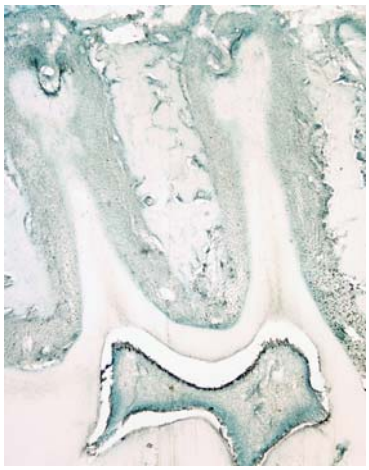
Expression and effects of IL-1 and TNF-α in different experimental models of dental inflammation

Athanasia Bletsa



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Scientific environment

The experimental work in this thesis was carried out at the Department of Physiology, Faculty of Medicine (later Institute of Biomedicine, Section for Physiology), University of Bergen, during the period of 2000-2005. I thank the University for providing excellent working facilities.

There was collaboration with the Department of Orthodontics and Facial Orthopaedics, and the Department of Oral Sciences, Faculty of Dentistry, University of Bergen.

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ITHACA

When you set out on your journey to Ithaca,
pray that the road is long,
full of adventure, full of knowledge.
The Lestrygonians and the Cyclops,
the angry Poseidon -- do not fear them:
You will never find such as these on your path,
if your thoughts remain lofty, if a fine
emotion touches your spirit and your body.
The Lestrygonians and the Cyclops,
the fierce Poseidon you will never encounter,
if you do not carry them within your soul,
if your soul does not set them up before you.

Pray that the road is long.
That the summer mornings are many, when,
with such pleasure, with such joy
you will enter ports seen for the first time;
stop at Phoenician markets,
and purchase fine merchandise,
mother-of-pearl and coral, amber and ebony,
and sensual perfumes of all kinds,
as many sensual perfumes as you can;
visit many Egyptian cities,
to learn and learn from scholars.

Always keep Ithaca in your mind.
To arrive there is your ultimate goal.
But do not hurry the voyage at all.
It is better to let it last for many years;
and to anchor at the island when you are old,
rich with all you have gained on the way,
not expecting that Ithaca will offer you riches.

Ithaca has given you the beautiful voyage.
Without her you would have never set out on the road.
She has nothing more to give you.

And if you find her poor, Ithaca has not deceived you.
Wise as you have become, with so much experience,
you must already have understood what Ithacas mean.

K.Kavafis

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Nancy

Bergen March 8, 2006

(Women's Day)

Abbreviations

^{51}Cr	Radioactive chromium
^{125}I	Radioactive iodine
BSA	Bovine serum albumin
b.w.	Body weight
CGRP	Calcitonin gene-related peptide
COP	Colloid osmotic pressure
cpm	Counts per minute
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GCF	Gingival crevicular fluid
HPA	Hypothalamic-pituitary adrenal axis
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IF	Interstitial fluid
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1α	Interleukin-1alpha
IL-1β	Interleukin-1beta
IL-1r	Interleukin-1receptor
i.p.	Intraperitoneally
IR	Immunoreactive
i.v.	Intravenously
KCl	Potassium chloride

LPS	Lipopolysaccharide
NaCl	Sodium chloride
NF-κB	Nuclear factor-kappa B
NK	Natural killer cells
NO	Nitric oxide
Non-SCGx	Non-sympathectomized
NPY	Neuropeptide Y
OTM	Orthodontic tooth movement
PA	Systemic arterial blood pressure
PBF	Pulpal blood flow
PBS	Phosphate buffered saline
PDL	Periodontal ligament
PGs	Prostaglandins
P_{if}	Interstitial fluid pressure
SCG	Superior cervical ganglion
SCGx	Sympathectomized
SNS	Sympathetic nervous system
TBS	Tris-buffered saline
Th	T helper lymphocytes
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
V_i	Interstitial fluid volume
V_v	Intravascular fluid volume
V_x	Extracellular fluid volume
w.w.	Wet weight

List of publications

This thesis is based on the following papers, which will be referred to by their roman numerals throughout the text:

I. Bletsa A, Heyeraas KJ, Haug SR, Berggreen E (2004): " IL-1α and TNF-α expression in rat periapical lesions and dental pulp after unilateral sympathectomy", *Neuroimmunomodulation* 11: 376-384.

II. Bletsa A, Nedrebø T, Heyeraas KJ, Berggreen E (2006): "Edema in Oral Mucosa after LPS or Cytokine Exposure", *Journal of Dental Research* 85(5): 442-446.

III. Bletsa A, Berggreen E, Fristad I, Tenstad O, Wiig H (2006): "Cytokine signalling in rat pulp interstitial fluid and transcapillary fluid exchange during LPS-induced acute inflammation", *Journal of Physiology* 573: 225-236.

IV. Bletsa A, Berggreen E, Brudvik P: "IL-1α and TNF-α expression during early phases of experimental orthodontic tooth movement", *European Journal of Oral Sciences* In press.

Contents

1. INTRODUCTION

1.1 Inflammation

Inflammation is the first response of living tissue to injury and may be referred to as the innate immunity. The cause of inflammation may be due to microbial infections, physical factors (trauma, radiation, temperature), chemical substances (irritant and corrosive chemicals**)**, as well as tissue necrosis and hypersensitivity reactions. Inflammation is characterised by the following quintet: redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and dysfunction of the organs involved (*functio laesa*). The first four characteristics were drawn up by Celsus nearly 2000 years ago; *functio laesa* was added to the definition of inflammation by Rudolf Virchow in 1858.

Generally, the inflammatory response consists of a vascular and a cellular component. The vascular component represents changes in vessel diameter (vasodilation) and consequently increased blood flow (causing redness and heat), and increased vascular permeability resulting in loss of plasma into the tissue and formation of fluid exudate. The exudate contains many valuable proteins such as fibrin and immunoglobulins but it also gives rise to edema. The swelling distends the tissues, it may compress nerve endings and thus causes pain.

The cellular component involves the movement of white blood cells (leukocytes) from the blood vessels into the inflamed tissue. They extravasate from the capillaries into tissue, and act as phagocytes, picking up bacteria and cellular debris. They may also aid by walling off an infection and preventing its spread.

Influx of neutrophils is one of the earliest stages of the inflammatory response. These cells mount a rapid, non-specific phagocytic response. At a later stage, monocytes/macrophages and cells of other lymphocyte lineages (specific subsets of T cells and B cells) appear at the site of injury. These cell types are associated with antigen-specific and more tightly regulated immune responses and once activated also produce protective and inflammatory molecules.

All the above described changes in blood flow, increased permeability of blood vessels and escape of cells from the blood into the tissues are essentially the same whatever the cause and wherever the site of inflammation is. The sequelae of acute inflammation depend upon the type of tissue involved and the amount of tissue destruction, which depend in turn upon the nature of the injurious agent. The possible outcomes of acute inflammation can be either healing or chronic inflammation. Chronic inflammation is characterised by a dominating presence of macrophages in the injured tissue. These cells provide a powerful defensive mechanism in the body, but the mediators they release are injurious to the organism's own tissues, as well as invading agents. This is why chronic inflammation is almost always accompanied by tissue destruction (Trowbridge & Emling, 1997).

1.2 Inflammatory mediators

The inflammatory response and its outcome are orchestrated by a plethora of chemical substances that are released as a result of the harmful stimuli. These regulatory molecules termed as inflammatory mediators, are released and act in sequence and they serve as vasomotor and edema-promoting substances, chemotaxins, and cellular activators. The inflammatory mediators are released in the

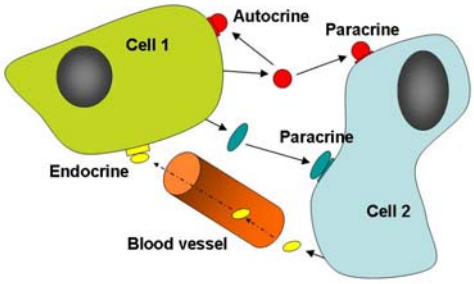
serum and/or tissue fluids by degranulation of cells, or they are secreted by activated inflammatory cells and/or endothelial cells at the site of inflammation. The plasma contains four enzymatic cascade systems, the complement, the kinins, the coagulation factors and the fibrinolytic systems, which are inter-related and produce various inflammatory mediators. Another group of mediators are neuropeptides such as tachykinins and VIP (vasoactive intestinal peptide), and the VEGF/VPF (vascular endothelial growth factor/vascular permeability factor). These substances enhance capillary permeability and have vasodilatory activity. Cell released inflammatory mediators include arachidonic acid metabolites such as prostaglandins (PGs) and leukotrienes (LTs), histamine, lysosomal compounds, 5-hydroxytryptamine (serotonin) and cytokines (Trowbridge & Emling, 1997). In this thesis, the focus has been on cytokines, particularly Interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α).

1.3 Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They are produced *de novo* in response to an immune stimulus. They generally, although not always, act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins including cytokine receptors, cell proliferation, and secretion of effector molecules.

Cytokine is a general name; other names used are lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes).

Fig. 1. Action of cytokines



Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances they enter the circulation and act on distant cells (endocrine action) (Fig. 1). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (pleiotropy). Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically (two or more cytokines potentiating each others effects) or antagonistically (cytokines causing contrasting effects) (Balkwill, 2001).

