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Abstract: Periodontitis is an inflammatory condition that destroys the tooth-supporting tissues, as a result of local bacterial infection. Aggregatibacter actinomycetemcomitans is a Gram-negative facultative anaerobic species, highly associated with aggressive periodontitis. Periodontal inflammation is dominated by cytokines of the Interleukin (IL)-1 family. Prior to their secretion by mononuclear cells, IL-1 cytokines are processed by intracellular protein complexes, known as "inflammasomes", which can sense the bacterial challenge. The aim of this study was to investigate which inflammasomes are regulated in mononuclear cells in response to A. actinomycetemcomitans. The D7SS strain and its derivative leukotoxin and cytolethal distending toxin knock-out mutant strains were used to infect human mononuclear cells at a 1:10 cell: bacteria ratio, for 3 h. The expression of various inflammasome components in the cells was investigated by TaqMan quantitative real-time polymerase chain reaction (qPCR). The expressions of NOD-like receptor protein (NLRP)1, NLRP2 and Absent In Melanoma (AIM)2 inflammasome sensors, as well as their effector Caspase-1 were not affected. However, NLRP3 was up-regulated, while NLRP6 was down-regulated. This effect was not dependent on the leukotoxin or the cytolethal distending toxin, as demonstrated by the use of specific gene knock-out mutant strains. IL-1 and IL-18 expressions were also up-regulated by the bacterial challenge. In conclusion, A. actinomycetemcomitans enhances NLRP3 and reduces NLRP6 inflammasome expression, irrespective of its major virulence factors, confirming the high pathogenic profile of this species, and providing further insights to the mechanisms of periodontal inflammation.

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Aggregatigacter actinomycetemcomitans targets NLRP3 and NLRP6 inflammasome expression in human mononuclear leukocytes

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Running title: Inflammasome regulation by *A. actinomycetemcomitans*

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Abstract

Periodontitis is an inflammatory condition that destroys the tooth-supporting tissues, as a result of local bacterial infection. *Aggregatibacter actinomycetemcomitans* is a Gram-negative facultative anaerobic species, highly associated with Aggressive Periodontitis. Periodontal inflammation is dominated by cytokines of the Interleukin (IL)-1 family. Prior to their secretion by macrophages, IL-1 cytokines are processed by intracellular protein complexes, known as "inflammasomes", which can sense the bacterial challenge. The aim of this study was to investigate which inflammasomes are regulated in mononuclear cells in response to *A. actinomycetemcomitans*. The D7SS strain and its derivative leukotoxin and cytolethal distending toxin knock-out mutant strains were used to infect human mononuclear cells at a 1:10 cell: bacteria ratio, for 3 h. The expression of various inflammasome components in the cells was investigated by TaqMan quantitative real-time polymerase chain reaction (qPCR). The expressions of NOD-like receptor protein (NLRP)1, NLRP2 and Absent In Melanoma (AIM)2 inflammasome sensors, as well as their effector Caspase-1 were not affected. However, NLRP3 was up-regulated, while NLRP6 was down-regulated. This effect was not dependent on the leukotoxin or the cytolethal distending toxin, as demonstrated by the use of specific gene knock-out mutant strains. IL-1β and IL-18 expressions were also up-regulated by the bacterial challenge. In conclusion, *A. actinomycetemcomitans* enhances NLRP3 and reduces NLRP6 inflammasome expression, irrespective of its major virulence factors, confirming the high pathogenic profile of this species, and providing further insights to the mechanisms of periodontal inflammation.

Keywords: *Aggregatibacter actinomycetemcomitans*, inflammasome, NLRP3, NLRP6, mononuclear leukocytes, interleukin-1β, aggressive periodontitis.

1. Introduction

Periodontitis is the most common chronic infectious inflammatory disease in man, characterised by the inflammatory destruction of the tooth supporting (periodontal) tissues. If left untreated, periodontitis will eventually lead to tooth loss. The etiological factor of the disease is oral bacteria colonizing the tooth surfaces as polymicrobial biofilm communities [1]. *Aggregatibacter actinomycetemcomitans* is a facultatively anaerobic Gram-negative bacterium, which is present frequently and in high numbers in biofilms from sites with localized aggressive periodontitis [2, 3]. This variant of periodontitis affects adolescents and young adults and is characterised by an early onset and rapid progression [4]. High proportions of *A. actinomycetemcomitans* in biofilms are strongly correlated with loss of periodontal tissue attachment in the affected sites [5]. This species may also be associated with systemic diseases, as it has occasionally been isolated from non-oral infections, including endocarditis, bacteremia, pericarditis, septicemia, pneumonia [6], as well as atherosclerotic plaque [7]. The pathogenic capacities of *A. actinomycetemcomitans* are well underlined by its virulence factors, including two protein exotoxins, which are prevalent among Gram-negative species [8, 9]. These are namely the leukotoxin and the cytolethal distending toxin (Cdt), belonging to the repeats-in-toxin (RTX) and Cdt superfamilies, respectively. The Cdt causes growth arrest and eventually apoptosis to virtually all eukaryotic cells [10-13]. Leukotoxin, predominantly associated with the bacterial surface [14, 15], can selectively kill human leukocytes by triggering apoptosis or lysis [16-19]. The lysis of human monocytes / macrophages by leukotoxin is mediated by the activation of the cysteine proteinase Caspase-1, which is also associated with the secretion of bio-active IL-1β [17, 20, 21]. In addition, the strong association between presence of the highly leuokotoxic clone (JP2) of *A.*

actinomycetemcomitans and disease progression indicates an important pathogenic role of the leukotoxin [22].

