Importance of lymph vessels in the transcapillary fluid balance in the gingiva studied in a transgenic mouse model

Lilian Ephrem Mkonyi,1 Athanasia Bletsa,1,2 Inge Fristad,2 Helge Wiig,1 and Ellen Berggreen1

Departments of 1Biomedicine and 2Clinical Dentistry, University of Bergen, Bergen, Norway

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Mkonyi LE, Bletsa A, Fristad I, Wiig H, Berggreen E. Importance of lymph vessels in the transcapillary fluid balance in the gingiva studied in a transgenic mouse model. Am J Physiol Heart Circ Physiol 299: H275–H283, 2010. First published May 14, 2010; doi:10.1152/ajpheart.01199.2009.—The gingiva is frequently challenged by oral bacterial products leading to inflammatory responses such as increased fluid filtration and edema formation. The role of initial lymphatics for transcapillary fluid balance in the gingiva is unknown and was therefore investigated in genetically engineered K14-VEGF receptor 3-Ig (K14) lymphedema mice. The mutant mice demonstrated a total lack of lymphatics in the gingiva, whereas lymphatics were found in the submucosal parts of the alveolar mucosa, although they were almost completely absent in the mucosa. In wild-type (WT) mice, lymphatic vessels were detected in mucosal and submucosal parts of the alveolar mucosa. Interstitial fluid pressure (Pif) measured with micropipettes was increased in the gingiva of K14 mice in the normal situation (P < 0.001) and after inflammation (P < 0.01) induced by lipopolysaccharide from the oral bacteria Porphyromonas gingivalis compared with WT littermates. Fluid volume expansion caused a >75% increase in interstitial fluid volume followed by a drop in Pif after recovery in both strains. Continuous measurements during the expansion showed an increase in Pif followed by a decline, suggesting that compliance is increased after the disruption of the extracellular matrix during edema formation. In the alveolar mucosa, no strain differences were observed in Pif in the normal situation or after fluid volume expansion, suggesting that lymph vessels in the mucosa are not critical for tissue fluid regulation in any situation. Our study demonstrates an important role of gingival lymphatics in transcapillary fluid balance in the steady-state condition and during acute perturbations.

interstitial fluid pressure; inflammation; alveolar mucosa

THE LYMPHATIC VASCULATURE is uniquely made to transport protein-rich fluid from the interstitium back to the blood circulation, thus maintaining tissue homeostasis. Moreover, the lymphatic vascular system serves an important role in immuno-surveillance, as lymphocytes and antigen-presenting cells are trafficking in lymphatics from the periphery toward the lymph nodes to elicit immune responses (22, 27). The initial lymphatics start as blind-ended vessels and are made of a single layer of overlapping endothelial cells that lack a continuous basement membrane, making them highly permeable. They are anchored to the extracellular matrix by elastic fibers, preventing the vessels from collapsing during changes in interstitial fluid pressure (Pif). This architecture furthermore facilitates lymph uptake even in a high-pressure environment (17). From the initial lymphatics, fluid drains into the collecting lymphatics. Lymph vessels and their function have been difficult to study in the past due to lack of specific lymphatic markers, but the discovery of several specific lymphatic vessel antigens has opened a new research field (2).

In the gingiva, the part of the oral mucosa that surrounds the tooth, lymph vessels are found in the lamina propria under the epithelium (31) and a submucosal layer is lacking. The gingiva has a free part and a part firmly attached to the underlying bone, and, from the gingiva, lymph vessels travel into the adjacent alveolar mucosa and palate (31). The alveolar mucosa consists of a mucosal layer and a submucosal layer, with the latter loosely attached to the alveolar bone. A description of lymphatic vessel organization in the alveolar mucosa is lacking. The palate and gingiva account for the keratinized masticatory mucosa, and the major part of the palate lacks a submucosal layer.

The gingiva is constantly exposed to oral microorganisms, making it susceptible to inflammation.

Gingivitis is the forerunner of periodontitis, which is characterized by a loss of alveolar bone, and is the most prevalent form of bone pathology in humans (15).