Nomenclature has always been a problem because these factors were originally named for the activity that they described. This resulted in a large number of three or four or occasionally five letter acronyms. The idea that the "interleukin" - between leukocytes - designation would simplify nomenclature has not proved to be

the case. A review in 1979 by Waksman listed almost 100 apparently distinct activities (Waksman, 1979). It was not known at the time whether these represented distinct cytokines or a few cytokines with multiple activities. The application of molecular technology allowed us to determine that some cytokines had multiple activities and different cytokines had similar overlapping activities and it also led to the discovery of even more cytokines. A search for new cytokines is now often conducted at the DNA level, identifying genes similar to known cytokine genes. New cytokines are still being identified and most are still being named based on their biological activities. Nomenclature continues to be a problem.

Pro-inflammatory cytokines: is a general term for those immunoregulatory cytokines that favor inflammation. The major pro-inflammatory cytokines that are responsible for early responses are: IL-1alpha (IL-1α), IL-1beta (IL-1β), IL-6, and TNF-α. Other pro-inflammatory mediators include interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-8, IL-11, IL-12, IL-17, IL-18, and a variety of other chemokines. The pro-inflammatory cytokines can act as endogenous pyrogens (IL-1, IL-6, TNF-α), up-regulate the synthesis of secondary mediators and other pro-inflammatory cytokines by both macrophages and mesenchymal cells (fibroblasts, epithelial and endothelial cells), stimulate the production of acute phase proteins or attract inflammatory cells (Billiau & Vandekerckhove, 1991; Cerami, 1992; Baumann & Gauldie, 1994).

Anti-inflammatory cytokines: is a general term for those immunoregulatory cytokines that counteract various aspects of inflammation, and thus contribute to the

control of the magnitude of the inflammatory responses *in vivo*. These mediators act mainly by the inhibition of the production of pro-inflammatory cytokines or by counteracting many biological effects of pro-inflammatory mediators in different ways. The major anti-inflammatory cytokines are IL-4, IL-10 and IL-13. Other anti-inflammatory mediators include IL-16, IFN-alpha (IFN-α), TGF-β, IL-1receptor antagonist (IL-1ra), granulocyte-colony stimulating factor (G-CSF), as well as soluble receptors for TNF or IL-6.

It should be pointed out that the common and clear-cut classification of cytokines as either anti-inflammatory or pro-inflammatory may be misleading. The net effect of the inflammatory response is determined by the balance between pro-inflammatory cytokines and anti-inflammatory cytokines. The type, duration, and also the extent of cellular activities induced by one particular cytokine can be influenced considerably by the nature of the target cells, the micro-environment of cells, depending for example on the growth and activation state of the cells, the type of neighboring cells, cytokine concentrations, the presence of other cytokines, and even on the temporal sequence of several cytokines acting on the same cell (Balkwill, 2001).

1.4 Lipopolysaccharide

One of the most common causes of inflammation is bacterial infection. Bacteria release specific exotoxins - substances synthesized by them which specifically initiate inflammation or endotoxins, which are components of their cell walls. Endotoxin is a term used to describe an essential lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria. Together with phospholipids and membrane-bound proteins it is a constituent of the outer cell membrane. The diversity of the bacterial

lipopolysaccharides explains the different characteristic antigenic properties of Gram-negative bacteria.

LPS consists of three structural elements (Fig. 2):

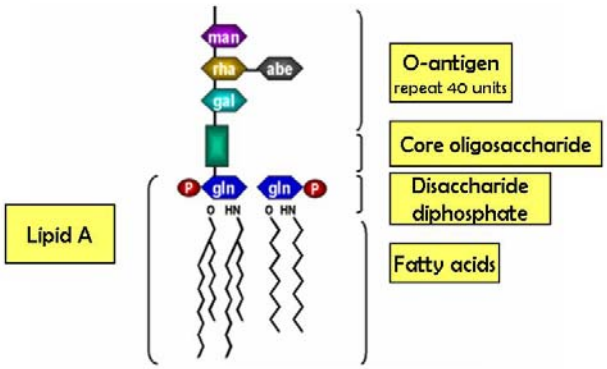


Fig. 2 Structure of lipopolysaccharide (LPS)

One is a hydrophobic component, called lipid A, which serves to attach the molecule into the membrane. The lipid A determines the endotoxin properties such as toxicity, pyrogenicity, macrophage and complement activation (Rietschel *et al.*, 1993). The second is a core oligosaccharide. The third component is a hydrophilic O-polysaccharide projecting into the extracellular space that determines the serotype specificity (Nester *et al.*, 2001). More than 150 different variants of the third component are known. Although Gram-negative bacterial endotoxins may differ considerably in their antigenicity they elicit the same physiological responses during infection of an organism. However, there is debate on the extend of host tissue injury that is due to the effects of LPS *per se,* relative to the effects that are secondary through induction of inflammatory mediators (Salgado *et al.*, 1994): LPS mediates cell activation of macrophages and activates the complement cascade, as well as the

synthesis of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 and non-protein mediators which in turn, are responsible for most pathophysiological consequences of a bacterial infection.

Toll-like receptors (TLRs) comprise a family of proteins that enhance certain cytokine gene transcription in response to various pathogenetic ligands (Akira *et al.*, 2001). TLR-4 is recognized as the LPS signaling receptor (Takeda *et al.*, 2003). However, LPS from some bacterial species, such as *Porphyromonas gingivalis*, activate cells through TLR-2 (Darveau *et al.*, 2004). The signaling events triggered by LPS in mammalian cells are believed to be initiated upon its binding with the LPS receptor molecule, CD14 (Schumann *et al.*, 1990). Formation of the complex between LPS and CD14 facilitates the transfer to the LPS receptor complex composed of TLR-4 and its accessory protein MD2 (da Silva Correia *et al.*, 2001; Takeuchi & Akira, 2001). Activation of these receptors initiates several intracellular signaling pathways, including the nuclear factor-kappa B (NF-κB), that regulate the balance between cell viability and inflammation (Takeda & Akira, 2004). Moreover, TLRs and IL-1β have common signaling pathways, since TLRs are part of the IL-1receptor (IL-1R) family (Zhang *et al.*, 1999). There is also evidence that TNF-α acts in an autocrine fashion in inducing NF-κB (Natoli *et al.*, 1997; Song *et al.*, 1997).

Under some conditions LPS can enter the circulation (septicemia) causing a systemic inflammatory response which is detrimental to the host (Pinsky, 2004). At the cellular level septic shock is elicited by endogenous mediators. The list of shock mediators is rather long including histamine, complement factor C5a, Beta-Endorphin, thromboxane B2, platelet activating factor (PAF), and oxygen free

radicals. In addition, pro-inflammatory cytokines but also anti-inflammatory cytokines are elevated in the blood stream and the condition has been referred to as a "Cytokine Storm". The major pro-inflammatory cytokines involved in septic shock are IL-1, IL-6 and TNF-α (Ertel *et al.*, 1991), which are released by macrophages following cell activation by bacterial endotoxins.

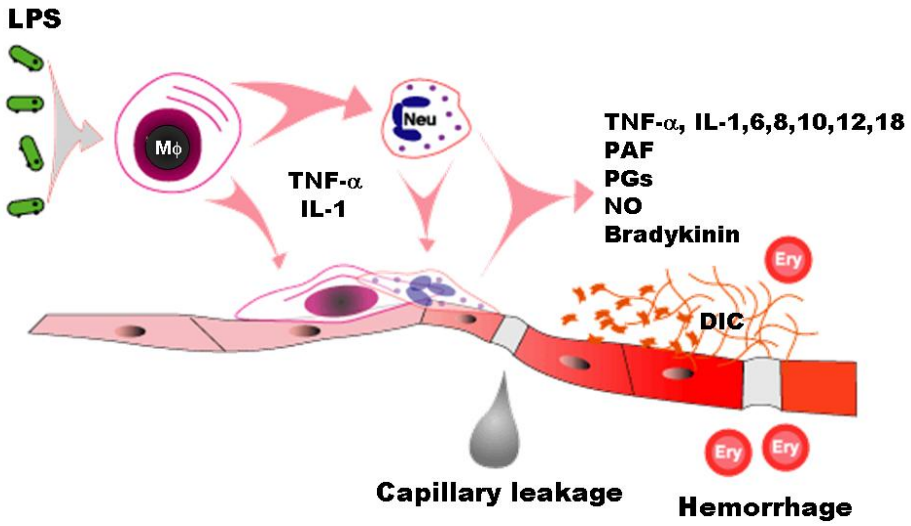


Fig. 3 During infection, the normal functions of the endothelium are perturbed by several inflammatory mediators, contributing to the organ failure characteristic of the end stage of sepsis. Mφ, macrophage; Neu, neutrophils; DIC, disseminated intravascular coagulation; Ery, erythrocytes. (Modified from Bendtzen, 1996).

Multiple organ dysfunction may represent the end stage of sepsis (Fig. 3). The symptoms are characterized by hypotension, insufficient tissue perfusion, uncontrollable bleeding, and multisystem organ failure caused mainly by hypoxia, tissue acidosis, and severe local alterations of metabolism (Mayer *et al.*, 1995).

