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Virulence Mechanisms of Leukotoxin from *Aggregatibacter actinomycetemcomitans*

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1. Introduction

Aggregatibacter actinomycetemcomitans is a gram-negative bacterium that is present in the oral cavity of a large proportion of the human population (Zambon et al., 1983; Henderson et al., 2010) The bacterium is acquired through transmission from infected individuals and thought to initially colonize oral mucosa as a facultative intracellular pathogen (Fine et al., 2006). When the bacteria translocate to a site in the subgingival crevices, a persistent colonization may lead to periodontal destruction and development of periodontitis in susceptible individuals (Fig. 1) (Philstrom et al., 2005; Darveau 2010). The prevalence of this bacterium shows a great variation depending on geographic origin, age and life stile of the examined population (Kinane et al., 2008; Habek 2010). *A. actinomycetemcomitans* is a part of the normal flora in many healthy individuals but it is also a major agent in some aggressive forms of periodontitis (Fine et al., 2006). Periodontitis is a chronic infection characterized by the destruction of tooth-supporting structures (Darveau 2010). The number and composition of bacteria in the subgingival dental plaque, as well as life stile and genetic predisposition are factors that determine the outcome of the disease activity (Philstrom et al., 2005; Darveau 2010). The genetic diversity among different isolates of *A. actinomycetemcomitans* is great and its ability to express and release virulence factors varies (Henderson et al., 2010). The different adhesins and fimbriae expressed by this bacterium have been shown to be important factors that promote colonization at the various ecological niches of the human oral cavity (Fine et al., 2006).

A. actinomyctemcomitans expresses two exotoxins, a cytolethal distending toxin (Cdt) and a leukotoxin. Cdt´s are expressed by a number of gram-negative bacteria and causes death of the host cells by blocking their proliferation (Belibasakis et al., 2004). The leukotoxin selectively affects human cells of hematopoetic origin by binding to the lymphocyte function associated receptor 1 (LFA-1) and cause disruption of the membrane integrity (Lally et al., 1999). Leukotoxin belongs to the Repeat in Toxin family (RTX) and shares genomic organization and molecular structures with RTX proteins produced by a number of other gram-negative bacteria (Linhortavá et al., 2010). The expression of leukotoxin and Cdt varies among different *A. actinomycetemcomitans* isolates and high leukotoxin expression has been shown to correlate with disease while the role of Cdt still is more unclear (Henderson et al.,

2010). The genetic features and the molecular structure of leukotoxin have recently been described in detail (Kachlany 2010; Johansson 2011). This chapter focuses on the functional aspects of the leukotoxin as a virulence factor associated with pathogenic cellular mechanisms.

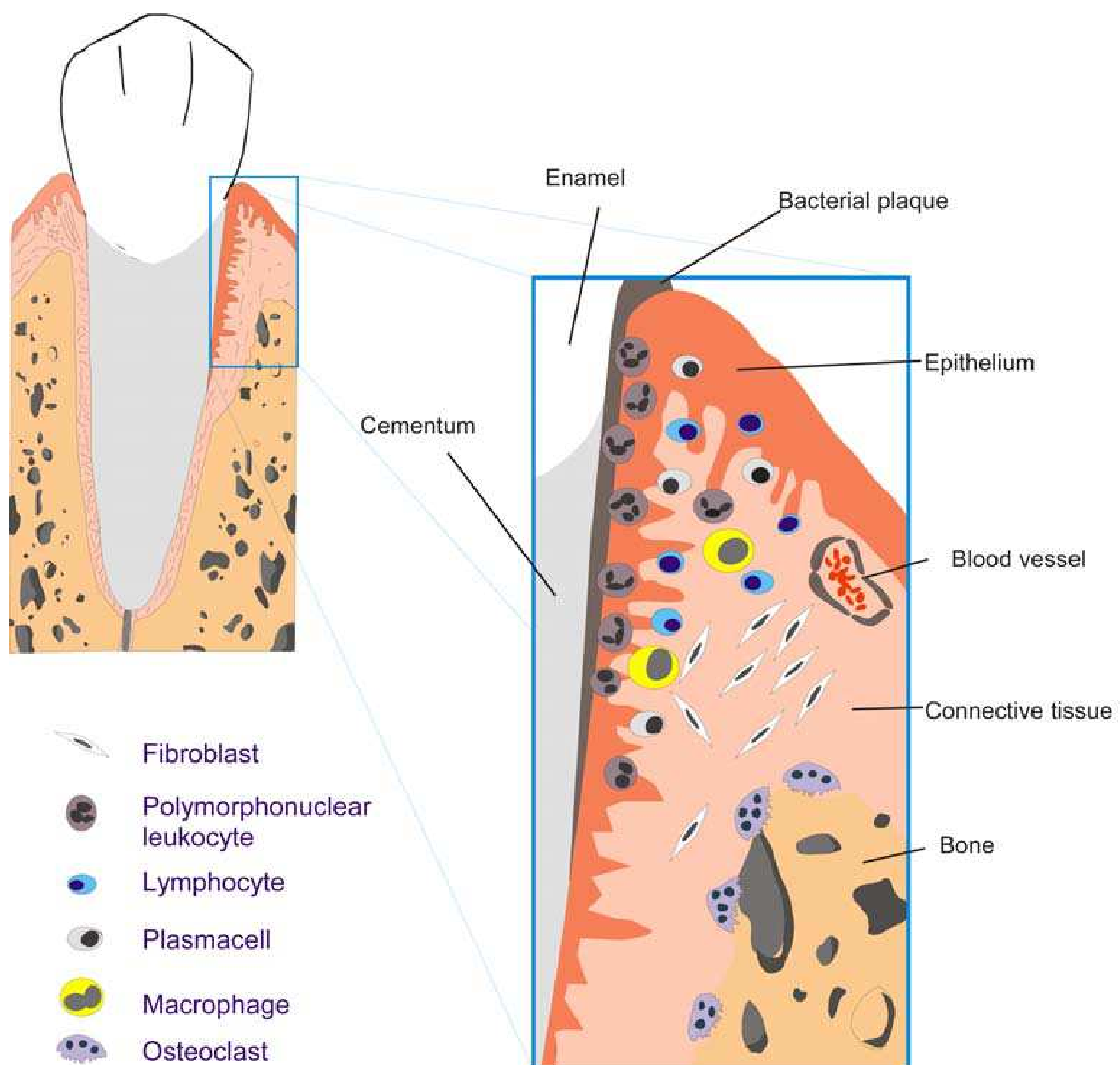

Fig. 1. Schematic illustration of cells and tissues involved in the pathogenesis of periodontal diseases. Microbes adhere to the epithelium and tooth surface and form a biofilm (microbial plaque). Persistent presence of this biofilm activates an inflammatory response in the surrounding tissues, which recruits a substantial number of immune cells from the peripheral circulation to the inflamed site. An imbalance in the host response might lead to degradation of the tooth supporting tissues, bone and connective tissue, and finally to tooth loss. A large number of microbial components, such as toxins and proteases that are released from the biofilm can affect the cellular response of the host.

2. *Aggregatibacter actinomycetemcomitans* and its association to disease

As mentioned, periodontitis is a chronic inflammatory condition in the periodontal tissues, which leads to periodontal attachment loss and destruction of the alveolar bone that houses the teeth. Based on clinical characteristics, several forms of periodontitis are recognized. Most prevalent is the slowly progressing chronic periodontitis while the so called aggressive form shows a more rapid tissue loss and often occurs in young subjects (Pihlstrom et al., 2005).

