes with artificial cerebrospinal fluid (aCSF in mM: 119 NaCl, 30 $NaHCO_3$, 11 glucose, 2.5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 2 $CaCl_2$, and the level of pH was adjusted to 7.4) at room temperature (RT). Then,

hemiskulls were washed four times with 150 µl aCSF for 15 minutes at 37°C to stabilize the basal condition. Samples of aCSF (100 µl each) from the last two washes were collected to assess the basal level of CGRP (control conditions). After the last wash, hemiskulls were bathed with an aCSF solution containing 30 mM KCl for 15 minutes. Thereafter, a 100 µl sample was collected to assess CGRP under KCl-treated condition.

2.5. Cytokine release assay

For cytokine release assay, we used fresh hemiskull preparations from both WT and K14 mice. One hemiskull/animal was used to assess the release of cytokine under the control condition, while the contralateral was used to measure the the release of cytokine under treatment condition (100 µM BzATP, a more stable analogue of ATP was used because of long treatment time – three and a half hours). After the collection of hemiskulls, all procedures were performed at 37°C. Hemiskulls were bathed in 150 µl of either aCSF or aCSF + BzATP for three and a half hours. aCSF samples (50 µl) were taken to assess the level of cytokines in control (treated with aCSF only) or stimulated (aCSF + BzATP) conditions. The levels of aCSF in the hemiskulls were monitored during all the incubation period and, if needed, refilled with the respective treatment solution to compensate for evaporation.
To assess the level of cytokines (Il-6, Il-10, MCP-1, TNFα, INFγ, Il-12p70), we used the CBA Mouse Inflammation Kit (Cytometric Bead Array, BD Biosciences, New Jersey, USA). All procedures (sample and standard beads preparation) were performed following instructions provided by the manufacturer. Data were acquired on the CytoFLEX S cytometer (Beckman Coulter Inc, USA) and analyzed with the Flow Cytometric Analysis Program (FCAP) Array software (BD Biosciences, USA).
Due to the low concentration of IL-12p70 and IFNγ in some samples, it was not possible to quantify the exact concentration with the abovementioned method. Thus, for these cytokines number of *n* was lower than the number of animals used for the assay.

2.6. Electrophysiology

Direct recording of action potentials in trigeminal nerve afferents innervating meninges was conducted as described earlier (Shatillo et al., 2013; Zakharov et al., 2015). Briefly, hemiskulls of WT and K14 animals were placed in a perfusion chamber with a constant flow of approximately 6 ml/min of oxygenated aCSF. In order to access the nervus spinosus of the mandibular branch of the trigeminal nerve, we made a small incision in the dura mater. Then, the nerve ending was sucked into a glass recording electrode filled with aCSF. We recorded 10 minutes of spontaneous activity to get a baseline and to assess the difference in basal activity between WT and K14 animals. We applied

100 μM ATP solution for 10 minutes to stimulate nociceptive firing in the trigeminal nerve (Zakharov et al., 2016), followed by 10 minutes of washout. The recording was conducted with a digital amplifier (ISO 80, WPI Inc, USA) with bandpass 300 Hz – 3 kHz, gain 10000. Signals were digitized at 125 kHz with NIPCI 6221 data acquisition (DA) board (National Instruments, USA) and visualized with WinEDR software (Strathclyde University, UK).

2.7. Meningeal nerve staining for β-tubulin III

For the meningeal nerve staining, meninges from PFA-fixed hemiskulls were isolated, avoiding damage, and stained as previously described (Louveau et al., 2015). Briefly, meningeal whole-mount preparations were incubated with 1x phosphate-buffered saline (PBS) containing 2% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 0.05% Tween 20 for 1 hour at RT. After that, meninges were incubated with rabbit anti-β-tubulin III (1:1000, Cat# T2200, Sigma-Aldrich, USA) overnight at 4°C in PBS containing 1% BSA and 0.5% Triton X-100. Whole-mounts were washed in PBS at RT (3x) followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:500, Cat# A11008, Invitrogen, USA) for 1 hour at RT in PBS with 1% BSA and 0.5% Triton X-100. After further washing in PBS (3x) and in phosphate buffer (PB; 2x), meninges were mounted with VECTASHIELD mounting medium (Vector Laboratories, USA), including DAPI, and coverslipped.

Images were acquired using a Leica TCS SP8 X confocal system (Leica Microsystems, Germany) using the LAS X software. Images of the region of interest, adjacent to the middle meningeal artery, were acquired with a 20x objective with 0.75 numerical aperture (NA), with an in-plane pixel size of 1.14×1.14 μm^2 and a z-step of 1 μm. The full images were created by merging 4×4 tile scans, covering a total area of 2034×2034 μm^2.

2.8. Axon segmentation and innervation analysis

We developed a semi-automated segmentation technique to annotate axons and analyze the innervation in acquired 3D confocal microscopy images. To segment an axon, an expert input two points along the desired axon on the maximum projection image. Given the two points, a minimal path algorithm detected the centerline of that axon, based on which an active contour segmented the desired axon. In detail, we formed the 2D maximum intensity projection image along the direction of focal planes, i.e., *z*-axis, and enhanced axons using Frangi filter (Frangi et al., 1998). We normalized the enhanced images to the range [0, 1] and complemented the intensity of the enhanced images, such that axons appeared darker than their background, denoting the image as P. We used Benmansour and Cohen method (Benmansour and Cohen, 2011) to define the minimal path between two given

points p_1 and p_2 as C_{p_1,p_2}, a path which minimizes an energy functional on *P*. The energy minimized by the minimal path is $E(\gamma) = \int_\gamma P(\gamma(s))\mathrm{d}s$, where γ is a path parametrized along its length by *s*. To find C_{p_1,p_2}, we computed the minimal action map $U: \Omega \to \mathbb{R}^+$ associated to p_1 on the domain $\Omega \subset \mathbb{R}^2$ which is the minimal energy integrated along a path between p_1 and any point $x \in \Omega$. To compute *U*, we used the fast marching method (Sethian, 1996) with the speed function $1 - P$. The map *U* has only one local minimum, the source point p_1. Thus, the minimal path C_{p_1,p_2} can be retrieved with a simple gradient descent on *U* from p_2 to p_1, solving an ordinary differential equation with a standard numerical Runge-Kutta method. The minimal path C_{p_1,p_2} is the axon centerline, because 1-*P* was constructed using Frangi filter, admitting higher velocity in the center of the axon.