1.5 Dental Inflammation

The relationship between bacterial infection and dental inflammation is very well established (Kakehashi *et al.*, 1965; Theilade *et al.*, 1966; Lindhe *et al.*, 1973). Pulpitis is the result of bacterial infection due to dental caries, trauma and operative procedures and it can be either reversible or irreversible. Gingivitis, the mildest form of periodontal disease, is caused by the dental plaque that accumulates on teeth adjacent to the gingiva. Gingivitis does not affect the underlying supporting structures of the tooth and is reversible. Apical and marginal periodontitis are chronic infectious diseases characterized by a destructive inflammatory process affecting the supporting tissues of the tooth. Periodontitis results in loss of connective tissue and bone support and is a major cause of tooth loss in adults. All of the above forms of dental inflammation have common feature that the primary etiologic factor is the bacterial biofilm. Bacteria found within the oral cavity possess a plethora of structural or secreted components that may cause direct destruction to dental tissues or stimulate host cells to activate a wide range of inflammatory responses. At birth, the oral cavity is sterile but bacterial colonization begins quickly, creating the so called oral microbial flora or microbiota. More than 500 species living in different ecosystems where anaerobic, strictly anaerobic (65%), saprophytic and pathogenic species all co-exist, are found in the mouth (Paster *et al.*, 2001). Under healthy conditions there is a natural balance (eubiosis) between bacteria and host. Disturbance of this equilibrium because of an increase in the number and/or virulence of the bacteria and/or because the host defenses are low, leads to the disease (dysbiosis). In other words, bacteria play an important role in the onset and

subsequent development of periodontal diseases but also a susceptible host is required.

In the human oral cavity, varieties of anaerobic gram negative bacteria exist and the LPS of these bacteria is thought to be a virulence factor that participates in the development of dental diseases (Slots & Genco, 1984; Warfvinge *et al.*, 1985; Larjava *et al.*, 1987). Once inflammation in terms of pulpitis, gingivitis, or periodontitis has been established, an inflammatory infiltrate is formed consisting of different cell types such as neutrophils, macrophages and lymphocytes that will produce different cytokine subtypes responsible for the immunopathology of the diseases. *In situ* production of pro-inflammatory cytokines is generally seen in inflamed pulp and/or gingiva as part of the inflammatory response (Van Dyke *et al.*, 1993; Zehnder *et al.*, 2003). In periodontitis, the bone resorption is mediated largely by the increased local production of pro-inflammatory cytokines, such as IL-1α, IL-1β, and TNF-α. LPS released from the periodontopathic bacteria triggers the synthesis of IL-1 and TNF-α from macrophages. IL-1 and TNF-α stimulate the production of a variety of other inflammatory mediators (cytokines, nitric oxide (NO), PGE2), matrix-degrading enzymes (metalloproteinases), and decrease the synthesis of glycosaminoglycans and thereby mediate bone destruction (Meyer, 2003; Hong *et al.*, 2004).

The cytokine production at the sites of inflammation in the dental tissues is part of the host response which is essentially protective in nature. Both under-activity (hypo-responsiveness) and over-activity (hyper-responsiveness) of the host response, such as unrestricted production of cytokine(s), can result in enhanced tissue

destruction. Periodontitis shares many pathologic features with other inflammatory diseases with concomitant bone resorption such as rheumatoid arthritis (RA). There is accumulative evidence that both conditions manifest as a result of an imbalance between pro-inflammatory and anti-inflammatory cytokines (Bartold *et al.*, 2005). The increased local production of pro-inflammatory cytokines either directly enhances the proliferation and/or activity of cells in the osteoclast lineage or indirectly affects the production of the essential osteoclast differentiation factor, receptor activator of NF-κB ligand, and/or its soluble decoy receptor, osteoprotegerin, by osteoblast/stromal cells (Boyce *et al.*, 2005).

Not only is the inflammatory bone destruction governed by local cytokine production but also the normal bone remodeling. Physiologically, bone undergoes continuous resorption and rebuilding. A negative balance between bone resorption and formation, frequently due to excessive resorption, is the basis of many bone diseases. Among the locally produced factors that regulate physiologic bone remodeling are PGs, IL-1, TNF-α and possibly IL-6 (Rodan, 1992). Resorption is carried out by osteoclasts, which are specialized multinucleated cells of hemopoietic origin and bone formation is carried out by osteoblasts. The main strategy in clinical orthodontics is the application of mechanical forces in order to produce an organized periodontal tissue remodeling with an ultimate goal; the tooth movement. The orthodontic forces are transmitted from the dental roots to the periodontium where cells are stimulated to remodel the matrices that surround them. Orthodontic movement causes bone resorption in the pressure sites and bone deposition in the tension sites (Reitan, 1954; Rygh, 1973, 1976; Brudvik & Rygh, 1993). Again,

cytokines such as IL-1α, IL-1β, and TNF-α have been implicated in the process (Davidovitch *et al.*, 1988; Saito *et al.*, 1991) (Fig. 4).

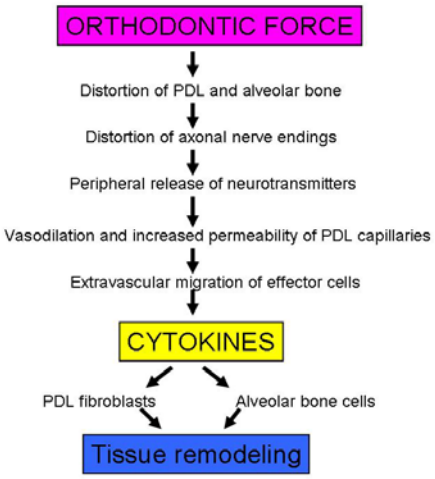


Fig. 4 A schematic model for the involvement of cytokines in tissue remodeling upon orthodontic tooth movement (Modified from Davidovitch *et al.*, 1988).

1.6 Sympathetic Nervous System and Cytokines

Traditionally, the immune functions have been though to be regulated by signals originating within the immune system. It is now evident that the immune system is regulated in part by the central nervous system (CNS), acting principally via the hypothalamic-pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS)(Elenkov *et al.*, 2000; Haddad *et al.*, 2002). The communication between immune system and brain is bidirectional and the ultimate goal of this communication is maintenance of homeostasis. The SNS provides a major integrative and regulatory pathway for this communication. The sympathetic innervation of lymphoid tissue (Felten *et al.*, 1988), the presence of adrenergic receptors on immune cells (B and T lymphocytes, macrophages) and studies of catecholamine interactions with the immune system (Madden *et al.*, 1995) provide substantial evidence for the role of SNS in immune regulation. Furthermore, the cellular products of an activated immune system, namely cytokines, can signal to the brain. The cytokines TNF-α, IL-

1 and IL-6 are involved in the cross-talk between brain and immune system via corticotropin-releasing hormone (CRH) secretion and hence, they activate both the HPA and the SNS (Besedovsky *et al.*, 1986; Chrousos, 1995; Elenkov *et al.*, 2000).

As previously mentioned, several infectious, autoimmune, but also allergic and neoplastic diseases have been attributed to the imbalance between pro-inflammatory and anti-inflammatory cytokines and therefore, the regulation of pro/anti-inflammatory cytokines has attracted considerable interest (Elenkov & Chrousos, 1999). Components of the innate immunity such as antigen presenting cells (APCs), dendritic cells, and monocytes/macrophages, as well as components of the acquired (adaptive) immunity such as T helper (Th) lymphocytes, are all involved in immunoregulation. The Th lymphocytes are divided into two subclasses; Th1 and Th2. Th1 cells primarily secrete IFN-γ, and IL-2 which enhance cellular immunity, whereas Th2 cells secrete a different set of cytokines, primarily IL-4, IL-10, IL-13 and IL-9, which enhance humoral immunity (Abbas *et al.*, 1996; Fearon & Locksley, 1996). The antigen –inexperienced (naïve) CD4+ Th0 cells can differentiate to either Th1 or Th2 and the differentiation is strongly dependent on the cytokines produced by cells of the innate immune system (Fearon & Locksley, 1996). IL-12 produced by activated monocytes/macrophages, represents a major inducer of Th1 differentiation and hence cellular immunity. IL-12, together with TNF-α and IFN-γ act synergistically in inflammation and further promote Th1 responses and hence considered major pro-inflammatory cytokines (Fearon & Locksley, 1996). Th1 and Th2 responses are mutually inhibitory. Thus IL-12 and IFN-γ inhibit Th2 and vice

versa, IL-4 and IL-10 inhibit Th1 responses and the production of pro-inflammatory cytokines (Abbas *et al.*, 1996; Fearon & Locksley, 1996). IL-4 and IL-10 are the major anti-inflammatory cytokines and an increasing body of evidence suggests that catecholamines inhibit selectively the Th1 functions and pro-inflammatory cytokines and favour the Th2 responses and anti-inflammatory cytokines (Elenkov *et al.*, 2000).

The *periapical lesion* represents a product of the body's immune response to bacterial infection of the pulp, with the purpose to wall-off the infection within the confines of the root canal system (Stashenko, 1990). In many respects this periapical inflammatory response recapitulates the pulpal response to infection, with the additional feature of periapical bone destruction. A mixed infiltrate of T and B lymphocytes, polymorphonuclear leukocytes (PMNs), macrophages, plasma cells, natural killer (NK) cells, eosinophils and plasma cells has been characterized (Cymerman *et al.*, 1984; Torabinejad & Kettering, 1985; Gao *et al.*, 1988; Kopp & Schwarting, 1989; Stashenko & Yu, 1989; Kawashima *et al.*, 1996). However, there are controversial results regarding the predominant infiltrating cell type in periapical lesions, with either lymphocytes (Bergenholtz *et al.*, 1983; Kontiainen *et al.*, 1986) or macrophages (Kopp & Schwarting, 1989; Kawashima *et al.*, 1996) generally reported to be most numerous. T cells consistently outnumber B cells (Kontiainen *et al.*, 1986; Yu & Stashenko, 1987). Of the T lymphocytes both Th and T suppressor cells have been identified with temporal differences regarding their relative concentration (Yu & Stashenko, 1987; Stashenko & Yu, 1989; Kawashima *et al.*, 1996). The variety of inflammatory cells reveals that both non-specific and specific immune responses are mediated in the pulp and periapical tissues in response to infection. Therefore, the

periapical lesion provides a suitable model for studying local immune responses. The superior cervical ganglion (SCG) supplies with sympathetic innervation the oral tissues (Anneroth & Norberg, 1968; Pohto, 1972). By removal of the SCG, the local control of immune responses by sympathetic nerves can be studied in periapical lesions.

