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Human gingival epithelial cells stimulate proliferation, migration, and tube formation of lymphatic endothelial cells in vitro

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Abstract

Objective: The aim of this study was to investigate the response of gingival epithelial cells to microbial and inflammatory signals.

Background: The gingival epithelial barrier provides the first line of defense and supports tissue homeostasis by maintaining the cross-talk between gingival epithelium, oral microbiota, and immune cells. Lymphatic vessels are essential to sustaining this homeostasis. The gingival epithelial cells have been shown to produce prolymphangiogenic factors during physiologic conditions, but their role in response to microbial and inflammatory signals is unknown.

Methods: Immortalized human gingival epithelial cells (HGEC) and human dermal lymphatic microvascular endothelial cells (LEC) were cultured. HGEC were exposed to *Porphyromonas gingivalis* derived-LPS, human IL-1 beta/IL-1F2 protein, or recombinant human IL-6/IL-6R. Levels of vascular growth factors (VEGF-A, VEGF-C, and VEGF-D) in cell supernatants were determined by ELISA. LEC were grown to confluence, and a scratch was induced in the monolayer. Uncovered area was measured up to 48 h after exposure to conditioned medium (CM) from HGEC. Tube formation assays were performed with LEC cocultured with labelled HGEC or exposed to CM.

Results: VEGF-A, VEGF-C, and low levels of VEGF-D were constitutively expressed by HGEC. The expression of VEGF-C and VEGF-D, but not VEGF-A, was upregulated in response to proinflammatory mediators. VEGF-C was upregulated in response to *P. gingivalis* LPS, but not to *Escherichia coli* LPS. A scratch migration assay showed that LEC migration was significantly increased by CM from HGEC. Both the CM and coculture with HGEC induced significant tube formation of LEC.

Conclusions: HGEC can regulate production of lymphangiogenic/angiogenic factors during inflammatory insults and can stimulate proliferation, migration, and tube formation of LEC in vitro in a paracrine manner.

KEYWORDS
conditioned medium, cytokines, keratinocytes, lymphatic endothelial cells, vascular endothelial growth factors

1 | INTRODUCTION

The gingival epithelial barrier separates the host from the environment and provides the first line of defense against pathogens, exogenous substances, and mechanical stress. Cross-talk between the gingival epithelium, the oral microbiota, and immune cells is crucial for maintaining tissue homeostasis and to prevent infection of deeper tissues.[1] Under clinically healthy conditions, the gingival epithelium acts as a physical barrier, senses microbial presence, and secretes various immune mediators including proinflammatory cytokines, chemokines, antibacterial peptides, and angiogenic factors, and thereby contributes to maintaining a mutualistic microbe-host relationship.[2–4]

Below, the gingival epithelium and the basement membrane lies the connective tissue, lamina propria. It is rich in collagen fibers and well vascularized. In addition, it provides firm attachment of gingiva to cementum and alveolar bone. Gingiva is supplied with lymphatic vessels located centrally in the gingival papilla along with blood vessels.[5] The lymphatic system is intimately linked to tissue fluid homeostasis, immune cell trafficking, and to the establishment and development of an inflammatory host response to infections.[6,7] Lymphatic vessels contribute to eliminate bacterial infections by transporting antigen and immune cells to lymph nodes and drain protein-rich fluid extravasated from blood vessels back into the blood circulation.[6]

Lymphangiogenesis, the formation of new lymphatic vessels from pre-existing ones, is observed during many inflammatory diseases and has been previously demonstrated during development of periodontal disease in mice.[8] Inflammation-induced lymphangiogenesis is expected to be actively involved in both the pathophysiology and resolution of various immune-mediated and inflammatory disorders including respiratory tract inflammation, diabetes, and inflammatory bowel disease as reviewed by Kim et al.[9] The gingival lymphatics have also been demonstrated to, in part, protect against periodontal disease development during *Porphyromonas gingivalis* induced periodontitis in mice.[8] Growth of both blood vessels and lymphatic vessels involves expression of vascular endothelial growth factors (VEGFs) signaling via VEGF receptors.[6] Lymphangiogenesis is mediated by VEGF-A signaling through VEGF receptor 2 (VEGFR2) and VEGF-C/D signaling through VEGFR3.[10] We have previously demonstrated that VEGF-C and VEGF-D, the main lymphatic vascular growth factors are expressed in epithelium, in blood vessels and in scattered cells with fibroblastic appearance in normal human gingiva.[11] Furthermore, epithelial expression of vascular growth factors has been observed in murine gingiva during infection-driven periodontal inflammation.[8]

Here, we test the hypothesis that human gingival epithelial cells produce vascular growth factors in response to microbial and inflammatory signals and we examine whether growth factors produced by epithelial cells during physiologic conditions and during inflammation may stimulate LEC functions necessary for lymphangiogenesis.

2 | METHODS

2.1 | Cell culture conditions

Telomerase immortalized human gingival epithelial cells (HGEC) (ATCC, #CRL-3397™)[12] were cultivated in keratinocyte growth medium (KGM): Dermal Cell Basal Medium (ATCC, #PCS-200-030) with the addition of Keratinocyte Growth Kit (ATCC, #PCS-200-040) and penicillin–streptomycin at 37°C in 5% CO_2. All experiments were performed in passage 35–37. Adult Human Dermal Lymphatic Microvascular Endothelial Cells (LEC) (Lonza™ #CC-2810) were cultivated in endothelial cell growth medium (EGM): Endothelial Cell Growth Basal Medium-2 (Lonza™ #CC3156) with the addition of EGMTM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuotsTM supplements (Lonza™, #CC-4147) at 37°C in 5% CO_2. Experiments were performed in passage 7.

2.2 | Testing medium combinations

To determine an appropriate medium to use for coculture experiments and as conditioned medium, 10×10^3 viable cells were seeded in 96-well plates in 100 µL medium according to cell type. Cells were given 4 h to adhere. Culture medium was then replaced either with fresh medium according to cell type or different combinations of EGM and KGM medium (20%, 40%, 60%, 80%, and 100%). Cells were incubated with the different medium combinations for 24, 48, and 72 h at 37°C under 5% CO_2 and cell proliferation determined using Cell Proliferation Kit I (MTT, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. 80% KGM/20% EGM was found to support the growth of both HGEC and LEC and were used in subsequent experiments involving conditioned medium or coculture.