We used the centerlines to segment the underlying axons using Chan-Vese active contours (Chan and Vese, 2001) (Matlab 2018b, Image Processing Toolbox, activecontour), initialized from the centerlines with the speed function $1-P$. We set the contraction bias equal to -0.2, expanding an initial contour, and the maximum number of iterations to 10 to perform the curve evolution. The initial centerlines were deformed on the speed function to adapt to the shape of axons.

We calculated the diameter of axons along their centerlines, as described in (Abdollahzadeh et al., 2019a). We calculated the length of the centerlines in 3D, by first projecting centerlines from $\mathbb{R}^2$ to $\mathbb{R}^3$, then smoothing the curves with an 11th-order one-dimensional median filter and a shape-preserving piecewise cubic polynomial. We reported the sum of the length of all centerlines, excluding centerlines, which corresponded with axon bundles. The underlying segment of an axon bundle has a diameter thicker than a certain threshold, where we set the threshold to 8 μm experimentally. We also quantified the innervation complexity by forming a graph from the union of the extracted centerlines as in (Abdollahzadeh et al., 2019b). We reported the number of graph terminal-vertices (endpoints) with the vertex degree equal to 1 and regular-vertices (branching points) with the vertex degree greater than 2.

2.9. Statistical analysis

We performed statistical analysis using the GraphPad Prism 8.4.2 software (GraphPad Software, California, USA). The data are presented as mean ± the standard error of the mean (SEM). We set the alpha-threshold defining the statistical significance as 0.05 for all analyses. We used paired or unpaired Student t-test and matched two-way ANOVA with Bonferroni post-hoc test for our data.

3. Results

3.1. Elevated number of mast cells in meninges of mice without lymphatic system

Meningeal mast cells recently emerged as important players in the initiation of a migraine attack (Giniatullin et al., 2019; Kilinc et al., 2017; Koyuncu Irmak et al., 2019; Levy, 2009). As the meningeal lymphatic vessels (mLVs) are important for the movement of immune cells (Ahn et al., 2019; Louveau et al., 2015), we expected that the lack of mLVs could affect the local accumulation of mast cells. We took advantage of toluidine blue labelling to characterize the profile of meningeal mast cells and assess whether lack of dural lymphatic system in K14 mice alters the number of meningeal mast cells.

In WT animals a substantial portion of mast cells was located along the meningeal blood vessels (**Figure 2.A**), where allegedly are located the mLVs (Antila et al., 2017; Aspelund et al., 2015). A similar pattern of mast cell positioning was observed in K14 animals (**Figure 2.B**).

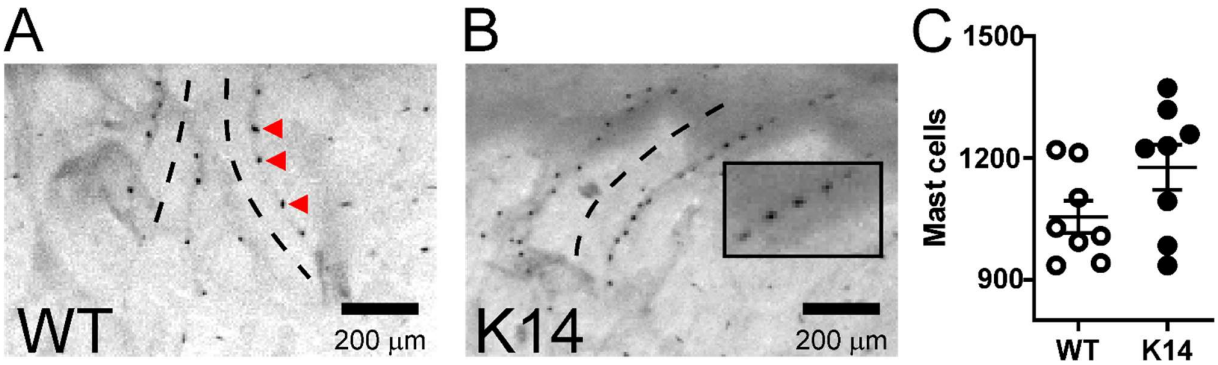

Figure 2. Dural mast cells in WT and K14 mice lacking the lymphatics. Mast cells localize in meninges both WT **(A)** and K14 mice **(B)**. Note the presence of mast cells (red triangles) alligned along the blood vessels (outlined with black dashed curves). **(C)** Mast cells were counted in WT and K14 animals. Transgenic animals show a non-significant trend towards a larger number of mast cells (1055 ± 40 cells in WT *vs.* 1177 ± 56 cells in K14, n = 8/experimental group, $t(14) = 1.784$, $p = 0.096$ by unpaired Student t-test). Scale bar = 200 µm.

K14 animals showed a non-significant trend towards increased number of meningeal mast cells (**Figure 2.C**). Since an increase in the number of mast cells may lead to a more abundant release of local mediators such as serotonin, histamine and ATP (Giniatullin et al., 2019; Levy, 2009; Suleimanova et al., 2020), resulting in meningeal inflammation, and stimulation of the trigeminal afferents (Kilinc et al., 2017; Koroleva et al., 2019) we analyzed the levels of the different migraine-

associated pro-inflammatory molecules and the ultimate activation of the trigeminal nerves in the K14 and their WT littermates mice.

3.2. Altered cytokine release in meninges

We first measured the level of cytokines (IL-6, IL-10, IL-12p70, MCP-1, TNFα, and IFNγ) released into meninges in basal conditions (**Figure 3**). The levels of the pro-inflammatory MCP-1 were significantly higher in K14 animals compared to their WT controls (**Figure 3.C**). On the contrary, the basal level of IL-12p70, which is also a pro-inflammatory cytokine, was significantly decreased in K14 animals compared to WT littermates (**Figure 3.E**). We failed to detect any differences between the genotypes in the levels of all the other measured cytokines in basal conditions (**Figure 3**).

