Role of gingival lymphatics in tissue fluid balance
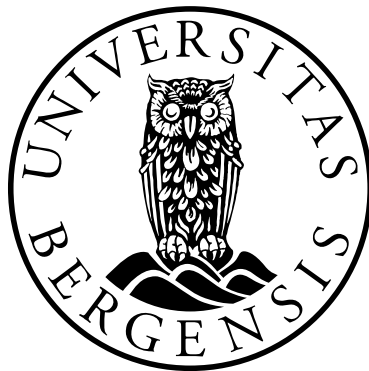
and periodontal disease development

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Abstract

Background: The gingiva is frequently challenged to oral bacteria which are able to induce inflammatory responses with increased fluid filtration and edema formation in the tissue. Under given circumstances periodontal pathogens can also promote periodontal disease characterized by bone resorption. Lymphatic vessels drain fluid from the interstitium and transport lymphocytes and antigen-presenting toward lymph nodes to elicit immune responses. In gingiva, lymphatic vessels are found, but their function in transcapillary fluid balance and their role in periodontal disease development was hitherto unknown.

Methods and Results: We demonstrated that the mouse model of lymphedema, the K14-VEGF receptor 3-Ig (K14) mice, lacked gingival lymphatic vessels and had an almost absence of lymphatics in the mucosal layer of alveolar mucosa. We therefore used this transgenic mouse model to investigate the role of gingival lymphatics intranscapillary fluid balance in steady state situation and after perturbation of the tissue. The gingiva of K14 mice had significantly higher interstitial fluid pressure (Pif) in normal situation and after inflammation-induced by lipopolysaccharide (LPS) from *Porphyromonas gingivalis (P. gingivalis)* compared with wild type (WT) controls. Overhydration caused more than 75% increase in interstitial fluid volume (IFV) 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 during edema formation. In the alveolar mucosa, no strain differences were observed in Pif and IFV in any situation, suggesting that mucosal lymphatics are not critical for tissue fluid regulation.

 Using WT mice, we further demonstrated that lymphatic growth take place in gingiva after *P. gingivalis* oral gavage. The lymphatics area fraction was increased in infected mice at both 10 and 42 days postinfection and modest bone loss was verified in the longest observation period, whereas proliferation of vessels was observed only in the shortest observation period. Higher numbers of immune cells expressing

vascular endothelial growth factor (VEGF)-C along with upregulation of IL-1β and TNF-β at protein levels were found in infected mice 10 days after infection.

We also demonstrated that K14 mice developed significantly more bone loss 42 days after infection with *P. gingivalis.* The mutant mice had also higher level of G-CSF, IL-1β and IFN-γ in periodontal tissues as well as increased number of macrophages and antigen presenting cells in bone resorptional areas compared to WT mice. However, significantly lower plasma level of *P. gingivalis* specific IgG was found in infected K14 mice compared to infected WT littermates. Our data demonstrate that *P. gingivalis* infection induced a strong periodontal inflammatory response accompanied by a weakened systemic humoral B-cell response in K14 mice.

Conclusion: Gingival lymphatics are crucial for transcapillary fluid balance in the steady-state condition and during acute perturbation. Lymphangiogenesis takes place in gingiva during periodontal disease development and up-regulation of VEGF-C in recruited immune cells is likely important for the growth of lymphatic vessels. Moreover, gingival lymphatic vessels protect against *P. gingivalis* induced periodontitis, probably by enhancing clearance of bacterial products and promoting humoral immune responses.

Acknowledgements

Finally, I thank God for keeping me strong and healthy throughout this journey and for surrounding me with such wonderful people.

Abbreviations

ABC   Alveolar bone crest

BSA   Bovine serum albumin

b.w.   Body weight

CEJ   Cementum–enamel junction

CFC   Capillary filtration coefficient

CMC   Carboxymethylcellulose

COP_{if} Colloid osmotic pressure in interstitial fluid

COP_c Colloid osmotic pressure in the capillary

^{51}Cr Radioactive chromium

cpm   Counts per minute

EDTA   Ethylenediaminetetraacetic acid

ELISA   Enzyme-linked immunosorbent assay

GM-CSF   Granulocyte-macrophage colony stimulating factor

HSA   Human serum albumin

^{125}I Radioactive iodine

IF   Interstitial fluid

IFV   Interstitial fluid volume

IFN-γ   Interferon-gamma

IL   Interleukin

i.p.   Intraperitoneally

IP-10   Interferon gamma-induced protein 10

i.v   Intravenously

J_v Transcapillary fluid flux

KCl   Potassium chloride

L   Lymph flow

LPS   Lipopolysaccharide

LYVE-1   Lymphatic vessel endothelial hyaluronan receptor

MIP-1   Macrophage Inflammatory Proteins- 1

MMP   Matrix metalloproteinases

M-CSF   Macrophage colony stimulating factor

NaCl   Sodium chloride

NF-κB   Nuclear factor-kappa B

NK   Natural killer cells

NO   Nitric oxide

OPG   Osteoprotegerin

PBS   Phosphate buffered saline

P_c Capillary blood pressure

PCR   Polymerase chain reaction

PDL   Periodontal ligament

P_{if} Interstitial fluid pressure

RANK   Receptor activator of nuclear factor-kappa B

RANKL   Receptor activator of nuclear factor-kappa B ligand

RANTES   Regulated on Activation, Normal T cell Expressed and Secreted

TBS   Tris-buffered saline

TGF   Transforming growth factor

Th   T helper lymphocytes

TLR   Toll-like receptor

TNF-α   Tumor necrosis factor-alpha

TRAP   Tartrate Resistant Acid Phosphatase

VEGF   Vascular endothelial growth factor

VEGFR   Vascular endothelial growth factor receptor

Vv   Intravascular fluid volume

Vx   Extracellular fluid volume

σ   Capillary reflection coefficient

List of publications

Paper I: 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 2010, 299:H275-283

Paper II: Mkonyi LE, Bakken V, Sovik JB, Mauland EK, Fristad I, Barczyk MM, Bletsa A, Berggreen E: **Lymphangiogenesis is induced during development of periodontal disease,** J Dent Res 2012, 91:71-77

Paper III: Mkonyi LE, Bletsa A, Bolstad AI, Bakken V, Wiig H, Berggreen E: **Gingival lymphatic drainage protects against *Porphyromonas gingivalis* induced bone loss in mice**, *Manuscript*.

Contents

1. INTRODUCTION

1.1 The gingiva

The periodontal tissues (periodontium) represent the supporting structures of the teeth (Bartold, Walsh et al. 2000). Tissues which form periodontium are gingiva, periodontal ligament, cementum and alveolar bone Fig. 1.

Gingiva which is also part of the masticatory mucosa surrounds the cervical neck of the tooth and is divided into *free gingiva* and *attached gingiva.* The *free gingiva* is relatively mobile and extends from the gingival margin to the base of gingival sulcus. The *attached gingiva* is firmly attached to the tooth and alveolar bone by dense collageneous fiber bundles and forms the bulky portion of the gingiva. The *attached gingiva* extends apically from *free gingiva* to merge with mucosa of the hard palate on the palatal aspect or adjacent alveolar mucosa on the vestibular and lingual aspect. The interdental gingiva is *free gingiva* that occupies the space between the adjacent teeth (Schroeder and Listgarten 1997).
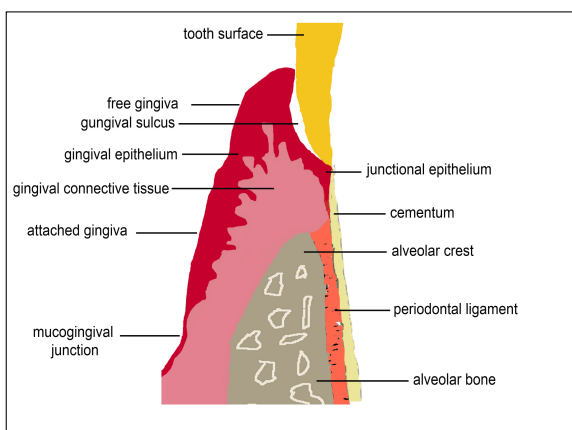
Figure 1. Schematic illustration of the periodontium. Modified from
http://www.dental.pitt.edu/informatics/periohistology/en/gu0102.ht)

The gingival epithelium is divided into oral epithelium, sulcus epithelium and junctional epithelium. The oral epithelium is a stratified, squamous epithelium comprised of layers of cells which lose their nuclei as they grow toward the surface. It lines the external surfaces of the gingiva and forms a physical barrier to oral bacteria. The sulcus epithelium is a parakeratinized stratified squamous epithelium that lines the lateral surface of the gingival sulcus. The junctional epithelium is a nonkeratinizing stratified epithelium in contact with the sulcus epithelium and thegingival connective tissue (Fig.1), and it forms the floor of the gingival sulcus (Schroeder and Listgarten 1997). The cells of the junctional epithelium have wide intercellular spaces between them, which makes the junctional epithelium more permeable than gingival and sulcus epithelium. As a results the junctional epithelium is a preferential route for bacteria/bacterial products from sulcus to the gingival connective tissue, and for fluid from the connective tissue to the sulcus (crevicular fluid) (Tanaka 1984). These morphological characteristics of the junctional epithelium make the gingival tissue highly susceptible to external agents that can lead to inflammation (Marchetti, Poggi et al. 1999).

