J Periodont Res 2009; 44: 503–510

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01141.x

Treponema denticola peptidoglycan induces the production of inflammatory mediators and matrix metalloproteinase 9 in macrophage-like cells

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Tanabe S-I, Bodet C, Grenier D. Treponema denticola *peptidoglycan induces the production of inflammatory mediators and matrix metalloproteinase 9 in macrophage-like cells. J Periodont Res 2009; 44: 503–510. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard*

Background and Objective: Treponema denticola is a key pathogen associated with periodontitis, a chronic inflammatory disease affecting tooth-supporting tissues. In the present study, we investigated the response of human macrophage-like cells to stimulation by peptidoglycan isolated from *T. denticola*. We also studied the effect of the peptidoglycan preparation on the phosphorylation state of kinases.

Material and Methods: Monoblastic leukemia cells (U937 strain) were differentiated into adherent macrophage-like cells using phorbol myristic acid prior to being stimulated for 6 or 24 h with various amounts of *T. denticola* peptidoglycan. Secreted inflammatory mediators were quantified by enzyme-linked immunosorbent assays. The phosphorylation state of kinases was determined by immunoblotting.

Results: The *T. denticola* peptidoglycan preparation, which was non-toxic for macrophage-like U937 leukemia cells at the concentration used, significantly increased, in a dose-dependent manner, the secretion of the pro-inflammatory cytokines tumor necrosis factor α, interleukin-1β and interleukin-6. It also increased the secretion of two potent chemokines, interleukin-8 (IL-8) and regulated on activation normal T cell expressed and secreted (RANTES). *T. denticola* peptidoglycan also induced a significant increase in the secretion of prostaglandin E_2 and matrix metalloproteinase 9 by macrophage-like cells. The phosphorylation state of several kinases, including extracellular regulated protein-serine kinase 2 (+99%), G protein-coupled receptor-serine kinase 2 (+50%), Yes-related protein-tyrosine kinase (+44%) and extracellular regulated protein-serine kinase 1 (+30%) also increased following stimulation with the peptidoglycan preparation.

Conclusion: T. denticola peptidoglycan activates intracellular signaling pathways, leading to an increased production of inflammatory mediators by macrophage-like cells.

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Key words: periodontitis; macrophage; *Treponema denticola*; peptidoglycan; cytokine; kinase

Accepted for publication June 9, 2008

Periodontitis is a multifactorial, polymicrobial infection characterized by a destructive inflammatory process affecting tooth-supporting tissues and resulting in periodontal pocket formation, alveolar bone resorption and, eventually, tooth mobility and tooth loss. It is initiated by an overgrowth of specific gram-negative anaerobic bacterial species, called periodontopathogens, colonizing the subgingival sites (1). The host response to periodontopathogens and their secreted and/or cell-associated products is a critical determinant in the progression of periodontitis (2). More specifically, the continuous, high secretion of cytokines, chemokines, prostaglandin E_2 (PGE_2) and matrix metalloproteinases (MMPs) by host cells following stimulation by periodontopathogens modulates periodontal tissue destruction and disease progression (3).

Treponema denticola and some other spirochetes play a major role in the development and progression of periodontitis (4–6). The fact that the serum of patients with periodontitis contains higher levels of anti-*T. denticola* antibodies than that of healthy subjects provides indirect support for this role (7). *T. denticola* produces virulence factors, including proteolytic enzymes, that can promote the invasion and destruction of periodontal tissues (8). It also produces a cell-associated lipooligosaccharide that stimulates osteoclastogenesis and MMP expression in a mouse calvaria–bone marrow cell co-culture model (9) and induces inflammatory mediator production by murine macrophages (10). In a previous study, we found that a significant proportion of the cells in *T. denticola* cultures possess wheat germ agglutinin (WGA)-binding activity and that this activity resides in the peptidoglycan fraction (11). Peptidoglycans are thought to be responsible for maintaining the coiled shape of spirochetes (12,13). While *T. denticola* lipooligosaccharide has a negligible toxic effect on periodontal ligament epithelial cells, *T. denticola* peptidoglycan is highly toxic in a time- and concentration-dependent manner (14). Peptidoglycans from other spirochetes, including *Leptospira interrogans*, induce human monocytes to release tumor necrosis factor α (TNF-α; 15). The present study was undertaken to evaluate the inflammatory response of human macrophage-like cells to stimulation by *T. denticola* peptidoglycan. The effect of the peptidoglycan preparation on the phosphorylation state of kinases was also studied.

