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Phenol/water extract of *Treponema socranskii* subsp. *socranskii* as an antagonist of Toll-like receptor 4 signalling

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Treponema socranskii is one of the most frequently found oral spirochaetes in periodontitis and endodontic infections. LPS or glycolipids from bacteria are potent stimulators of innate immune and inflammatory systems. In this study the bioactivity of a phenol/water extract from *T. socranskii* subsp. *socranskii* (TSS-P) was analysed. TSS-P showed minimal endotoxicity and no inducing potential for proinflammatory cytokines (TNF-α and IL-8) or for intercellular adhesion molecule-1 (ICAM-1) in human monocyte cell line THP-1 cells and primary cultured human gingival fibroblasts. Rather, it inhibited ICAM-1 expression and IL-8 secretion from cells stimulated by the LPS of *Escherichia coli* and *Actinobacillus actinomycetemcomitans*, which are known to be Toll-like receptor 4 (TLR4) agonists. However, this antagonistic activity was not shown in cells stimulated by peptidoglycan or IL-1β. As its antagonistic mechanism, TSS-P blocked the binding of *E. coli* LPS to LPS-binding protein (LBP) and CD14, which are molecules involved in the recruitment of LPS to the cell membrane receptor complex TLR4–MD-2 for the intracellular signalling of LPS. TSS-P itself did not bind to MD-2 or THP-1 cells, but inhibited the binding of *E. coli* LPS to MD-2 or to the cells in the presence of serum (which could be replaced by recombinant human LBP and recombinant human CD14). The results suggest that TSS-P acts as an antagonist of TLR4 signalling by interfering with the functioning of LBP/CD14.

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INTRODUCTION

Periodontitis is a polymicrobial disease caused by predominantly anaerobic Gram-negative bacteria. Oral spirochaetes comprise a diverse range of *Treponema* species, which have cell walls similar to those of Gram-negative bacteria. Currently 10 species have been cultivated and their differential distribution in periodontitis has been reported (Ellen & Galimanas, 2005; Moter *et al.*, 1998; Wyss *et al.*, 1999, 2004).

Treponema socranskii is one of the most frequently found oral spirochaetes in endodontic infections as well as in chronic and aggressive periodontitis (Baumgartner *et al.*, 2003; Ellen & Galimanas, 2005; Moter *et al.*, 1998). Its presence is correlated with clinical parameters such as pocket depth and attachment loss, and thus it is associated with the severity of periodontal tissue destruction (Socransky *et al.*, 1991; Takeuchi *et al.*, 2001). Riviere *et al.* (2002) detected several species of oral spirochaetes including *T. socranskii* in the brain cortex of patients with Alzheimer's disease by PCR and immunohistochemical analyses. A protease showing chymotrypsin-like activity was identified in this bacterium (Correia *et al.*, 2003; Heuner *et al.*, 2001). More recently we observed that *T. socranskii* induced osteoclastogenesis by upregulating prostaglandin E_2, which stimulated the receptor activator of nuclear factor-κB ligand (Choi *et al.*, 2005).

LPS localized in the outer membrane of Gram-negative bacteria is a representative pathogen-associated molecular pattern that exerts biologically diverse activities and is one of the most powerful stimulators of innate immune and inflammatory responses (Miller *et al.*, 2005). Biological activities of LPS or LPS-like materials from the outer

Abbreviations: HGFs, human gingival fibroblasts; HRP, horseradish peroxidase; HS, heat-inactivated human serum; ICAM-1, intercellular adhesion molecule-1; LAL, Limulus amoebocyte lysate; LBP, LPS-binding protein; mCD14, membrane-bound CD14; rhCD14, recombinant human CD14; rhLBP, recombinant human LBP; sCD14, soluble forms of CD14; TIR, Toll-interleukin-1 receptor; TL-P, phenol/water extract of *Treponema lecithinolyticum*; TLR4, Toll-like receptor 4; TMB, 3,3′,5,5′-tetramethylbenzidine; TSS-P, phenol/water extract of *Treponema socranskii* subsp. *socranskii*.

membrane of oral spirochaetes, also named lipooligosaccharides, glycolipids or glycoconjugates, have been reported in different species. The *Treponema denticola* lipooligosaccharide induced TNF-α and nitric oxide in macrophages prepared from the peritoneal cavity of endotoxin-responsive and unresponsive mice (Rosen *et al.*, 1999). It also induced osteoclastogenesis by upregulating the osteoclast differentiation factor in a co-culture system using mouse calvaria and bone marrow cells, and matrix metalloproteinase-9 expression in mouse calvaria-derived osteoblastic cells (Choi *et al.*, 2003). Glycolipids from *Treponema maltophilum* induced IL-6 and TNF-α in human monocytes and a murine macrophage cell line, and NF-κB translocation in Chinese hamster ovary cells transfected with human CD14 (Opitz *et al.*, 2001). LPS from *Treponema pectinovorum* induced IL-6, IL-8 and monocyte chemoattractant protein 1 in human gingival fibroblasts (HGFs) (Kesavalu *et al.*, 2002).

LPS signalling is initiated by the combined actions of LPS-binding protein (LBP), the membrane-bound (mCD14) or soluble forms (sCD14) of CD14 and the Toll-like receptor 4 (TLR4)–MD-2 complex (Akashi *et al.*, 2003; Mancek *et al.*, 2002; Miyake 2003; Re & Strominger 2003; Palsson-McDermott & O'Neill, 2004). LBP, a serum protein, enhances the binding of LPS to CD14, and CD14 facilitates the recognition of LPS by the TLR4–MD-2 complex. MD-2, a glycoprotein, is an adaptor molecule that is associated with the extracellular leucine-rich repeats of TLR4 (Mancek *et al.*, 2002; Miyake, 2003). LPS, presented by CD14, binds to TLR4–MD-2, and the LPS interaction with TLR4–MD-2 induces the TLR4 oligomerization on which TLR4 signalling is dependent (Saitoh *et al.*, 2004). The intracellular signalling of TLR4 is homologous to the signalling systems of IL-1, as they possess a common cytoplasmic Toll-interleukin-1 receptor (TIR) domain (Martin & Wesche, 2002). Although TLR4 is known to be a predominant receptor mediating LPS activation, recent studies have suggested a role for TLR2 as a receptor for some LPS (Darveau *et al.*, 2004; Hirschfeld *et al.*, 2001; Opitz *et al.*, 2001; Werts *et al.*, 2001). Furthermore, the LPS of some other bacteria show antagonism against the activity induced by typical LPS of Gram-negative bacteria (Coats *et al.*, 2003; Jarvis *et al.*, 1997; Lepper *et al.*, 2005; Yoshimura *et al.*, 2002). The agonistic and antagonistic activities of LPS are known to be correlated with the chemical structure of lipid A in the LPS molecule (Brandenburg *et al.*, 2003). Glycolipids of small-sized oral spirochaetes like *T. denticola* and *T. maltophilum* have been suggested to be structurally similar to lipoteichoic acid (LTA; Schröder *et al.*, 2000; Schultz *et al.*, 1998). In *T. maltophilum*, LTA-like glycolipids are the major membrane components responsible for various biological effects, and these glycolipids use TLR2 rather than TLR4 (Opitz *et al.*, 2001). Recently, the glycolipids of the medium-sized oral spirochaete *T. medium* showed antagonized cell activation induced by *Escherichia coli* LPS as well as by peptidoglycan (Asai *et al.*, 2003a). Their structure is also similar to LTA (Hashimoto *et al.*, 2003).

In our preliminary study comparing the effect of glycolipids of several oral spirochaetes on the host innate immune response, a phenol/water extract of *T. socranskii* subsp. *socranskii* (TSS-P) revealed antagonism against *E. coli* LPS activity. In this study we examined TSS-P antagonism in more detail, including the antagonistic mechanism, mainly using the human monocytic THP-1 cell line but also using primary cultured HGFs in some experiments.

