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Major surface protein complex of *Treponema denticola* induces the production of tumor necrosis factor α, interleukin-1β, interleukin-6 and matrix metalloproteinase 9 by primary human peripheral blood monocytes

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Background and Objective: Treponema denticola is a micro-organism that is involved in the pathogenesis of periodontitis. Major surface protein complex (MSPc), which is expressed on the envelope of this treponeme, plays a key role in the interaction between *T. denticola* and gingival cells. The peptidoglycan extracted from *T. denticola* induces the production of a large variety of inflammatory mediators by macrophage-like cells, suggesting that individual components of *T. denticola* cells induce the inflammatory response during periodontal disease. This study was designed to demonstrate that MSPc of *T. denticola* stimulates release of proinflammatory mediators in primary human monocytes.

Material and Methods: Primary human monocytes were separated from the blood of healthy donors and incubated for up to 24 h with varying concentrations of MSPc. The production of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and matrix metalloproteinase 9 (MMP-9) was measured at different time points with commercially available enzyme-linked immunosorbent assays.

Results: T. denticola MSPc induced the synthesis of TNF-α, IL-1β, IL-6 and MMP-9 in a dose- and time-dependent manner. Similar patterns of TNF-α, IL-1β and IL-6 release were observed when cells were stimulated with 100 and 1000 ng/mL of MSPc. The production of MMP-9 was significant only when cells were treated with 1000 ng/mL of MSPc.

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Conclusion: These results indicate that *T. denticola* MSPc, at concentrations ranging from 100 ng/mL to 1.0 μg/mL, activates a proinflammatory response in primary human monocytes.

Key words: monocyte; *Treponema denticola*; major surface protein; cytokine

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Periodontitis is a chronic inflammatory process in the gingival tissues that results in bone resorption and destruction of the connective tissue. Periodontitis is characterized by a polymicrobial infection by several species of anaerobic gram-negative bacteria (1). Spirochetes are frequently found within the periodontal pocket in patients suffering from periodontitis (2), and *Treponema denticola* is among the most frequently isolated oral spirochetal species in these patients. This treponeme is frequently involved in the pathogenesis and progression of periodontal diseases (3).

The enzymes on the outer membrane of *T. denticola* contribute to the tissue damage that accompanies periodontal disease. Chymotrypsin-like protease (CTLP; 4), major surface protein complex (MSPc) and cystalysin (5) are the major bacterial factors responsible for the cytopathic effect of *T. denticola* (6–8). Chymotrypsin-like protease is a cytotoxic molecule that exhibits specific enzymatic activity for a large spectrum of substrates (4,6,9). Major surface protein complex is a transmembrane antigen with pore-forming and adhesion activity that elicits a specific cytotoxic effect in different cell types (6,8). In addition, MSPc stimulates the release of the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β; 10) from murine macrophages and induces murine erythrocyte lysis, *in vitro* (6).

Cytokines are released in the periodontium following the accumulation of bacteria (3,11–13). Recently, Tanabe *et al*. demonstrated that the peptidoglycans of *T. denticola* activate a macrophage-like cell line to secrete TNF-α, IL-1β, interleukin-6 (IL-6), interleukin-8 (IL-8), regulated on activation, normal T expressed and secreted, prostaglandin E_2 and matrix metalloproteinase 9 (MMP-9; 14). Since peptidoglycans induced a proinflammatory response in a macrophage-like cell line, we were prompted to investigate the role of *T. denticola* MSPc in stimulating human peripheral blood monocytes that are present in periodontal lesions (15) to produce proinflammatory cytokines and MMP-9.

Material and methods

Bacterial culture and preparation of MSPc

The *T. denticola* strain ATCC 35405 (kindly provided by Jacques Izard, The Forsyth Institute, Boston, MD, USA) was grown in new oral spirochetes medium at 37°C in anaerobic conditions for 4 d. The extraction and purification of MSPc was performed as previously described (6,8).