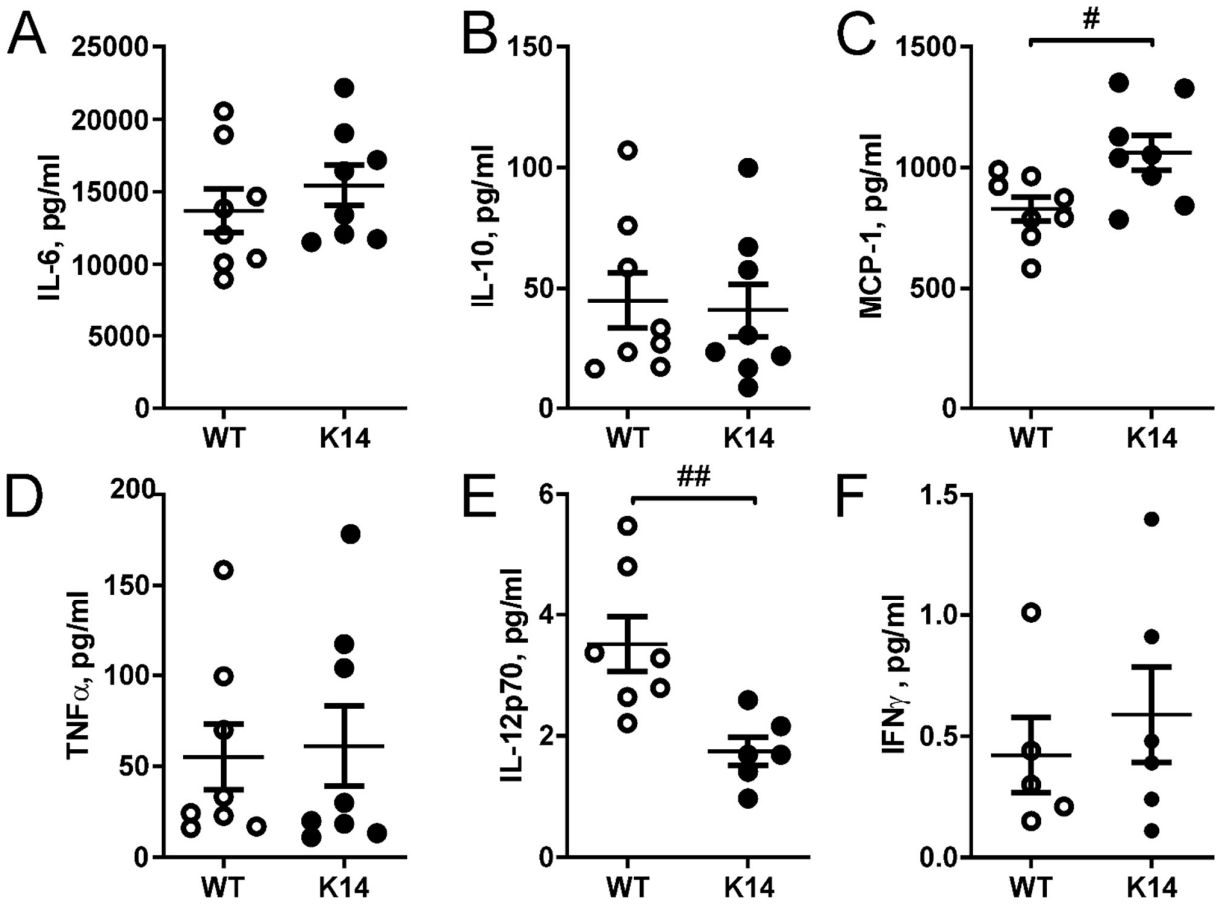

Figure 3. Baseline cytokines release from meninges. Concentrations (pg/ml) of following cytokines are depicted: IL-6 **(A)**, IL-10 **(B)**, MCP-1 **(C)**, TNFα **(D)**, IL-12p70 **(E)** and IFNγ **(F)**. In K14 animals, we found a significant increase in the levels of MCP-1 **(C)** (829 ± 48 pg/ml in WT *vs.* 1061 ± 72 pg/ml in K14, $t(14) = 2.675$, #$p = 0.0181$, unpaired Student t-test), whereas the levels of IL-12p70 **(E)** were significantly lower compared to

the one measured in WT littermates (3.5 ± 0.5 pg/ml in WT *vs.* 1.8 ± 0.2 pg/ml in K14, t(11) = 3.283, ##p = 0.0073, unpaired Student t-test). No difference was found for other interleukines. Number of replicates were: for IL-6, IL-10, MCP-1, TNFα n = 8/experimental group; for IL-12p70: n = 7 for WT, n = 6 for K14; for IFNγ: n = 5 for WT, n = 6 for K14.

To mimic inflammatory conditions, we used benzoyl ATP (Baraldi et al., 2005) (BzATP), a stable ATP analogue to stimulate the P2X7 receptors (Takenaka et al., 2016) in dural immune cells, including mast cells (Nurkhametova et al., 2019), and measured the cytokine release from meninges of both WT and K14 animals. Indeed, 100 μM BzATP significantly increased the levels of IL-10 and TNFα **(Figure 4.B, D)**. Interestingly, while the IL-10 increase did not differ between the WT and K14 mice, BzATP-stimulated release of the pro-inflammatory cytokine TNFα was significant only in the WT mice (**Figure 4.D**) and not in K14 animals. BzATP stimulation did not alter the levels of other measured cytokines.