METHODS

Bacterial strains. *Treponema socranskii* subsp. *socranskii* ATCC 35536 and *Treponema lecithinolyticum* ATCC 700332 were cultured anaerobically in OMIZ-Pat (Wyss *et al.*, 1999) or NOS broth (Chan *et al.*, 1993) for 3 to 5 days. *Actinobacillus actinomycetemcomitans* ATCC 33384 and *Escherichia coli* DH5α were also included for LPS isolation. *A. actinomycetemcomitans* was grown anaerobically in brain heart infusion broth for 2 days. *E. coli* was grown aerobically in Luria–Bertani broth.

Bacterial phenol/water extraction. The bacterial cells (50 ml cultures for *Treponema* species and 5 ml cultures for *A. actinomycetemcomitans* and *E. coli*) were harvested by centrifugation at 5000 ***g*** for 10 min at 4 °C. The cells were then washed with phosphate-buffered saline (PBS). Bacterial phenol/water extracts were prepared by using an LPS extraction kit (iNtRON Biotechnology) according to the manufacturer's protocol with a slight modification. Briefly, cells were mixed with 1 ml lysis buffer containing phenol from the kit and vortexed vigorously until the cell clump disappeared. After adding 200 µl chloroform, the mixture was vortexed vigously for 30 s and incubated at room temperature for 5 min. The mixture was centrifuged at 13 200 ***g*** at 4 °C for 10 min and the upper phase (400 µl) was transferred to a new tube. This crude preparation was incubated with endonucleases (100 µg DNase I ml^{-1} and 100 µg RNase A ml^{-1}, 1 h at 37 °C) and subsequently proteinase K (250 µg ml^{-1}, 1 h at 50 °C). After adding 800 µl lysis buffer, the same procedure as described above was repeated. The second preparation was mixed well with the purification buffer (800 µl) from the kit, incubated at −20 °C for 10 min, and centrifuged at 13 200 ***g*** at 4 °C for 15 min. The pellets were washed with 1 ml 70 % ethanol, air-dried, and dissolved in endotoxin-free distilled H_2O. The phenol/water extracts were quantified by lyophilization and dry weight measurement. They were dissolved in endotoxin-free distilled H_2O at the concentration of 1 mg ml^{-1}; dissolution was ensured by sonication. To visualize and compare the patterns, the phenol/water extracts (10 µg each) were subjected to SDS-PAGE (15 % polyacrylamide gel) and silver stained. The phenol/water extracts of *E. coli* and *A. actinomycetemcomitans* showed typical ladder-like LPS patterns, whereas those of *T. socranskii* and *T. lecithinolyticum* did not. In this study, we called the phenol/water extracts of *E. coli* and *A. actinomycetemcomitans* LPS, and those of *T. socranskii* and *T. lecithinolyticum* phenol/water extracts TSS-P (for the phenol/water extract of *T. socranskii*) and TL-P (for the phenol/water extract of *T. lecithinolyticum*).

E. coli O127 : B8 LPS was purchased from Sigma; possible contaminants were removed by treatment with endonucleases and proteinase K and subsequent phenol/water extraction with the LPS extraction kit, as described above. This repurified *E. coli* LPS was used throughout this study.

Endotoxin assay. Endotoxin activity of the LPS or phenol/water extracts was determined by the Limulus amoebocyte lysate (LAL) assay using an LAL Endochrome kit (Charles River Endosafe) according to the manufacturer's protocol. In principle, endotoxin activated a proenzyme in LAL. In the presence of a colourless

substrate, the activated enzyme rapidly catalysed the cleavage of the chromophore *p*-nitroaniline, which produced a yellow colour and was measured at 405 nm. A standard curve was run with *E. coli* control standard endotoxin from the kit.

Culture and treatment of cells. The human monocytic THP-1 cell line was purchased from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with L-glutamine, 10 % heat-inactivated fetal bovine serum, and antibiotics (100 U penicillin ml^{-1} and 100 μg streptomycin sulfate ml^{-1}). The cells were cultured overnight, plated in 35 mm-diameter cell culture dishes at a concentration of 1×10^6 cells ml^{-1}, and treated with LPS (0·01–10 μg ml^{-1}), phenol/water extracts (TSS-P or TL-P, 0·01–50 μg ml^{-1}), peptidoglycan from *Staphylococcus aureus* (0·1–10 μg ml^{-1}, Sigma) or human recombinant IL-1β (300 pg ml^{-1}; R&D Systems) in the presence or absence of 2 % heat-inactivated human serum (HS, Sigma) for 12–24 h. In some experiments, THP-1 cells were treated with TSS-P or TL-P in the presence of LPS, peptidoglycan or IL-1β at the indicated concentrations. In another set of experiments, the 2 % HS in the cell culture medium was replaced by recombinant human LBP (rhLBP, 50 ng ml^{-1}, R&D Systems) and recombinant human CD14 (rhCD14, 200 ng ml^{-1}, R&D Systems). Cells were harvested for flow cytometry analysis and RNA isolation for RT-PCR. The culture supernatants were collected and stored at −70 °C for cytokine measurement by ELISA. To examine the tolerance induction by TSS-P, THP-1 cells (1×10^6 cells ml^{-1}) cultured in 2 % HS were pretreated with TSS-P (10 μg ml^{-1}) for 12 h. The cells were then washed three times with RPMI medium and treated with *E. coli* LPS (10 ng ml^{-1}) for 12 h. ICAM-1 expression was measured by flow cytometry.

To ensure that the cell-stimulating activity of the *E. coli* LPS used in this study was not caused by contaminants, THP-1 cells were treated with *E. coli* LPS (10 ng ml^{-1}) in the presence of polymyxin B (50 μg ml^{-1}) and assessed for ICAM-1 expression. Polymyxin B completely inhibited ICAM-1 expression induced by the *E. coli* LPS.

To confirm the LPS signalling via TLR4, THP-1 cells were preincubated with mouse anti-human TLR4 mAb HTA125 (5 μg ml^{-1}), a TLR4-blocking antibody, or an isotype-matched antibody for 30 min and were then stimulated with *E. coli* LPS (10 ng ml^{-1}) or *A. actinomycetemcomitans* LPS (10 ng ml^{-1}) for 12 h. IL-8 levels in the culture supernatants were determined by ELISA.

In order to determine the effect of TSS-P on primary cultured cells, HGFs were obtained from explants of a healthy donor, cultured in alpha-minimal essential medium (α-MEM) as described previously (Choi *et al.*, 2001), and treated with TSS-P (10 μg ml^{-1}) or TL-P (10 μg ml^{-1}) in the presence of *E. coli* LPS (10 ng ml^{-1}).

Flow cytometry analysis. The expression of mCD14, TLR2, TLR4 and ICAM-1 by THP-1 cells, unstimulated or stimulated as described above, was analysed by flow cytometry. THP-1 cells were collected by centrifuging and washed with PBS. The cells (1×10^6 cells in 100 μl) were reacted with mouse anti-human CD14 mAb, mouse anti-human TLR2 mAb, mouse anti-human TLR4 mAb or mouse anti-human ICAM-1 mAb at 4 °C for 20 min. After washing, the cells were stained with FITC-labelled goat anti-mouse IgG at 4 °C for 20 min. The cells were washed with PBS, and expression of the proteins was analysed using a fluorescence-activated cell sorter (FACS). The data were obtained by counting 15 000 cells. As a control for nonspecific binding, the cells were stained with isotype-matched IgG; no nonspecific reactivity was observed.

RT-PCR. MD-2 expression in the THP-1 cells was analysed by RT-PCR. RNA was isolated from the THP-1 cells using TRIzol reagent (Invitrogen Life Technology) according to the manufacturer's protocol. RT-PCR was performed as described previously (Lee *et al.*, 2005) using the following primers: 5′-AGG GGC ACG AGG TAA ATC TT-3′ for the sense primer and 5′-GGC TCC CAG AAA TAG CTT CA-3′ for the anti-sense primer (annealing temperature 52 °C). PCR without reverse transcription was performed as a negative control. The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for gene expression.

ELISA for cytokines (TNF-α and IL-8). The culture supernatants of the cells treated with LPS and other stimuli were assayed to determine TNF-α and IL-8 levels using ELISA kits from R&D Systems.