1.7 Interstitial fluid and Transcapillary fluid balance

The interstitial fluid (IF) represents extracellular fluid located outside the blood vessels. Interstitial fluid bathes the cells of the tissues and it provides a mean of delivering materials to the cells, intercellular communication, as well as removal of metabolic waste. Plasma, the major component of blood, communicates with the interstitial fluid through pores and intercellular clefts in the capillary endothelium. The capillary wall acts as a filtration "barrier". Most of the fluid within the capillaries is retained, but some fluid filters normally through pores between the cells, pushed by the pressure difference between the capillary blood and the IF. Water and small solutes can pass freely through these pores. The net effect of the hydrostatic pressure alone is a net loss of water and solutes from plasma to the IF. The capillary wall is, however, nearly impermeable to the plasma proteins and lipids but following inflammation, the capillary permeability for proteins increases. Because the capillary wall is permeable to water, but essentially impermeable to the plasma proteins, these molecules generate a colloid osmotic pressure gradient that draws water out of the interstitium and into the plasma. This pressure is proportional to the difference in protein concentration between the plasma and the IF.

The British physiologist Starling (1896) first identified the interrelationship between the hydrostatic pressure (P) and the colloid osmotic pressure (COP) between blood vessels and interstitium according to the equation:

Transcapillary fluid flux (J_v) = CFC [(P_c-P_{if})-σ(COP_c-COP_{if})], where the subscripts "c" and "if" represent the capillary and the interstitium respectively, while σ is the reflection coefficient for proteins (σ =1 for impermeable vessels and σ =0 when the capillary wall is freely permeable for proteins). CFC is the capillary filtration coefficient that relates to the surface area subject to filtration and the wall's hydraulic conductivity. In other words, the Starling's law tells us that normally ΔP tends to cause fluid to leave the capillary, and ΔCOP pulls it back. These two forces tend to balance each other. Furthermore, IF may drain into the lymphatic vessels and increased lymph flow occurs subsequent to increased IF volume and pressure. When the fluid filtration across the capillary wall is increased above that which can be removed by lymph flow, then edema is generated.

Traditionally, there are four mechanisms for edema formation:

1. Increased vascular permeability (caused by changes in the structural integrity of the vascular wall)
2. Increased P_c (e.g. in vasodilation or venous obstruction)
3. Decreased COP_c
4. Lymphatic obstruction

5. In addition, lowering of P_{if} is an extremely potent factor leading to increased fluid flux and edema formation during acute inflammation, first described by Lund *et al*., in skin burn injuries (Lund *et al.*, 1987)

Compliance is defined as the change in IF volume (ΔIFV) divided by the corresponding change in P_{if} (ΔP_{if})(Guyton, 1965). In low-compliant tissues such as brain, rat tail, dental pulp, and attached gingiva, a modest change in IFV will be counteracted by a marked change in P_{if} acting as edema preventive mechanism (Tønder & Kvinnsland, 1983; Wiig & Reed, 1983; Aukland & Wiig, 1984; Aarli & Heyeraas, 1991). The increase in tissue pressure triggers an increase in lymphatics' flow that helps to keep interstitial fluid volume close to physiological levels. On the other hand, lowering of P_{if} leads to rapid edema formation during acute inflammation in several tissues (eg. skin, trachea, nasal mucosa) (Rodt *et al.*, 1990; Koller & Reed, 1992; Reed *et al.*, 1992; Koller *et al.*, 1993; Berg *et al.*, 1998).

In dental tissues, there is limited information about transcapillary fluid exchange upon acute inflammation. Measurements of P_{if} in dental pulp, oral mucosa, gingiva and PDL have been performed mainly with micropuncture, a fluid equilibration technique (Tønder & Kvinnsland, 1983; Johannessen *et al.*, 1987; Kristiansen & Heyeraas, 1989; Fjaertoft *et al.*, 1992; Jacobsen & Heyeraas, 1997; Del Fabbro *et al.*, 2001). COP_{if} measurements in the gingiva and oral mucosa, as well as protein concentration in the gingival crevicular fluid (GCF), have been measured with the wick-fluid technique (Aarli & Heyeraas, 1995; Del Fabbro *et al.*, 2001). However, there are no data available regarding intra- and extra-vascular fluid volumes in dental tissues (oral mucosa, pulp) during health and disease. The lack of

such information from the dental pulp is mainly due to the nature of the tissue that does not allow easy access to the interstitium. Nevertheless, attempts have been made to isolate dentinal fluid, which is a pulpal IF that moves across the dentinal tubules, in order to measure COP and protein concentration (Brown *et al.*, 1969; Maita *et al.*, 1991; Knutsson *et al.*, 1994). A prerequisite for collection of dentinal fluid is drilling a deep cavity in the dentin and removal of the smear layer. These are rather invasive procedures that may affect the pulpal blood flow and subsequently the rate and direction of dentinal fluid flow (Vongsavan & Matthews, 1992b, a). Furthermore, the radius of the exposed tubules (Pashley, 1985) and the disruption of the odontoblast layer as a result of the preparation trauma (Bishop, 1992), may yet influence the dentinal fluid composition regarding plasma proteins. For the above reasons, it is evident that there is a need for a less invasive and traumatic method for isolation of pulp IF in order to explore the pulp microenvironment.

As previously mentioned, changes in the structural integrity of the vascular wall is one mechanism for edema formation. Increased vascular permeability can be induced by cytokines such as IL-1 and TNF-α acting either directly at the endothelial cells (Martin *et al.*, 1988; Wong *et al.*, 1999)or indirectly by inducing other inflammatory mediators that affect the vascular endothelium (Rossi *et al.*, 1985; Bussolino *et al.*, 1986). In addition, LPS can increase vascular permeability not only through induction of pro-inflammatory cytokines, but also affecting directly the endothelial barrier (Bannerman & Goldblum, 1999, 2003). It has been shown recently in rat skin that the pro-inflammatory cytokines IL-1β and TNF-α and IL-6 induce lowering of P_{if} and therefore, contribute to edema formation in an experimental

model of acute inflammation (Nedrebø *et al.*, 1999). The oral mucosa and the gingiva are constantly challenged by bacteria and bacteria by-products. The clinical signs and symptoms of gingivitis have been attributed to pathological tissue changes that take place at the level of gingival microcirculation (Del Fabbro *et al.*, 2001). LPS and cytokines have only been implicated in the vascular component of edema formation (Daffonchio *et al.*, 2002), however their possible effect on the interstitium has not been explored.

2. AIMS OF THE PRESENT STUDY

Tissue inflammation is the first response of the body to infection or injury. It is a rather complex phenomenon with many different mediators participating in the process and cytokines playing a central role. The overall aim of this thesis was to study the involvement of cytokines in various aspects of oral inflammation (Fig. 5).

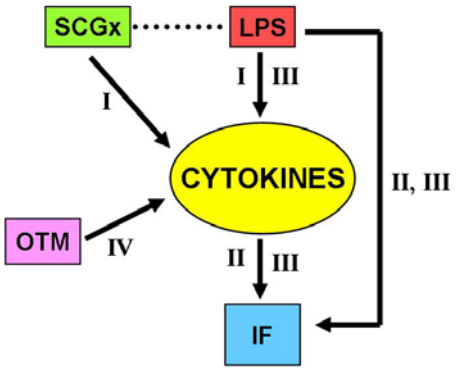


Fig. 5 Cytokines, especially IL-1 and TNF-α, played a central role in this thesis. This illustration points out the different topics investigated in the individual papers.

Specific aims

1. To study the role of sympathetic innervation in IL-1α and TNF-α production in bacterial-induced periapical lesions and intact dental pulp (Paper I)
2. To explore if LPS and the pro-inflammatory cytokines IL-1β and TNF-α have a mechanistic role in development of edema in oral mucosa (Paper II)
3. To establish a method for isolating dental pulp IF (Paper III)
4. To investigate transcapillary fluid exchange in the dental pulp under LPS-induced acute inflammation (Paper III)
5. To study cytokine signaling in pulp IF during LPS-induced acute inflammation (Paper III)

6. To investigate the effect of mechanical force application in the expression of the pro-inflammatory cytokines IL-1α and TNF-α in the dental tissues (Paper IV)

3. MATERIALS AND METHODS

A brief review of the experimental material and methods is presented here. The methodological details are given in the individual papers.

3.1 Animal Experiments

Male Sprague-Dawley (Paper I) or Wistar-Møller (Paper IV) as well as female Wistar-Møller (Paper II, III) rats were used. All rats were housed in polycarbonate cages at the animal facility unit at least one week prior to the experiments. Standard food pellets and tap water were given *ad libitum*. The housing unit was temperature controlled (22 ± 2 ^{o}C) with a constant 12-hour light and 12-hour darkness cycle.

All the experiments described in this thesis have been carried out in accordance with the recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee.