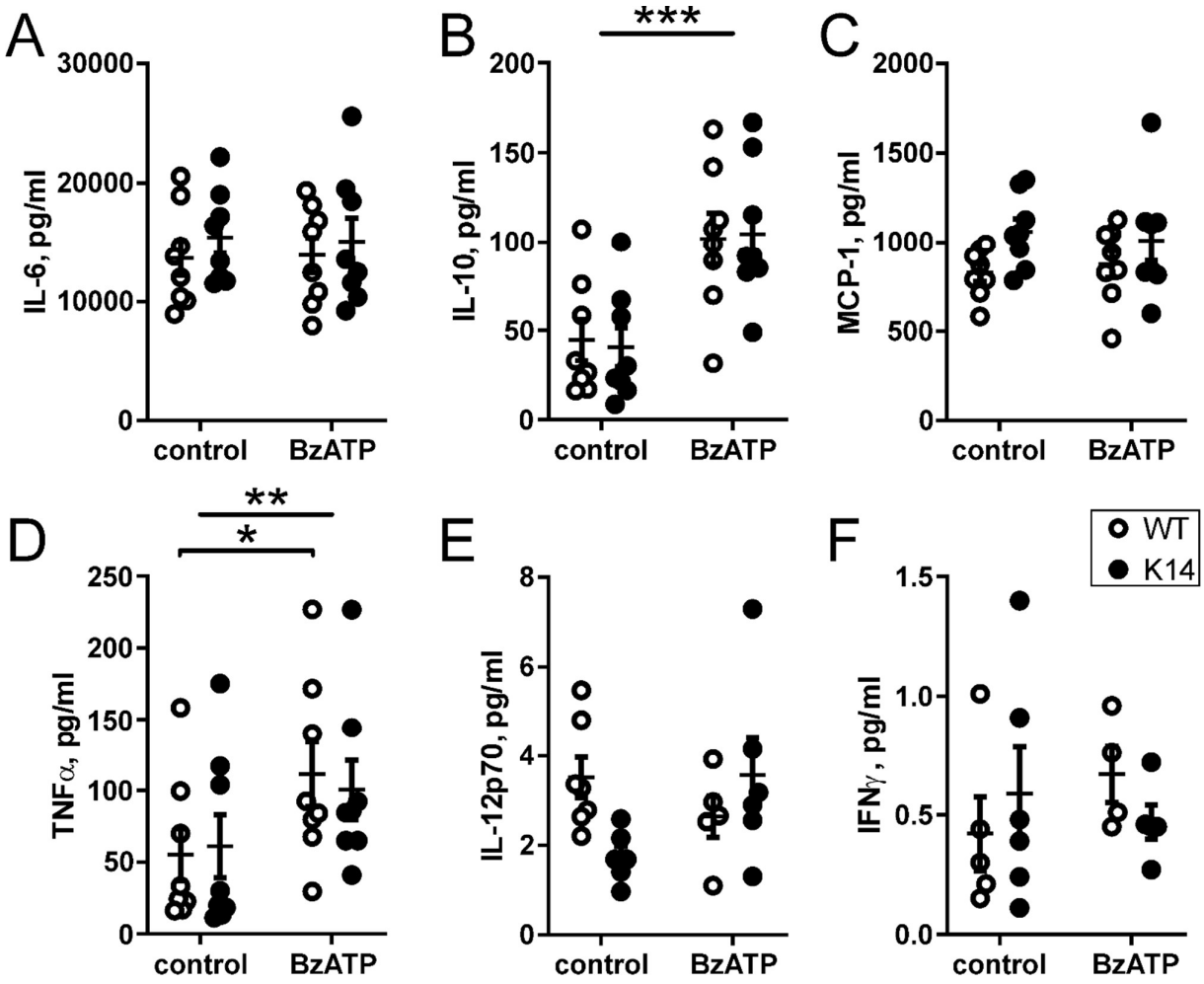

Figure 4. Cytokines release from meninges after BzATP stimulation. Scatter plots reporting the concentrations of cytokines released from meningeal preparation after stimulation with BzATP: IL-6 **(A)**, IL-10 **(B)**, MCP-1 **(C)**, TNFα **(D)**, IL-12p70 **(E)** and IFNγ **(F)**. BzATP induced an increase in the release of IL-10 **(B)** (WT- K14-control *vs.* WT- K14-BzATP, $F(1, 14) = 35.75$, ***$p < 0.0001$ by matched two-way ANOVA) and TNFα **(D)** (WT- K14-control *vs.* WT- K14-BzATP, $F(1, 14) = 12.43$, **$p = 0.0034$ by matched two-way ANOVA). Post-hoc comparisons revealed that IL-10 increase did not differ between WT and K14, whereas BzATP-stimulated release of TNFα was significant only in the WT mice (55 ± 18 pg/ml in WT-control *vs.* 112 ± 23 pg/ml in WT-BzATP, $t(14) = 2.933$, *$p = 0.0218$ by Bonferroni post-hoc test). Number of replicates were: for IL-6, IL-10, MCP-1, TNFα n = 8/experimental group for both treatments; for IL-12p70: control n = 7 for WT, n = 6 for K14, BzATP n = 5 for WT, n = 6 for K14; for IFNγ: for control n = 5 for WT, n = 6 for K14, for BzATP n = 4 for WT, n = 5 for K14.

3.3. Decreased CGRP release in meninges of mice lacking lymphatic system

We measured the level of CGRP released in meninges in both basal and stimulated conditions (**Figure 5**). CGRP levels measured at two consecutive samples collected at basal condition (baselines¸ BL) were similar in both genotypes. Interestingly, stimulation with 30 mM KCl, which leads to CGRP release through stimulation of peptidergic nerve fibers, in K14 mice shows significantly lower levels of CGRP compared to WT hemiskulls (**Figure 5.B**).