2.3 | Stimulating HGEC with LPS or inflammatory mediators

For dose–response assays, 50×10^3 HGEC were plated in 24-well plates and cells were allowed to adhere for 4 h. Cells were then stimulated by fresh medium containing lipopolysaccharide (LPS) or proinflammatory cytokines in the following concentrations: 10 µg/mL, 1 µg/mL, or 0,1 µg/mL *E. coli* O111:B4 LPS (Sigma-Aldrich, L4391-1MG) or *P. gingivalis* LPS (Sigma-Aldrich, SMB00610); 100 ng/mL, 10 ng/mL, or 1 ng/mL recombinant human IL-1 beta/IL-1F2 Protein (R&D Systems, 201-LB) or 500 ng/mL, 50 ng/mL, or 5 ng/mL recombinant human IL-6/IL-6 R alpha protein chimera (R&D, 8954-SR/CF). Only fresh medium was added to negative control. Levels of vascular growth factors (VEGF-C, VEGF-A, and VEGF-D) in cell supernatants were determined by DuoSet ELISA (DuoSet, R&D Systems # DY752B, #DY622, #DY293B) following the manufacturer's recommendations.

2.4 | Testing effects of HGEC conditioned medium on LEC proliferation

To characterize the effect of HGEC secreted growth factors on LEC proliferation, and to determine an optimal concentration of HGEC conditioned medium for use in assays, LEC were grown in various concentrations of conditioned medium and cell proliferation was determined. To prepare conditioned medium, 75×10^4 viable HGEC were seeded in KGM in a T75 flask and allowed to grow to 80% confluence. Medium was removed and replaced by 80% KGM/20% EGM with no added VEGF-A or FGF2. HGEC were allowed to condition the medium for 24 h. Concentrations of vascular growth factors in conditioned medium were determined by ELISA as described above. This conditioned medium will be referred as CM in the following experiments. To identify additional cytokines and growth factors present, CM collected from two independent cultures was also screened by a Proteome Profiler Array Kit (ARY022B) Human XL Cytokine Array Kit, a membrane-based sandwich immunoassay that can simultaneously detect 105 human cytokines following the manufacturer's instruction.

For the proliferation assay, LEC were seeded in 96-well plates (10^4 cells/well) in 100 µL EGM, and cells were given 4 h to adhere. Medium was removed and replaced by CM (100%) or CM diluted in 80% KGM/20% EGM into 90%, 80%,70%, 60%, 40%, and 20% concentrations. Nonconditioned 80% KGM/20% EGM was added to negative control wells. Cells were stimulated for 24, 48, and 72 h at 37°C, 5% CO_2. Cell proliferation in the different concentrations of CM or control medium was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay System (Promega) following the manufacturer's instructions.

2.5 | Scratch migration assay

To test whether CM from HGEC stimulates the migration of LEC, a scratch assay was performed. In brief, 10^4 LEC/well were seeded in 96-well plates, 10 wells per treatment. Cells were cultured in EGM (Lonza) at 37°C/5% CO_2 and allowed to grow to 100% confluence. A single scratch was made in each confluent cell layer using a 200 µL pipette tip. Cells were washed once in 80% KGM/20% EGM and images of the lesion were captured at 10× magnification at time 0 (initial) using a Zeiss Primo Vert™ inverted microscope (Carl Zeiss Microscopy, Berlin, Germany). Medium was then replaced by either 80% KGM/20% EGM (negative control), 80% KGM/20% EGM w/ 1(X) Positive Control Angiogenesis Supplement (# 634968, Merck) containing VEGF-A and FGF2 and 20 ng/mL VEGF-C (positive control), or CM diluted in 80% KGM/20% EGM to 50% or 80%. Cells were incubated at 37°C/5% CO_2. Images were captured again after 24, 48, and 72 h at 10× magnification. Uncovered area at T0 and after 24 h and 96 h was measured using ImageJsoftware (National Institute of Health).

2.6 | Tube formation assay

Real-time tube formation assays were performed in µ-slides (Ibidi, Alsace, FR) coated with 10 µL growth factor reduced Matrigel (9.4 mg/mL)(Corning, NY, US). LEC were labelled with cell tracker red dye (10 µM, Invitrogen, Carlsbad, US) and were seeded in the gel at a density of 10^4 cells/well. LEC were either (1) cocultured with HGEC labelled by green CMFDA dye (10 µM, Invitrogen) in different ratios [1:1 and 2:1] or (2) cultured in the presence of CM further diluted in 80% KGM/20% EGM to 50% or 80%. LEC cultivated in their growth medium supplemented with 800 ng/mL recombinant human VEGF-C and 400 ng/mL recombinant human bFGF were used as positive control and LEC grown in 80% KGM/20% EGM without lymphangiogenic growth factors were used as negative control. Tube formation was assessed in three different fields in each well by time-lapse imaging using Andor Dragonfly fluorescence microscope (Leica, Wetzlar, DE). The experiment was repeated two times and images shown are representation of the experiment at different time points (Figure 5). Tube forming capacity of LEC was assessed by counting number of enclosed meshes and area covered by mesh after 5 and 10 h after seeding, using angiogenesis analyzer plugin in ImageJ (Fiji, NIH-US).

2.7 | Gingival sections from periodontitis patients

Subjects were recruited amongst patients (>18 years of age) referred to the Oral Health Centre of Expertise, Western Norway. Gingival tissue was collected from patients undergoing periodontal surgery as part of their treatment scheme and buccal oral mucosa from healthy patients during surgery of impacted molars. The study protocol was approved by the Regional Ethical Committee (REK Vest project number 19621) and followed the Helsinki Declaration. All patients read and signed a written consent prior to enrolment in the study. Collected gingival tissue was fixed in formalin overnight. Tissue used for paraffin sections underwent graded dehydration, paraffin embedding, and was sliced into 4 or 10 µm sections.