Limulus test

Preparations of MSPc were tested for contamination by lipopolysaccharide-

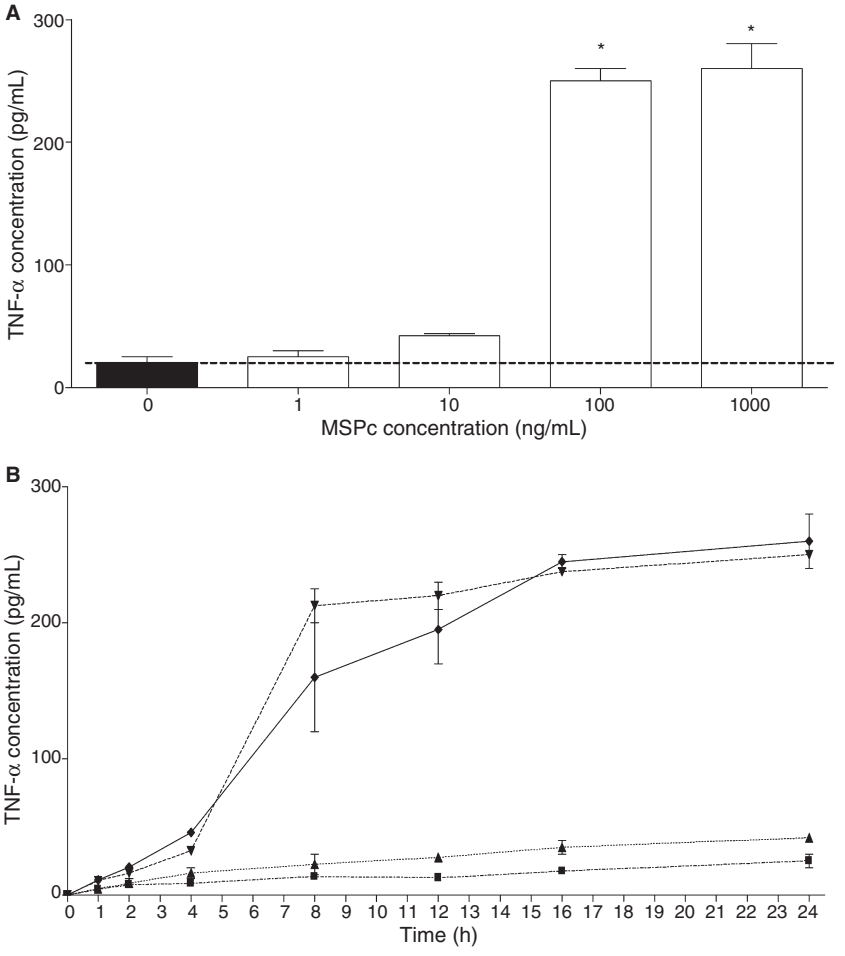

Fig. 1. (A) Tumor necrosis factor α release by human peripheral blood monocytes stimulated with different concentrations of *T. denticola* MSPc after 24 h. The results are representative of three independent experiments and are expressed as the mean + SD (error bars) of three replicates. The asterisks indicate which MSPc concentrations achieved statistically significant differences ($p < 0.01$) in the cytokine response between the MSPc-stimulated monocytes and unstimulated (control) monocytes according to the ANOVA and Bonferroni *post hoc* test. (B) Tumor necrosis factor α levels in the cell supernatant at different time points after stimulation with different concentrations of MSPc. The graph shows production of TNF-α by monocytes stimulated with 1 (■), 10 (▲), 100 (▼) and 1000 ng/mL (♦) of MSPc.

like material using the Limulus amebocyte lysate assay (LAL test; International PBI, Milan, Italy), following the manufacturer's instructions.

Monocyte isolation

Peripheral blood monocytes, obtained from healthy blood donors spontaneously attending the Sant'Orsola-Malpighi central blood bank, were separated from the buffy coats by Histopaque-1077 (Sigma-Aldrich Chemicals, St Louis, MO, USA) density gradient centrifugation according to the manufacturer's instructions. The cells were then separated using the Miltenyi monocyte isolation kit II human (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions, and characterized by flow cytometric analysis with anti-CD14 fluorescein isothiocyanate-labeled monoclonal antibody and anti-CD163 phycoerythrin-labeled monoclonal antibody (BD Bioscience, Milan, Italy). The resulting enriched CD14-positive cell fraction was resuspended in Dulbecco's modified minimal essential medium (DMEM; EuroClone–Celbio, Milan, Italy) supplemented with 10% fetal bovine serum, 1% (vol/vol) L-glutamine (EuroClone–Celbio) and 1% (vol/vol) penicillin plus streptomycin (EuroClone). The cell suspension was stained with Trypan blue to determine the number of surviving cells. The cells were plated into individual wells of a 24-well plate at a concentration of 1.5×10^5 cells per well and incubated for 2 h.