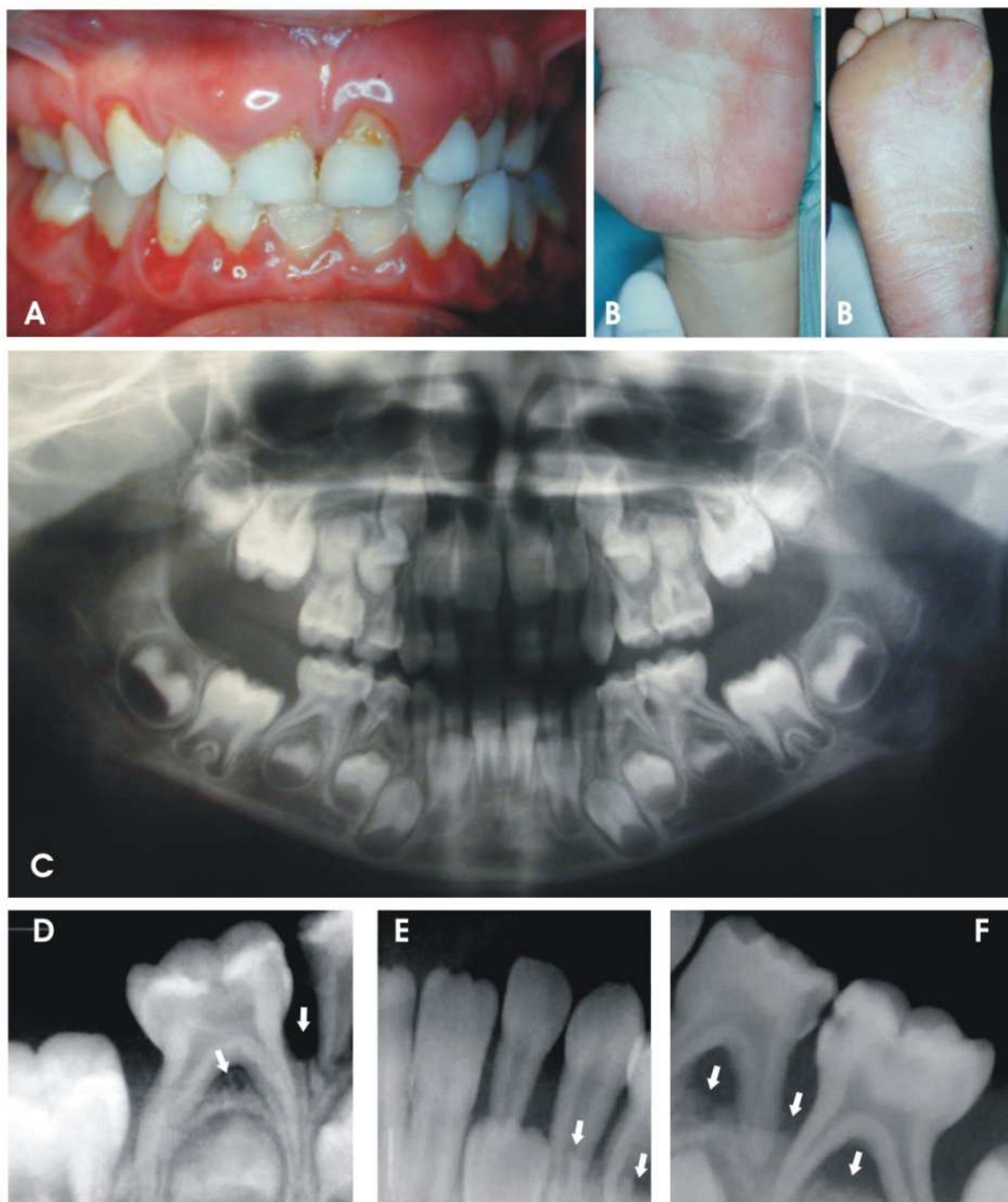

Fig. 2. Papillon-Lefèvre syndrome in a 6-year old child. A. Clinical manifestation of the extended periodontal inflammation affecting the primary dentition. B. Hyperkeratotic palmo-plantar skin lesions C-F. Radiographs showing the severe bone loss (arrows) around the teeth. Cultures of samples from the periodontal pockets revealed the rich presence (up to 70% of the total sample flora) of *A. actinomycetemcomitans.* (unpublished data of author SK).

Periodontitis can also be developed in conjuction with systemic diseases or medication and its severity depends on the underlying condition, the most severe forms being found in patients with disorders in the cellular defense, mostly in neutrophils, e.g. Papillo-Lefèvre syndrome, Kostmann syndrome, various neutropenias, and Chediak-Higashi syndrome (Fig. 2) (Reichart & Dornow 1978; Deasy et al. 1980; Deas et al. 2003; Tempel et al. 1972; Saglam et al. 1995; Defraia & Marinelli 2001; De Vree et al. 2000).

The etiology of periodontitis is microbial. The infection is caused in most cases by consortia of bacteria with a predominance of gram-negative anaerobic rods that colonize the periodontal pocket (Pihlstrom et al., 2005; Darveau 2010). Approximately 700 different bacterial species can be detected in samples from the subgingival plaque biofilm (Socransky & Haffajee 2005). The majority of these species can be isolated from samples of both healthy and periodontally diseased subjects. In plaque samples from diseased sites, the number and proportion of pathogenic organisms are elevated (Berezew & Darveau 2011; Nishihara & Koseki 2004).

In some forms of aggressive periodontitis *A. actinomycetemcomitans* is often found in high numbers in subgingival plaque samples from the affected tooth sites (Fine et al., 2007). Especially in patients with certain neutrophil disorders, *A. actinomycetemcomitans* is the main pathogen colonizing the periodontal area (Stabholz et al. 1995; Kleinfelder et al. 1996; Velazco et al. 1999; Pütsep et al., 2002). These epidemiological data indicate that establishment of *A. actinomycetemcomitans* in the periodontium as the main pathogen and development of inflammation depends on the lack of functional neutrophils in this area.

Besides the pathogens in the oral biofilm, genetic and environmental host factors contribute to periodontitis development (Nishihara & Koseki 2004). Furthermore, an association between periodontal infections and other inflammatory systemic diseases, such as cardiovascular diseases and diabetes, is well established, but the underlying specific mechanism is still unknown (Pihlstrom et al., 2005).

The prevalence of *A. actinomycetemcomitans* in subgingival plaque samples can be estimated by traditional culture methods, as well as by molecular (PCR-based) techniques (Fine et al., 2007). In an examined population, the proportion of *A. actinomycetemcomitans* positive individuals varies with periodontal status, age, ethnicity and geographic origin (Fine et al., 2007). Genetic differences of both patients and potential pathogens are of importance for a better insight into the etiology of periodontal diseases (Rylev & Kilian 2008). Longitudinal studies have shown that periodontally healthy children that harbour *A. actinomyctemcomitans* have an increased risk to develop Localized aggressive periodontitis (LAP) (Van der Velden et al., 2006; Fine et al., 2007). A specific clone (JP2) is strongly associated with LAP in subjects of African origin, and differs from other clones of this species by several genetic peculiarities, including a 530-bp deletion in the promotor region of the leukotoxin gene operon, which results in an enhanced expression of leukotoxin (Brogan et al. 1994; Haubek et al., 2007 & 2008). Healthy adolescents harbouring this clone were shown to have an 18-fold increased risk to develop periodontal attachment loss within a 2-year follow up period compared to the *A. actinomycetemcomitans* negative controls (Haubek et al., 2008). In contrast to the promoter deletion in the JP2, in a Japanese strain was shown that an insertion mutation increases the expression of the leukotoxin operon (He et al., 1999). However, no reports are yet available that associates the presence of this specific highly toxic clone with the onset and progression of aggressive periodontitis in this population.

3. Genetic characteristics of *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a member of the bacterial family *Pasteurellaceae* (Kilian et al., 2006). Recently, it was reclassified in the new genus *Aggregatibacter* together with its close relatives *Aggregatibacter* (*Haemophilus*) *aphrophilus* and *Aggregatibacter* (*Haemophilus*) *segnis* (Nørskov-Lauritsen & Kilian 2006). Molecular genetics has demonstrated a degree of biodiversity in the oral microflora and *A. actinomycetemcomitans* is genetically heterogeneous and comprises distinct clonal lineages that may have different virulence potentials (Kittichotirat et al., 2011). Seven serotypes have been identified among *A. actinomycetemcomitans* isolates; each serotype represents a distinct clonal lineage (Kaplan et al., 2002; Kilian et al., 2006; Takada et. al., 2010). The complete genome sequence of *A. actinomycetemcomitans* serotype b strain HK1651 from the JP2 clone has been available since 2002 (http://www.genome.ou.edu/act.html). Genome sequencing of 14 different strains from *A. actinomycetemcomitans* species have identified a pangenome consisting of 3301 genes, including 2034 core genes and 1267 flexible genes (Kittichotirat et al., 2011). The natural competence of this bacterium for horizontal gene transfer might explain the substantial genetic diversity shown within this species (Wang et al., 2002). The within-species variable virulence may be attributed to a strain-to-strain variation in genome content and regulation of virulence gene expression (Kittichotirat et al., 2011). Future work with genomic characterization of *A. actinomycetemcomitans* might contribute to identify specific virulent clones other than the already well characterized highly leukotoxic JP2 clonal types (Haubek 2010; Kittichotirat et al., 2011). In addition, population genetic analyses of this bacterium have been giving information about global dissemination of this species and its strict horizontal transfer pattern together with the presence of several genetic peculiarities give also information about population trades (Kilian et al., 2006; Habek et al., 2007).

4. Expression and secretion of *A. actinomycetemcomitans* leukotoxin

The leukotoxin operon consists of four coding genes designated *ltx*C, *ltxA, ltx*B and *ltx*D and an upstream promoter gene (Lally et al., 1989; Kraig et al., 1990). *ltxA* is encoding for the structure of the toxin, *ltx*C for components required for posttranslational acylation of the toxin and *ltxB* and *D* for transport of the toxin to the bacterial outer membrane. The leukotoxin operon is organized as illustrated in fig. 3, this pattern being similar to the gene organization found for other proteins of the RTX-family (Welch et al., 2001; Linhartová et al., 2010).