Edema represents an increase in interstitial fluid volume (Vif) sufficient to manifest with swelling, and visible edema is a common finding in gingivitis. Edema is due to an imbalance between capillary filtration and lymph drainage, and in periods of increased filtration in the gingiva, interstitial fluid is also filtered through the sulcular epithelium into the sulcus as a crevicular fluid (1, 7). Oral bacteria can be colonize the tooth surface, and, via the sulcus epithelium, they induce gingivitis. In the acute phase of gingivitis, vascular inflammatory responses such as vasodilation and increased fluid filtration take place (7). Pif has been reported to increase in the inflamed gingiva (7, 8), a situation that favors lymphatic flow in the tissue. However, the contribution of initial lymphatics in normal tissue fluid balance or under conditions with increased fluid filtration has never been previously investigated in either the gingiva or its adjacent alveolar mucosa.

The formation of new lymph vessels occurs when VEGF-C and VEGF-D bind to VEGF receptor 3 (VEGFR3) (16, 21). Genetically engineered K14-VEGFR3-Ig (K14) mice have an overexpression of soluble VEGFR3 that competes for VEGF-C/D binding, resulting in the regression of lymphatic vessels and lymphedema (18). In the dermis, keratinocytes express the promoter VEGFR3 connected to the IgG Fc domain. Engineered mice have visible swelling of the limbs due to hypoplasia of the lymphatics in the skin, whereas the blood vessel supply to the dermis is normal (18). In all parts of the oral mucosa, the basal epithelial cells have the keratin K14 gene, suggesting that this model can be used for functional studies of lymphatics in this area.

The aim of the present study was to use the K14 mouse model to gain new insights into lymphatic function in the gingiva and adjacent alveolar mucosa. Our findings suggest...
that in the alveolar mucosa, lymphatics are not important for the transcapillary fluid balance. We demonstrated an important role of initial lymphatics in the control of $P_g$ in the normal situation and after an acute perturbation of the fluid balance in the gingiva, suggesting that tissue compliance is increased during edema formation.

**METHODS**

**Experimental animals.** All experiments were performed with the approval of and in the accordance with the Norwegian State Commission for Laboratory Animals. In the present study, we used K14 mice, as previously described in detail (18), which were generously provided by Dr. Kari Alitalo (Molecular/Cancer Biology Laboratory and Haartman Institute, University of Helsinki, Helsinki, Finland). The mutation in K14 mice was verified by PCR analysis before the experiments using 5'-GAAAGCCCAAAACACTCCAAACAATG-3' as the forward primer and 5'-AGGCCAAAGTCGCAGAA-3' as the reverse primer, where the 3'-nucleotide of the reverse primer matches with the mutant allele but not with the wild-type (WT) allele.

All experiments were performed when female and male K14 and WT mice (C57/B16) were 12–16 wk old. They were fed a standard pellet diet and had tap water ad libitum before the experiments. All animals were anesthetized intramuscularly with a 1:1 mixture (0.1 ml/10 g body wt) of midazolam (Dormicum, Roche, France) and fentanyl-fluanison (Hypnorm, Janssen). The depth of anesthesia during the experiments was controlled by testing the withdrawal reflex of the paws by applying a pinch between the toes. An additional subcutaneous injection of anesthetic was supplied when needed. Euthanasia was induced by an overdose of anesthesia.

**Immunostaining.** K14 (n = 8) and WT (n = 8) mice were transcardiacally perfused with fixative (4% paraformaldehyde with 10% EDTA 7.5% polyvinylpyrrolidone) or EDTA. Demineralization of hard tissue was done in 10% EDTA 7.5% polyvinylpyrrolidone. The specimens were rinsed in PBS, saturated in 30% sucrose, and stored at −80°C until being cryosectioned. The frozen sections were then embedded in Tissue-Tek OCT compound (Sakura, Zoeterwoude, The Netherlands), and 8- to 20-μm-thick sagittal sections were cut in a freezing (−20°C) slide microtome. Sections were used for immunoperoxidase staining using a Vectastain ABC kit (Vector Laboratories, Burlingam, CA) or immunofluorescence staining, which was performed on precoated glass slides (Superfrost Plus, Menzelglaser, Braunschweig, Germany). Block of the endogenous peroxidase was performed using 0.3% H2O2 in 90% methanol. Peroxidase activity was developed with 3,3'-diaminobenzidine (Sigma, St, Louis, MO) and enhanced with nickel. Detailed protocols for immunostaining were as previously described (4).