Cytokines of the IL-1 family, including IL-1β and IL-18, are central to the inflammatory host response in periodontal diseases [23, 24], as levels of IL-1β are higher in gingival crevicular fluid [25] or gingival tissues [26], in periodontitis. Macrophages are key cells in the local immune responses towards infection, and their main functions involve phagocytosis, antigen presentation and production of IL-1β. In fact, macrophages are the main source of IL-1β in inflamed tissue [27]. This cytokine is produced as an inactive precursor (pro-IL-1β) that is subsequently activated by Caspase-1 before being secreted as biologically active IL-1β [28]. Bacterial products, such as the lipopolysaccharide (LPS), can increase the expression of pro-IL-1β in macrophages [29]. However, the activation of Caspase-1 that further induces the secretion of biologically active IL-1β requires auxiliary stimuli, known as pathogen-associated or danger-associated molecular patterns (PAMPs and DAMPs). These are sensed by "inflammasomes", which are intracellular pattern recognition receptors (PRRs) that form oligomeric molecular platforms [30]. Inflammasome oligomeres consist of the "sensor" (or "scaffold") nucleotide-binding oligomerization domain-like receptor (NLR) proteins, the mediating "adaptor" apoptotic speck protein containing a C-terminal caspase recruitment domain (ASC), and the "effector" pro-Caspase-1. Recognition of PAMPs or DAMPs by inflammasomes triggers the cleavage of pro-Caspase-1 into its active form, in turn activating IL-1β into its mature secreted form.

The most well characterised is the NLRP3 inflammasome that is activated in response to cell stress signals [31], whole bacteria and viruses, or their virulence factors [32-35], and is likely to play a key role in inflammatory periodontal disease [26]. NLRP2 is less well studied, but it is considered to inhibit the NLRP3-ASC

interaction [36, 37]. NLRP1 does not require the mediation of ASC for the activation of Caspase-1 [38], it can also activate Caspase-5 [39], and can be activated by the anthrax lethal toxin, causing cell death [40]. NLRP6 is as a critical regulator of pro-inflammatory signal transduction, particularly by activating NF-κB and Caspase-1 [41]. Recent studies demonstrated that it is and important PRR for developmental control of intestinal colonization by bacteria [42], epithelial self-renewal [43] and prevention of inflammation-induced tumorgenesis in the gut [44] or colitis [45]. Hence, this inflammasome may convey anti-inflammatory signals by commensal bacteria, crucial for the homeostatic maintenance [46]. Absent In Melanoma (AIM)2 is the only non-NLR inflammasome that senses a broad spectrum of cytosolic double-stranded DNA from bacteria, viruses, or even the host [47]. Similarly to NLRP3, the AIM2 inflammasome platform also consists of ASC and Caspase-1 [48].

The inflammasome responses to *A. actinomycetemcomitans* infection are not known. Taken the ability of leukotoxin to activate an abundant secretion of bio-active IL-1β, its is important to further examine the regulation of the up-stream molecules responsible for this activation by *A. actinomycetemcomitans* [49]. Therefore, the present *in vitro* study aims to investigate the effects of *A. actinomycetemcomitans* on the expression of various inflammasomes in human mononuclear leukocytes, and to evaluate the potential involvement of the leukotoxin and the Cdt.

2. Materials and Methods

2.1 Bacterial strains

The *A. actinomycetemcomitans* strain D7S-smooth wild-type (D7SS WT), serotype a, with a complete leukotoxin promoter and Cdt operon was used in this study. To evaluate the relative role of leukotoxin and Cdt in the effects, derivative D7SS

leukotoxin and/or Cdt knock-out mutant strains were used. These mutant strains were were namely the D7SS Δ*ltxA*, the D7SS Δ*cdtABC* and the D7SS Δ*ltxA/CdtABC*, in which, respectively, the *ltxA* gene, the *CdtABC* operon, or both, had been deleted and replaced with a spectinomycin resistance cassette [50]. The determination of the leukotoxic activity and Cdt-related growth inhibitory activity of the strains were previously determined [11, 21]. The strains were cultivated on blood agar plates at 37 °C in aerobic atmosphere containing 5% CO_2. After 72 h incubation, the bacterial colonies were harvested with a sterile cotton swab and suspended in phosphate buffered saline (PBS), at pH 7.2. The concentration of bacteria in the suspensions were quantified by measuring the optical density (OD 600^{nm}) and counting the colonies cultivated on blood agar plates [51].

2.2 Leukocyte preparations

Human mononuclear leukocytes (MNLs) were isolated from an enriched leukocyte fraction (buffy coat) obtained from 450 ml venous blood of one donor, at Norrland's University Hospital blood bank (Umeå, Sweden). Ethical clearance was obtained by the committee of Umeå University, Sweden. MNLs were isolated by isopycnic centrifugation in Lymphopreps (Nycomed AB, Lidingo, Sweden) as described previously [21]. The monocytic and lymphocytic content of the MNL fraction was defined by measuring the forward scatter (FSC) and side scatter (SSC) by FACS. Approximately 30% of the cells corresponded to monocytes, whereas 70% of the cells to lymphocytes (Figure 1). The fraction containing MNLs was collected, washed 3 times with PBS at 250 g for 10 min to remove the platelets. The cell pellet was then re-suspended in RPMI-1640 containing 10% FBS and penicillin–streptomycin to

yield 5 x 10^6 cells/ml. One ml of suspension was distributed to 1 or 2 cm^2 culture dishes (NUNC A/S) and cultured for 20 h at 37 °C in 5% CO_2 to equilibrate the cells.

