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Inflammatory cell death of human macrophages induced by *Aggregatibacter actinomycetemcomitans* leukotoxin

av

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ABSTRACT

Aggregatibacter (Actinobacillus) actinomycetemcomitans is a bacterium mainly associated with aggressive forms of periodontitis. Among its virulence factors, a leukotoxin is suggested to play an important role in the pathogenicity. Periodontal infections with strains producing high levels of the leukotoxin are strongly associated with severe disease. Leukotoxin selectively kills human leukocytes and can disrupt the local defense mechanisms. Previous studies examining the role of the leukotoxin in host-parasite interactions have mainly focused on polymorphonuclear leukocytes (PMNs). In the inflamed periodontium, macrophages play a significant role in the regulation of the inflammatory reactions and the tissue breakdown and remodeling.

Thus, the aim of this dissertation was to investigate death mechanisms of human macrophages exposed to leukotoxin.

Human lymphocytes, PMNs, and monocytes/macrophages isolated from venous blood were exposed to purified leukotoxin or live *A. actinomycetemcomitans* strains producing variable levels or no leukotoxin. Different target cells were characterized by their expression of cell surface molecules. Cell death and viability were studied by examining cell membrane integrity and morphological alterations. Further, processes and cellular markers involved in apoptosis and necrosis were investigated. The expression and activation of pro-inflammatory cytokines of the leukotoxin-challenged leukocytes were examined at the mRNA and protein level. The biological activity of the secreted cytokines was investigated by testing the culture supernatants in a bone resorption assay. Additionally, different intracellular signaling pathways involved in the pro-inflammatory response from the macrophages were examined.

Monocytes/macrophages were the most sensitive leukocytes for *A. actinomycetemcomitans* leukotoxin-induced lysis. This process in monocytes/macrophages involved caspase-1 activation, and in addition, leukotoxin triggered abundant activation and secretion of IL-1β from these cells. The secreted IL-1β was mainly the 17 kDa bioactive protein and stimulated bone resorption. This activity could be blocked by an IL-1 receptor antagonist. When live bacteria were used, the *A. actinomycetemcomitans*-induced IL-1β secretion from human macrophages was mainly caused by the leukotoxin. Closer examination of the macrophages exposed to leukotoxin revealed that the induced cell death proceeded through a process that differed from classical apoptosis and necrosis. Interestingly, this process resembled a newly discovered death mechanism termed pyroptosis. The extensive leukotoxin induced IL-1β secretion did not correlate to increased levels of mRNA for IL-1β. It was mainly mediated by caspase-1 activation, since blocking it by a specific inhibitor also abolished the secretion of IL-1β. A similar pattern, but at much lower level, was seen for IL-18.

In conclusion, these results show that *A. actinomycetemcomitans* leukotoxin induces a death process in human macrophages leading to a specific and excessive pro-inflammatory response. Our results indicate that this novel virulence mechanism of leukotoxin may play an important role in the pathogenic potential of *A. actinomycetemcomitans*.

Key words: *Aggregatibacter actinomycetemcomitans*, Leukotoxin, IL-1β, Caspase-1, Inflammatory cell death, Pyroptosis

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"*The ascent triggered something in me. As I churned upward, I reflected on my life, back to all points, my childhood, my early races, my illness and how it changed me… I saw my life as a whole. I saw the pattern and the privilege of it, and the purpose of it, too. It was simply this: I was meant for a long, hard climb*."

Lance Armstrong

American cancer survivor and the record-breaking seven times winner of the "Tour de France"

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Aggregatibacter (Actinobacillus) actinomycetemcomitans is a bacterium mainly associated with aggressive forms of periodontitis. Among its virulence factors, a leukotoxin is suggested to play an important role in the pathogenicity. Periodontal infections with strains producing high levels of the leukotoxin are strongly associated with severe disease. Leukotoxin selectively kills human leukocytes and can disrupt the local defense mechanisms. Previous studies examining the role of the leukotoxin in host-parasite interactions have mainly focused on polymorphonuclear leukocytes (PMNs). In the inflamed periodontium, macrophages play a significant role in the regulation of the inflammatory reactions and the tissue breakdown and remodeling.

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Monocytes/macrophages were the most sensitive leukocytes for *A. actinomycetemcomitans* leukotoxin-induced lysis. This process in monocytes/ macrophages involved caspase-1 activation, and in addition, leukotoxin triggered abundant activation and secretion of IL-1β from these cells. The secreted IL-1β was mainly the 17 kDa bioactive protein and stimulated bone resorption. This activity could be blocked by an IL-1 receptor antagonist. When live bacteria were used, the *A. actinomycetemcomitans*-induced IL-1β secretion from human macrophages was mainly caused by the leukotoxin. Closer examination of the macrophages exposed to leukotoxin revealed that the induced cell death proceeded through a process that differed from classical apoptosis and necrosis. Interestingly, this process resembled a newly discovered death mechanism termed pyroptosis. The extensive leukotoxin induced IL-1β secretion did not correlate to increased levels of mRNA for IL-1β. It was mainly mediated by caspase-1 activation, since blocking it by a specific inhibitor also abolished the secretion of IL-1β. A similar pattern, but at much lower level, was seen for IL-18.

In conclusion, these results show that *A. actinomycetemcomitans* leukotoxin induces a death process in human macrophages leading to a specific and excessive pro-inflammatory response. Our results indicate that this novel virulence mechanism of leukotoxin may play an important role in the pathogenic potential of *A. actinomycetemcomitans*.

Key words: *Aggregatibacter actinomycetemcomitans*, Leukotoxin, IL-1β, Caspase-1, Inflammatory cell death, Pyroptosis

TABLE OF CONTENTS

ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AgP	aggressive periodontitis
ATP	adenosine triphosphate
CD	cluster of differentiation
Cdt	cytolethal distending toxin
DC	dendritic cell
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell scanner
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCF	gingival crevicular fluid
ICE	IL-1β-converting enzyme
IL-1	interleukin-1
IL-1R AcP	IL-1 receptor accessory protein
IL-1R I	interleukin-1 receptor type I
IL-1R II	interleukin-1 receptor type II
IL-1Ra	interleukin-1 receptor antagonist
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-6	interleukin-6
IRAP	interleukin-1 receptor antagonist (i.e. IL-1Ra) protein
IκB	inhibitor of NF-κB
LAP	localized aggressive periodontitis
LDH	lactate-dehydrogenase
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
Ltx	*A. actinomycetemcomitans* leukotoxin (i.e. LtxA)
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
OPG	osteoprotegerin
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
PRR	pattern recognition receptor
RANK	receptor activator of NF-κB
RANKL	RANK-ligand
RTX	repeats-in-toxin
TEM	transmission electron microscopy
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
WB	western blot
WT	wild-type

LIST OF ORIGINAL PAPERS

This doctoral dissertation is based on the following four individual papers, which will be referred to by their Roman numerals in the text. Figures in the dissertation from these papers will be referred to by the Roman numerals of the papers followed by the number of the figures (e.g. Paper III: Fig. 2B).

I. **Caspase-1 involvement in human monocyte lysis induced by *Actinobacillus actinomycetemcomitans* leukotoxin.**
Kelk P, Johansson A, Claesson R, Hänström L, Kalfas S.
Infection and Immunity 2003; **71**: 4448-4455

II. **Abundant secretion of bioactive interleukin-1β by human macrophages induced by *Actinobacillus actinomycetemcomitans* leukotoxin.**
Kelk P, Claesson R, Hänström L, Lerner U, Kalfas S, Johansson A.
Infection and Immunity 2005; **73**: 453-458

III. **IL-1β secretion induced by *Aggregatibacter (Actinobacillus) actinomycetemcomitans* is mainly caused by the leukotoxin.**
Kelk P, Claesson R, Chen C, Sjöstedt A, Johansson A.
Int J Med Microbiol. 2008; **298:** 529-41

IV. **Inflammatory cell death of human macrophages in response to *Aggregatibacter actinomycetemcomitans* leukotoxin.**
Kelk P, Abd H, Claesson R, Sandström G, Sjöstedt A, Johansson A.
(Manuscript)

1. INTRODUCTION

1.1 General introduction

More than 700 bacterial species or phylotypes have been detected in the oral cavity of which over 50% have not been cultivated. Over 400 of these 700 species have been identified from the periodontal pocket, while the remaining 300 species have been identified from other oral sites such as the tongue, oral mucous membranes, carious lesions and endodontic infections. One individual can have roughly 100–200 of these 700 species, meaning that there is considerable diversity among different persons. However, there is a characteristic bacterial flora in subjects with periodontal disease compared to healthy subjects [Aas et al., 2005; Paster et al., 2006].

Periodontitis is one of mankind's most common inflammatory diseases, and the initiation of it by Gram-negative bacteria is well-accepted today. Nevertheless, the disease progression is believed to be mainly caused by the host response to the infection. In other words, the severity of the progession is caused by the induced inflammation [Graves, 2008; Slots and Ting, 1999; Van Dyke, 2007].

Today we know that one important part of the host's defense against infection is the macrophages. These cells are specialized phagocytic cells and are essential for the innate as well as for the acquired response. Their molecular mechanisms leading to phagocytosis and pathogen killing appear to be important for the modulation of inflammation [Kantari et al., 2008].

Aggregatibacter (Actinobacillus) actinomycetemcomitans is a bacterium mainly associated with severe forms of periodontitis [Pihlstrom et al., 2005; Slots and Ting, 1999]. This species has several virulence factors that enable it to colonize, invade, avoid the host defense system and cause tissue destruction. Among these traits, an exotoxin (leukotoxin) is suggested to play an important role for the disease initiation [Fine et al., 2006].

The topic of this dissertation is the host-parasite-interactions of human macrophages with this toxin.

Periodontitis is a complex and multi-factorial disease. My ambition is therefore to give the readers of this dissertation a thorough background before I get to the main focus of the dissertation. Thus, a substantial part of the introduction is probably well-known to those dentists and periodontists that read this dissertation. For those readers who are not in the field of odontology the initial parts of the introduction (sections 1.2-1.2.7) will hopefully be helpful to get familiar with the subject. It will also elucidate why we have chosen to study pathogenic mechanisms induced by the *A. actinomycetemcomitans* leukotoxin.

1.2 Periodontal diseases

1.2.1 Anatomy of the periodontal tissues

The periodontium (peri=around, odontos=tooth) is a term used for a combination of tissues around the tooth. It consists of the gingival epithelium, the gingival connective tissue, the periodontal ligament, the root cementum, and the alveolar bone [Hassell, 1993; Schroeder, 1971] (**Fig. 1**).

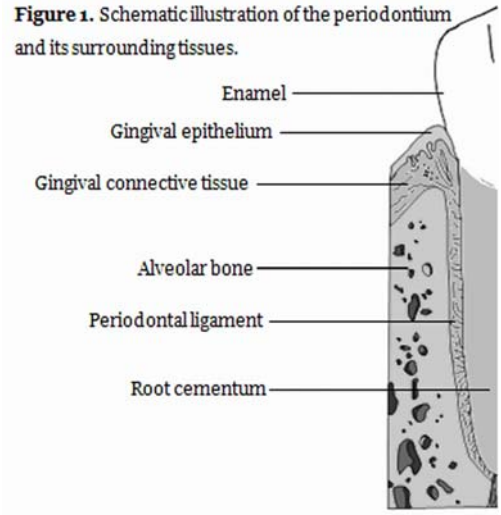


Figure 1. Schematic illustration of the periodontium and its surrounding tissues.

Its main function is to attach the tooth to the bone tissue; another function is to adapt the tooth to daily mechanical exposure.

Although the teeth and the periodontal tissues form a fairly "simple" organ, compared to other organs in the body, it is a very unique collection of tissues. The tooth is the only mineralized tissue that passes through a soft tissue so that a part of the tooth is exposed to the external environment (oral cavity) while the rest is surrounded by the connective tissue and alveolar bone. The presence of various bacterial species in the oral cavity and the nature of the tooth provide a surface which is well suited for bacterial colonization. Bacteria can adhere to the tooth, the epithelium of the gingiva, the epithelium in the periodontal pocket, the underlying connective tissues and to the whole complex, i.e. biofilm [Lindhe et al., 2008].

1.2.2 General background about periodontal diseases

All inherited or acquired disorders of the periodontium can be termed as periodontal diseases, with different sources such as developmental, inflammatory, traumatic, neoplastic, genetic, or metabolic [Armitage, 2004b; Jordan, 2004].

Periodontal diseases are extremely prevalent and can affect up to 90% of the worldwide population [Pihlstrom et al., 2005]. Nevertheless, the term periodontal disease in the literature in general refers to gingivitis and periodontitis that are caused by the pathogenic microflora in the biofilm or dental plaque. Gingivitis and periodontitis comprise the vast majority of the periodontal diseases [Pihlstrom et al., 2005].

The strict medical definitions of these periodontal diseases according to the Medical Subject Headings (MeSH) created and updated by the United States National Library of Medicine (NLM) are as follows:

- Gingivitis is inflammation of gum tissue (gingiva) without loss of connective tissue.
- Periodontitis is inflammation and loss of connective tissues supporting or surrounding the teeth.

Gingivitis can be acute or chronic and it can be aggravated by pregnancy, diabetes, puberty, contraceptives, ascorbic acid deprivation, and menstruation [Armitage, 2004b; Jordan, 2004; Pihlstrom et al., 2005].

Periodontitis can also be acute or chronic. Moreover, it can be generalized or localized. It is mainly found in adults as a slowly progressive chronic form. In contrast, it can be rapidly progressive and occurs mostly in children, adolescents and young adults, but it can also occur in adults [Armitage, 2004b; Jordan, 2004; Pihlstrom et al., 2005].

There are diseases and genetic disorders in which periodontal manifestations can be found. These diseases and disorders will not be discussed in this dissertation. However, some of the most important will be listed below [Armitage, 2004b; Jordan, 2004; Pihlstrom et al., 2005]:

- Diseases with periodontal manifestations:
  Diabetes, lichen planus, pemphigoid, pemphigus, leukemia, neurtropenia, Wegener's granulomatosis, erythema multiforme, candidiasis, HIV/AIDS, psoriasis, tuberculosis, gonorrhoea, primary and recurrent herpes simplex infection, lupus erythematosus, histoplasmosis, linear IgA disease, primary and metastatic carcinoma, Crohn's disease, and drug-associated gingival enlargement
- Genetic disorders with periodontal manifestations:
  Familial and cyclic neutropenias, granulomatous disease, agranulocytosis, Langerhans' cell disease, glycogen storage disease, hypophosphatasia, leukocyte adhesion deficiency, Papillon-Lefèvre, Chédiak-Higashi, Cohen, Ehlers-Danlos, Marfan's, Down's, Haim-Munk, and Kindlers syndromes

In addition to the widely accepted causal factor of microorganism and the above mentioned genetic and systemic factors/diseases, several environmental factors have been identified that can affect the periodontal health [Van Dyke and Sheilesh, 2005]. Tobacco smoking is the most accepted environmental factor that clearly leads to periodontal disease with a risk factor equal to that of lung cancer for long-term smokers [Bergstrom, 2004].

More precise sub-classifications of periodontal diseases will be presented in the next section.

1.2.3 Classification of periodontal diseases

The classification of the periodontal diseases has changed repeatedly over the years. In 1999, the International Workshop for "Classification of Periodontal Diseases and Conditions" decided to classify periodontitis by disease progression (chronic or aggressive) instead of the previous age of onset [Armitage, 1999; Kinane and Hodge, 2001].

The current classification from 1999 separates the periodontal disease categories into eight main groups [Consensus report, 1999]:

I. Gingival diseases
II. Chronic periodontitis (localized or generalized)
III. Aggressive periodontitis (localized or generalized)
IV. Periodontitis as a manifestation of systemic disease
V. Necrotizing periodontal diseases
VI. Abscesses of the periodontium
VII. Periodontitis associated with endodontic lesions
VIII. Developmental or acquired deformities and conditions

1.2.4 Diagnosis of gingivitis and periodontitis

Before anything can be mentioned about the prevalence of gingivitis and periodontitis, the diagnostic criteria for these conditions must be explained briefly.

Gingivitis and periodontitis, usually, show a low grade of symptoms, particularly if the disease has a slow progression and is not severe. However, there are signs that can be noticed by patients. Gingivitis often leads to bleeding from the gums during tooth brushing that is normally only a minor problem for the affected individuals. Periodontitis is usually without symptoms until the disease reaches a point where the teeth shift, loosen, or are lost. Moreover, persons with advanced periodontitis may have frequent periodontal abscesses and halitosis [Pihlstrom et al., 2005].

The clinical signs for gingivitis are classical signs of inflammation such as redness, swelling and bleeding. For making the right diagnosis of periodontitis, the entire constellation of signs and symptoms associated with the disease is taken into account before arriving at a diagnosis. For the diagnosis of an ongoing periodontitis, gingivitis that is the first phase of periodontitis must be present [Armitage, 2004b].

The clinical diagnosis does not primarily depend on the etiological factors such as microorganisms. Instead, the clinical diagnosis of periodontitis is based on visual and radiographic evaluation of the periodontal tissues and on measurements of the space between the tooth and gum (the periodontal pocket). These pockets in healthy individuals are 1–3 mm in depth, and deepen as periodontium and bone are lost due to inflammation [Armitage, 2004a;

Pihlstrom, 1992]. In a complete clinical examination, periodontal pocket depths and tissue support (attachment level) are measured at four to six locations around every tooth. If found, the quantity of supragingival periodontal biofilm (plaque), dental calculus, gingival bleeding, tooth mobility, and pus are recorded [Armitage, 2004a; Pihlstrom, 1992]. In epidemiological studies, these measurements are often simplified and the number of measurement sites is reduced. Although these partial examinations have revealed disease severity, they can significantly underestimate disease prevalence [Stoltenberg et al., 1993].

1.2.5 Prevalence of gingivitis and periodontitis

Gingivitis, the first phase and the mildest form of periodontal disease, is highly prevalent and easily reversible by appropriate oral hygiene on a daily basis. Gingivitis affects 50–90% of adults worldwide, depending on its exact definition [Albandar and Rams, 2002].

Two large epidemiological studies from Sweden and Norway showed prevalence of 17%-18% with moderate and 6-7% with severe periodontitis [Hugoson et al., 2008; Skudutyte-Rysstad et al., 2007]. A collection of studies from the National Health and Nutrition Examination Surveys (NHANES) in the USA, covering several thousand individuals from 1988–2000, showed an overall prevalence of periodontitis from 2.5% up to 11.4% [Borrell et al., 2005]. Another large survey from 1988-1994 estimated that about 22% of US adults had mild disease and 13% had moderate or severe disease. It was also shown that periodontitis is more common in men than women and is more common in African- and Mexican-Americans than Caucasian-Americans [Albandar et al., 1999].

Clearly, prevalence calculations might differ on the basis of how the disease is defined. However, the prevalence, severity, and rate of disease progression show clear discrepancies worldwide. In general, periodontitis is more common in developing countries [Loe et al., 1986; Loe et al., 1978; Rylev and Kilian, 2008; van Palenstein Helderman et al., 1996].