Monocyte stimulation

Before performing each stimulation experiment and after 24 h of incubation, one well per series was stained with Trypan blue to determine the number of surviving cells. The CD14-positive cells were stimulated with 1, 10, 100 or 1000 ng/mL of *T. denticola* MSPc in DMEM at 37°C in air containing 5% CO_2. The culture supernatants were collected after 2, 4, 8, 12, 16 and 24 h of incubation and stored at −80°C until use for cytokine and MMP-9 measurement. Cells incubated in culture medium in the absence of *T. denticola* MSPc were used as negative controls.

Cytokine and MMP-9 assay

The amounts of TNF-α, IL-1β, IL-6 and MMP-9 in the supernatants were determined at each time point with an enzyme-linked immunosorbent assay (Instant ELISA Bender MedSystem, Wien, Austria) according to the manufacturer's instructions.

Statistical analysis

Each experiment was performed three times in triplicate. Means and standard deviation were calculated for group comparisons within and between experiments. The one-way ANOVA test was performed using GRAPHPAD 4.0 software (GrapPad Software Inc., La Jolla, CA, USA). To confirm the results, a Bonferroni *post hoc* test was also performed.

Results

The MSPc, obtained by sequential detergent extraction and autoproteolysis of the *T. denticola* membrane, existed primarily as an oligomeric peptide that had a molecular weight of 53 kDa, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after heating at 100°C for 5 min (data not shown). The MSPc preparations did not contain any lipopolysaccharide-like material, as measured by Limulus amebocyte lysate assay.

After peripheral blood monocyte isolation, more than 87% of the cells were CD14-positive and were 100% viable. After the 24 h incubation, more than 80% of the cells were still viable,

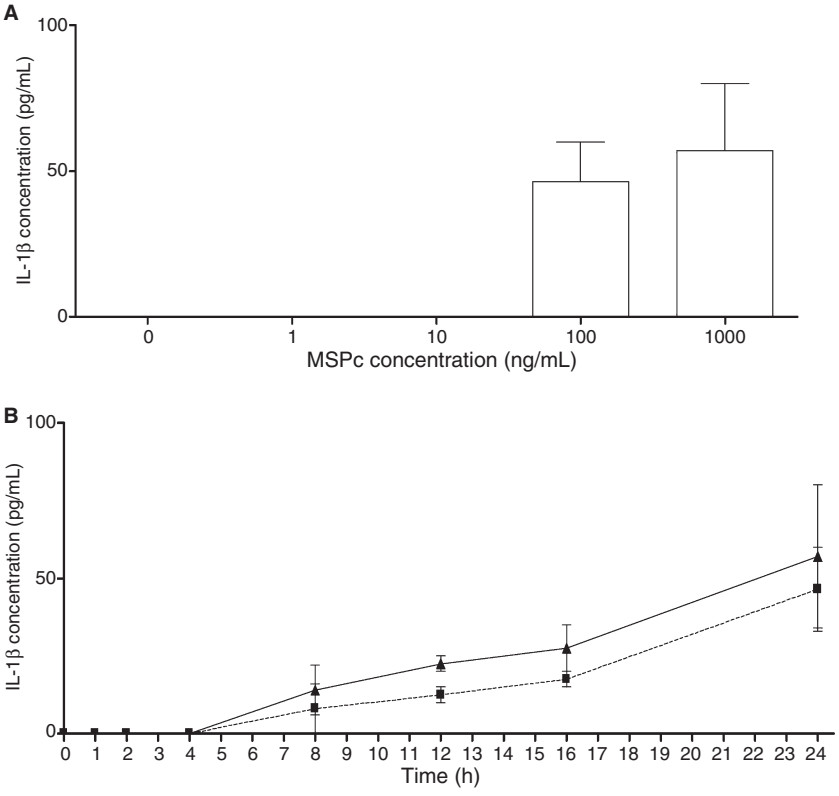

Fig. 2. (A) Interleukin-1β release by human peripheral blood monocytes stimulated with different concentrations of *T. denticola* MSPc after 24 h. The results are representative of three independent experiments and are expressed as the mean + SD (error bars) of three replicates. (B) Interleukin-1β levels in the cell supernatant at different time points after stimulation with 100 and 1000 ng/mL of MSPc. The continuous line indicates IL-1β production by monocytes stimulated with 100 ng/mL MSPc; the dashed line indicates IL-1β production by monocytes stimulated with 1000 ng/mL MSPc.

even at MSPc concentrations as high as 1 μg/mL (data not shown).