2.8 | Immunohistochemical staining of human gingival sections

Vascular structures, vascular growth factors, and proliferating cells were detected in human sections by immunohistochemistry. For paraffin sections, heat-induced epitope retrieval was performed by immersing slides in either Citrate Buffer, pH 6.0, 10×, Antigen Retriever (Sigma-Aldrich) or Tris-EDTA buffer pH 9.0 (Abcam) kept at 97°C for 20 min (according to antibody). After antigen retrieval, sections were washed, and nonspecific binding blocked by incubation with serum corresponding to the host species of the secondary antibody. Primary antibodies used were mouse anti-podoplanin, D2-40

antibody (DAKO, #M3619), and rabbit polyclonal VEGF-C antibody (Abcam, #ab9546). Gingival tissue sections were incubated with the unconjugated primary antibodies overnight at 4°C. Slides were then washed and secondary antibody added.

For immunohistochemical detection, antigen-specific binding was detected by a streptavidin–biotin–peroxidase (ABC) method, using Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Abcam) or Mouse specific HRP/DAB (ABC) Detection IHC Kit (Abcam) depending on the host species of primary antibody used, according to the manufacturer's instructions. Finally, sections were counterstained by Hematoxylin Solution (Mayer's, Modified) (Abcam, #ab220365) dehydrated in a graded alcohol series, cleared with xylene, and mounted in Histokitt mounting medium (Karl Hecht GmbH & co). Sections were examined by light microscopy using a Nikon Eclipse E600 microscope (Nikon Corporation) and Nikon image software (NIS-Elements AR3.22).

2.9 | Statistical analysis

Statistical analyses and graphing of results were performed using GraphPad Prism (Version 8.1.2 (332) GraphPad Software Inc., La jolla, CA). Results are reported as mean levels, and variability is reported as standard error of the mean. Data were tested for normal distributions by D'Agostino–Pearson normality test and then analyzed by (nonparametric) Kruskal–Wallis test followed by Dunn's multiple comparisons test or (parametric) one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons when significant differences were found. $^{*}p<.05$, $^{**}p<.01$, $^{****}p<.0001$.

3 | RESULTS

3.1 | HGEC produce vascular growth factors under control condition and in response to microbial and inflammatory stimuli

Cell culture supernatants from HGEC exposed to microbial- or inflammatory stimulus at various doses were analyzed for VEGF-A, VEGF-C, and VEGF-D. VEGF-A, VEGF-C, and low levels of VEGF-D were found in all culture supernatants from HGEC grown in negative control medium, showing that these growth factors are constitutively expressed by HGEC in vitro (Figure 1).

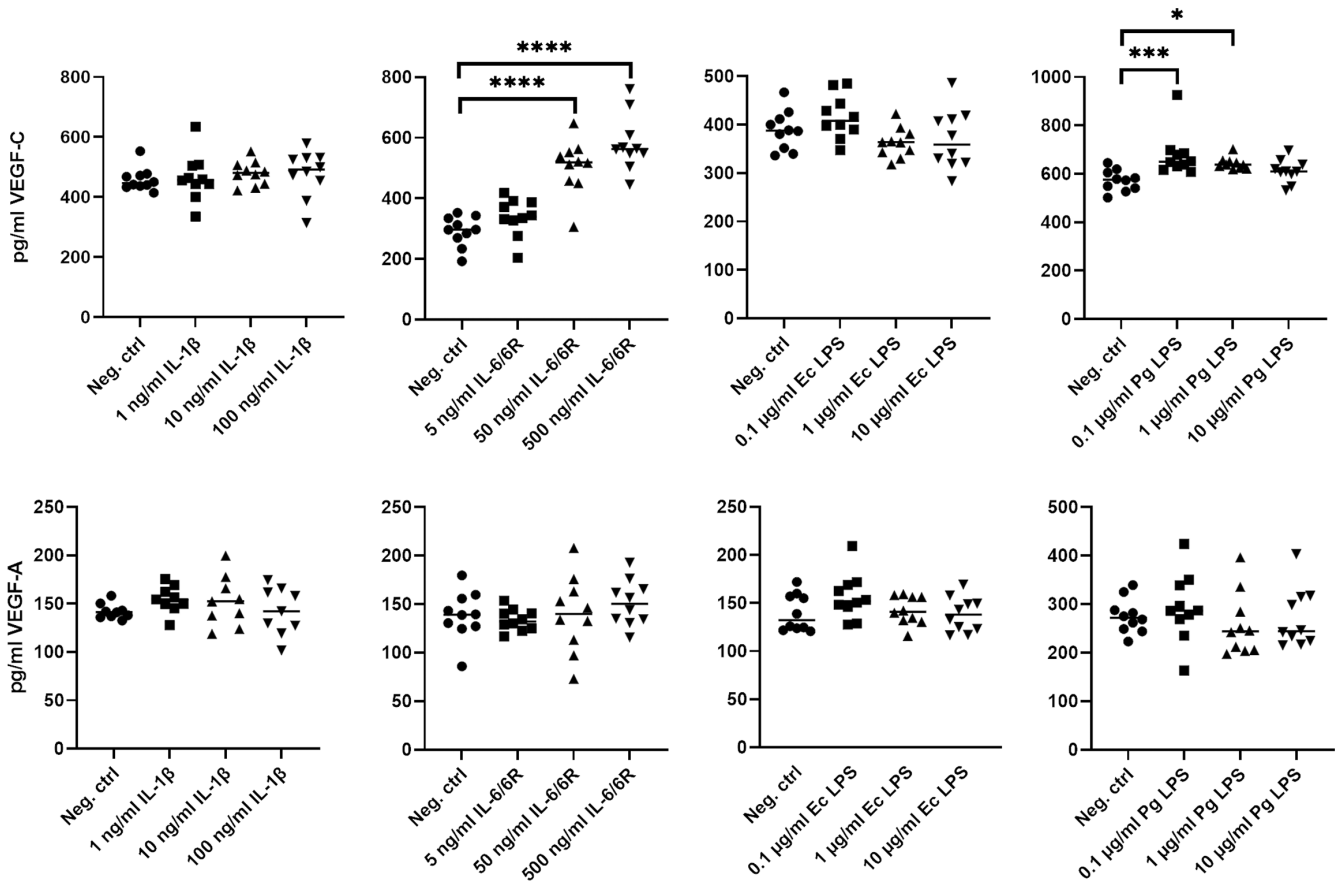

FIGURE 1 VEGF-C, VEGF-D, and VEGF-A levels in supernatant of HGEC stimulated by proinflammatory mediators or LPS. Bars represent mean levels of growth factors in supernatants of 10 parallel wells per treatment after 24 h of stimulation. One-way ANOVA followed by Dunnett's post hoc test was applied to evaluate differences among groups. ($^{*}p<.05$, $^{***}p<.001$, $^{****}p<.0001$). VEGF-C data are representative of two independent experiments.