Material and methods

Bacteria and peptidoglycan preparation

T. denticola ATCC 35405 was grown in new oral spirochete (NOS) medium (16) at 37°C for 4 days in an anaerobic chamber (N_2–H_2–CO_2, 80:10:10) at 37°C. A peptidoglycan fraction of *T. denticola* was prepared using a slight modification of the method of Heckels & Virji (17). The protocol is adapted for gram-negative bacteria and provides pure protein-, lipoprotein- and lipopolysaccharide (LPS)-free peptidoglycan (17). Briefly, 1 g of bacterial cells (dry weight) was suspended in 15 mL of cold water and added dropwise to 15 mL of boiling 8% sodium dodecyl sulfate (SDS). The suspension was boiled for 30 min and then incubated overnight at room temperature. The suspension was then centrifuged at $90{,}000 \times g$ for 2 h and the pellet resuspended in 10 mL of cold water. The SDS treatment was repeated twice, and the final pellet was resuspended in 9 mL of 10 mM Tris-HCl (pH 7). Pronase and proteinase K (2 mg each) were added to 1 mL of the suspension, and the mixture was incubated for 2 h at 50°C and then at 37°C for 16 h. The SDS treatment was repeated once more and the suspension was centrifuged at $90{,}000 \times g$ for 2 h. The pellet, which contained the peptidoglycan fraction, was washed three times in 50 mM phosphate-buffered saline (PBS, pH 7) and resuspended in 5 mL of distilled water. The fraction was extensively dialyzed against distilled water, freeze-dried, and stored at −20°C until used. A *Limulus* amebocyte lysate assay (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), which detects 0.05 endotoxin units/mL, was used to show that the peptidoglycan preparation was free of lipopolysaccharide-like contaminants. Using the method described by Hadzija (18), the preparation was found to contain muramic acid, a specific marker for peptidoglycan. This latter method is based on the colorimetric determination of lactyl groups following acid hydrolysis to remove peptides in peptidoglycan and treatment with NaOH to hydrolyze the ether bond of the lactyl groups in muramic acid. Commercial colorimetric tests (Bio-Rad Laboratories, Ltd, Mississauga, ON, Canada) were used to confirm that the peptidoglycan preparation contained no detectable DNA or protein. The Complex Carbohydrate Research Center (Athens, GA, USA) determined glycosyl composition of the peptidoglycan preparation by combined gas chromatography and mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. This analysis revealed that *N*-acetyl glucosamine and glucose represented 75 and 13% of total carbohydrates, respectively. Lastly, the colorimetric assay of Arand *et al*. (19) was used to confirm that no residual SDS remained in the preparation.

Monocyte cultures and differentiation into macrophage-like cells

A monoblastic leukemia cell line, U937 cells (ATCC CRL-1593.2), were cultivated at 37°C in a 5% CO_2 (95% air) atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; RPMI-FBS, HyClone Laboratories, Logan, UT, USA) and 50 μg/mL of penicillin-streptomycin (Sigma-Aldrich Canada Ltd). Monocytes (2×10^5 cells/mL) were incubated in RPMI-FBS containing 10 ng/mL of phorbol myristic acid (PMA; Sigma-Aldrich Canada Ltd) for 48 h to induce differentiation into adherent macrophage-like cells. Phorbol myristic acid induces the appearance of cell characteristics consistent with mature macrophages, as previously reported (20). Following the PMA treatment, the medium was

replaced with fresh medium, and the differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophage-like cells were suspended in RPMI-FBS, centrifuged at $300 \times g$ for 5 min, washed, and suspended in RPMI supplemented with 1% heat-inactivated FBS to a density of 1×10^6 cells/mL. The cells were incubated in six-well plates (2×10^6 cells per well in 2 mL) at 37°C in a 5% CO_2 (95% air) atmosphere for 2 h prior to stimulation.