The gingival connective tissue, also known as lamina propria, is found underneath the epithelium. The lamina propria is made of a network of dense collagen fiber bundles; the supra-alveolar fiber apparatus. The fiber apparatus, which is mainly composed of collagen Type I and III, provides gingival attachment to the tooth and alveolar bone and gives gingiva the rigidity and resilience against masticatory forces (Narayanan and Page 1983). In the connective tissue lies the ground substance, fibroblasts, immune cells such as lymphocytes, macrophages and some plasma cells (Schroeder and Listgarten 1997). In addition, numerous blood vessels and also lymphatic vessels are present in the tissue (Marchetti, Poggi et al. 1999). The blind- ended lymphatics travel in the lamina propria toward the alveolar crest and pass along the external surface of the alveolar bone (Marchetti, Poggi et al. 1999; Ushijima, Inoue et al. 2008), as demonstrated in Fig. 2.
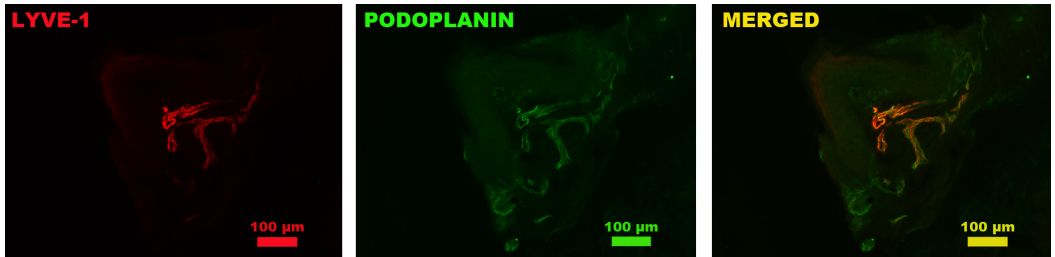
Figure 2. **Localization of lymphatic vessels in gingiva in WT mice. The lymphatic vessels were identified by double labeling with Lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and podoplanin antibodies, both markers for lymphatic endothelium.**

1.2 Lymph drainage

Lymphatic vasculature maintains tissue homeostasis by carrying fluid and protein from the interstitial space and returns them to the blood circulation and is also a trafficking route for extravasated leukocytes and antigens. Activated antigen-presenting cells travel in lymphatic vessels towards the draining lymph nodes where they initiate adaptive immune responses. Lymphatic vessels are also involved in fat absorption from the digestive system (Alitalo, Tammela et al. 2005; Tammela and Alitalo 2010).

The lymphatic capillaries originate in the interstitium as blind-ended vessels and are the smallest vessels in the lymphatic vasculature. The lymphatic capillaries are made of a continuous single layer of overlapping endothelial cells and lack a continuous basement membrane making them highly permeable. They are attached to the extracellular matrix by anchoring filaments which keep them open during periods of increased interstitial fluid pressure and thus facilitate fluid drainage (Tammela, Petrova et al. 2005; Baluk, Fuxe et al. 2007; Maby-El Hajjami and Petrova 2008). Lymphatic capillaries carry the lymph to the collecting ducts. The collecting ducts unlike the lymphatic capillaries are surrounded by smooth muscle cell layer, have basement membrane and posses valves that prevent lymph backflow. From here the

lymph passes through the chain of lymph nodes and eventually reach the systemic circulation via the thoracic duct (Alitalo, Tammela et al. 2005).

Failure of lymph transport results in accumulation of fluid and protein in the interstitial space with eventual edema formation. The protein- rich fluid in the tissue initiates an inflammatory reaction leading to fibrosis and accumulation of fat. Impairment of lymphatic vessels function also weakens immune responses and increases the host's susceptibility to infection (Rockson 2001; Witte, Bernas et al. 2001; Alitalo, Tammela et al. 2005).

There are several lymphatic endothelial cells specific markers that enable the characterization and tissue localization of lymphatics. Among them are the vascular endothelial growth factor receptor 3 (VEGFR-3) (Kaipainen, Korhonen et al. 1993); podoplanin, a membrane mucoprotein (Breiteneder-Geleff, Soleiman et al. 1999); lymphatic endothelial hyaluronan receptor-1(LYVE-1), a hyaluronic acid receptor (Banerji, Ni et al. 1999) and the prospero-related homeobox gene-1(Prox-1), a transcription factor (Wigle and Oliver 1999). Some of these lymphatic markers have been used this thesis.

1.3 Interstitial fluid pressure and transcapillary fluid balance

Interstitial space is the space that surrounds the cells of tissues. This space has a solid component; the extracellular matrix and a fluid component; the interstitial fluid. The interstitial fluid together with the blood plasma constitutes the extracellular fluid. Interstitial fluid acts as a medium through which nutrients from the blood plasma diffuse and can be taken up by the cells. Furthermore, waste products from the cells pass through the interstitial fluid and are absorbed into the blood capillaries. In this way interstitial fluid allows the communication between the cells and blood plasma which is crucial for cells vitality and normal function (Aukland and Nicolaysen 1981).

Fluid is transported between the blood vessels and the interstitial space. This

fluid flow is governed by Starling forces namely, hydrostatic pressure (P) and colloid osmotic pressure (COP) (Starling 1896). The Starling forces can be summarized in the following equation:

$$(J_v) = CFC \ [(P_c - P_{if}) - \sigma \ (COP_c - COP_{if})] - L$$

The J_v is net fluid flux across the capillary, CFC is the capillary filtration coefficient, P_c is the capillary blood pressure, P_{if} is the interstitial fluid pressure, σ is the reflection coefficient for proteins, COP_c and COP_{if} are the colloid osmotic pressures in plasma and interstitial fluid, respectively and L is the lymph flow.

Under normal situation the P_{if} is lower than the P_c; this pressure difference creates a hydrostatic force which favors filtration of fluid into the interstitial space. On the other hand, since the capillary wall acts as a semipermeable membrane, the plasma proteins are retained inside the vessels. Consequently, the concentration of proteins will increase and create a higher COP_c than COP_{if}, which encourage fluid absorption into the vessels.

CFC is the constant of proportionality and is a product of capillary surface area and capillary hydraulic conductance. A high CFC value indicates that the capillary wall is highly water permeable whereas the low value indicates low permeability. The reflection coefficient for proteins σ is a factor which describes the permeability to proteins of capillary wall. If the vessel is impermeable for proteins, the σ equal to 1 whereas $\sigma = 0$ shows that the vessel is freely permeable for proteins.

Under control condition, the net filtration of fluid (J_v), is balanced by equal lymph flow (L), and therefore a constant interstitial fluid volume (IFV) is maintained. However, if the J_v is increased (either by an increase in hydrostatic pressure or decrease in oncotic pressure) above the lymph flow, the IFV will increase. The latter may be followed by an increase in P_{if} which may prevent or reduce edema generation, depending upon the compliance of the tissue (Aukland and Reed 1993).

Interstitial compliance is defined as the change in IFV (ΔIFV) divided by the

corresponding change in P_{if} (ΔP_{if}) (Guyton 1965). Interstitial compliance is an important factor for the IFV regulation as it determines how much P_{if} will change in response to changes in IFV. In a tissue with a relatively low interstitial compliance, a modest increase in IFV resulting from an increase in capillary filtration, will lead to a rise in P_{if} and thereby counteract further capillary filtration.

Measurement of P_{if} in a low compliant tissue such as dental pulp (Tonder and Kvinnsland 1983; Heyeraas, Kim et al. 1994; Berggreen and Heyeraas 1999), brain (Wiig and Reed 1983), rat tail (Aarli and Aukland 1991), hard palate and attached gingiva (Johannessen, Fjartoft et al. 1987; Fjaertoft, Johannessen et al. 1992) ; and PDL (Kristiansen and Heyeraas 1989) demonstrated P_{if} to be higher compared to the tissues with high compliance such as skin and muscle, which have Pif below ambient pressure (Aukland and Reed 1993).