1.2.6 Etiopathogenesis of periodontitis

Causal factors, such as specific microorganisms, are crucial for initiation of periodontitis. However, the progression of this disease is also related to various host-based risk factors. Based on epidemiological evidence, the term “risk factor” refers to a personal behavior or lifestyle, an environmental exposure, or an inborn or inherited characteristic. In fact, periodontitis is now recognized to be a eco-genetic disease that reflects its multi-factorial nature [Kinane and Mark Bartold, 2007].

The search for the etiology of periodontitis has led to several different hypotheses during the last 130 years. These hypotheses have been continually modified or developed as we started to understand more about the anatomy, histology, microbiology, and molecular biology of the oral cavity. Technological improvements have also helped. Four major hypotheses regarding the

etiology of periodontitis have been presented throughout the last 130 years [Marsh, 2003; Socransky and Haffajee, 1994]:

- Ameabe, fusiforms, spirochetes and streptococci were considered as etiological factors up to the 1930s.
- The "non-specific plaque hypothesis", which was presented in the 1950s, focused more on the quantity of the dental plaque.
- The "specific plaque hypothesis", which was presented in the 1960s and 1970s, proposed that only a few species could cause periodontitis. Thus, the quality of the plaque was shown to be more important than the quantity.
- The "ecological plaque hypothesis", a modification of the previous hypothesis, was introduced in the 1990s. In that version, the relationship between plaque and the host in health and disease was the main concept. The disease can be prevented not only by directly inhibiting the supposed pathogens, but also by interfering with the ecosystem's factors.

Infectious diseases in humans can be caused by one or more restricted sets of pathogens. Nevertheless, variants of the same pathogen can be more or less virulent or the host can be more or less susceptible to the disease. This is also the case for periodontitis [Lindhe et al., 2008]. Periodontitis seems to be a polymicrobial disease and can be caused by a relatively restricted set of pathogens, either alone or in combination. Such species include*: Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Eubacterium nodatum, Fusobacterium nucleatum, Parvimonas micra, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Streptococcus intermedius, Tannerella forsythia,* and *Treponema sp.* [Haffajee and Socransky, 1994].

Fundamentally, as mentioned previously, periodontitis results from the host's response to the masses of oral bacteria. These microorganisms initially colonize the tooth surface above the gingival margin where salivary proteins provide the nutrition [Groenink et al., 1998]. Eventually, the supragingival bacteria migrate to the subgingival region where the nutrition source is switched to serum proteins, resulting in the survival of the adapted subgingival flora in the periodontal pocket [Delima and Van Dyke, 2003].

In this environment (**Fig. 2**), the bacteria are confronted with professional phagocytes. Occasionally these bacteria may descend into the underlying connective tissue. Finally, bacterial factors and abundant host responses such as antigens, toxins, enzymes and cytokines lead to tissue destruction. Eventually this leads to separation of the tooth from its underlying bone and connective tissue support, i.e. periodontitis [Graves, 2008; Page et al., 1997].

Most of the bacterial species in the plaque do not cause disease. This commensal flora normally can overcome or reject the "foreign" invaders. However, if these invaders "get a grip" they can disrupt the homeostasis and invade the weakened community. This disrupted host-flora equilibrium can be intensified by early host responses that can allow the "invaders" to progress

from the supragingival space to the subgingival area. As the progression of the disease continues, an anaerobic population shift in the subgingival biofilm occurs [Fine et al., 2006; Page et al., 1997].

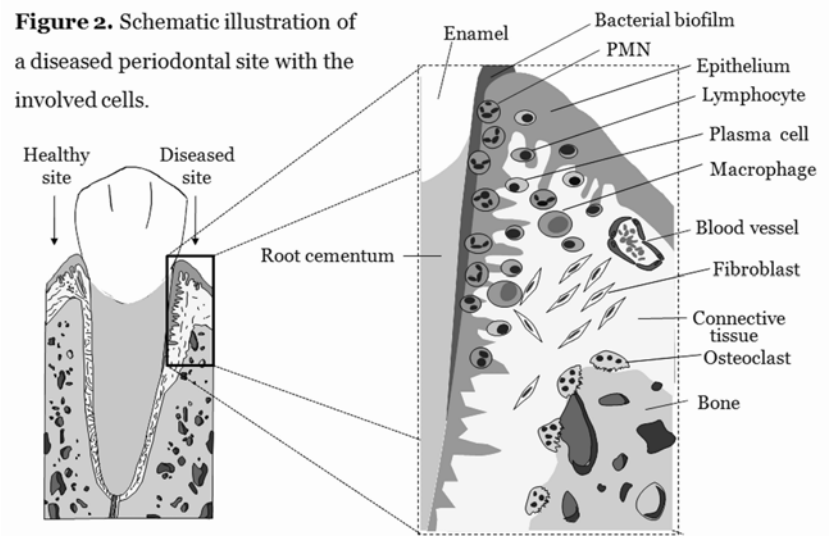


Figure 2. Schematic illustration of a diseased periodontal site with the involved cells.

The search for which of the invaders that could cause periodontitis led to a modified version of the famous "Koch's Postulates". In this version, Socransky proposed a principle to systematically identify those bacteria that are periodontal pathogens. These modified postulates consisted of the following standards [Socransky, 1977]:

- The presence of high numbers of the microorganism in the destructive periodontal lesion as compared to either its absence or its small presence in healthy or non-progressive sites.
- Elimination of the potential pathogen from the periodontal lesion results in healing and clinical improvement.
- Host-specific immune response against the supposed pathogen.
- Pathogenic bacteria express virulence factors that can contribute to the manifestation of clinical disease.
- Appropriate experimental models demonstrate similar tissue destruction in the presence of the reputed pathogen.

An extensive worldwide research for finding the key periodontal pathogens according to Socransky's modified postulates led to a consensus report in 1996. In that report, three of the previously mentioned bacterial species were identified as more common in diseased subjects, and those were designated as periodontal pathogens [Consensus report, 1996]:

- *P. gingivalis* and *T. forsythia* (mostly associated with adult/chronic periodontitis)
- *A. actinomycetemcomitans* (mainly associated with aggressive periodontitis)

1.2.7 Aggressive periodontitis

As mentioned earlier, aggressive periodontitis (AgP) can be divided in two major groups:

- Localized aggressive periodontitis (LAP)
- Generalized aggressive periodontitis (GAP)

The term "aggressive" was introduced because of the rapid bone and tissue destruction compared to many of the adult forms of periodontal disease [Tonetti and Mombelli, 1999]. The term LAP (previously termed localized juvenile/early onset periodontitis, LJP/EOP) refers to the fact that the destruction is mainly restricted to a few teeth (usually, the first molars and incisors). It also refers to the fact that the disease has its onset early in life [Kinane and Hodge, 2001].

Although the proportion of AgP is small as compared to other forms of periodontitis, a small but significant number of patients suffer from this disease worldwide [Lindhe et al., 2008; Slots and Ting, 1999]. What makes AgP interesting, besides its rapid progression, is that the etiological factors are much fewer than those normally seen in chronic periodontitis. This is particularly true in the case of LAP where the disease seems to be associated mainly with a single pathogen [Fine et al., 2006; Lindhe et al., 2008; Slots and Ting, 1999].

The prevalence of LAP is not consistent among the world's populations [Rylev and Kilian, 2008]. In USA the mean prevalence is 0.53% among adolescents of all racial origins. However, adolescents of African-American descent have a 15-fold higher incidence of disease than Caucasian Americans [Loe and Brown, 1991]. In Brazil 3.7% of 15- to 16-year-old adolescents have LAP, while in Nigeria a prevalence of 0.8% is found [Gjermo et al., 1984; Harley and Floyd, 1988; Macgregor, 1980].

Interestingly in adolescents with AgP, loss of periodontal support is the primary cause of tooth loss. In contrast, dental caries is the main cause of tooth extraction in the adolescents that do not suffer from AgP [Albandar et al., 1996]. Moreover, subjects with AgP have significantly less caries experience than subjects with chronic periodontitis [Al Omari et al., 2008].

Unlike caries (the "other" prevalent dental infection), where the bacteria colonize the tooth, consume carbohydrates and create acid that leads to tooth demineralization, LAP is an infectious disease that seems to require bacterial colonization of at least three separate environments [Fine et al., 2006; Loesche, 1986; Ximenez-Fyvie et al., 2000]. Accordingly, in the case of LAP, *A. actinomycetemcomitans* appears to have the machinery that allows it to colonize and survive in these three environments: on the oral mucosa, on the tooth surface and in the subgingival area [Henderson et al., 2003].

In the next section this bacterium will be more thoroughly presented.

1.3 *Aggregatibacter actinomycetemcomitans*

1.3.1 General background and taxonomy

In 1912, Klinger discovered a previously undescribed gram-negative coccobacillary microorganism that was mostly found together with *Actinomyces* in the actinomycotic granules [Klinger, 1912]. He named these organisms as "*Bacterium actinomycetem comitans*":

- actes (Greek), meaning ray, because of the star-shape that the colonies of this species formed when they were cultured on agar plates
- mycetes (Greek), meaning fungus, because *Actinomyces* was originally thought to be a fungus
- comitans (Latin), meaning in common with (refers to *Actinomycetes spp).*

Topley & Wilson in 1929 reclassified this bacterium to *Actinobacillus actinomycetemcomitans* [Topley, 1929]. The phylogenetic (evolutionary relatedness) analyses based on 16S rRNA (ribosomal RNA) gene showed that *A. actinomycetemcomitans* was closely related to *Haemophilus aphrophilus* and *Haemophilus paraphrophilus*, all belonging to the *Pasteurellaceae* family [Olsen et al., 1999]. In addition, further investigation of the phylogenic and phenotypic characteristics of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* led to a new genus in 2006. This new genus is termed *Aggregatibacter* since all these genetically related rod-shaped bacterial species have a tendency to aggregate [Norskov-Lauritsen and Kilian, 2006].

Fortunately, the abbreviation of both *Aggregatibacter* and *Actinobacillus* is an "*A.*", making the reading of this dissertation less confusing. The new genus, *Aggregatibacter*, has been accepted and used since 2007.

1.3.2 Association with disease

A. actinomycetemcomitans, in particular, has caught the attention of scientists because of its association with LAP [Fine et al., 2007; Haubek et al., 2008; Van der Velden et al., 2006; van Winkelhoff et al., 1994].

This bacterium's role and associations with periodontitis and its involvement in systemic diseases are based on three major lines of support [Fine et al., 2006]:

- Clinical and microbiological findings linking *A. actinomycetemcomitans* to the start, development, and reappearance of disease in LAP [Zambon, 1985].
- Genetic and experimental findings linking discovered virulence factors of *A. actinomycetemcomitans* to known pathogenic events in LAP [Fives-Taylor et al., 1999].

- Reports demonstrating that members of the HACEK group (*Haemophilus spp., A. actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens and Kingella kingae*), especially *A. actinomycetemcomitans*, are associated with systemic diseases far from the oral cavity [Das et al., 1997].

In 1951, Holm was the first to suggest the possibility that *A. actinomycetemcomitans* could help the progression of actinomycotic lesions and encouraged the thought that *A. actinomycetemcomitans* could be an organism of virulence [Holm, 1951]. Thjotta and Sydnes were the first to report that *A. actinomycetemcomitans,* by itself, could cause an infection [Thjotta and Sydnes, 1951]. Then, for some years, a common mix up of *A. actinomycetemcomitans* with *Haemophilus aphrophilus* occurred [King and Tatum, 1962]. The final identification of *A. actinomycetemcomitans* as a single infecting pathogenic organism was presented by Mitchell and Gillespie in a case of endocarditis after a dental extraction [Mitchell and Gillespie, 1964].

In 1959 it was shown that *A. actinomycetemcomitans* could be present in the normal oral flora and that it could colonize teeth, oral mucosa, and the oropharynx [Heinrich and Pulverer, 1959]. Kilian and Schiott were the first to find this bacterium in dental plaque [Kilian and Schiott, 1975]. The first associations of *A. actinomycetemcomitans* with juvenile periodontitis were shown by two groups in 1976 [Newman et al., 1976; Slots, 1976]. These and other studies were cross-sectional, meaning that the association between *A. actinomycetemcomitans* with disease was shown when bone loss had already taken place [Slots et al., 1980]. In addition, several longitudinal studies have shown a correlation of *A. actinomycetemcomitans* with disease progression where "stable" diseased sites had transformed to "active" diseased sites [Ebersole et al., 1995; Haubek et al., 2004; Mandell, 1984; Mandell et al., 1987]. Subjects with diseased sites infected with *A. actinomycetemcomitans* have shown progressing disease activity over a short observation period, compared to diseased sites where this bacterium is not detectable [Christersson et al., 1985; Mandell, 1984; Novak et al., 1991].

Moreover, treatment of subjects with LAP with the objective of reducing *A. actinomycetemcomitans* to undetectable levels resulted in obvious clinical improvement and disease progression was correlated with a failure to significantly reduce the levels of *A. actinomycetemcomitans* [Christersson et al., 1985]. Problems with removing this bacterium from the subgingival sites and repopulation or re-infection by *A. actinomycetemcomitans* from other locations in the oral cavity have been reported [Renvert et al., 1990; Takamatsu et al., 1999]. Disease recurrence after treatment has also been shown to correlate with the reappearance of *A. actinomycetemcomitans* [Renvert et al., 1990; Van der Velden et al., 1989].

A. actinomycetemcomitans can also be found in other forms of periodontitis, but its strongest association is still with AgP, in particular with LAP [Slots and Ting, 1999]. Despite some uncertainties about clinical diagnosis and prior periodontal therapy, studies have isolated *A. actinomycetemcomitans* from 75-100% of LAP lesions [Slots and Ting, 1999]. Clearly, this organism has been found more often in samples obtained from subjects with LAP than from

periodontally healthy subjects, subjects with gingivitis or other forms of periodontal disease [Slots et al., 1980]. Moreover, subjects with LAP consistently have shown elevated serum and locally produced antibody titers against *A. actinomycetemcomitans* [Ebersole et al., 1985a; Ebersole et al., 1985b; Slots et al., 1982].

Taken together, there is sufficient evidence for a correlation between rapid loss of attachment of the periodontal tissue and high proportion of *A. actinomycetemcomitans* in the periodontal pocket [van Winkelhoff et al., 1994].

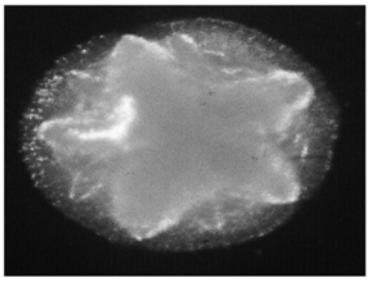
Additionally, this bacterium has, although rarely, been responsible for some non-oral infections including endocarditis, bacteremia, pericarditis, septicemia, pneumonia, infectious arthritis, osteomyelitis, synovitis, skin infections, urinary tract infections and various types of abscesses [van Winkelhoff and Slots, 1999].

Furthermore, a link between periodontitis and cardiovascular diseases seems to exist, and *A. actinomycetemcomitans* has been detected in atherosclerotic plaque samples [Haraszthy et al., 2000b; Kozarov et al., 2005; Pihlstrom et al., 2005]. Overall, while there are studies that fail to report *A. actinomycetemcomitans* in atherosclerotic plaques, the majority of the literature has shown this association [Fine et al., 2006]. In contrast to other oral microorganisms, *A. actinomycetemcomitans* is seldom (<20%) reported as a inhabitant of dental plaque obtained from periodontally healthy subjects, and it is found in the dental plaque in 20–30% of patients with chronic periodontitis [Zambon et al., 1983].

1.3.3 Morphology, biochemistry, and serotypes

A. actinomycetemcomitans is a gram-negative coccobacillus (rod-shaped), approximately 0.4±0.1 x 1.0±0.4 μm in size. When primary isolated, it forms small translucent or transparent colonies about 0.5-1.0 mm in diameter. These colonies appear smooth, circular or oval, and many are convex with irregular edges. The average colony appearance on agar resembles an internal "star" (**Fig. 3**) that is typical for this bacterium [Zambon, 1985].

Figure 3. "star-shaped" colony of *A. actinomycetemcomitans*



Microscopically, cultures appear mainly bacillary with a few coccal forms. *A. actinomycetemcomitans* is a facultative anaerobic species and also capnophilic, i.e. its growth is enhanced in the presence of 5-10% CO2. [Pulverer and Ko, 1970]. It is unable to grow on MacConkey's agar (used for distinguishing lactose fermentation) [Slots, 1982]. *A. actinomycetemcomitans* is non-sporulating, non-motile, oxidase negative and catalase positive. Moreover, it can degrade galactose, dextrin, maltose, mannitol and xylose, properties which can be used to distinguish this organism from other members of the oral flora [Zambon, 1985].

Besides having a star-shaped internal structure, colonies of fresh isolates are rough-surfaced and repeated subculture leads to two types of colonies. The first is smooth-surfaced, transparent and is an intermediate form before it changes to the second form that is smooth-surfaced and opaque [Inouye et al., 1990; Rosan et al., 1988; Scannapieco et al., 1987; Slots et al., 1982]. The colonial variation is associated with lower or loss of fimbriation. Wild-type (WT) *A. actinomycetemcomitans* strains are rough-surfaced and clump in suspension. Thus, most experiments are from strains that are smooth-colony variants. The problem with this selection is that these strains can be less virulent [Fine et al., 1999; Fives-Taylor et al., 1999].

A. actinomycetemcomitans strains are separated into six serotypes (a–f), based on the structure of the O-antigen (polysaccharide side chain) section of their lipopolysaccharide (LPS) molecules [Kaplan et al., 2001; Lakio et al., 2003]. Different serotypes have been shown to be associated with periodontal health and periodontitis. Serotype b seems to predominate in diseased subjects and serotype c is the most common serotype in healthy subjects [Asikainen et al., 1995; Asikainen et al., 1991; Dogan et al., 1999]. However, serotype differences can also depend on racial and ethnical factors, and carriers of multiple *A. actinomycetemcomitans* serotypes seem more common in the Far East than in Europe [Holtta et al., 1994; Mombelli et al., 1999; van der Reijden et al., 2008; Yoshida et al., 2003].

In addition, binding via co-aggregation of *A. actinomycetemcomitans* to other oral bacteria can be serotype-specific, and that can be important for colonization of the oral cavity and plaque development [Kolenbrander et al., 2002; Rosen et al., 2003].

1.3.5 Transmission, colonization, and localization

For transmission of an oral bacterial species, the prevalence of it in different populations is an important parameter. Of possible significance is the fact that studies among the tested subjects have shown large differences in the prevalence of *A. actinomycetemcomitans*: in China 69%, in Thailand 83%, in Africans 53%, and in USA and Sweden <15% [Alaluusua and Asikainen, 1988; Papapanou et al., 2002; Rodenburg et al., 1990; Van der Weijden et al., 1994]. With respect to these prevalence differences, transmission studies have shown that *A. actinomycetemcomitans* can aggregate in families, and family members usually share the same strains [Asikainen et al., 1997; Gunsolley et al., 1990; Van Winkelhoff and Boutaga, 2005].