2.3 Bacterial exposure

The cell culture medium was replaced by 0.25 ml fresh medium per well, containing the *A. actinomycetemcomitans* D7SS bacterial strains described above at concentrations 1:10 cell: bacteria ratio, for 3 h at 37 °C in 5% CO_2. The selection of this ratio was based on previous work in this experimental system, demonstrating that the cells are induced to produce IL-1β, without exhibiting significant cytotoxicity [21]. At the end of the incubation period cells and supernatants were separated by centrifuging at 250g at 20 °C for 5 min. The control group consisted of cells cultured in the absence of bacteria.

2.4 Cell lysis

Cell lysis was determined as the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture media from damaged cells, and its activity was measured spectophotometrically as described previously [14, 52] and expressed as absorbance at 340 nm. Data are normalized to control cultures exposed to culture medium alone, in the absence of bacteria.

2.5 Propidium Iodide staining of the cells

These cells were further analyzed for early detection of apoptosis/necrosis. For this purpose, the isolated MNLs were incubated in culture medium for 3 h, at 37 °C in the presence of 10 *A. actinomycetemcomitans* bacteria/cell. MNLs in plain culture medium served as controls. Upon termination of the experiment, the cell cultures were

placed on ice and Propidium Iodide (PI) was added according to the Vybrant® Apoptosis Assay Kit #4 protocol (Molecular Probes/Invitrogen Labeling and Detection). The cells were incubated further for 30 min, detached with a sterile cell scraper (Corning Incorporated), transferred to FACS tubes and analyzed by FACS as soon as possible using 488 nm excitation with fluorescence emission at 575 nm (e.g., FL3) (Calibur, Becton Dickinson Immunocytometry Systems). Dead cells were stained positive for PI and their proportion was calculated in a population of 10000 cells in each sample.

2.6 Quantification of secreted Interleukin-1β

Upon challenge of the macrophages with the various *A. actinomycetemcomitans* strains as described above, the cell culture supernatants were collected after the cells were removed by centrifugation. The concentrations of secreted IL-1β were then measured in the cell-free culture supernatants by using a commercially available ELISA kit (R&D systems, Abingdon, UK) that recognizes only the active form of this cytokine.

2.7 RNA extraction and cDNA synthesis

After completion of the experiments and removal of the cell-culture supernatants by centrifugation, the cell pellets were washed twice in PBS before being lysed. Total RNA was extracted by using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. The concentration of total RNA was measured by a spectrophotometer. Total RNA (400 ng) was then reverse transcribed into cDNA by using the High-Capacity cDNA Reverese Transcription Kit (Applied Biosystems) in a

volume of 20 µl, according to the manufacturer's protocol. The resulting cDNA was stored at -20°C, until further use.

2.8 Quantitative real-time Polymerase Chain Reaction (qPCR)

For gene expression analyses, qPCR was performed in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems). GAPDH and UBC were used as endogenous RNA control in the samples (house-keeping genes). For the amplification reactions, the TaqMan Gene Expression Master Mix and Gene Expression Assay kits from Applied Biosystems were used (assay IDs NLRP1: Hs00248187-m1 , NLRP2: Hs001546938-m1, NLRP3: Hs00918085-m1, NLRP6: Hs00373246-m1, ASC: Hs01547324-m1, Caspase-1: Hs00354836-m1, AIM2: Hs00915710-m1, IL-1β: Hs00174097-m1, IL-18: Hs01038787-m1, GAPDH: Hs99999905-m1, and ubiquitin C (UBC): Hs00824723-m1). The standard PCR conditions were 10 min at 95ºC, followed 40 cycles at 95ºC for 15 seconds and 60ºC for 1 min. The expression levels of the target transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to the average of the two housekeeping genes.

2.9 Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze the statistical significances of the results, using Bonferroni post-hoc test for comparisons between individual groups. The data were considered significant at $P<0.05$.

3. Results

After 3 h of challenge, the effect of *A. actinomycetemcomitans* on MNL death by cell lysis was determined by measuring the extracellularely released LDH activity. The results indicate no significant differences between the control and the *A. actinomycetemcomitans*-challenged groups (Table 1), indicating a low cytotoxic effect of the strain and concentrations used, within the short period of challenge. A slight increase in nuclear PI staining (corresponding to apoptosis) of the cells was observed in the presence of Cdt-producing strains (i.e. D7SS wild-type and D7SS Δ*ltxA*) but this proved to be only at a 5% range (Table 1).

To investigate the effect of *A. actinomycetemcomitans* on inflammasome expression, the MNL cultures were challenged for 3 h with the wild-type or the respective mutants strains, at 1:10 cells:bacteria ratio. The gene expression levels of the various inflammasome components were thereafter measured by qPCR. It was found that, compared to the unchallenged control, the expressions of the inflammasome sensors NLRP1 (Figure 2A), NLRP 2 (Figure 2B), AIM2 (Figure 2C) were not affected by *A. actinomycetemcomitants* challenge, irrespective of the strain used. On the contrary the expression of the inflammatory sensors NLRP3 and NLRP6 were affected by *A. actinomycetemcomitans* challenge. NLRP3 expression was significantly up-regulated by all *A. actinomycetemcomitans* strains used, between 3.4-fold and 5.0-fold, compared to the control (Figure 2D). Nevertheless, NLRP6 expression was significantly down-regulated by all strains used, by 50% - 60% (Figure 2E). Interestingly, no significant differences were observed among the four *A. actinomycetemcomitans* strains in their capacity to induce NLRP3 or reduce NLRP6 expression.

The expression of the adaptor molecule ASC, as well as the effector Caspase-1 was also investigated (Figure 3). ASC was down-regulated by *A.*

actinomycetemcomitans challenge by 33% - 58% (Figure 3A), whereas the expression of Caspase-1 was not affected (Figure 3B). Again, there were no significant differences in the capacity of the different *A. actinomycetemcomitans* strains to down-regulate ASC expression.