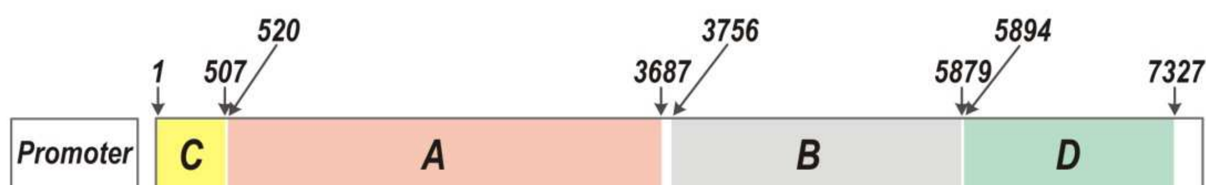

Fig. 3. Schematic illustration of the operon organization of *A. actinomycetemcomitans* leukotoxin.

There is a great variation in leukotoxin expression *in vitro* (Fig. 4), although all *A. actinomycetemcomitans* strains harbor a complete leukotoxin operon (Henderson et al., 2010).

Zambon and co-workers (1983) showed that *A. actionomycetemcomitans* isolated from periodontally diseased subjects exhibited significantly enhanced leukotoxicity compared with isolates from periodontally healthy subjects. Interestingly, certain clones of the bacterium with enhanced leukotoxin expression have been shown to have a modified promoter in the leukotoxin operon (Brogan et al., 1994; He et al., 1999). The cellular and molecular mechanisms in which a modified leukotoxin promoter enhances the expression of leukotoxin are not known. The most well known phenomenon is the highly leukotoxic JP2 clonal strains of *A. actinomycetemcomitans* characterized by a 530-bp deletion in the promoter of the leukotoxin operon (Haubek 2010). Hypertonic NaCl extracts of bacteria from this clone analyzed by SDS-PAGE and Comassie staining reveald a protein pattern that was dominated by a 116 kDa band shown to be the leukotoxin (Johansson et al., 2000a) (Fig. 6). Presence of the JP2 clone is highly associated to aggressive forms of periodontitis and shown to correlate with disease onset of adolescents in Morocco (Haubek et al., 2008). This highly leukotoxic clone (JP2) has recently been reported to also colonize subjects with, by genotyping confirmed, North-European origin (Claesson et al., 2011). Clonal diversity analysis of JP2-like isolates have shown that all strains of this clone have a common ancestor from Northern Africa (Haubek et al., 2007). The high accumulation of this clone in subjects of African origin has indicated a possible host tropism, but could also be a result of the strict vertical transmission pattern of this bacterium (Kilian et al., 2006; Haubek 2010).

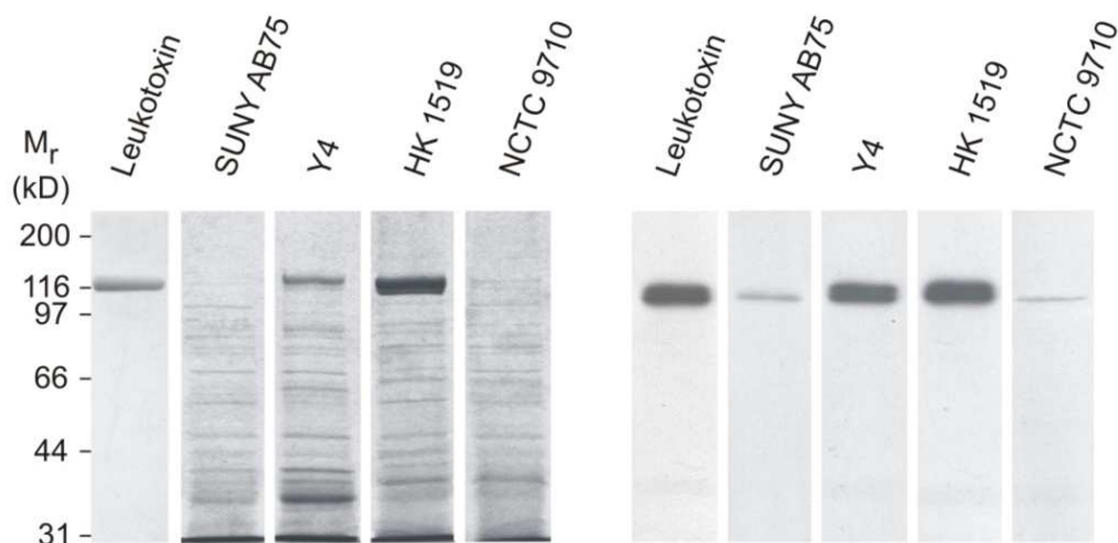

Fig. 4. The left figure shows NaCl extracts from different strains of *A. actinomycetemcomitans* separated on 8% SDS-PAGE and stained with Comassie blue. The left lane contain the purified leukotoxin and the other 4 lanes contain extract from 3 strains representing 3 different serotypes (SUNY AB75 serotype a, Y4 serotype b and NCTC 9710 serotype c) and having intact leukotoxin promoter and 1 strain serotype b from the highly leukotoxic JP2 clonal type (strain HK1519). The right figure shows the same SDS-PAGE separated extracts blotted on a PVDF membrane and visualized by western blot technique with a leukotoxin specific rabbit antiserum.

The expression of leukotoxin is also regulated by environmental factors, such as growth conditions and substrates (Kachlany et al., 2010). The expression of leukotoxin by various strains of *A. actinomycetemcomitans* at the infected site of the host is still unknown.

The expressed leukotoxin is transported to the bacterial outer membrane by a type I secretion system (Kuhnert & Christensen 2008). Three proteins, LtxB, LtxD and TdeA, are reported to be required for export of the toxin to the bacterial outer membrane and are organized in accordance to the figure below (Fig. 5) (Crosby & Kachlany 2007).

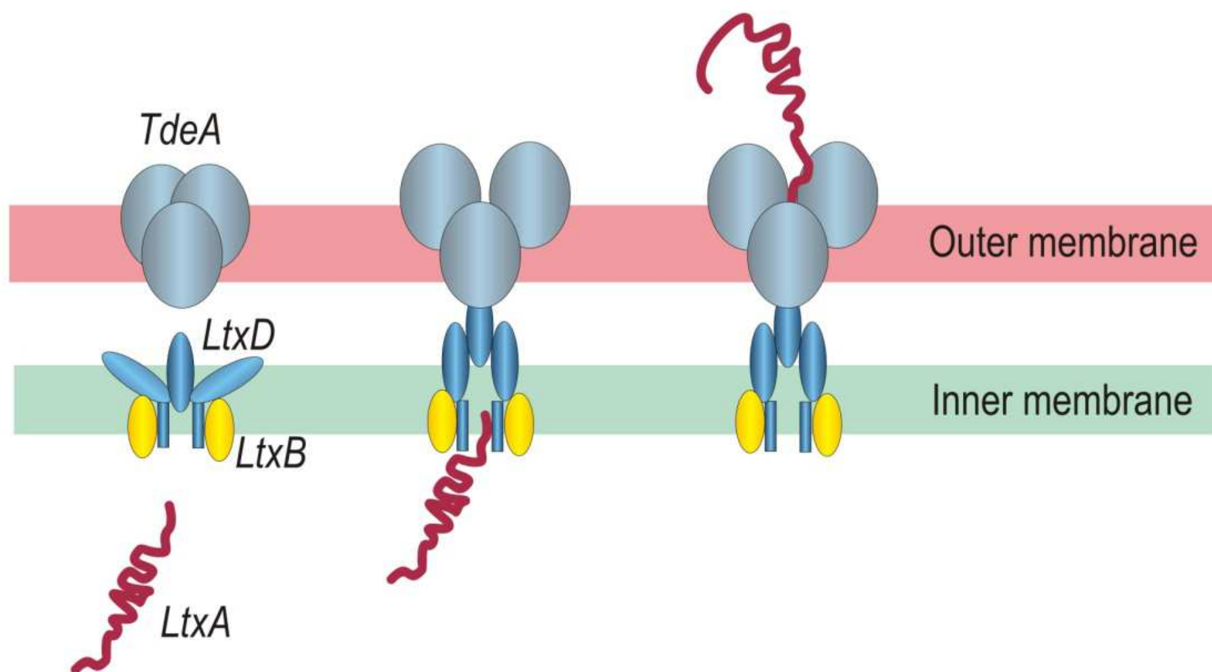

Fig. 5. Schematic illustration of the type I secretion system required for export of the expressed *A. actinomycetemcomitamns* leukotoxin to the bacterial outer membrane (OM). IM = Inner membrane and TdeA = a TolC like proten.

