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Correlation between chemical structure and biological activities of *Porphyromonas gingivalis* synthetic lipopeptide derivatives

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Summary

We recently separated a PG1828-encoded triacylated lipoprotein (Pg-LP), composed of two palmitoyl and one pentadecanoyl groups at the N-terminal of glycerocysteine from *Porphyromonas gingivalis*, a periodontopathic bacteria, and found that Pg-LP exhibited definite biological activities through Toll-like receptor (TLR) 2. In the present study, we synthesized 12 different Pg-LP N-terminal peptide moieties (PGTP) using four combinations of glyceryl (*R* and *S*) and cysteinyl (L and D) stereoisomers, and three different acyl group regioisomers, *N*-pentadecanoyl derivative (PGTP1), *S*-glycero 2-pentadecanoyl derivative (PGTP2) and *S*-glycero 3-pentadecanoyl derivative (PGTP3). All the PGTP compounds (RL, SL, SD, RD) tested showed TLR2-dependent cell activation. The activating capacities of the PGTP-R compounds were more potent than those of the PGTP-S compounds, whereas there were no differences between the PGTP-L and -D compounds. Furthermore, the production of interleukin (IL)-6 following stimulation with the PGTP1-RL, PGTP2-RL and PGTP3-RL compounds was impaired in peritoneal macrophages from TLR2 knock-out (KO), but not those from TLR1 KO or TLR6 KO mice. These results suggest that *P. gingivalis* triacylated lipopeptides are capable of activating host cells in a TLR2-dependent and TLR1-/TLR6-independent manner, and the fatty acid residue at the glycerol position in the PGTP molecule plays an important role in recognition by TLR2.

Keywords: lipoprotein, *Porphyromonas gingivalis*, stereoisomer, synthetic lipopeptide, Toll-like receptor

Introduction

Porphyromonas gingivalis, a Gram-negative, obligate anaerobic oral black-pigmented bacterium, is considered to be one of the major pathogens in the development and progression of periodontal diseases [1,2]. The bacterium is well known to possess potential virulence factors on its surface, such as fimbriae and lipopolysaccharide (LPS), which have been shown to activate host cells, such as macrophages, fibroblasts, epithelial cells and endothelial cells [3–7]. *P. gingivalis* LPS is known to have a lower endotoxic property than enterobacterial LPS because of the chemical structure of its lipid A, an active centre of LPS [8,9]. Furthermore, *P. gingivalis* LPS/lipid A have been reported to have a unique feature that activates cells derived from LPS-hyporesponder C3H/HeJ mice as well as those from LPS-responder mice [10].

A pattern-recognition receptor family, Toll-like receptors (TLRs), have been identified in mammals based on their homology with the Drosophila protein, Toll, and implicated in the recognition of a variety of microbial components [11]. Mammalian TLRs are composed of a large family with extracellular leucine-rich repeats and a cytoplasmic Toll/interleukin (IL)-1 receptor homology domain, and alert the host to the existence of infection through direct recognition of conserved structural moieties of bacteria, viruses and fungi [12]. Among TLRs identified, it was indicated that TLR4 acts as LPS receptor concomitant with its associated molecule MD-2 [13] and C3H/HeJ mice possess a natural point mutation of TLR4 [14]. Furthermore, TLR3, 5 and 9 recognize viral double-stranded RNA, bacterial flagellin and bacterial unmethylated CpG DNA, respectively [15]. In contrast to other TLRs, TLR2 has been demonstrated to mediate cellular responses to a much wider variety of microbial components, including peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipoprotein/lipopeptide and zymosan [16]. *P. gingivalis* LPS was also indicated to activate host cells through TLR2 rather than TLR4 [17]. However, it was demonstrated that *P. gingivalis* lipid A was

correlated with TLR4 but not TLR2 [5,18]. Thus, the relationship between *P. gingivalis* LPS/lipid A and TLRs has been controversial.

Lipoprotein/lipopeptide appear to be representative of TLR2 ligands, because of their well-defined chemical structures. Namely, the cysteine residue in bacterial lipoprotein/lipopeptide is triacylated at the N-terminus, whereas mycoplasmal lipoprotein/lipopeptide possess a diacylated cysteine residue [19,20]. In addition, studies of peritoneal macrophages derived from TLR knock-out (KO) mice have demonstrated that murine TLR1 and TLR6 in collaboration with TLR2, predominantly mediate responses to triacylated and diacylated lipopeptides, respectively [21,22].