Further on, the effect of *A. actinomycetemcomitans* on the transcription of IL-1 family cytokines, namely IL-1β and IL-18, was investigated (Figure 4). There was a clear induction of IL-1β expression by *A. actinomycetemcomitans* by 17-fold to 21-fold (Figure 4A). In the case of IL-18, a numerical enhancement of was observed, which however did not proved to be statistically significant, due to the high standard deviation between experiments (Figure 4B).

The levels of IL-1β secretion by the MNLs in response to *A. actinomycetemcomitans*, were further investigated by ELISA (Table 2). It was found that *A. actinomycetemcomitans* challenge enhanced IL-1β secretion by the cells. However, no differences were observed between the wild-type and mutant strains in this capacity.

4. Discussion

This study demonstrated that *A. actinomycetemcomitans* induced NLRP3 and reduced NLRP6 inflammasome gene expression in human MNLs, whereas it did not affect the expressions of NLRP1, NLRP2 or AIM2. As NLRP3 is a sensor associated with PAMP or DAMP recognition [53], whereas NLRP6 is associated with homeostatic or "protective" molecular signals [46], the findings denote that *A. actinomycetemcomitans* is perceived by the macrophages as a pathogen. Interestingly, the expression of the adaptor molecule ASC, that mediates NLR binding to Caspase-1, is down-regulated by *A. actinomycetemcomitans* infection. These results are in

agreement with the up-regulation of NLRP3 [26] and down-regulation of ASC [26, 54] in myelomonocytic Monomac-6 or monocytic THP1 cells, by the major periodontal pathogen *Porphyromonas gingivalis*. Similar findings were are also demonstrated in another experimental system, in which *Listeria monocytogenes* is shown to up-regulate NLRP3, but down-regulate NLRP6 and ASC expression in human peripheral blood mononuclear cells [55]. In contrast, in gingival epithelial cells *P. gingivalis* down-regulates NLRP3 expression, but it does not affect ASC [56], denoting that inflammasome responses to a given species may as well be cell-type specific. Although it is difficult to assess the net functional effect of the differential regulation of NLRP3 and ASC, bacterial pathogens may strategically perturb the expression of various inflammasome components, in order to manipulate the innate immune responses [57]. To this extent, supragingival and subgingival biofilms were shown to differentially regulate the expression of the NLRP3 and AIM2 inflammasomes in gingival fibroblasts, denoting the different pathogenic potential of these two biofilm variants for periodontal diseases [58].

Inflammasomes regulate the activation of Caspase-1, which itself constitutes the effector molecule of these oligomeric platforms. The end-point outcome of Caspase-1 activation is IL-1 cytokine secretion. The effect of *A. actinomycetemcomitans* on the transcriptional expression of Caspase-1, as well as IL-1β and IL-18 was also investigated in the present experimental system. Caspase-1 expression in MNLs was not affected in response to any of the *A. actinomycetemcomitans* strains used, but IL-1β and IL-18 expressions were up-regulated. In line with these findings, infection of macrophages with *A. actinomycetemcomitans* does not affect the intracellular levels of pro-Caspase-1 [20], although its leukotoxin can promote Caspase-1 activation [17] and enhanced IL-1β

production. In the absence of leukotoxin, transcribed pro-IL-1β is withheld intracellularly [20, 21].

The present study also investigated the potential role of leukotoxin and Cdt, the two major virulence factors of *A. actinomycetemcomitans*, in the observed effects. It was found that neither toxin was involved in the differential regulations of inflammasome components, namely of NLRP3, NLRP6 and ASC. Hence, it appears that *A. actinomycetemcomitans* is sensed by inflammasomes irrespective of these two major virulence factors, albeit for the activation of the effector molecule Caspase-1 and subsequent IL-1β secretion, leukotoxin is required [17, 20]. The relative effect of *A. actinomycetemcomitans* leukotoxin and Cdt on IL-1β and IL-18 expressions by the cells was also considered in this study. Of note, IL-18 was expressed at lower levels that IL-1β, also in line with recent findings in macrophages [59] and gingival fibroblasts [58]. Although none of the *A. actinomycetemcomitans* knock-out mutant strains differentially regulated IL-1β expression compared to the wild-type strain, they all appeared to enhance IL-18 expression levels by the cells, but this did not prove to be statistically significant, due to the large standard deviation between experiments. Moreover, leukotoxin was previously shown not to be able to cause a significant effect on IL-1β gene expression [59]. Therefore, the leukotoxin and Cdt at sub-lethal concentrations do not appear to be clearly involved in the *A. actinomycetemcomitants*-induced IL-1β expression by MNLs.

The potential effect of *A. actinomycetemcomitans* and its virulence factors on IL-1β secretion by the MNLs was also considered in the present experimental study. Although IL-1β secretion was enhanced, no differences were observed between the effects of the wild-type and knock-out mutant strains. The lack of additive effect on IL-1 β in the presence of leukotoxin may be surprising. However, it is likely to be due

to the fact that the D7SS strain is a low leukotoxic strain [21], and therefore may not further enhance IL-1β at the low (1:10) concentrations used. Accordingly, no significant differences were observed in the cytotoxic effects of the different strains, as indicated by the LDH release and PI staining data, indicating further the lack of any pronounced effect by the leukotoxin. It can therefore be concluded that in the present experimental system, *A. actinomycetemcomitans* did not induce the "pyroptotic" cell death and associated IL-1β release attributed to the leukotoxin [21, 59]. This could either be attributed to the low leukotoxin-producing activity of the D7SS strain, or to the fact that a whole MNL population was used, rather than isolated more sensitive macrophages. The MNL mixture is dominated by cells of lymphoid origin, which are less sensitive to the leukotoxin and might compete with the sensitive monocytes in leukotoxin binding.