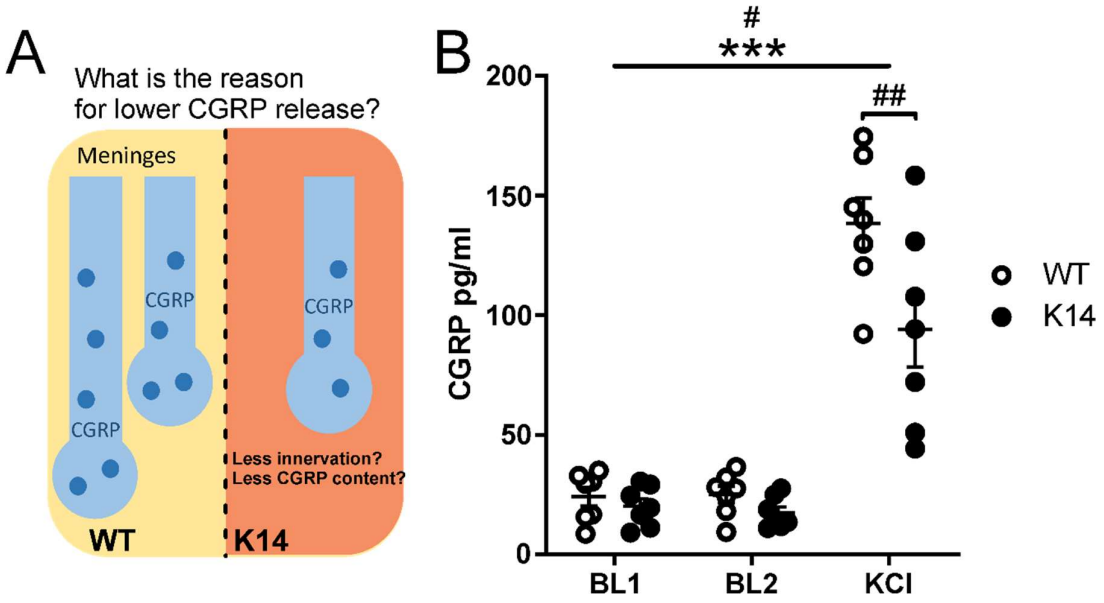

Figure 5. CGRP released from trigeminal nerve endings in the meninges, from hemiskull preparation. (A) Scheme proposing trigeminal nerve innervation and CGRP release in WT and K14 mice. **(B)** CGRP release was not altered in K14 hemiskulls under basal conditions: 24 ± 4 pg/ml in WT-BL1 *vs.* 20 ± 3 pg/ml in K14-BL1, and 25 ± 3 pg/ml in WT-BL2 *vs.* 17 ± 2 pg/ml in K14-BL2 (n = 7/experimental group, $p > 0.05$ by matched two-way ANOVA with Bonferroni post-hoc test). The application of 30 mM KCl triggered a significant increase in the CGRP level in both genotypes (WT- and K14-KCl *vs.* WT- and K14-BL1/BL2, n = 7/experimental group, $F(2, 24) = 103.3$, ***$p < 0.0001$ by matched two-way ANOVA). Overall CGRP release was higher in WT animals (WT *vs.* K14, n = 7/experimental group, $F(1, 12) = 6.025$, #$p = 0.0303$ by matched two-way ANOVA). Post-hoc comparisons revealed that the concentration of the released CGRP under the stimulation was significantly lower in the hemiskulls lacking the lymphatic system compared to the WT preparations (138 ± 11 pg/ml in WT-KCl *vs.* 94 ± 16 pg/ml in K14-KCl; n = 7/experimental group, $t(36) = 3.816$, ##$p = 0.0015$ by Bonferroni post-hoc test).

3.3. Increased spontaneous and ATP induced spiking activity in meningeal nerves of mice lacking meningeal lymphatic system

To address the state of trigeminal nociception in animals without the lymphatic system, we recorded spiking activity from meningeal afferents. First, we recorded spiking activity in trigeminal nerve fibers in hemiskulls under the basal conditions. Thereafter, we stimulated nerve terminals with ATP, a powerful trigger of local nociception (Koroleva et al., 2019; Yegutkin et al., 2016) (**Figure 6.A**).

ATP induced a significant increase in the nociception in both genotypes. Notably, meninges lacking lymphatic vessels (both in basal conditions and under ATP stimulation), showed a significant increase in nociceptive spiking compared to WT (**Figure 6.B**).

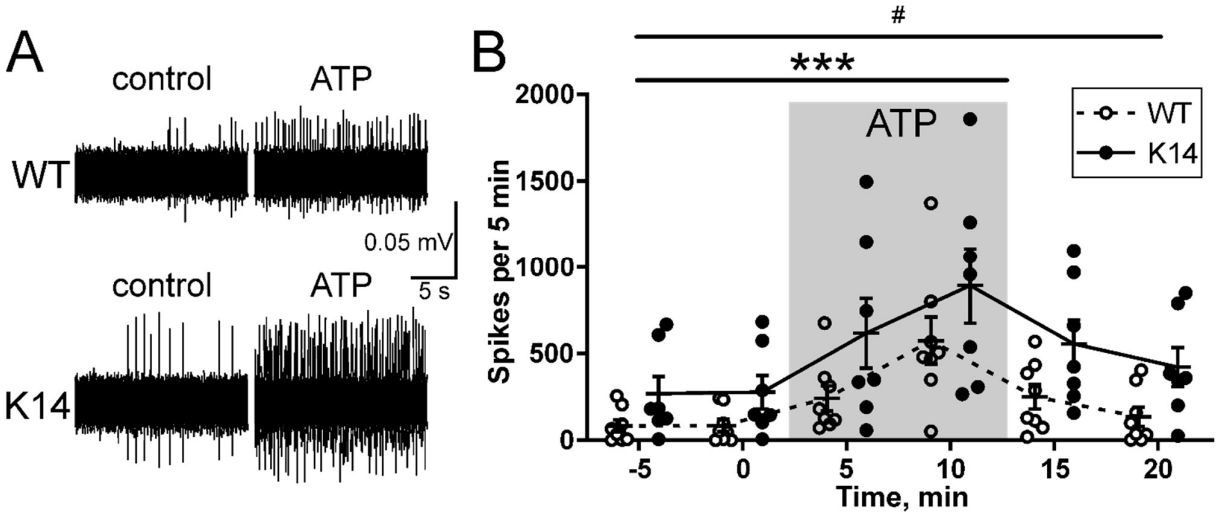

Figure 6. Direct spikes were recorded in WT and K14 hemiskulls under basal conditions and ATP stimulation. (A) Sample traces of nociceptive recordings in WT and K14 animals under basal and stimulated conditions. **(B)** The plot represents an average number of pain spikes recorded in 5 minutes (mean ± SEM) under basal conditions, during the application of 100 μM ATP, and during the following washout. ATP induced a significant increase in nociception activity in both genotypes (baseline *vs.* ATP, WT: n = 8, K14: n = 7, $F(3, 39) = 15.74$, ***$p < 0.0001$ by matched two-way ANOVA). Overall spiking activity was higher in K14 hemiskulls (WT *vs.* K14, WT: n = 8, K14: n = 7, $F(1, 13) = 4.752$, #$p = 0.0482$ by matched two-way ANOVA).

3.5. Unchaged patterns of meningeal innervation

To evaluate the density of trigeminal innervation in hemiskull preparations from WT and K14 animals we stained meningeal preparations with the antibodies specific to the neuronal marker β-tubulin III (**Figure 7**) (Cáceres et al., 1986). After that, we applied a semi-automated segmentation method to evaluate the following parameters: total innervation length (indirect evaluation of the innervation density in the meninges), number of branching points (*i.e.*, the complexity of innervation network) and the number of terminal points (i.e., trigeminal fiber terminal endings, likely releasing CGRP). None of the analyzed parameters showed a difference between the genotypes.