Sections were evaluated in a Nikon photomicroscope (Nikon Eclipse E600, Nikon Instruments) connected to a digital camera using Lucia imaging software (Ludwig Maximilian University, Munich, Germany) or viewed with a fluorescence microscope (Axiolab, Carl Zeiss MicroImaging) connected to a AxioCam MRM camera (Carl Zeiss MicroImaging).

The following antibodies were used: mouse monoclonal antibody to the human IgG Fc domain (1:1,000 dilution, Zymed Invitrogen, San Francisco, CA), rabbit anti-mouse lymphatic vessel endothelial receptor-1 (LYVE-1; 1:1,000 dilution, Abcam, Cambridge, UK), Syrian hamster anti-mouse podoplanin (1:50 dilution, Abcam), rat anti-mouse CD31 (1:50 dilution, Abcam), rat anti-mouse CD4 (1:500 dilution, AbD-Serotec, Oxford, UK), rat anti-mouse CD45 (1:500 dilution, Abcam), and rat anti-mouse CD3 (1:500 dilution, AbD-Serotec). The corresponding secondary antibodies for immunofluorescences were Cy3-conjugated IgGs (1:200 dilution, Jackson ImmunoResearch, West Grove, PA) and Alexa-488 conjugated IgG (Molecular Probes, Invitrogen). Controls for the specificity of the immunoreactions were included by isotype control Ig incubation and by substitution of the primary antiseraum with PBS.

**Quantification of immune cells.** To quantify immune cells, images were captured digitally under ×40 magnification. Cells were counted in a 100 × 100-μm grid, and positively stained cells were counted in longitudinal sections of the maxilla from three predetermined fields in the masticatory mucosa. The fields included were 1) between the incisor and first molar (epithelium and lamina propria) and 2) the gingiva mesially and 3) distally to the first molar (lamina propria close to the sulcus epithelium). For each antibody, counting of cells was performed in six sections per animal where all three fields were found. The percentage of positive immune cells was calculated as the mean percentage of the total cell number in the field.

**Collagen fiber visualization and content.** For collagen visualization, jaws were fixed in 4% phosphate-buffered formalin and decalcified in EDTA for 7 days followed by a routine laboratory technique for paraffin embedding. Sections (6 μm thick) were stained with Sirius red F3Ba (Sigma) according to Junqueira et al. (12) and studied under polarizing light.

Collagen content in the alveolar mucosa and masticatory mucosa was quantified based on hydroxyproline content (33) as previously described (18). The absorbance was read at 557 nm on a Specord M400 plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The hydroxyproline concentration was quantified by comparison with a standard curve of l-4-hydroxyproline (Fluka Chemie, Buchs, France) in 1 mM HCl. Collagen content was calculated based on a 6.94:1 collagen-to-hydroxyproline ratio (11).

**$P_g$ measurements.** Mice were placed on a thermostatically controlled heating pad to maintain temperature at 37.5–38.5°C. The right femoral artery was cannulated with polyethylene-25 catheters for systemic arterial blood pressure recordings. Mice were tracheotomized, and the head was immobilized and fixed to the operating table by a stereotactic frame. The upper lip was gently pulled away with a suture to increase accessibility to the attached gingiva and the alveolar mucosa distally to the left incisor. Pipettes were made from glass capillaries (1.00 mm outer diameter × 0.58 mm inner diameter, Harvard Apparatus, Kent, UK) and sharpened to achieve a tip diameter of 2–7 μm (MB3/TPS5 microbeveller, World Precision Instruments, Aston, UK).

Pipettes were filled with 0.5 M NaCl solution stained with Evans blue and inserted into the tissue with a micromanipulator (Leica, Heerbrugg, Switzerland). Measurements were performed at a depth of 0.2–0.6 mm below the mucosal surface under visual guidance via a stereomicroscope (MZ16, Leica, Wetzlar, Germany). The transducer was calibrated before each experiment, and zero pressure was measured in a cup filled with saline in the same height as the tissue. An injection of a small amount of Evans blue after measurements had been performed, to observe if the color remained in the tissue, showed that the pipette was not placed intravascularly.

$P_d$ and systemic blood pressure were monitored with a pressure transducer (model 1280C, Hewlett-Packard Medical Electronics Division, Waltham, MA) connected to an amplifier and recorder (model 8188 2201 06, Gould Instrument Systems, Valley View, OH).