5. Conclusion

In conclusion, the present study indicates that *A. actinomycetemcomitans* enhances the expression of NLRP3 and reduces the expression of NLRP6 in MNLs, irrespective of its two major exotoxins. Given the PAMP-sensing role of the former inflammasome [53] and the commensal-sensing role of the latter [46], this dual regulation is commensurate with the high pathogenic profile of *A. actinomycetemcomitans* [8] in Aggressive Periodontitis.

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Tables

Table 1

	LDH release (mean ± SEM)	PI staining (% of total)
Control	1.00 ± 0.35	9.77
D7SS WT	1.01 ± 0.49	14.06
D7SS D*LtxA*	0.92 ± 0.14	12.98
D7SS D*CdtABC*	0.85 ± 0.16	9.23
D7SS D*LtxACdtABC*	0.60 ± 0.57	10.11

***LDH release and PI staining in MNLs challenged with* A. actinomycetemcomitans.** MNLs were cultured for 3 h in the presence or absence of the *A. actinomycetemcomitans* D7SS wild-type (WT) or the knock-out mutant strains D7SS D*LtxA*, D7SS D*CdtABC*, or double mutant D7SS D*LtxACdtABC*. The released LDH was measured colorimetrically and the results are presented as mean absorbance ± standard error of mean (SEM) from triplicate cultures. The nuclear PI staining was measured by flow cytometry and the results are expressed as the percentage of positively stained cells in the culture, in a population of 10000 cells in each sample (one experiment).

Table 2

	IL-1β concentration (mean ± SD)	
Control	108.4 ± 28.3 pg/ml	
D7SS WT	200.3 ± 47.5 pg/ml	*
D7SS D*LtxA*	191.4 ± 12.2 pg/ml	*
D7SS D*CdtABC*	183.9 ± 9.6 pg/ml	*
D7SS D*LtxACdtABC*	206.5 ± 25.4 pg/ml	*

***IL-1β secretion by human MNLs in response to* A. actinomycetemcomitans.** MNLs were cultured for 3 h in the presence or absence of the *A. actinomycetemcomitans* D7SS wild-type (WT) or the knock-out mutant strains D7SS D*LtxA*, D7SS D*CdtABC*, or double mutant D7SS D*LtxACdtABC* (1:10 cell: bacteria ratio). The secreted IL-1β in the culture media was measured by commercially available ELISA. The concentrations are measured in pg/ml and the results are presented as mean concentrations ± standard deviation (SD) from three independent experiments. The asterisk represents statistically significant difference compared to the control group ($P<0.05$).

Figure Legends

Figure 1

Analysis of the MNL fraction by FACS. Flow cytometric analysis was used to determine the cell populations in the MNL fraction isolated from human blood, by combined FSC/SSC measurement. The sub-fraction of lymphocytes comprises approximately 70 % of the total MNL population, whereas the sub-fraction of monocytes comprises approximately 30 % of the total MNL population. Representative results from the MNL isolation process are shown.

Figure 2

Effect of A. actinomycetemcomitans on the expression of inflammasome sensors in MNLs. The various *A. actinomycetemcomitans* strains were used to challenge macropghages at 1:10 cell: bacteria ratio, for 3 h. The gene expression levels of NLRP1 (A), NLRP2 (B), AIM2 (C), NLRP3 (D) and NLRP6 (E) were measured by qPCR analysis, normalized against the expression levels of the average of GAPDH

and UBC (housekeeping genes). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference compared to the control group ($P<0.05$). Un-challenged control (Con), D7SS wild-type strain (WT), D7SS D*LtxA* strain (DLtx), D7SS D*CdtACB* strain (DCdt), D7SS D*LtxACdtABC* strain (DLtxCdt).

Figure 3

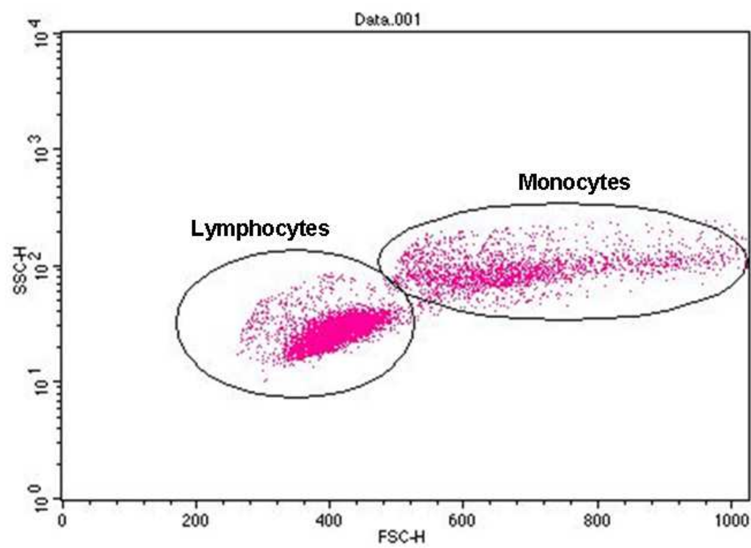
Effect of A. actinomycetemcomitans on ASC and Caspase-1 expression in MNLs. The various *A. actinomycetemcomitans* strains were used to challenge macropghages at 1:10 cell: bacteria ratio, for 3 h. The gene expression levels of ASC (A) and Caspase-1 (B) were measured by qPCR analysis, normalized against the expression levels of the average of GAPDH and UBC (housekeeping genes). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent experiments. The asterisk represents statistically significant difference compared to the control group ($P<0.05$). Un-challenged control (Con), D7SS wild-type strain (WT), D7SS D*LtxA* strain (DLtx), D7SS D*CdtACB* strain (DCdt), D7SS D*LtxACdtABC* strain (DLtxCdt).