We recently separated a novel PG1828-encoded triacylated *P. gingivalis* lipoprotein (Pg-LP), composed of two palmitoyl and one pentadecanoyl groups at N-terminal glycerocysteine, from LPS preparation of *P. gingivalis* strain 381 and found that it exhibited definite biological activities through TLR2 [23]. In the present study, we synthesized 12 kinds of Pg-LP N-terminal peptide moieties (PGTP), which consisted of four combinations of glyceryl (*R* and *S*) and cysteinyl (L and D) stereoisomers, and three types of acyl group regioisomers, to examine the relationship between their structures and biological activities. We also investigated the recognition receptor for PGTP.

Materials and methods

Mice

Wild-type, TLR1 KO, TLR2 KO and TLR6 KO mice, engineered as described previously [21,22,24], were kindly provided by Dr S. Akira (Research Institute for Microbial Diseases, Osaka University, Japan). The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

Reagents

The racemic mixtures as well as pure *R*- and *S*-stereoisomers of the synthetic bacterial lipopeptides, purchased from EMC Microcollections (Tuebingen, Germany) were: Pam_3CSK_4, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteinyl-Ser-Lys-Lys-Lys-Lys; FSL-1, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteinyl-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam_2CGDPKHPKSF (lipopeptide from *Mycoplasma salivarium*); and MALP-2, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-L-cysteinyl-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys, Pam_2CGNNDESNISFKEK (macrophage-activating 2-kDa lipopeptide from *Mycoplasma fermentans*). *Escherichia coli* LPS was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA). *E. coli*-type lipid A (compound 506) was chemically synthesized as described by Imoto *et al.* [25].

Synthesis of triacyl *S*-glycerocysteine derivatives as Pg-LP N-terminus

Stereoisomer mixture of Pg-LP N-terminal glycerocysteine moieties; *N*-pentadecanoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-DL-cysteine is shown in Fig. 1a. *N*-palmitoyl-*S*-[2-pentadecanoyloxy, 3-palmitoyloxy-(2*RS*)-propyl]-DL-cysteine is shown in Fig. 1b. *N*-palmitoyl-*S*-[2-palmitoyloxy, 3-pentadecanoyloxy-(2*RS*)-propyl]-DL-cysteine is shown in Fig. 1c. Ser benzyl ester (Bachem AG, Bubendorf, Switzerland) was used as a starting material after being *N*-selective acylated using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (WSC; Wako Pure Chemical, Osaka, Japan) and pentadecanoic acid (Wako Pure Chemical) in pyridine/methanol gave *N*-pentadecanoyl Ser derivative (Fig. 1d). Ser benzyl ester was also *N*-selective acylated in a similar manner using palmitic acid (Wako Pure Chemical), and gave *N*-palmitoyl Ser derivative (Fig. 1e). The hydroxyl group of *N*-pentadecanoyl Ser derivative (Fig. 1d) was converted to bromide using *N*-bromosuccinimide (NBS; Wako Pure Chemical) and triphenylphosphine (Wako Pure Chemical) in CH_2Cl_2. The reactant was directly conjugated with thioglycerol with triethylamine in CH_2Cl_2, and gave *N*-pentadecanoyl *S*-glycerocysteine derivative (Fig. 1f). The hydroxyl group of *N*-palmitoyl Ser derivative (Fig. 1e) was also converted to bromide and conjugated with thioglycerol in a similar manner, and gave *N*-palmitoyl *S*-glycerocysteine derivative (Fig. 1g). Non-selective acylation of *N*-pentadecanoyl *S*-glycerocysteine derivative (Fig. 1d) was carried out with palmitic chloride in pyridine at room temperature and gave triacylated *N*-pentadecanoyl *S*-glycerocysteine derivative (Fig. 1h). Selective acylation of *N*-palmitoyl *S*-glycerocysteine derivative (Fig. 1g) was carried out in a stepwise fashion using palmitic acid with a 1·5 equivalent of WSC in pyridine/CH_2Cl_2 at 0°C, and 3-palmitoyloxy derivative (Fig. 1i) was obtained. *N*-palmitoyl *S*-glycerocysteine derivative (Fig. 1g) was also carried out in a similar manner using pentadecanoic acid, and 3-pentadecanoyloxy derivative (Fig. 1j) was obtained. 3-Palmitoyloxy derivative (Fig. 1i) was acylated using pentadecanoic acid and WSC in pyridine, and gave triacylated 2-pentadecanoyloxy *S*-glycerocysteine derivative (Fig. 1k). 3-Pentadecanoyloxy derivative (Fig. 1j) was also acylated in a similar manner, and gave triacylated 3-pentadecanoyloxy *S*-glycerocysteine derivative (Fig. 1l). The benzyl ester group of triacylated *N*-pentadecanoyloxy *S*-glycerocysteine derivative (Fig. 1h) was removed by treatment with palladium catalyst and gave the *N*-terminus of PGTP1 (Fig. 1a). The benzyl ester group of 2-pentadecanoyloxy *S*-glycerocysteine derivative (Fig. 1k) and 3-pentadecanoyloxy *S*-glycerocysteine derivative (Fig. 1l) were also removed in a similar manner, and N-terminus of PGTP2 (Fig. 1b) and PGTP3 (Fig. 1c) were obtained.