A. actinomycetemcomitans has been categorized as a tertiary colonizer (an organism that attaches to bacteria that have already colonized tooth surfaces) [Kolenbrander, 2000]. However, this bacterium has also been isolated from newly cleaned tooth surfaces of humans and macaque monkeys after 2–6 h [Kilian et al., 1976; Kilian and Rolla, 1976; Li et al., 2004]. In addition, *A. actinomycetemcomitans* can colonize predentate children, meaning that the oral mucosa may actually be the initial colonization site in the oral cavity [Lamell et al., 2000; Tanner et al., 2002]. Moreover, it has been described that this bacterium can “hide” in buccal

epithelial cells for future tooth colonization [Rudney et al., 2005]. This capability can make the buccal epithelial cells into a protected reservoir for *A. actinomycetemcomitans* [Fine et al., 2006]. These reports indicate that this species has the ability to colonize a healthy oral cavity and can be an early colonizer of the teeth.

A. actinomycetemcomitans has been recovered mostly from the buccal mucosa of children and young adults, but can also less frequently be found from tongue, subgingival plaque, supragingival plaque, tonsillar crypts and saliva in these subjects [Eger et al., 1996; Gafan et al., 2004; Lamell et al., 2000; Muller et al., 2001]. To date, the natural habitat of *A. actinomycetemcomitans* is the human oral cavity, and no extra-oral habitat of this bacterium has been found. Interestingly, two independent virulence factors of this bacterium (leukotoxin and the Aae adhesin) have been shown to have high specificity for human and Old World monkey tissue [Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003; Henderson et al., 2002]. The current data suggest that the narrow host ranges exhibited by Aae and leukotoxin evolved separately and that the evolution of host-range specificity may be a complex process that involves several host-specific factors [Fine et al., 2005]. These findings also suggest that the natural history of *A. actinomycetemcomitans* may date back at least 35 million years to the time when humans and Old World primates last had a common ancestor [Fine et al., 2005; Vogel et al., 1999].

1.3.6 Virulence factors

Many studies have focused on the virulence of *A. actinomycetemcomitans* because of the strong link between this bacterium, AgP and associated extra-oral infections.
A. actinomycetemcomitans has several virulence factors that have been characterized as secreted and/or bacterial membrane-associated components. Some factors can promote colonization and persistence of the bacteria in the oral cavity. Other factors can interfere with the host defense system. In addition, some factors can inhibit host repair or can even destroy host tissues [Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003; Henderson et al., 2002; Wilson and Henderson, 1995].

In vitro studies have shown that many phenotypes of *A. actinomycetemcomitans* contribute to the colonization of the oral cavity. These phenotypes can lead to attachment to human epithelial cells, attachment to hydroxyapatite, invasion of non-phagocytic human cells, aggregation with themselves/other bacteria, and biofilm formation. In addition, *A. actinomycetemcomitans* cells secrete two cytolethal toxins, leukotoxin and cytolethal distending toxin (cdt), that promote bacterial survival during the colonization process and may also contribute to pathogenesis [Belibasakis, 2004; Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003].
Some of the reputed virulence traits of *A. actinomycetemcomitans* and their suggested induced host-effects are summarized in **Table 1**.

Table 1. Summary of host functions induced by virulence factors of *A. actinomycetemcomitans*.

	Function(s)	Factor(s)	Review(s)
Factors that promote colonization and persistence in the oral cavity	Attachment to abiotic surface	Flp-1 pili	[Fine et al., 2006]
	Inter-bacterial adhesion	Flp, Fibrils, Fimbriae	[Fine et al., 2006]
	Auto-aggregation	Flp-1 pili, PGA polysaccharide	[Fine et al., 2006]
	Biofilm formation	Flp-1 pili	[Fine et al., 2006]
	Attachment to epithelium	Aea, Flp-1 pili, ApiA	[Fine et al., 2006]
	Attachment to collagen	EmaA	[Fine et al., 2006]
	Resistance to tetracyclines	Not determined	[Fives-Taylor et al., 1999]
	DNA binding and uptake	PilA pili	[Fine et al., 2006]
Factors that interfere with the host's defenses	Chemotactic inhibitors	Not determined	[Fives-Taylor et al., 1999]
	Invasion of epithelium Invasion of endothelium	ApiA Phosphorylcholine	[Fine et al., 2006]
	Lysis of PMNs and macrophages	Leukotoxin	[Fives-Taylor et al., 1999]
	Resistance to phagocytic killing	Leukotoxin	[Fives-Taylor et al., 1999]
	Affecting IgG	Fc-binding proteins	[Fives-Taylor et al., 1999]
	Lymphocyte apoptosis	Leukotoxin, Cdt	[Fine et al., 2006]
	Lymphocyte proliferation	Cdt	[Fine et al., 2006]
Factors that destroy host tissues	PMN degranulation	Leukotoxin	[Fine et al., 2006]
	Inflammatory response	Bacterial extract, LPS	[Fives-Taylor et al., 1999]
	Collagen degradation	Collagenase	[Fives-Taylor et al., 1999]
	MMP-release	Leukotoxin	[Fives-Taylor et al., 1999]
	Bone resorption	Cdt, GroEL, LAP, LPS, bacterial extract	[Fine et al., 2006] [Henderson et al., 2003]
Factors that inhibit host repair of tissues	Inhibition of collagen and extracellular matrix production	Bacterial extract	[Fine et al., 2006] [Henderson et al., 2003]
	Inhibition of fibroblast and epithelium proliferation	Cdt	[Fine et al., 2006] [Henderson et al., 2003]

Aae, EmaA, and ApiA (types of autotrasporter proteins)
Cdt (cytolethal distending toxin)
Flp, fibrils, fimbriae (variants of adhesins)
GroEL (belongs to family of molecular chaperones)
LAP (lipid A-associated proteins)
PGA (polyglycosamine)

Some of the virulence factors of *A. actinomycetemcomitans* have been shown to be more important than others for the pathogenic processes in the early stages of infection [Belibasakis, 2004; Fine et al., 2006; Fives-Taylor et al., 1999; Henderson et al., 2003]. These virulence factors can be categorized into three important groups:

- Attachment to tissue (via adhesins and other cell-associated proteins)
- Evasion of the local defenses (via leukotoxin and cdt)
- Invasion of tissue (possibly through cdt and ApiA)

In the next section, the leukotoxin, the virulence factor of interest for this dissertation, will be presented more comprehensively.

1.3.6.1 Leukotoxin

Perhaps the most extensively studied virulence factor of *A. actinomycetemcomitans* is its leukotoxin that was discovered in 1979 [Baehni et al., 1979].

Studies on *A. actinomycetemcomitans´* capacity to express the leukotoxin, i.e. its leukotoxicity, have focused mainly on *ltxA* and its gene product, LtxA, the leukotoxin [Fine et al., 2006]. Leukotoxin belongs to the Repeats in Toxin (RTX) family, which includes *Escherichia coli* α-hemolysin, *Bordetella pertussis* adenylate cyclase, and *Mannheimia haemolytica* leukotoxin (Lally, Hill et al. 1999; Narayanan, Nagaraja et al., 2002).

A. actinomycetemcomitans leukotoxin (LtxA) shares considerable molecular homology with toxins of the RTX family [Ludwig, 1996; Welch, 2001]. LtxA is 51% similar to *E. coli* α-hemolysin and 43% similar to *M. haemolytica* leukotoxin [Kraig et al., 1990; Lally et al., 1989a; Lally et al., 1989b].

RTX toxins are divided into two categories based on their target cell specificity. RTX hemolysins, such as *E. coli* α-hemolysin (HlyA) and *Actinobacillus pleuropneumoniae* ApxIA, are toxic to a wide range of cell types from many species. In contrast, leukotoxins of *A. actinomycetemcomitans* (LtxA) and *M. haemolytica* (LktA), are toxic to restricted groups of cells in a species-specific manner [Lally et al., 1999].

M. haemolytica LktA has specificity for bovine lymphoid cells [Shewen and Wilkie, 1982; Strathdee and Lo, 1989]. *A. actinomycetemcomitans* leukotoxin exhibits unique specificity for primate leukocytes [Taichman et al., 1987; Tsai et al., 1984]. Although the mechanism of RTX-toxin-mediated cytotoxicity is preserved, differences in host-cell specificity show that certain regions of RTX toxins have undergone significant changes [Lally et al., 1999].

ApxIA, HlyA, and LtxA, all are reported to possess strong cytotoxic activity, and the structural genes of them show strong similarities. However, the bacterial species harboring these three related RTX toxins are phylogenetically far from each other. *E. coli*, belonging to the

Enterobacteriaceae, is not even a member of the *Pasteurellaceae*. This indicates that a horizontal transfer of RTX toxin genes has occurred not only among different species of *Pasteurellaceae* but also across bacterial families [Frey and Kuhnert, 2002].

1.3.6.2 Transcription, translation, regulation, and secretion

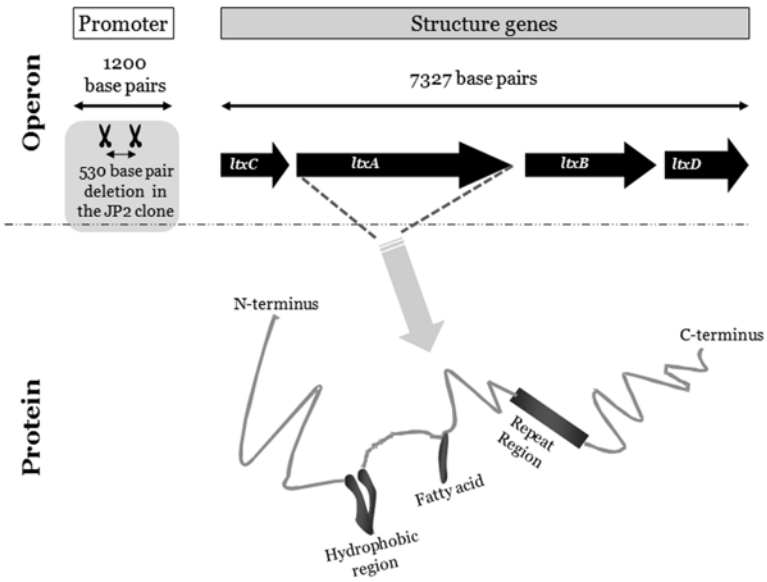
Members of the RTX toxin family, including cytolytic toxins, metalloproteases and lipases, all share a widespread gene organization and characteristic structural traits [Lally et al., 1999]. Even though variations do exist, the generic RTX toxin operon consists of four genes that are termed in transcriptional order: *rtxC*, *rtxA*, *rtxB*, and *rtxD*. RTX toxins need post-translational modification to become biologically active [Lally et al., 1999]. This activation step is achieved by fatty acid acylation of the *rtxA* gene product and requires the contribution of the *rtxC* gene product and an acyl carrier protein [Issartel et al., 1991; Stanley et al., 1998].

The products of *rtxB* and *rtxC* are believed to be involved in the secretion (type 1) of *rtxA* gene product [Frey and Kuhnert, 2002].

Consequently, the functions of the genes for *A. actinomycetemcomitans* leukotoxin are (**Fig. 4**):

- *ltxC* is the post-translational activator gene.
- *ltxB* and *ltxD* genes are responsible for transport of the LtxA to the cell surface.
- *ltxA* is the structural gene for its product, LtxA (i.e., the leukotoxin).

Figure 4. Schematic illustration of leukotoxin operon and protein.



The final modified protein has three important regions (**Fig. 4**) [Frey and Kuhnert, 2002; Lally et al., 1999]:

- The glycine-rich repeat region that is thought to function as target cell membrane recognizer.
- The fatty acid part that seems to provide initial protein-anchoring to the target cell.
- The hydrophobic region that is believed to be responsible for pore formation of target cell membrane.

Like other RTX toxins, the leukotoxin is a large molecule. It consists of 1055 amino acids with a molecular weight of ≈ 116 kDa [Fine et al., 2006; Kraig et al., 1990]. The protein crystal structure of leukotoxin has not to date been resolved. Thus, information about the structural biology of leukotoxin is limited [Fine et al., 2006].

It has been shown that the leukotoxin is associated with the outer cell membrane and outer membrane vesicles [Berthold et al., 1992]. Due to culture conditions the leukotoxin is released from the membrane and vesicles [Kato et al., 2002]. Treatment of the bacteria with nucleases results in release of the leukotoxin [Ohta et al., 1993; Ohta et al., 1991; Welch, 2001]. This indicates that DNA in the cell membrane may be involved in association of leukotoxin to the bacteria. Also the high isoelectric point (pI) of the leukotoxin (8.9) is of importance for its interaction with the bacteria and the environment. By increasing the ion strength the electrostatic forces between leukotoxin and nucleic acids can be broken resulting in a release of the leukotoxin from the bacterial surface [Johansson et al., 2000b]. A similar effect can be achieved in the presence of serum, indicating that at physiological conditions the leukotoxin can be secreted from the bacteria [Johansson et al., 2003].

Bacterial membrane-associated leukotoxin can be important for the bacteria when they are in direct contact with immune cells, while secreted toxin may be important for the pathogenesis [Fine et al., 2006].

The intra-species diversity of *A. actinomycetemcomitans* results in clones with different leukotoxin-producing abilities [Fine et al., 2006; Guthmiller et al., 1995]. All tested strains have the leukotoxin operon, but one clone, the JP2, with enhanced leukotoxin expression due to a 530 base pair deletion in the leukotoxin promoter region has attracted much interest (**Fig. 4**) [Brogan et al., 1994]. Most studies on leukotoxin expression have focused on the two types of leukotoxin promoters. Strains that produce large quantities of leukotoxin are referred to as highly-leukotoxic and contain the JP2 promoter.

The strains with an intact promoter region, non-JP2 strains, produce smaller amounts of leukotoxin. Thus, they are called minimally-leukotoxic [Brogan et al., 1994; Fine et al., 2006]. However, there have also been reports on other non-JP2 clone strains of *A. actinomycetemcomitans* that produce relatively high levels of leukotoxin [Claesson et al., 2004; He et al., 1999]. The expression of leukotoxin can also depend on anaerobic conditions or

accessible levels of carbohydrate [Hritz et al., 1996; Mizoguchi et al., 1997]. In addition, iron appears to play an important role in the regulation of leukotoxin independently from gene regulation [Balashova et al., 2006].

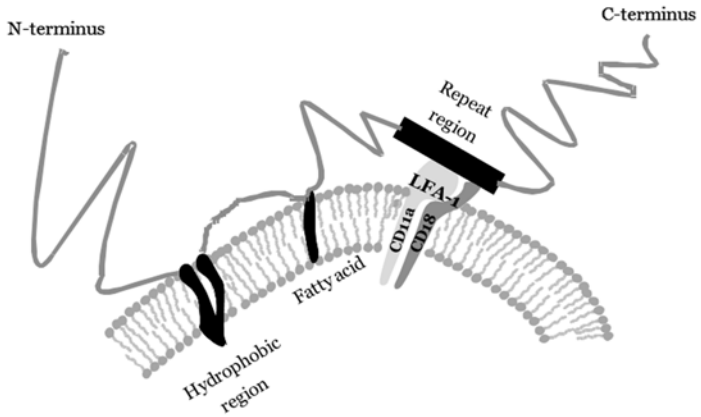
1.3.6.3 Target cell receptor and pore formation

RTX-induced cell lysis is assumed to be due to the formation of pores by the toxin in the cytoplasm membrane of the susceptible cells [Lear et al., 1995]. It has been assumed that several RTX molecules, including leukotoxin, can oligomerize to form a channel in the host cell membrane [Lally et al., 1999]. High doses of RTX molecules cause oligomerization that leads to the fusion of transmembrane pores and rapid destruction of the cell membrane, with little time for activation of apoptotic pathways. However, at lower toxin concentrations, transmembrane pores would be smaller and fewer in number and target cells could survive long enough for apoptosis to be observed [Korostoff et al., 1998; Taichman et al., 1991].

The leukotoxin has been shown to be a calcium-binding protein. It has been proposed that Ca^{2+} ions may link the negatively charged regions of the leukotoxin to a cell surface receptor. Alternatively, conformational changes of leukotoxin induced by Ca^{2+} ions may enable it to bind to the surface of the target cell [Lally et al., 1991; Sato et al., 1993].

The domain of the leukotoxin that binds to the human target cells has been mapped and a β_2-integrin, the lymphocyte function-associated antigen-1 (LFA-1), was shown to be a target cell receptor involved in leukotoxin-induced cell lysis (**Fig. 5**) [Lally et al., 1994; Lally et al., 1997]. Human LFA-1 comprises the CD11a and CD18 subunits (**Fig. 5**) [Springer, 1990]. Recently it was shown that CD18 serves as the functional receptor for leukotoxin that confers species-specific susceptibility to leukotoxin [Dileepan et al., 2007].

Figure 5. Schematic illustration of the proposed target cell receptor for leukotoxin.



1.3.6.4 Toxin expression and associations with disease

As mentioned previously, leukotoxin is primate-specific, and many studies have shown that the specificity is restricted to human and Old World Primate white blood cells [Berthold et al., 1992; Stanley et al., 1998; Taichman et al., 1987; Tsai et al., 1984]. However, recently also β-hemolytic activity was shown for purified leukotoxin, where it could kill erythrocytes of both sheep and humans [Balashova et al., 2006].

Consequently, the leukotoxin is assumed to protect the bacterium from the local defense mechanisms through its capacity to lyse human immune cells [Guthmiller et al., 2001; Johansson et al., 2000c]. This ability can significantly contribute to the pathogenesis of periodontitis [Fives-Taylor et al., 1999; Kinder-Haake and Huang, 2002]. Leukotoxin selectively kills human leukocytes by inducing apoptosis and lysis [Fong et al., 2006; Korostoff et al., 1998; Mangan et al., 1991; Nalbant et al., 2003]. Beside lysing polymorphonuclear leukocytes (PMNs) and monocytes, it also induces degranulation of PMNs and apoptosis in T-lymphocytes [Claesson et al., 2002; Johansson et al., 2000a; Mangan et al., 1991; Taichman et al., 1980; Tsai et al., 1979].

Most of the studies aimed to examine the leukotoxic activity of *A. actinomycetemcomitans* have mainly been focused on the interactions of the leukotoxin with PMNs and promyelocytic carcinoma cell lines [Baehni et al., 1979; Johansson et al., 2000a; Johansson et al., 2000b; Lally et al., 1994; Lally et al., 1997; McArthur et al., 1981; Tsai et al., 1979].

There are also animal models for studying the LAP caused by *A. actinomycetemcomitans*. These models usually use rats that ingest live bacteria, but leukotoxin does not affect rat cells and the pathogenic involvement of leukotoxin cannot be examined in these models [Fine et al., 2006; Fine et al., 2009].

However, many clinical studies strongly suggest that leukotoxin is, in fact, a critical virulence factor. Strains isolated from patients with AgP have been shown to produce more leukotoxin than those isolated from periodontally healthy subjects or patients with chronic adult periodontitis [Haraszthy et al., 2000a; He et al., 1999; Zambon et al., 1983]. There is a clear relationship between increased leukotoxin production and disease initiation and progression [Haubek et al., 2001; Haubek and Westergaard, 2004]. Examination of different clonal types of *A. actinomycetemcomitans* in patients with LAP suggests that the highly-leukotoxic JP2 clone with deletion in the leukotoxin promoter region (**Fig. 4**) is strongly correlated to disease onset in certain populations [Bueno et al., 1998; Haraszthy et al., 2000a; Haubek et al., 2008].