Figure 4

.***Effect of A. actinomycetemcomitans on IL-1β and IL-18 expression in MNLs.*** The various *A. actinomycetemcomitans* strains were used to challenge macropghages at 1:10 cell: bacteria ratio, for 3 h. The gene expression levels of IL-1β (A) and IL-18 (B) were measured by qPCR analysis, normalized against the expression levels of the average of GAPDH and UBC (housekeeping genes). The results are expressed as the $2^{-\Delta CT}$ formula. Bars represent mean values ± SEM from three independent

experiments. The asterisk represents statistically significant difference compared to the control group ($P<0.05$). Un-challenged control (Con), D7SS wild-type strain (WT), D7SS D*LtxA* strain (DLtx), D7SS D*CdtACB* strain (DCdt), D7SS D*LtxACdtABC* strain (DLtxCdt).

Figure 1



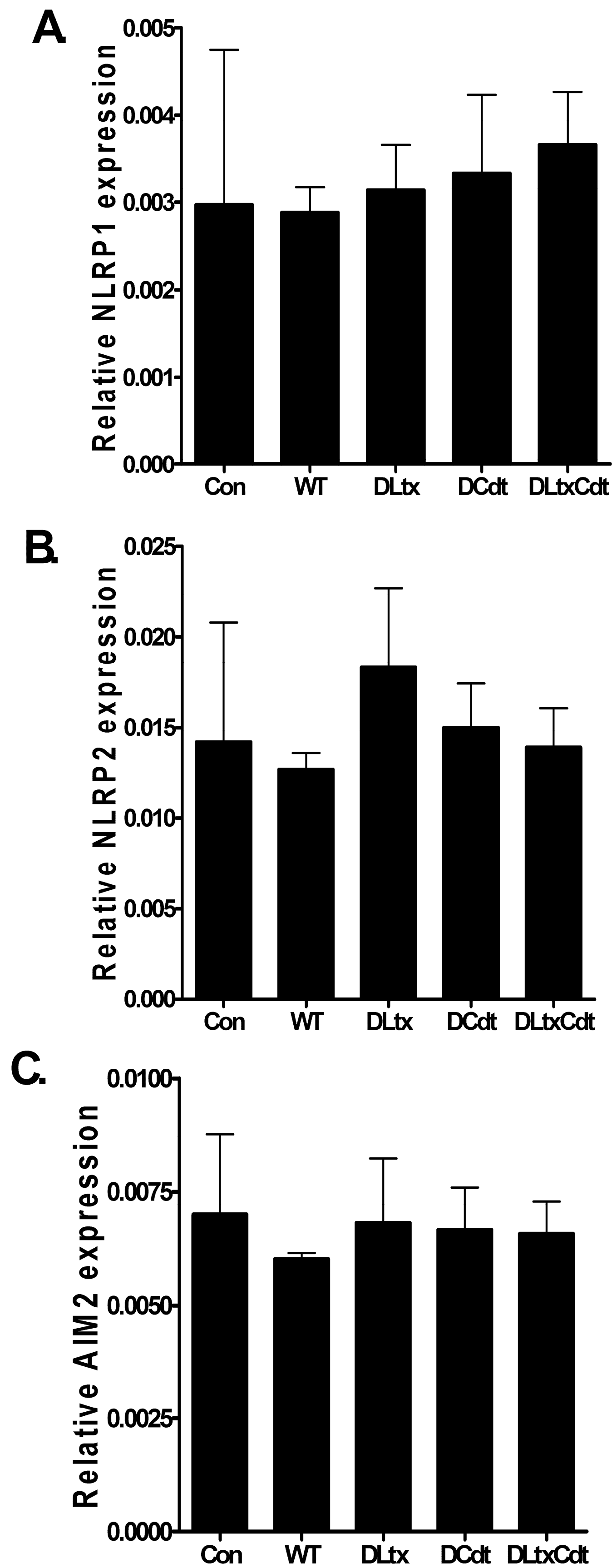
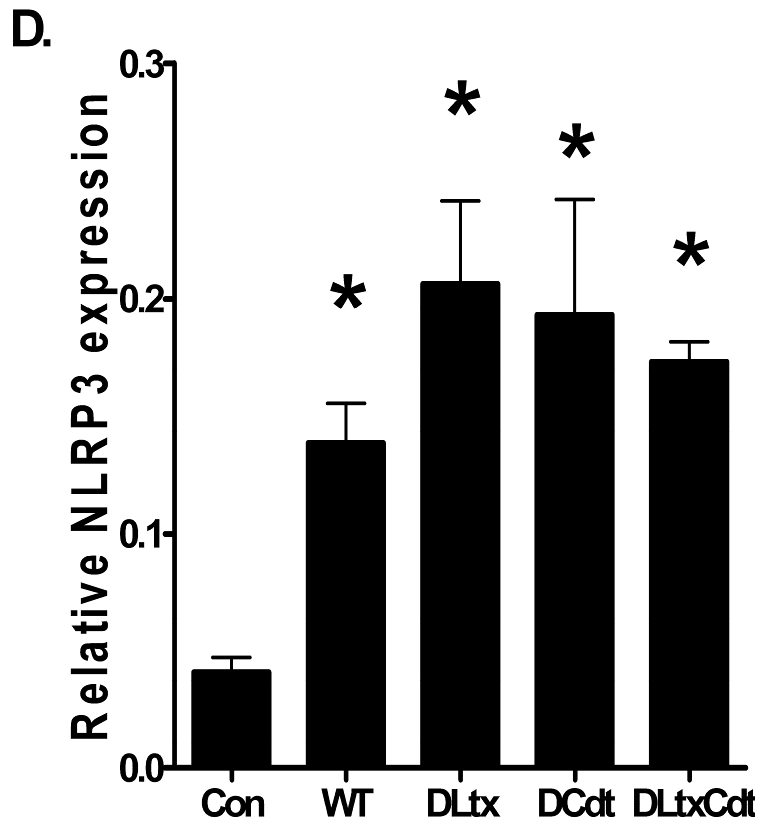
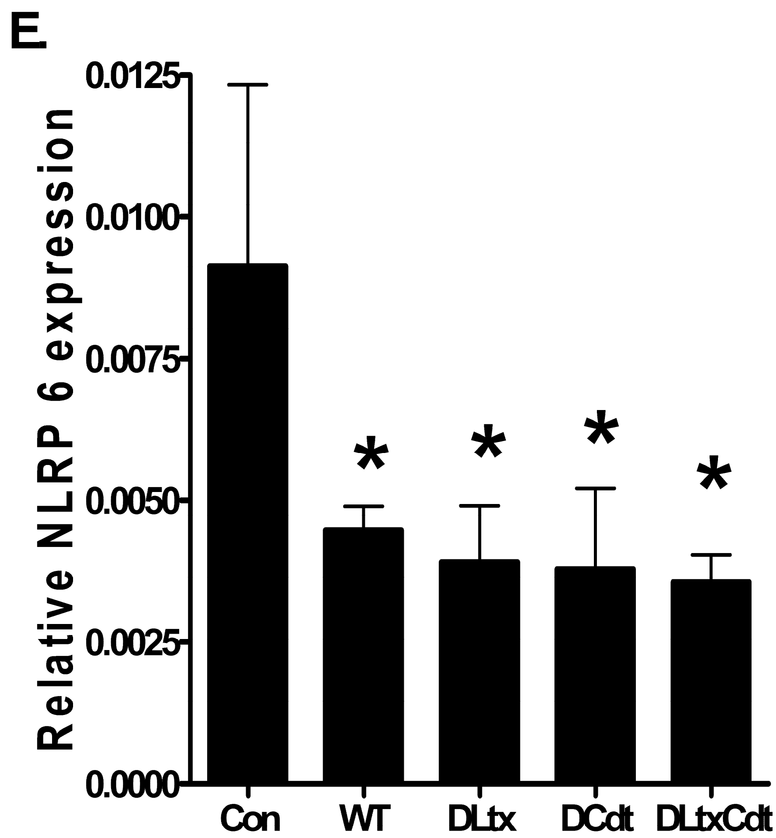