The MSPc induced the production of proinflammatory cytokines in a dose- and time-dependent manner. After 24 h of incubation in the presence of MSPc at 100 ng/mL and 1 μg/mL, monocytes released TNF-α at a concentration of 240 ± 8 and 260 ± 10 pg/mL, respectively. In contrast, the incubation of monocytes with MSPc at concentrations lower than 100 ng/mL did not stimulate TNF-α production (no difference from basal levels) (Fig. 1A). These differences were statistically significant ($p < 0.0001$ as demonstrated by the ANOVA test). With regard to the kinetics of cytokine production from cells that were stimulated with MSPc at 100 ng/mL and 1.0 μg/mL, TNF-α production increased dramatically up to 8 h and then rose modestly between the remaining time points (Fig. 1B).

The effect of MSPc stimulation on the production of IL-1β by CD14-positive cells was also evaluated. At 100 ng/mL and 1.0 μg/mL, MSPc induced a pattern of IL-1β release that was similar to that of TNF-α. At low MSPc concentrations (1.0 and 10 ng/mL), no IL-1β was released into the supernatant (Fig. 2A). A significant level of IL-1β was detected in the supernatant only after 8 h of incubation with the MSPc (at concentration of 100 ng/mL and 1.0 μg/mL), which increased linearly up to 24 h (Fig. 2B).

Interleukin-6 was detectable in the supernatant as early as 4 h after CD14-positive cells were incubated with MSPc at concentrations ranging from 100 to 1000 ng/mL. The release of this cytokine increased rapidly up to 8 h of incubation and slowed between 8 and 24 h with MSPc concentrations of 100 and 1000 ng/mL (Fig. 3B). Concentrations of MSPc of 100 ng/mL or greater induced significant IL-6 release, resulting in statistically significant differences ($p < 0.0001$ as demonstrated by the ANOVA test; Fig. 3A).

Matrix metalloproteinase 9 was released at a high concentration (240 μg/mL) when the cells were stimulated with MSPc at 1000 ng/mL. This result was statistically different from that obtained with lower concentrations of MSPc ($p < 0.0023$ as demonstrated by the ANOVA test). Lower concentrations of MSPc induced MMP-9 release in amounts comparable with basal levels (Fig. 4A). The MMP-9 release started to increase 2 h after stimulation with 1000 ng/mL. At 100 ng/mL of MSPc or lower, the curve showed a marked increase after 4 h of incubation (Fig. 4B).