In Africans suffering from AgP, the highly-leukotoxic JP2 clone is thought to act as an exogenous pathogen [Bueno et al., 1998; Haubek et al., 1997]. In contrast to this distinct subpopulation, the non-JP2-strains (the vast majority of the strains) are considered to be rather opportunistic pathogens [Lindhe et al., 2008]. Interestingly, it has recently been shown in a

population-based longitudinal study that adolescents who carry the JP2 clone of *A. actinomycetemcomitans* have a significantly (18 fold) increased risk of periodontal attachment loss after two years as compared to control subjects without this bacterium [Haubek et al., 2008]. Moreover, enhanced prevalence of systemic antibodies to leukotoxin can be found in patients with LAP [Califano et al., 1997; Engstrom et al., 1999].

Taken together, convincing evidence suggests that highly-leukotoxic clones of *A. actinomycetemcomitans* are more virulent. However, so called minimally-leukotoxic strains are also frequently isolated from patients with LAP [Fine et al., 2007; Kaplan et al., 2002; Van der Velden et al., 2006].

1.4 Initial host defense in the periodontal tissues

In this section a brief introduction to initial host defense in the periodontal tissue is summarized. The initial host defense system in the periodontium can be divided into three lines of defense [Lindhe et al., 2008]:

1. Epithelium:
    - intact barrier with attachment to the tooth
    - keratinization and high tissue turnover
    - antimicrobial peptides (e.g. β-defensins)
    - cytokine and chemokine secretion
    - elevated expression of surface molecules for leukocyte recruitment

2. Saliva and gingival crevicular fluid (GCF):
    - flushing action
    - bactericidal effects directly or indirectly (via chemotaxis) in presence of agglutinins, antibodies, proteases, complement, lactoferrin, opsonins, and other plasma components

3. Innate immunity (works without any previous contact with the pathogens):
    - migration of PMNs, macrophages, and dendritic cells (DCs) and later on lymphocytes
    - detection by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), e.g. via the toll receptor (TLR) family
    - phagocytosis and antigen presentation
    - secretion of prostaglandins (particularly PGE_2) and cytokines, such as proinflammatory cytokines (Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) primarily by macrophages

If the pathogenic burden is too large and overwhelms this initial host defense, then acquired immune responses are initiated resulting in the generation of antigen-specific cellular and humoral immunity [Twigg, 2004]. However, despite some protective evidence of antibodies, the mechanisms behind the magnitude and quality of the humaoral response in periodontitis are not fully understood [Lindhe et al., 2008]. Nevertheless, the proportions of leukocytes (>50% B and plasma cells) in the advanced lesions of chronic periodontitis reveal that the acquired immune responses dominate the late phase of this disease. The different leukocyte proportions are virtually consistent in chronic periodontitis, while larger differences are found in studies on aggressive forms of periodontitis [Berglundh and Donati, 2005].

Variation in human susceptibility to periodontitis has long been accepted, but the responsible pathological mechanisms are poorly understood [Rylev and Kilian, 2008]. The differences, if any, between the pathology of chronic and aggressive periodontitis are unclear [Kinane and Lappin, 2002].

Macrophages are professional phagocytic cells that are able to phagocytose and destroy infectious agents. These cells are key anti-infectious actors in host defense but can also cause tissue damages. In addition, it is now clear that the role of these cells is far more than just phagocytosis and pathogen killing [Kantari et al., 2008].

The focus of this dissertation is on the interactions of *A. actinomycetemcomitans* leukotoxin with human monocytes/macrophages. Thus, a closer presentation of these cells will follow in the next section.

1.5 Monocytes/Macrophages

1.5.1 Monocytes

Macrophages differentiate from circulating blood monocytes that migrate through the vessel wall into tissue in the steady state or as a response to inflammation [Gordon and Taylor, 2005]. Monocytes develop in the bone marrow from a general hematopoietic stem cell. Further differentiation results in a common myeloid progenitor cell in the bone marrow that is the precursor of many different cell types, including neutrophils, eosinophils, basophils, macrophages, dendritic cells (DCs) and mast cells [Gordon and Taylor, 2005]. During monocyte development, myeloid progenitor cells (also called granulocyte/macrophage colony-forming units, GM-CFU) develop in the following order: from myeloid progenitor cells into macrophage colony-forming units (M-CFU), then into monoblasts, and then into pro-monocytes under the influence of the macrophage colony-stimulating factor (M-CSF) [Gordon and Taylor, 2005; Mosser and Edwards, 2008].

Finally, monocytes exit the bone marrow and enter the bloodstream. In fact, monocytes are by definition monocytes as long as they exist in the circulation, and as soon as they enter a tissue,

through the endothelium in the blood vessel wall, they become tissue-specific macrophages or DCs.

1.5.2 Monocyte subsets

Monocytes show morphological heterogeneity, such as variability of size, granularity and nuclear morphology. These cells were initially identified by their expression of large amounts of cluster of differentiation (CD) 14 but additional identification of CD markers showed that human monocytes are heterogeneous [Gordon and Taylor, 2005]. Differential expression of CD14 and CD16 (also known as FcγRIII) divided monocytes into two subsets: $CD14^{hi}CD16^{-}$ cells (termed as "classic monocytes"), and $CD14^{+}CD16^{+}$ cells [Passlick et al., 1989]. It was consequently shown that the $CD14^{+}CD16^{+}$ monocytes express higher amounts of major histocompatibility complex (MHC) class II molecules and CD32 (also known as FcγRII), and it was later proposed that these cells resemble mature tissue macrophages [Ziegler-Heitbrock et al., 1993]. Distinct chemokine-receptor expression profiles are also among the phenotypic differences that were recognized between these subsets. For instance, $CD14^{+}CD16^{+}$ monocytes express CC-chemokine receptor 5 (CCR5), whereas $CD14^{hi}CD16^{-}$ monocytes express CCR2 [Weber et al., 2000]. An additional monocyte subset that is defined by the expression of CD14, CD16 and CD64 (also known as FcγRI) has also been detected [Grage-Griebenow et al., 2001].

In summary, there are three major subsets of monocytes: "Classical" ($CD14^{+}$ $CD16^{-}CD64^{+}$), "Intermediate" subset ($CD14^{+}$ $CD16^{+}$ $CD64^{+}$), and "Non-classical" monocytes ($CD14^{lo}$ $CD16^{+}$ $CD64^{-}$) [Tacke and Randolph, 2006]. Approximately 90% of human monocytes express the classical markers [Passlick et al., 1989]. Although the origin and renewal of macrophages and DCs have been intensively investigated, the precise responsible monocyte subset is still unclear in many ways [Fogg et al., 2006].

Monocytes represent about 5–10% of peripheral blood leukocytes in humans [Tacke and Randolph, 2006]. The half-life of monocytes in blood is believed to be only 3 days in humans [Whitelaw, 1972]. This short half-life in blood has raised the concept that blood monocytes may constantly repopulate macrophage or DC populations to preserve homeostasis and, during infection and inflammation, execute actions needed in innate and adaptive immunity [Ziegler-Heitbrock, 2000]. However, some evidence suggests that macrophages in several different organs are self-renewed [Gordon and Taylor, 2005]. Nevertheless, the fact that monocytes give rise to macrophages in practically any organ in the case of inflammation is unquestionable [Tacke and Randolph, 2006].

1.5.3 Macrophages

Macrophages are extraordinarily phagocytic cells that clear approximately 2×10^{11} erythrocytes each day and remove cellular debris that is generated through tissue remodeling. They can rapidly and efficiently clear apoptotic cells. These processes occur separately of immune-cell

signaling, and the removal of apoptotic cells seems to result in little or no production of immune mediators by unstimulated macrophages [Mosser and Edwards, 2008]. The receptors for this clearance processes include scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors [Erwig and Henson, 2007]. These receptors either fail to induce cytokine-gene transcription or actively produce inhibitory signals and/or cytokines, so most of the phagocytosis that occurs is independent of other immune cells. Therefore, the primary function of macrophages is to clear the tissue environment of extraneous cellular material [Mosser and Edwards, 2008].

On the other hand, the clearance of necrotic debris clearly changes the physiology of macrophages. In many cases the debris from necrosis is full of endogenous danger signals, such as heat-shock proteins, nuclear proteins, histones, DNA, other nucleotides, and components of the extracellular matrix [Zhang and Mosser, 2008]. Phagocytosis of these components by macrophages leads to expression of surface proteins and the production of cytokines and proinflammatory mediators.

Macrophages discover the endogenous danger signals that are present in the debris of necrotic cells via several mechanisms: Toll-like receptors (TLRs) [Chen et al., 2007; Kono and Rock, 2008; Park et al., 2004], intracellular pattern-recognition receptors, and the interleukin-1 receptor (IL-1R), all mainly signaling through the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88) [Chen et al., 2007]. Therefore, macrophages are one of the initial sensors of danger in humans, and the stimulation of macrophages by cellular debris can occur in experimental animals that lack lymphocytes, confirming that adaptive immune responses are not involved [Kono and Rock, 2008].

1.5.4 Macrophage populations

Macrophages exhibit extraordinary flexibility and can change their physiology in response to environmental signals. These changes result in different populations of cells with individual functions [Mosser and Edwards, 2008]. Monocytes migrate from the blood into the tissue to reload long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum [Gordon and Taylor, 2005; Mosser and Edwards, 2008].

In general, macrophages are classified into two different functional subtypes: M1 and M2. In this classification, the M1 stands for classically activated macrophages, and the M2 represents alternatively activated macrophages [Gordon and Taylor, 2005]. Identification of many subgroups within the M2 subtype has led to a more informative foundation for macrophage classification that was recently presented by Mosser and Edwards, based on the fundamental macrophage functions. The three proposed “functionally organized” populations of macrophages are:

- Host defense macrophages (same as M1)

- Wound-healing macrophages
- Regulatory macrophages

Further, it is suggested that subpopulations of macrophages can have characteristics from more than one of these major three macrophage populations [Mosser and Edwards, 2008].

1.5.5 The role of macrophages in periodontitis

As described previously, periodontitis is initiated by bacteria that colonize the tooth surface and periodontal pocket. In this situation, macrophages may play the same roles as PMNs, which are protection through phagocytosis and damage through the release of lysosomal material. Further, the macrophages may enhance the antimicrobial actions of PMNs. Roughly 2–3% of immune cells found in the GCF are monocytes or macrophages [Delima and Van Dyke, 2003].

Regarding their role in the periodontal tissues, macrophages have two distinct functions: some act as phagocytes, destroying pathogens and removing damaged or aged tissues. Other macrophages can function as antigen-presenting cells and help to regulate cellular immunity. Activated macrophages are essential for innate resistance to infection and can mediate inflammation in the connective tissue infiltrate, where they produce several cytokines and also present antigens to T-cells [Delima and Van Dyke, 2003]. Moreover, macrophages are important cells for connective tissue and bone remodeling. In addition, osteoclast-differentiation is closely related with the differentiation of macrophages and DCs [Lerner, 2004; Lerner, 2006].

The key factor of periodontitis progression is the breakdown of connective tissue and bone. This is believed to be mainly due to the host response. An intermediate mechanism that lies between bacterial stimulation and tissue destruction is the production of cytokines, which stimulate inflammatory events that activate effector mechanisms. Regarding this aspect, macrophages are one of the major sources of pro-inflammatory cytokines such as IL-1 and TNF. These cytokines improve the activation of cellular immunity and intensify the inflammatory cascade. However, if an imbalance in the host response occurs these cytokines can even harm host tissues [Delima and Van Dyke, 2003; Graves, 2008].

Production of proinflammatory cytokines, including TNF-α, and IL-1β by monocytes/ macrophages in response to extracts and components of *A. actinomycetemcomitans*, has been shown [Henderson et al., 2003]. However, the contribution of leukotoxin to these findings has never been examined, probably because leukotoxin has mainly been considered to disrupt the host defense system by killing the immune cells.

1.6 Cytokines

Cytokines are mainly involved in host responses to disease or infection, but they can also play a role in homeostasis. They influence many biological processes including embryonic

development, disease pathogenesis, non-specific response to infection, and specific response to antigen. Moreover, they can affect cognitive functions and development of the degenerative processes of aging. These molecules can bind to specific cell-surface receptors, and as a consequent they induce intracellular signaling pathways. These cascade reactions can additionally modify cell functions such as up-/or down regulation of different genes and their transcription factors. The outcome of these processes can result in the construction of new cytokines, inhibition of other cytokines, and effects on surface receptors [Dinarello, 2000; Dinarello, 2007].

Cytokines (from the Greek "cyto", cell; and "kinos", movement) are small, nonstructural proteins with molecular weights of 8-40 kDa. Depending on their cellular source, initially they were termed as lymphokines and monokines. However, today it is a fact that nearly all cells (apart from the erythrocytes) can produce as well as respond to cytokines. Cytokines include interferons, the interleukins, the chemokine family, mesenchymal growth factors, the tumor necrosis factor family and adipokines. To date, 35 cytokines are termed as interleukins and these are further divided in cytokine "families". The members of these families are closely related (e.g. IL-1 family). There is no amino acid sequence motif or crystalline protein structure that can be used to classify cytokines. Instead, they are categorized in different classes depending on their functions that include lymphocyte growth factors, proinflammatory, and anti-inflammatory cytokines. In addition, some cytokines polarize the immune response to antigens. They act mostly in an autocrine or paracrine manner in the vicinity of their source. However, during a systemic inflammatory response, cytokines can have endocrine-like functions distant from the site of infection or inflammation. For instance, cytokines can induce synthesis of hepatic acute phase proteins and release of leukocytes from the bone marrow. Nevertheless, they are not "hormones" since they are produced virtually by all cells. Cytokines can even be more potent than hormones and can induce effects in very low concentrations. One good example is IL-1 that can induce gene expression and synthesis of cyclooxygenase-2 at only 10 pM [Dinarello, 2000; Dinarello, 2007].

Most cytokine genes are not expressed at the translational level until they are specifically triggered. This activation can be achieved by cell stressors such as ultraviolet light, heat-shock, hyperosmolarity, or adherence to a foreign surface. In addition, the mitogen-activated protein kinases (MAPKs) are activated and can phosphorylate transcription factors for gene expression. These processes can also be triggered by infection and inflammatory products. In fact, some cytokines such as IL-1, TNF-α and IL-6 evidently promote inflammation and are termed as proinflammatory cytokines. In contrast, other cytokines such as IL-4, IL-10, and IL-13 suppress the activity of proinflammatory cytokines and are consequently called anti-inflammatory cytokines.

Today, cytokines are used for diagnosis and to estimate prognosis of different diseases. Moreover, modulation of cytokines' activity can even be used as therapeutic agents in some diseases. Cytokine research is predominantly in the fields of inflammation, immunology, atherosclerosis and cancer [Dinarello, 2000; Dinarello, 2007].

1.6.1 Important ligands of the IL-1 family

One important group of interleukins is the IL-1 family. The members of this family, consisting of both ligands and receptors, possess several properties that modulate the inflammatory and immune responses [Dinarello, 1996]. To date, the ligands of the IL-1 family have eleven members and include IL-1α (IL-1F1), IL-1β (IL-1F2), IL-1 receptor antagonist (IL-1Ra also called IL-1F3), IL-18 (IL-1F4), and the recently discovered IL-33 (IL-1F11) [Dinarello, 2007; Pizarro and Cominelli, 2007]. There is a separate gene for every member of the IL-1 family. However, their products can have common characteristics in their functions as proinflammatory cytokines [Dinarello, 1996; Dinarello, 2002; Dinarello, 2005b]. The IL-1 family members have a common β-barrel structure with 12 β-strands [Priestle et al., 1989; Schreuder et al., 1997; Vigers et al., 1997]. The four most important and extensively studied ligands of this family are IL-1α, IL-1β, IL-18, and IL-1Ra [Dinarello, 2002].

IL-1α, IL-1β, and IL-18 are agonists, while IL-1Ra is the specific receptor antagonist for IL-1α and IL-1β but not for IL-18. IL-1Ra is a highly specific and naturally occurring receptor antagonist for IL-1 (i.e. IL-1α and IL-1β). This is a unique characteristic in cytokine biology and seems to only exist for the IL-1 family. Although IL-1 and TNF-α have many shared biological properties, it is important to mention that the large family of TNF-α-like cytokines and related proteins have no naturally occurring antagonist like IL-1Ra [Dinarello, 2002].

IL-1 and IL-18 are mainly proinflammatory cytokines since they can stimulate the expression of genes associated with inflammation and autoimmune diseases. For IL-1 (i.e. IL-1α and IL-1β), the most important properties are the initiation of cyclooxygenase-2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). Exposure to naturally produced IL-1, or IL-1 injection in animals or humans, leads to high levels of prostaglandin-E2 (PGE2), platelet activating factor and nitric oxide (NO). IL-18 is also an important player in autoimmune disease because of its ability to induce IFN-γ, particularly in combination with IL-12 or IL-15 [Dinarello, 2002]. Both IL-1 and IL-18 enhance the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on mesenchymal cells. Moreover, IL-1 and IL-18 can increase vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells. IL-1 and IL-18 can also be seen as angiogenic factors due to their ability to increase the expression of vascular endothelial growth factor [Dinarello, 1996; Dinarello, 2002].

1.6.1.1 IL-1β

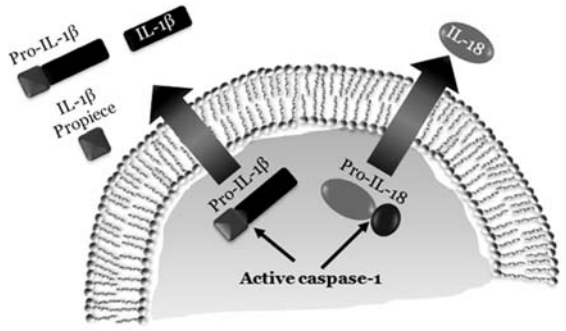
IL-1 was originally found as many different biologically active factors such as endogenous pyrogen, leukocyte endogenous mediator, mononuclear cell factor, and lymphocyte-activating factor. It was first when the molecular cloning of IL-1 was performed that it became clear that all previously mentioned factors were two related but distinct cytokines. These are termed as IL-1α and IL-1β [Dinarello, 1989].

Both IL-1α and IL-1β are produced as 31 kDa pro-forms [Auron et al., 1984; Giri et al., 1985]. These cytokines can be cleaved to a 17.5 kDa "mature" form by intracellular or extracellular proteases. However, approximately 90% of IL-1α remains in the cytosol as precursor or is transported to the cell surface where it remains membrane associated [Endres et al., 1989; Lonnemann et al., 1989]. In contrast to IL-1α, proteolytic cleavage is needed for biological activity of IL-1β whereby more than 80% of the processed IL-1β is secreted [Dinarello, 1996]. This is executed by caspase-1, previously known as the IL-1β-converting enzyme (ICE). IL-1β lacks a signal peptide for secretion and requires this cytosolic cysteine protease for its activation and secretion [Dinarello, 1996; Edgeworth et al., 2002; Monack et al., 2001a; Thornberry et al., 1992]. Although caspase-1 is primarily responsible for activation of pro-IL-1β, trypsin, elastase, chymotrypsin, a mast cell chymase, and other proteases can process the pro-IL-1β to its biologically active form [Fantuzzi et al., 1997].