In normal conditions, P_{if} was about 3.5 mm Hg in rat free gingiva and ranged from 6.0 to 7.4 mm Hg in attached gingiva (Aarli and Heyeraas 1991; Fjaertoft, Johannessen et al. 1992). A significant rise in P_{if} after gingival inflammation induced by tooth-ligation have been demonstrated in rats (Fjaertoft, Johannessen et al. 1992) and in rabbits (Del Fabbro, Francetti et al. 2001) .

 The findings demonstrated that gingiva is a relatively low compliant tissue, meaning that a fluid volume increase is reflected in an elevated P_{if}. The increase in P_{if} during inflammation is a result of an increased fluid filtration from capillaries to the interstitium due increase in P_c and/or to an increase capillary permeability. Del Fabbro et al. (Del Fabbro, Francetti et al. 2001), further showed that during inflammation, proteins were accumulating in the crevicular fluid derived from inflamed gingiva and /or from dental plaque bacteria, which create a pressure gradient driving fluid from the gingiva interstitium into the sulcus area (Del Fabbro, Francetti et al. 2001). In addition to increased crevicular fluid formation, it is also likely that gingival lymph flow increases in this situation due to the increased P_{if}. The role, however, of lymphatic vessels in regulation of transcapillary fluid balance in gingiva during steady state or during periods of increased fluid filtration, was not investigated and at the time unknown.

1.4 The K14-VEGFR3-Ig mouse model

A K14-VEGFR3-Ig mouse is a genetically engineered mouse model in which the ligand-binding domain of vascular endothelial growth factor receptor (VEGFR)-3 fused to the Fc-region of human immunoglobulin γ chain, is overexpressed under the control of the keratin-14 promoter in the basal keratinocytes of the skin (Makinen, Jussila et al. 2001).

The soluble VEGFR-3 inhibits the binding of vascular endothelial growth factor-C (VEGF-C)/VEGF-D ligands to their endogenous receptor VEGFR-3, resulting in hypoplasia of dermal lymphatic vessels. The K14-VEGFR3-Ig mice have visible swelling of the limbs due to lymphatic vessel regression in the skin, whereas the growth of blood vessels is not affected (Makinen, Jussila et al. 2001). The keratin -14 gene is also expressed in the epithelial cells of all parts of the oral mucosa (Barrett, Selvarajah et al. 1998).

1.5 Lymphangiogenesis

Lymphangiogenesis is the formation of new lymphatic vessels from preexisting vasculature. It occurs when lymphatic endothelial cells sprout, migrate and proliferate to form new lymphatic capillaries. Lymphatic vessel growth also involves vessel enlargement resulting from proliferation of lymphatic endothelial cells without sprouting and migration. (Jeltsch, Kaipainen et al. 1997; Tammela, Saaristo et al. 2005).

During embryonic development lymphatic vasculature arises from the blood vasculature by sprouting from cardinal veins (Oliver 2004; Srinivasan, Dillard et al. 2007), a process which involves Prox-1 and VEGF-C as the key players (Wigle and Oliver 1999; Karkkainen, Haiko et al. 2004). In adults, lymphatic vessels grow during physiological wound healing, but it has also been associated with inflammation and tumour growth (Achen, McColl et al. 2005; Alitalo, Tammela et al. 2005). The formation of new lymphatics in inflamed tissue facilitate the drainage of

accumulated interstitial fluid and clear up leukocytes and cytokines (Mouta and Heroult 2003). However, lymphangiogenesis has been shown to actively participate in maintance of chronic inflammatory diseases (Cueni and Detmar 2006) and is demonstrated in rejected renal transplants (Kerjaschki, Regele et al. 2004; Kerjaschki 2006), in psoriatic skin lesions (Kunstfeld, Hirakawa et al. 2004), in joints with inflammatory arthritis (Zhang, Lu et al. 2007), in ulcerative colitis (Kaiserling, Krober et al. 2003) as well as in chronic airway inflammation (Baluk, Tammela et al. 2005).

Adult lymphangiogenesis takes place mainly by sprouting from pre-existing lymphatics and is mediated by VEGF-C and VEGF-D after they bind to their lymphatic endothelial receptor, VEGFR- 3 (Alitalo, Tammela et al. 2005). These lymphangiogenic factors are derived mainly from immune cells such as macrophages, but resident tissue cells such as fibroblasts and keratinocytes also secrete the factors (Ristimaki, Narko et al. 1998). The VEGF-A, which is mainly a proangiogenic factor, can also promotes lymphangiogenesis by binding to VEGFR- 2 expressed in low level in lymphatic endothelium (Lohela, Bry et al. 2009). In addition, VEGF-A recruits inflammatory cells to the inflamed tissue, which secrete VEGF-C and VEGF-D (Schoppmann, Birner et al. 2002; Cursiefen, Chen et al. 2004).

There are other growth factors such angiopoietin -1 (Morisada, Oike et al. 2005; Tammela, Saaristo et al. 2005), fibroblast growth factor -2 (Chang, Garcia-Cardena et al. 2004), hepatocyte growth factor (Kajiya, Hirakawa et al. 2005), insulin-like growth factor- 1 and 2 (Bjorndahl, Cao et al. 2005) and platelet derived growth factors (Cao, Bjorndahl et al. 2004) which also promote lymphatics growth indirectly via the VEGF-C/VEGFR-3 signaling pathway.Besides vessel sprouting, new lymphatic vessels can be generated from bone marrow–derived cells such as macrophages which transdifferentiate into lymphatic endothelial cells (Maruyama, Ii et al. 2005).

1.6 Periodontal diseases

Periodontal diseases are chronic inflammatory conditions caused by bacterial infection of the periodontium. The diseases are initiated by accumulation of bacterial plaque in the gingival sulcus adjacent the root surface of the tooth (Figure 3). The pathogenic bacteria in the plaque produce a wide range of virulence factors which penetrate the gingival epithelium and the underlying connective tissue (Pihlstrom, Michalowicz et al. 2005).

Although the bacterial products can directly damage the host's tissues, most of tissue destruction occurs by the stimulation of host's immune response resulting in secretion of inflammatory mediators such cytokines and chemokines into the tissues (Graves 2008; Preshaw 2008). These mediators increase blood vessel permeability causing fluid to move from the vascular compartment into the interstitium which can results in edema formation (Page 1986). Furthermore, the inflammatory mediators reinforce the recruitment of various inflammatory cells including neutrophils, macrophages and lymphocytes which further release more of the pro-inflammatory cytokines into the connective tissues (Taubman, Valverde et al. 2005; Graves 2008; Preshaw 2008; Hernandez, Dutzan et al. 2011).

In most cases the host responses cause reversible tissue destruction and the condition is termed *gingivitis*. In some instances, however, gingival inflammation continue and lead to irreversible breakdown of periodontal tissues including alveolar bone a condition called *periodontitis* (Graves, Oates et al. 2011).
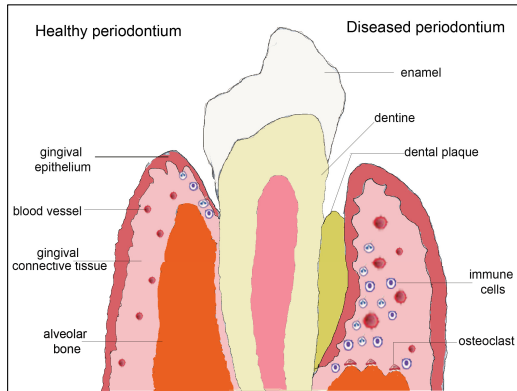
Figure 3. **Diagrammatic representation showing healthy and diseased periodontium. Note the accumulation of immune cells, dilated blood vessels, presence of bone resorbing-osteoclasts and loss of alveolar bone in diseased side.**

1.7 Porphyromonas gingivalis

Bacterial plaque initiates periodontal diseases but which species cause the irreversible breakdown of periodontal tissues is still controversial (Pihlstrom, Michalowicz et al. 2005; Sakamoto, Umeda et al. 2005). There are certain organisms in the bacterial plaque which have been demonstrated to have a strong relationship with periodontal disease development. Among them *P. gingivalis,* a gram-negative black-pigmented anaerobe bacteria commonly associated with chronic periodontitis in human adults (Lamont and Jenkinson 1998; Holt and Ebersole 2005; Silva, Dutzan et al. 2008; Byrne, Dashper et al. 2009).

P. gingivalis possesses several virulence factors which are important for successful bacterial invasion and survival inside the host`s periodontal tissue. These include proteases that degrade proteins to peptides and provides bacterial nutrition, fimbriae which is important for the bacterial attachment to various host components and the lipopolysaccaride (LPS) which is essential for the bacteria`s structural and functional integrity (Lamont and Jenkinson 2000; Tribble, Mao et al. 2006). However, these virulence factors cause direct damage to the tissues of the periodontium as well as stimulate host immune responses (Graves 2008; Liu, Lerner

et al. 2010).The latter ment to eliminate the bacterial infection, but have also been shown to contribute to the periodontal tissue destruction (Lamont and Jenkinson 1998; Yilmaz 2008).