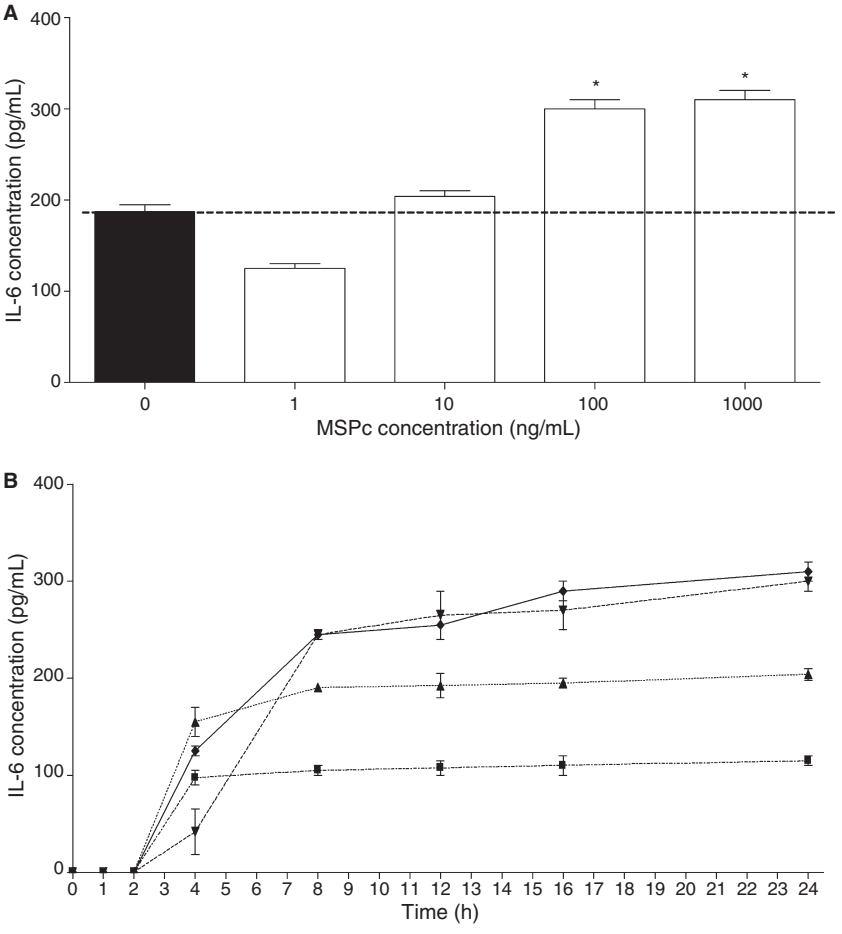

Fig. 3. (A) Human peripheral blood monocytes incubated with different concentrations of MSPc after 24 h. Supernatants were analyzed for IL-6 release after 24 h. The asterisks indicate which MSPc concentrations achieved statistically significant differences ($p < 0.01$) in the cytokine response between the MSPc-stimulated monocytes and unstimulated (control) monocytes according to the ANOVA and Bonferroni *post hoc* test. (B) Interleukin-6 levels in the cell supernatant at different time points after stimulation with different concentrations of MSPc. The graph shows interleukin-6 production by monocytes stimulated with 1 (■), 10 (▲), 100 (▼) and 1000 ng/mL (♦) of MSPc. The data presented are from three independent experiments and are expressed as the means ± SD.

Discussion

Monocytes are involved in the response against bacteria during infections and are among the cell populations that are detected at sites of active periodontitis (15). Immune cells synthesize proinflammatory mediators that can cause the destruction of tissues (10–13). Several bacterial products stimulate host cells to secrete proinflammatory mediators; this process is implicated in the development of bone resorption and tissue damage (10,13,14). Previous studies have shown that the release of cytokines from gingival cells correlates with the progression of periodontitis.

Different components of *T. denticola* envelopes, such as CTLP, MSPc, lipo-oligosaccharide and peptidoglycan, play specific roles in the progression of periodontal diseases (3,7,8).