Each stereoisomer (RL, RD, SL, and SD) of PGTP1-3 N-terminus (Fig. 1a–c) was purified using high-pressure liquid

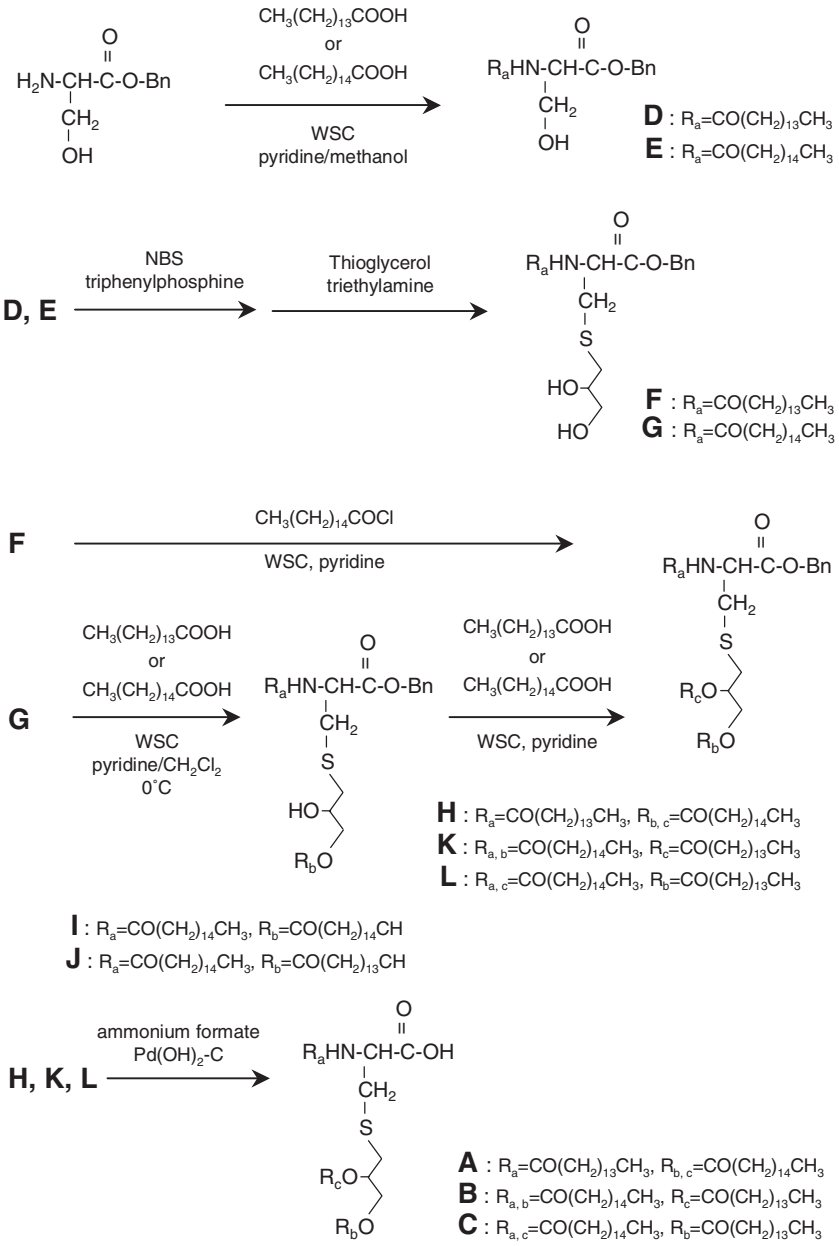

Fig. 1. Synthetic scheme of triacyl S-glycerocysteine derivatives.

chromatography (HPLC) with a chiral column (Chiralpak 1 A; Daicel Chemical Industries, Ltd, Osaka, Japan) (Fig. 2). Each optical configuration was determined by comparison with standard materials (*N*-pentadecanoyl-*S*-[2,3-bis (palmitoyloxy)-(2*R*)-propyl]-L-cysteine and *N*-pentadecanoyl-*S*-[2,3-bis(palmitoyloxy)-(2*S*)-propyl]-L-cysteine) were synthesized according to the method of Roth [26] using optical isolated 3-chloro-1,2-propanediol (Wako Pure Chemical).

Peptide synthesis

The general procedure used for the synthesis of peptides was a build up to H-Asn-Ser(ᵗBu)-Ala-Gln-Lys(Boc)-wang resin using the fluorenylmethoxycarbonyl (Fmoc) protocol for solid phase synthesis in an organic synthesizer (CCS-1200; EYELA, Tokyo, Japan). Each triacyl *S*-glycerocysteine derivative