VEGF-C was significantly upregulated in response to IL-6/IL6R while VEGF-D was downregulated in response to IL-1β in a dose-dependent manner. VEGF-C was also upregulated in response to *P. gingivalis* LPS, with the most pronounced effect after exposure to the lowest dose. In contrast, LPS from *E. coli* O111:B4 did not affect VEGF-C expression in HGEC. VEGF-A was not regulated in response to any proinflammatory mediators (Figure 1).

3.2 | Testing medium combinations for HGEC and LEC

To identify a growth medium that can sustain growth of both HGEC and LEC in coculture, both cell types were cultivated in their standard medium (KGM or EGM, respectively) or in medium combinations, and cell proliferation was measured after 24, 48, and 72 h. No significant difference in proliferation was found between HGEC cultivated

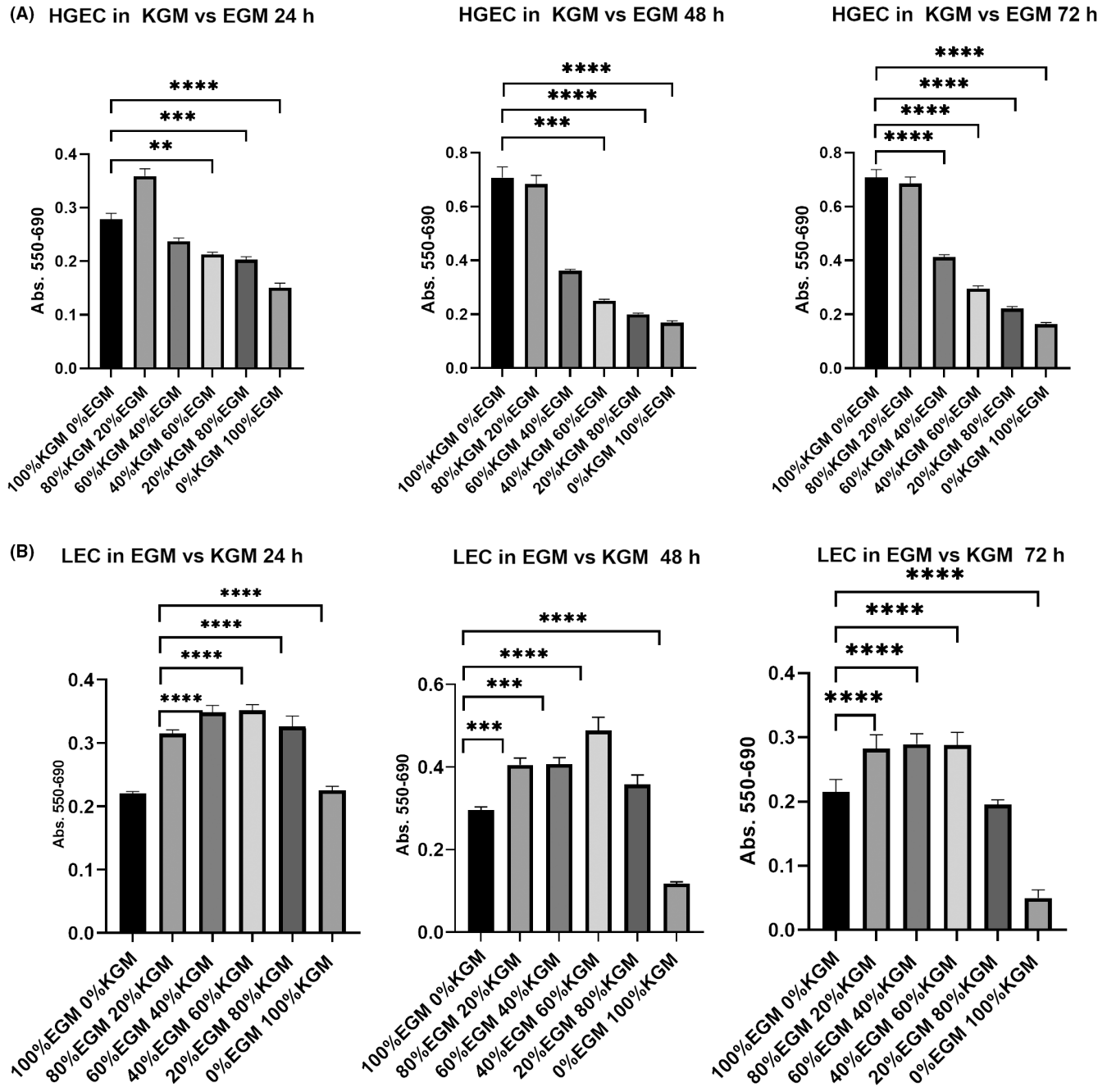

FIGURE 2 Testing medium combinations for HGEC and LEC to be used for CM or coculture. Proliferation tests of HGEC (A) and LEC (B) cultivated in different combinations of endothelial cell growth medium (EGM) and keratinocyte growth medium (KGM) for 24, 48, and 72 h. Graphs show mean values of eight biological replicates. Error bars represent SEM. (**$p < .01$, ***$p < .001$, ****$p < .0001$) Kruskal–Wallis with Dunn's multiple comparison test or one-way ANOVA with Dunnett test.

in 100% KGM and HGEC cultivated in KGM with 20% EGM medium after 24, 48 or 72 h. At higher concentrations of EGM, HGEC proliferation was reduced over time (Figure 2A). Increased LEC proliferation was seen at 24–72 h when EGM was replaced by 20%–60% KGM. 80% KGM resulted in increased proliferation after 24 h and no significant changes in growth after 48–72 h, whereas replacing EGM with 100% KGM resulted in reduced growth over time (Figure 2B). Both cell types grew well in 80% KGM with 20% EGM up to 72 h, and this combination was used in subsequent coculture and to produce CM by HGEC.