Macrophage-like cell stimulation

The monocyte-derived macrophage-like cells were stimulated with 0.1, 1 or 5 μg/mL of the *T. denticola* peptidoglycan preparation for 6 or 24 h at 37°C in a 5% CO_2 (95% air) atmosphere. The culture medium supernatants were collected and stored at −20°C until used. Cells incubated in culture medium in the absence of *T. denticola* peptidoglycan were used as controls.

Cell viability

Viability of macrophage-like cells was evaluated using an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Briefly, macrophage-like cells were incubated in 96-well plates (1×10^5 cells per well in 100 μL) at 37°C in a 5% CO_2 (95% air) atmosphere for 2 h prior to stimulation with the *T. denticola* peptidoglycan preparation, as described in the previous subsection. After 6 or 24 h, the cells were incubated with MTT for 4 h, and the insoluble formazan dye was solubilized overnight at 37°C. The absorbance was read at 550 nm using a microplate reader (Modal-680, Bio-Rad Laboratories, Ltd, Missasauga, ON, Canada) with the wavelength correction set at 650 nm.

Cytokine, chemokine and MMP-9 production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-8, TNF-α, regulated on activation normal T cell expressed and secreted (RANTES) and matrix metalloproteinase 9 (MMP-9) concentrations in the cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (Modal-680, Bio-Rad Laboratories, Ltd, Missasauga, ON, Canada) with the wavelength correction set at 550 nm. The rated sensitivities of the commercial ELISA kits were 3.9 pg/mL for IL-1β, 9.3 pg/mL for IL-6, 31.2 pg/mL for IL-8, 15.6 pg/mL for TNF-α and RANTES, and 0.31 ng/mL for MMP-9.

Prostaglandin E_2 production

A competitive enzyme immunoassay was performed on the supernatant fluids according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance at 415 nm was read using a microplate reader (Modal-680, Bio-Rad Laboratories, Ltd, Missasauga, ON, Canada). The rated sensitivity of the kit was 7.8 pg/mL.

Analysis of kinase phosphorylation

Kinetworks™ Phosphosite Screen 11.0 (KPSS 11.0), which provides quantitative information on 37 phosphorylation sites in protein kinases, was performed by Kinexus Bioinformatics (Vancouver, BC, Canada). The Kinetworks™ analysis uses highly specific immunological reagents and has been specially optimized to detect band shifts in signaling proteins on SDS-PAGE gels that are caused by phosphorylation. Additional details can be found at http://www.kinexus.ca. Macrophage-like cells were treated with 5 μg/mL of the *T. denticola* peptidoglycan preparation for 90 min and compared with unstimulated control cells. After the incubation, cell lysates were prepared according to the Kinexus protocol. Briefly, cells were washed twice with ice-cold PBS and homogenized at 4°C in a buffer containing 20 mM 3-(*N*-morpholino)-propanesulfonic acid (Mops; pH 7.0), 2 mM ethylene glycol tetraacetic acid (EGTA), 5 mM ethylene glycol tetraacetic acid (EDTA), 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin and 0.5% Nonidet P-40 (Sigma-Aldrich Canada Ltd). The cells were lysed by sonication on ice, and the lysates were centrifuged at $136{,}000 \times g$ for 30 min at 4°C in an LE-80K ultracentrifuge (Beckman, Mississauga, ON, Canada). Protein concentrations in the lysate supernatant fractions were estimated using the Bradford assay (Bio-Rad Laboratories, Ltd) and were adjusted to 2 mg/mL. The KPSS 11.0 analyses of the supernatants were performed by Kinexus. Changes in phosphorylation states were determined based on the intensities of individual phosphoprotein bands. The levels of phosphorylated proteins were expressed as counts per minute (c.p.m.) normalized to correct for differences in the amount of protein. As recommended by the manufacturer, differences greater than 25% were considered significant.

Statistical analyses

Two independent experiments were performed, and assays were carried out in triplicate. Statistical analyses were performed using Student's paired *t*-test. Results were considered significant at $p < 0.02$.