Anesthesia: The rats were anesthetized with either subcutaneous injection of a 1:1 mixture of fluanizon midazolam/fentanyl (Hypnorm/Dormicum) 2.7 ml/kg body weight (Paper I, IV) or with sodium pentobarbital (Mebumal) 50 mg/kg body weight given intraperitoneally (Paper II, III).

Sympathectomy (Paper I): A vertical incision was made on the ventral surface of the neck adjacent to the midline under anesthesia. The right SCG was identified under a stereomicroscope and subsequently excised. Successful sympathectomy was

confirmed by ptosis of the ipsilateral eyelid. Sham surgery consisted of incision and localization of the SCG.

Pulp exposure (Paper I): The first and second maxillary molars in both sides were drilled with a round bur until the pulp was exposed. All pulp exposures were left open to the indigenous oral microflora for 3 weeks and periapical lesions were developed.

P_{if} measurements (Paper II): The P_{if} in the rat oral mucosa was performed 1-2 mm distal-buccally of the maxillary left incisor. Sharpened glass capillaries with tip diameter 2-6 μm filled with colored 0.5 M NaCl were inserted into the tissue with a micromanipulator under the guidance of a stereomicroscope. The micropipettes were connected to a servo-controlled counter-pressure system (Fig. 6).

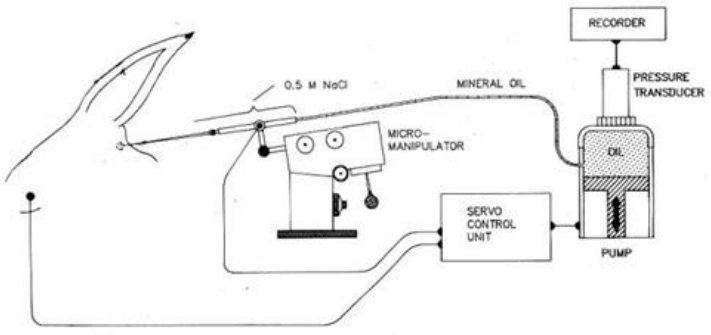


Fig. 6 Experimental set-up for measuring P_{if} with the micropuncture technique

The set-up was calibrated before each experiment and zero pressure was checked repeatedly throughout the measurements.

PA recordings (Paper II, III): The femoral artery was cannulated with polyethylene catheter connected to a pressure transducer and recorder for continuous measurements of the systemic blood pressure.

PBF recordings (Paper III): A laser-Doppler flow meter was used to continuously record PBF in the left maxillary incisor of animals treated with LPS for 3 hours. The head of the rat was immobilized and fixed on the operating table and the laser-Doppler probe was positioned 3-5 mm above the level of the gingiva on the distal aspect of the tooth.

Administration of substances (Paper II, III): Doses and protocol for substance administration are presented in detail in each individual paper. Briefly, in paper II, IL-1β and TNF-α were given either systemically or locally at the oral mucosa of rats. LPS was administered only locally at the oral mucosa. One to 3 min following injection of the substances, cardiac arrest was induced with saturated potassium chloride (KCl) i.v. Control rats received equivalent volume of vehicle (0.9% NaCl with 0.1-1% BSA) with the respective route of administration.

In Paper III, endotoxaemia was induced with i.v. administration of LPS. Controls received the equivalent volume of vehicle (0.9% NaCl with 0.1% BSA) i.v. At the end of the experimental periods (1.5 hr and 3 hr groups) the rats were killed with saturated KCl. When isotopes were used, the extracellular marker ^{51}Cr-EDTA was

circulating for at least 120 min whereas the intravascular marker ^{125}I-HSA was circulating for 5 min only prior to euthanasia with KCL.

Orthodontic tooth movement (Paper IV): The right first maxillary molar was moved mesially by means of a fixed orthodontic appliance consisting of a coil spring ligated to the molar and connected to an orthodontic band cemented onto the incisors. The delivering force was 50 g upon insertion and there was no reactivation of the spring during the experimental periods (3 hours, 1 day and 3 days after force application).

3.2 Enzyme-linked immunosorbent assay (ELISA) (Paper I)

Periapical lesions and incisor pulp were collected, weighed and immediately frozen and stored at -80 ^{o}C until extract preparation. The tissue was thereafter homogenized in lysis buffer and the tissue extract was used for quantification of IL-1α and TNF-α with commercially available ELISA kits according to the manufacturer's instructions (BioSource International Cytoscreen^{TM} Rat IL-1α and TNF-α, Camarillo, CA, USA). All samples were assayed in duplicate and the concentration of cytokines present was calculated with reference to a standard curve. The levels of cytokines were expressed as pg of cytokine/mg of tissue, calculated from the known weight of the tissue sample and the volume of lysis buffer in which the sample was homogenized.

3.3 Immunohistochemistry (Paper I, III, IV)

In Papers I and IV the rats used for immunohistochemistry were transcardiacally perfused through the aorta with heparinized saline followed by 4% paraformaldehyde

with 0.2% picric acid. All tissues were post-fixed overnight. In paper III, the rats were not perfused; the pulp from the incisor teeth was removed and fixed in the above fixative solution for 2 hr at 4 ^{o}C. The jaws (Paper I, IV) were decalcified in either Kristensen's decalcification solution (Paper I) or EDTA (Paper IV) for approximately 5-7 weeks. Tissues were rinsed in 0.1 M phosphate buffer, soaked overnight in 30% sucrose solution and stored at -80 ^{o}C until use.

Serial sections of the investigated tissues were made on a freezing slide microtome after embedding the tissues in mounting compound (Tissue-Tek OCT). The maxillary jaw sections were 40 (Paper I) or 20-25 (Paper IV) μm thick; pulp tissue was at 25 μm. Immunohistochemistry was performed either on free floating sections (Paper I) or on precoated glass slides (Paper III, IV).

After incubation for 2 hr in 2.5% normal goat serum, the sections were incubated for 72 hr in anti-rat IL-1α (dilution 1:400; Endogen, MA, USA) or anti-TNF-α (dilution 1:300, Endogen) polyclonal antibodies raised in rabbit. In paper I, anti-rat CGRP (dilution 1:6,000; Diagnostika, Falkenberg, Sweden) and anti-NPY (dilution 1:4,000; Diagnostika) primary antibodies (polyclonal raised in rabbits) were also used. The specificity of the immunoreactions was tested by omission of the primary antibody. Antigen-antibody complexes were detected by the avidin-biotin peroxidase (ABC) method and visualized by nickel enhanced 3,3'-diaminobenzidine (DAB). Finally, the sections were counterstained with methylene blue/azure IV. All sections were evaluated in light microscope.

The immunohistochemical analysis was performed for localization of IL-1α and TNF-α expression (Paper I, III) and confirmation of the success of sympathectomy (paper I). In paper III, a semiquantitative analysis was performed on selected areas, namely the mesial side of the distal root and the distal side of the mesial root of the first maxillary molar, as well as the gingiva and PDL mesially and distally to the first maxillary molar. The coronal and the root pulp of the first maxillary molar were only qualitatively evaluated. All of the above investigated areas were observed in the orthodontically moved (right) maxillary molars and compared with the contralateral (left) molars that served as untreated controls.

3.4 Fluid volume measurements (Paper II, III)

Oral mucosa and pulp IF volume was calculated as the difference between total extracellular fluid and plasma volume according to the equation: V_i (ml g^{-1}) = V_x - V_v. The measurements of fluid volumes require a tracer molecule that is distributed in the extracellular space and reach equilibrium between plasma and interstitial fluid, and a tracer molecule that is confined in the vascular compartment only. ^{51}Cr-EDTA was used as the extracellular marker since this probe is not metabolized and is not taken up by cells (Løkken, 1970). In addition, the rats were subjected to ligation of both kidney pedicles to avoid tracer removal by the kidneys, in order to achieve equilibrium between plasma and interstitial fluid with respect to ^{51}Cr-EDTA. ^{125}I-HSA was used as reference for the intravascular volume. This tracer was circulating for 5 min only before induction of cardiac arrest to minimize tracer extravasation. Tissue and blood samples were harvested after cardiac arrest, weighed and the

calculation of the parameters was based on the radioactive counts from both tissue and plasma, detected by a gamma radiation detecting equipment.

3.5 Isolation of IF (Paper III)

Based on a reliable method for isolation of IF from tumors (Wiig et al, 2003) we tested if it is possible to isolate pulp IF by centrifugation. Incisor pulp was carefully removed under 100% relative humidity and placed in a centrifuge tube provided with a basket of nylon mesh with pore size ~ 15-20 μm. The tube was spun at 1500 r.p.m. (239 *g*) for 10 min. The fluid (0.5-5.0 μl) collected at the bottom of the tube was representative for pulp IF.

Validation of the method was done by use of the extracellular tracer ^{51}Cr-EDTA and the intravascular tracer ^{125}I-HSA. Furthermore, the distribution of macromolecules in the centrifugate was determined by HPLC and compared with plasma, pulp tissue eluate and pulp tissue extract.

The isolated pulp IF was stored at -80 ^{o}C until further analysis.

3.6 Multiplex Analysis (Paper III)

Rat IF and serum was used for simultaneous quantitative analysis of six cytokines, namely IL-1α, IL-1β, IL-2, IL-6, IFN-γ and TNF-α with a commercially available multiplex kit [Lincoplex kit (Linco Research, St. Charles, Missouri, USA)] according to the manufacturers instructions. This kind of multiplex analysis is a microsphere-based flow cytometric immunoassay using the Luminex technology (Luminex Corporation Austin, TX, USA). Briefly, the principles of the multiplexed sandwich

immunoassay are: cytokine-specific capture antibodies are coupled to polystyrene microspheres internally dyed. The internal color of each bead is translated to different and unique emission spectra. These antibody-coupled beads are incubated with serum or interstitial fluid (containing antigen). Then, fluorochrome-labeled detection antibodies are added. The multiplexed assays are analyzed on a flow cytometer equipped with two lasers to excite the internally dyed beads and the fluorescent reporter. A digital signal processor is used to transform thousands of signals into manageable data output. Quantitation of the cytokines concentration in the samples is done with the aid of a broad range of standards (4.8-20000 pg/mL) provided in the multiplex kit with great sensitivity (Fig. 7).