3.3 | CM from HGEC stimulates LEC proliferation

To determine whether secreted factors from HGEC stimulate LEC proliferation and to identify an optimal concentration of CM to use in LEC assays, proliferation assay of LEC cultured in CM at various concentrations was performed. 100% CM from HGEC significantly reduced the proliferative response of LEC after 24, 48, and 72 h suggesting that keratinocytes had depleted nutrients from the medium. Diluted CM induced higher proliferation than negative control at concentrations between 10% and 90% after 48 h and between 30% and 80% after 72 h (Figure 3). Stimulatory effect of CM was stronger than that positive control medium containing a combination of VEGF-A, FGF2, and VEGF-C. Levels of VEGF-A, VEGF-C, and VEGF-D were determined in CM of eight independent wells by ELISA. VEGF-A and VEGF-C were detected in all CMs: mean level of 378.6 ± 33.50 pg/mL for VEGF-A and 2830 ± 462.6 pg/mL for VEGF-C. VEGF-D was present at very low levels (1.4 ± 1.2 pg/mL). To identify additional cytokines, chemokines, and growth factors in the CM potentially contributing to proliferation effects and/or migratory effects, undiluted CM was screened using a cytokine array kit. This approach allowed the simultaneous detection of 15 different cytokines/chemokines/growth factors including several proteins with previously reported stimulatory or inhibitory effects on vascular growth (Figure S1, Table S1).

3.4 | HGEC conditioned medium stimulates LEC migration

A scratch migration assay showed that LEC migration was significantly increased in LEC stimulated by 80% or 50% HGEC CM compared with cells cultivated in unconditioned medium after 24 and 48 h. CM stimulated migration activity to a greater extent than the positive control containing VEGF-C, VEGF-A, and FGF2 (Figure 4).

3.5 | HGEC support LEC tube formation through direct interactions and soluble factors

Formation of capillary-like structures (LEC-meshes) was seen in the presence of HGEC or CM. A tube formation assay showed that LEC cultivated in the presence of VEGF-C and FGFb (positive control medium), co-cultivated with HGEC, or cultivated in the presence of CM

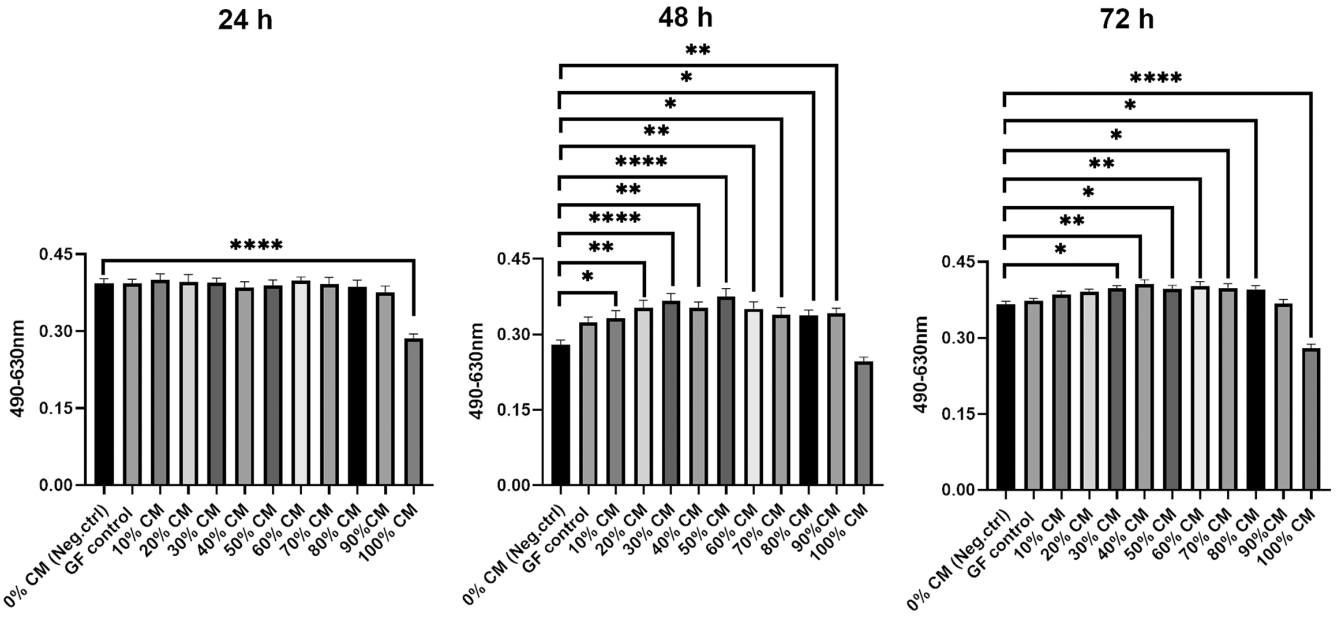

FIGURE 3 Effects of different concentrations of CM on the proliferation of LEC. To determine an optimal dilution of CM to use in LEC assays, and to determine the effect of CM on LEC proliferation, LECs were cultivated in different dilutions of CM for 24, 48, or 72 h before cell proliferation was determined by colorimetric method (MTS Assay). Graphs show mean values of eight biological replicates. Error bars represent SEM. (*p<.05, **$p < .01$, ***$p < .001$, ****$p < .0001$) Kruskal–Wallis with Dunn's multiple comparison test or one-way ANOVA with Dunnett test. Data are representative of two independent experiments.