Results

The *T. denticola* peptidoglycan preparation was minimally toxic for macrophage-like U937 leukemia cells after 6 and 24 h. At the highest concentration tested (5 μg/mL), cell viability decreased by less than 18% after 24 h (data not shown). The ability of the preparation to induce the secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 was examined first (Fig. 1). The *T. denticola* peptidoglycan-induced secretion of IL-1β and IL-6 was time dependent. Significant increases in the secretion of TNF-α,

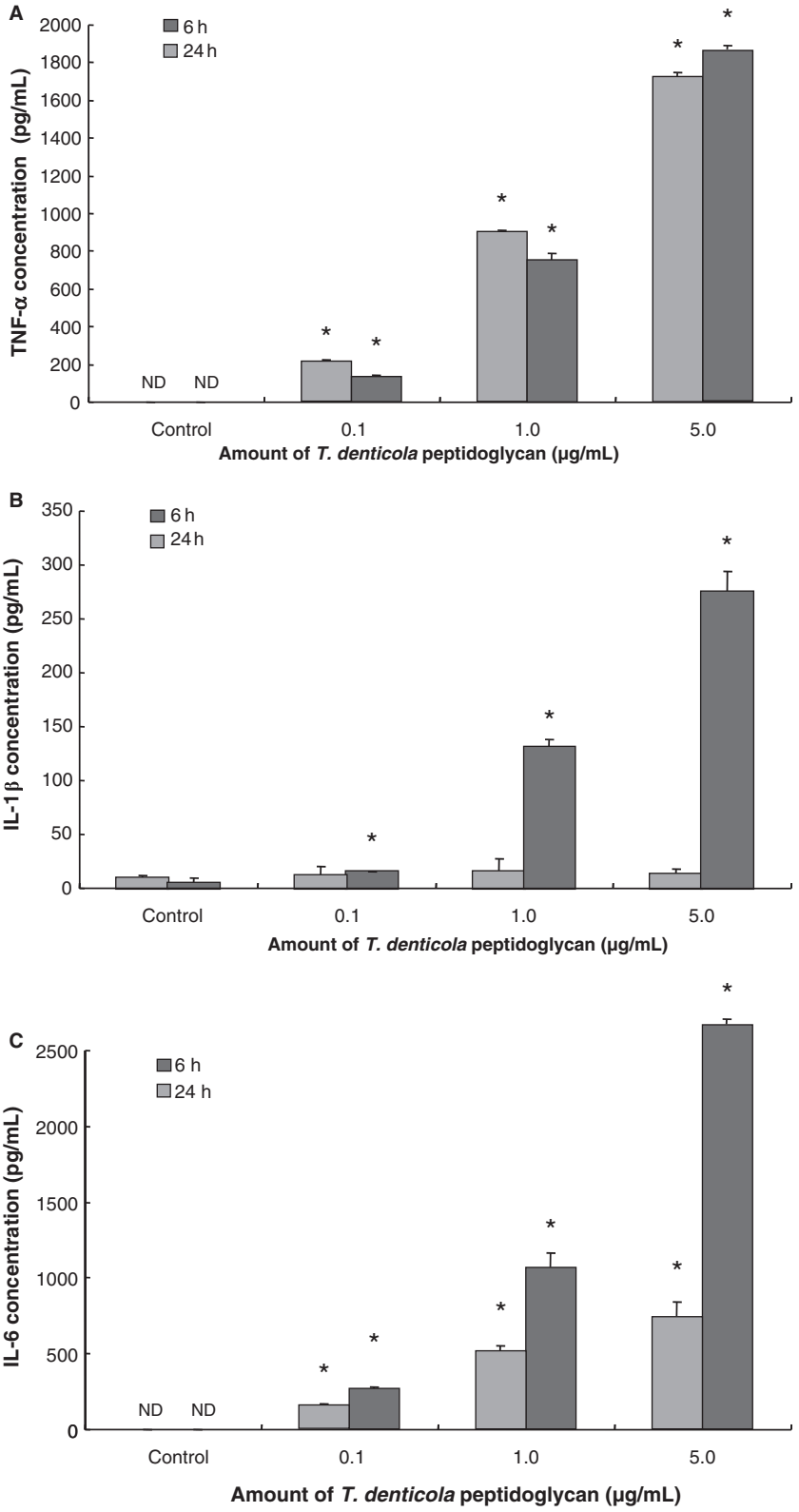

Fig. 1. Secretion of pro-inflammatory cytokines TNF-α (A), IL-β (B) and IL-6 (C) by macrophages stimulated with *T. denticola* peptidoglycan. Cytokine concentrations were determined by ELISA. Data are the means + SD of triplicate assays. Asterisks indicate a significant difference between control and stimulated macrophages ($p < 0.02$). ND, not detected.

IL-1β and IL-6 by macrophage-like cells were observed following stimulation with 1 and 5 μg/mL of the peptidoglycan preparation for 6 and 24 h (Fig. 1). The amounts of TNF-α and IL-6 secreted following stimulation with 5 μg/mL of peptidoglycan for 24 h were 1872 ± 16 and 1973 ± 35 pg/mL, respectively, whereas no TNF-α or IL-6 was detected in the absence of peptidoglycan (Fig. 1A,C). The amount of IL-1β secreted following stimulation with 5 μg/mL of peptidoglycan for 24 h increased approximately 55-fold compared with unstimulated macrophage-like cells (Fig. 1B).

