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Macrophage tolerance response to *Aggregatibacter actinomycetemcomitans* lipopolysaccharide induces differential regulation of tumor necrosis factor-α, interleukin-1β and matrix metalloproteinase 9 secretion

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Background and Objective: The lipopolysaccharide of *Aggregatibacter actinomycetemcomitans*, a potent stimulator of the immune system, induces the secretion of inflammatory mediators that modulate periodontal tissue destruction. In this study, we investigated the tolerance response of human macrophages to stimulation with *A. actinomycetemcomitans* lipopolysaccharide.

Material and Methods: U937 monocytes were differentiated into adherent macrophage-like cells by treatment with phorbol myristic acid. Macrophage-like cells were then pretreated for 24 h with either 0.01 or 0.1 μg/mL LPS *A. actinomycetemcomitans*. Culture medium supernatants were removed and cells were restimulated with LPS at 1 μg/mL. Cell-free supernatants were collected after 24 h of stimulation and analyzed by ELISA for TNF-α, IL-1β, IL-6, IL-8, PGE_2 and MMP-9.

Results: Phorbol myristic acid-differentiated U937 macrophages treated with low doses of lipopolysaccharide developed tolerance to subsequent lipopolysaccharide treatments, resulting in significantly reduced secretion of tumor necrosis factor-α. However, this tolerance response was associated with increased secretion of interleukin-1β and matrix metalloproteinase 9, whereas the secretion of interleukin-6, interleukin-8 and prostaglandin E_2 was unaffected. Phosphatidylinositol-3′-kinase inhibitors added during the tolerance-induction period markedly attenuated the increase in interleukin-1β secretion but had no effect on tumor necrosis factor-α.

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Conclusion: This study showed that *A. actinomycetemcomitans* lipopolysaccharide can induce a tolerance response in macrophages that alters the secretion of two important inflammatory mediators as well as of the tissue-degrading enzyme matrix metalloproteinase-9. This phenomenon may play a role in modulating the host inflammatory response and the progression of periodontitis.

Periodontitis is a chronic destructive inflammatory disease that leads to the loss of tooth support. A group of approximately 10 bacterial species, known as periodontopathogens, induce and modulate periodontal tissue destruction through complex interactions with mucosal and immune cells (1). The host response to these bacteria and their products is critical for periodontitis progression. A continuous high rate of secretion of various cytokines, including interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α, by host cells is observed following stimulation by periodontopathogens (2). These cytokines are involved in the expression of several matrix metalloproteinases (MMPs), which in turn can degrade extracellular matrix proteins and ultimately cause bone resorption (3).

The development of localized aggressive periodontitis is generally associated with the presence of high numbers of the gram-negative bacterium *Aggregatibacter actinomycetemcomitans* (4,5). Lipopolysaccharide, the major constituent of the outer membrane of gram-negative bacteria, stimulates host cells through the Toll-like 4/CD14/MD2 cell receptor complex (6). The lipopolysaccharide of *A. actinomycetemcomitans* is well known as a potent inducer of pro-inflammatory mediator secretion in a variety of cells, including epithelial cells, fibroblasts and macrophages (7–9). Nishida *et al.* (10) reported that the injection of *A. actinomycetemcomitans* lipopolysaccharide into mice gingival caused inflammation associated with the destruction of connective tissue and the resorption of alveolar bone.

The pre-exposure of host cells to nonlethal doses of lipopolysaccharide can affect various postreceptor signaling proteins and induce a tolerance response (11–14). The tolerance response, which results in an inhibition of excessive inflammatory mediator secretion, was initially thought to be a beneficial adaptive innate immune response. However, data also suggested that impaired cytokine release as a result of stimulation with lipopolysaccharide is associated with the presence of sepsis (14). Tumor necrosis factor-α is probably the best marker of lipopolysaccharide tolerance given its response in *in vivo* and *in vitro* models (11,14). In contrast, the pre-exposure of host cells to low doses of lipopolysaccharide may be associated with an enhanced secretion of certain inflammatory mediators upon subsequent stimulation with lipopolysaccharide (11,14). Lipopolysaccharide tolerance is not specific to the action of lipopolysaccharide, and cross-reactivity with other exogenous bacterial components can occur (12). In addition to lipopolysaccharide, exposure to a wide variety of stimuli, including lipoteichoic acid (15) and heat shock protein 60 (16), can alter the response to subsequent lipopolysaccharide stimulations. Although few data are available, the tolerance response is probably of biological significance during chronic inflammatory diseases such as periodontitis. In this study, we investigated the tolerance response of human macrophages to *A. actinomycetemcomitans* lipopolysaccharide.