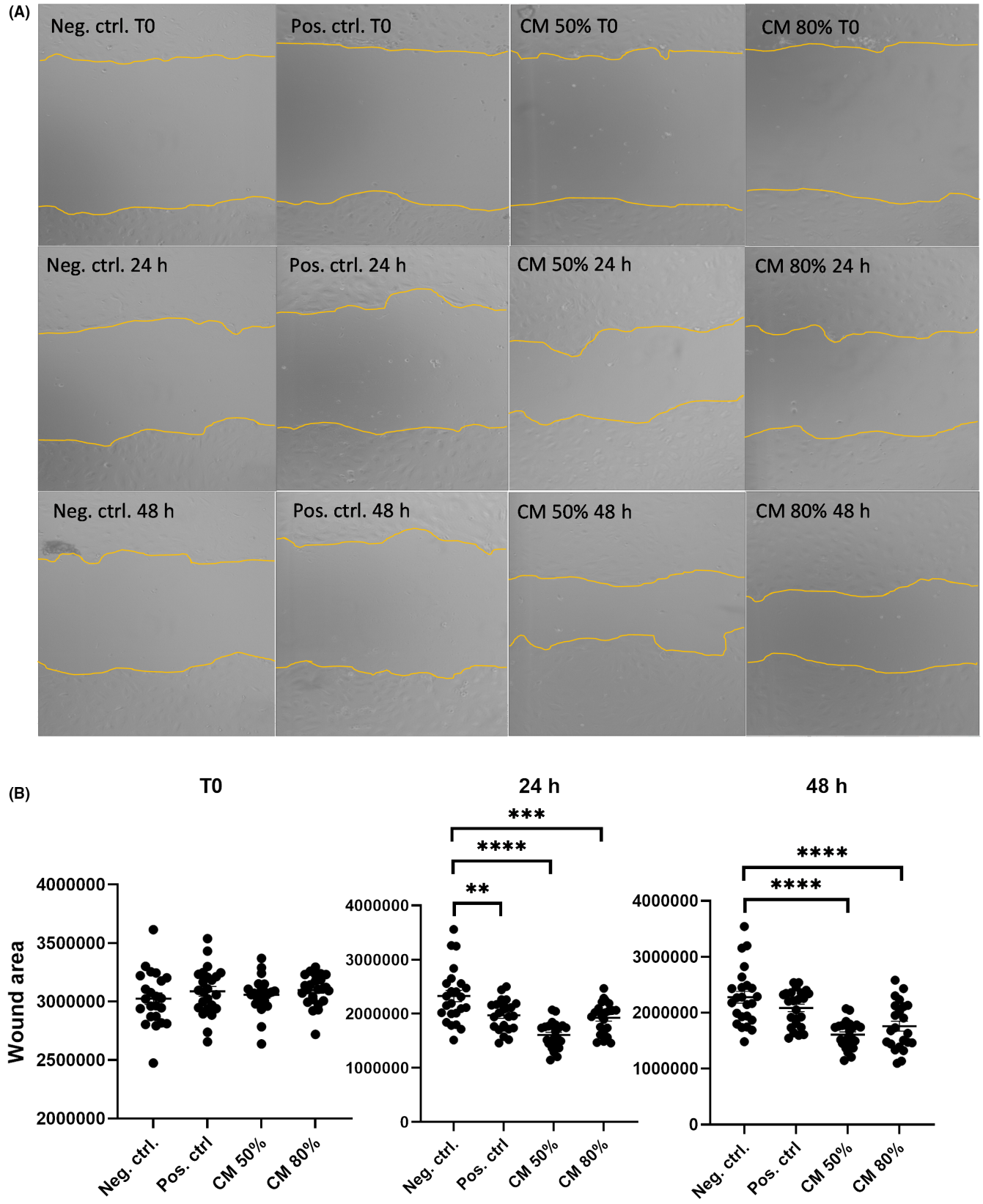

FIGURE 4 Scratch migration test for lymphatic endothelial cells. All LEC were seeded in EGM medium and allowed to grow until confluent. Cells were washed and medium replaced with 80% KGM/20% EGM without added growth factors (Neg. ctrl), 80% KGM/20% EGM containing VEGF-A, FGF-2, and VEGF-C (Pos. ctrl), or HGECs CM diluted to 50% or 80% with 80% KGM/20% EGM. (A) Edges of the scratch was indicated (in yellow) and uncovered area measured using the ImageJ software. Area is provided as a dimensionless number. (B) One-way ANOVA followed by Dunnett's post hoc test was applied to evaluate differences among groups. (**$p < .01$, ***$p < .001$, ****$p < .0001$). Data are representative of two independent experiments.