In addition, presence of serum proteins also mediates a similar release of the toxin from the bacterial outer membrane, which indicates involvement of competitive mechanisms (Johansson et al., 2003). Different culture conditions have been shown to determine the distribution of the expressed toxin between the bacterial outer membrane and the culture supernatant (Kachlany et al., 2000; 2010). Whether leukotoxin remains associated to the bacteria in the periodontal pocket is not known. However, the serum mediated release of the toxin (Johansson et al., 2003), as well as the intense systemic immune response to the toxin (Brage et al., 2011), possibly indicate a release of the toxin from bacteria in the biofilm *in vivo*. Among the different proteins of the RTX family, *A. actinomycetemcomitans* leukotoxin differ from the other toxins by its high isoelectric point, as well as the membrane association of the expressed protein (Welch 2001; Linhortavá et al., 2010). This property of leukotoxin further supports the importance of electrostatic forces for its association to the bacterial outer membrane.
The secreted leukotoxin has been shown to be easily inactivated by environmental proteases and superoxide radicals (Johansson et al., 2000b, 2001; Balashova et al., 2007). This degradation of the toxin molecule can be inhibited by the presence of superoxide dismutase

(SOD) produced by *A. actinomycetemcomitans* and the naturally occurring protease inhibitors of human serum (Johansson et al., 2001; de Haar et al., 2006; Balashova et al., 2007). In 1981, McArthur and co-workers showed that the activity of leukotoxin in interaction with polymorphonuclear leukocytes (PMNs) was enhanced by the presence of human serum (McArthur et al., 1981). This phenomenon could later be explained by the protective effect of the serum protease inhibitors on leukotoxin degradation caused by lysosomal enzymes released by the affected PMNs (Johansson et al., 2001).

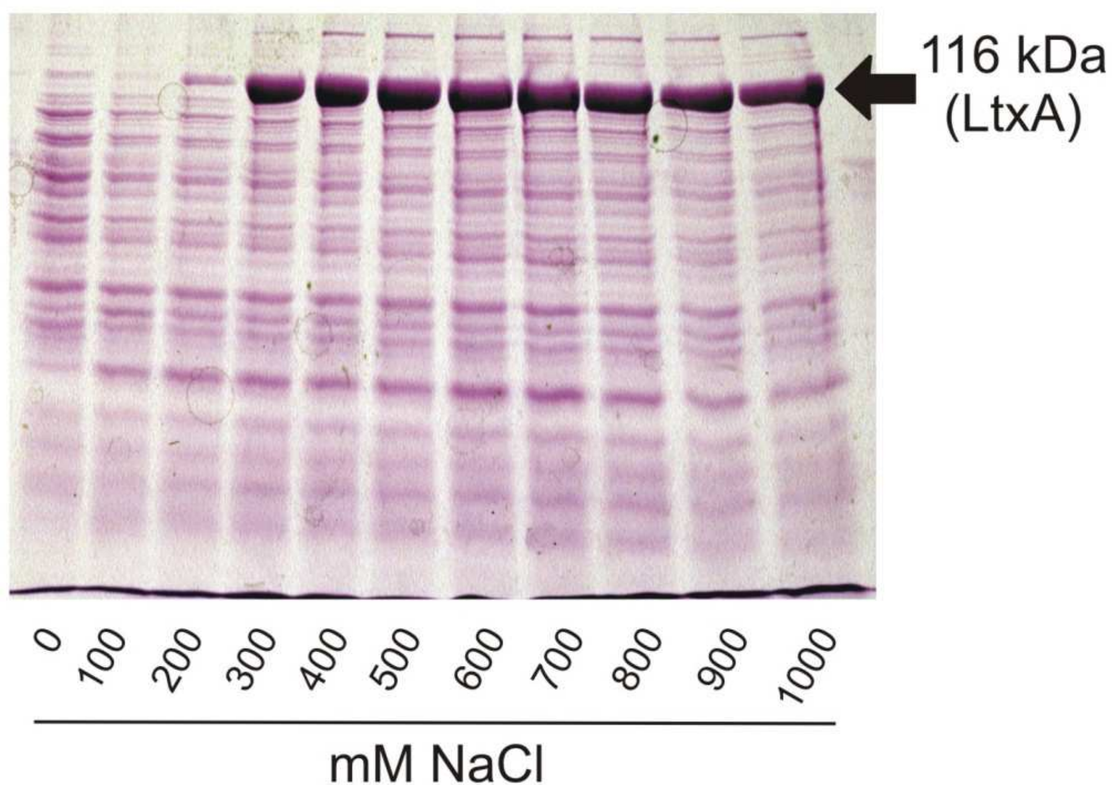

Fig. 6. Surface extract of bacteria from a highly leukotoxic (JP2) strain of *A. actinomycetemcomitans* (HK1619) separated by SDS-PAGE and stained with Comassie blue. The 116 kDa leukotoxin is released from the bacterial surface at NaCl concentration ≥300 mM, becoming the dominant band in the protein profile of the extracts (Johansson et al., 2000a).

5. Molecular aspects of *A. actinomycetemcomitans* leukotoxin

5.1 Structure

Leukotoxin (LtxA) expressed by *A. actinomycetemcomitans* is a large pore-forming protein that belongs to the RTX family of proteins. Leukotoxin consists of 1055 amino acids encoded by the leukotoxin gene in the leukotoxin operon (Lally et al., 1989; Kraig et al., 1990). The molecule can be divided into four regions based upon analysis of the amino acid sequence, the N-terminal region, the central region, the repeat region and the C-terminal region (Fig. 7) (Lally et al., 1996).

The N-terminal region at residues 1-408 exhibits alternating hydrophobic and hydrophilic clusters. The pore-forming regions of RTX proteins have been suggested to be mediated by

the hydrophobic clusters located between residues 175-400 (Welch et al., 2001). The central region at residues 409-729 contains large hydrophilic domains and the two acylation sites of leukotoxin located at $lysine_{562}$ and $lysine_{687}$ (Balashova et al., 2009). The fatty acids at these positions have been shown necessary for the activity of the toxin and suggested to contribute to the anchorage at the target cell membrane. The repeat region consists of tandem repeats of a cassette with nine amino acids located between residues 730-900 and 14 such repeats have been identified in this region of leukotoxin (Stanley et al., 1994). The target cell receptor LFA-1 binds to the repeat region and this interaction has been shown to determine the host cell specificity of leukotoxin (Stanley et al., 1994; Lally et al., 1994). In addition, the glycine rich repeats in this region have a strong capacity to bind Ca^{2+} and presence of these cations mediates increased binding of the toxin to leukotoxin-sensitive LFA-1 expressing cells (Lally et al., 1997). Finally, residues 901-1055 at the C-terminal end have been shown to be needed for export of the toxin to the bacterial outer membrane by interactions with secretory proteins (Stanley et al., 1991; Sato et al., 1993). This region of leukotoxin contains 20 additional basic amino acid residues, which differs the leukotoxin from the other RTX-proteins and confers its high isoelectric point (9.7) (Kraig et al., 1990). The four regions of leukotoxin described above are shared among the various toxins in the diverse family of pore forming RTX proteins but their amino acid sequence homology is limited to about 40-50%, with the highest homology between their repeat regions and the lowest between their C-terminal regions (Kraig et al., 1990). A partial denaturation of the leukotoxin molecule has been reported to enhance its leukotoxicity, which indicates that conformational changes affect the activity of the toxin (Lear et al., 2000). Some minor differences have been identified on the leukotoxin genes between different *A. actinomycetemcomitans* isolates but whether these differences interfere with leukotoxicity is not known (Lally et al., 1989; Kraig et al., 1990; Chen et al., 2009, Kittichotirat et al., 2011). The crystalline structure of leukotoxin has not yet been solved, which limits the available information about the molecular structures of the protein.

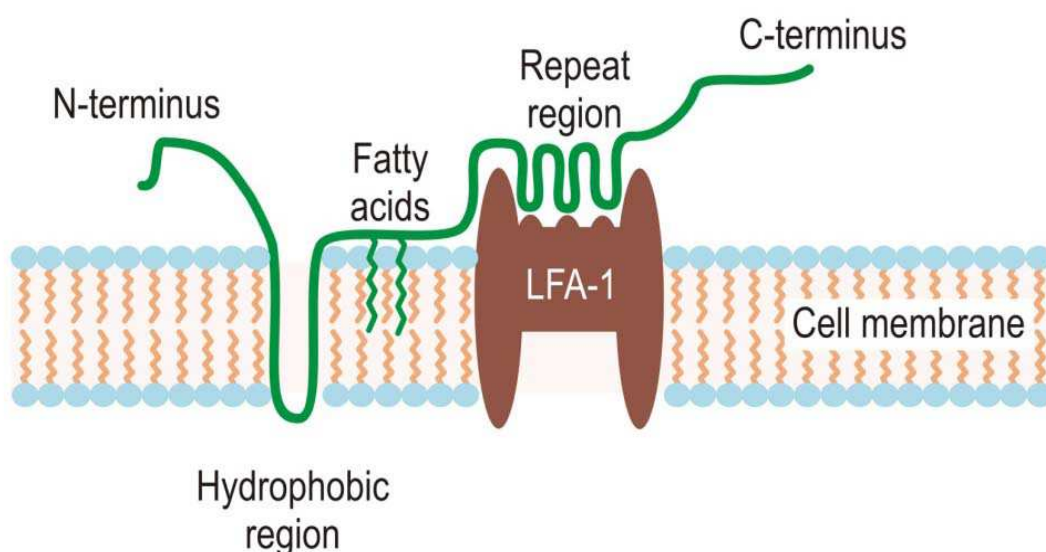

Fig. 7. Schematic illustration of the molecules involved in the interaction between *A. actinomycetemcomitans* leukotoxin and the target cell membrane.