Material and methods

Bacteria and lipopolysaccharide preparation

A. actinomycetemcomitans ATCC 29522 was grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% yeast extract. The bacterial cultures were incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 2 d. Lipopolysaccharide was isolated using the protocol of Darveau & Hancock (17), which is based on the digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The lipopolysaccharide preparation was freeze dried and stored at −20°C. Contaminating protein in the lipopolysaccharide preparations was established to be $< 0.005\%$ (w/w) using a protein assay kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) with bovine serum albumin as a control.

Monocyte culture and differentiation into macrophage-like cells

U937 cells (ATCC CRL-1593.2 human monoblastic leukemia cell line) were cultured at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of penicillin/streptomycin. Monocytes (2×10^5 cells/mL) were incubated for 48 h in RPMI-1640/10% fetal bovine serum containing 10 ng/mL of phorbol myristic acid (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) to induce differentiation into adherent macrophage-like cells. Phorbol myristic acid is known to induce the appearance of cell characteristics consistent with mature macrophages (18). Following the treatment with phorbol myristic acid, the RPMI-1640/10% fetal bovine serum was replaced with fresh medium and the differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-1640/10% fetal bovine serum and centrifuged at 300 *g* for 5 min at room temperature. The cells were washed, suspended in RPMI supplemented with

1% heat-inactivated fetal bovine serum, seeded in six-well plates (2×10^6 cells/well in 2 mL) and incubated at 37°C in a 5% CO_2 atmosphere for 2 h prior to stimulation.

Macrophage stimulation

The macrophages were pretreated with *A. actinomycetemcomitans* lipopolysaccharide, at a final concentration of 0.01 or 0.1 μg/mL, for 24 h at 37°C in a 5% CO_2 atmosphere. The culture medium supernatants were removed, new medium was added and the cells were restimulated with 1 μg/mL of *A. actinomycetemcomitans* lipopolysaccharide for 24 h. The culture supernatants were collected and stored at −20°C until used. Two independent experiments were performed to ensure reproducibility of the observations.

Determination of interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α, MMP-9 and prostaglandin E_2 concentrations

Commercial enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN, USA) were used, according to the manufacturer's protocols, to quantify interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α and MMP-9 concentrations in the cell-free culture supernatants. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The rated sensitivities of the commercial enzyme-linked immunosorbent assay kits were 3.9 pg/mL for interleukin-1β, 9.3 pg/mL for interleukin-6, 31.2 pg/mL for interleukin-8, 15.6 pg/mL for tumor necrosis factor-α and 0.31 ng/mL for MMP-9. A competitive enzyme immunoassay was used to quantify the prostaglandin E_2 concentration according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance at 415 nm was read using a microplate reader. The prostaglandin E_2 concentrations were determined in triplicate. The rated sensitivity of the kit was 7.8 pg/mL. All the above assays were performed in triplicate.

Effect of phosphatidylinositol-3′-kinase inhibitors

The effects of the phosphatidylinositol-3′-kinase inhibitors wortmannin and LY294002 (Calbiochem, San Diego, CA, USA) on the lipopolysaccharide tolerance response were investigated. The inhibitors were added at a final concentration of 50 nM during the first lipopolysaccharide (0.1 μg/mL) stimulation (tolerance-induction period) of the macrophages. Following a subsequent lipopolysaccharide challenge, the secretion of tumor necrosis factor-α and interleukin-1β was determined as described above.

Statistical analyses

Statistical analyses were performed using the Student's *t*-test for paired values. The results were considered significant at a *p*-value of < 0.02.

Results and discussion

We first determined the concentration of *A. actinomycetemcomitans* lipopolysaccharide that induces a significant pro-inflammatory mediator response and MMP-9 secretion in phorbol myristic acid-differentiated U937 macrophages without affecting cell viability (data not shown). In naïve macrophages, stimulation for 24 h with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/mL) induced a significant ($p < 0.02$) secretion of tumor necrosis factor-α (360 ± 20 pg/mL), interleukin-1β (66 ± 22 pg/mL), interleukin-6 (376 ± 60 pg/mL), interleukin-8 (13,837 ± 110 pg/mL), MMP-9 (444 ± 31 ng/mL) and prostaglandin E_2 (89 ± 6 pg/mL). With the exception of MMP-9 (179 ± 46 ng/mL) and prostaglandin E_2 (26 ± 3 pg/mL), no basal levels were detected with unstimulated macrophages.