   P. gingivalis proteases such as gingipains are able to degrade tissue proteins and peptides and thereby damage tissue integrity (Stathopoulou, Galicia et al. 2009; Taylor 2010). In addition, the protein digestion by the gingipains not only destroy cytokines and thus weakens the host immune responses against the microbes (Stathopoulou, Benakanakere et al. 2009), but also induces cytokine secretion in host cells via the cleavage and activation of protease-activated receptors (Lourbakos, Potempa et al. 2001; Uehara, Imamura et al. 2008).

   P. gingivalis has two forms of fimbriae (major form A and the minor form Mfa,). The major form A interact with various host cells and induces cytokine secretion and modulates immune responses (Hajishengallis, Sojar et al. 2004). For example, major fimbriae inhibit IL-12 secretion (Hajishengallis, Shakhatreh et al. 2007) , a cytokine which is important for activation of natural killer cells and CD8^{+} cytotoxic T-cells. These T-cells are important for killing of P. *gingivalis* infected cells and the lack of their activation will therefore promote survival of the organism (Hajishengallis and Harokopakis 2007; Wang, Shakhatreh et al. 2007).

   LPS is a major component of the outer membrane of gram negative bacteria and is important for the bacterial integrity (Raetz and Whitfield 2002; Nikaido 2003). It consists of O- antigen polysaccharide, a core oligosaccharide and lipid A. Lipid A, also known as endotoxin, is a biologically active region of LPS which is responsible for activation of immune responses (Jain and Darveau 2010). When the bacteria interact with the host cells, their components are recognized by toll-like receptors (TLRs), a group of pattern-recognition receptors which are expressed on various cells of immune and non-immune cell types, thereby governing immune responses (Akira and Takeda 2004; Gribar, Richardson et al. 2008). LPS is commonly recognized by TLR-4 which in addition requires molecules such as CD14 and MD-2 to form a complex that triggers intracellular signaling cascade resulting in the release of pro-inflammatory cytokines (Mahanonda and Pichyangkul 2007). The structure of *P. gingivalis* LPS is different from that of the much studied *Escherichia coli* (Ogawa

1993). *P. gingivalis* LPS is heterogeneous, with variation in the lipid A structure (Diya, Lili et al. 2008) allowing to signal mainly via TLR-4 and sometimes through TLR-2 (Darveau, Pham et al. 2004). The lipid A from this microbe is capable of antagonizing the action of agonist forms of *P. gingivalis* LPS as well as the agonist activity of LPS from other bacterial species thus enabling the pathogen to evade immune responses and persist in the oral tissue (Jain and Darveau 2010).

1.8 Host immune response and contributions to periodontal diseases

Both the innate and adaptive immune responses are crucial for protecting the host against invading pathogens (Garlet, Cardoso et al. 2007; Garlet, Cardoso et al. 2008).However, during periodontal infection activation of leukocytes from both arms of immunity results in the expression of various inflammatory mediators such as cytokines which are capable of stimulating alveolar bone resorption and soft tissue destruction *via* host-derived matrix metalloproteinases (MMPs) (Garlet, Martins et al. 2004; Goncalves, Oliveira et al. 2008). In addition, receptor activator of nuclear factor kappa-B ligand (RANKL), a key mediator of the alveolar bone destruction is also upregulated (Garlet, Martins et al. 2004; Cochran 2008) . RANKL binds to the its receptor RANK, found on the surfaces of pre-osteoclasts leading to their maturation and activation.OPG is a decoy receptor that inhibits the binding of RANKL to RANK and thus hinders bone resorption (Leibbrandt and Penninger 2008).

The innate immune responses are initiated after the bacterial components are recognised by TLRs which are present on resident cells and leukocytes (neutrophils and monocytes/macrophages) in the periodontal tissues (Mahanonda and Pichyangkul 2007). Activation of these receptors stimulates intracellular signalling cascade leading to activation transcription factors including nuclear factor-k B (NF-kB) and to subsequence release of various inflammatory cytokines and chemokines (Gelani, Fernandes et al. 2009; Lima, Gelani et al. 2010). Innate immunity cytokines

such as TNF-α, IL-1, and IL-6 are host mediators produced after microbial recognition and which have been associated with the enhanced RANKL production, osteoclastogenesis and connective tissue and alveolar bone destruction (Graves 2008). Neutrophils and monocytes/macrophages also release enzymes such as elastases and collagenases which cause degradation of the connective tissue (Lee, Aitken et al. 1995). Besides production of inflammatory mediators subset of monocytes have been shown also to express RANK on their surface and serve as pre-osteoclastic cells (Chapple, Srivastava et al. 1998; Leon, Martinez del Hoyo et al. 2004; Mormann, Thederan et al. 2008).

The adaptive immune response is also initiated by the invading microbe through activation of TLRs found on the surface of antigen presenting cells, particularly dendritic cells (Cutler and Jotwani 2004). The dendritic cells capture and process the pathogen and present the bacterial peptides to lymphocytes. The dendritic cells also release cytokines and chemokines that determine the differentiation of the naïve T helper (Th) cells into appropriate mature effector cells (Cutler and Jotwani 2004).

Initially Th cells were divided into two subclasses Th1 and Th2 according to the pattern of the cytokines they produce (Murphy and Reiner, 2002). Recently two more subsets of Th cells have been discovered namely Th17 and Tregs (Appay, van Lier et al. 2008). Both these subsets are found in periodontal lesions suggesting their involvement in pathogenesis of periodontal disease (Garlet, Martins et al. 2003; Gemmell and Seymour 2004; Honda, Domon et al. 2006; Cardoso, Lobato et al. 2009).

Th1 lymphocytes secrete IFN-γ and are associated with cellular immunity. IFN-γ activates macrophages which are important for killing of microorganisms, but these cells also produce TNF-α and IL-1β which participate in alveolar bone destruction (Gao, Grassi et al. 2007; Garlet, Cardoso et al. 2008). Th2 cells are involved in humoral immune responses and secrete IL-4 among other cytokines. IL-4 is crucial for stimulation of B cell and antibody production (Murphy and Reiner 2002; Appay, van Lier et al. 2008; Sallusto and Lanzavecchia 2009). The B cells also secrete RANKL in response to oral bacteria (Han, Lin et al. 2009), and in periodontal lesions they express RANKL (Kawai, Matsuyama et al. 2006) suggesting their involvement

in alveolar bone destruction. Th17 lymphocytes are characterized by their production IL-17 which in turn induces production of RANKL (Kotake, Udagawa et al. 1999; Sato, Suematsu et al. 2006) and MMPs by secreting IL-6 and induce TNF-α and IL-1β upregulation (Beklen, Ainola et al. 2007).

1.9 Cytokines

Cytokines are small soluble proteins that are secreted by variety of cell types. Their primary function is to regulate host immune responses in diseases, but they are also involved in many biological activities such as cell growth and differentiation, inflammation and repair (Okada and Murakami 1998; Seymour and Gemmell 2001).Most cytokines are produced upon cell activation and usually act locally on the target cells. They exert their effect at very low concentrations (picomolar) in an autocrine or paracrine fashion.

Cytokines act by binding to high affinity surface receptors on target cells and initiate intracellular signaling cascades that alter cell functions through the up-regulation and/or down-regulation of several genes and their transcription factors. As a results, specific proteins are produced which can be involved in a variety of biological activities, including inflammation (Schindler 1999; Handfield, Baker et al. 2008; Preshaw and Taylor 2011).

Included in the cytokine group are the interleukins, interferons, tumor necrosis factor family and chemokine family which are crucial for leukocyte migration. In addition, growth factors and adipokines, such as transforming growth factors-β (TGF-β) and , leptin are capable of modifying immune responses and thus, are also considered as cytokines (Preshaw and Taylor 2011).

Cytokines are divided into pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines are those cytokines produced during an inflammatory response which favors its progression. Included in this group are the major cytokines interleukin-1 (IL-l), IL-6, IL-8, and tumor necrosis factor- α (TNF-α). Other pro-inflammatory cytokines are IL-12, IL-18, interferon-γ (IFN- γ) and granulocyte-macrophage colony stimulating factor (GM-CSF).

In contrast, anti-inflammatory cytokines suppress the inflammation process, by their ability to suppress gene expression for pro-inflammatory cytokines and further counteract the effects of inflammatory mediators (Dinarello 2000). Anti-inflammatory cytokines includes IL-4, IL-10, IL-13, IL-16 and TGF-β (Dinarello 2000).