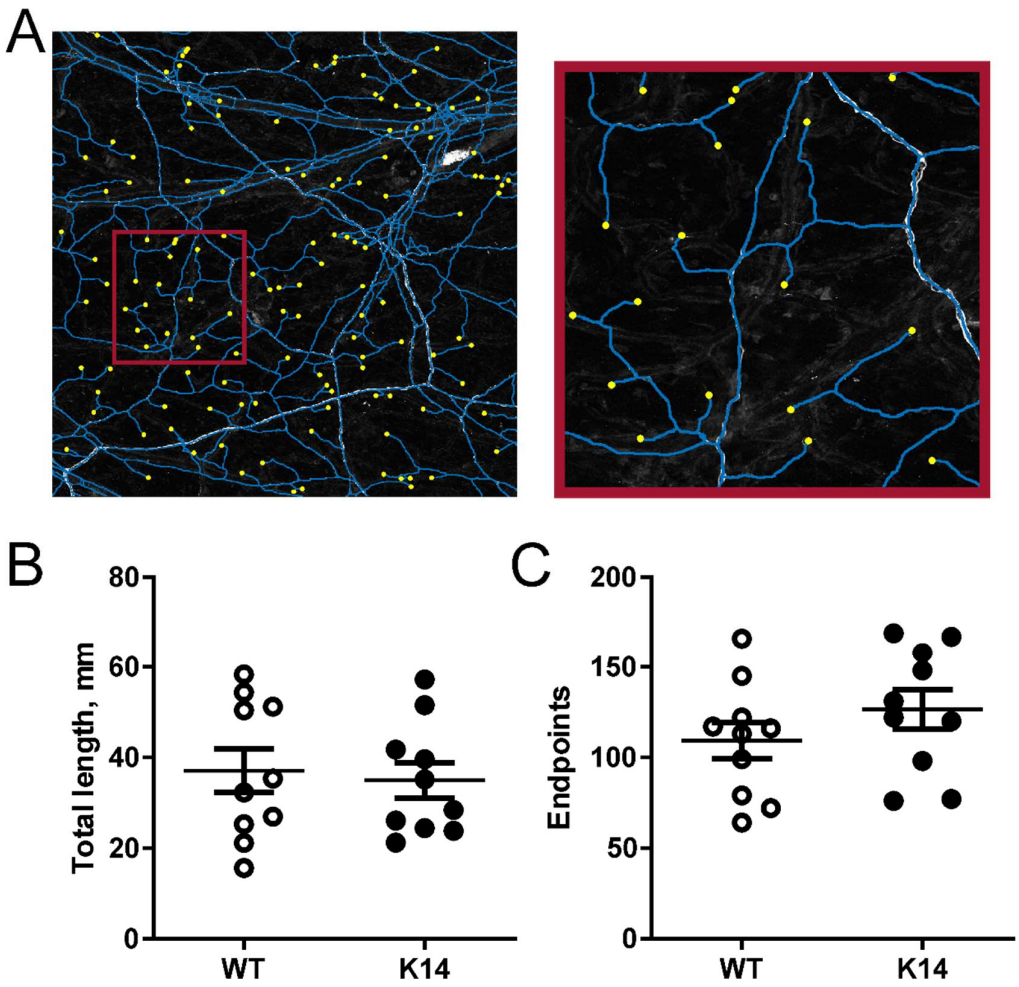

Figure 7. Segmentation analysis of meningeal innervation. (A) A representative image of meninges stained for β-tubulin III (white), segmented for morphological analyses. Blue lines represent axons, magenta filled-circles represent branching points, and yellow filled-circle represent terminal points (*i.e.*, CGRP-releasing trigeminal fiber terminal endings). **(B)** Scatter plot depicting the total axonal length (axons highlighted with blue on panel **A**): 37 ± 5 mm in WT *vs.* 35 ± 4 mm in K14, n = 10/experimental group, t(18) = 0.3498, p = 0.7306 by unpaired Student *t*-test. **(C)** Scatter plot depicting the number of terminal points (yellow dots on panel **A**): 109 ± 10 in WT *vs.* 127 ± 11 in K14, n = 10/experimental group, t(18) = 1.160, p = 0.2614 by unpaired Student t-test. We analyzed meningeal innervation from the right hemiskulls of 10 independent animals in each experimental group (WT and K14; one image per animal).

4. Discussion

Here we evaluated for the first time the role of the meningeal lymphatic system in the regulation of the neurochemical profile and functional properties of the meningeal trigeminovascular system

(TVS), implicated in migraine pain using the K14 mice lacking meningeal lymphatics. Our data show that the level of CGRP is decreased in K14 animals, the sensitivity to the algogen ATP is higher. In addition, in animals lacking meningeal lymphatic system, we found decreased release of pro-inflammatory IL-12p70 and TNFα and increase in pro-inflammatory MCP-1 (summarized in **Figure 8**).