Pretreating the macrophages with *A. actinomycetemcomitans* lipopolysaccharide modified the secretion levels of tumor necrosis factor-α, interleukin-1β and MMP-9 (Figs 1–3) in response to subsequent lipopolysaccharide treatments but had no significant effect on the secretion levels of interleukin-6, interleukin-8, or prostaglandin E_2 (data not shown). Subsequent stimulation of macrophages that had been pretreated with 0.01 or 0.1 μg/mL of

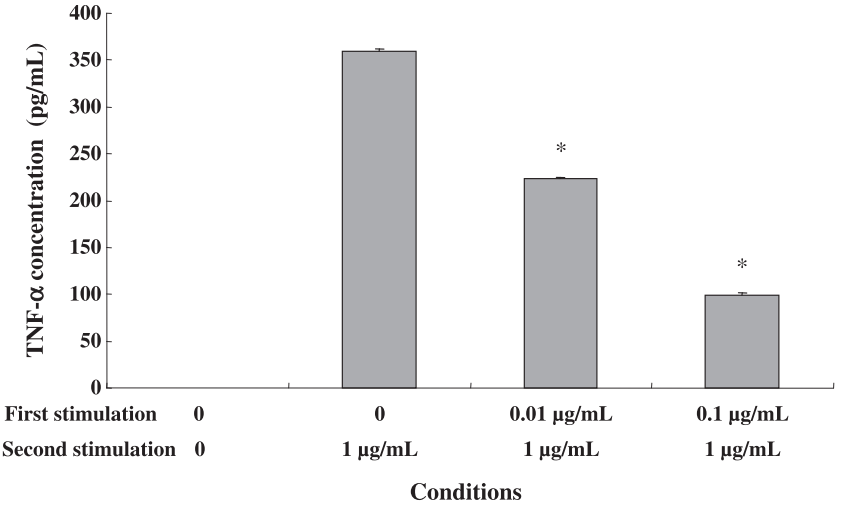

Fig. 1. Effect of pretreatment with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide on tumor necrosis factor-α secretion by macrophages subjected to subsequent lipopolysaccharide stimulation. Phorbol myristic acid-differentiated U937 macrophages were pretreated for 24 h with either 0.01 or 0.1 μg/mL of lipopolysaccharide. Culture medium supernatants were removed and the cells were restimulated with lipopolysaccharide at 1 μg/mL. Cell-free supernatants were collected after 24 h of stimulation and analyzed by enzyme-linked immunosorbent assay. *$p < 0.02$ compared with naive stimulated cells. TNF-α, tumor necrosis factor-α.

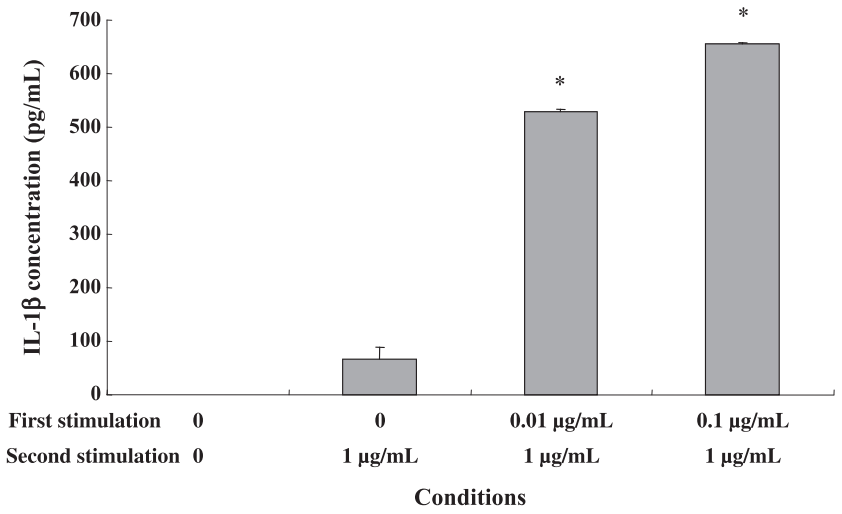

Fig. 2. Effect of pretreatment with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide on interleukin-1β secretion by macrophages subjected to subsequent lipopolysaccharide stimulation. Phorbol myristic acid-differentiated U937 macrophages were pretreated for 24 h with either 0.01 or 0.1 μg/mL of lipopolysaccharide. Culture medium supernatants were removed and the cells were restimulated with lipopolysaccharide at 1 μg/mL. Cell-free supernatants were collected after 24 h of stimulation and analyzed by enzyme-linked immunosorbent assay. $*p < 0.02$ compared with naive stimulated cells. IL-1β, interleukin-1β.