**Fluid volume measurements.** $V_d$ was calculated as the difference between the total extracellular fluid volume ($V_e$) and intravascular volume ($V_v$) as previously described by Bletsas et al. (5). After the anesthesia and femoral artery cannulation, $V_v$ was measured by a continuous intravenous infusion of an extracellular tracer as described by Borge et al. (6). A bolus of 0.13 ml of 58Cr-labeled EDTA (3.7 MBq/ml) was injected into the femoral artery followed by a continuous infusion of 1.5 μl/min throughout the equilibration period. Ninety minutes after the start of the infusion period, 125I-labeled human serum albumin (125I-HSA; 3.5 kBq in 0.1 ml saline) was injected through the femoral artery for $V_v$ measurement. 125I-HSA circulated for 5 min before a final blood sample was collected. A blood sample of 0.5–0.7 ml was obtained through cardiac puncture after the thoracic cavity had been opened. The mouse was transferred to an infant incubator kept at 20–24°C and 100% relative humidity. The alveolar and masticatory mucosa from the maxilla were excised and transferred to preweighed airtight tubes to avoid evaporation of
fluid from the tissue. All tubes were reweighed to obtain wet weights of the tissues. Blood samples were centrifuged at 14,000 rpm for 10 min. Known volumes of plasma were removed and used for further analysis. All samples were counted in an LKB-counter (Wallac 1282, Compugamma, Turku, Finland) with window settings of 15–75 keV for $^{125}$I and 290–350 keV for $^{51}$Cr-labeled EDTA. Standards were counted in every experiment, and all samples were corrected for background and spillover. After being counted, samples were dried in a heating chamber that kept a temperature of 65°C until constant weight was obtained. Weighing was performed to the nearest 0.1 mg. Total tissue water ($V_w$) was calculated as milliliters of water per gram dry weight $\left(\frac{\text{wet weight}}{\text{dry weight}}\right)/\text{dry weight}$.

**Experimentally induced overhydration.** To study the response in $P_r$ with increased volume in a tissue with reduced lymphatic drainage capacity, we induced overhydration by infusion through the right femoral artery of Ringer solution corresponding to 15% of body weight over a period of 30 min using an infusion pump. Surgical procedures were similar to those described above. $P_r$ was measured in the attached gingiva and alveolar mucosa of anesthetized mice in the control situation before the infusion and after a 30-min equilibration period. The distribution of volumes after overhydration was measured as described above. Tissue samples were excised from the masticatory and alveolar mucosa. In addition, $P_r$ in the attached gingiva was measured continuously during fluid expansion and recovery in a separate group of WT mice.

**Experimental protocol of LPS-induced acute inflammation.** Acute inflammation in the gingiva was studied using injections of LPS from *Porphyromonas gingivalis*, an oral pathogen involved in development of gingivitis (28, 30). The test substance (1 μl LPS, 5 mg/ml, InvivoGen, San Diego, CA) or vehicle (1 μl of 0.9% NaCl with 1% BSA), which were both colored with Evans blue for visualization, was given locally at the border between the alveolar mucosa and attached gingiva. $P_r$ was recorded for 10 min before any injections were made in the attached gingiva. Immediately after the administration of the test substance or vehicle, $P_r$ was measured continuously in the gingiva in the periphery of the blue-stained area for up to 90 min.

**Statistical analysis.** Results are given as means ± SD unless otherwise stated. Student’s $t$-test or Mann-Whitney test was used for comparisons between two groups, and one-way ANOVA was used for comparisons between more than two groups. For repeated pressure measurements within a group and between groups, data were analyzed with one-way repeated-measures ANOVA followed by Bonferroni’s $t$-test or Dunn’s method (if the normality test failed). $P$ values of <0.05 were considered statistically significant.

**RESULTS**

**Verification of the K14 mouse phenotype in the oral mucosa.** The existence of transgenic protein was verified in keratinocytes in the basal layer in all parts of the oral mucosa of K14 mice (Fig. 1A).