Inhibition assay for LBP and CD14 binding. In order to analyse the antagonistic mechanism, we evaluated whether the binding of biotinylated *E. coli* LPS to CD14 or LBP was inhibited by TSS-P. Biotinylation was performed using EZ-Link Biotin-LC-Hydrazide (Pierce Biotechnology). *E. coli* LPS (500 μl of 2 mg ml^{-1}) or TSS-P (500 μl of 2 mg ml^{-1}) was incubated with 20 mM cold sodium metaperiodate at 4 °C for 30 min and then dialysed overnight in coupling buffer (0·1 M sodium acetate, pH 5·5). The mixture was transferred to a new tube and biotin hydrazide solution was added to a final concentration of 5 mM. After agitation at room temperature for 2 h, the mixture was dialysed three times in distilled H_2O for 24 h.

For LBP binding, anti-human LBP mAb (50 ng per well in 50 μl) was coated on EIA plates (Corning) at 4 °C overnight. The plates were washed with PBS and blocked with 2 % BSA in PBS at room temperature for 1 h. The plates were washed five times with PBS/0·1 % Tween 20 (PBST), then rhLBP (62·5 ng per well in 50 μl) was added and incubated at room temperature for 1 h. After washing the plates five times with PBST, the biotinylated LPS, alone or with nonbiotinylated *E. coli* LPS, TSS-P or TL-P, was added at the indicated dose and incubated at room temperature for 1 h. The plates were washed five times with PBST and biotinylated LPS captured by rhLBP was reacted with 50 μl horseradish peroxidase (HRP)-labelled streptavidin (1 μg ml^{-1}) in PBST containing 2 % BSA for 1 h. The plates were washed five times with PBST, and then 50 μl 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added as a substrate for HRP. The enzyme reaction was stopped after 20 min by adding 50 μl 0·5 M sulfuric acid, and the absorbance at 450 nm was measured using an ELISA reader. For sCD14 binding, anti-human CD14 mAb (125 ng per well in 50 μl) was coated on EIA plates at 4 °C overnight. The plates were washed with PBS, blocked with 2 % BSA in PBS at room temperature for 1 h, and washed five times with PBST. In parallel, 500 ng of the biotinylated *E. coli* LPS and rhCD14 (100 ng) were incubated with or without the indicated dose of nonbiotinylated *E. coli* LPS, TSS-P or TL-P at room temperature for 1 h in a total volume of 50 μl. This mixture was then added to each well of the anti-human CD14 mAb-coated plates and incubated at room temperature for 1 h. The plates were washed five times with PBST and binding of biotinylated *E. coli* LPS to CD14 captured by anti-CD14 mAb was detected as described above. To perform a binding assay to immobilized CD14, EIA plates were directly coated with rhCD14 (100 ng per well in 50 μl) at 4 °C overnight, and subsequent procedures were carried out as described above. Biotinylated *E. coli* LPS was used at a concentration of 500 ng per well in 50 μl, which was saturated by rhLBP (62·5 ng per well in 50 μl) or rhCD14 (100 ng per well in 50 μl).

LPS binding assay to MD-2 and THP-1 cells. Since MD-2 physically associated with TLR4 on cells is known to interact with LPS, we analysed whether *E. coli* LPS and TSS-P bound to MD-2 and THP-1 cells. The *E. coli* LPS or TSS-P was biotinylated as described above. EIA plates were coated with recombinant human MD-2 (rhMD-2, 50 ng per well in 50 μl, R&D Systems) at 4 °C overnight. The plates were washed with PBS and blocked with 2 % BSA in PBS at room temperature for 1 h. After washing five times with PBST, the plates were incubated with the biotinylated *E. coli* LPS (0·01–1 μg per well) or TSS-P (0·01–1 μg per well) in the presence

of rhLBP (62·5 ng per well in 50 µl) and rhCD14 (100 ng per well in 50 µl). The plates were washed five times with PBST; the bound LPS or TSS-P was detected with HRP-labelled streptavidin as described above. For the binding inhibition assay, biotinylated *E. coli* LPS (500 ng per well) was incubated with nonbiotinylated *E. coli* LPS, TSS-P or TL-P at the indicated doses in the presence or absence of rhLBP and rhCD14.

For the cell binding assay by *E. coli* LPS and TSS-P, THP-1 cells (1×10^6 cells ml^{-1}) cultured in RPMI 1640 medium with or without 2 % HS were incubated with the biotinylated *E. coli* LPS or TSS-P at various concentrations at 37 °C for 1 h in a CO_2 incubator. The cells were collected by centrifugation at 360 ***g*** for 3 min, washed three times with PBS, resuspended in 100 µl DPBS, and incubated with FITC-labelled streptavidin (1 µg) for 30 min. After three washes, the cells were analysed by FACS. For the binding inhibition assay, biotinylated *E. coli* LPS (0·01–1 µg ml^{-1}) was incubated with nonbiotinylated *E. coli* LPS (10 µg ml^{-1}) or TSS-P (10 µg ml^{-1}).

Statistical analysis. The statistical significance of the differences between nontreated and LPS- or phenol/water extract-treated cells was evaluated by a one-way analysis of variance (ANOVA). The statistical significance of the differences between agonist (LPS)-treated and inhibitor-treated cells was evaluated using Student's *t*-test. A *P*-value of less than 0·05 was considered significant.

RESULTS

Endotoxin activity of LPS and phenol/water extracts

LPS-like materials were purified using a phenol/water extraction technique. In order to remove possible contamination with nucleic acids and proteins, the extracts were further treated with DNase and RNase, and subsequently treated with proteinase K, followed by re-extraction with phenol. *T. socranskii* subsp. *socranskii*, *T. lecithinolyticum*, *A. actinomycetemcomitans* and *E. coli* were included. The endotoxicity of phenol/water extracts was determined by LAL assay. The two treponemes showed minimal endotoxicity compared to *A. actinomycetemcomitans* and *E. coli*: the endotoxicity was 1·4 EU mg^{-1} for TSS-P and 0·5 EU mg^{-1} for TL-P, while the LPS from *A. actinomycetemcomitans* and *E. coli* showed endotoxicity of 137 and 13 300 EU mg^{-1}, respectively. The repurified form of the commercially available *E. coli* LPS revealed a nearly identical endotoxicity (13 200 EU mg^{-1}) to that of the LPS extracted from the *E. coli* culture in this study. Therefore, we believed that the phenol/water-extracted components were LPS or LPS-like materials.

Expression of mCD14, TLR2, TLR4 and MD-2 on THP-1 cells

In this study, we mainly used THP-1 cells to investigate cell response to LPS. In order to ensure that the molecules necessary for LPS response in the cell were expressed, the expression of mCD14, TLR2, TLR4, and MD-2 was analysed by flow cytometry or RT-PCR. As shown in Fig. 1, THP-1 cells expressed all of these molecules.