5.2 Interaction with the target cell membrane

Leukotoxin exhibits a unique specificity to cells of haematopoetic origin from humans and some other primates (Lally et al., 1994). This restricted host cell specificity indicates that the species-specific effect of leukotoxin is mediated through a unique receptor on the target cells and that a precise region in the toxin recognizes and interacts with the receptor (Dileepan et al., 2007; Kieba et al., 2007). The principal feature of this species recognition region of leukotoxin is a series of 14 tandemly repeated amino acid sequences in the repeat region of the toxin (Stanley et al., 1994).

The leukotoxin has been shown to bind to surfaces of toxin-sensitive LFA-1-expressing cells, as well as toxin-resistant cells without LFA-1 expression (Lally et al., 1997). It has been suggested that the role of LFA-1 in leukotoxin-mediated cell lysis is to help the protein to have a correct orientation on the target cell membrane (Fig. 7) (Lally et al., 1999). Further, the two fatty acids strengthen the anchorage of the toxin when inserted in the target cell membrane and the hydrophobic domain forms small pores in the membrane. It has been stated that low concentration of the toxins might induce apoptosis through loss of membrane integrity caused by the small pores and that higher concentration of the toxin allows oligomerization of leukotoxin-LFA-1 complexes on the target cell membrane mediating a rapid and complete membrane collapse (Lally et al., 1999). In addition, leukotoxin has been shown to require lipid rafts for target cytotoxicity, which also indicates the importance of a high mobility on the target cell membrane (Fong et al., 2006).

The domain of leukotoxin that recognizes the target cell receptor has been determined to residues 688-941 examined by epitope mapping with monoclonal antibodies (Stanley et al., 1994). The LFA-1 molecule identified as the leukotoxin target cell receptor is a heterodimer consisting of the α_L (CD11a) and β_2 (CD18) subunits. The residues 1-128 on human CD11a has been shown to determine the human specificity of leukotoxin-induced cell lysis (Kieba et al., 2007). In addition, the extracellular region of human CD18 (residues 500-600) has been shown critical for conferring susceptibility to leukotoxin-induced cell lysis (Dileepan et al., 2007). The most important ligand of LFA-1 is the intercellular adhesion molecule 1 (ICAM-1), but this interaction does not coincide with the residues identified for leukotoxin binding (Dileepan et al., 2007; Kieba et al., 2007, Dustin et al., 2004). This finding indicates that the intracellular signaling mediated by the LFA-1 ligand binding is not activated by the leukotoxin binding. Three different affinity states (low, intermediate, high) of LFA-1 that interfere with ligand binding have been described (Shimaoka et al., 2003). If these different affinity states of the leukotoxin receptor interfere with the interactions between leukotoxin and its target cells is not known.

6. Cellular and molecular host response against *A. actinomycetemcomitans* leukotoxin

The ability of *A. actinomycetemcomitans* extracts to cause death of leukocytes was first shown more than 30 years ago (Baehni et al., 1979; Tsai et al., 1979). A protein named leukotoxin was indentified as the responsible molecule for this effect that was restricted to affect human PMNs and monocytes (Baehni et al., 1979; Tsai et al., 1979; Taichman et al., 1980). It has later been shown that leukotoxin also can affect human lymphocytes and erythrocytes from human and animal origin, however, at higher toxin concentrations than those lysing

PMNs and monocytes (Mangan et al., 1991; Balashova et al., 2006). Even though leukotoxin affects all subsets of human immune cells it is highly immunogenic and induces a specific acquired systemic immune response in the infected host (Ebersole 2003; Brage et al., 2011).

6.1 Acquired humoral immune response against leukotoxin

The specific role of humoral immunity in periodontal disease progression has not been fully elucidated, although the production of antibody response is suggested to be beneficial to the host in fighting periodontal infections (Ebersole 2003). On the other hand, the acquired immune response against periodontal pathogens has been shown to mediate disease associated mechanisms, such as bone resorption (Taubman et al., 2005; Teng 2003). It has clearly been shown that leukotoxin specific antibodies are present in the peripheral circulation of both periodontally healthy and diseased subjects (Kachlany et al., 2000; Califano et al., 1997). Plasma samples from the subjects with specific immunoreactivity against leukotoxin have been shown to neutralize leukotoxin activity and have also enhanced antibody titers against whole cells of *A. actinomycetemcomitans* in comparison with samples from subjects without immunoreactivity to leukotoxin (Califano et al., 1997). It has also been shown that systemic leukotoxin neutralization is correlated to the presence of this bacterium in the subgingival plaque (Källestål et al., 1991; Sjödin et al., 1995; Carlsson et al., 2006). The prevalence of systemic leukotoxin antibodies has been shown to be present in >50% in samples from adults and without significant differences in relation to periodontitis (Brage et al., 2011; Johansson et al., 2011). Interestingly, systemic leukotoxin neutralization correlates to decreased risk of the incidence of stroke in woman (Johansson et al., 2005). The mechanism behind this phenomenon has not yet been determined but a possible role of leukotoxin is suggested in the association seen between peridontitis and cardiovascular diseases (Pihlstrom et al., 2005).

A general opinion is that the humoral immune response against antigens of the oral subgingival microbiota is both local and systemic (Ebersole 2003). Whether the leukotoxin activity can be neutralized in the gingival pocket by specific antibodies is not known and there has been no report about the presence of leukotoxin neutralizing antibodies in the gingival crevicular fluid. The strong correlation between prevalence of higly leukotoxic *A. actinomycetemcomitans* and the development of attachment loss (Haubek et al., 2008) indicates a minor role of neutralizing antibodies in the infected periodontal pocket. However, it can be assumed that systemic leukotoxin neutralizing antibodies are an important protection against the systemic side effects, such as increased risk for diabetes and cardiovascular diseases that are associated with periodontitis. It has previously been shown that systemic antibodies against leukotoxin completely neutralize its activity even at high dilutions of the positive sera (Brage et al., 2011). In addition, the systemic leukotoxin-neutralizing capacity negatively associates to stroke while the systemic immunoreactivity to *A. actinomycetemcomitans* also shows a negative association to rheumatoid arthritis (Johansson et al., 2005; Okada et al., 2011). The mechanism behind these negative associations has not been elucidated. It could be speculated that the ability of leukotoxin to specifically affect the immune cells, in particular the antigen presenting monocytes/macrophages, causes a delayed acquired immune response in a primary *A. actinomycetemcomitans* infection.

6.2 Polymorphonuclear leukocytes

PMNs are the first defense cells to be recruited in the acute phase of an inflammation, as in a periodontal infection (Kantarci & van Dyke 2005). These defense cells are often found at high numbers in the infected periodontal pocket, attracted from the peripheral circulation through chemotaxis towards a gradient of molecules released from the dental plaque, as well as activated host cells. Although PMNs are crucial for phagocytizing and killing bacteria, they also release substances that mediate tissue destruction in aggressive forms of periodontitis (Kantarci et al., 2003). PMNs in the periodontium have been described as a "double-edged sword", capable of producing periodontal disease as well as protecting against the disease (Lamster et al., 1992). Leukotoxin as well as leukotoxic bacteria have been shown to efficiently cause death of human PMNs and consequently the leukotoxin is assumed to protect *A. actinomycetemcomitans* against phagocytic killing (Henderson et al., 2010). The protection occurs in relation to the leukotoxin production of the bacterial population (Johansson et al., 2000c). In a mixture of low-leukotoxic bacteria, human serum and PMNs (25 bacteria/PMN), which is agitated at 37°C under anaerobic condition, the bacteria are efficiently phagocytized and killed (Johansson et al., 2000c). In contrast, in the presence of highly leukotoxic (JP2-clone) bacteria and under the same conditions, the PMNs failed to phagocytize and kill the bacteria. Transmission electron microscopy pictures of the exposed PMNs showed a peripheral translocation of the granules in cells exposed to the highly leukotoxic bacteria (Fig. 8). Further analysis of PMNs exposed to leukotoxin showed an extracellular release of proteolytic enzymes from both primary and secondary granules (Johansson 2000b). More over, the interaction between leukotoxin and PMNs mediates activation and release of matrix metalloproteinase 8 (Claesson et al., 2002). Taken together these findings indicate that leukotoxin before causing death of the PMNs induces activation and release of proteolytic enzymes from these cells, which can contribute to periodontal tissue destruction.