It should be noted that, most of immune cytokines tend to have both pro-and anti-inflammatory activity (Dinarello 2000) and further they interact and work in network to drive immune responses necessary for host protection. However, if disruption of this network occurs it can lead to destructive inflammation observed in many pathological conditions including periodontal diseases (Preshaw and Taylor 2011). The outcome of inflammatory response is therefore determined by a balance between the effects of pro- and anti-inflammatory cytokines (Dinarello 2000; Preshaw and Taylor 2011).

1.10 Mouse periodontitis model

It is difficult to establish causal relationships between various factors and periodontal tissues breakdown by using human studies (Graves, Fine et al. 2008), therefore the use of animal models has provided useful information about different aspects of host-bacteria interaction and disease process (Graves, Fine et al. 2008). A mouse periodontitis model was developed to assess the alveolar bone resorption after oral bacterial infection (Baker, Evans et al. 1994). In this model, the mouse is initially treated with antibiotic to reduce murine normal flora and is then orally infected by a human strain of oral bacteria such as *P. gingivalis* (Fig. 4). The alveolar bone loss around the molars is detected 6 weeks or more after bacterial challenge either histologically, or with morphometric analysis or microcomputed tomography (Hart, Shaffer et al. 2004; Wilensky, Gabet et al. 2005; Garlet, Cardoso et al. 2006).
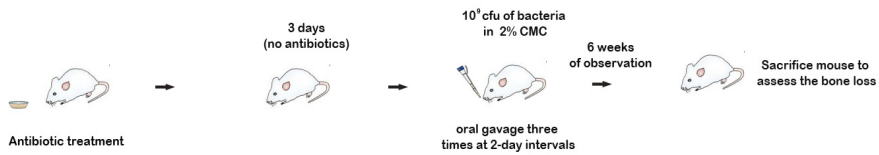
Figure 4. **In a mouse periodontitis model mice are pretreated with antibiotics, orally infected with bacteria and observed for 6 weeks before they are sacrificed**

1.11 Rational of the thesis

Gingiva is constantly challenged by bacteria and changes in the host responses in addition to the pathogenicity of the microflora, frequently leads to inflammatory processes. The lymphatic system in gingiva has previously been under-investigated due to the lack of specific lymphatic markers which made it difficult to differentiate it from the blood vessels. As a result little was known about the functional effect of the lymphatic system in gingiva in health and disease. Therefore, the focus of this thesis has been to investigate lymphatic function in normal situation and its function and responses after onset of periodontal disease.

2. AIMS OF THE THESIS

2.1 GENERAL AIMS

The overall aim of this thesis was to investigate the role of gingival lymphatics for tissue fluid balance and during development of periodontal disease.

2.1.1 SPECIFIC AIMS

To describe the phenotype of oral mucosa in the K14-VEGFR3-Ig mouse model, (Paper I).

To determine the functional effects of missing gingival lymphatics in relation to tissue fluid balance in steady state condition and after fluid overload, (Paper I).

To investigate the role of gingival lymphatics during acute inflammation, (Paper I).

To study if lymphangiogenesis occur in inflamed gingiva during development of periodontal disease, (Paper II).

To investigate if lack of lymphatics in gingiva may cause altered immune responses and affect alveolar bone resorption after bacterial challenge with *P. gingivalis* (Paper III).

3. METHODS

Here, experimental material and methods are briefly presented. Detail descriptions of specific procedure are found in individual papers.

3.1 Animals

Experiments were performed in male and female K14 mice (Paper I, III) and wild type mice (Paper I, II, and III) both of C57/Bl6 background. The mice were housed in polycarbonate cages at the animal facility unit and received a standard food pellets and tap water *ad labium*. The mutation in K14 mice was verified by PCR analysis prior to the experiments. The housing unit was temperature controlled (22 ± 2 ˚C) with a constant 12-hour light and 12-hour darkness cycle. All the experiments were performed according to the recommendations outlined by Norwegian State Commission for Laboratory Animals with the local ethical committee's approval.

Anesthesia: In paper I, mice were anesthetized intramuscularly with a 1:1 mixture (0.1ml/10g body wt) of midazolam (Dormicum, Roche, France) and fentanyl/fluanison (Hypnorm; Janssen)*.* In paper II and III isoflurane was administered for 2 min during the oral gavage of the bacteria. In all three papers mice were sacrificed with an overdose of anaesthesia.

3.2 Measurement of interstitial fluid pressure (Paper I)

P_{if} was measured by the micropunture method (Wiig, Reed et al. 1981) using a sharpened glass capillaries (micropipettes) with tip diameter of 2-6 μm filled with colored 0.5 M NaCl. Under the guidance of a stereomicroscope the micropipettes were inserted into the gingiva and alveolar mucosa with a micromanipulator at a depth of 200-600 μm below the surface. The micropipettes were connected to a

servo-controlled counter-pressure system and P_{if} was measured by the counter
pressure method developed by Wiederhielm and collaborators (Wiederhielm,
Woodbury et al. 1964).

3.3 Systemic blood pressure recordings (Paper I)

Systemic blood pressure was continuously measured in the femoral artery which was
first exposed and then cannulated with polyethylene catheter connected to a pressure
transducer and recorder.

3.4 Measurements fluid volume (Paper I)

Fluid volumes were determined using ^{51}Cr-EDTA and [125]I- labeled human serum
albumin. Total extracellular fluid volume (V_x) was measured using ^{51}Cr-EDTA as the
extracellular marker. ^{51}Cr-EDTA distributes uniformly in the plasma and interstitial
fluid without uptake in cells (Løkken, 1970). A bolus of intravenous (i.v) injection of
[51]Cr-EDTA was given, and the tracer was then infused (i.v) continuously during an
equilibrium period (90 min) to compensate for loss in urine. ^{125}I-HSA was used as
the intravascular marker to measure intravascular volume (V_v). ^{125}I-HSA was allowed
to circulate for 5 min only before the blood was collected (Linderkamp, Holthausen
et al. 1977). Blood samples, alveolar and masticatory mucosa were weighed and the
radioactivity was counted. IFV of the tissues was calculated as the difference
between V_x and V_v.

3.5 Experimentally induced overhydration (Paper I)

The effect of increased volume in a tissue with reduced lymphatic drainage capacity
was studied by measuring the P_{if} response. By using an infusion pump, Ringer
solution corresponding to 15% of body weight was infused through right femoral
artery over a period of 30 min. P_{if} 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. In separate groups, mice were infused with isotopes (as described above) to determine volume distribution after overhydration. In a separate group of WT mice, P_{if} was measured continuously in the attached gingiva during fluid expansion and recovery period.

3.6 Acute inflammation induction (Paper I)

P_{if} was recorded for 10 min in the attached gingiva before induction of gingival inflammation using injection of LPS from *P. gingivalis* (1 μl LPS (5 mg/ml, InvivoGen, San Diego, CA, USA) in experimental group or equivalent volume of vehicle (1 μl of 0.9% NaCl with 1% bovine serum albumin (BSA)) in controls. Thereafter P_{if} was continuously measured for 90 min, before mice were sacrificed with an overdose of anaesthesia.

3.7 Collagen fiber visualization and content (Paper I)

Collagen fibers were visualized under polarizing light in 6μm thick paraffin embedded sections stained with Picro-Sirius Red. Measurement of collagen content in the alveolar mucosa and masticatory mucosa was based on hydroxyproline content (Woessner 1961). The absorbance was read at 557 nm on a spectrophotometer (Molecular Devices, Sunnyvale, CA). Quantification of hydroxyproline concentration was done by comparison to a standard curve of L-4- hydroxyproline (Fluka Chemise GmbH, Bucks, France) in 1mM Hal. Collagen content was calculated based on a 6.94 to 1 collagen to hydroxyproline ratio (Jackson and Cleary 1967).

3.8 Oral infection with *P. gingivalis* (Paper II,III)

Periodontal infection was induced in WT mice (Paper II) and in both strains of mice (Paper III) by *P. gingivalis* ATCC 53978 (W50) oral gavage (Baker, Evans et al.

1994). Briefly, mice were treated with antibiotics in drinking water for 10 days to reduce oral flora, followed by a 4-day antibiotic-free period. *P. gingivalis* suspended in 100 µL PBS with 2% carboxymethylcellulose (CMC; Sigma) was given to experimental animals while controls received the vehicle CMC without the bacteria. Ten days (Paper II) and forty-two days (Paper II, III) after the last gavage tissue and blood samples were harvested for further analysis.