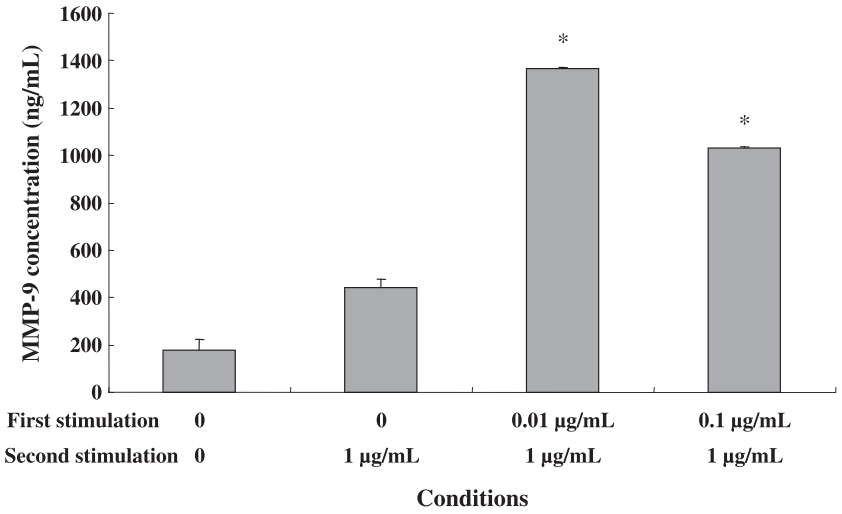

Fig. 3. Effect of pretreatment with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide on matrix metalloproteinase 9 secretion by macrophages subjected to subsequent lipopolysaccharide stimulation. Phorbol myristic acid-differentiated U937 macrophages were pretreated for 24 h with either 0.01 or 0.1 μg/mL of lipopolysaccharide. Culture medium supernatants were removed and the cells were restimulated with lipopolysaccharide at 1 μg/mL. Cell-free supernatants were collected after 24 h of stimulation and analyzed by enzyme-linked immunosorbent assay. $*p < 0.02$ compared with naive stimulated cells. MMP-9, matrix metalloproteinase 9.

lipopolysaccharide with 1 μg/mL of lipopolysaccharide caused a 38 and 72% decrease, respectively, in tumor necrosis factor-α levels (Fig. 1). This is in agreement with previous studies suggesting that the inhibition of *in vitro* tumor necrosis factor-α secretion may be a universal characteristic of lipopolysaccharide tolerance (14). In contrast, pretreating the macrophages with *A. actinomycetemcomitans* lipopolysaccharide (0.01 and 0.1 μg/mL) significantly increased the interleukin-1β response to a second stimulation with lipopolysaccharide (Fig. 2). More specifically, pretreatment with 0.01 μg/mL of lipopolysaccharide increased the secretion of interleukin-1β by approximately eight-fold. This supports the results of the study of Nakamura *et al.* (19), who showed that pretreatment of whole blood, which contains a mixed population of immune cells, with a substimulatory concentration of *A. actinomycetemcomitans* lipopolysaccharide significantly enhances the secretion of interleukin-1β following a subsequent stimulation with lipopolysaccharide. Both RelB (20) and interleukin-1R antagonist (21) are usually expressed in the endotoxin-tolerant state and are involved in interleukin-1β repression. In our tolerance assays we did not observe decreased production of interleukin-1β, indicating that signals provided by *A. actinomycetemcomitans* lipopolysaccharide do not allow expression of RelB and interleukin-1R antagonist. The results reported in the literature on the interleukin-1β response in lipopolysaccharide-tolerant cells are ambiguous. Some researchers have reported that interleukin-1β secretion increases when lipopolysaccharide-treated cells are restimulated with lipopolysaccharide, whereas others have reported that interleukin-1β secretion decreases (22–24). These discrepancies are probably related to the nature of the lipopolysaccharide and cell type used in the studies (14).

The effect of pretreatment with an *A. actinomycetemcomitans* lipopolysaccharide on MMP-9 secretion was also investigated (Fig. 3). Pretreatments with 0.01 and 0.1 μg/mL of lipopolysaccharide increased MMP-9 secretion by macrophages by 3-fold and 2.3-fold, respectively. To our knowledge, this is the first time that such a phenomenon has been associated with a lipopolysaccharide tolerance response.