Whether leukotoxin can exist as a biologically active protein in the infected periodontal pocket has not yet been examined. The presence of serum proteins and the relatively high pH (≈8) in the pocket indicates that leukotoxin is released from the bacterial surface in this ecological niche (Kraig et al., 1990; Johansson et al., 2003). The released toxin is an easy target for inactivation by several of the components present in the periodontal pocket, such as superoxide radicals and proteinases released from the host defense cells or the colonizers of the subgingival biofilm (Johansson et al., 2000a, 2003; Balashova et al., 2007). In addition, systemic leukotoxin specific antibodies neutralize leukotoxic activity, but if these antibodies are functional in the environment of the infected periodontal pocket is not known (Brage et al., 2011). There are also molecules that can protect leukotoxin from inactivation, such as the serum proteinase inhibitors and SOD expressed by *A. actinomycetemcomitans* (Johansson et al., 2001; Balashova et al., 2007). Probably, the great variation over time in the balance between these factors and the leukotoxin produced at a site of infection affects the progression of periodontal destruction.

As mentioned above, impaired PMN function is closely associated with periodontitis and functional PMNs seem to be of certain importance for combating *A. actinomycetemcomitans* establishment in the subgingival biofilm (de Haar et al., 2006; Kantarci et al., 2003; Carlsson et al., 2006; Pütsep et al., 2002). For instance, PMNs of subjects with Kostmann´s syndrome are immature and expresses truncated LL37, a cathelicidin with antibacterial effect against

A. actinomycetemcomitans (Pütsep et al., 2002). Furthermore, subjects with Papillon-Lefèvre syndrome have truncated PMN serine proteases, this causing an enhanced leukotoxin sensitivity due to impaired capacity to degrade extracellular leukotoxin by the released lysosomal PMN enzymes (de Haar et al., 2006).

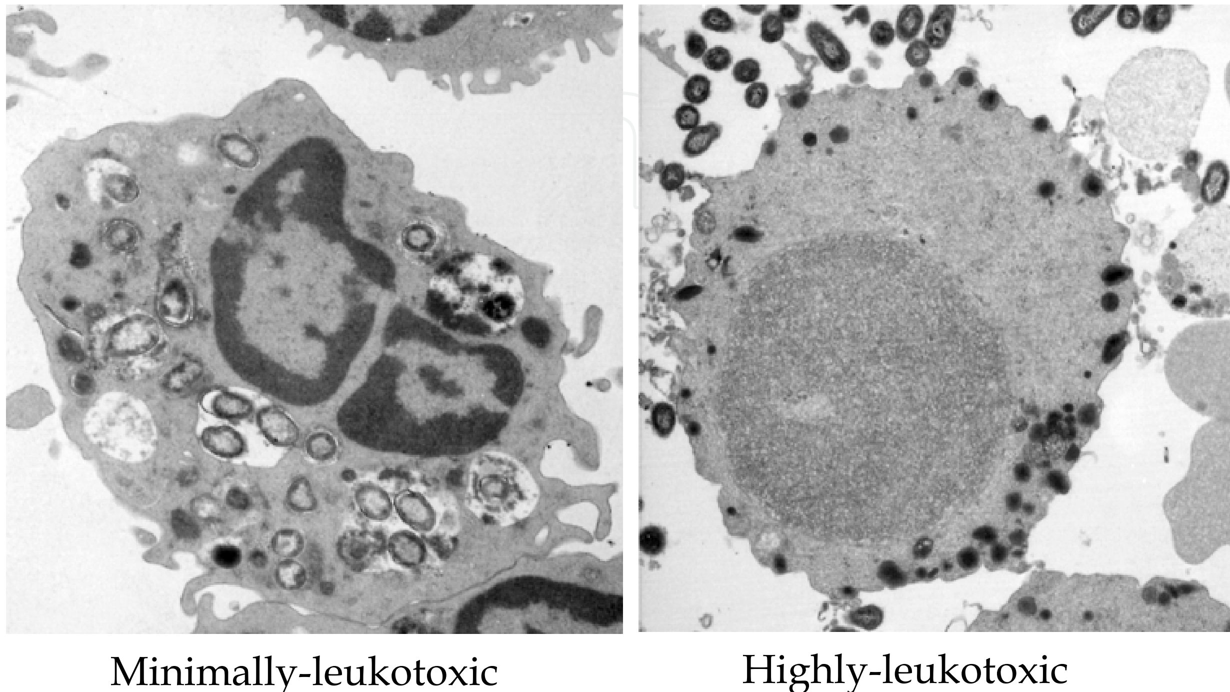

Fig. 8. PMNs exposed to alive *A. actinomycetemcomitans* (25 bacteria/PMN) for 10 min in the presence of 10% human non-immune sera. The mixture was gently agitated at 37°C in an anaerobic atmosphere. The minimally leukotoxic bacteria are phogocytized and killed by the PMN, while the highly leukotoxic bacteria (JP2) resist PMN phagocytosis and causes extracellular release of lysosomal components (Johansson et al., 2000c).

6.3 Lymphocytes

The lymphocytes were initially described as leukotoxin resistant cells (Baehni et al., 1979; Tsai et al., 1979). The first observation of leukotoxin susceptible cells of lymphocytic origin was by Simpson and co-workers (Simpson et al., 1988) who showed that several lymphoid cell lines were killed in the presence of leukotoxin. In addition, leukotoxin was shown to suppress function of peripheral blood lymphocytes (Shenker et al., 1994). A few years later, Mangan and co-workers (1991) showed that T-cells isolated from human peripheral blood were affected by leukotoxin. This leukotoxin-induced T-cell death was a slow process compared to the lysis of human cells of myeloid origin, the death being caused through apoptosis (Mangan et al., 1991). Human natural killer (NK) cells are affected by leukotoxin in a similar way as the T-cells, while the effects of leukotoxin to human B-cells or plasma-cells have not been studied (Shenker et al., 1994). Human lymphocytes show a great heterogeneity in regard to leukotoxin sensitivity and a subgroup of these cells are shown to be lysed at approximately the same concentrations as human PMNs (Kelk et al., 2003). The

lymphocytes with different leukotoxin sensitivity was not further characterized in this study but analysis of CD11a expression on the cell membrane showed a heterogenic distribution pattern for this cell population. The reason for the variation in leukotoxin sensitivity between PMNs and lymphocytes is not known. The suggested oligomerization of leukotoxin-LFA-1 complex and the need of lipid rafts on the target cell membrane may indicate that the composition of membrane molecules on the target cells determines the source of leukotoxin-induced death mechanisms (Lally et al., 1999). In cells from the human myeloid carcinoma cell line HL-60, low concentrations of leukotoxin cause apoptosis while higher concentrations lead to necrosis (Korostoff et al., 1998).

Cells of lymphoid origin are rare in the infected periodontal pocket but they reside at high numbers in the surrounding tissues as well as in the lymph glands (Kinane et al., 2002). It has been known for >30 years that the development of periodontitis involves a switch from a T cell lesion to one involving large numbers of B-cells and plasma cells. A shift in the balance between Th1 and Th2 subsets of T-cells is found in periodontal inflammation, with the Th2 cells to associate with chronic periodontitis (Ohlrich et al., 2009). More recently, T regulatory (Treg) and Th17 cells have been detected in periodontal tissues indicating that these cells also are of importance in the host response and pathogenesis of periodontal disease (Garlet 2010). The strong humoral immune response induced by leukotoxin indicates direct contact between this molecule and cells of lymphoid origin (Brage et al., 2011; Califano et al., 1997). The ability of leukotoxin to induce apoptosis in lymphocytes might impair the acquired immune response of periodontal infections. The ability of leukotoxin to affect the lymphocytes also indicates a possible role of this molecule in Th1/Th2/Th17 differentiation, a process that seems to be of importance in the pathogenesis of inflammatory diseases.

6.4 Monocytes/macrophages

It was early shown that human monocytes were as sensitive to leukotoxin, as human PMNs (Tsai et al., 1979). Killing of monocytes by the toxin proceeds through three distinct phases 1) cessation of the membrane undulating folding and an accumulation of granulae in the perinuclear area, 2) abnormal membrane movement and strings of cytoplasm projecting from the cell, and 3) explosive release of cytoplasmic material from the cells (Taichman et al., 1980). However, it should be taken into consideration that these studies were made with a crude leukotoxin extract that contained a lot of other bacterial components. Rabie and co-workers (Rabie et al., 1988) showed that purified leukotoxin caused a rapid death of human monocytes in mixtures of the toxin with peripheral blood mononuclear leukocytes (MNL).