3.9 Enzyme-linked immunosorbent assay (Paper II,III)

Specific serum IgG to *P. gingivalis* ATCC 53978 (W50) was measured by ELISA (Baker, Evans et al. 1994). A ninety six well polystyrene plates were coated overnight with formalin-killed bacteria followed overnight incubation at 4°C of serial dilutions (2-fold) of test or positive control sera (2^2 to 2^{12} or 2^{14}). The plates were washed and the bounded antibody was detected using alkaline phosphatase–conjugated goat anti-mouse IgG antibodies (Paper II, III). The reaction was quantified by P-nitrophenyl phosphate substrate and absorbance was determined at OD_{405} by microplate reader. In addition, the level of different cytokines in the periodontal tissue was detected using commercially available ELISA kits obtained from R&D Systems (DuoSets, Minneapolis, USA) according to the manufacturer's instructions (Paper II and III).

3.10 Alveolar bone loss (Paper II,III)

Assessment of horizontal bone loss was performed by the morphometric method .The maxillary jaws were defleshed, stained with 1% methylene blue and positioned so that the occlusal face of the molars was perpendicular to the base. Images of the buccal view of the jaws were captured under a stereomicroscope equipped with a digital camera. Using Lucia imaging software, alveolar bone loss was measured directly as the distance from cementum–enamel junction (CEJ) to the alveolar bone

crest (ABC) (Paper II) or as a polygonal area made by tracing the CEJ, marginal ABC of the three molars, and the distal margin of the visible root of 3rd molar and the mesial margin of the visible root of 1st molar (Paper III).

3.11 Immunohistochemistry (Paper I,II,III)

In paper I, mice were transcardiacally perfused through the aorta with heparinized saline followed by 4% paraformaldehyde with 0.2% picric acid. The jaws were post-fixed for 2 hr at 4 ºC and then decalcified in EDTA for 7 days. In papers II and III jaws were collected and immediately decalcified in EDTA for 4 days without fixation. All jaws were soaked in 30% sucrose overnight, sectioned (8-20µm) and stored at -80 ºC. The sections were used for immunoperoxidase staining, using a Vectastain ABC kit, or for immunofluorescence staining. Sections from EDTA treated jaws were acetone fixed for 10 min on the glass before immunostaining procedures. Peroxidase activity was developed with 3, 3`-diaminobenzidine and enhanced with nickel.

The following antibodies were used; mouse monoclonal antibody to human IgG Fc domain (dilution 1:1000), rabbit anti-mouse LYVE-1 (dilution 1:1000), syrian hamster anti-mouse podoplanin (dilution 1: 50), rat anti-mouse CD3 (dilution 1:500), rat anti-mouse CD4 (dilution 1:500), rat anti-mouse CD8 (dilution 1: 50), rat anti-mouse CD 20 (dilution 1:200), rat anti-mouse CD31 (dilution 1:50), rat anti-mouse CD45 (dilution 1:500), rat anti-mouse CD31 (dilution 1:100), rat anti-mouse CD4 (dilution 1:100) , rat anti-mouse Ki-67 (dilution 1:50), rat anti-mouse Ly-6B.2 (dilution 1: 4000) and rat anti-mouse MHC class II (dilution 1:1500), goat polyclonal anti-mouse VEGF-C (dilution 1:50) and VEGF-A (dilution 1:50), rabbit anti-mouse VEGF-D (dilution 1:50).

The corresponding secondary antibodies for immunofluorescense includes Cy[TM]3-conjugated IgGs (dilution1:200), Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 647conjugated species-specific (dilution 1:250) used for visualization of the immune reactions. Controls of the specificity of the immunoreactions were included by isotype control immunoglobulin incubation and by substitution of the primary

antiserum with PBS. Sections were evaluated in a photomicroscope or in a fluorescent microscope.

3.12 Quantification of gingival immune cells (Paper I,II,III)

Immunolabeled cells counts were performed in the gingival connective tissue mesial to first molar and in the papillas between the molars in a grid (150 μmX100 μm) under the light microscope, connected to a digital camera using Lucia imaging software. In paper II, VEGF-C $^{+}$/CD45^{+} cells were counted in gingival lamina propria mesial to 1st and distal to 3rd molars using a in a 100 μmX200 μm grid. The pooled value for each animal was obtained by averaging the number of positive stained cells detected in three to five sections.

3.13 Quantification of lymphatic and blood vessels (Paper II)

Lymphatic and blood vessels were identified 10 days post-infection using LYVE-1 and CD31 immunofluorescent double labeling and images captured using a digital camera. In addition, LYVE-1 positive lymphatics were identified with the ABC method forty two days after infection. Lymphatic and blood vessels area fraction was evaluated by Lucia imaging software (Lucia v.145 480, Laboratory Imaging, Hostiva, Check Republic) in the connective tissue of the gingiva (mesial to 1st and distal to 3rd) molars. The area fraction was calculated as the ratio between the area occupied by lymphatics or blood vessels and the total tissue area (μm^{2}).The vessel profiles per area in the same areas were also obtained.

3.14 RNA isolation and quantitative RT-PCR (Paper II)

Total RNA was extracted from the gingival tissues using the RNeasy® mini kit (Qiagen; Chatsworth, CA).The quality control was analyzed by 1% agarose gel

electrophoresis. RNA concentration was quantified by a spectrophotometer. 2µg of total RNA was used for the reversed transcription into cDNA using synthesis kit (Fermentas GmbH, Germany). Primers for detection of VEGF-C and VEGF-D were added to eighty nanograms of cDNA and then Q-PCR reactions were performed using iQ SYBR Green Supermix (BioRad) in LightCycler 480®. VEGF-A gene expression was quantified by using a pre-designed TaqMan® gene expression assay (Applied Biosystems) and a TaqMan Universal PCR Master Mix (Applied Biosystems). All samples were assayed in triplicate and normalized to Gapdh content.

3.15 TRAP staining (Paper III)

Histochemical analysis using tartrate resistant acid phosphatase (TRAP) staining was performed in order to quantify osteoclasts and their precursors. The TRAP$^+$ cells were counted in a grid (100x200µm) placed on top of the approximal bone between the molars.

3.16 Multiplex analysis (Paper III)

The concentration of cytokines in the periodontal tissues was measured using a multiplex kit (Millipore Corporation, Billerica, MA, USA). A panel of the following cytokines was simultaneously quantified: G-CSF, IFN-γ, IL-10, IL-17, IL-1α, IL-2, IL-6, IP-10, KC, M-CSF, MIP-1 α, RANTES and TNF- α according to manufacturer's instructions. Total surface fluorescence was measured with a flow – based dual laser system (Luminex [100], Luminex corporation, Austin, TX, USA) for the detection of different colour-coded beads and quantitation of cytokines. Cytokines concentration was calculated with reference to standard curve based on broad range of standards (4.8- 20 000 pg ml^{-1}) provided in the kit.

3.17 Flow Cytometry of immune cells in cervical lymph nodes

After the dissection, the cervical lymph nodes were incubated with 0.28 Wunsch units/ml of Liberase thermolysin medium ™ for 50 min at 37°C, to liberate leukocytes. The cells were filtered through a 70-μm filter , washed three times in PBS and incubated for 30 min at 4 °C with the following conjugated antibodies for antigen detection: Pacific Blue^{TM} CD4 (1: 25), PE CD8 (1:25), APC CD19 (1: 50), PerCP-CY^{TM}5.5 CD11c (1: 25), FITC F4/80 (1:25) and Alexa Fluor® 700 Ly-6B.2 (1: 10) .Cells were washed again and approximately 300,000 cells were analyzed by flow cytometry. Negative controls were unstained cells from lymph nodes, and for control of gate settings each antibody was incubated with microparticles from an anti-rat Ig κ/ negative Control Compensation Particles Set.

3.18 Statistical analysis

Results were given as means± SD or SEM. Student's t test or Mann Whitney-test was used for comparison between two groups (paper I and II) and analysis of variance (ANOVA) for comparisons between more than two groups (paper I and III) followed by Holm-Sidak or Bonferroni post-hoc tests. For repeated pressure measurements within a group and between groups, data were analysed with one-way repeated measures analysis of variance (RM-ANOVA) (paper I). $P < 0.05$ was considered statistically significant in all three papers.

4. SUMMARY OF THE RESULTS

4.1 Paper I

In this paper, the role of initial lymphatics in gingival transcapillary fluid balance was investigated in genetically engineered K14 mice. Immunohistochemistry showed that lymphatic vessels were absent in gingiva of K14 mice. On contrary, in alveolar mucosa lymphatics were present in the submucosal area but were almost completely absent in the mucosa. Blood vessel distribution, tissue histology and collagen content in masticatory mucosa and alveolar mucosa in K14 mice were normal, indicating that only lymphatic vasculature was defected in the mutant mice.

K14 mice had increased P_{if} in gingiva in normal situation and after gingival inflammation induced by *P. gingivalis* LPS compared to WT mice. Furthermore, overhydration resulted in increased tissue volume by more than 75% in both strains, followed by a drop in P_{if} after recovery. When P_{if} was continuously measured in the gingiva during overhydration, an increase in P_{if} was observed in early phase of fluid expansion followed by a decline. In alveolar mucosa, there was no significant difference neither in P_{if} nor IFV between the strains under normal situation and after fluid volume expansion.