Effects of LPS and phenol/water extracts on cytokine production

In order to examine the cellular activity of LPS and phenol/water extracts, we analysed TNF-α and IL-8 production from THP-1 cells and HGFs after stimulation with these molecules. As shown in Fig. 2, TSS-P (10 µg ml^{-1}) and TL-P (10 µg ml^{-1}) did not induce TNF-α or IL-8 production in

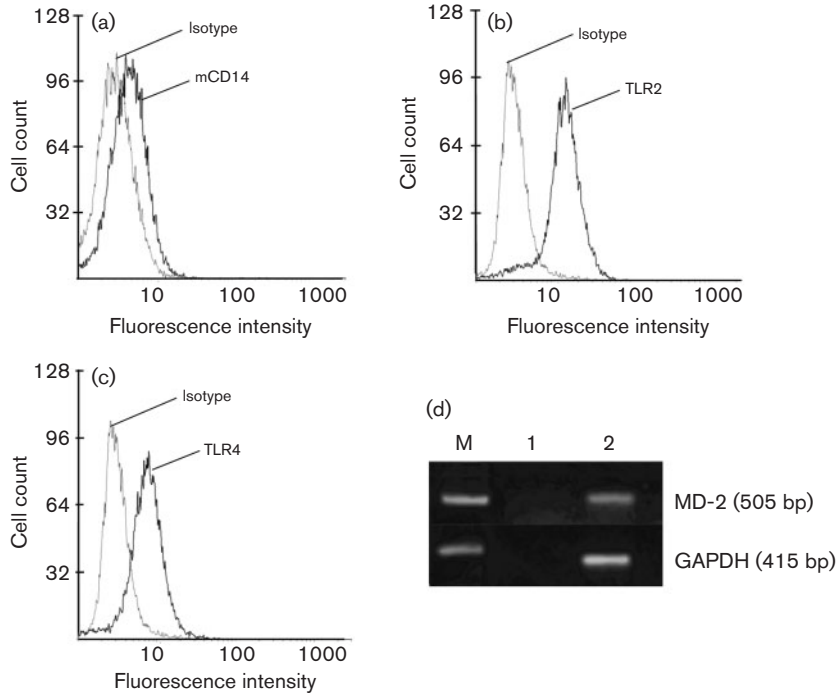

Fig. 1. Expression of mCD14, TLR2, TLR4 and MD-2 in THP-1 cells. THP-1 cells (1×10^6 ml^{-1}) were cultured in 35 mm-diameter culture dishes in RPMI 1640 medium containing 2 % HS, harvested, and washed with PBS. The cells (1×10^6 in 100 µl) were reacted with mouse anti-human CD14 mAb (a), mouse anti-human TLR2 mAb (b), or mouse anti-human TLR4 mAb (c), and subsequently with FITC-labelled goat anti-mouse IgG. The cells were analysed by flow cytometry. (d) MD-2 mRNA expression was determined by RT-PCR after isolation of the total RNA of the THP-1 cells (lane 2); PCR without RT was used as a negative control (lane 1). Lane M, DNA size marker.

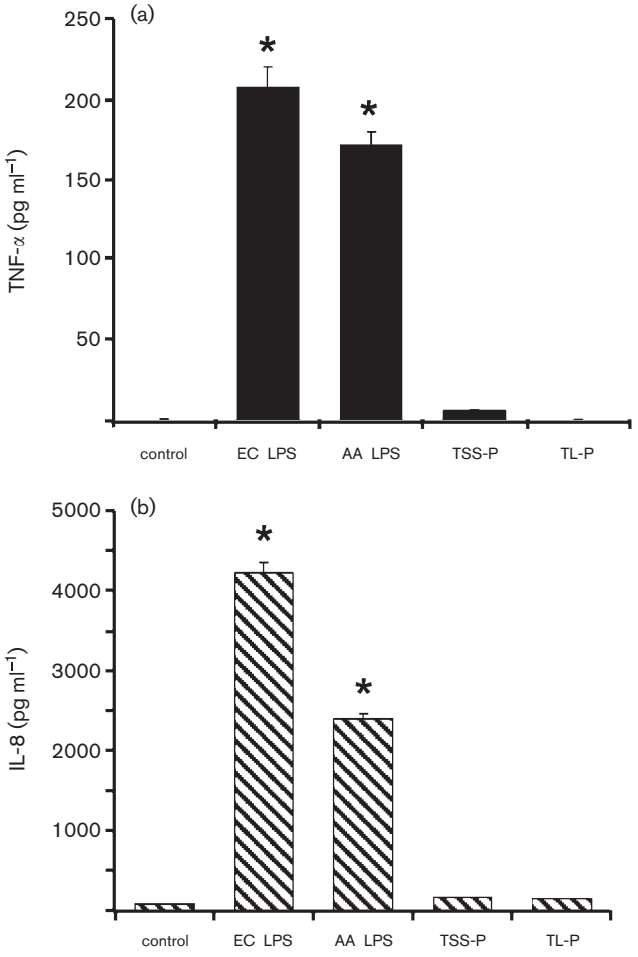

Fig. 2. Test for induction of TNF-α and IL-8 production by LPS and phenol/water extracts. THP-1 cells (1×10^6 ml^{-1}) were cultured in 35 mm-diameter culture dishes in RPMI 1640 medium containing 2 % HS and treated with *E. coli* LPS (EC LPS, 10 ng ml^{-1}), *A. actinomycetemcomitans* LPS (AA LPS, 10 ng ml^{-1}), TSS-P (10 μg ml^{-1}) or TL-P (10 μg ml^{-1}) for 12 h. The culture supernatants were assayed for TNF-α (a) and IL-8 (b) by ELISA. Nontreated cell were used as a control. The experiments were performed three times in triplicate for each assay. The data are representative means and standard deviations. The asterisks indicate significant differences ($P<0{\cdot}05$) compared with the nontreated control values.

THP-1 cells, whereas the LPS from *E. coli* (10 ng ml^{-1}) and *A. actinomycetemcomitans* (10 ng ml^{-1}) significantly induced the production of these cytokines. The induction of IL-8 was not observed in HGFs stimulated with TSS-P or TL-P either (data not shown).

Effects of LPS and phenol/water extracts on ICAM-1 expression

Next, we analysed the effects of LPS and phenol/water extracts on ICAM-1 expression. ICAM-1 is a cell-surface adhesion molecule that is involved in the innate immune response and is expressed in various cell types including endothelial cells and leukocytes. *E. coli* LPS induced ICAM-1 expression at concentrations as low as 10 ng ml^{-1}. However, TSS-P and TL-P did not induce ICAM-1 expression, even at concentrations up to 50 μg ml^{-1} (data not shown). Rather, TSS-P (10 μg ml^{-1}) antagonized the *E. coli* LPS-induced ICAM-1 expression in THP-1 cells. We analysed the antagonism of TSS-P under three different conditions. First, in the presence of 2 % HS in the cell culture medium, *E. coli* LPS induced ICAM-1 expression in a dose-dependent manner at concentrations of 0·01–10 μg ml^{-1}, and TSS-P at 10 μg ml^{-1} significantly inhibited the ICAM-1 expression induced by *E. coli* LPS (Fig. 3a). Second, in serum-free medium, the THP-1 cell response to *E. coli* LPS was drastically reduced (Fig. 3b). ICAM-1 expression was not induced in the cells with 10 ng ml^{-1} of *E. coli* LPS, was slightly induced with 100 ng ml^{-1}, and was significantly induced with 1 μg ml^{-1}, although the response was less than that observed with the same concentration in the presence of 2 % HS. A high concentration of *E. coli* LPS (10 μg ml^{-1}) showed the same cell-stimulating activity regardless of the presence of serum. Significant inhibition by TSS-P was not observed under the serum-free condition. Third, in the presence of rhLBP and rhCD14 instead of 2 % HS in the cell culture medium, *E. coli* LPS showed the same stimulatory effect as in the presence of serum and the inhibitory effect by TSS-P was also the same (data not shown). Fig. 3(c) shows the inhibitory effect of TSS-P on the ICAM-1 expression induced by 10 ng ml^{-1} of *E. coli* LPS under three conditions. TL-P showed neither an agonistic nor an antagonistic effect. The addition of either rhLBP or rhCD14 alone instead of human serum showed only limited stimulation by *E. coli* LPS (data not shown).

TSS-P purified from bacteria cultured in NOS medium retained the antagonistic effects, and heating TSS-P (10 min at 100 °C) did not reduce its ability to antagonize the activity of *E. coli* LPS (data not shown).

As *E. coli* LPS is known to transduce signals via TLR4, we tested whether TSS-P inhibits the ICAM-1 induced by another known TLR4 ligand, *A. actinomycetemcomitans* LPS. This molecule stimulated ICAM-1 in the same concentration-dependent manner as found with the *E. coli* LPS, and TSS-P (10 μg ml^{-1}) completely inhibited the ICAM-1 expression induced by 10 ng ml^{-1} of *A. actinomycetemcomitans* LPS (Fig. 3d). The inhibitory effect of 10 μg ml^{-1} of TSS-P was not observed at the highest concentration (10 μg ml^{-1}) of either *E. coli* or *A. actinomycetemcomitans* LPS.