Figure 2
A
0.005
0.004
0.003
0.002
0.001
0.000
Relative NLRP1 expression
Con
WT
DLtx
DCdt
DLtxCdt
B.
0.025
0.020
0.015
0.010
0.005
0.000
Relative NLRP2 expression
Con
WT
DLtx
DCdt
DLtxCdt
C.
0.0100
0.0075
0.0050
0.0025
0.0000
Relative AIM2 expression
Con
WT
DLtx
DCdt
DLtxCdt

Figure 2





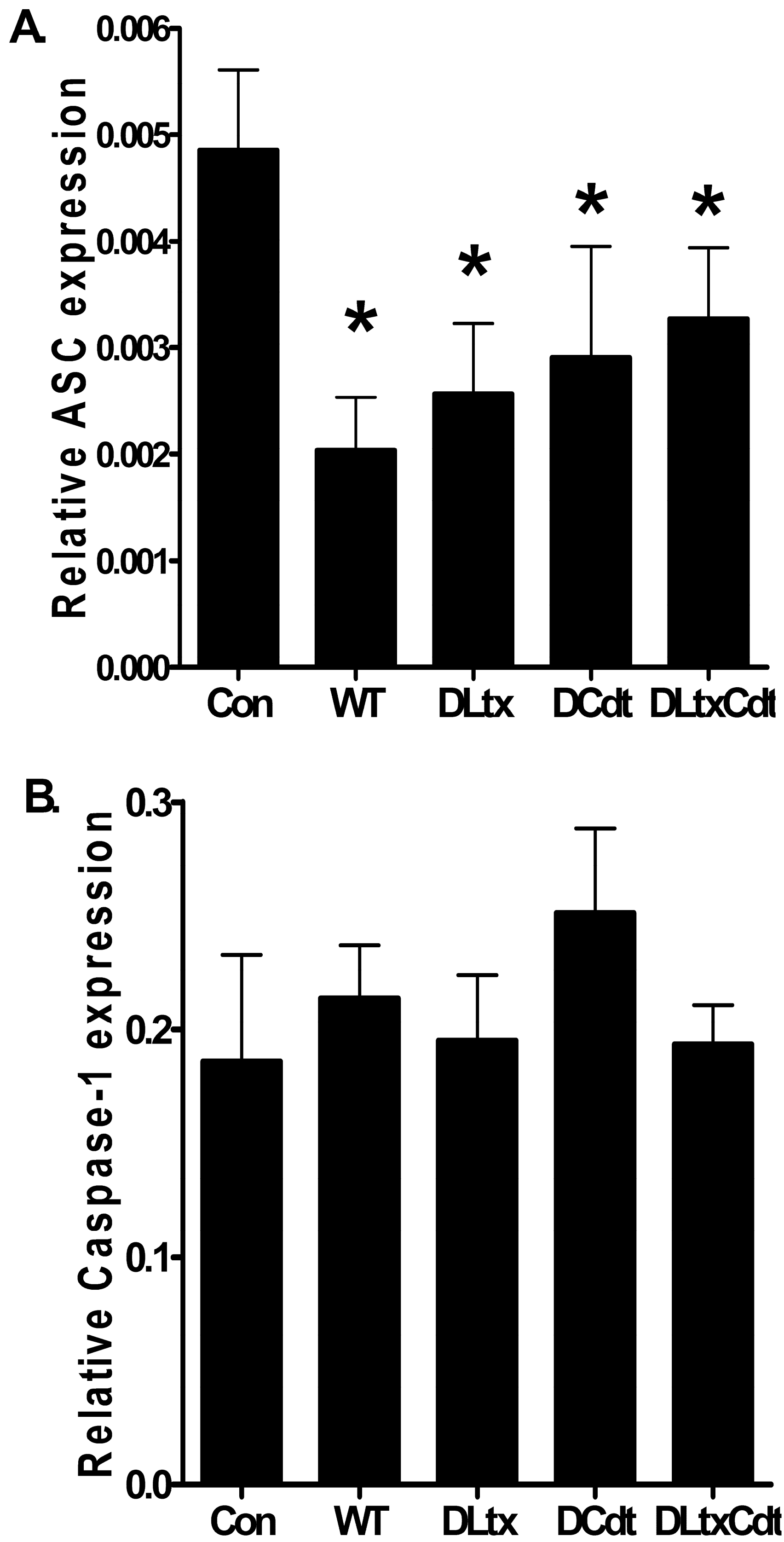


Figure 3
A.
Relative ASC expression