Immunohistochemical analysis of immune cells in the masticatory mucosa using CD3, CD4, and CD45 markers revealed that the $CD45^+$ cell number was significantly higher in K14 mice compared to WT mice. However, T cell ($CD3^+$ and $CD4^+$) numbers were similar in both strains.

4.2 Paper II

In this study, we investigated if lymphangiogenesis occurs in inflamed gingiva during periodontal disease development. Gingival inflammation was induced in mice by *P. gingivalis* oral gavage and the animals were observed for 10 and 42 days.

Immunostaining with LYVE-1 demonstrated that lymphatic vessel area fractions were significantly increased in infected mice compared to sham mice, whereas the profiles were not different between the groups 42 days post-infection. LYVE-1$^+$ / CD45$^+$ leukocytes were also detected close to LYVE-1$^+$ vessels in infected mice. The infected mice had also significantly greater CEJ-ABC distances 42 days post-infection than sham mice, demonstrating that bone loss had occurred.

Immunofluorescent labeling with LYVE-1 and CD31 showed that lymphatic and blood vessel area fractions and profiles in the gingiva were increased 10 days post-infection. The lymphangiogenic response was shown in infected mice by the presence of LYVE-1$^+$/Ki-67$^+$ endothelial cells.

VEGF- C protein was expressed in epithelial cells and CD45$^+$ immune cells in gingiva in both groups of mice 10 days post-infection. VEGF-D and VEGF-A staining was found in the epithelium and also in a few CD45$^+$ immune cells.VEGF-A protein was also expressed in blood vessels. The VEGF-C$^+$/CD45$^+$ cell number in lamina propria was significantly higher in infected mice than sham 10 days post-infection. Gene analysis revealed that the expressions of VEGF-A and D were significantly down-regulated, whereas that of VEGF-C was slightly increased but not statistically significant at 10 days post-infection.

Higher levels of pro-inflammatory cytokines IL-1β and TNF-α were found in the infected mice 10 days post infection, but only TNF-α was statistically significant elevated.

4.3 Paper III

This study was undertaken to investigate if lack of gingival lymphatics in K14 mice may cause altered immune responses and alveolar bone resorption 42 days after *P. gingivalis* challenge. The results demonstrated that infected K14 mice had significantly more alveolar bone loss compared to their own shams as well as to WT infected mice. No signinificant difference in bone loss was found within the WT strain.

TRAP$^+$ cell number on the alveolar bone surface tended to be higher in K14 infected mice but was not significantly different compared to K14 shams as well as infected WT.

The number of F4/80$^+$ cells and MHC class II $^+$ cells in the gingival connective tissue of infected K14 mice were significantly higher compared to infected WT mice. Numerous CD20$^+$ B cells were also found close to the alveolar bone in infected K14 mice, however their number was not significantly different compared to infected WT mice. Few neutrophils were found adjacent to the alveolar bone, but in two infected K14 mice a periodontal abscess surrounded by neutrophils was found, suggesting a more severe infection in the K14 strain.

The level of the cytokines IFN-γ, IL-1β and G-CSF were significantly elevated in infected K14 mice compared to infected WT mice. The M-CSF level was significantly higher in K14 sham compared to WT sham and was also high in both infected strains.

The levels of *P. gingivalis* specific IgG in the serum were elevated in both strains after infection , but significantly lower IgG levels was found in infected K14 mice than in infected WT mice.

There were no significant differences in immune cell distribution in cervical lymph nodes between the strains in any condition.

5. GENERAL DISCUSSION

Lymphatic vessels are present in the gingiva (Ushijima, Inoue et al. 2008), a tissue which is frequently associated with inflammatory responses due to infectious disease. Yet, the function of lymphatic vessels in transcapillary fluid balance and during development of periodontal disease was hitherto unknown. In this thesis therefore, the role of gingival lymphatic vessels in transcapillary fluid exchange in normal and during periods of increased fluid filtration was investigated. Additionally, we investigated whether lymphangiogenesis occurs during development of periodontal disease. Finally, the function of lymphatic vessels during periodontal disease development was also investigated.

5.1 The role gingival lymph vessels in transcapillary fluid exchange

In paper I, we demonstrated that K14 mice lack lymphatics in the gingiva, whereas in the alveolar mucosa lymphatics were found in the submucosal layer and almost completely absent in the mucosal layer.
The K14 mice had significantly higher Pif in the gingiva compared to WT mice in the normal situation. The finding indicates that there is accumulation of fluid in the gingival interstitium of K14 mice, resulting in a higher P_{if} Pif .The latter counteract further fluid filtration into the interstitium and a new steady state is established. Furthermore, injection of LPS in the gingiva resulted in an increase in Pif in both strains of mice, which remained significantly higher in K14 mice compared to WT mice. This finding suggests increased fluid filtration into the interstitium in LPS induced inflammation, and the fluid accumulated to a greater extent in K14 mice where lymphatics were missing in gingiva. Together, these results demonstrate that gingival lymphatics play an important role in tissue fluid homeostasis in the gingiva in both conditions.

The finding that overhydration led to increase of IFV by more than 75% in the masticatory mucosa (gingiva included) in both strains, which was accompanied by fall in P_{if} in the gingiva, was an unexpected observation. P_{if} is expected to rise in response to increase IFV in order to prevent further fluid filtration across the capillary. We therefore decided to continuously measure P_{if} during the infusion and recovery period. We observed a rise in P_{if} in the early phase of fluid loading, which was followed by a drop in P_{if} as the infusion continued. A likely explanation for this observation is ; as overhydration continued there was increased filtration which resulted in fluid accumulation in the extracellular space, followed by rise in P_{if}. Because the cells and extracellular matrix components are bound together by integrin receptors which keep them in tension (Hynes 1992) we speculate that the rise in Pif may have lead to perturbation of the receptors and thus release of the tension between the cells and the matrix. This will result in an increase in tissue compliance followed by a drop in P_{if} (Wiig, Rubin et al. 2003). The drop in Pif will further enhance fluid filtration out of the capillary and lead to increase in IFV, as observed in our experiments. Antibody blocking of integrin receptors has been demonstrated to be followed by lowering of P_{if} and oedema formation (Reed, Laurent et al. 1996). We speculate that the edema we observed was caused by disruption of the extracellular matrix, a hypothesis that is supported by Negrini *et al.,* who found that fluid expansion that caused drop in P_{if} and edema formation in the lung, was associated with loss of interaction between proteoglycan and other extracellular matrix components (Negrini, Tenstad et al. 2006).

We could not measure difference in IFV in the masticatory mucosa between strains in any conditions. One possible explanation is that the higher Pif in the gingiva

in the mutant mice creates pressure gradients which favour fluid flux toward the alveolar mucosa and also into the gingival sulcus. Increased fluid conductance in the gingival interstitium in K14 mice may also take place, as it has been demonstrated that tissue conductance is increased in tissue with lymphatic vessel impairment (Hedbys and Mishima 1962; Guyton, Scheel et al. 1966). An increased conductance may favour fluid movement toward the alveolar mucosa in the mutant mice and

thereby contribute to reduce the IFV in the masticatory mucosa. Lack of sensitivity in our method and inclusion of palatinal mucosa with lymphatics in the submucosal layer, may also be a reason for not detecting IFV differences between the strains. In the alveolar mucosa, there were no significant differences neither in Pif nor in IFV in K14 mice compared to WT mice in any situation. This may be due to the presence of lymphatics in the submucosal layer which can compensate for impaired lymph drainage of mucosal lymphatic vessels.

Lymphangiogenesis in periodontal disease

The main finding in paper II was that oral infection with *P. gingivalis* resulted in lymphatic vessel growth which was accompanied by angiogenesis. In addition to increase in both vascular area fractions and profiles 10 days post-infection, there was also presence of LYVE-1^{+}/Ki-67^{+} endothelial cells in the gingiva of infected mice. The observation indicates that lymphatic vessel proliferation take place in the first weeks after infection in this periodontitis model. The growth of lymphatics in inflamed gingiva may promote lymph flow and antigen presentation, and contribute to protect mice from severe inflammation. This speculation is supported by our recent finding in paper III, where the mutant K14 mice developed severe inflammation accompanied by increased alveolar bone loss after *P. gingivalis* infection.

The lymphatic area fraction remained significantly increased 42 days post-infection (paper II). Persistent enlarged lymphatic vessels have also been demonstrated in *Mycoplasma pulmonis* (*M. pulmonis*) infected airways in which elimination of bacteria by antibiotic treatment did not regress the enlarged lymphatics vessels (Baluk, Tammela et al. 2005). However, the reason for the resistance of lymphatics regression is unknown (Yao, Baluk et al. 2010).