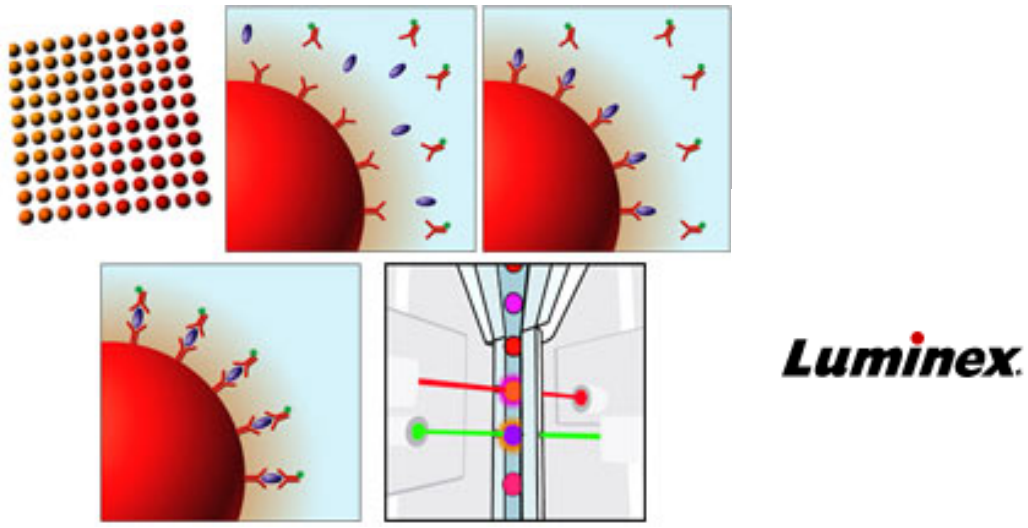


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Fig. 7 The multiplex immunoassay measures multiple analytes in a single reaction well employing the xMAP® technology of Luminex® Corporation. The xMAP® technology is based on 100 distinct sets of color-coded tiny beads, called microspheres. Each bead set can be coated with an antibody specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex system, lasers excite the internal dyes that identify each microsphere, and also any reporter dye captured during the assay. In this way, the xMap technology allows multiplexing of up to 100 analytes within a single sample.

3.7 COP measurements (Paper III)

A colloid osmometer designed for submicrolitre samples (lower limit for successful application was 0.1-0.2 μl) (Wiig et al., 1988) was used for measurements of COP in IF and plasma. The samples (0.5-2 μl) were applied to the membrane with cut-off size of 30 kDa, providing minimal exposure to air. The colloid osmometer was connected to a pressure transducer and calibrated with fluid of known COP before the sample measurements.

3.8 Statistical analyses (Paper I, II, III)

Differences within groups were evaluated using Student's paired *t*-test or one-way analysis of variance with repeated measures (RM-ANOVA) followed by Bonferroni or Fisher LSD or Dunn's post-hoc tests. For differences between groups unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni or Dunn's post-hoc tests were performed. A $p<0.05$ was considered as statistically significant.

4. RESULTS

4.1 Expression of IL-1α and TNF-α in dental tissues

Dental pulp: Immunohistochemical analysis of normal rat pulp (Paper I, III, and IV) revealed differences in the staining pattern for these two cytokines. The odontoblast layer was intensely stained for IL-1α but not for TNF-α. In the pulp body, scatter cells were stained for TNF-α but no staining for IL-1α was observed. Low levels of both IL-1α and TNF-α were detected with ELISA in the normal incisor pulp of all rats (Paper I). Multiplex analysis of the pulp IF showed low levels of both IL-1α and TNF-α in the control rats (Paper III) in agreement with the ELISA results. When the pulp was inflamed, the staining pattern for the above cytokines changed. The odontoblasts lose the IL-1α-staining capacity and increased numbers of IL-1α- and TNF-α-IR cells were seen in the pulp body (Paper I, III and IV). The stained cells resembled immune cells or fibroblasts (Paper IV). During the LPS-induced acute pulpitis, a tremendous increase in IL-1α and TNF-α in the pulp IF was observed (Paper III).

Periapical lesions: IL-1α and TNF-α were both detected with ELISA in the tissues from the periapical area of the exposed 1st and 2nd maxillary molars (Paper I).

Gingiva: Intense staining of the rat gingiva for both IL-1α and TNF-α was observed under normal conditions (Paper I, IV). The staining was mainly localized at the gingival epithelium. IL-1α and TNF-α were also detected with ELISA in the rat gingiva, in higher concentrations than the ones measured in the incisor pulp (Bletsa,

unpublished data). Heavier staining for the above cytokines was observed in the gingiva around the orthodontically moved first molar (Paper IV).

Periodontal Ligament and Alveolar Bone: Immunohistochemical staining of untreated maxillae (Paper IV) showed no staining for IL-1α in all the rats, whereas a few sections exhibited light staining for TNF-α (2 out of total 7 rats) at the PDL and the alveolar bone. When mechanical irritation was introduced, increased staining for these cytokines was observed (Paper IV).

4.2 Effect of sympathectomy on IL-1α and TNF-α production (Paper I)

Dental Pulp: Significantly lower levels of TNF-α were detected with ELISA in the incisor pulp of the SCGx-side compared with the contralateral one (non-SCGx) ($p < 0.05$), whereas no differences were seen in IL-1α.

Periapical lesions: Significantly higher levels of IL-1α were detected with ELISA in the periapical lesions of the SCGx-side compared with the non-SCGx-side ($p < 0.006$), whereas no differences were seen in TNF-α levels.

4.3 Effect of IL-1β, TNF-α and LPS on edema formation in oral mucosa (Paper II)

P_{if}: The pro-inflammatory mediators IL-1β and TNF-α lowered P_{if} significantly following both intravenous and submucosal administration. Submucosal injection of LPS induced an immediate increase in P_{if} followed by lowering after 40 min. No changes in P_{if} were seen after systemic or local injections of vehicle.

Fluid volumes: 1.5 hr after systemic administration of LPS, V_i was increased significantly from 0.41 ± 0.02 to 0.51 ± 0.03 ml/g wet weight ($p < 0.05$) whereas V_v remained unchanged. This 24.4% increase in V_i confirmed edema formation.

4.4 Isolation of pulp IF (Paper III)

After testing different centrifugation parameters (G-force and centrifugation time), the optimal conditions were set at 239 *g* (1500 r.p.m.) for 10 min. This kind of centrifugation gave a pulp fluid to plasma ^{51}Cr-EDTA ratio not different from 1.0, demonstrating that intracellular fluid was not diluting the fluid obtained by centrifugation. The centrifugate was found to contain 8% of the intravascular tracer. The HPLC pattern of pulp fluid resembled closely to that of plasma and pulp eluate, indicating that the centrifugation process per se did not trigger critical cell destruction.

All of the above implied that the fluid isolated by the proposed centrifugation method, was representative for pulp IF.

4.5 Effect of LPS-induced endotoxaemia on transcapillary fluid exchange and cytokine levels in the pulp (Paper III)

Fluid volumes: In the rat incisor pulp $V_i = 0.60 \pm 0.03$ ml/g w.w. and $V_v = 0.03 \pm 0.01$ ml/g w.w. There were no changes in the distribution of fluid volumes 1.5 and 3 hr after LPS exposure.

PBF: PA and PBF decreased significantly within 10 min after LPS administration compared to baseline values ($p < 0.001$). PBF remained significantly

($p < 0.001$) low throughout the experimental period whereas PA was restored at the end of the 3 hr period.

COP: Under normal conditions, the pulp IF COP averaged 19 ± 1.3 mmHg, corresponding to 83% of plasma COP ($p < 0.05$). Three hours after LPS exposure, there was no difference in COP between pulp IF and plasma (Fig. 8).

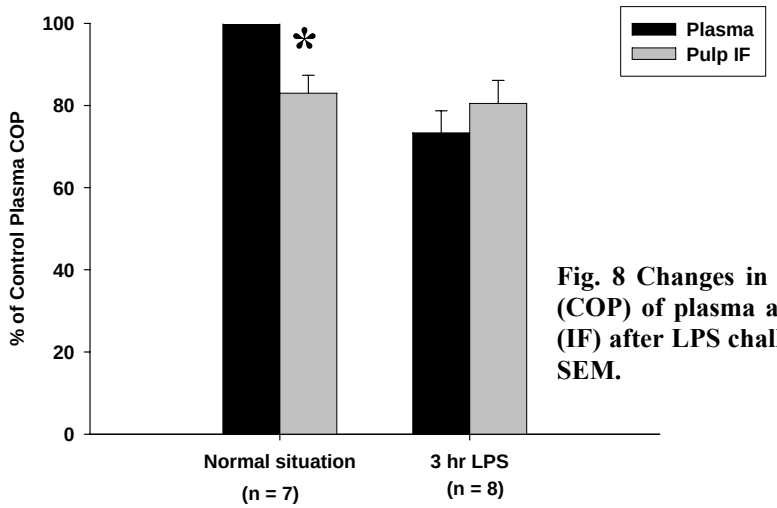

Fig. 8 Changes in colloid osmotic pressure (COP) of plasma and pulp interstitial fluid (IF) after LPS challenge. Values are mean ± SEM.