![Fig. 1. Phenotypic characterization of mutant K14-VEGF receptor 3-Ig (K14) mice. A: immunoperoxidase staining of the gingiva of a transgenic K14 mouse and a wild-type (WT) littermate mouse with antibody against the human IgG Fc domain. Transgenic protein was detected in the basal cells of the mucosal epithelium (arrow). B: staining for a marker of lymphatic endothelium [lymphatic vessel endothelial receptor-1 (LYVE-1)] in gingival sections of K14 and WT mice. Note the absence of lymphatic vessels in the transgenic mouse.](http://ajpheart.physiology.org/ by 10.220.32.247 on October 30, 2017)
LYVE-1\(^+\) staining of lymphatic vessels in the gingiva demonstrated a complete lack of initial lymphatics in K14 mice (Figs. 1A and 2), whereas in WT mice initial lymphatics travelled from the top of the papilla in the lamina propria and followed the soft tissue into the alveolar mucosa (Figs. 1B and 2A). In the alveolar mucosa of WT mice, initial lymphatic vessels were found in the rete pegs under the epithelium and in the submucosal layer (Fig. 2A). In contrast, lymphatic vessels were practically devoid from the mucosa in K14 mice, whereas they were found in the submucosal layer (Fig. 2A). The palate lacked initial lymphatics in the lamina propria of K14 mice, and initial lymphatics were only found in areas with a submucosal layer (Fig. 2B), in contrast to WT mice, where lymphatic vessels were found in all areas. In general, the palatine mucosa had less initial lymphatic vessels than the alveolar mucosa in WT mice.

The existence of lymphatic vessels was confirmed with colocalization of LYVE-1 and podoplanin (data not shown). In addition, podoplanin was found in epithelial cells in the oral mucosa, as also previously described by Miyazaki et al. (19), in contrast to LYVE-1, which was only found in vessels colocalized with podoplanin. LYVE-1 was therefore chosen for the demonstration of lymphatic vessels in this study. The blood vessel distribution also appeared to be normal in K14 mice, as demonstrated by double labeling with a pan-endothelial marker (CD31) and LYVE-1 (Fig. 3B). The mutant mice seemed to have a normal histological appearance in all areas of the oral mucosa investigated, as shown in Fig. 3A.

**Collagen staining and content.** Since impairment of lymphatic drainage has been suggested to lead to the development of fibrosis with increased collagen content (26) followed by reduced tissue compliance, collagen analysis was performed. For collagen content measurements, the masticatory mucosa was collected since the gingiva only represents very small tissue samples. Sirius red staining showed a dense distribution of collagen in the oral mucosa, particularly in the masticatory mucosa, in both strains (Fig. 4A). Collagen in the masticatory mucosa averaged 320.2 ± 39.3 and 398.7 ± 19.4 µg/mg tissue in K14 and WT mice, respectively (\(P = 0.10;\) Fig. 4B), whereas the corresponding values for the alveolar mucosa were 190.2 ± 12 and 209 ± 11.5 µg/mg in K14 and WT mice, respectively (\(P = 0.28;\) Fig. 4B).

**P\(_{if}\) in the normal situation and in overhydration.** Systemic arterial blood pressure in the normal situation was not significantly different when K14 and WT mice were compared (74.6 ± 11.7 and 72.8 ± 13.7 mmHg, respectively, \(P = 0.7\)). After fluid expansion, systemic blood pressure was 70.8 ± 11.1 and 81.3 ± 20.9 mmHg in K14 and WT mice, respectively, and the difference between strains after loading was not significant (\(P = 0.23\)). Compared with WT mice, K14 mice had significantly higher \(P_{if}\) in the attached gingiva (15.6 ± 1.2 mmHg, \(n = 7\), vs. 9.6 ± 1.9 mmHg, \(n = 6\), respectively, \(P < 0.001;\) Fig. 5A), whereas no significant differences (\(P = 0.8\) by Mann-Whitney test) between the two strains were observed in the alveolar mucosa (4.1 ± 0.9 mmHg, \(n = 7\), in K14 mice vs. 4.0 ± 0.5 mmHg, \(n = 6\), in WT mice; Fig. 5B). Surprisingly, after fluid load representing 15% of body weight, \(P_{if}\) in the gingiva dropped from the control level to 10.6 ± 2.5 in K14 mice (\(n = 7, P = 0.011\)) and 4.9 ± 1.9 mmHg in WT mice (\(n = 6, P = 0.002;\) Fig. 5A). \(P_{if}\) between the two strains of mice remained significantly different also after fluid loading (Fig. 5A).

In contrast, in the alveolar mucosa, \(P_{if}\) increased after fluid loading to 6.3 ± 1.5 mmHg in WT mice, which was not significantly different from the corresponding pressure of 5.3 ± 1.4 mmHg found in K14 mice (\(P = 0.29;\) Fig. 5B).