More recently, analyses of different subsets of leukocytes separated from peripheral blood of a single donor showed that monocytes had an enhanced sensitivity to leukotoxin compared to PMNs and lymphocytes (Kelk et al., 2003). The leukotoxin-induced monocyte lysis was shown to involve activation of caspase-1, which indicates involvement of pro-inflammatory intracellular signalling (Fig. 9). Caspase-1 is a cytosolic cysteine proteinase that specifically induces activation and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Dinarello, 2009a, 2010). The two cytokines are expressed as biologically inactive precursors and have to be cleaved by caspase-1 for activation and secretion. Caspase-1 is activated by incorporation in a cytosolic multimer

complex named the inflammasome (Latz 2010). The intracellular signalling pathways involved in leukotoxin-induced inflammasome activation in human monocytes/macrophages have not yet been determined. A partial characterization of this process indicates involvements of activation of the purogenic receptor $P2X_7$ (Kelk et al., 2011). Caspase-1 activation is also caused by several other gram-negative pathogens, such as *Salmonella* and *Shigella* species, and has been shown to be an important innate immune effector mechanism against intracellular bacteria (Miao et al., 2010).

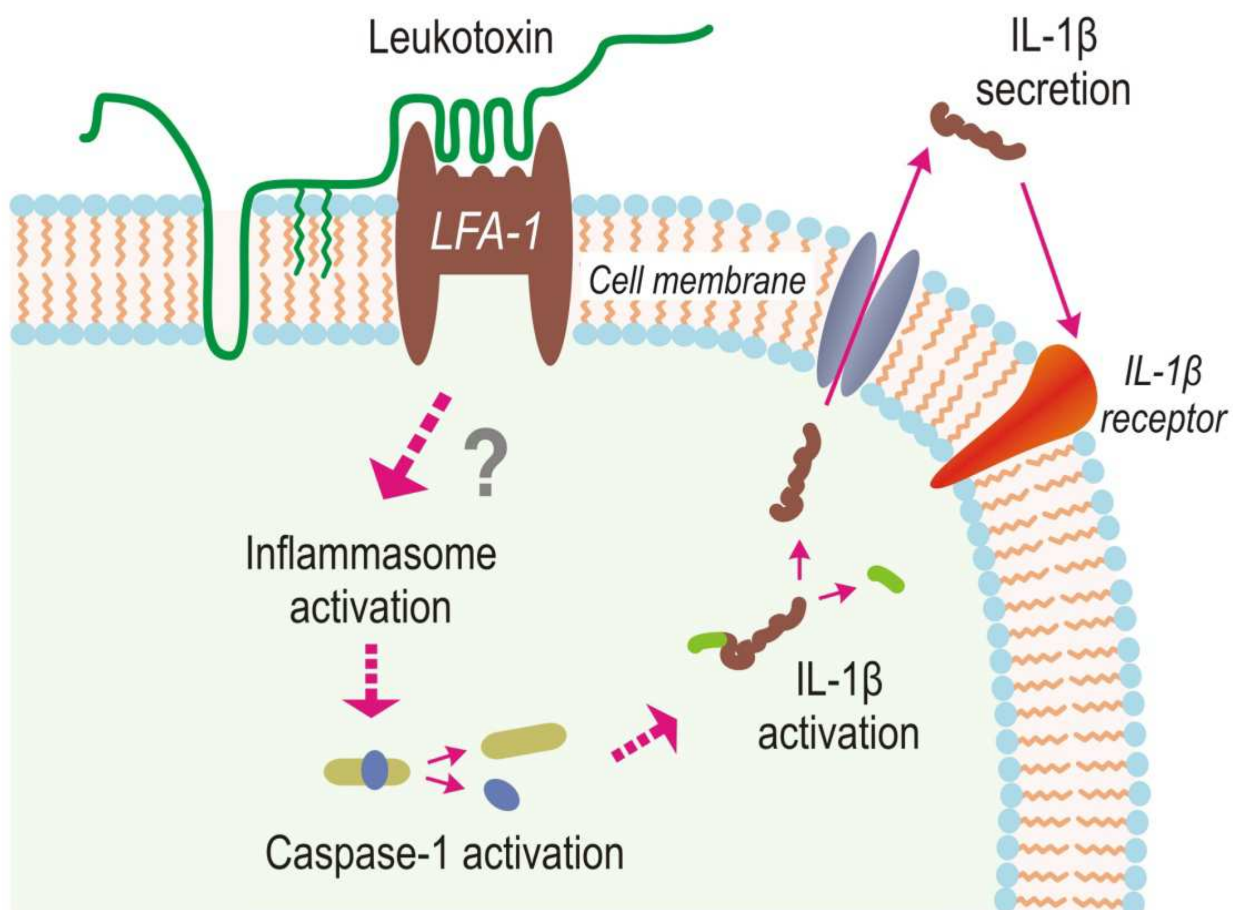

Fig. 9. Schematic illustration of the leukotoxin interaction with macrophages. Leukotoxin adheres to the cell membrane through binding to the LFA-1 dimer and it further anchors by inserting the fatty acid into the membrane lipid bilayer. The hydrophobic domain of the toxin is thought to cause small pores in the cell membrane. Through still undefined intracellular signalling pathways, this interaction with the target cell causes activation of caspase-1 and IL-1β that is secreted in a bioactive form from the affected cell.

IL-1β is a key component involved in acute and chronic inflammation, which makes the discovery of the leukotoxin induced IL-1β activation relevant and important (Kelk et al., 2005; Dinarello, 2011). IL-1 is an important regulator of bone resorption, which associates this cytokine to the alveolar bone loss seen in periodontitis (Schett, 2011; Dinarello, 2011;

Preshaw & Taylor, 2011). Analysis of gingival exudates have shown increased IL-1 concentration associated to periodontitis (Reinhardt et al., 2010; Preshaw & Taylor, 2011). Periodontal pockets examined from the same subject have indicated an association between high levels of *A. actinomycetemcomitans* and increased IL-1β concentration (Kelk et al., 2008).

The finding that leukotoxin induces activation of caspase-1 in human inflammatory defence cells indicates a new role of this virulence factor as a mediator of pro-inflammatory host response. Human macrophages (adherent blood monocytes) exposed to leukotoxin activates a rapid and abundant secretion of bioactive IL-1β (Kelk et al., 2005). Culture supernatants of leukotoxin-exposed macrophages activate bone resorption in cultured mouse calvaria, while presence of monoclonal antibodies against IL-1β abolishes this activation. This data indicate that bone resorption caused by culture supernatants of leukotoxin-exposed macrophages is mainly caused by released IL-1β. Moreover, exposure of human macrophages to components of gram-negative oral pathogens causes an increased accumulation of cytosolic pro-IL-1β that was not activated and released (Kelk et al., 2008). Leukotoxin or leukotoxic *A. actinomycetemcomitans* activates cleavage and secretion of this accumulated macrophage IL-1β, while *A. actinomycetemomitans* mutants without leukotoxin expression fail to cause this phenomenon. IL-1β secretion was activated already at a ratio of 1 bacterium/macrophage when using a highly leukotoxic *A. actinomycetemcomitans* strain or at a 10 times higher ratio when strains with low leukotoxicity were used (Kelk et al., 2008). Taken together, these data show that leukotoxin is the major *A. actinomycetemcomitans* component that induces activation and release of IL-1β from human macrophages and that this effect is further enhanced by priming the macrophages with other bacterial components.