The tolerance response has also been investigated in *Porphyromonas gingivalis*, a major etiologic agent of chronic periodontitis (25–27). More specifi-

cally, Hajishengallis *et al*. (25) reported that *P. gingivalis* lipopolysaccharide primes differentiated THP-1 cells for enhanced nuclear factor-κB-dependent transcription and tumor necrosis factor-α release upon restimulation with the same lipopolysaccharide. To explain this nonclassical tolerance response in regard to tumor necrosis factor-α secretion, the authors suggested that with relatively weak inducers of inflammation, such as *P. gingivalis* lipopolysaccharide, which are unlikely to cause excessive inflammation on their own, the innate immune system might be programmed to keep sensing their presence and maintaining cytokine responses. More recently, Muthukuru *et al*. (27) showed that a challenge of human peripheral blood monocytes with *P. gingivalis* lipopolysaccharide, following a first stimulation, induced the downregulation in expression of Toll-like receptor-2 and -4 and a reduction in tumor necrosis factor-α secretion. This discrepancy between both studies suggests that the tolerance response associated with *P. gingivalis* lipopolysaccharide is probably influenced by the cell type and the lipopolysaccharide preparation.

The cellular and molecular mechanisms of tolerance induced by the pretreatment of immune cells with low doses of lipopolysaccharide are complex. The decreased secretion of mediators associated with the tolerance phenomenon has been linked to alterations in signal events (14,28). Phosphatidylinositol-3′-kinase, an enzyme complex, regulates a pathway involved in the expression of various lipopolysaccharide-induced inflammatory genes and lipopolysaccharide tolerance (28,29). It behaves as an early inhibitor of Toll-like receptor signaling (30). Both phosphatidylinositol-3′-kinase inhibitors (wortmannin and LY294002) tested attenuated the interleukin-1β response to subsequent lipopolysaccharide stimulation when added during the tolerance-induction period (Table 1). In contrast, the phosphatidylinositol-3′-kinase inhibitors had no significant effect on the tumor necrosis factor-α response to subsequent lipopolysaccharide stimulation. Our data suggest that when macrophages are rechallenged with *A. actinomycetemcomitans* lipopolysaccharide, the secretion of tumor necrosis factor-α (decreased) and interleukin-β (increased) is probably modulated through at least two independent signaling pathways.

Table 1. Effect of the presence of phosphatidylinositol-3′-kinase inhibitors during the pretreatment with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide on the secretion of tumor necrosis factor-α and interleukin-1β by macrophages following a subsequent lipopolysaccharide stimulation

	Relative secretion	
Assay conditions	TNF-α	IL-1β
No LPS pretreatment	100%	100%
With LPS pretreatment		
No inhibitor	63%*	333%*
+ wortmannin	66%*	167%*,†
+ LY294002	61%*	143%*,†

*$p < 0.02$ compared with cells not pretreated with lipopolysaccharide.
†$p < 0.02$ compared with lipopolysaccharide-pretreated cells in the absence of inhibitors
IL-1β, interleukin-1β; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

Monocytes and macrophages, which are present in higher numbers in active periodontal lesions than in inactive sites (31), are key members of the innate immune system and play a critical role in the host response during chronic infections such as periodontitis (1). Lipopolysaccharide is a potent stimulator of these cells and can induce the secretion of numerous inflammatory mediators. Pro-inflammatory cytokine induction may be a double-edged sword. Beyond their beneficial effect, the uncontrolled secretion of such mediators may have deleterious effects on the host (32). Lipopolysaccharide tolerance is characterized by a state of transient hyporesponsiveness of immune cells to subsequent challenges by lipopolysaccharide and may potentially limit excessive inflammation. However, one should consider that although the secretion of specific pro-inflammatory cytokines may be reduced, the production of other inflammatory mediators or tissue-degrading enzymes may be enhanced, as demonstrated in our study. It is still not clear whether lipopolysaccharide tolerance has protective and/or deleterious effects on the host. On the one hand, endotoxin tolerance has been associated with increased resistance to and protection against tissue damage in various models (11). On the other hand, other studies suggested that the induction of lipopolysaccharide tolerance may impair resistance to infectious processes (11).

In summary, our study showed that the pretreatment of phorbol myristic acid-differentiated U937 macrophages with low doses of *A. actinomycetemcomitans* lipopolysaccharide resulted in a significantly decreased secretion of tumor necrosis factor-α and an increased secretion of interleukin-1β and MMP-9 following subsequent lipopolysaccharide stimulation of the macrophages. This phenomenon may modulate the inflammatory status of periodontitis and contribute to the episodic nature of the disease, which involves active and inactive phases of tissue destruction.

Acknowledgements

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