Cytokine concentrations: The IL-1α, IL-1β, IL-6, IFN-γ and TNF-α levels were increased compared to control values in both serum and pulp IF after exposure to LPS. There was a differential pattern of cytokine expression in pulp IF and serum. IL-1α, IL-1β and TNF-α exhibited a significantly higher concentration in pulp IF compared with serum ($p < 0.05$), indicating local production of this cytokine in the pulp tissue whereas IFN-γ was significantly higher in serum ($p < 0.05$), suggesting systemic production of this cytokine. IL-6 showed parallel increase in pulp IF and serum after LPS exposure.

4.6 Effect of orthodontic tooth movement on IL-1α and TNF-α production (Paper IV)

Periodontal Ligament and Alveolar Bone: Increased IL-1α and TNF-α staining intensity was observed 1 day and 3 days after mechanical force application with the sites of pressure (mesial aspect of distal root) exhibiting heavier staining than the areas of tension (distal aspect of the mesial root) for both cytokines. In addition, staining for the above cytokines was observed in the PDL and bone mesially and distally to the orthodontically moved teeth.

Gingiva: Placement of the orthodontic appliances caused disfiguration of the interdental papillae. Very intense staining that extended to the subjunctional area and the PDL was observed. The gingiva exhibited heavier staining for IL-1α and TNF-α after OTM than the pressure (mesial aspect of the distal root) and tension (distal aspect of the mesial root) sites of the orthodontically moved tooth.

Dental Pulp: Mild inflammatory changes were seen in the pulp at the early stages of OTM (Fig. 9)

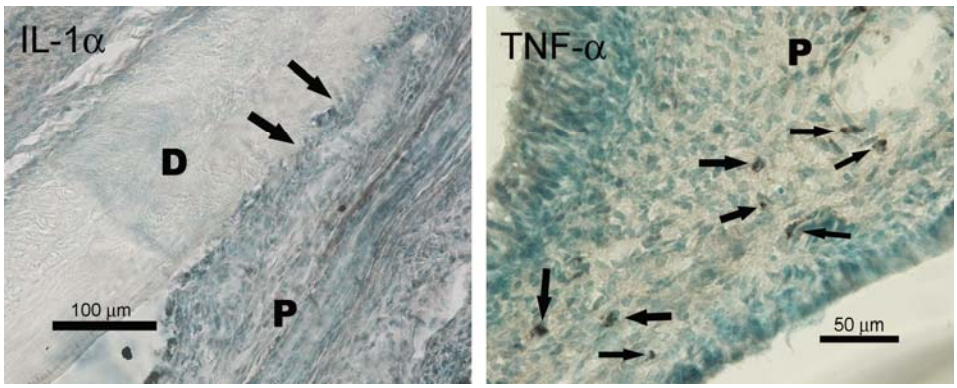

Fig. 9 Sporadically, the odontoblasts (arrows) of the root pulp adjacent to the pressure site (mesial aspect of the distal root) were losing their staining capacity for IL-1α. Three days after mechanical force application, more TNF-α-IR cells (arrows) were seen at the pulp floor of orthodontically moved 1st molars compared with the pulp in the untreated control side. The above findings were classified as mild inflammatory changes in the pulp. P, pulp; D, dentin.

5. GENERAL DISCUSSION

In this thesis, the focus was on the role of cytokines in different aspects of inflammation in dental tissues. A method for isolation of pulp IF was established and used for measurements of cytokines during acute pulpitis, as well as for COP measurements. Furthermore, information on transcapillary fluid exchange was provided by measurements of P_{if} in the oral mucosa and measurements of fluid volumes in the intra- and extra-vascular compartments of pulp and oral mucosa during LPS-induced acute inflammation.

5.1 Pro-inflammatory cytokines in dental tissues in response to bacterial or mechanical stimuli

IL-1 and TNF-α are pro-inflammatory cytokines involved in bone resorption (Roodman, 1993) and bone resorption is a common finding in several inflammatory diseases such as rheumatoid arthritis, osteomyelitis, marginal and apical periodontitis (Haynes, 2004; Bartold *et al.*, 2005). The IL-1α isoform seems to be of greater importance in the rat periodontium regarding bone resorption and therefore chosen in the current studies (Paper I, IV). A model of inflammatory bone resorption after bacterial challenge (Paper I), as well as bone resorption after mechanical stress (Paper IV) were utilized. In the periapical lesions, a different localization of IL-1α- and TNF-α-IR cells was observed immunohistochemically, with IL-1α-IR cells localized centrally and TNF-α-IR cells in the periphery of the lesion (Paper I). On the other hand, there were no spatial differences in cytokine expression during orthodontic

tooth movement at the sites of compression and tension (Paper IV). There was, however, difference in the time frame for cytokine up-regulation, with TNF-α up-regulation occurring as early as 3 hr after force application, whereas IL-1α up-regulation was observed after 1 day (Paper IV). As TNF-α staining was occasionally found at the PDL and the bone along the investigated roots in the non-treated control sections (Paper IV), the findings of the current study indicate involvement of TNF-α in physiological bone remodeling during the distal drift of the rat molars (Vignery & Baron, 1980; Kawahara & Takano, 1995). Furthermore, the earlier up-regulation of TNF-α compared with IL-1α, suggested an indirect role of TNF-α in bone resorption via IL-1α induction (Paper IV).

In the first paper, we explored the sympathetic modulation of IL-1α and TNF-α production. Sympathectomy did not affect the levels of TNF-α in the periapical lesions, whereas IL-1α was significantly increased in the SCGx side. This finding was attributed to the different localization of cytokine producing cells, as previously mentioned, and/or to the different time frame for the above cytokine production (Paper I). If the hypothesis of the indirect role of TNF-α in bone resorption is correct, then the more plausible explanation for the discrepancy in the TNF-α levels after sympathectomy is the different time frame. Cytokines have been detected in the periapical area as early as 3 days after pulp exposure (Wang *et al.*, 1997), and the observation period in the current study was as long as 21 days.

The anti-inflammatory role of sympathetic nerves, demonstrated with higher levels of IL-1α in periapical lesion of the SCGx side, is in agreement with previous

findings from studies on sympathetic nerves and periapical lesions. Larger periapical lesions and more ED1-IR osteoclasts lining the periapical lesions were seen after surgical sympathectomy in rats (Haug & Heyeraas, 2003). The increased production of IL-1α in this thesis (Paper I), can certainly explain the increased size of lesions after sympathectomy. A selective inhibitory control of the SNS in pro-inflammatory cytokines is not uncommon. Chemically sympathectomized rats challenged with LPS exhibited enhanced serum levels of IL-1β and IL-6, whereas TNF-α levels were not affected (De Luigi *et al.*, 1998).

Expression of IL-1α and TNF-α was also observed in the gingiva and the dental pulp under normal conditions (Paper I, IV). The gingiva, especially the interdental papillae, exhibited distinct staining for both cytokines, revealing some degree of inflammation (Paper I, IV). When cavities were drilled in teeth (Paper I) or orthodontic appliances were placed and activated (Paper IV) the expression of the above cytokines was increased and the staining was not confined at the epithelial layers of the gingiva but it was extended at the subepithelial connective tissue, indicating a severe inflammatory reaction. Upon tissue collection for the ELISA measurements of cytokine levels (Paper I), gingiva and oral mucosa were dissected free of the jaws. Considerable bone destruction was seen around the drilled molars showing that not only apical but also marginal periodontitis was developed, probably due to plaque retention at the area. In the orthodontically moved molars (Paper IV), the gingiva proved to be a significant source of cytokines. A combination of orthodontic forces together with a rigorous bacterial challenge has triggered a quite

high cytokine production. It is therefore, possible that the gingival tissues contribute significantly in bone remodeling during OTM.

Under physiological conditions, the gingival epithelium is continuously invaded by bacterial irritants from gingival sulcus, and hence the constitutive expression of pro-inflammatory cytokines is totally reasonable. In analogy, the odontoblasts are the outermost cells of the pulp and they represent the first line of defense against dental pathogens. Therefore, the expression of IL-1α in the odontoblast layer of normal pulp (Paper I) may be of significant importance in pulp homeostasis. Constitutive expression of other pro-inflammatory cytokines in odontoblasts, namely IL-8, has been previously reported (Levin *et al.*, 1999) and it was significantly increased after LPS challenge showing that the odontoblasts participate actively in inflammatory reactions. However, the odontoblasts capacity to express IL-1α was lost during pulpal inflammation (Paper I) indicating irreversible damage of the odontoblasts. Furthermore, the dental pulp is well-equipped with immunocompetent cells (Jontell *et al.*, 1987; Okiji *et al.*, 1992) possible responsible for the low expression of TNF-α under normal conditions. This low TNF-α expression is under sympathetic modulation (Paper I). During acute pulpitis there is a high local production of both IL-1 and TNF-α (Paper III) that should be accredited to both odontoblasts and cells of the pulp tissue proper. In the same model of retrograde pulpitis, increased levels of other pro-inflammatory cytokines such as IFN-γ and IL-6 were also detected in the pulp IF (Paper III) in agreement with other studies reporting elevated levels of IL-6 and IFN-γ in pulpal inflammation

(Barkhordar *et al.*, 1999; Hahn *et al.*, 2000; Tokuda *et al.*, 2001). During OTM, minor changes in IL-1α and TNF-α expression were seen in the pulp (Paper IV), showing that application of mechanical stress affects pulpal cytokine production. This finding is in line with previous reports showing inflammatory reactions in the dental pulp in response to OTM (Vandevska-Radunovic *et al.*, 1994; Haug *et al.*, 2003).