The effect of the peptidoglycan preparation on the secretion of the chemokines IL-8 and RANTES is presented in Fig. 2. The preparation induced a strong dose-dependent IL-8 response over the 24 h stimulation period. After 24 h, 5 μg/mL of peptidoglycan had induced the secretion of 344 ± 9 ng/mL of IL-8, whereas no IL-8 was detected in the absence of peptidoglycan (Fig. 2A). In the same stimulatory conditions, the amount of RANTES secreted was approximately 17-fold greater than that of unstimulated macrophage-like cells (Fig. 2B).

The effects of the *T. denticola* peptidoglycan preparation on PGE_2 and MMP-9 secretion are presented in Fig. 3A,B. The amount of PGE_2 secreted increased significantly after a 6 or 24 h stimulation in the presence of 1 and 5 μg/mL of the peptidoglycan preparation, while the amount of MMP-9 secreted only increased significantly (2-fold) after 24 h (Fig. 3B). Secretion of MMP-9 was comparable for all the peptidoglycan concentrations tested.

The U937 cells, differentiated into adherent macrophages by a 48 h treatment with PMA, were washed and further incubated (24 h) in fresh medium prior to stimulation with *T. denticola* peptidoglycan. Changes in the phosphorylation state of signaling proteins were then monitored by detecting band shifts following SDS-PAGE immunoblotting analysis. Table 1 reports kinases showing an increase greater than 25% between non-treated and peptidoclycan-treated macrophage-like cells. *T. denticola* peptidoglycan induced an