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**Fig. 2. Overview of the gingiva and adjacent alveolar mucosa and palate.** A and B: sagittal sections in the molar region from WT and K14 mice immunostained with LYVE-1 demonstrating the localization of lymphatic vessels in the gingiva (g), alveolar mucosa (am), and palate (p). In alveolar mucosa (A) and palate (B) of WT mice, LYVE-1\(^-\) lymphatic vessels were found in the rete pegs of the mucosa (arrowheads) and in the submucosal layer (arrows), whereas in K14 mice, only submucosal lymphatic vessels were detected (arrows, A and B). s, Sulcus epithelium.
To observe the dynamic changes in Pif during infusion and to test the hypothesis that an initial increase in Pif took place before Pif dropped, as observed after the recovery period in both strains, we performed continuous measurements of Pif during fluid expansion and recovery in WT mice (n = 6). Pif measurements in the attached gingiva during the infusion of fluid demonstrated an increase in Pif starting between 2 and 7 min after the initiation and reached a mean maximal value in the 10- to 20-min period (P < 0.001 compared with baseline; Fig. 6). Maximal Pif values ranged from 15 to 24 mmHg. Pif started to decline during the rest of the infusion period (up to 30 min), which continued during the recovery phase. On average, Pif returned to the baseline level at the end of the recovery period (up to 60 min), and, in three animals, Pif dropped below their baseline levels.

**Tissue fluid volume in the normal situation and response to fluid loading.** To quantify the possible effect of lymphatic regression on fluid distribution in the normal situation as well as after fluid load of the tissue, we determined Vx, Vv, and Vif. Unfortunately, we were not able to measure fluid volumes in the gingiva solely, merely due to the small size of this tissue in mice. We therefore found it necessary to pool the tissue along with the oral mucosa from the palate (masticatory mucosa) to obtain enough tissue for reliable fluid volume measurements. Vif in the masticatory mucosa in control animals averaged 1.4 ± 0.2 ml/g dry wt in K14 mice (n = 6) and 1.5 ± 0.2 ml/g dry wt in WT mice (n = 6) and increased to 3.0 ± 0.5 and 3.4 ± 1.1 ml/g dry wt after Ringer solution infusion in K14 (n = 6) and WT (n = 6) mice, respectively. In the alveolar mucosa, Vif averaged 1.2 ± 0.4 ml/g dry wt in K14 mice (n = 6) and 1.3 ± 0.4 ml/g dry wt in WT mice (n = 6) and increased to 3.1 ± 0.9 and 2.4 ± 0.8 ml/g dry wt after Ringer solution infusion, respectively.

There were no significant differences between K14 and WT mice in the two tissues in either the control situation or after fluid volume loading in Vif or in Vv (data not shown). Vv averaged 0.17 ± 0.07 and 0.18 ± 0.06 ml/g dry wt (P = 0.72) in the masticatory mucosa and 0.21 ± 0.09 and 0.19 ± 0.06 ml/g dry wt (P = 0.84) in the alveolar mucosa in K14 and WT mice, respectively. No differences in Vv were found between strains in the control situation or after fluid expansion in any tissue investigated.
Response to LPS-induced acute inflammation. In an attempt to mimic the clinical situation, which is characterized by inflammation induced by bacterial products, we injected bacterial LPS in the gingiva. Similar to what was observed during the induced overhydration, $P_f$ in the control situation was significantly higher in K14 mice than in WT mice (14.1 ± 1.3 mmHg, $n = 8$, and 9.4 ± 1.3 mmHg, $n = 14$, respectively). Injection of LPS raised $P_f$ to 22.3 ± 3.9 mmHg in K14 mice and 14.6 ± 2.9 mmHg in WT mice ($P < 0.05$) at 0–10 min (Fig. 7). The elevation of $P_f$ lasted up to 60 min in both strains and returned thereafter to the control level (Fig. 5). In all observation periods, $P_f$ was significantly higher compared with WT mice (Fig. 7).

In the sham groups, $P_f$ averaged 13.4 ± 0.5 mmHg ($n = 8$) and 9.7 ± 1.7 mmHg ($n = 10$) in K14 and WT mice, respectively ($P = 0.017$), and remained stable before the injection of saline with 1% BSA. Local injection of the vehicle did not change $P_f$ through the 90-min observation period in any strain (data not shown).