IL-18 also lacks a signal peptide, and it is processed by caspase-1 from a 24-kDa precursor to the active 18 kDa peptide [Dinarello, 1998a]. Recently IL-33 has also been shown to be processed by caspase-1 *in vitro* [Schmitz et al., 2005].

In contrast to IL-18, where only the "mature" form is secreted, all three forms of IL-1β (pro-IL-1β, IL-1β, and IL-1β-propiece) can be found extracellulary [Dinarello, 1996; Dinarello, 1998a] (**Fig. 6**).

Figure 6. Schematic illustration of IL-1β and IL-18 activation and secretion. See text for details. Adapted from [Dinarello, 1998a].



It is not fully understood how IL-1β is secreted. However, the activation of caspase-1 is a crucial step for this process, and it has been shown that this activation occurs in specialized caspase-1 multi-protein complexes referred to as inflammasomes [Martinon et al., 2002].

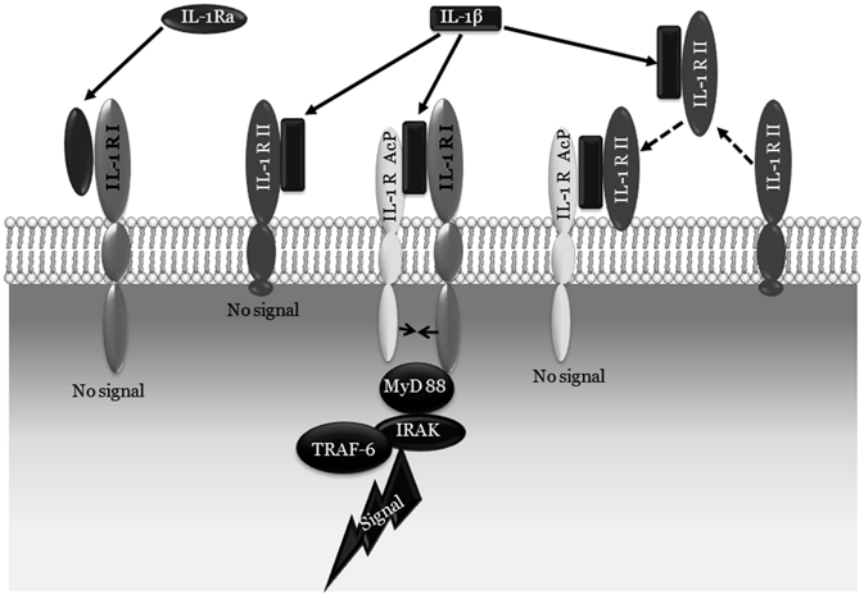
The investigation of mechanisms involved in the caspase-1 inflammasome-activation is a new interesting field in cytokine research. There are several different pathways for activation of the caspase-1 inflammasome by PAMPs and danger associated molecular patterns (DAMPs). Various factors for initiation of these pathways have been identified and include: extracellular adenosine triphosphate (ATP), different crystals (monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD), silica, asbestos, aluminum salts, and ameloid-β), bacterial products (muramyl dipeptide (MDP) and flagellin), and toxins (Nigericin, Maitotoxin and aerolysin) [McIntire et al., 2009; Yu and Finlay, 2008]. Moreover, a newly discovered cell death, designated pyroptosis, also leads to specific caspase-1 activation [Bergsbaken et al., 2009].

It has been suggested that cells such as macrophages require a second stimulus to activate and release IL-1β. The initial stimulus, e.g. LPS, causes large accumulation of pro-IL-1β in the cytosol and only a modest IL-1β secretion [Dinarello, 1998b]. In the case of LPS, production of IL-1β can be induced via pathways that activate p38-activation. Following LPS-induced IκBα phosphorylation and degradation, NF-κB is translocated to the nucleus, which can induce proinflammatory cytokine production, including IL-1β [Guha and Mackman, 2001; Hsu and Wen, 2002].

IL-1β secretion is induced robustly by extracellular ATP, which signals via the purinergic receptor, $P2X_7R$. That causes K^+ efflux from cells which activates pro-caspase-1 and consequently pro-IL-1β processing [Ferrari et al., 2006]. The efflux of potassium ions causes an influx of calcium ions that in turn activates phospholipases [Dinarello, 2005b].

Although mature IL-1α and IL-1β only share <30% homology, both can exhibit a basically identical collection of functions [Dinarello, 1998a]. When IL-1β binds to its receptor, which is the IL-1 receptor type I (IL-1R I), an interaction with IL-1 receptor accessory protein (IL-1R AcP) occurs and leads to downstream signaling pathways (**Fig. 7**). Among IL-1α, IL-1β and IL-1Ra, IL-1β has the lowest affinity for the cell-bound form of IL-1R I. IL-1Ra has the greatest binding affinity for the IL-1R I. Moreover, the off-rate of IL-1Ra from IL-1R I is slow and binding of IL-1Ra to the cell-bound IL-1R I is nearly irreversible [Dinarello, 2002].

Figure 7. Schematic illustration of IL-1β signal transduction. See text for details. Adapted from [Dinarello, 2002].



A second receptor, designated IL-1 receptor type II (IL-1R II), can also bind IL-1β, but due to lack of the intracellular domain, it cannot signal. This latter receptor works biologically as an inhibitor for secreted IL-1β function since it has a 10–100 fold lower affinity for IL-1α. Thus, IL-1R II has been classified as a decoy receptor [Colotta et al., 1993; Dinarello, 1998b]. As mentioned previously, IL-1Ra is a receptor antagonist of IL-1β (and IL-1α). IL-1Ra binds to IL-1R I, but high concentrations of IL-1Ra (in relation to IL-1β) are needed to block IL-1-mediated signaling. Further, the ligand binding portion of IL-1 receptors (both R I and R II) can be released from the plasma membrane as soluble receptors (**Fig. 7**). These released receptors are also able to bind to free IL-1 with high avidity [Dinarello, 1998b]. Despite all these "roadblocks" for IL-1β, this cytokine is highly potent and requires only activation of a few receptors for biological activity [Orencole and Dinarello, 1989].

Many proinflammatory factors induce IL-1 cytokine transcription such as PAMPs, e.g. LPS, and proinflammatory cytokines such as TNF-α, IFN-α, IFN-β and IL-1β itself [Barksby et al., 2007]. The receptors for IL-1 cytokines are structurally related to PRRs such as TLRs, which recognize LPS and other PAMPs [O'Neill, 2000]. The intracellular signaling molecules that mediate the proinflammatory action of PAMPs are the same as those involved in IL-1 signaling via IL-1R I [Akira and Takeda, 2004]. Activation of IL-1R I results in recruitment of adaptor molecules such as myeloid differentiation primary-response gene 88 (MyD88) and activation of IL-1R-associated kinases (IRAK) (**Fig. 7**). These processes eventually lead to activation of NF-κB and MAPK-regulated transcription factors such as c-jun n-terminal kinase (JNK) and p38 [Akira et al., 2006].

It has been shown that many inflammatory diseases and medical conditions are linked to IL-1 activity. Specific blockage of IL-1 receptors leads to rapid and continued resolution in these diseases and conditions [Dinarello, 2004; Dinarello, 2005a].

IL-1β is also important in the pathological processes involved in periodontitis. In 1972 it was shown that dental plaque-exposed peripheral blood leukocytes could stimulate bone resorption by releasing factors into the culture supernatants [Horton et al., 1972]. This activity was consequently called osteoclast-activating factor (OAF). Later it was shown that this factor was identical to IL-1β [Dewhirst et al., 1985]. In fact, IL-1β has been shown to be one of the most potent stimulators of bone resorption (Lorenzo et al., 1987).

Alveolar bone resorption, involved in the periodontal attachment loss, is caused by enhanced local formation and activation of osteoclasts [Taubman and Kawai, 2001]. Osteoclasts are derived from myeloid hematopoetic stem cells and their differentiation is closely related with the differentiation of macrophages and DCs. For fully differentiated osteoclasts, receptor activator of NF-κB (RANK) expressed by osteoclast progenitor cells/macrophages need to be activated. This is obtained by binding of the ligand for RANK, i.e. RANK-ligand (RANKL). RANKL is expressed by some restricted cell types, such as stromal cells, osteoblasts and T-lymphocytes. One important regulator/inhibitor of this activation is the ubiquitously expressed osteoprotegerin (OPG) that serves as decoy receptor for RANKL. Thus, OPG inhibits the

interaction between RANKL and RANK. The role of the RANK-RANKL-OPG-system is crucial for osteoclast formation. Proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, can enhance the expression of RANKL in periosteal osteoblasts. Thereby these cytokines can indirectly affect osteoclast progenitor cells/macrophages towards fully differentiated osteoclasts [Lerner, 2004; Lerner, 2006].

Interestingly, elevated expression of IL-1 in periodontal tissue as well as increased concentrations of this cytokine in GCF, correlate with periodontitis [Boch et al., 2001; Gravallese and Goldring, 2000; Holmlund et al., 2004; McGee et al., 1998; Rasmussen et al., 2000; Roberts et al., 1997]. The role of *A. actinomycetemcomitans* leukotoxin in this association remains to be determined. In addition, the use of IL-1 antagonists inhibits inflammatory cell recruitment, osteoclast formation, and it prevents loss of periodontal tissues in primate models of experimental periodontitis [Assuma et al., 1998; Delima et al., 2002; Delima et al., 2001; Graves and Cochran, 2003].

1.7 Pyroptosis

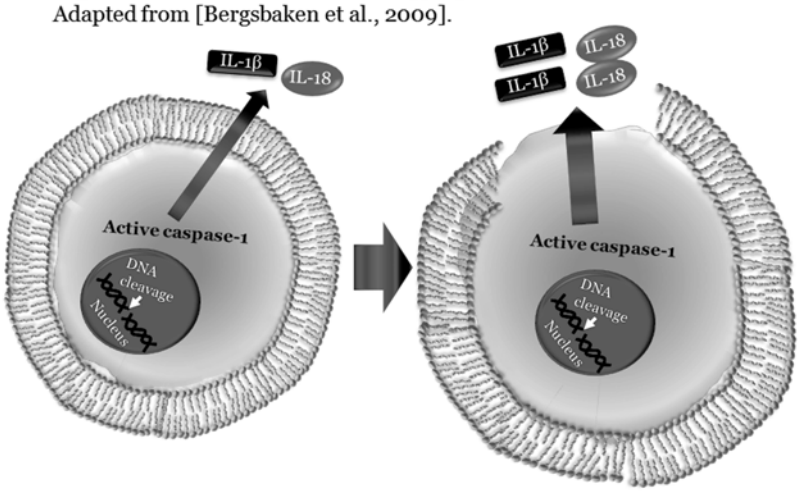
Cells can die through different biochemical pathways that result in diverse morphological and physiological endings. Apoptosis is the most widely recognized cell death process and involves activation of specific caspases [Samali et al., 1999]. Caspases are a group of cysteine proteases that, physiologically, can be divided into two groups: those involved in the initiation and execution of apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10) and those that trigger inflammation (caspase-1, -4, -5 and -12 in humans and -11 in rodents) [Bergsbaken et al., 2009; Scott and Saleh, 2007]. Apoptotic caspases cleave cellular substrates. These cascade reactions eventually lead to characteristic features of apoptosis, such as cytoplasmic and nuclear condensation. Other typical signs of cell death through apoptosis are DNA cleavage and intact plasma membrane [Albert, 2004]. Although apoptosis is a well-recognized "programmed cell death", increasing data suggest that there are other forms of programmed cell deaths, such as autophagy, oncosis and caspase-1-dependent programmed cell death (i.e. pyroptosis) [Fink and Cookson, 2005].

Pyroptosis is a more recently identified pathway of host cell death that is stimulated by a range of microbial infections such as *Salmonella*, *Francisella,* and *Legionella* [Bergsbaken et al., 2009]. As mentioned previously, caspase-1 was first recognized as the IL-1β-converting enzyme (ICE). Caspase-1 belongs to the inflammatory caspase subfamily. The members in that family have a predilection for hydrophobic amino acids, such as a tryptophan or tyrosine, and they can in addition cleave synthetic substrates with the sequence WEHD or YVAD [Garcia-Calvo et al., 1998; Scott and Saleh, 2007]. Caspase-1's "natural" substrates are certain members of the IL-1 family, which include pro-IL-1β, pro-IL-18, and pro-IL-33 [Dinarello, 1998a; Schmitz et al., 2005].

Beside that caspase-1 activation can result in the activation of these proinflammatory cytokines, it can also lead to rapid cell death characterized by plasma membrane rupture and release of

proinflammatory intracellular contents [Bergsbaken et al., 2009; Cookson and Brennan, 2001; Fink and Cookson, 2006]. The term pyroptosis (from the Greek "pyro", relating to fire or fever, and "ptosis", meaning a falling), is used to describe the inflammatory process of caspase-1-dependent programmed cell death that leads to specific secretion of IL-1β and IL-18 (**Fig. 8**) [Brennan and Cookson, 2000; Fink and Cookson, 2006].

Figure 8. Schematic illustration of pyroptosis. See text for details. Adapted from [Bergsbaken et al., 2009].



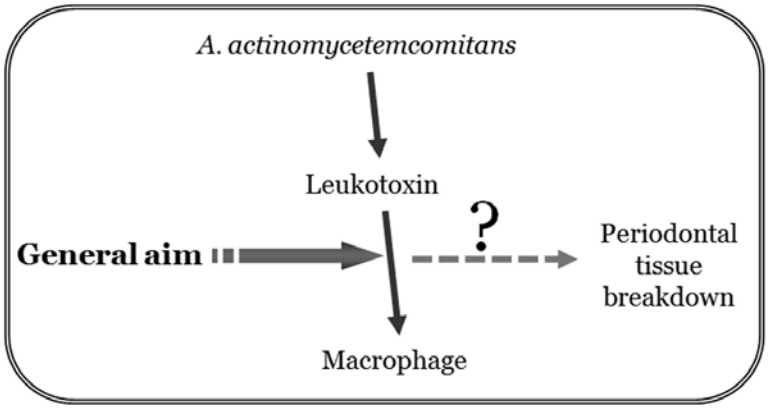
Pyroptosis is morphologically and mechanistically distinct from other forms of cell death. The apoptotic caspases, including caspase-3, -6 and -8, are not involved in pyroptosis [Bergsbaken et al., 2009; Cookson and Brennan, 2001; Fink and Cookson, 2006]. DNA fragmentation is a characteristic that usually is associated with apoptosis. However, pyroptosis also leads to DNA cleavage, but unlike apoptosis, nuclear integrity is maintained [Bergsbaken and Cookson, 2007]. In fact, DNA cleavage and cell lysis are both typical caspase-1-dependent features of pyroptosis, but cell lysis does not require DNA cleavage [Fink and Cookson, 2005].

Cells dying by pyroptosis increase in size and show signs of plasma-membrane pores. These pores cause cellular ion dispersion that eventually leads to increased osmotic pressure, water influx, and cell swelling. Finally, these processes lead to osmotic lysis and specific abundant secretion of IL-1β and IL-18 [Fink and Cookson, 2005]. The cytoprotective glycine, which acts on the cell surface, non-specifically inhibits pyroptosis by blockage of ion fluxes [Bergsbaken et al., 2009; Fink and Cookson, 2005; Fink and Cookson, 2006].

The Nomenclature Committee on Cell Death (NCCD) has recently accepted the term pyroptosis and has stated that macrophages undergoing pyroptosis exhibit features of both apoptosis and necrosis [Kroemer et al., 2008].

2. HYPOTHESIS AND AIMS

As it has been pointed out in the introduction of this dissertation, macrophages play an important role in tissue remodeling, both in physiological and pathological conditions. There is also convincing evidence for a correlation between a rapid loss of attachment of the periodontal tissue and a high proportion of *A. actinomycetemcomitans* in the periodontal pocket. In addition, highly-leukotoxic strains of this bacterium are considered to act as an exogenous pathogen in certain populations. Thus, the objective of this dissertation was to investigate the interactions of *A. actinomycetemcomitans* leukotoxin and human monocytes/macrophages. The main hypothesis was the assumption that this host-parasite interaction could induce processes that would be important for the pathogenic abilities of leukotoxin.



The specific aims of this dissertation were:

- To investigate leukotoxin-induced cell death of macrophages and to study responsible cellular and molecular mechanisms involved in this process.
- To identify possible leukotoxin-induced responses from macrophages that could help explain the clinical associations of leukotoxin with disease.
- To determine the role of leukotoxin-expression in interactions between live *A. actinomycetemcomitans* strains and macrophages.

3. MATERIALS AND METHODS

The study design of this dissertation is basically a host-parasite-interaction model. The detailed description of the materials used and methods are presented in Papers I-IV. Consequently, only a short summary of the materials and methods will be presented here.

3.1 Target cells

Human leukocytes were isolated from an enriched leukocyte fraction (buffy coat) of venous blood. The blood was taken from donors visiting the University Hospital blood bank in Umeå. Informed approval was given by all subjects, and authorization for the project was granted by the Human Studies Ethical Committee of Umeå University, Sweden (§67/3, dnr 03-019).

Cells of the human carcinoma promyelocytic cell line HL-60 (ATCC CCL-240) were also used.

The isolated human leukocytes were further treated to separate different cell types. In this dissertation isolated "adherent peripheral mononuclear leukocytes" are referred to as "monocytes" (**Paper I**). In addition, "macrophages" refer to "overnight culture-adapted adherent peripheral mononuclear leukocytes" (**Papers II-IV**).

3.2 Leukotoxin preparation

Leukotoxin was purified from *A. actinomycetemcomitans* strain HK 1519 belonging to the highly-leukotoxic JP2-like clone with a deletion in its promoter region. This strain was originally isolated from a patient with LAP. The leukotoxin-purification procedure included extraction of leukotoxin from the bacteria with a 300 mM NaCl solution and purification of the toxin from the bacterial extract by liquid chromatography. The leukotoxin preparation was essentially free of LPS (<0.0001% of total protein) (**Papers I, II & IV**).

In some experiments, LPS from *E. coli* (026:B6) or LPS-enriched leukotoxin-free fraction from *A. actinomycetemcomitans* (HK 1519) were used as positive controls (**Papers II & IV**).

3.3 Bacterial strains

P. gingivalis (ATCC33277), *A. actinomycetemcomitans* (HK 1519, serotype b), and two variants of *A. actinomycetemcomitans* strain D7S-smooth (D7SS, serotype a) were used in this dissertation. These strains were originally isolated from patients with periodontitis. The variants of the D7SS strain were a wild type (D7SS WT), with a complete leukotoxin promoter, and a mutant with the leukotoxin structure gene deleted and replaced with a spectinomycin resistance cassette (D7SS *ΔltxA*). Moreover, leukotoxin production of these strains was analyzed (**Paper III**).

The *A. actinomycetemcomitans* strains were cultivated in aerobic atmosphere, while *P. gingivalis* was cultivated in anaerobic atmosphere.