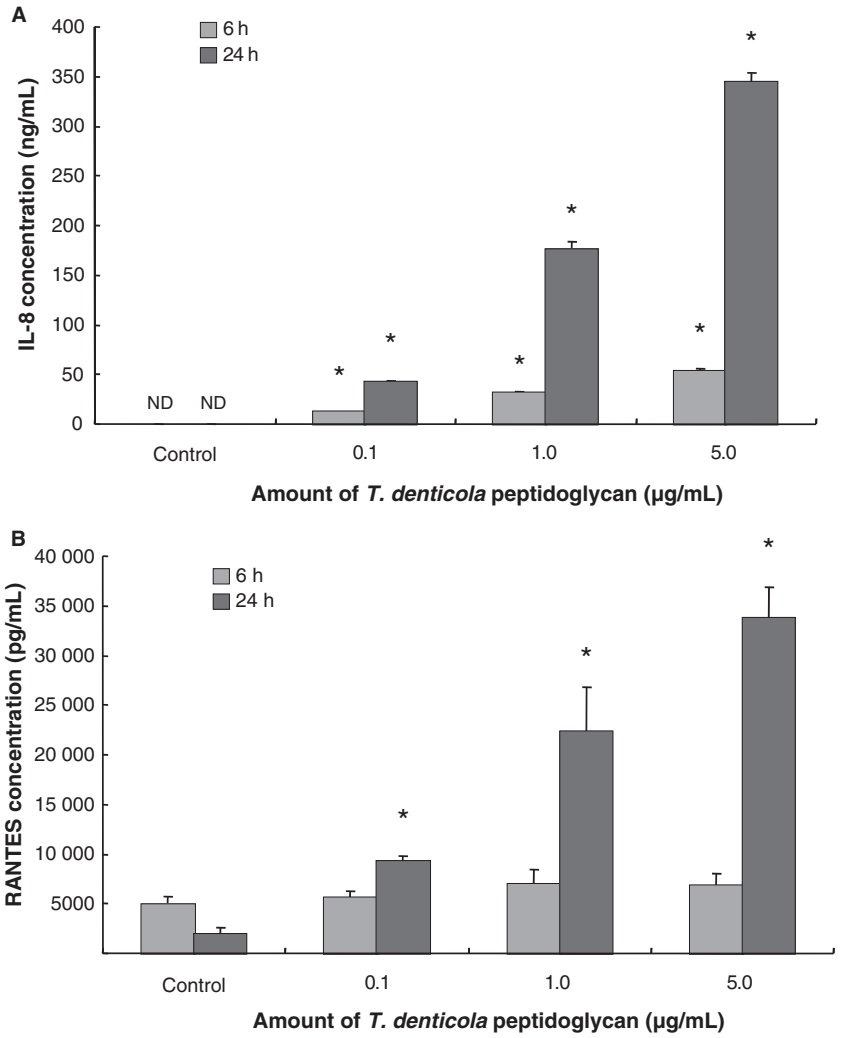

Fig. 2. Secretion of chemokines IL-8 (A) and RANTES (B) by macrophages stimulated with *T. denticola* peptidoglycan. Chemokine concentrations were determined by ELISA. Data are the means + SD of triplicate assays. Asterisks indicate a significant difference between control and stimulated macrophages ($p < 0.02$). ND, not detected.

increase in the phosphorylation state of extracellular regulated protein-serine kinase 2 (ERK2, +99%), G protein-coupled receptor-serine kinase 2 (GRK2, +50%), Yes-related protein-tyrosine kinase (Lyn, +44%) and extracellular regulated protein-serine kinase 1 (ERK1, + 30%).

Discussion

Monocytes and macrophages, which are present in higher numbers in active periodontal lesions than in inactive sites (21), are key members of the innate immune system and play a critical role in the host response during chronic infections such as periodontitis (3). Lipopolysaccharides from gram-negative periodontopathogenic bacteria are highly inflammatory and activate responsive cells, leading to production of inflammatory mediators (22). Bacterial peptidoglycan, which was once thought to be an inert material, is now considered a potent immunostimulatory constituent (23). Both gram-negative and gram-positive bacteria possess peptidoglycans with comparable features. However gram-negative peptidoglycans are much thinner (one or two layers) than peptidoglycans from gram-positive bacteria (≥ 10 layers; 24). Peptidoglycans trigger innate immune cells and induce the release of cytokines via interactions with Toll-like receptors and nucleotide-binding oligomerization domain proteins (23,25). Peptidoglycans from a number of periodontopathogens have deleterious effects on macrophages, including loss of viability and increased secretion of PGE_1 and PGE_2 (26).

A positive relationship between the frequency of detection and population load of spirochetes, more particularly *T. denticola*, and the severity of periodontitis has been previously reported (4). Since cell surface peptidoglycan is an important component of *T. denticola* (11,13) which is likely to come into contact with host cells during periodontitis, there is a need for a better knowledge of its biological properties. In this study, we assessed the capacity of *T. denticola* peptidoglycan to induce an inflammatory response in macrophage-like cells. More specifically, we characterized the secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokines (IL-8 and RANTES), MMP-9 and PGE_2, by human monocyte-derived macrophage-like cells in response to a challenge by *T. denticola* peptidoglycan. We also investigated the phosphorylation of macrophage-like cell kinases induced by the peptidoglycan preparation.