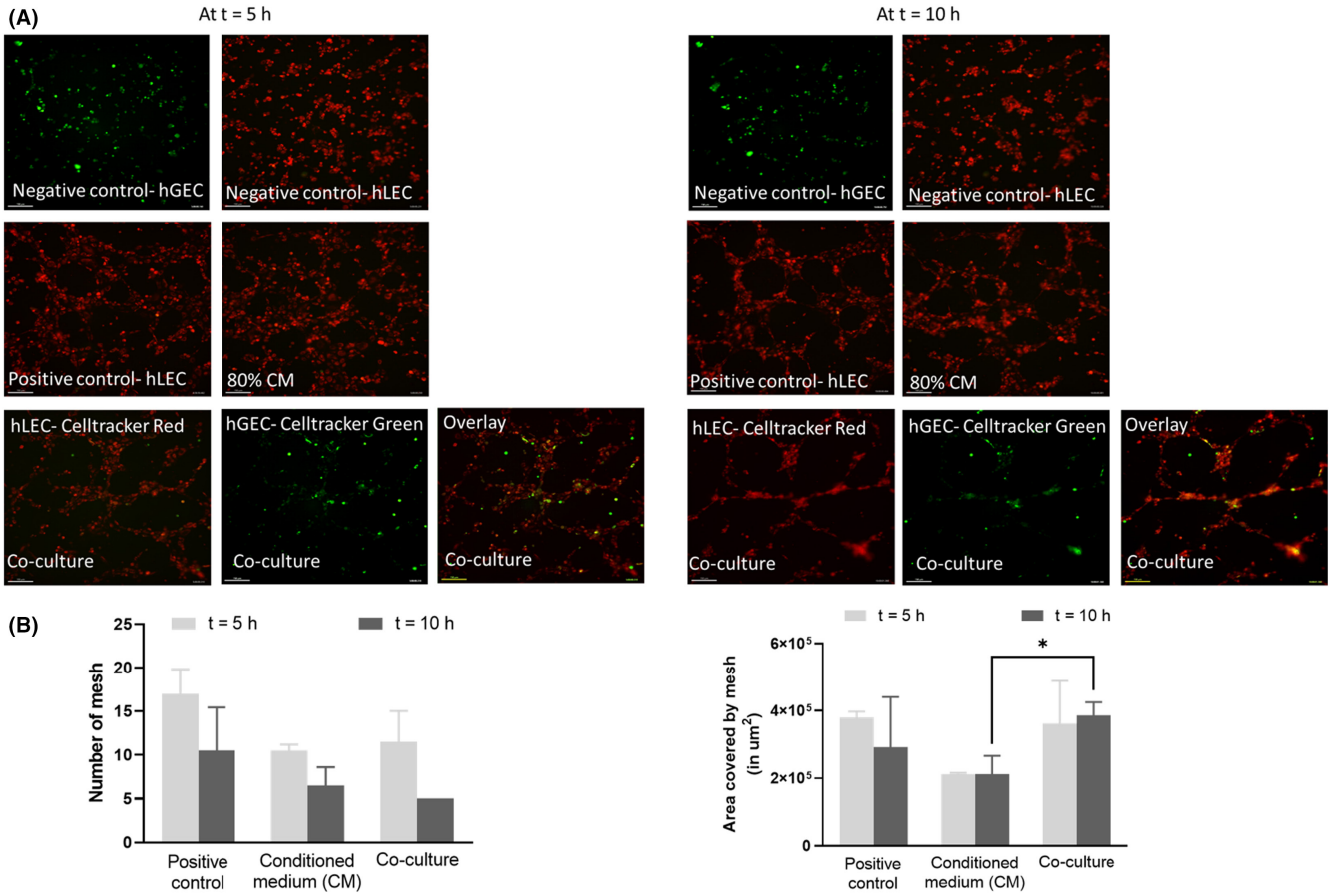

FIGURE 5 LEC tube formation assay. Images show effects of HGEC CM, and cocultivating LEC with HGEC on LEC tube formation. LEC were either cultivated in positive control medium with added VEGF-C and FGF2, negative control medium without added growth factors, CM diluted to 80%, or in the presence of HGEC. LEC were labelled in red, HGEC in green. (A) Representative images of LEC tube formation after 5 and 10 h. (B) Graphs show area covered by mesh number in positive control medium, in CM, and in coculture after 5 and 10 h (right). LEC did not form closed meshes in the presence in negative control medium without added vascular growth factors, and negative control is therefore not included in the graph. (*$p < .05$). Data are representative of two independent experiments.

from HGEC formed enclosed meshes of vessel-like structures over time, while LEC cultivated in medium without added growth factors or HGEC present did not form such structures. LEC cultivated in the presence of HGEC formed fewer meshes, but longer, narrower tubes than positive control cells (Figure 5).

3.6 | Lymphatic vessels are closely associated with VEGF-C$^+$ oral epithelium in chronically inflamed gingiva

Podoplanin$^+$ lymphatic vessels were found in lamina propria (LP) extending into gingival papilla. Localization of lymphatic vessels in close contact with epithelium was found in both chronically inflamed gingiva (Figure 6) and in healthy control (data not shown).

Immunostaining of VEGF-C showed expression in vessels and some stromal cells in LP as well as in the epithelium. The strongest immunostaining was in the basal part of the epithelium and in the pocket epithelium. (Figure 6).

4 | DISCUSSION

4.1 | HGEC expression of vascular growth factors

Here, we show that the growth factors VEGF-A, VEGF-C, and low levels of VEGF-D are constitutively expressed by HGEC in culture, suggesting a potential regulatory role under non-inflammatory conditions.

Whether vascular growth factors are regulated in response to inflammatory insults or microbial ques in HGEC has not previously been investigated. To test the responses of HGEC under inflammatory conditions, we stimulated them with IL6/IL6R, IL-1β, and *P. gingivalis* LPS. Proinflammatory cytokines including IL-1β and IL-6 play a prominent role in the development of bacteria-induced periodontal bone resorption.[13,14] High levels of IL-1β in gingival crevicular fluid (GCF) have been associated with disease progression and severity, and its levels are reduced after periodontal therapy.[15–17] Similarly, elevated levels of IL-6 have been demonstrated in the GCF and saliva of periodontitis patients compared with healthy controls.[18,19]