was manually coupled to the peptide-wang resin using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3- tetramethyluronium, tetrafluoroborate and 1-hydroxybenzotriazole, monohydrate. The peptide and all protecting groups were cleaved from the resin with trifluoroacetic acid: H_2O: triisopropylsilane: thiophenol (90 : 4 : 4 : 2). Each lipopeptide was purified by HPLC with a reverse-phase column (YMC-Pack TMS; YMC, Inc., Milford, MA, USA) (Fig. 3) and gave 12 different PGTP derivatives (Fig. 4). These lipopeptides were dissolved at a concentration of 10 mg/ml in dimethyl sulphoxide and diluted with each medium.

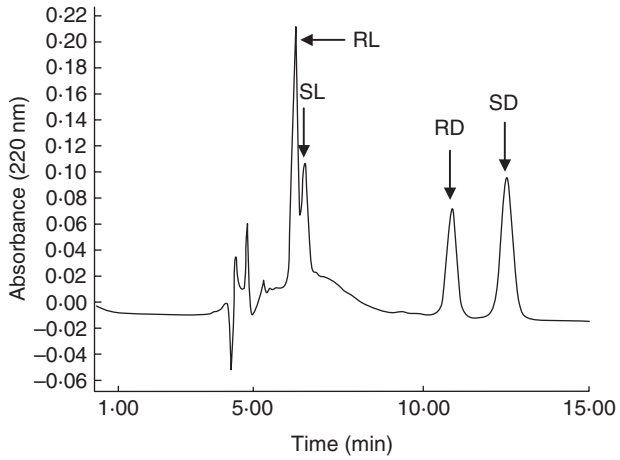

Fig. 2. Isolation of *N*-pentadecanoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-DL-cysteine (RL, RD, SL and SD). High-pressure liquid chromatography (HPLC) elution conditions: column; Daicel chiralpak 1 A (4·8 mm × 250 mm) 94·95% t-butyl methyl ether, 5% methanol, and 0·05% trifluoroacetic acid for 15 min at a flow rate of 0·8 ml/min.