Endotoxin tolerance is the phenomenon that prior exposure of the cells to LPS induces a transient state of reduced ability to respond to second LPS exposure. In experiments to test whether TSS-P made the cells tolerant to LPS by treating THP-1 cells with TSS-P and subsequently with *E. coli* LPS, we observed that TSS-P pretreatment did not affect the ICAM-1 expression induced by *E. coli* LPS, whereas TSS-P significantly inhibited *E. coli* LPS-induced

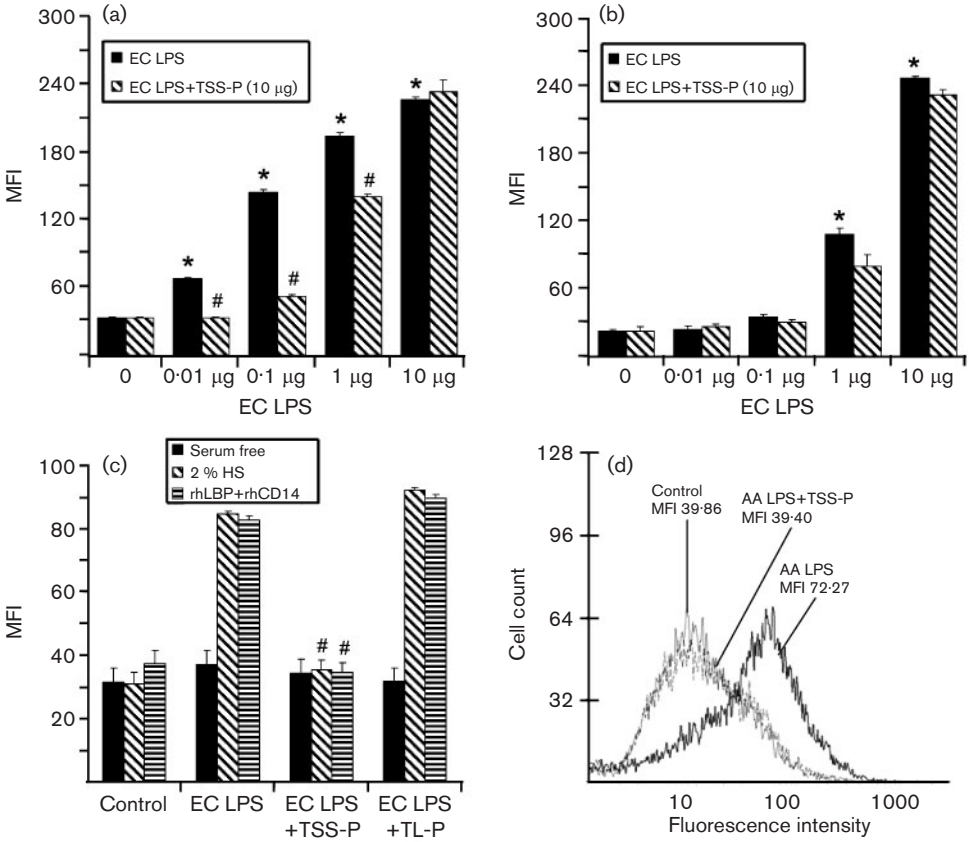

Fig. 3. Inhibition of LPS-induced ICAM-1 expression by TSS-P. (a, b) THP-1 cells (1×10^6 cells ml^{-1}) cultured in RPMI 1640 medium with (a) or without 2 % HS (b) were treated with TSS-P (10 μg ml^{-1}) in the presence of *E. coli* LPS (EC LPS, 0·01–10 μg ml^{-1}) for 12 h. (c) THP-1 cells (1×10^6 ml^{-1}) cultured in RPMI 1640 medium with or without 2 % HS, or with rhLBP (50 ng ml^{-1}) and rhCD14 (200 ng ml^{-1}) were treated with TSS-P (10 μg ml^{-1}) or TL-P (10 μg ml^{-1}) in the presence of *E. coli* LPS (10 ng ml^{-1}). The cells were harvested and ICAM-1 expression was assayed by flow cytometry using mouse anti-human ICAM-1 mAb and FITC-labelled goat anti-mouse IgG. The experiments were performed three times, and representative data are shown. (d) THP-1 cells cultured in RPMI 1640 medium with 2 % HS were treated with *A. actinomycetemcomitans* LPS (AA LPS, 10 ng ml^{-1}) alone or with TSS-P (10 μg ml^{-1}), and analysed for ICAM-1 expression. The asterisks (*) indicate significant differences ($P<0·05$) compared with the nontreated control values and the hash signs (#) indicate significant differences ($P<0·05$) compared with the *E. coli* LPS-treated cells. MFI, mean fluorescence intensity.

ICAM-1 expression in the cells treated with TSS-P and *E. coli* LPS simultaneously (data not shown).

Effects of anti-TLR4 mAb and TSS-P on IL-8 production

To confirm the functional role of TLR4 in IL-8 secretion induced by LPS, THP-1 cells were pre-incubated with a TLR4-blocking antibody, HTA125. This antibody significantly blocked the LPS-induced production of IL-8, while an isotype-matched control antibody did not (Fig. 4a). In addition to the inhibitory effects on ICAM-1 expression, TSS-P significantly inhibited IL-8 production in THP-1 cells and HGFs stimulated with *E. coli* LPS, whereas TL-P did not affect the activity (Fig. 4b).

Effects of TSS-P on ICAM-1 induction by peptidoglycan and IL-1β

The inhibitory effect of TSS-P was tested with peptidoglycan, which is also a pathogen-associated molecular pattern, but does not interact with TLR4. Peptidoglycan significantly induced ICAM-1 expression at 100 ng ml^{-1} in the presence (Fig. 5a) or absence (data not shown) of serum. This stimulation was not affected by TSS-P (10 μg ml^{-1}). In order to differentiate the target for antagonism by TSS-P, we analysed its effect on IL-1β-induced ICAM-1 expression, as the IL-1 receptor (IL-1R) and TLR4 have a common intracellular TIR domain. As shown in Fig. 5(b), TSS-P (10 μg ml^{-1}) did not affect the ICAM-1 expression stimulated by IL-1β (300 pg ml^{-1}). This result indicates that TSS-P antagonism is specific to the TLR4 pathway.

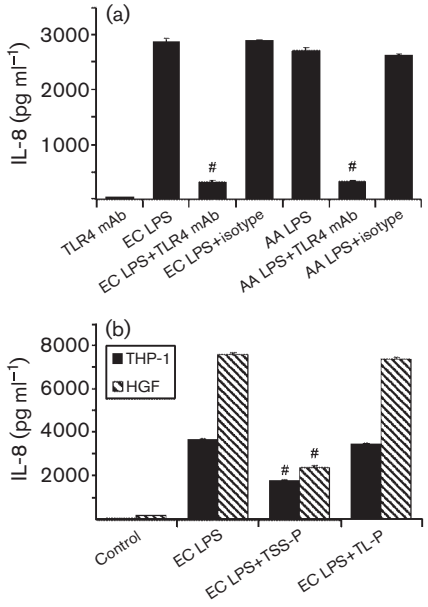

Fig. 4. Inhibition of LPS-induced cytokines by anti-TLR4 mAb and TSS-P. (a) THP-1 cells (1×10^6 ml^{-1}) cultured in RPMI 1640 medium were preincubated with anti-TLR4 mAb HTA125 (5 μg ml^{-1}) or an isotype-matched antibody for 30 min, and were then stimulated with *E. coli* LPS (EC LPS, 10 ng ml^{-1}) or *A. actinomycetemcomitans* LPS (AA LPS, 10 ng ml^{-1}) for 12 h. (b) THP-1 cells and HGFs were incubated with *E. coli* LPS (10 ng ml^{-1}) alone or with TSS-P (10 μg ml^{-1}) or TL-P (10 μg ml^{-1}) for 12 h. The culture media were collected and measured for IL-8 levels by ELISA. The experiments were performed three times in triplicate for each assay. The data are representative means and standard deviations. The hash signs (#) indicate significant differences ($P<0{\cdot}05$) compared with the values for LPS-treated cells.