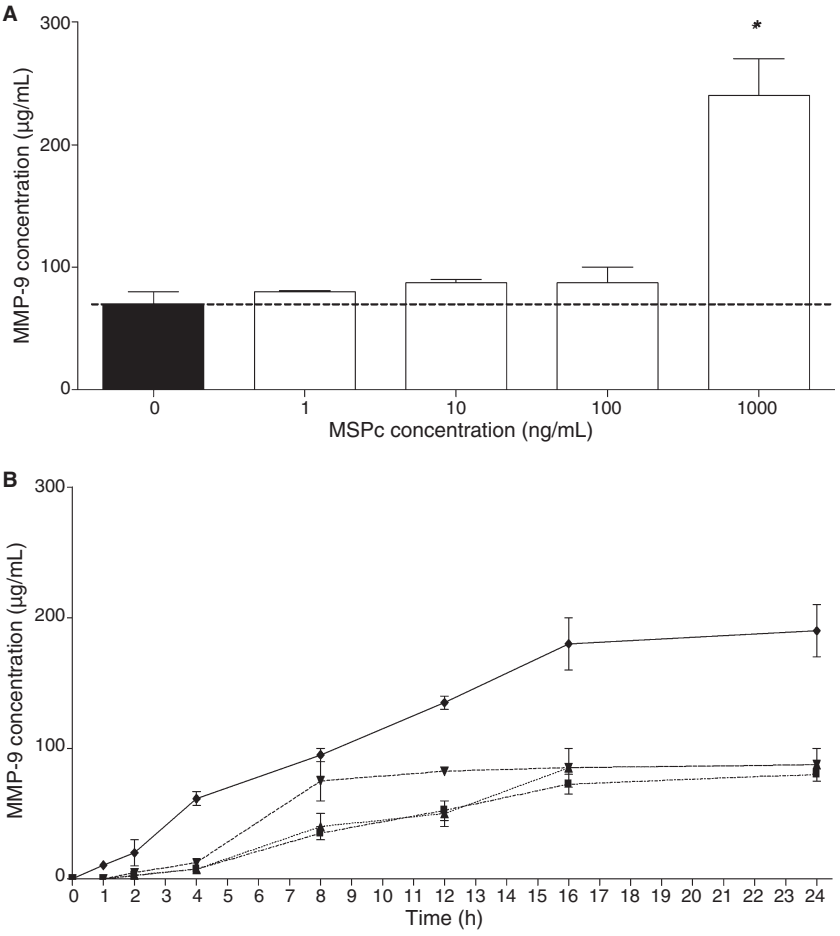

Fig. 4. (A) Matrix metalloproteinase 9 release by monocytes stimulated with different concentrations (1, 10, 100 and 1000 ng/mL) of *T. denticola* MSPc. The results are expressed as means + SD of three different cultures. The asterisk indicates which MSPc concentration achieved a statistically significant difference ($p < 0.01$) in the cytokine response between the MSPc-stimulated monocytes and unstimulated (control) monocytes according to the ANOVA and Bonferroni *post hoc* test. (B) Matrix metalloproteinase 9 levels in the cell supernatant at different time points after stimulation with different concentrations of MSPc. The graph shows MMP-9 production by monocytes stimulated with 1 (■), 10 (▲), 100 (▼) and 1000 ng/mL (♦) of MSPc.

In vitro studies have demonstrated that the MSPc–CTLP complex can adhere to and lyse gingival epithelial cells (7). In addition, isolated MSPc is highly cytotoxic to fibroblasts and epithelial cells *in vitro* (6,16). *T. denticola* lipo-oligosaccharide induces the release of mediators that are involved in the differentiation of osteoclasts (17) from bone marrow cells. In a very recent study by Tanabe and coworkers (14), peptidoglycan extracted from *T. denticola* (ATCC 35405) induced the production of several proinflammatory cytokines by macrophage-like cells that were derived from a monoblastic cell line (U937).

In this study, we demonstrated that a single polypeptide component of the outer membrane of *T. denticola*, MSPc, stimulated the release of proinflammatory cytokines from human primary CD14-positive cells. The absence of contaminating lipo-oligosaccharide-related activity in the protein preparation strongly indicated that this effect was likely to be due to MSPc stimulation.

As expected, the production of cytokines was a dose- and time-dependent phenomenon that increased up to 24 h of stimulation. The minimal concentration of MSPc that induced significant production of TNF-α, IL-1β and IL-6 by CD14-positive cells was 100 ng/mL. These proinflammatory cytokines have been demonstrated to play an important role in the progression of periodontitis (11–13), particularly in mechanisms that involve osteoclast differentiation (18,19) and consequent bone resorption (20). The findings of the present study confirm the previously reported proinflammatory activity of spirochetal outer membrane proteins (21–23) and clearly suggest that MSPc is a strong inducer of the proinflammatory response of monocytes. We conclude that MSPc is one of the pathogenic factors that contribute to the pathogenesis of *T. denticola*-related periodontitis.

Collagen and numerous extracellular matrix proteins are among the most prominent components of the periodontium (7). The degradation of non-mineralized portions of periodontal tissues affects the extracellular activity of matrix metalloproteases that are released by intragingival cells (17,20). This process is the initial step in the progression of periodontal disease (24).

Our study demonstrated that MSPc is able to release MMP-9, which, indeed, can contribute to the proinflammatory response of human blood-derived monocytes. Peptidoglycan extracted from *T. denticola* is also a potent proinflammatory stimulus (14). Both macrophage-like cells and human primary monocytes released similar amounts of cytokines when they were stimulated with identical concentrations of either MSPc or peptidoglycans. Collectively, these findings clearly demonstrate that the *T. denticola* bacterial envelope plays an important role in the pathogenesis of periodontal disease.

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