The *T. denticola* peptidoglycan preparation used in this study was found to be devoid of LPS-like components and to contain both muramic acid and *N*-acetyl glucosamine, two important constituents of peptidoglycan. Using a similar protocol, Caimano and co-workers (13) prepared a peptidoglycan fraction from *T. denticola* and reported that ornithine, glutamine or glutamic acid, glycine and alanine were the principal amino acids and were present at molar ratios characteristic of spirochetal peptidoglycans. *T. denticola* peptidoglycan stimulated the secretion of TNF-α and IL-1β by macrophage-like cells. These two pro-inflammatory cytokines are critical determinants in the progression of periodontitis (27). They can induce the expression of adhesion molecules and mediators that facilitate and amplify the inflammatory response, MMP production and bone resorption (27). Stashenko and colleagues (28) reported a highly significant correlation between the levels of TNF-α

and IL-1β in the periodontal tissues of diseased sites, suggesting that the expression of these two mediators is coordinated. The *T. denticola* peptidoglycan preparation also induced the secretion of significant amounts of IL-6 by macrophage-like cells. Interleukin-6 promotes bone resorption (29) and acts as a potent inducer of osteoclast formation *in vitro* (30), suggesting that it may contribute to the bone resorption associated with periodontitis. There have been few studies on the pro-inflammatory cytokine-stimulating ability of spirochetal peptidoglycans. However, the peptidoglycan of *Borrelia burgdorferi* has been shown to be a powerful immunomodulator that stimulates macrophages to produce IL-1 (31), while *Leptospira interrogans* peptidoglycan can trigger human monocytes, leading to release of TNF-α (15).