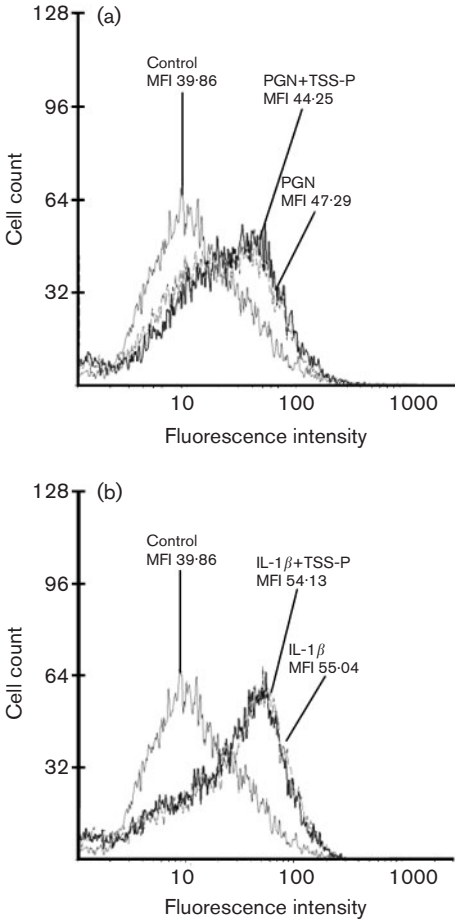

Fig. 5. Effects of TSS-P on ICAM-1 expression induced by peptidoglycan or IL-1β. THP-1 cells (1×10^6 ml^{-1}) cultured in RPMI 1640 medium containing 2% HS were treated with peptidoglycan (PGN, 100 ng ml^{-1}) (a) or IL-1β (300 pg ml^{-1}) (b) alone or with TSS-P (10 μg ml^{-1}) for 12 h. The cells were harvested, and ICAM-1 expression was assayed by flow cytometry using mouse anti-human ICAM-1 mAb and FITC-labelled goat anti-mouse IgG. The experiments were performed three times, and representative data are shown. Nontreated cells were used as a negative control.

Involvement of LBP and CD14 in TSS-P antagonism

TSS-P antagonism was observed in the signalling of the LPS from *E. coli* or *A. actinomycetemcomitans*, which interact with LBP and CD14, but not in that of peptidoglycan or IL-1β. No significant TSS-P antagonism was observed under the serum-free condition. These results indicated that the antagonistic target may be LBP and/or CD14, which are components of serum. To address this issue, we performed an experiment to determine whether TSS-P was in competition with the *E. coli* LPS for binding to LBP and CD14. Biotinylated *E. coli* or TSS-P bound to LBP and to both forms of CD14 (immobilized and soluble) in a concentration-dependent manner (data not shown). Since the binding saturation to rhLBP and rhCD14 was reached at 500 ng per well of biotinylated *E. coli* LPS, this concentration was used for the inhibition study. As shown in Fig. 6, TSS-P inhibited the binding of biotinylated *E. coli* LPS to both forms of CD14 as well as to LBP, in a dose-dependent manner. TL-P, which showed no antagonistic effect, did not inhibit the *E. coli* LPS binding to LBP and CD14. Nonbiotinylated *E. coli* LPS also inhibited the binding of biotinylated LPS to LBP and CD14. This inhibition was more efficient than that by TSS-P.

Effects of TSS-P on LPS binding to MD-2 and THP-1 cells

LPS is known to interact with MD-2 that is physically associated with TLR4 on the cell surface. We examined whether *E. coli* LPS and TSS-P bound to the rhMD-2 immobilized on microtitre plates or to THP-1 cells, and whether TSS-P interfered with the binding of LPS to them. As expected, the *E. coli* LPS bound to MD-2 and THP-1 cells in a dose-dependent manner, and the addition of rhCD14 plus rhLBP, or serum, significantly enhanced the binding capacity of the LPS (Figs 7a and 8a). In contrast, TSS-P bound neither to

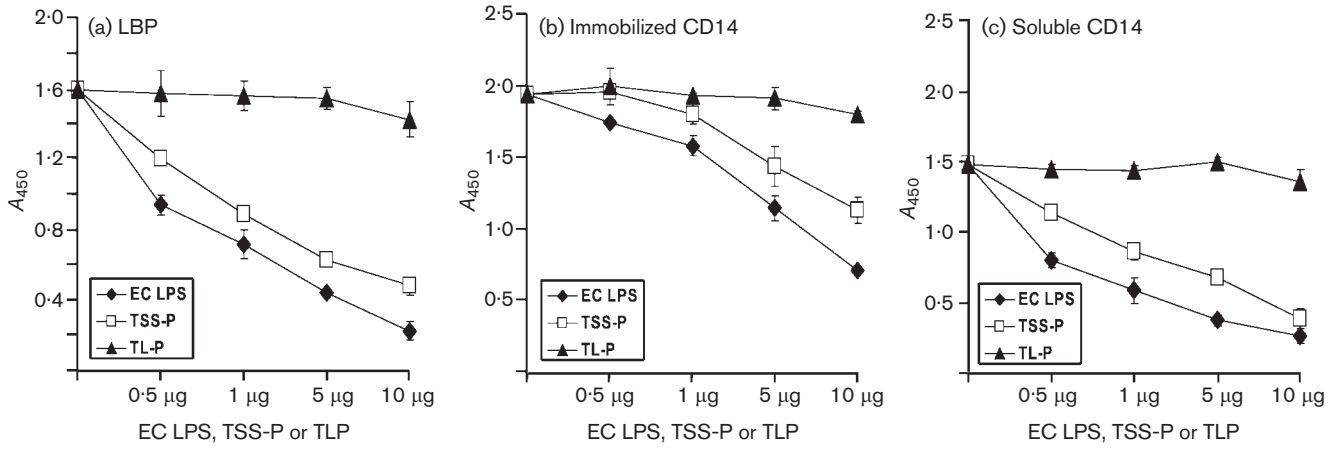

Fig. 6. Inhibition of *E. coli* LPS binding to LBP and CD14 by TSS-P. (a, b) EIA plates were coated with anti-human LBP mAb–rhLBP (a) and rhCD14 (b). The plates were incubated with 0·5–10 μg per well of TSS-P, TL-P or *E. coli* LPS (EC LPS) in the presence of biotinylated *E. coli* LPS (500 ng per well). (c) For binding to sCD14, the preincubated mixture containing rhCD14 and 0·5–10 μg per well of TSS-P, TL-P or *E. coli* LPS in the presence of biotinylated *E. coli* LPS (500 ng per well) was added to plates coated with anti-human CD14 mAb. The bound *E. coli* LPS was detected using HRP-labelled streptavidin and TMB as a substrate for HRP. The experiments were performed three times, and representative data are shown as means and standard deviations.

MD-2 nor to the THP-1 cells, regardless of the presence of serum (data not shown). However, TSS-P inhibited the binding of the *E. coli* LPS to MD-2 and THP-1 cells in the presence of rhLBP/rhCD14 or serum (Figs 7b and 8a). Nevertheless, under the serum-free condition, TSS-P did not affect the binding of LPS to MD-2 or THP-1 cells (Figs 7c and 8b).

Effect of TSS-P on TLR4 level

Some LPS are known to modulate TLR expression. To determine whether TSS-P downregulated TLR4 expression as an antagonistic mechanism, its effect on TLR4 expression was analysed by flow cytometry. TSS-P did not change the expression levels of TLR4 (data not shown).