3.4 Exposure of target cells

Isolated human lymphocytes, PMNs, monocytes/macrophages, and HL-60 cells were exposed to the above described bacterial products or bacterial strains. The exposure time varied from 5 min to 20 h, depending on the features and factors that were to be analyzed.
At the end of the experiments, cell death, cell viability, morphological alternations, induced responses and their responsible pathways were analyzed by various techniques.

3.5 Analyses

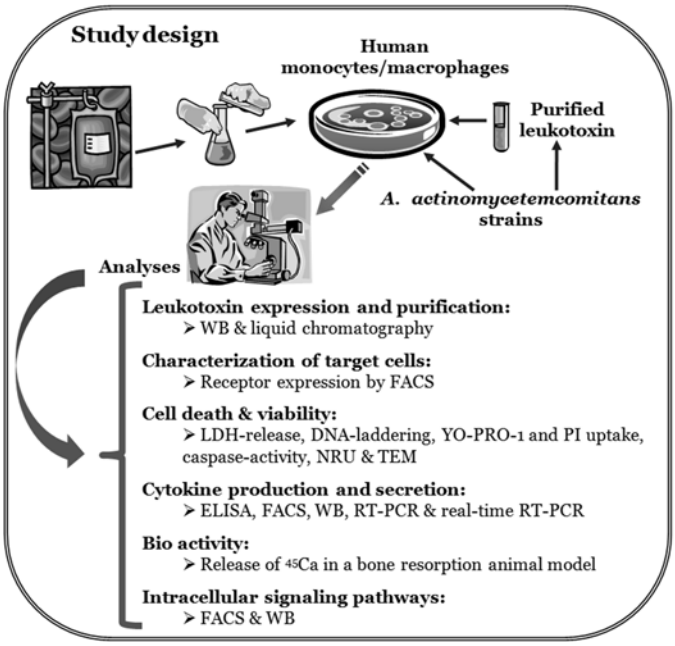
- Different target cells were characterized by their expression of cell surface molecules (**Paper I**).
- Cell death and viability were studied by examining cell membrane integrity and morphological alterations (**Papers I-IV**).
- Processes and cellular markers involved in apoptosis and necrosis were investigated (**Papers I & IV**).
- The expression and activation of pro-inflammatory cytokines of the leukotoxin-challenged macrophages were examined at mRNA and protein levels (**Papers II-IV**).
- Biological activity of the secreted cytokines was investigated by adding the culture supernatants to a bone resorption assay (**Paper II**).
- Different intracellular signaling pathways and activated proteins involved in the pro-inflammatory response from the macrophages were examined (**Papers I-IV**).

3.6 Methodology

- Cell death/viability were examined by:
    - morphological alterations detected by transmission electron microscopy (TEM)
    - release of the cytosolic enzyme, lactate dehydrogenase (LDH)
    - DNA-fragmentation by laddering and TEM
    - caspase-1 and caspase-3 activity
    - neutral red uptake
    - the uptake of early apoptotic (Yo-PRO-1) and necrotic/late apoptotic (propidium iodide, PI) markers, analyzed by fluorescence-activated cell scanner (FACS)
- Cytokine production, activation, and secretion were analyzed by:
    - enzyme-linked immunosorbent assay (ELISA)
    - semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)
    - quantitative real-time RT-PCR
    - western blot (WB)

- Surface molecule expression, intracellular expression of proteins and activated proteins were studied by:
    - FACS
    - WB
- Biological activity of the released components in culture supernatants from leukotoxin-exposed macrophages was examined by:
    - measuring the release of ^{45}Ca from prelabeled neonatal mouse calvariae in a bone resorption assay
- Intracellular activation pathways were further analyzed by using:
    - selective inhibitors

An overview of the study design, analyses and methodology is summarized below.



3.7 Statistical analyses

The significance of differences was assessed with Student's *t*-test (**Paper I**), one-way analysis of variance (ANOVA) (Dunnett two-sided test and Dunnett T3 test) (**Paper II**), multiple comparisons with one-way ANOVA (**Paper III**), and two-tailed *t*-test (**Paper IV**). The statistical analyses were performed in SPSS software (**Papers II-IV**).

4. RESULTS

4.1 The role of leukotoxin in cell survival and cell death of macrophages

The morphology of the macrophages exposed to leukotoxin for 1 h was analyzed by TEM (**Paper IV: Fig. 1c**). Exposing the macrophages to leukotoxin decreased the proportion of normal cells (**Paper IV: Fig. 1c-i**), while the proportions of necrotic cells (**Paper IV: Fig. 1c-ii & 1c-iv**) and apoptotic cells (**Paper IV: Fig. 1c-iii & 1c-v**) increased. Distinguishing features for apoptosis were chromatin condensation and DNA fragmentation. Features of necrosis were plasma membrane disruption and nuclear disintegration.

Exposure of the cultures of leukocytes to leukotoxin for 1 h revealed that the leukotoxin concentration required for cytolysis (LDH-leakage) varied for the different tested leukocyte populations (**Paper I: Fig. 1**). Monocytes were the most sensitive cells followed by PMNs and lymphocytes, while the tested cell line (HL-60 cells) appeared to be resistant. Maximal LDH leakage from PMNs occurred at approximately 10 times higher leukotoxin concentrations than the level that caused leakage from monocytes, and only half of the lymphocyte population was susceptible to leukotoxin-induced LDH-leakage (**Paper I: Fig. 1A**). LDH leakage from monocytes was induced at leukotoxin concentrations >1 ng/ml (**Paper I: Fig. 1B**). Challenging the macrophages with leukotoxin for 3 h caused similar sensitivity to leukotoxin-induced LDH leakage (**Paper II: Fig. 1a**). The observed leukotoxin-induced LDH leakage of human monocytes/macrophages indicated that a dose-dependent disruption of the membrane integrity occurred when leukotoxin concentrations were ≥1 ng/ml (**Paper I: Fig. 1B, Paper II: Fig. 1a and Paper IV: Fig. 1a**).

When whole bacteria were used, analysis of LDH leakage showed that only the leukotoxin-producing bacteria caused cytolysis when incubated with macrophages at concentrations 1-100 bacteria/macrophage (**Paper III: Fig. 2A**). The leukotoxin content of the highly-leukotoxic strain (HK1519) was approximately 10 times higher than that of the minimally-leukotoxic strain (D7SS WT), while the leukotoxin knockout mutant (D7SS *ΔltxA*) did not contain any detectable leukotoxin (**Paper III: Fig. 1**). The highly-leukotoxic strain (HK1519) required only 1 bacterium/macrophage to lyse the majority of the macrophages, while the minimally leukotoxic strain (D7SS WT) had a similar lytic effect at approximately 10 bacteria/macrophage (**Paper III: Fig. 2A**). Deletion of the leukotoxin structure gene (*ΔltxA*) from the minimally leukotoxic *A. actinomycetemcomitans* (D7SS WT) completely abolished the lytic capacity of this strain (**Paper III: Fig. 2A**).

Leukotoxin concentrations ≤1 ng/ml induced no classical apoptotic events in monocytes (**Paper I: Fig. 4**). The intracellular activity of caspase-3 was not significantly affected by the leukotoxin exposure for 6 h (**Paper I: Fig. 4A**). DNA fragmentation was observed in plain medium and cultures exposed to leukotoxin concentrations ≤1 ng/ml after a 20-h incubation period. DNA fragmentation was decreased in higher leukotoxin concentrations (**Paper I: Fig. 4B**). Toxin

concentrations of ≤1 ng/ml present in the culture for 3 h left the macrophages viable and their capacity for neutral red uptake was similar to the control cultures (**Paper II: Fig. 1a**).

The LDH leakage from human macrophages, exposed to various concentrations of *A. actinomycetemcomitans* leukotoxin for 1 h, indicated the presence of sublytical leukotoxin concentrations ≥1 ng/ml (**Paper I: Fig. 1B, Paper II: Fig. 1a and Paper IV: Fig. 1a**). Unexpectedly, testing the cell death by more sensitive markers for apoptosis and necrosis showed no marked difference in the uptake of early apoptotic (Yo-PRO-1) and necrotic/late apoptotic (propidium iodide, PI) markers. The lack of difference was seen both in a LDH-releasing leukotoxin concentration (10 ng/ml) and in a non-LDH releasing concentration of leukotoxin (1 ng/ml) (**Paper IV: Fig. 1b**).

Taken together, these results indicated that monocytes/macrophages were the most sensitive cells for leukotoxin-induced lysis. There was a threshold level for *A. actinomycetemcomitans* leukotoxin sensitivity for each monocyte/macrophage subset, and each of the affected cells activated signaling pathways that eventually led to cell death. Further, only leukotoxic-strains of *A. actinomycetemcomitans* induced macrophage lysis.

4.2 Mechanisms involved in leukotoxin-induced cell lysis of macrophages

The expression of the reputed leukocyte target receptor (LFA-1) for leukotoxin was measured by the expression of its unique α_L chain (CD11a) in all tested leukocytes. This expression was found in all leukocytes isolated from peripheral blood (**Paper I: Fig. 2**). More than 90% of the cells in each population expressed the receptor. In contrast, the leukotoxin-resistant HL-60 cells showed negligible expression of CD11a. Interestingly, differentiation of HL-60 cells towards monocytes induced by phorbol 12-myristate 13-acetate (PMA), activated LFA-1 expression and increased the leukotoxin sensitivity. Nevertheless, differentiation by dimethyl sulfoxide (DMSO) towards PMNs left the cells resistant to the toxin despite the increased LFA-1 expression (**Paper I: Fig. 3A & B**).

As mentioned in a previous section, no significant increase of caspase-3 was found in leukotoxin-challenged monocytes even though the exposure time lasted for 6 h. In contrast, a 5-fold increase in caspase-1 activity was recorded after only 20 min exposure of macrophages to 10 ng/ml of leukotoxin (**Paper I: Fig. 5A**). The amount of caspase-1 varied in different leukocyte populations. Monocytes contained approximately a 3-fold greater amount of caspase-1 than PMNs and lymphocytes. Interestingly, the presence of a caspase-1 inhibitor (Ac-YVAD-CMK) only decreased the leukotoxin-induced lysis of monocytes, while lysis of PMNs and lymphocytes was unaffected (**Paper I: Fig. 6**). In addition, a cytoprotectant (glycine) had an inhibitory effect on the leukotoxin-induced lysis of macrophages (**Paper IV: Fig. 5a**). Further, a combination of glycine and the caspase-1 inhibitor (Ac-YVAD-CMK) caused an additive decrease in the induced lysis of macrophages by leukotoxin (**Paper IV: Fig. 5a**).

In summary, these findings indicated that the β2-integrin receptor, LFA-1, served as a marker for *A. actinomycetemcomitans* leukotoxin susceptibility. However, the high sensitivity of monocytes/macrophages for leukotoxin-induced lysis could not be explained by the expression of this receptor. Leukotoxin-induced macrophage lysis seemed to involve caspase-1 activation, and the toxin seemed to act through surface interactions by pore-formation as well as via intracellular signaling pathways.

4.3 Caspase-1-dependent IL-1β secretion from leukotoxin-exposed macrophages

Compared to the other bacterial stimuli, such as LPS from *E. coli* and *A. actinomycetemcomitans*, the leukotoxin exhibited a limited capacity to induce production and secretion of IL-6 and TNF-α (**Paper II: Fig. 2 and Paper IV: Fig. 4c**). In the case of IL-1β (active form), two different patterns were observed. Predictably, *E. coli* LPS stimulation resulted in the production of mainly cell-associated cytokine. Unexpectedly, leukotoxin induced a substantial production of IL-1β that was mainly secreted (**Paper II: Fig. 2 and Paper IV: Fig. 2a).** It should be emphasized that after 3 h of exposure, the amount of IL-1β secreted by leukotoxin-challenged (1 ng/ml) macrophages was 25-fold enhanced compared to that caused by the *E. coli* LPS preparations at a 100 times higher concentration (100 ng/ml) (**Paper II: Fig. 2).** The high levels of IL-1β secretion were also found in leukotoxin concentrations that clearly induced rapid cell lysis. In fact, cultures of macrophages exposed for 3 h to leukotoxin (10 ng/ml) showed a 10-fold increase in total IL-1β protein as compared to cultures without stimulus and a 6-fold increase as compared to cultures exposed to *E. coli* LPS (100 ng/ml) (**Paper IV: Fig. 2a**).

When macrophages were exposed to live strains of *A. actinomycetemcomitans*, similar results were found. In fact, a significant increase in the secretion of IL-1β from the macrophages was induced by the two tested leukotoxin-producing strains (HK1519 and D7SS WT) (**Paper III: Fig. 2B**). The highly-leukotoxic strain (HK1519) required only a ratio of one bacterium per ten macrophages to induce considerable IL-1β secretion from these cells. A similar level of IL-1β secretion was induced by the minimally-leukotoxic strain (D7SS WT) at one bacterium per macrophage (**Paper III: Fig. 2B**). The secretion of IL-1β induced by the mutant strain without leukotoxin production (*ΔltxA*) was extremely limited as compared to the corresponding wild type strain (D7SS WT). This indicated that leukotoxin is the major factor inducing IL-1β secretion also for the minimally-leukotoxic strains of *A. actinomycetemcomitans* (**Paper III: Fig. 2B**).

Although exposure to non-leukotoxic bacteria (D7SS *ΔltxA* and *P. gingivalis)* at a ratio of ten bacteria per macrophage caused a negligible secretion of IL-1β, this exposure caused an increased accumulation of intracellular pro-IL-1β in macrophages (**Paper III: Fig 2B & 6**). The macrophages challenged by the non-leukotoxic strain of *A. actinomycetemcomitans* (D7SS *ΔltxA*) were exposed for an additional 3 h to the corresponding wild type strain (D7SS WT). This

treatment had no effect on the lysis of the macrophages (**Paper III: Fig. 7A**). On the other hand, this "priming" of macrophages enabled these cells to secrete 3-5 times more IL-1β after the secondary exposure to the wild type strain (**Paper III: Fig. 7B**). Analysis of cell-associated IL-1β in these cells showed that the majority of this cytokine was secreted in the presence of leukotoxic *A. actinomycetemcomitans* strains (**Paper III: Fig. 7C**). Quite the opposite, the secondary exposure did not cause increased secretion or elevated intracellular levels of TNF-α and IL-6 (**Paper III: Fig. 7 D-G**).

To study whether this excessive secretion could be due to higher production of IL-1β, the mRNA levels for this cytokine in macrophages were studied by semi-quantitative RT-PCR and quantitative real-time RT-PCR. First, the mRNA levels were studied by semi-quantitative RT-PCR after 3 h exposure to leukotoxin. The levels of mRNA for IL-1β were slightly increased in the presence of leukotoxin (1 ng/ml) or *E. coli* LPS (100 ng/ml) (**Paper II: Fig. 3**). However, a more precise quantification of IL-1β mRNA by quantitative real-time RT-PCR revealed very interesting results. Time-course experiments aimed to study the mRNA levels of IL-1β showed no sign of any marked increase of the mRNA levels during the 60 min leukotoxin exposure (**Paper IV: Fig. 2c**). On the other hand, the time-course of the secreted active protein showed that already after 20 min of leukotoxin exposure (10 ng/ml) a substantial amount of IL-1β was secreted and reached its maximum after 40 min (**Paper IV: Fig. 2d**). The IL-1β secretion caused by the *E. coli* LPS (100 ng/ml) after 60 min exposure was negligible as compared to that caused by the leukotoxin, even though LPS exposure had led to approximately 30 times higher levels of IL-1β mRNA (**Paper IV: Fig. 2c and Fig. 2d**).

To distinguish between the pro-IL-1β and IL-1β, both of these forms of IL-1β were analyzed. In fact, leukotoxin caused an extracellular release of both pro-IL-1β and active IL-1β (**Paper II: Fig. 1b**). A statistically significant increase in the secretion of active IL-1β was found after a 3-h incubation with ≥1 ng/ml leukotoxin. The secretion of active IL-1β peaked at 3 ng/ml leukotoxin, while the release of pro-IL-1β peaked at a toxin concentration of 100 ng/ml. At toxin concentrations ≥30 ng/ml, the secretion of the active IL-1β decreased (**Paper II: Fig. 1b**). Leukotoxin exposure (10 ng/ml) of macrophages for 1 h decreased the cell-associated pro-IL-1β and increased the secretion of pro-IL-1β (**Paper IV: Fig. 2e**). In addition, the secreted IL-1β was substantially increased by leukotoxin. Heat inactivation of leukotoxin completely abolished its ability to cause secretion of the pro-form, as well as the active IL-1β (**Paper IV: Fig. 2e**). The total level of cell-associated and secreted IL-1β (pro- and active forms, taken together) from macrophages exposed to leukotoxin (10 ng/ml) for 1 h, increased 2 times as compared to the unchallenged cells (**Paper IV: Fig. 2e**). Confirmation of these results by western blot analysis of macrophage cultures exposed to leukotoxin (10 ng/ml) or LPS (100 ng/ml) for 1 h, showed that leukotoxin exposure caused secretion of the active IL-1β (17 kDa), while LPS exposure mainly increased the cell-associated inactive precursor (31 kDa) (**Paper IV: Fig. 2f**). The kinetics of leukotoxin-induced caspase-1 activation showed that IL-1β secretion started when ≥80% of caspase-1 activation was reached (10 min after leukotoxin addition). The secretion of IL-1β peaked about 20 min later (**Paper I: Fig. 7**).

Among the tested cytokines, leukotoxin-induced cytokine secretion from macrophages was mainly restricted to IL-1β, while the effects on TNF-α and IL-6 secretion were limited (**Paper II: Fig. 2 and Paper IV: Fig. 4c**). Blocking of the caspase-1 with a specific inhibitor radically decreased the leukotoxin–induced IL-1β secretion to levels similar to those found in unchallenged cells. (**Paper II: Fig. 4 and Paper IV: Fig. 4a & b**). Furthermore, this inhibition reduced the leukotoxin-induced secretion of IL-6 and TNF-α.

Exposure to leukotoxin (1 or 10 ng/ml) activated phosphorylation of p38 MAPK in macrophages (**Paper IV: Fig. 3**). Time course registration of p38 phosphorylation showed a more rapid effect by the leukotoxin (10 ng/ml) than by the *E. coli* LPS (100 ng/ml) (**Paper IV: Fig. 3c**). Macrophages exposed to 10 ng/ml leukotoxin for 5 min showed a 10-fold increase in phosphorylated p38 as compared to the unchallenged control cells (**Paper IV: Fig. 3d**). However, neither of the two inhibitors used for p38 (SB 203580 or SKF-86002) affected the leukotoxin-induced IL-1β secretion (**Paper IV: Fig. 4a & b**). In fact, p38 inhibition did not interfere with the secretion of any of the tested cytokines (IL-1β, TNF-α, and IL-6) from leukotoxin-challenged macrophages. (**Paper IV: Fig. 4c**). On the other hand, this inhibitor (SB 203580) decreased the *E. coli* LPS-induced cytokine secretion. This means that the inhibitor actually did function and blocked known described pathways in LPS-induced cytokine activation where p38 is involved (**Paper IV: Fig. 4c**). In contrast to *E. coli* LPS, leukotoxin did not activate the NF-κB-system in macrophages since the total levels of IκBα were not affected by the leukotoxin after 15 or 30 min exposure. Neither was the NF-κB (p65) phosphorylated by leukotoxin after 5 min exposure (**Paper IV: Fig. 3a & d**).