Immune cells in the masticatory mucosa. Since a complete lack of lymphatics and a higher $P_f$ was found in the gingiva, we investigated if the number of immune cells (CD45$^+$) was altered in K14 mice compared with WT mice in the masticatory mucosa. In tissue with fluid and/or protein accumulation, an inflammatory immune cell response has previously been reported (23, 29). We sought also to determine if T cell numbers (CD3$^+$ and CD4$^+$) were different in K14 mice compared with WT mice, as T cell recruitment depends on lymphatic immune cell transport. Immunohistochemical analysis of CD3$^+$, CD4$^+$, and CD45$^+$ cell staining was performed as shown in Fig. 8A. The mean percentage of CD45$^+$ immune cells to the total cell number was significantly higher in the masticatory mucosa (8.5 ± 4.5% vs. 2.8 ± 1.9%) in K14 mice ($n = 6$) compared with WT mice ($n = 6$, $P = 0.018$; Fig. 8B). T cells (CD3$^+$ and CD4$^+$) were only occasionally found in the masticatory mucosa in both strains, and no differences were detected. The total cell numbers in the fields averaged 39.1 ± 10.6 and 39.9 ± 12.8 ($P = 0.84$) in WT and K14 mice, respectively, suggesting that K14 mice have normal histology in the mucosa.

**DISCUSSION**

The main aim of this study was to use a genetically engineered mouse model to acquire new insights into the functional role of lymphatics in the gingiva in normal fluid homeostasis and during periods of increased fluid filtration. In the gingiva of K14 mice, complete regression of lymphatics was found, whereas in the alveolar mucosa, lymphatic vessels were missing in the mucosa but found in the submucosal layer. No changes in histology or collagen content were found between the two strains of mice, in
either the masticatory or alvolar mucosa, demonstrating that fibrosis did not develop in the mucosa of the K14 model. The normal blood vessel distribution and normal plasma volume further indicate that only lymphatic vessel distribution was affected in the mutant mice.

Our measurements of $P_{if}$ in the attached gingiva and alveolar mucosa in WT mice in the control situation are in the same range as previously reported in rats (1, 5), where a significant increase in gingival $P_{if}$ was found after the induction of increased capillary filtration by venous stasis (1), paralleling our observation after LPS application and in the first phase of fluid expansion.

Measurements of $V_{if}$ in the masticatory mucosa have not previously been reported in any species, but in the rat alveolar mucosa, it has been reported to represent 40% of total tissue weight (5). When $V_{if}$ in the alveolar mucosa was calculated on a wet weight basis, it averaged 25% of tissue weight in WT mice, a difference that can be ascribed to species variations.

An interesting finding was that gingival $P_{if}$ was significantly increased in K14 mice compared with WT mice, demonstrating the involvement of lymphatics in the normal transcapillary fluid balance and edema prevention in the low-compliant gingiva, as a high $P_{if}$ is necessary for counteracting further fluid filtration in the absence of lymphatics. A new steady-state situation was established in K14 mice, probably due to a limited fluid accumulation in the tissue, a notion supported by the accumulation of immune cells in the tissue, a common finding in lymphedema (23, 29).

In contrast to the gingiva, no differences in $P_{if}$ were found in the alveolar mucosa between K14 and WT mice, and $V_{if}$ was not altered in transgenic K14 mice compared with WT mice. These results may indicate that lymphatic vessels in the mucosa are not critical for normal tissue fluid homeostasis as long as the submucosal lymphatics exist. This finding corresponds to observations in the thigh and back skin in the Chy mouse model of lymphedema (13), which resembles the present model, where lymphatic vessels are lacking in the dermis but present in the subcutis. In Chy mice, normal $P_{if}$ and tissue fluid volumes were found in most skin areas except the dependent forelimb and hindlimb when compared with WT littermates, indicating that vessels in the subcutis can compensate for the impaired lymphatic function in the dermis (14).

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Measurements of $V_{if}$ in the masticatory mucosa have not previously been reported in any species, but in the rat alveolar mucosa, it has been reported to represent ~40% of total tissue weight (5). When $V_{if}$ in the alveolar mucosa was calculated on a wet weight basis, it averaged ~25% of tissue weight in WT mice, a difference that can be ascribed to species variations.