DISCUSSION

T. socranskii was first identified more than 20 years ago (Smibert *et al.*, 1984), and many studies have reported its association with periodontitis and endodontic infections (Baumgartner *et al.*, 2003; Ellen & Galimanas, 2005; Moter

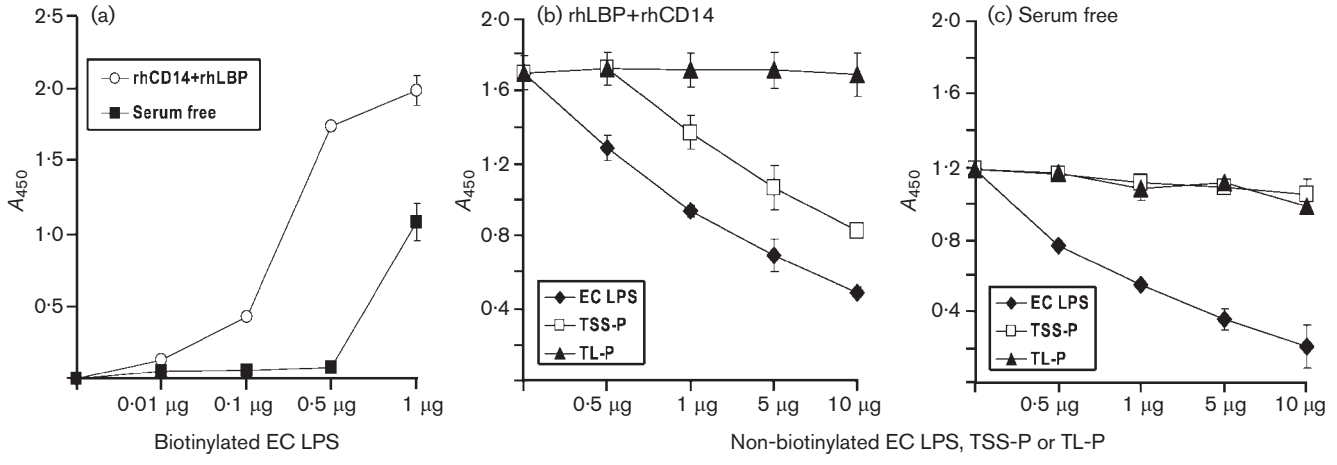

Fig. 7. Inhibition of *E. coli* LPS binding to MD-2 by TSS-P. (a) rhMD-2 was immobilized on EIA plates and incubated with biotinylated *E. coli* LPS (EC LPS, 0·01–1 μg per well). (b, c) For the binding inhibition assay, biotinylated *E. coli* LPS (500 ng per well) was incubated with nonbiotinylated *E. coli* LPS, TSS-P or TL-P (0·5–10 μg per well) in the presence or absence of rhLBP and rhCD14.

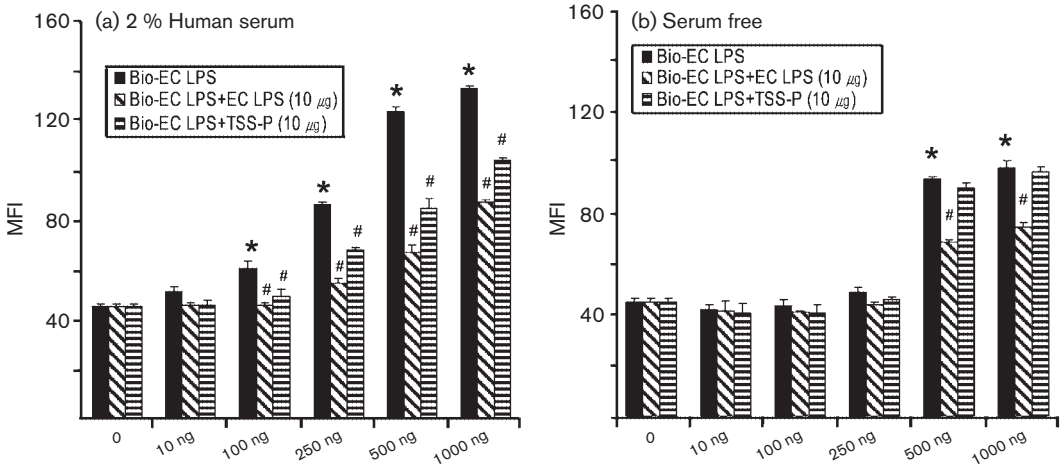

Fig. 8. Inhibition of *E. coli* LPS binding to THP-1 cells by TSS-P. THP-1 cells (1×10^6 cells ml^{-1}) cultured in RPMI 1640 medium were incubated with biotinylated *E. coli* LPS (10–1000 ng ml^{-1}) alone or with TSS-P (10 μg ml^{-1}) or *E. coli* LPS (EC LPS, 10 μg ml^{-1}) in the presence (a) or absence (b) of 2 % HS at 37 °C for 1 h. The cells were stained with FITC-labelled streptavidin and analysed by flow cytometry. The experiments were performed three times, and representative data are shown as means and standard deviations. The asterisks (*) indicate significant differences ($P<0{\cdot}05$) compared with the nontreated control values and the hash signs (#) indicate significant differences ($P<0{\cdot}05$) compared with the values for biotinylated *E. coli* LPS-treated cells. MFI, mean fluorescence intensity.

et al., 1998; Socransky *et al.*, 1991; Takeuchi *et al.*, 2001). However, its virulence has not been throughly investigated. Here, we studied the biological activity of a phenol/water extract of *T. socranskii*, by analysing its effect on the expression of proinflammatory cytokines and ICAM-1 in THP-1 cells and HGFs. The molecules of *E. coli* and *A. actinomycetemcomitans* prepared by phenol/water extraction revealed a typical LPS pattern on silver-stained SDS-PAGE gels, and the endotoxicity of both commercial and the extracted *E. coli* LPS was nearly identical. From these results, we concluded that the phenol/water extracts of oral spirochaetes are also LPS-like molecules. However, the chemical composition of these molecules remains to be characterized. The LPS-like materials in oral spirochaetes prepared by phenol/water extraction are termed glycolipids (Schröder *et al.*, 2000; Schultz *et al.*, 1998), lipooligosaccharides (Choi *et al.*, 2003; Rosen *et al.*, 1999) and glycoconjugates (Asai *et al.*, 2003a) in various reports.

Although the cell-stimulating activities of glycolipids in a few oral spirochaetes have been reported (Choi *et al.*, 2003, Kesavalu *et al.*, 2002; Opitz *et al.*, 2001; Rosen *et al.*, 1999), TSS-P showed minimal endotoxicity (about 10 000-fold less endotoxicity than *E. coli* LPS) and no cell-stimulating activity. Rather, it exhibited antagonistic activity against the TLR4-mediated signalling of LPS from *E. coli* and *A. actinomycetemcomitans*. The ICAM-1 expression stimulated by the LPS of these bacteria was significantly inhibited by a 10- to 1000-fold mass excess of TSS-P in the presence of serum. In contrast, TSS-P did not affect the ICAM-1 expression induced by peptidoglycan or IL-1β in THP-1 cells. TSS-P pretreatment did not affect the cellular response to subsequent *E. coli* LPS treatment. This suggests that TSS-P-dependent antagonism of *E. coli* LPS in THP-1 cells did not occur via an indirect mechanism like endotoxin tolerance.