In summary, exposure of macrophages to leukotoxin (purified as well as in the case of whole bacterial challenge) caused an abundant secretion of IL-1β. The leukotoxin-induced cytokine secretion from macrophages was particularly specific for IL-1β. This secretion was mediated by caspase-1 activation. Moreover, leukotoxin-induced IL-1β activation and secretion differed from known LPS-induced pathways for production and secretion of this cytokine.

Surprisingly, the experiments with the strains showed that *A. actinomycetemcomitans*-induced IL-1β secretion was mainly due to the leukotoxin. Primary exposure of macrophages to the non-leukotoxic strains resulted mainly in increased accumulation of the intracellular pro-IL-1β in macrophages (**Paper III: Fig. 5**). Further, the additional secondary exposure to the leukotoxic strain significantly enhanced the ability of these macrophages to secrete IL-1β.
The total IL-β protein in the macrophage cultures (both cell-associated and secreted pro-IL-1β and IL-1β) was increased. Thus, leukotoxin seemed to make more pro-IL-1β available for activation than what normally exists in the cells, and the toxin also acted as the secondary stimulus needed for activation and secretion of IL-1β. Interestingly, leukotoxin's effects on IL-1β mRNA level could not explain the increase of total IL-1β protein. The mechanism behind this phenomenon might involve post-transcriptional changes of the pro-IL-1β synthesis initiated by the leukotoxin-induced cell stress.

4.4 Bioactivity of culture supernatants from leukotoxin-exposed macrophages

Leukotoxin clearly caused excessive secretion of the active (17 kDa) IL-1β. These findings were conducted by ELISA and western blot. However, we wanted to further investigate if the secreted IL-1β was bioactive. Thus, to test the bioactivity of the released IL-1β from leukotoxin-challenged macrophages, the supernatants from these cultures were diluted 100 times and added in a bone resorption assay. In this assay the bioactivity was tested by measuring the release of ^{45}Ca from mouse calvarial bone. Intriguingly, the culture supernatants from leukotoxin-challenged macrophages significantly released ^{45}Ca at levels similar to the one obtained with 100 pg/ml of recombinant human IL-1β (**Paper II: Fig. 5**). The bone-resorbing activity caused by the leukotoxin-challenged supernatants was inhibited when IRAP (IL-1Ra) or antibodies to IL-1β were present. In contrast, addition of antibodies to IL-1α had no effect on the ^{45}Ca release (**Paper II: Fig. 5**). Nor did leukotoxin alone (≤1 μg/ml) stimulate ^{45}Ca-release of calvarial bone incubated in plane medium.

These results indicated that the IL-1β in culture supernatants from leukotoxin-induced macrophages was bioactive and the major activator of bone resorption in these mixtures.

4.5 Pyroptosis-like cell death of macrophages in response to leukotoxin

As mentioned in previous sections, the proinflammatory cytokine profile induced by leukotoxin was strikingly limited to IL-1β. The outcome of this leukotoxin-induced activation was excessive secretion of IL-1β, and that secretion was completely dependent on the activation of caspase-1. The cell death of macrophages in response to leukotoxin, as described previously in this dissertation, involved both traits associated with necrosis and apoptosis. In addition, leukotoxin did not induce a classical apoptosis of macrophages because of the lack of caspase-3 activity despite DNA-fragmentation at low-toxin concentrations. Further, caspase-1 was involved in the leukotoxin-induced lysis.

Interestingly, these processes resembled the newly described death process termed pyroptosis. This death mechanism is caspase-1 dependant and leads to release of abundant levels of IL-1β and IL-18. Since we knew that leukotoxin activated caspase-1, we also examined if leukotoxin-exposed macrophages could secrete IL-18. As expected, we found that IL-18 from leukotoxin-challenged macrophages showed an almost identical pattern as seen for IL-1β (**Paper IV: Fig. 6a, cf. Fig. 2d**). However, the absolute levels of secreted IL-18 from leukotoxin-challenged macrophages were about 30 times lower than the levels of the secreted IL-1β. In the tested concentrations of macrophages (10^6 cells) obtained from eight different donors, leukotoxin caused secretion of IL-1β at 5-6 ng/10^6 cells and IL-18 at 0.1-0.2 ng/10^6 cells (**Paper IV: Fig. 6b & c**).

Taken together, the leukotoxin seemed to induce a pyroptosis-like cell death in macrophages, and this process consequently led to excessive release of IL-1β and IL-18 from these cells.

4.7 High proportion of *A. actinomycetemcomitans* and elevated levels of IL-1β in GCF

Two periodontitis sites from a young patient with LAP were chosen for analysis. The microbiological analysis showed that in the sample taken from one site *A. actinomycetemcomitans* constituted 75% of the bacterial population whereas in the other site, it constituted only 0.09% (**Paper III: Table 1**). In the site with the high proportion of *A. actinomycetemcomitans*, an elevated concentration of IL-1β was also found in the GCF, while the concentrations of IL-6 and total protein in the GCF were at similar levels in both samples (**Paper III: Table 1**).

5 DISCUSSIONS

5.1 Initial remarks

The results in this dissertations show, for the first time, that leukotoxin causes a specific inflammatory cell death of human macrophages.

Among all of the various virulence factors from *A. actinomycetemomitans*, leukotoxin has been shown to be particularly interesting. The reason for this is basically based on two different types of findings. First, the clear clinical association of highly-leukotoxic strains of *A. actinomycetemcomitans* with LAP. Second, the studies showing that leukotoxin can cause lysis of human leukocytes.

However, the central question about why the highly-leukotoxic strains of *A. actinomycetemcomitans* are so strongly associated with disease cannot simply be explained by the immune defense-evading abilities of these strains compared to other strains of this bacterium. One important question that we raised was if leukotoxin by itself could cause the ultimate phase of periodontitis that is the alveolar bone resorption? And if it can, the next question would be how?

Perhaps the results from this dissertation can give some answers to these questions.

The interactions of *A. actinomycetemcomitans* leukotoxin with PMNs have been extensively studied. It is also known that leukotoxin kills macrophages, but little is known about the mechanisms involved. It is generally accepted that macrophages play a decisive role in the regulation of the inflammatory reactions. Moreover, many of the soluble mediators of macrophages, including cytokines, regulate the activities of osteoblasts and osteoclasts. In fact, macrophages are the main source for secreted IL-1 that is one of the most potent stimulators of bone resorption. Activated osteoclasts are evidently an important part of the physiological as well as the pathological bone remodeling. Additionally in periodontitis, bone resoption is the final devastating process, and it is almost impossible to replace periodontitis-induced resorbed bone.

Therefore, the basic hypothesis - that the interaction of leukotoxin with macrophages could induce processes that would be important for the pathogenic abilities of leukotoxin – formed the basis for this dissertation.

First, the results in this dissertation showed the fact that monocytes/macrophages were the most sensitive leukocytes for leukotoxin-induced lysis. In the next step we turned our focus on an investigation of the underlying mechanism behind this phenomenon. We discovered unique leukotoxin-induced pathways in human macrophages involving activation of caspase-1 and excessive secretion of bioactive IL-1β. During the time when the four studies of this dissertation

were conducted, new interesting data have been presented regarding caspase-1 activation by various factors. For instance, some bacteria or bacterial products have been shown to induce specific activation of caspase-1. In addition, a recently described form of cell death, pyroptosis, has helped us to understand our intriguing results. At the time of finalizing this dissertation, it is now clear that leukotoxin induces a specific inflammatory cell death in macrophages that is stunningly similar to the processes described for pyroptosis. Pyroptosis and other caspase-1-dependent processes seem to be important in diseases for which inflammation is central for pathogenesis.

In the following sections, a closer explanation of the results in this dissertation in relation to the other findings in the literature will be presented.

5.2 Can leukotoxin interact with macrophages in the periodontium?

The intensity of the inflammatory reactions in periodontitis seems to be decisive to the extent of tissue damage caused during the course of the disease. AgP differs from the chronic periodontitis in terms of the rapid bone destruction that is observed in the former disease type. In young subjects, AgP is strongly associated with a high prevalence of *A. actinomycetemcomitans* [Novak and Novak, 1996; Slots and Ting, 1999], and especially with the presence of highly-leukotoxic strains [Bueno et al., 1998; Haraszthy et al., 2000a; Haubek et al., 1997; Haubek et al., 2008]. Strains isolated from infected subjects have higher leukotoxin expression than strains isolated from healthy subjects [Haraszthy et al., 2000a]. In Africans suffering from AgP, the JP2 clone of *A. actinomycetemcomitans* that produces copious amounts of leukotoxin is thought to act as an exogenous pathogen [Bueno et al., 1998; Haubek et al., 1997]. Interestingly, in a recent large randomized controlled trial (RCT) study it was shown that adolescents who carry the JP2 clone of *A. actinomycetemcomitans* have a significantly (18 fold) increased risk of periodontal attachment loss after only 2 years as compared to control subjects without the prevalence of this bacterium [Haubek et al., 2008]. These findings indicate a role of leukotoxin for disease onset in the clinical situation.

Most of the studies on the interactions of *A. actinomycetemcomitans* leukotoxin with leukocytes have been focused on PMNs [Baehni et al., 1979; Johansson et al., 2000a; Johansson et al., 2000b; Lally et al., 1994; Lally et al., 1997; McArthur et al., 1981; Tsai et al., 1979]. These cells comprise the major leukocyte population in the periodontal pocket. Positioned between the bacterial plaque on the tooth surface and the epithelial lining, PMNs are thought to protect the soft tissues against massive bacterial invasion and the rapid spread of the infection. Nevertheless, their inability to eliminate the entire plaque by phagocytosis leads to the inflammation becoming chronic and its apical proliferation with concomitant destruction of the alveolar bone. Besides being lytic, leukotoxin was also shown to induce a rapid degranulation of PMNs [Claesson et al., 2002; Johansson et al., 2000a]. This active process was suggested to contribute to the local tissue destruction through increased proteolysis caused by the released lysosomal enzymes. The protective importance of PMNs in periodontitis is evident since

subjects with no or impaired PMN-function, such as in Kostmann and Papillon-Lefèvre syndromes, are more susceptible to develop aggressive forms of periodontitis early in life. This is particularly true if these subjects are infected with *A. actinomycetemcomitans* [Carlsson et al., 2006; de Haar et al., 2006; Putsep et al., 2002].

It is debatable whether leukotoxin can further intensify the inflammatory response by interacting with leukocytes in the soft periodontal tissues behind the PMNs layer. The vulnerability of the toxin to proteolytic enzymes [Johansson et al., 2001; Johansson et al., 2000a], and the relatively high concentration of the toxin required for cytolytic activity, support the hypothesis that the pathogenic action of leukotoxin is exerted in the vicinity of the PMN layer. In addition, systemic antibodies to leukotoxin can be found in patients with LAP [Califano et al., 1997; Engstrom et al., 1999], meaning that the toxin must have been introduced for the antigen presenting cells such as macrophages at some point during the infection.

In the tissues surrounding the inflamed periodontal pocket, macrophages are increased in numbers. These cells play a decisive role in the regulation of the inflammatory reactions and the tissue breakdown and remodeling [Hassell, 1993]. Alveolar bone loss, involved in the periodontal attachment loss, is caused by the enhanced local formation and activation of osteoclasts [Taubman and Kawai, 2001], and their differentiation is believed to be partially caused by pro-inflammatory cytokines, including IL-1 [Lerner, 2006]. Tissues in the vicinity of a periodontal infection have increased expression of IL-1 that correlates with disease progression [Boch et al., 2001; Gravallese and Goldring, 2000; McGee et al., 1998; Roberts et al., 1997]. The specific role of *A. actinomycetemcomitans* and its leukotoxin in these studies was not investigated.

The present dissertation demonstrated that human monocytes are the most sensitive leukocytes for leukotoxin-induced lysis (**Paper I**). The critical toxin concentration is approximately 10 times lower than that found for other leukocytes (**Paper I**). This concentration could reasonably be reached in the periodontal tissues provided that the toxin is released from the bacterial surface and that it is not immediately destroyed by proteases. The first condition is likely to occur in a protein-rich environment, like the one in GCF, that promotes the complete release of active leukotoxin from the surface of *A. actinomycetemcomitans* [Johansson et al., 2003]. Serum can also protect the toxin against proteolytic degradation either competitively due to its protein content, or mainly by its protease inhibitors [Johansson et al., 2001]. Thus, it seems possible that interactions of leukotoxin with tissue macrophages can occur in the periodontium.

5.3 Mechanisms involved in leukotoxin-induced cell death of macrophages

Expression of the β_2-integrin LFA-1 on the leukocyte plasma membrane has been shown to be crucial for the target cell specificity to leukotoxin-induced cytolysis [Lally et al., 1997]. However, we found a similar expression of LFA-1 in all human leukocytes tested (**Paper I**). The non-

sensitive human promyelocytic carcinoma cells, HL-60, showed no LFA-1 expression. Interestingly, using a differentiation agent towards monocytes led to higher receptor expression in HL-60 cells and consequently these cells became leukotoxin susceptible (**Paper I**). It seems that LFA-1 is a membrane protein that is necessarily involved in the cytolytic interaction of leukotoxin with human leukocytes [Lally et al., 1997]. However, expression of this integrin may not be the only condition required, since the type and possibly the level of differentiation in our experiments appeared to affect the sensitivity of HL-60 cells to *A. actinomycetemcomitans* leukotoxin (**Paper I**). All populations of the human leukocytes expressed LFA-1 at a high level (>90%). Remarkably, the expression did not correlate with the sensitivity of the target cells to the toxin (**Paper I**). This finding also supports the hypothesis that other molecules may be involved in this cytolytic interaction.

Mammalian integrins form a number of subfamilies sharing common β subunits that associate with different α subunits. β_2 integrins include four different heterodimers: CD11a/CD18 (i.e. LFA-1, $\alpha_L\beta_2$), the predominant β_2-integrin, CD11b/CD18 restricted to granulocytes and monocytes (Mac-1, $\alpha_M\beta_2$), CD11c/CD18 (CR4, $\alpha_X\beta_2$), and CD11d/CD18 (aDb2, $\alpha_D\beta_2$) [Gahmberg et al., 1998]. A correlation has been reported between cytolysis induced by another RTX toxin, the leukotoxin of *M. haemolytica*, and the expression of CD18, the common β subunit of the β_2-integrins. CD18 was suggested to mediate leukotoxin-induced lysis of bovine leukocytes [Deshpande et al., 2002]. In fact, recently it was shown that CD18 serves as the functional receptor for *A. actinomycetemcomitans* leukotoxin [Dileepan et al., 2007]. Since the functional receptor for leukotoxin seems to be CD18, it is possible that other β_2 integrins than LFA-1, such as Mac-1, are involved in the monocyte/macrophage interaction with leukotoxin.

The existence of different cytolytic mechanisms induced by the leukotoxin in the various leukocytes could constitute another possible explanation. Low concentrations of leukotoxin activate apoptotic events in HL-60 cells. However, with increasing concentrations, the plasma membrane integrity totally collapses and results in an uncontrolled leakage of cytosolic components [Korostoff et al., 1998]. It has been suggested that leukotoxin at low concentrations causes small pores in the plasma membrane that allow influx of Ca^{2+} that activates apoptotic pathways. In contrast, the presence of high concentrations of leukotoxin leads to toxin oligomerization in the plasma membrane and the formation of large pores that cause rapid cytolysis [Lally et al., 1999]. Human monocytes/macrophages differ in this aspect from HL-60, since the monocytes/macrophages do not undergo a “classic” apoptosis in the presence of leukotoxin (**Papers I & IV**). Furthermore, the lytic concentration for monocytes/macrophages is much lower than that for HL-60, which may indicate that the concentrations are probably too low to allow oligomerization.

A relatively new cytotoxic mechanism has been described in macrophages in response to *Salmonella typhimurium* [Brennan and Cookson, 2000; Monack et al., 2001b]. The *Salmonella* invasive protein B activates caspase-1 when released intracellularly in the infected leukocytes and leads to a rapid cell necrosis through the so-called inflammatory death pathway [Boise and

Collins, 2001; Hersh et al., 1999]. A similar phenomenon has been reported for *Shigella* species [Hilbi et al., 1998]. Initially, the cytotoxic effect was characterized as apoptosis but a more extensive analysis led to the suggestion that the phenomenon is a necrotic rather than an apoptotic event [Brennan and Cookson, 2000]. In caspase-1-knockout mice, *Shigella* infection cannot induce macrophage death, and the animals develop a chronic inflammation without clearing the bacterial infection [Sansonetti et al., 2000].

Although apoptosis and necrosis are thought to be distinct forms of cell death, there is increasing evidence that the morphological and biochemical characteristics of these events represent only the extreme ends in a wide range of possible cell deaths [Nicotera et al., 1999]. The *S. typhimurium*-induced cytolysis involves no activation of caspase-3 and is characterized by rapid changes in the plasma membrane integrity. This caspase-1-dependent cell death involves both apoptotic and necrotic pathways, which indicates a mechanism for cytotoxicity at the interface between apoptosis and necrosis. Later the same research group introduced a name for this caspase-1-dependent programmed cell death that was termed pyroptosis [Bergsbaken et al., 2009; Cookson and Brennan, 2001; Fink and Cookson, 2006]. The results of this dissertation indicated pyroptosis-like features in monocytes/macrophages exposed to leukotoxin. We found lack of caspase-3 activity and at the same time leukotoxin exposure led to activation of caspase-1 and an irreversible loss of cell membrane integrity of macrophages exposed to ≥1 ng/ml leukotoxin (**Papers I & IV**). We also found that low concentrations of leukotoxin caused DNA-fragmentation in macrophages (**Papers I & IV**). Usually, DNA fragmentation is a lethal event that is often assumed to indicate apoptotic cell death. However, DNA damage also occurs during pyroptosis [Bergsbaken et al., 2009; Fink and Cookson, 2005]. Further, our results showed a dose-dependent increase of affected cells correlated to the concentration of leukotoxin and not to the concentration of the bacteria (**Papers I-IV**).

Our results are the first report on an RTX-toxin-induced inflammatory death pathway similar to the one described for the invasins of *Salmonella* and *Shigella* species. The interaction of leukotoxin with monocytes resulted in a rapid increase of the caspase-1 activity (**Paper I**). Caspase-1 activates IL-1β and IL-18, and this activation leads to an acute inflammation that is responsible for severe tissue destruction [Sansonetti et al., 2000; Zychlinsky and Sansonetti, 1997]. Monocyte/macrophage lysis by the leukotoxin of *A. actinomycetemcomitans* involved caspase-1 activation, since the caspase-1 inhibitor (Ac-YVAD-CMK) could block it partially (**Papers I & IV**). On the other hand, the inhibitor protected neither PMNs nor lymphocytes, indicating the involvement of different pathways in the cytolytic interactions of these leukocytes with the leukotoxin (**Paper I**). The different cellular markers for both apoptosis and necrosis followed a similar pattern of dose-dependency and indicated a heterogenic cell population with regard to leukotoxin sensitivity (**Paper IV**). TEM-analysis confirmed the presence of both apoptotic and necrotic cells in the macrophage populations exposed to the leukotoxin (**Paper IV**). The similar morphology of the affected cells exposed to different concentrations of leukotoxin indicated a threshold level for leukotoxin sensitivity for each cell subset. Each of the affected cells activated signaling pathways that finally ended up in cell death. An increase of leukotoxin enhanced the proportion of affected cells. Moreover, the additive inhibitory effect of

a cytoprotectant (glycine) that acts on the cell surface, and a caspase-1 inhibitor on leukotoxin-induced macrophage lysis indicated that this process involved both pore-formation and activation of intracellular signaling pathways (**Papers I & IV**).