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When the gingiva was challenged with a fluid load, no increase in $P_{if}$ was measured after the recovery phase in any of
the strains, whereas $V_{if}$ increased as much as 81% and 76% in WT and K14 mice, respectively. This observation is surprising since we would have expected that an increase in $V_{if}$ would be followed by a corresponding increase in $P_{if}$ in a relatively low compliant tissue as the attached gingiva. By continuous measurements of $P_{if}$ during infusion and recovery, we demonstrated that $P_{if}$ increased initially but reached a maximal, elevated level and started to decline before the infusion period was ended. At the end of the recovery phase, $P_{if}$ returned to normal levels despite the substantial increase in $V_{if}$. A possible explanation may be that the high $P_{if}$ caused disruption of the extracellular matrix followed by a lowering of the compliance in the tissue. This explanation is supported by data from the lung where saline infusion alone caused a marked edema formation. Subsequent analysis of extracellular matrix proteoglycans showed a significant weakening of proteoglycan interactions with other extracellular matrix components and proteoglycan degradation per se, resulting in increased interstitial compliance and fluid accumulation similar to what was observed here (20).

Blockade of collagen binding receptors has been shown to cause edema formation through lowering of $P_{if}$ in the skin (25), a tissue with a comparable composition and a collagen content (32) in the same range as measured in the gingiva in this study. We speculate that collagen attachment may be weakened in the gingiva during fluid loading, resulting in a drop in $P_{if}$ in both strains of mice. The reduction in $P_{if}$ reduced the counterpressure to capillary filtration (according to Starling’s equation) and allows further fluid filtration. The drop in $P_{if}$ in the gingiva in both strains probably contributed to a further increase in $V_{if}$ of the same magnitude as observed in the high-compliant alveolar mucosa. Edema is defined as a >50% increase in $V_{if}$ (3), a situation established in both strains after fluid volume expansion in this study. It is possible that such matrix degradation also takes place in patients during the development of edema in the inflamed gingiva and may thus have a mechanistic role in edema development in gingivitis.

In the alveolar mucosa, a modest and equal elevation of $P_{if}$ was found in both strains after tissue fluid expansion. This finding, together with the parallel and similar increase in $V_{if}$ in the two strains, may indicate that lymphatics in the alveolar submucosa in K14 mice can compensate for the impaired mucosal lymphatic drainage in the tissue during periods of increased fluid filtration. Whether this is the case remains to be tested in future experiments.

We wanted to challenge the gingiva with LPS to mimic the clinical situation in gingivitis. In the gingiva, acute inflammation caused a marked elevation of tissue pressure, which was most pronounced in the 0- to 10-min period after LPS injection in both strains. For all time periods investigated, $P_{if}$ remained significantly higher in K14 mice than in WT mice, suggesting that the extra fluid that was filtered in this situation as a result of the inflammation accumulated to a greater extent in the tissue where lymphatics were missing. This observation demonstrates the important role of lymphatics in fluid homeostasis during an acute perturbation, e.g., during the early phase of an inflammatory reaction.

The duration of the elevation of $P_{if}$ was not different in the two strains (up to 60 min), and might be explained by a higher net pressure difference between the gingiva and alveolar mucosa in K14 mice. Such a pressure difference may also increase the fluid flux into the sulcus and lead to increased crevicular fluid formation in this situation. The increased pressure difference in K14 mice promotes interstitial fluid flux from the gingiva toward the alveolar mucosa and into the sulcus and could, in part, compensate for the lack of lymphatics and thus lymph flow in K14 mice. Another factor that may play a role in this situation is the increased tissue fluid conductance.
reported in tissues with impaired lymphatic drainage (9, 10) that could accommodate a fluid load in the gingiva in K14 mice. Enhanced fluid conductance would further promote fluid flux from the interstitial space toward the alveolar mucosa. The increased number of CD45+ but not CD3+/4+ immune cells indicates a T cell-independent inflammatory response in the masticatory mucosa in K14 mice compared with WT mice that may be a result of protein accumulation in the tissue (2, 26). Furthermore, the very low numbers of cells and no differences between the strains indicate that there is no substantial recruitment of T cells that require immune cell trafficking in lymphatic vessels. In summary, we suggest that lymphatic vessels in the alveolar mucosa are not critical for tissue fluid regulation in the normal situation or during periods of increased filtration. We demonstrated an important role of gingival initial lymphatics in the transcapillary fluid balance under steady-state conditions and during acute perturbation, suggesting that tissue compliance is increased during edema formation.

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