An antagonistic effect of LPS has been reported in other bacteria such as *Rhodobacter sphaeroides* (Jarvis *et al.*, 1997), *Rhodobacter capsulatus* (Loppnow *et al.*, 1990), *Helicobacter pylori* (Lepper *et al.*, 2005) and some periodontal pathogens. Among periodontal pathogens, *Porphyromonas gingivalis* LPS antagonized the response to LPS of TLR4 ligands in human endothelial cells (Coats *et al.*, 2003) and HGFs (Yoshimura *et al.*, 2002). *Capnocytophaga ochracea* LPS showed the same antagonistic effect in HGFs as did the *P. gingivalis* LPS (Yoshimura *et al.*, 2002). *T. medium* glycolipid has been reported to show antagonism against a TLR4 ligand, and the antagonistic mechanism has been suggested to be competitive binding to LBP and CD14 (Asai *et al.*, 2003a). In our study, TSS-P exhibited similar antagonistic effects, showing competitive binding to LBP and CD14. Since TSS-P did not inhibit the intracellular signalling of IL-1β, and TLR4 and IL-1R have a common intracellular TIR domain, the inhibition targets of TSS-P should be upstream of the convergence point of TLR4 and IL-1β signalling. LBP monomerizes LPS molecules to prevent them from forming aggregates and transfers them to CD14, which in turn presents LPS to TLR4–MD-2. In the presence of LBP, the presentation of LPS by CD14 is facilitated. In our experimental system, despite expression of mCD14 on the THP-1 cells, the addition of sCD14 along with LBP was necessary for the cellular response to a low dose of LPS and for the antagonistic effect of TSS-P. It seems that a low dose

of LPS forms a complex with sCD14 more efficiently than with mCD14. Thomas *et al.* (2002) also observed that sCD14 and LBP enhanced the sensitivity of human monocytes to minute amounts of LPS. They suggested that minute concentrations of LPS are better delivered to the TLR4–MD-2 complex by sCD14 than by mCD14. In the binding inhibition assay, we used soluble and immobilized CD14; the latter was intended to mimic mCD14. Although TSS-P inhibited LPS binding to immobilized CD14, it did not significantly antagonize the induction of ICAM-1 expression by LPS under the serum-free condition, in which mC14 was the only CD14 available. Furthermore, TSS-P did not bind to THP-1 cells independent of serum and inhibited *E. coli* LPS binding to the cells only in the presence of sCD14 and LBP. These results suggest that TSS-P antagonized LPS signalling by interfering with the function of sCD14 rather than that of mCD14, and matrix-immobilized molecules could not represent membrane-bound forms. Further studies are necessary to address the difference between sCD14 and mD14 in LPS binding.

Although peptidoglycan was dependent on sCD14 for its cellular signalling (Asai *et al.*, 2003a), we did not observe an enhanced cellular response by the addition of rhCD14 and the inhibition of peptidoglycan-induced activity by TSS-P. This discrepancy could be due to the peptidoglycan used. Asai *et al.* (2003a) used peptidoglycan that was extracted from *S. aureus* in their laboratory, whereas we used commercial *S. aureus* peptidoglycan. The molecules involved in the cellular response by peptidoglycan were believed to be TLR2 and possibly CD14. However, the concept of TLR2-mediated peptidoglycan signalling is challenged by the fact that this activity did not originate from peptidoglycan but from contaminating cell wall components like lipopeptides and lipoteichoic acid (Travassos *et al.*, 2004). The different response to sCD14 observed by us and by Asai *et al.* (2003a) could be due to a difference in the contamination degree and molecules in the peptidoglycan used by the two groups. Shimizu *et al.* (2004) showed that cells expressing mCD14 did not need exogenous sCD14 for TLR2-mediated peptidiglycan signalling. This result is consistent with our observation that peptidoglycan stimulated cells in an sCD14-independent manner. Shimizu *et al.* (2004) also suggested the presence of CD14-independent peptidoglycan signalling by observing that mCD14-deficient epithelial cells responded to peptidoglycan to some extent under serum-free conditions. Recent investigations revealed that peptidoglycan recognition proteins (PGRP) and nucleotide-binding oligomerization domain (NOD) proteins play a key role in peptidoglycan-induced cell signalling (Girardin & Philpott, 2004).

It is known that LPS presented by CD14 binds to cells via the receptor TLR4–MD-2, forming LPS–TLR4–MD-2 complexes (Mancek *et al.*, 2002; Miyake 2003; Palsson-McDermott & O'Neill, 2004). MD-2 is a molecule that is associated with the extracellular domain of TLR4. Therefore, TLR4–MD-2 could be another target of TSS-P antagonism, in addition to LBP and CD14. TSS-P bound to neither immobilized rhMD-2 nor THP-1 cells, whereas the *E. coli* LPS bound to both of them in a dose-dependent manner. However, TSS-P interfered with the binding of *E. coli* LPS to immobilized MD-2 and to THP-1 cells in the presence of 2 % HS or rhCD14/rhLBP. This binding inhibition was not observed in the absence of serum. These results confirmed that TSS-P antagonized LPS signalling by interfering with the interaction of LPS with CD14 and LBP.

The agonistic and antagonistic effects of various LPS molecules appear to be due to their structural differences. The presence and the structure of fatty acids in lipid A play a key role in the biological activities of LPS. Lipid A with less than six acyl chains showed little or no cell-stimulating activity in human cells or antagonistic activity against active LPS (Schromm *et al.*, 2000). In addition to the number, length and position of the acyl chains, the saturation degree and the charge density determine the intramolecular conformation of amphiphilic LPS molecules, which affects their biological activity. Lipid A from *R. sphaeroides* and lipid IVa, a lipid A precursor with a tetra-acyl group, are antagonistic, but are concurrently agonistic to TLR4, although the differential response is species-specific (Hajjar *et al.*, 2002, Lohmann *et al.*, 2003; Saitoh *et al.*, 2004). The characteristics of lipid A from *R. sphaeroides* are penta-acyl chains with shorter chain lengths, including one unsaturated chain (Brandenburg *et al.*, 2003). *P. gingivalis* LPS showed an antagonistic effect to TLR4 and an agonistic effect to TLR2 (Coats *et al.*, 2003; Hirschfeld *et al.*, 2001). Also, a single strain of *P. gingivalis* showed multiple lipid A forms that activate cells via both TLR2 and TLR4 (Darveau *et al.*, 2004).

The structures of glycolipids in oral spirochaetes like *T. medium*, *T. maltophilum* and *T. denticola* have been suggested to resemble lipoteichoic acid (Hashimoto *et al.*, 2003; Schröder *et al.*, 2000; Schultz *et al.*, 1998). The structural components essential for typical LPS like heptose, 2-keto-3-deoxyoctonate (KDO) and β-hydroxy fatty acids were absent in *T. denticola* (Schultz *et al.*, 1998) and *T. medium* (Schröder *et al.*, 2000). In *T. pectinovorum* LPS, KDO was found, whereas heptose and β-hydroxy fatty acids were absent (Walker *et al.*, 1999). All these observations indicate structural differences between glycolipids of oral spirochaetes and typical LPS, suggesting the possible use of different receptors and possibly different functions. *T. maltophilum* glycolipids used TLR2 as a main receptor to induce TNF-α in a murine macrophage cell line and in a human embryonic kidney cell line, HEK293, transfected with TLR2 (Opitz *et al.*, 2001). Asai *et al.* (2003b) reported that outer-membrane extracts of *Treponema vincentii*, *T. medium* and *T. denticola* induced IL-8 in human gingival epithelial cells, and this induction was inhibited by anti-human TLR2 monoclonal antibody. However, the single active component in the outer-membrane extracts in this organism was not elucidated. In other spirochaetes, LPS from *Leptospira interrogans* has been reported as a TLR2 agonist (Werts *et al.*, 2001). TSS-P did not show

cell-stimulating activity in THP-1 cells and HGFs that expressed TLR2.

Growth conditions are known to influence the biochemical and antigenic properties of the bacterial outer membrane. LPS of *P. gingivalis* grown in haemin-containing medium showed an additional antigenic determinant (Cutler *et al.*, 1996) as compared with that of cells grown in haemin-depleted medium. Different medium compositions resulted in heterogeneous LPS types (Darveau *et al.*, 2004). To address this problem, we used two different media (OMIZ-Pat and NOS) for *T. socranskii* culture and found no difference in the antagonistic effects of TSS-P extracted from the two cultures.

In summary, TSS-P exhibited no cell-stimulating activity in cells expressing TLR4 and TLR2. By blocking the function of LBP/CD14, it potentially antagonized LPS activity, which normally would stimulate proinflammatory cytokines and ICAM-1 in THP-1 cells and HGFs. These properties indicate that TSS-P may allow the bacteria to escape the innate host defence system during the initiation and progression of periodontitis. The antagonistic property may be a great advantage for the bacteria, enabling them to modulate the innate immune system and therefore to facilitate host tissue colonization and disease progression. This may contribute to prolonged persistent chronic periodontitis. The elucidation of the structure of TSS-P in the future may provide more insight into the correlation between the structure and function of glycolipids of oral spirochaetes.

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