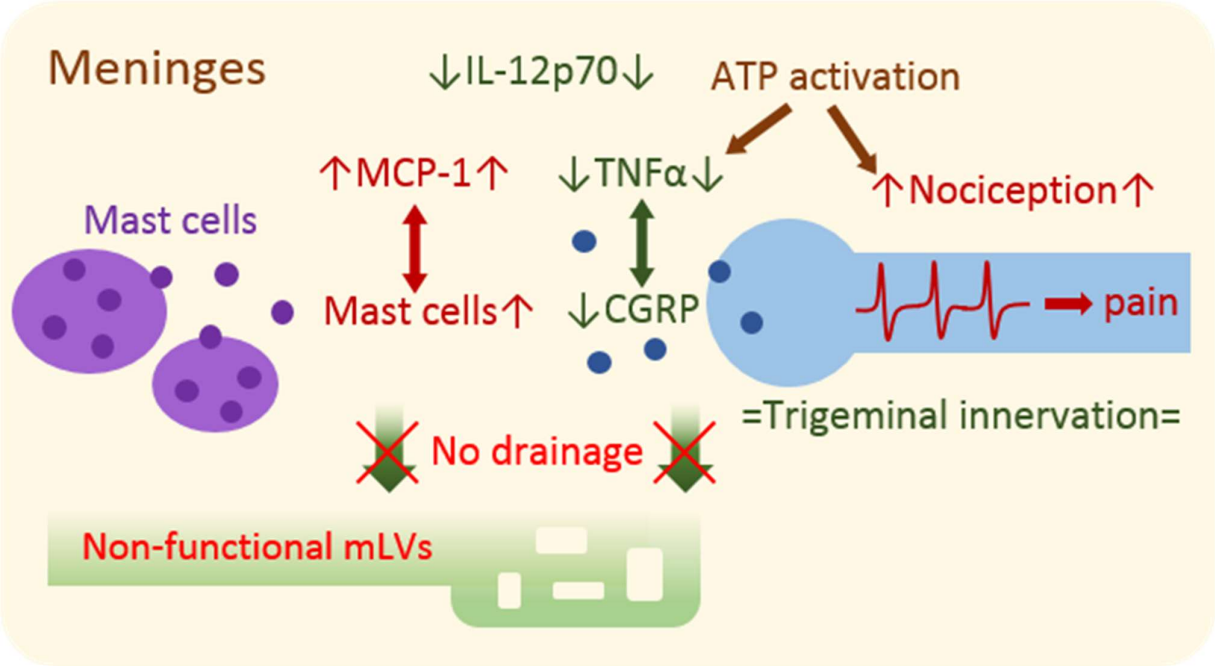

Figure 8. Experimental findings summary. Dysfunctional lymphatic system triggered several alterations in meninges. Number of mast cells was increased, supporeted by increased level of mast cell-activating cytokine MCP-1. CGRP release was decreased, supported by decreased level of TNFα, cytokine triggering CGRP transcription. Level of pro-inflammatory IL-12p70 was decreased. Trigeminal innervation of the meninges was not changed, however, trigeminal nociception was elevated. Red indicates pro-inflammatory and pro-nociceptive changes, green indicates anti-inflammatory and anti-nociceptive changes. Arrows up (↑) indicate increase in parameters, arrows down (↓) – decrease, equals sign (=) indicates unchanged parameter.

4.1 Dural inflammation responding to the deficiency of the lymphatic system

Meningeal tissues represent the main site for the generation of migraine pain. They comprise of blood vessels, sensory nerve fibers and a high diversity of local immune cells (McIlvried et al., 2017; Olesen et al., 2009; Pietrobon and Moskowitz, 2013). Among the latter, lymphocytes and mast cells have been reported to be present in dura mater membranes (McIlvried et al., 2017). In migraine-like

conditions, meninges are involved in so-called 'neurogenic inflammation,' which is induced and maintained by neuropeptides, such as CGRP, released from trigeminal nerve fibers (Eftekhari et al., 2013; Mason et al., 1984). CGRP is currently recognized as one of the central migraine mediator, with multiple targets including dural vessels (Goadsby et al., 1988), meningeal nerve fibers (Cady et al., 2011) and immune cells (Ottosson and Edvinsson, 1997). Interestingly, it was proposed, that impaired clearance of CGRP from perivascular space can be a reason for post-traumatic headache after TBI (Piantino et al., 2019). Surprisingly, here we demonstrate that the release of CGRP in the meninges from K14 mice lacking mLVs is lower compared to meninges in the WT control group suggesting that K14 animals are less prone to triggering migraine attacks via this particular mechanism. Interestingly, CGRP is vital for lymphatic capillary formation (in particular, alleviating the severity of postoperative lymphedema) (Matsui et al., 2019). These data imply a potential positive crosstalk between CGRP signaling and activity of the meningeal lymphatic system. As the source of meningeal CGRP are local trigeminal nerve fibers (Eftekhari et al., 2013; Mason et al., 1984), we propose that the lymphatic impairment causes either reduced storage of CGRP in trigeminal nerve terminals or lowered meningeal innervation by 'peptidergic' nerve fibers (Keller and Marfurt, 1991). Previous studies on patients have mainly focused on cytokine profiling in the systemic circulation (Fidan et al., 2006; Khaiboullina et al., 2017; Oliveira et al., 2017; Perini et al., 2005; Sarchielli et al., 2004; Yücel et al., 2016), whereas the production of cytokines in the meninges have not been fully characterized. Various immune cells can be identified in the meninges, including cells transported within the mLVs (McIlvried et al., 2017; Alitalo, 2011; Hayday et al., 2001). These cells constitute the primary and major source of multiple proinflammatory and anti-inflammatory cytokines. Here we demonstrate the presence at detectable levels of IL-6, IL-10, MCP-1, TNFα, IL-12p70, and IFNγ even in the basal conditions in meninges. In particular, we found the presence of the pro-inflammatory cytokines TNFα, IL-1β and IL-6 known to be associated with migraine pathology (Bø et al., 2009; Khaiboullina et al., 2017; Perini et al., 2005; Sarchielli et al., 2006; Wang et al., 2015; Yücel et al., 2016). Further, the absence of functional mLVs was associated with the increased levels of the pro-inflammatory cytokine MCP-1 (Wood et al., 2014). MCP-1 is known as the activator of mast cells, provoking release of mast cells derived components (Lv et al., 2012). In addition, MCP-1 delays mast cell death (Lopes et al., 2019). This supports our finding of higher number of mast cells in meninges lacking mLVs. Notably, an increase in MCP-1 was reported in the cerebrospinal fluid of patients during a migraine attack (Bø et al., 2009). In contrast, the levels of the pro-inflammatory cytokine (IL-12p70) (Martins et al., 2011), which is typically released from macrophages and dendritic cells (Müller-Berghaus et al., 2004), were reduced in the meninges from K14 animals lacking mLVs. Interestingly, it has been shown that IL12-p70 plasma level was increased in migraine patients during

the interictal phase (Oliveira et al., 2017). The reduction of IL12-p70 in K14 animals lacking mLVs probably suggests a decreased inflammatory state in the meninges. Notably, mast cells can release IL12-p70 (Mukai et al., 2018) in response to CGRP-induced degranulation (Ottosson and Edvinsson, 1997; Theoharides et al., 2005). Our data showing decreased levels of CGRP in animals lacking mLVs may imply that the absence of a functional meningeal lymphatic system leads to a reduced tendency to develop a migraine through several downstream mechanisms controlled by this neuropeptide.