5.4 Mechanisms involved in leukotoxin-induced IL-1β secretion from macrophages

The present findings together with the results from previous studies regarding the invasins of *Salmonella* and *Shigella* species [Edgeworth et al., 2002; Fink and Cookson, 2006; Monack et al., 2001a] indicated that the interaction of *A. actinomycetemcomitans* leukotoxin with human macrophages may lead to two different events depending on the toxin concentration. At low (<30 ng/ml) concentrations and after 1 or 3 h of exposure time, the leukotoxin induced a rapid activation and secretion of IL-1β, before the cells finally died. At higher concentrations, the toxin caused a rapid cell death with limited secretion of IL-1β (**Papers II & IV**). Compared to the classic inflammatory stimulus, LPS, the leukotoxin appears to be a more potent inducer of cytokine release, since it caused a substantially larger secretion of bioactive IL-1β at a 100 times lower concentration (**Papers II & IV**). The presence of specific antibodies to IL-1β partially inhibited the leukotoxin-stimulated production and secretion of this cytokine. This is probably due to the inhibition of the autocrine and paracrine loop of IL-1β. On the other hand, a complete inhibition of IL-1β secretion from leukotoxin-exposed macrophages was obtained with an inhibitor for caspase-1 (**Papers II & IV**).

Caspase-1 is responsible for activation and secretion of IL-1β [Dinarello, 1996]. It seemed that the release of IL-6 and TNF-α from leukotoxin-exposed macrophages was a secondary event induced by the secreted bioactive IL-1β (**Paper II**). The precursor of IL-1β is synthesized as a 31 kDa biologically inactive protein that is stored intracellularly. During activation, caspase-1 cleaves the precursor to a 17 kDa bioactive protein, which is then secreted [Dinarello, 1996]. Leukotoxin appeared to trigger not only the mechanism for production but also for secretion of active IL-1β, the latter involving activation of caspase-1. The mechanism for the abundant secretion of the active cytokine seemed to involve two separate steps. The first step was the activation of caspase-1, which was leukotoxin-dependent. The active enzyme cleaved intracellular pro-IL-1β, which was then secreted in its active form (**Papers II & IV**). The second step is the synthesis of new IL-1β, which is stimulated by the secreted cytokine in an autocrine manner [Dinarello, 1996]. Secretion of active IL-1β was dose-dependent and significantly enhanced at low-lytic concentrations of leukotoxin (1-30 ng/ml). At higher concentrations, the proportion of pro-IL-β increased, which indicates an insufficient conversion of the precursor to the active cytokine (**Paper II**). Possibly, cell lysis at high toxin concentrations proceeds too rapidly to allow the mechanisms for activation and secretion to operate fully. Perhaps the rapid cell death involves other mechanisms independent of caspase-1 activation such as the one that has been described previously by Lally and co-workers [Lally et al., 1999]. Even though major cell damage to the macrophages was initiated by leukotoxin (≤10 ng/ml), these cells were able to activate and secrete excessive amounts of IL-1β during the death process. In relation to cells

exposed to *E. coli* LPS (one of the most widely used inducers of IL-1β production and secretion), the leukotoxin-challenged cells were able to induce >10-fold higher secretion of the active IL-1β after a short exposure time (60 min) (**Paper IV**). The leukotoxin-induced IL-18 secretion seemed to follow the same pattern as for IL-1β. This is reasonable since both cytokines require caspase-1 activation for their activation and secretion. However, the leukotoxin-induced secretion of IL-1β from macrophages was 30 times higher than the IL-18 secretion. Thus, we proposed that leukotoxin causes a specific inflammatory cell death of human macrophages that resembles the one designated pyroptosis (**Paper IV**).

The activation by the leukotoxin was mainly mediated by caspase-1 activation and was not correlated to any changes in the mRNA levels for IL-1β up to 60 min after leukotoxin-exposure (**Paper IV**). Exposure of macrophages to leukotoxin also caused an increase of the total IL-β protein in the macrophage cultures (both cell-associated and secreted pro-IL-1β and IL-1β). In other words, leukotoxin seemed to make more pro-IL-1β available for activation than what normally exists in the cells, and the toxin also acted as the secondary stimulus needed for activation and secretion of this cytokine. Interestingly, we did not find any corresponding increase of IL-1β at the mRNA level that could explain the increased protein levels caused by the leukotoxin (**Paper IV**). The mRNA levels for IL-1β were only marginally increased after longer exposure time (3 h) of macrophages to leukotoxin, while the protein levels were greatly enhanced (**Paper II**).

The mechanism behind this phenomenon is not fully understood. However, it might involve post-transcriptional changes of the pro-IL-1β synthesis initiated by the leukotoxin-induced cell stress. It has been proposed that significant changes in translational efficiency occur for IL-1β mRNA, since there is a lack of correlation between IL-1β mRNA and protein levels [Schindler et al., 1990]. In addition, adherence of peripheral blood monocytes (like the ones used in this dissertation) to a surface activates transcription of the IL-1β gene without necessarily resulting in production of the protein [Dinarello, 1996]. On the other hand, the mRNA levels of IL-6 and TNF-α in the different cultures of macrophages were more in line with the production of the corresponding proteins. We found a slight increase for these cytokines due to leukotoxin exposure of macrophages, while a substantial enhancement of IL-6 and TNF-α was caused by LPS in these cells (**Papers I & IV**).

Our results show the importance of examining the protein levels instead of just relying on mRNA levels of the protein of interest. This statement is particularly accurate regarding the IL-1β, since there is an inconsistency of protein levels in comparison with mRNA levels.

Since leukotoxin is primate-specific [Fine et al., 2006], conducting experiments on animals are in many ways of little biological interest. Thus, we decided to as an alternative test the already secreted IL-1β from leukotoxin-challenged human macrophages in an animal bone resorption model. Interestingly, we found that culture supernatant of macrophages that was exposed to low concentrations of leukotoxin (1 ng/ml) stimulated bone resorption in an assay with cultured mouse calvariae (**Paper II**). The activity was nearly completely blocked by IL-1β antibodies or

an IL-1 receptor antagonist, while IL-1α antibodies had no significant effect. Thus, we concluded that secreted IL-1β from leukotoxin-induced macrophages was the most important factor for bone resorption in the used model (**Paper II**).

Several bacterial products, such as LPS, are known to increase expression of pro-IL-1β in macrophages [Mosser and Edwards, 2008]. However, a secondary stimulus is needed to induce a substantial secretion of the bioactive IL-1β. It has been shown that this activation can be induced by various bacterial species through activation of the inflammasome (intracellular signal-induced multiprotein complex) that leads to caspase-1 activation and IL-1β secretion [Mariathasan et al., 2006]. This secondary activation can be induced by direct interactions of bacteria/bacterial compounds with the inflammasome, such as proteins secreted by *Salmonella typhimurium* [Mariathasan et al., 2006] or *Francisella tularensis* invasion [Mariathasan et al., 2005]. The other system for stimulating this secondary activation has been described through cell surface interactions as for *Listeria monocytogenes* and *Staphylococcus aureus* [Mariathasan et al., 2006]. The physiological properties of the leukotoxin as a large protein that involves interaction with a target cell receptor, the CD18 subunit of the β2-integrin [Dileepan et al., 2007], indicates that this toxin also acts on the cell surface and involves transmembrane signaling mechanisms.

Leukotoxin-exposed macrophages showed a rapid increase of phosphorylated p38, but this activation was not correlated to cell lysis or IL-1β activation and secretion (**Paper IV**). Moreover, the lack of activation of NF-κB p65, IκBα, and mRNA levels for IL-1β in leukotoxin-challenged macrophages indicated that the toxin mainly acted at a post-transcriptional level (**Paper IV**). These findings are in contrast to the LPS-induced pathways in which p38-activation is involved in the production of IL-1β. In addition, LPS-induced IκBα-phosphorylation and degradation allow translocation of NF-κB to the nucleus, which can induce proinflammatory cytokine production such as IL-1β [Guha and Mackman, 2001; Hsu and Wen, 2002].

To further examine the leukotoxin-induced IL-1β activation and secretion from macrophages, we decided to compare this process with macrophages exposed to live bacteria. Consequently, we used different strains of *A. actinomycetemcomitans* with various levels of, or no, leukotoxin production. The results from the selected leukotoxic strains of *A. actinomycetemcomitans* also showed that leukotoxin was, in fact, the primary component from this bacterium that induced secretion of IL-1β from the macrophages. Remarkably for the highly-leukotoxic strain, less than one bacterium per macrophage was needed for excessive secretion of IL-1β (**Paper III**). Even in the case of minimally-leukotoxic strain, low moi (multiplicity of infection) was required for substantial IL-1β secretion (**Paper III**). The use of a *ltxA* mutant without production of leukotoxin dramatically reduced the ability of *A. actinomycetemcomitans* to induce secretion of IL-1β from the macrophages (**Paper III**). In addition, the presence of a leukotoxin antiserum in mixtures with leukotoxic bacteria and macrophages decreased the IL-1β secretion to similar levels as for the corresponding leukotoxin mutant (**Paper III**).

Interestingly, the ability of *P. gingivalis* to induce IL-1β production and secretion was also limited as compared with leukotoxic *A. actinomycetemcomitans* strains. We found that leukotoxin was also the primary means by which even strains with low toxin production induced secretion of IL-1β from macrophages (**Paper III**). However, a highly-leukotoxic strain of *A. actinomycetemcomitans* was about 10 times more effective in inducing IL-1β secretion from macrophages. Accordingly, our model showed that the quantity of leukotoxin produced by a specific *A. actinomycetemcomitans* strain is more important for the induced IL-1β secretion from macrophages than the bacteria/macrophage ratio (**Paper III**). The non-leukotoxic bacteria caused a negligible secretion of IL-1β from macrophages. At the same time an increased accumulation of intracellular pro-IL-1β due to this exposure was found (**Paper III**). However, a secondary exposure to a leukotoxic strain of *A. actinomycetemcomitans* was able to trigger secretion of the accumulated pro-IL-1β. In fact, this treatment of macrophages resulted in a 3-5 fold enhanced secretion of IL-1β. On the other hand, it seemed that the presence of the leukotoxic bacteria inhibited the secretion of TNF-α and IL-6. This indicated, once again, that the inflammatory response of macrophages to leukotoxic bacteria is mainly limited to activation and secretion of IL-1β (**Paper III**).

6 CONCLUDING REMARKS

In this dissertation we have identified a novel proinflammatory response from leukotoxin-exposed macrophages. Our results, based on the used host-parasite model, show that leukotoxin has the potential to induce cellular and molecular mechanisms that may play an important role in the pathogenesis of LAP.

A. actinomycetemcomitans can be found in all forms of periodontitis, but its strongest association is with AgP, especially with LAP [Slots and Ting, 1999]. In fact, *A. actinomycetemcomitans* is isolated from the majority of LAP lesions [Slots and Ting, 1999]. In addition, an increasing number of large, longitudinal clinical studies show that this bacterium is frequently associated with LAP [Fine et al., 2007; Haubek et al., 2008; Van der Velden et al., 2006]. Among the various virulence factors that this bacterium possesses, leukotoxin is presumed to be a critical virulence factor. Strains isolated from patients with AgP produce more leukotoxin [Haraszthy et al., 2000a; He et al., 1999]. Moreover, there is an obvious relationship between increased leukotoxin production and disease initiation and progression [Haubek et al., 2001; Haubek and Westergaard, 2004]. Examination of different clonal types of *A. actinomycetemcomitans* in patients with LAP suggests that the highly-leukotoxic JP2 clone is strongly correlated to disease onset in certain populations [Bueno et al., 1998; Haraszthy et al., 2000a; Haubek et al., 2008].

Even though periodontitis is primarily an infectious disease, the duration and intensity of the induced inflammation is the primary cause of the disease progression. This process ultimately leads to loss of tooth supporting tissues.

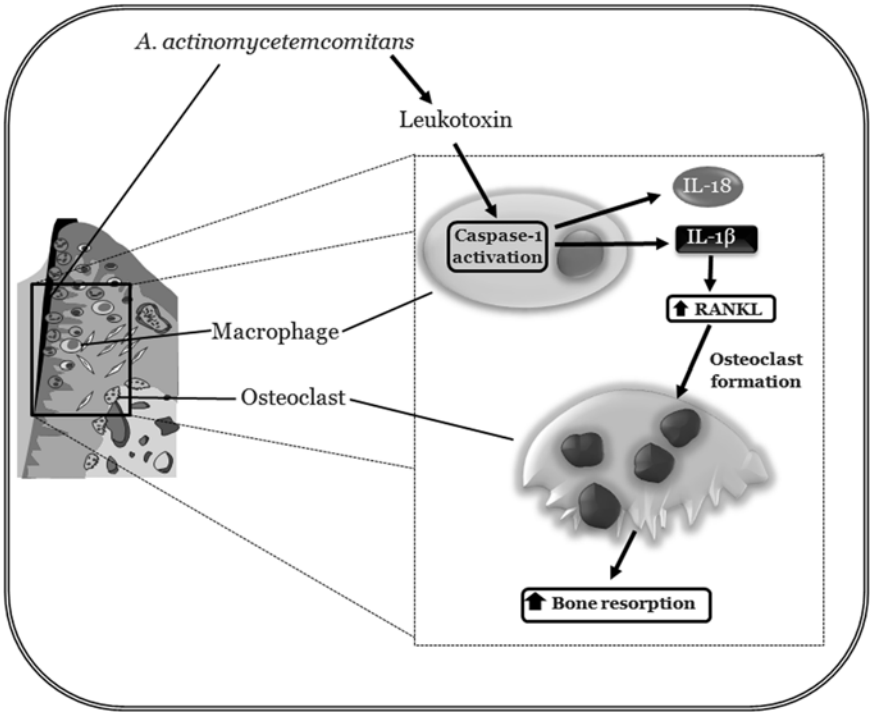
Possibly, the rapid tissue destruction observed in aggressive *A. actinomycetemcomitans*–associated periodontitis is the result of the interaction of leukotoxin with the macrophages accumulated in the periodontal tissues. Moreover, infection load and leukotoxin expression might determine the pathogenic potential of an *A. actinomycetemcomitans* infection.

Elevated levels of secreted IL-1β can indirectly promote bone resorption. For instance, IL-1β enhances the expression of RANKL that results in increased osteoclast formation, which consequently leads to bone resorption.

Clearly, our results show that leukotoxin has the potential to trigger an inflammatory response by causing pyroptosis-like cell death in human macrophages. Consequently, this process leads to excessive and specific secretion of both IL-1β and IL-18. This novel virulence mechanism of the leukotoxin may play an important role in explaining the pathogenic potential of *A. actinomycetemcomitans*.

In conclusion, the major findings of this dissertation are:

- Macrophages were the most sensitive cells to leukotoxin-induced cell lysis and this process caused a specific cell death that resembled pyroptosis. This death process involved a specific activation of caspase-1 that consequently lead to excessive secretion of IL-1β.
- The secreted IL-1β, from leukotoxin-exposed macrophages, was mainly the 17 kDa bioactive form and acted as the major activator of bone resorption in the tested bioactivity assay.
- Exposing macrophages to live strains of *A. actinomycetemcomitans* showed, in fact, that this rapidly induced IL-1β secretion from the macrophages was mainly due to the leukotoxin.



7 CLINICAL RELEVANCE AND FUTURE PERSPECTIVES

In a site of inflammation, monocytes are recruited from the peripheral circulation and need to pass through the blood vessel wall to enter the infected tissue. During this migration and later in the tissue they differentiate into macrophages and are primed by a gradient of inflammatory components from the host [Perregaux et al., 2002] and microbial components from the infection [Loesche, 1993]. It is tempting to speculate that this also happens in the inflamed periodontium, where monocytes increase their levels of pro-IL-1β during migration and differentiation towards macrophages in the tissue. An additional exposure of the macrophages to bacterial components such as LPS may increase their level of pro-IL-1β even more. If the macrophages then are exposed to a secondary factor (in our case, leukotoxin) that activates caspase-1, the level of IL-1β secretion from these primed macrophages might be substantial and devastating for the tissue.

Macrophages play an important role in periodontal tissue remodeling. The ability of these cells to secrete large amounts of IL-1 indicates their involvement in the enhanced bone loss seen in periodontitis [Boch et al., 2001; Gravallese and Goldring, 2000; McGee et al., 1998; Roberts et al., 1997]. Moreover, IL-1 levels in GCF from subjects with periodontitis are generally decreased after treatment. The levels of these cytokines in GCF samples seem to play important roles for the bone resorption activity seen in periodontally diseased subject [Holmlund et al., 2004].

In the clinical case presented in this dissertation (**Paper III**), there was a correlation between enhanced levels of IL-1β in GCF samples and high proportion of *A. actinomycetemcomitans*. One can hypothesize if the mechanism involved in this case regarding the IL-1β secretion could be due to leukotoxin-induced processes presented in this dissertation. However, it must be emphasized that this is only one case and further clinical studies are needed to investigate if the correlation between elevated levels of IL-1β and high proportion of *A. actinomycetemcomitans* can be consistently found.

If future clinical studies can confirm this correlation, new possibilities for therapeutic agents arise. Perhaps, local inhibition of IL-1β secretion in AgP patients infected by *A. actinomycetemcomitans* can be a useful treatment in the future.

The idea of treating periodontitis with specific inhibitors to IL-1 is a relatively new approach. For instance, in primate models of experimental periodontitis, antagonists to IL-1 inhibit bone loss and inflammation [Assuma et al., 1998; Delima et al., 2002; Delima et al., 2001]. In other fields, research on this topic has gone much further. In a multicenter trial on the treatment of rheumatoid arthritis (RA), clinical improvement and decrease in disease progression including bone erosions is found in patients treated with an IL-1Ra as compared to patients treated with a placebo [Bresnihan et al., 1998].

As mentioned previously, clinical studies are needed to verify our findings in relation to the complex *in vivo* situation. This is one aspect of future investigations that can be initiated due to our results. The other important issue which should be studied is leukotoxin-induced processes up-stream and down-stream from caspase-1 activation. One major question would be to find out which of the caspase-1 inflammasomes that are activated by leukotoxin. Moreover, the resemblances and perhaps differences between leukotoxin-induced cell death of macrophages and pyroptosis need further characterization. These cellular and molecular studies can be of importance for understanding how leukotoxin activates caspase-1. In addition, these future studies can identify new targets for therapeutic agents aimed to inhibit the dysregulated leukotoxin-induced IL-1β secretion.

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