5.2 IL-1β, TNF-α and LPS affect P_{if}

The increased expression of pro-inflammatory cytokines observed in dental tissues in response to bacterial stimuli, is not surprising. It is due to the inflammatory reactions that aimed at eliminating the bacteria and protecting the host. In paper II, the potential effect of IL-1β and TNF-α, as well as LPS in the gingival interstitium was investigated.

Measurements of P_{if} with the micropuncture method were performed *post-mortem*. The reason was that if the investigated substances induced lowering of P_{if} then fluid filtration would have occured as part of the inflammatory process and a rapid increase in IFV and subsequently increase in P_{if} would have taken place. As a result, the initial lowering of P_{if} as driving force for edema development would have been reduced or remain undetected with the current technique. Therefore, circulatory arrest was induced as part of the experimental protocol, as it does not change P_{if} for up to 90 min after death (Wiig & Reed, 1981).

The main finding of Paper II was that both IL-1β and TNF-α induced lowering of P_{if} and therefore, contribute to rapid edema by creating “suction” on the fluid in

the capillaries. A proposed explanation for the phenomenon is perturbation of the β1-integrin receptors based on studies where *in vivo* blockade of these receptors caused local edema in rat skin associated with lowering of P_{if} (Reed *et al.*, 1992). As these receptors bind the cells and the extracellular matrix proteins together (Hynes, 1992), it has been suggested that they are responsible for keeping the connective tissue under tensile forces and thus, any disturbance or loss of such binding may cause tissue expansion and concomitant decrease in P_{if} (Wiig *et al.*, 2003b). There is evidence that cytokines such as IL-1β and TNF-α affect the integrin expression and hence, the interaction with extracellular matrix proteins such as fibronectin (Rinaldi *et al.*, 1994; Kao *et al.*, 2002). Changes of the β1-integrin organization are seen in advanced stages of periodontitis in human gingival epithelial cells (Del Castillo *et al.*, 1996).

Local administration of LPS had an immediate increasing effect on P_{if} followed by a lowering of P_{if} in the same time interval as the injected cytokines did. Systemic administration of LPS induces lowering of P_{if} in the rat paw skin but local injection does not have the same effect (Nedrebø *et al.*, 1999). This discrepancy was partly attributed to the lack or low number of cytokine producing cells after LPS stimulation in the skin. This is not the case in the oral mucosa/gingiva where a plethora of cytokine positive cells reside under normal conditions (Paper I, IV). However, the LPS effect in P_{if} cannot be solely ascribed to the release of IL-1β and/or TNF-α since the response was slightly different with a more rapid and pronounced effect of LPS.

Besides P_{if} recordings, edema formation after LPS exposure was confirmed with measurements of the fluid volumes in the intra- and extra-vascular compartments of the rat oral mucosa showing an increase of ~24% in V_i compared with controls (Paper II). When the same LPS model was used to induce retrograde pulpitis, there were no changes in the distribution of fluid volumes in the pulp (Paper III), probably due to the low compliance of this organ.

5.3 Pulp IF and haemodynamic changes during acute inflammation

In paper III the core interest was the microenvironment around the pulpal cells. The dental pulp houses a number of tissue elements but its cellular component is relatively low. Since the total tissue water in the pulp is 75% of w.w. (Berkovitz *et al.*, 2002) and measurements of fluid volumes in the rat pulp showed that 63% of tissue w.w. is water localized extracellularly (Paper III), then only 12% of tissue w.w. is water in the intracellular compartment, pointing out that the pulp interstitium represents a significant component of the tissue.

Based on a successful technique used for isolation of IF from rat mammary tumors (Wiig *et al.*, 2003a), a centrifugation method for isolation of IF from rat incisor teeth was established. The main advantage of this method is that no inflammatory changes are introduced in the pulp due to the procedure, as no drilling, no smear layer removal, no dentin-traumatic procedures are used that could have damaged the odontoblast layer (Turner *et al.*, 1989; Ohshima, 1990) and thereby affected the composition of the IF. Therefore, the centrifugation method used in this

thesis has strong advantages over the dentinal fluid isolation methods used in the past.

Approximately 0.5-5 μm of pulp IF could be collected by centrifugation and six different pro-inflammatory cytokines were measured. Traditionally, protein concentrations in plasma/serum and tissue homogenates have been measured with ELISA (Paper I). Polymerase chain reaction (PCR) and different blotting techniques have been used mostly for qualitative studies. A problem that arises with ELISA is sample size. For measurements of six or more different cytokines we must have a substantial amount of IF in order to perform the analysis or to increase significantly the number of experimental animals. In paper III, a multiplex assay was used to perform simultaneous measurements of different cytokines in only few micro liters of sample. Considering the significantly reduced working time and relative cost of the multiplex assay compared with ELISA, as well as the small sample size and therefore, the low number of rats, we can greatly appreciate the benefits of this method.

The main finding from the cytokine measurements after LPS-induced endotoxaemia was the differential cytokine response in serum and pulp IF. The cytokines IL-1α, IL-1β and TNF-α were locally produced in this model of retrograde pulpitis, whereas IL-6 and IFN-γ were systemically produced and most likely spilled over to the pulp interstitium. It is very interesting that although the whole body is under a systemic inflammatory response due to sepsis, some organs e.g. the dental pulp exhibit an exaggerated response as shown by the increased local production of

pro-inflammatory cytokines. The differential cytokine response in acute inflammation has been previously reported in the rat skin where IL-1β was locally produced and TNF-α was systemically produced after experimental endotoxaemia (Nedrebø *et al.*, 2004). The current results are partly in agreement with the results from the skin showing that there are tissue differences in cytokine responses and pointing out that exploring the interstitium gives a better understanding of the mechanisms involved in inflammatory processes in each tissue.

Taking into consideration the findings of paper II regarding the effect of IL-1β and TNF-α on P_{if}, we can imagine the harmful consequences of the local production of cytokines in the low-compliant pulp. Although no changes in V_i were measured, the capillary permeability is likely increased as the COP in pulp IF and plasma were similar after the LPS exposure (Paper III). Both LPS and locally produced cytokines are known to increase capillary permeability. In addition, the pulp exhibited very low perfusion after LPS challenge. The low perfusion probably results in reduced P_c favoring fluid reabsorption in the capillary and hence, increased COP_{if}, partly explaining the equilibration of the COP between pulp IF and plasma. All of the above haemodynamic and transcapillary changes during the current model of pulpitis implied that the pulp was severely damaged. Pulp necrosis as a result of bacterial ingress in the tissue is a very common phenomenon in clinical dentistry and results in the need for root canal therapy. The current study provides evidence for organ dysfunction development in the dental pulp after LPS exposure, in apparent association with the organ failure commonly seen at the end stage of sepsis.

6. CONCLUSIONS

1. The sympathetic nerves inhibit the production of the bone-resorptive cytokine IL-1α in rat periapical lesions. The odontoblast layer of intact rat pulp expresses constitutively IL-1α. This capacity is lost upon inflammation and it is not controlled by sympathetic nerves. Low expression of TNF-α is seen in intact rat incisor pulp and sympathetic nerves have a stimulatory effect on this cytokine in the pulp under normal conditions.
2. IL-1β, TNF-α and LPS induce lowering of P_{if} in the rat oral mucosa and thereby, contribute to edema formation during acute inflammation. The LPS-induced lowering of P_{if} in the rat oral mucosa is probably mediated via IL-1β and/or TNF-α dependent and independent mechanisms.
3. V_i and V_v in the rat oral mucosa average 41 % and 2 % of tissue w.w. respectively. LPS-induced acute inflammation results in a V_i of 51 % of tissue w.w., whereas V_v remains unchanged. This 24.4 % increase in IFV confirms edema formation in the oral mucosa after LPS exposure.
4. Centrifugation of the rat incisor pulp at 239 *g* for 10 min in a basket of nylon mesh with pore size ~ 15x20 μm is a reliable method for isolation of pulp IF.
5. V_i and V_v in the rat incisor pulp average 60 % and 3 % of tissue w.w. respectively and COP_{if} corresponds to 83 % of plasma COP. LPS-induced acute pulpitis does not affect the distribution of fluid volumes in the rat pulp whereas COP_{if} equilibrates with plasma COP. In the same model of pulpitis,

the pulp develops organ dysfunction resembling the clinical situation of pulpal necrosis after bacterial infection.

6. The pulp IF provides more accurate information regarding extracellular signaling mechanisms during inflammation. In a model of LPS-induced retrograde pulpitis elevated levels of several pro-inflammatory cytokines is found in the pulp IF. A differential pattern of cytokine expression in pulp IF and serum with locally produced IL-1α, IL-1β and TNF-α, and systemically produced IFN-γ and IL-6, is seen.
7. OTM affects IL-1α and TNF-α expression in supportive dental tissues almost immediately after mechanical force application. TNF-α up-regulation occurs first and probably induces IL-1α up-regulation in the compression and tension sites. Increased expression of IL-1α and TNF-α is seen at the gingiva of the orthodontically moved teeth and it extends toward the marginal bone implying involvement in bone remodeling. Minor changes in IL-1α and TNF-α expression are also seen in the dental pulp during the very first stages of OTM.

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