Macrophages are rare cells in the healthy periodontium but are often found in high numbers in tissues from periodontal lesions (Kinane & Lappin, 2002). These cells are recruited to the infected site from the peripheral blood monocytes that are attracted by ICAM-1 expressing endothelial cells. The monocytes are passing through the vessel wall and are migrating in the connective tissue towards a gradient of chemoattractants that are released from the biofilm and the activated host cells (Geissmann et al., 2010). During diapedesis the monocytes differentiate into macrophages with a concomitant up-regulation of their inflammatory machinery, which continues during the migration. This process involves an accumulation of pro-inflammatory precursor molecules, such as IL-1β and IL-18, in the migrating macrophages (Dinarello, 2009b). A secondary stimulus is needed to induce activation and release of the accumulated precursors of IL-1β and IL-18 in the primed macrophages (Dinarello, 2010). In the case of an infection containing *A. actinomycetemcomitans,* the gradient of bioactive components in the connective tissue will contain leukotoxin, and the migrating macrophages will sooner or later meet leukotoxin concentrations that activate secretion of cytokines in the surrounding tissues. If this process is activated in the tooth supporting tissues in vicinity to the infection it might cause imbalance in the host inflammatory response and it might promote pathogenic cellular mechanisms. Some preliminary results indicate an association between enhanced IL-1β levels in gingival crevicular fluid and high number of *A. actinomycetemcomitans* in the periodontal pocket (Kelk et al., 2008). Interestingly, recent results suggest that IL-1β is transported into the *A. actinomycetemcomitans* cells and binds to the trimeric form of

intracellular ATP synthase subunit β (Paino et al., 2011). This specific mechanism might universally enhance biofilm resistance to host defence by binding IL-1β during inflammation.
The strong systemic immune response of the host to leukotoxin in *A. actinomycetemcomitans* infected subjects indicates direct contact between the antigent-presenting macrophages and leukotoxin (Califano et al., 1997; Brage et al., 2011). The enhanced leukotoxin-sensitivity of human macrophages indicates that these antigen presenting cells might be affected during a primary infection with leukotoxic *A. actinomycetemcomitans,* which might cause a delayed acquired immune response.
The pro-inflammatory response associated to degenerative diseases is at focus of research in many different medical disciplines (Dinarello, 2010). A variety of safe and effective anti-inflammatory agents are available today and commonly used in treatments of many autoimmune or auto-inflammatory disorders, neurodegenerative disease, or cancer. Increased knowledge of the cellular and molecular mechanisms involved in the pathogenesis of periodontitis might open up possibilities for new specific therapeutic agents and strategies in the future. The cellular and molecular targets for specific blockage of the inflammatory response to infection, as well as the possible therapeutic agents now and in the future, have recently been extensively reviewed (Dinarello, 2011).

6.5 Erythrocytes

The ability of some strains of *A. actinomycetemcomitans* to cause β-hemolysis on blood agar plates has been known for many years (Kimuzuka et al., 1996). Later, it was found that red blood cell lysis caused by *A. actinomycetemcomitans* involved an interaction with leukotoxin (Balashova et al., 2006). Different strains of the bacterium with various expressions of leukotoxin show a specific pattern when cultured on blood agar plates containing fresh horse blood. Red blood cells lack the receptor LFA-1, a key molecule for leukotoxin-induced leukocyte lysis (Lally et al., 1994). The cellular and molecular mechanisms for the hemolytic effect of leukotoxin are unknown. The lysis of erythrocytes by the leukotoxin has recently been reviewed (Kachlany 2010). It remains to be answered whether the hemolytic capacity of leukotoxin is an important virulence factor in periodontitis.

7. Conclusions

The ability of leukotoxin to cause death of all subsets of cells with hematopoetic origin might contribute to help the bacterium to survive the host immune response and also to release compounds essential for bacterial growth (Fig. 10). The more recent discoveries that leukotoxin mediates activation and release of proteolytic enzymes from PMNs and pro-inflammatory cytokines from monocytes/macrophages indicate a more direct role of leukotoxin in the disease pathogenesis. Unfortunately there is no animal model available for studying the virulence mechanisms of leukotoxin because of its specificity against defense cells of human or old world monkey origin. However, the strong correlation between presence of highly leukotoxic (JP2-clone) *A. actinomycetemcomitans* and development of attachment loss in adolescents indicates an important role of leukotoxin in the pathogenesis of aggressive periodontitis (Haubek, 2010).

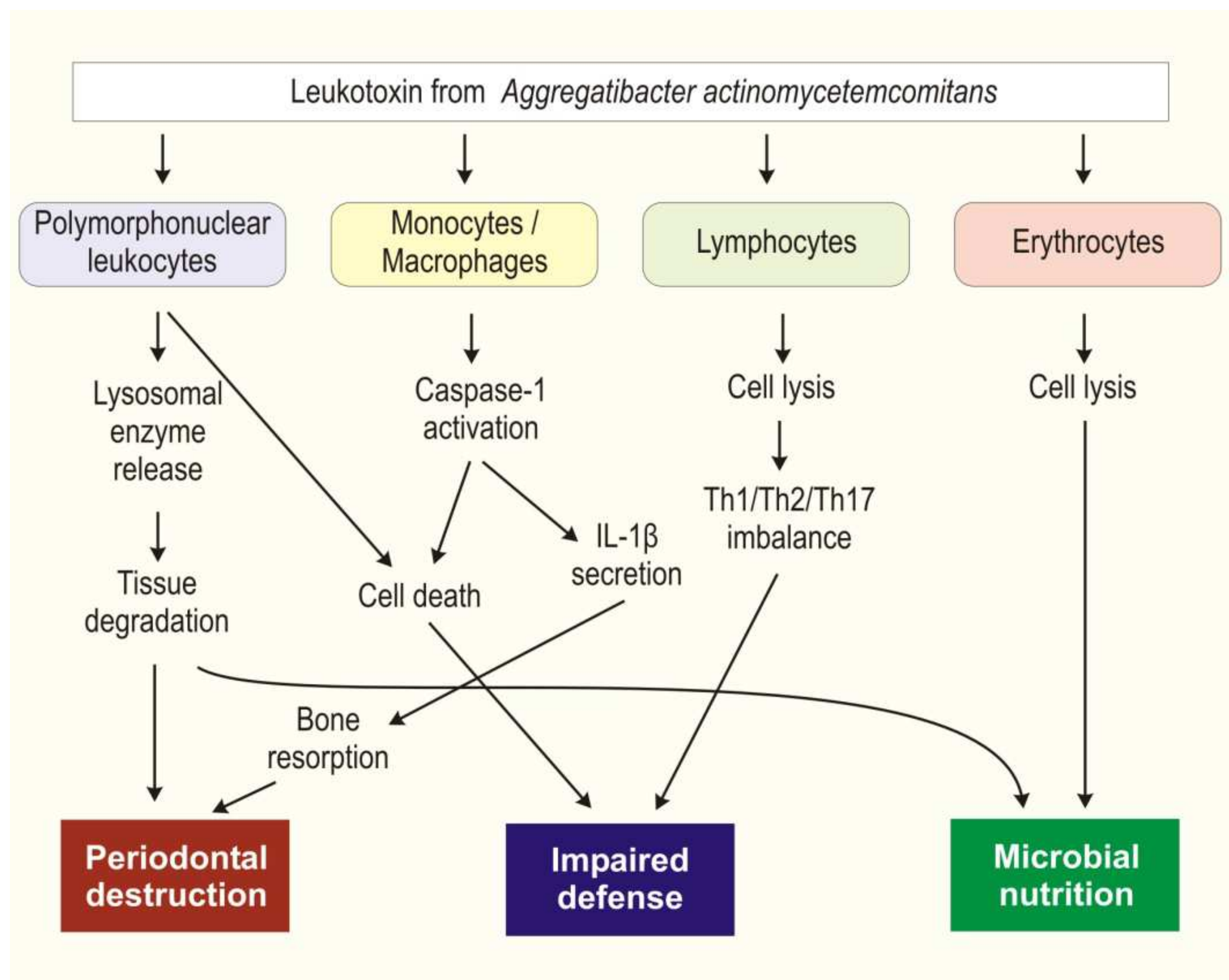

Fig. 10. Effects of importance for development of periodontal inflammation and local tissue destruction derived from the interactions of *A. actinomycetemcomitans* leukotoxin with human blood cells.

8. Acknowledgment

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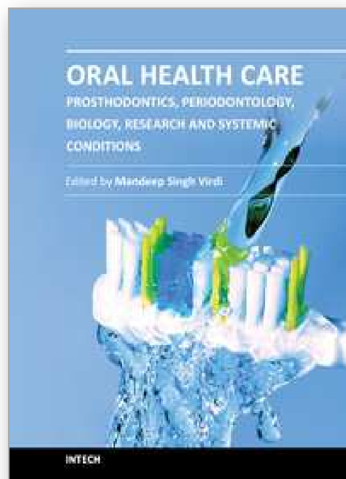

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Geriatric dentistry, or gerodontics, is the branch of dental care dealing with older adults involving the diagnosis, prevention, and treatment of problems associated with normal aging and age-related diseases as part of an interdisciplinary team with other healthcare professionals. Prosthodontics is the dental specialty pertaining to the diagnosis, treatment planning, rehabilitation, and maintenance of the oral function, comfort, appearance, and health of patients with clinical conditions associated with missing or deficient teeth and/or oral and maxillofacial tissues using biocompatible materials. Periodontology, or Periodontics, is the specialty of oral healthcare that concerns supporting structures of teeth, diseases, and conditions that affect them. The supporting tissues are known as the periodontium, which includes the gingiva (gums), alveolar bone, cementum, and the periodontal ligament. Oral biology deals with the microbiota and their interaction within the oral region. Research in oral health and systemic conditions concerns the effect of various systemic conditions on the oral cavity and conversely helps to diagnose various systemic conditions.

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