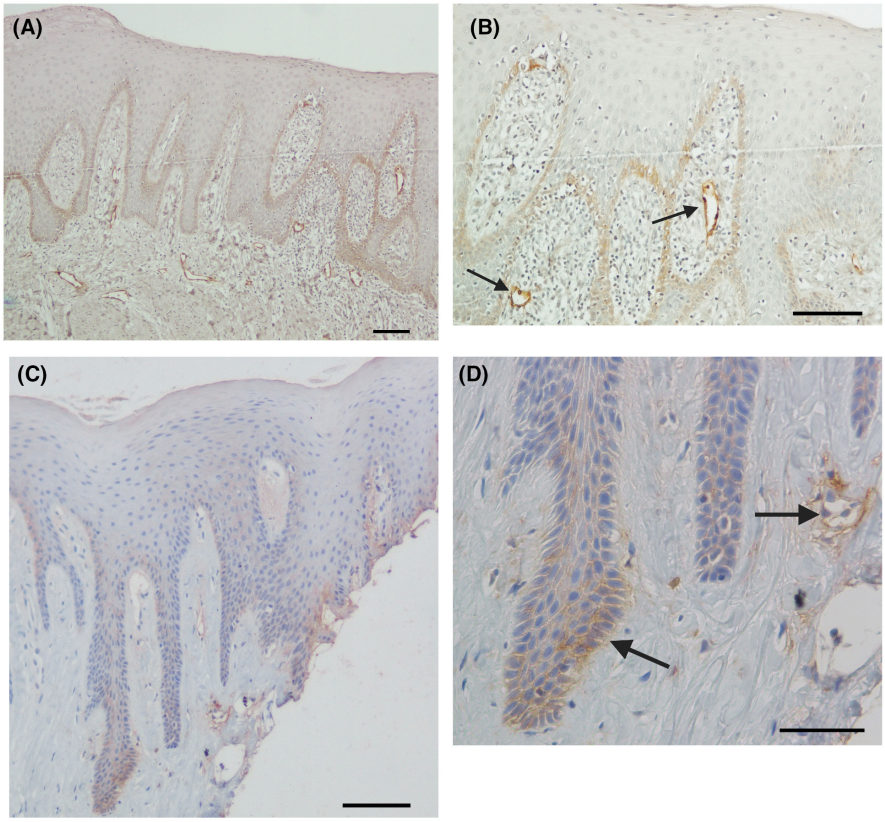

FIGURE 6 Immunohistochemical staining of inflamed gingiva. (A + B) Podoplanin$^+$ lymphatic vessels (arrows, B) located in connective tissue papilla in inflamed gingiva surrounded by infiltrates of immune cells. (C + D) VEGF-C expression (arrows, D) in gingival epithelium was strongest in the basal part of epithelium and was also found in vessel walls. Bar represents 100 μm (A, B), 200 μm (C), and 50 μm (D). B + D represents higher magnification of area in A + C, respectively.

VEGF-C and VEGF-D are structurally and functionally closely related,[20] but their expression was differentially regulated in response to proinflammatory cytokines in HGEC cultures. IL-6/IL-6R induced upregulation of VEGF-C, but not VEGF-D, whereas only VEGF-D expression was reduced in response to IL-1β. A negative regulatory effect of IL-1β on VEGF-D expression has previously been noted in cardiac microvascular endothelial cells, mediated through MAPKs and PKCalpha/beta1 signaling.[21] While the expression of VEGF-A, a key regulator of angiogenesis has previously been shown to be regulated in response to IL6/IL6R and *E. coli* LPS exposure in gingival fibroblasts,[11] HGEC did not respond to inflammatory insults by increased expression of VEGF-A. This finding is in line with previous results from Kasnak et al., 2019 [22] showing that *P. gingivalis* LPS fail to modify VEGF-A expression in HGEC.

The gingival epithelium is constantly exposed to both mutualistic microbes and opportunistic pathogenic oral bacteria such as *P. gingivalis*. *P. gingivalis* is considered major etiological agent of chronic periodontitis and is highly prevalent in periodontal pockets.[23] LPS, a major constituent of the outer membrane of gram-negative bacteria, is important for this periodontal pathogen's virulence.[24] Interestingly, we found increased VEGF-C production in gingival epithelial cells in response to LPS from *P. gingivalis*, but not in response to *E. coli* LPS. *P. gingivalis* LPS and *E. coli* LPS have structural differences affecting their immune stimulatory capacities.[24] The two have previously been shown to modulate the surface expression of LPS binding pattern recognizing receptors (CD14, TLR2, and TLR4) and cytokine production in human gingival fibroblasts and monocyte cell lines, in a different way.[25–27]

In summary, VEGF-C was upregulated in response to both inflammatory and microbial stimulation in HGEC, proposing a functional role for the epithelium in supporting lymphatic vessel expansion during gingival infection and inflammation. Our results confirm the ability of gingival epithelial cells to differentiate between different pathogen-associated molecular patterns to produce microbe-specific responses in expression of vascular growth factors.

4.2 | LEC responses

Lymphangiogenesis on the cellular level requires proliferation, migration, and tube formation of LEC. Having established that HGEC produce lymphangiogenic growth factors, we wanted to investigate whether secreted factors from gingival epithelial cells or direct interactions between HGEC and LEC may contribute to regulate LEC functions necessary for lymphangiogenesis in vitro. Initial tests showed that both HGEC and LEC grow well in a combination of 80% keratinocyte growth medium and 20% endothelial cell growth medium and this combination was used for CM. CM from gingival epithelial cells stimulated both the proliferation and migration of LEC to a higher extent than control medium containing VEGF-A, FGF2, and VEGF-C. Undiluted CM repressed LEC proliferation, suggesting that the HGEC had depleted the medium of nutrients necessary for LEC growth. However, diluted conditioned with fresh medium significantly improved LEC proliferation and growth compared with unconditioned medium. Furthermore, both

CM and direct interactions between HGEC and LEC stimulated LEC tube formation. In addition to lymphangiogenic growth factors of the VEGF family, several cytokines/chemokines and growth factors with previously reported pro- and antilymphangiogenic properties, were detected in the CM and may have further contributed to the stimulatory effect of HGEC on LEC migration, proliferation, and tube formation.

4.3 | Human gingival sections from periodontitis patients

While we could establish that HGEC express vascular growth factors, it is not clear what the function of this production is in the local environment in vivo. To evaluate the results from cell models in a biologically relevant context, we immune-stained human gingival sections from patients suffering from periodontal disease for the main lymphatic growth factor VEGF-C, and the lymphatic marker podoplanin.

Lymphatic vessels were found closely associated with the basal part of gingival epithelium in human tissue. A close association between lymphatic vessels and gingival epithelium has previously been described in murine models[28] and was confirmed in human tissues in this study, supporting a possible regulatory function for the epithelium on adjacent lymphatic vessels. What is more, VEGF-C expression was found in the basal epithelial layer further supporting that lymphangiogenic signaling may occur between epithelium and underlaying tissues and may be maintained by inflammatory ques from infiltrating immune cells. However, the expression of VEGFR3, the main receptor for VEGF-C and VEGF-D in basal epithelial cells of normal gingiva[11] and inflamed gingiva opens the possibility that VEGF-C and VEGF-D, may also have autocrine functions in the epithelium itself.

5 | CONCLUSIONS

Our results showed that the expression of vascular growth factors was regulated in response to inflammatory insults in gingival epithelial cells. Furthermore, we showed that gingival epithelial cells supported LEC proliferation, migration and tube formation, cellular functions important for lymphangiogenesis. VEGF-C expression in the epithelium was also found in sections from chronically inflamed gingiva from periodontitis patients. Results suggest that gingival epithelial cells may play a role in supporting lymphatic function during inflammation.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest in this project.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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