0.006
0.005
0.004
0.003
0.002
0.001
0.000
*
*
*
*
Con
WT
DLtx
DCdt
DLtxCdt
B.
Relative Caspase-1 expression
0.3
0.2
0.1
0.0
Con
WT
DLtx
DCdt
DLtxCdt

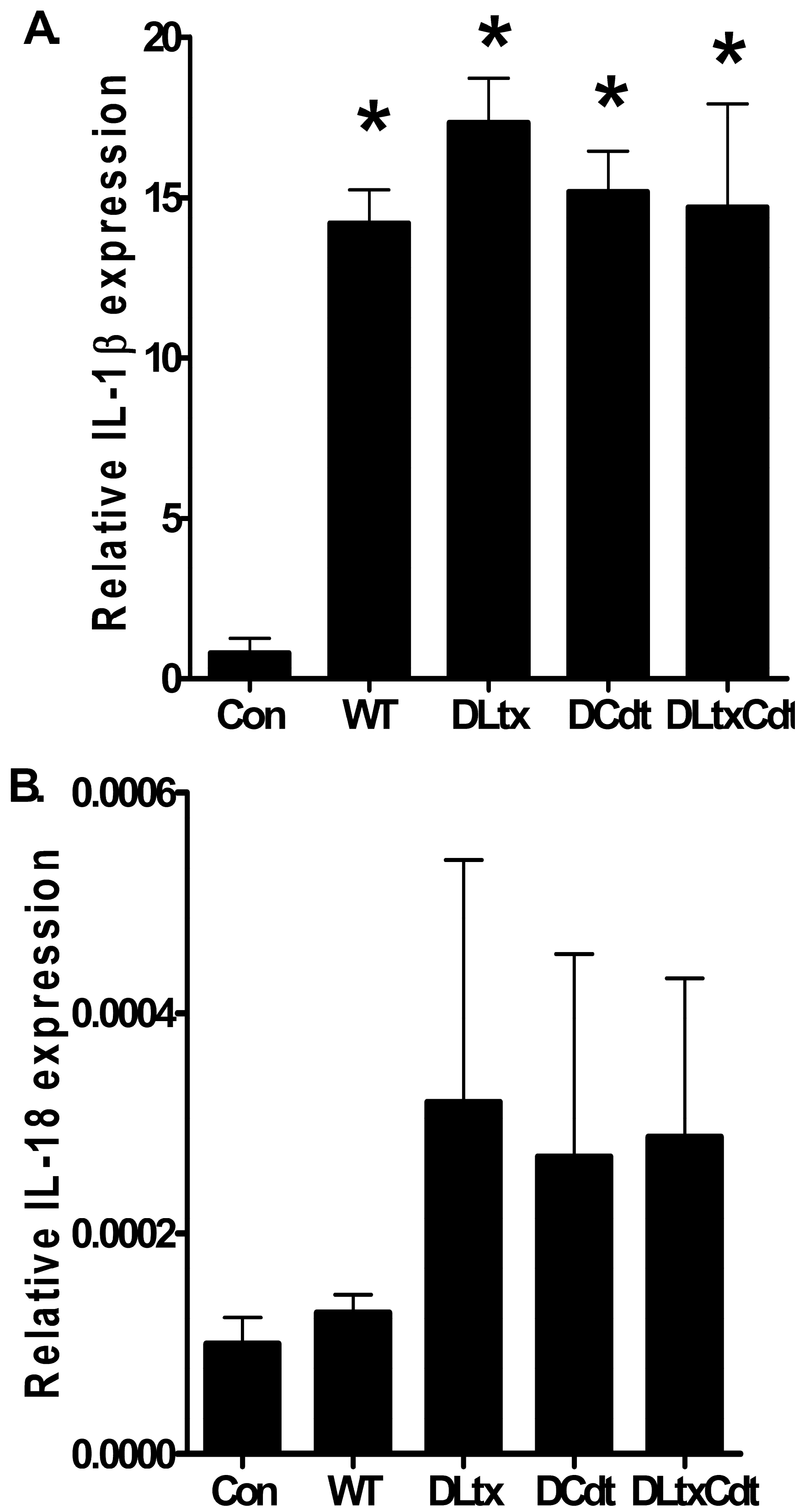


Figure 4
A.
20
15
10
5
0
Relative IL-1β expression
Con
WT
DLtx
DCdt
DLtxCdt
B.
0.0006
0.0004
0.0002
0.0000
Relative IL-18 expression
Con
WT
DLtx
DCdt
DLtxCdt