Apart from CGRP, many immune cells, including mast cells, are controlled by purinergic mechanisms mediated by extracellular ATP which is serving as a powerful trigger of inflammatory reactions acting primarily via P2X7 receptors (Burnstock, 2016; Karmakar et al., 2016; Nurkhametova et al., 2019, 2018). Consistent with this view, we also found that the stimulation of meninges with the P2X7 agonist BzATP stimulated the release of IL-10 and TNFα, demonstrating the pro-inflammatory action of purinergic signaling in meninges. We used BzATP as a stable ATP analogue, because of the long, 3.5 hour treatment time and because of fast degradation of ATP. Notably, ATP receptor stimulation resulted in the significant release of TNFα only in the meninges from WT animals, but not in the meninges lacking the mLVs. TNFα is the main migraine-related pro-inflammatory cytokine (Martami et al., 2018; Oliveira et al., 2017; Perini et al., 2005; Yücel et al., 2016). In line with this, TNFα has been reported as the pro-inflammatory agent, which can stimulate CGRP transcription (Bowen et al., 2006). The lack of increase in TNFα levels after ATP receptor stimulation in the meninges of the K14 mice lacking the lymphatic system is consistent with our other findings on the reduced level of CGRP in these animals K14. Taken together, the lack of meningeal lymphatic system may induce a local shift in the balance of various humoral factors (i.e., CGRP and pro-inflammatory cytokines such as TNFα), suggesting a reduction in the signalling of neurogenic inflammation.

4.3 Meningeal nociception and morphological properties of nerve terminals

Our recordings of nociceptive activity from trigeminal afferents demonstrated a significant increase in nociceptive spiking in response to the powerful trigger of meningeal nociception ATP in K14 mice. Notably, ATP can operate either directly via its own P2X3 receptors at meningeal nerve terminals (Yegutkin et al., 2016), or by degranulating mast cells and release of serotonin, which acts via it own 5-HT3 receptors (Koroleva et al., 2019). Therefeore, one possible explanation for higher spiking frequency in K14 mice could be the involvement of mast cells in ATP-induced nociceptive spiking. Another possibility is the more extensive innervation of meninges, which are heavily innervated by somatic and autonomous nerve terminals, respectively originating from the trigeminal ganglion

(Ramachandran, 2018; Zakharov et al., 2015) and sphenopalatine ganglion (Delépine and Aubineau, 1997). Sensory nerve fibers are characterized mainly by nociceptive Aδ and peptidergic C-fibers (Levy and Strassman, 2002), with the latter considered as the primary source of the key migraine mediator CGRP (Eftekhari et al., 2013). Thus, since CGRP release in meninges without mLVs was lower than in healthy meninges, we speculate that sensitization of dural afferents, which depends on the level of CGRP (Fabbretti et al., 2006), was lower in K14 animals compared to WT animals, leading to a less prominent difference in nociceptive activity.

To clarify if the presence of functional mLVs affects the innervation of dura mater, we analyzed several parameters of meningeal innervation. However, we did not find a difference in the total length of axonal fibers or in the number of terminal segments of meningeal afferents. This suggests that the higher activity of ATP or the lowered level of CGRP released in K14 mice are not due to a reduced number of meningeal innervation or a small number of nerve terminals but rather present the different functionality of local innervation.

4.4 Limitations of the model

Our data for the first time demonstrates the interactions between the meningeal lymphatic system, neurogenic inflammation, and nociception, which determine the predisposition to the generation of the migraine attack. However, the model used in our study has some limitations. First, all our experiments have been conducted in *ex vivo* conditions. Thus, the meningeal lymphatic system was not active in this preparations (e.g., recruitment and trafficking of immune cells and clearance of migraine mediators were not functioning in our samples). Second, our animals were not sensitized to mimic migraine attacks. Third, it has been previously reported that K14-VEGFR3-Ig mice have a defect in systemic immunity (Thomas et al., 2012), involving mainly the CD8+ T lymphocytes, which were not studied here. Third, to study alterations between WT and K14 meninges we used ATP, which is only one of the numerous pro-inflammatory compounds released in meninges. Thus, the impact of the lymphatic system in migraine state in meninges requires further tests with injections of nitroglycerin (Demartini et al., 2019) or induction of CSD representing a model for migraine with aura (Van Den Maagdenberg et al., 2004) and considering other immune cells, such as T cells, known to be involved in migraine (Nurkhametova et al., 2018).

4.5 Pathophysiological implications

The discovery of meningeal lymphatic system revealed new aspects for the functioning of the healthy brain and proposed new mechanisms of several brain diseases such as stroke, epilepsy, TBI, Alzheimer's Disease (Kwon et al., 2019; Noé and Marchi, 2019; Patel et al., 2019; Rasmussen et al.,

2018; Tamura et al., 2019). Here we propose a possible contribution of mLVs also in migraine pathology, given the generally accepted central role of the meningeal tissues in this disorder. Our main working hypothesis was that the lack of the meningeal lymphatic system exaggerates the neurogenic inflammation, leading to more severe symptoms of migraines because such animals are not able to clean up the dural environment. Indeed, we found that the lack of the meningeal lymphatic system produced several pro-nociceptive changes in the trigeminovascular system relevant to migraine pathology. This resulted in the increased nociceptive activity and enhanced expression of the pro-inflammatory MCP-1 cytokine. Yet surprisingly, the expression of key migraine mediator CGRP and other pro-inflammatory TNFα and IL-12p70 cytokines was decreased. This suggest that the absence of meningeal lymphatic system caused not uniform, but rather more complex, and eventually functionally opposite, changes in the nociceptive and inflammatory balance in the meninges.

Our findings highlight the importance of the meningeal lymphatic drainage in the pathophysiology of migraine, suggesting it as a potential target in the search for new therapeutic anti-migraine strategies, or in the re-evaluation of the existing ones.

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6. Author Contributions

NM, FMN and RashidG originally conceived the study; NM, FMN, RashidG elaborated the study design; NM, KK, RG performed experiments; NM, AA, OG, AS, JT analyzed the data; NM, AA, OG, JT performed the statistical analysis; NM, AA, AS, FNM, RashidG drafted a significant portion of the manuscript and figures. TM supported the study and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

7. Conflict of Interest Statement

None of the authors has any conflict of interest to disclose. The authors confirm that have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

8. Data Availability

The raw data supporting the conclusions of this manuscript will be made available by the corresponding author, upon reasonable request, to any qualified researcher.

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