The finding that infected mice had higher numbers of CD45^{+} immune cells expressing VEGF-C protein 10 days post-infection, suggests that these cells contribute to the lymphangiogenic response. Similar findings that inflammatory cells secrete VEGF-C which promoted lymphangiogenesis were observed in mice with

chronic airway inflammation induced by *M. pulmonis* infection (Baluk, Tammela et al. 2005)

The VEGF-C activation of VEGFR-3 expressed in lymphatic endothelium is followed by proliferation of these cells (Makinen, Veikkola et al. 2001; Wirzenius, Tammela et al. 2007). Mature form of VEGF-C can also bind and activates VEGFR-2 expressed in lymphatics and in blood vessels (Joukov, Sorsa et al. 1997; Wirzenius, Tammela et al. 2007). VEGF-D[+] and VEGF-A[+] proteins were strongly expressed in the epithelial layer and in some few immune cells in gingiva (paper II). VEGF-A staining was also found in gingival blood vessels. However, the gene expression for VEGF-D and A was down-regulated and the number of immune cells expressing VEGF-D and VEGF-A was not different after bacterial challenge. *P. gingivalis* has been reported to induce gene down regulation in an early phase after infection (Sasaki, Okamatsu et al. 2004), possibly through inhibition of cell activation or induction of apoptosis. Several studies have shown apoptosis *in vitro* (Johansson and Kalfas 1998) and *in vivo* (Isogai, Isogal et al. 1996; Graves, Oskoui et al. 2001) and affect various cells as epithelial cells, fibroblasts and immune cells.

The elevated expression of TNF-α and IL-1β in the infected mice 10 days post-infection indicates ongoing inflammatory responses. Both cytokines, have been shown to promote lymphangiogenesis by inducing gene expression of VEGF-C (Ristimaki, Narko et al. 1998). TNF-α also activates recruited immune cells and may therefore further reinforcing their role in lymphangiogenesis (Baluk, Yao et al. 2009).

5.2 The role of gingival lymph vessels in periodontal disease

In paper III, we showed that K14 mice which lack gingival lymphatics exhibit significantly more alveolar bone loss compared to WT mice 42 days after *P.gingivalis* infection. The increased bone loss in infected K14 mice was accompanied by increased number of macrophages and MHC II antigen presenting

cells adjacent to alveolar bone. Furthermore, the level of IL-1β, IFN-γ and G-CSF in the gingival tissue in transgenic mice was also increased compared to infected WT mice.

Macrophages produce several cytokines that stimulate destruction of connective tissue and alveolar bone (Graves 2008). Among them, IL-1β is involved in alveolar bone destruction through stimulating RANKL expression in osteoblasts and lymphocytes (Kwan Tat, Padrines et al. 2004). IL-1 induces also production of colony stimulating factors (Bagby, Dinarello et al. 1986; Tanabea, Maenob et al. 2005) for example M-CSF, a chemokine crucial for the survival and longevity of osteoclast precursors (Lorenzo, Horowitz et al. 2008). Furthermore, M-CSF`s interaction with RANKL results in the differentiation of pre-osteoclasts to mature osteoclasts (Hodge, Collier et al. 2011).

Macrophages may also express major histocompatibility complex (MHC) class II molecules in bony lesion (Kopp and Schwarting 1989) and may therefore contribute to lesion development through activation of T-cell-mediated immune responses. In addition, macrophages can act as osteoclast precursors and differentiate into mature osteoclasts (Takeshita, Kaji et al. 2000).

In the present study (paper III), there was a tendency towards increased number of B-cells adjacent to the alveolar bone in infected K14 mice, although not significant compared to infected WT mice. The presence of B-cells together with T cells in periodontal lesions is associated with periodontal disease development as these cells contribute substantially to production of RANKL (Kawai, Matsuyama et al. 2006), a cytokine known to induce osteoclast activity (Wei and Siegal 2008).

The level of IFN-γ was significantly elevated in infected K14 mice. IFN-γ is mainly produced by Th1 cells, but also macrophages, B cells (Harris, Haynes et al. 2000), Natural Killer T cells (Carnaud, Lee et al. 1999) and professional antigen-presenting cells (Frucht, Fukao et al. 2001) may secrete IFN-γ. Concerning periodontal disease, IFN-γ has been shown to inhibit osteoclastogenesis *in vitro*

(Takayanagi *et al*., 2005; Ji *et al*., 2009), but this inhibitory effect is overcome *in vivo* because IFN-γ upregulates the levels of TNF-α and IL-1β which results in an increase in RANKL (Gao, Grassi et al. 2007; Graves 2008). Furthermore, IFN-γ stimulates upregulation of IFN-γ inducible protein-10 (IP-10), a chemoattractant for T- lymphocytes (Taub, Lloyd et al. 1993). In the present study infected K14 mice had insignificant but elevated level of the chemokine IP-10 in the periodontal tissue compared to infected littermates. IP-10 protein is secreted by various cell types including endothelial cells, monocytes, fibroblasts, and keratinocytes in response to IFN- γ (Luster 1987; Weng, Siciliano et al. 1998). IP-10 has shown to increase the infiltration of macrophages (and CD4 T-cells) into inflamed joints and act together with RANKL to stimulate bone destruction (Kwak, Ha et al. 2008).

G-CSF was also significantly increased of in the gingival tissues of infected K14 mice. G-CSF is crucial for granulocyte production (Villunger, Scott et al. 2003) and is secreted by many different cells including bone marrow stromal cells, endothelial cells, macrophages and fibroblasts. Pro-inflammatory cytokines such as IL-1 induce G-CSF production (Demetri and Griffin 1991). Besides prolonging neutrophil survival (Villunger, Scott et al. 2003), G-CSF also increases macrophages number and enhances neutrophil and macrophage phagocytosis (Bermudez, Petrofsky et al. 1998; Fattorossi, Battaglia et al. 2001). Although neutrophil number was not significantly increased in infected K14 mice, they were in close proximity to microabcesses found in this strain only. Neutrophils degrade soft tissue and bone through degranulation and release of superoxide anion, and are associated with the pathogenesis of inflammatory arthritis (Bombini, Canetti et al. 2004). Microabcess formation indicates an impaired ability to clear the infection in the mutant infected mice.

The finding that specific *P. gingivalis* IgG serum level was lower in infected K14 mice than in WT mice is indicative of lower level of adaptive B cell response in the former, probably caused by impaired transport of antigen-presenting cells from the oral mucosa toward the lymph nodes after *P. gingivalis* infection. The weakened

humoral immune response may have left the K14 mice more susceptible to develop periodontal disease, because a high specific serum IgG is shown to be protective against *P. gingivalis* induced bone loss in mice (Momoi, Hashizume et al. 2008).

There were no differences in immune cell distribution in cervical lymph nodes neither within the strains nor between the strains in any condition. The finding that the shams had similar immune cells distribution indicates that presence of lymphatics in the gingiva and oral mucosa does not affect lymph nodes immune cells distribution in steady state. Furthermore the observation that there were no difference in immune distribution in either strain after infection may be due a reduced antigen presenting ability in the K14 mice that may have hinder or reduced the infection-induced responses. In contrast in the WT mice antigen presentation should be efficient after *P. gingivalis* infection, but the 42 days post-infection observation period was too long to observe significant immune cell responses in the local lymph nodes.

Together, the findings from paper III indicates that the K14 strain which lack gingival lymphatics developed a stronger inflammatory response toward alveolar bone accompanied by a weakened humoral immune response after P. *gingivalis* infection which might have resulted in enhanced alveolar bone loss observed. Lymphatics vessels are therefore crucial for protection against periodontal disease probably by clearing the bacteria and bacterial products from gingiva as well as by promoting humoral immune responses.

6. CONCLUSIONS

The presented studies in this thesis led to the following conclusions.

- The gingival lymphatic vessels are important for the transcapillary fluid balance under steady-state condition and during acute perturbation.

- The lymphatic vessels in the alveolar mucosa are not critical in transcapillary fluid balance when lymphatic vessels exist in the submucosal layer.

- Fluid volume expansion is followed by a significant drop in P_{if} in gingiva, suggesting that compliance is increased after disruption of the tissue structure during edema formation.

- Lymphangiogenesis take place in gingival during periodontal disease development induced by *P.gingivalis*, and the lymphatic vessels remain expanded after establishment of chronic periodontitis.

- Vascular endothelial growth factor C in recruited immune cells may contribute to the lymphangiogenic response observed after *P.gingivalis* infection in gingiva.

- The gingival lymphatic vessels are protective in *P. gingivalis* induced periodontitis in mice, probably by enhancing clearance of bacterial products and through promotion of specific humoral IgG responses

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