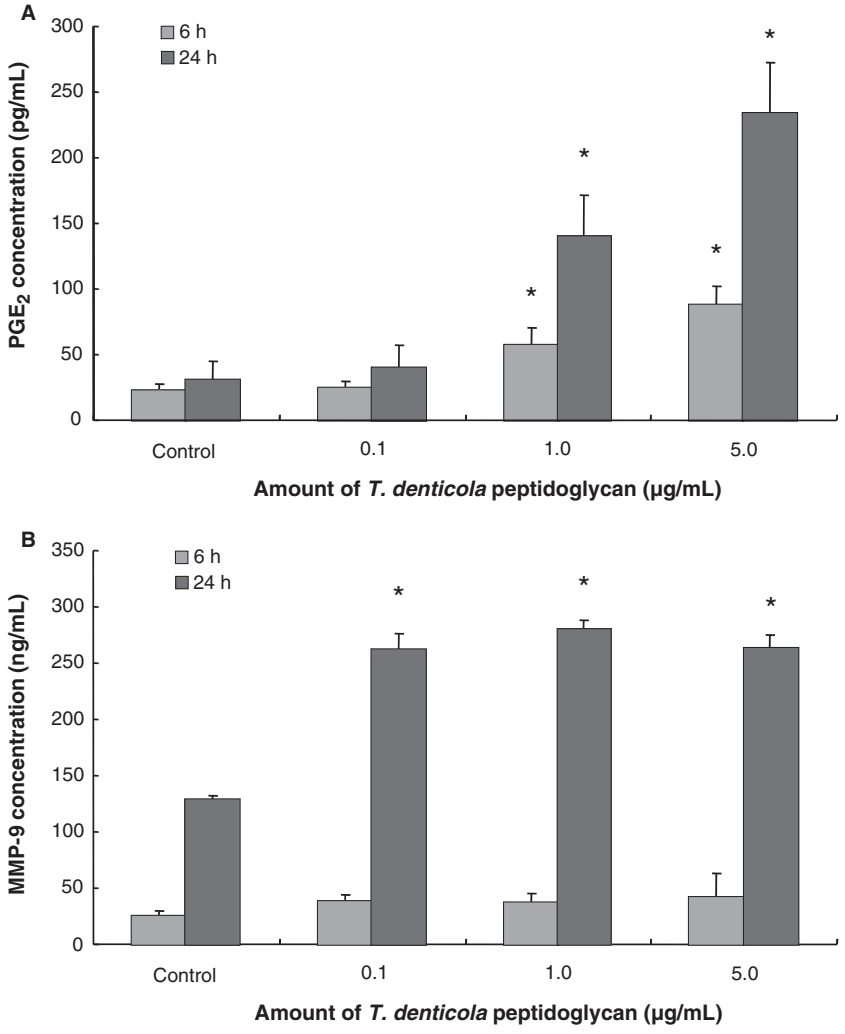

Fig. 3. Secretion of PGE_2 (A) and MMP-9 (B) by macrophages stimulated with *T. denticola* peptidoglycan. The PGE_2 and MMP-9 concentrations were determined by ELISA. Data are the means + SD of triplicate assays. Asterisks indicate a significant difference control and stimulated macrophages ($p < 0.02$).

T. denticola peptidoglycan induced an IL-8 and RANTES response in macrophage-like cells. Interleukin-8 and RANTES are potent chemokines that direct the migration of neutrophils, eosinophils, monocytes and T-helper 1 cells to sites of infection (32). The stimulation of chemokine production by periodontopathogens favors the accumulation of leukocytes during active inflammation, which contributes to periodontal tissue destruction. High levels of RANTES have been detected in gingival crevicular fluid and in inflamed gingival tissue adjacent to periodontal pockets of adult patients with periodontitis, indicating that RANTES is important in the initiation and progression of periodontitis (33,34). Higher levels of IL-8 are also found in the gingival crevicular fluid of inflamed periodontal sites (35).

Immunoblotting analyses showed that *T. denticola* peptidoglycan induced changes in the phosphorylation state of macrophage-like cell kinases. Our peptidoglycan preparation increased the

Table 1. Changes in the phosphorylation state of macrophage kinases induced by *Treponema denticola* peptidoglycan

Protein				Signal (% change from control)
Full name	Abbreviation	Epitope	Molecular weight (kDa)	Control vs. *T. denticola* peptidoglycan
Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)	ERK2	T185 + Y187	40	+99
G protein-coupled receptor-serine kinase 2 (BARK1)	GRK2	S670	75	+50
Yes-related protein-tyrosine kinase	Lyn	Y507	46	+44
Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)	ERK1	T202 + Y204	42	+30

Kinexus Phosphosite Kinase Screen 11.0 (KPSS 11.0) was used to screen for these changes. Unstimulated macrophages were used as a control. Experimental macrophages were stimulated with 5 μg/mL *T. denticola* peptidoglycan for 90 min.

phosphorylation of GRK2 on serine 670, suggesting that GRK2 is involved in the signal transduction induced by *T. denticola* peptidoglycan. G protein-coupled receptor-serine kinase 2 is known to selectively phosphorylate G protein-coupled receptors (GPCRs), which play a critical role in inflammatory process signaling (36) and thus determine the rate and extent of GPCR desensitization and resensitization (37). *T. denticola* peptidoglycan also activated ERK1 and ERK2, as well as Lyn, which has been involved in production of inflammatory mediators (38–40). This is consistent with previous studies that reported that ERK1, ERK2 and Lyn are phosphorylated following peptidoglycan stimulation of macrophages (41,42). The fact that the phosphorylation state of other important kinases was not significantly increased by *T. denticola* peptidoglycan may be related to the PMA treatment used to induce differentiation into macrophage-like cells prior to stimulation.

The present study showed that *T. denticola* peptidoglycan can activate various intracellular signaling pathways in macrophage-like cells, leading to increased production of pro-inflammatory cytokines and chemokines. In addition to peptidoglycan, it is likely that other cell surface virulence factors of *T. denticola*, such as hemin-binding proteins (43), major outer sheath protein (44) and dentilisin (45), also induce inflammatory responses in macrophages. *T. denticola* peptidoglycan, by stimulating the secretion of pro-inflammatory cytokines and chemokines in macrophages, can activate the host-mediated destructive processes observed during periodontitis. This suggests that *T. denticola* has the potential to participate in periodontal tissue destruction, probably by acting in concert with other periodontopathogens.

Acknowledgements

This study was supported in part by the Canadian Institutes of Health Research (MOP-79364) and the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

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