Luciferase assay

Interleukin (IL)-3-dependent murine Ba/F3 pro-B cells stably expressing p55IgκLuc, as well as a nuclear factor (NF)-κB/DNA binding activity-dependent luciferase reporter construct (Ba/κB), murine TLR2 and a p55IgκLuc reporter construct (Ba/mTLR2) and murine TLR4/MD-2 and a p55IgκLuc reporter construct (Ba/mTLR4/mMD-2), were kindly provided by Dr K. Miyake (Institute of Medical Science, University of Tokyo, Japan) and used to detect NF-κB-dependent luciferase activity, as described previously [27]. Briefly, the cells were inoculated onto 96-well plates with 1×10^5/100 µl of RPMI-1640 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; Sigma Chemical Co.), and stimulated separately with various doses of the test specimens. After 4 h at 37°C, 100 µl of Bright-Glo™ luciferase assay reagent (Promega, Madison, WI, USA) was added to each well and luminescence was quantified with a luminometer (Turner Designs Luminometer Model TD-20/20; Promega).

Human cell culture and stimulation assay

This experiment was performed with four healthy adult volunteers (two males, two females; average age 35·25 years). All subjects were informed regarding the study and each signed an informed consent form approved by the Ethics Committee of Asahi University (reference number 15007). Heparinized venous blood drawn from healthy donors was subjected to fractionation using a Histopaque-1077 (Sigma Chemical Co.) to obtain human peripheral blood mononuclear cells (PBMC). These cells were stimulated with various doses of the test specimens for 24 h at 37°C. Following incubation, the culture supernatants were collected and analysed for secreted IL-8 using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Murine cell culture and stimulation assay

A mouse macrophage cell line, J774·1 (Dainippon Pharmaceutical, Osaka, Japan), was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co.) supplemented with 10% FBS, 50 µg/ml of gentamicin and 50 ng/ml of amphotericin B, after which the cells were distributed to each well of a 96-well flat-bottomed plate at 2×10^5 cells per 200 µl. In some experiments, elicited peritoneal macrophages were obtained from mice 72 h after intraperitoneal inoculation with 1·0 ml of 3% sterile Brewer's thioglycolate broth (BD Biosciences, San Jose, CA, USA). Peritoneal exudate cells were centrifuged and suspended in RPMI-1640 supplemented with 10% FBS, 50 µg/ml of gentamicin and

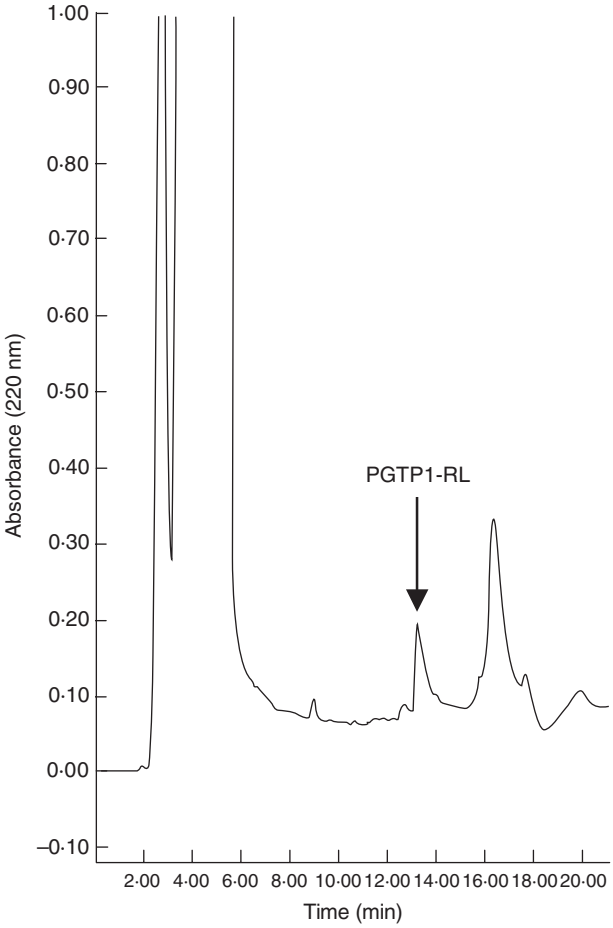

Fig. 3. High-pressure liquid chromatography (HPLC) profile of *Porphyromonas gingivalis* lipopeptide derivative (PGTP1-RL). HPLC elution conditions: column; YMC-Pack TMS (6 mm × 150 mm) with a linear increase of 0·05% trifluoroacetic acid-acetonitrile concentration from 70% to 90% in 0·05% trifluoroacetic acid a.q. for 20 min at a flow rate of 1 ml/min.

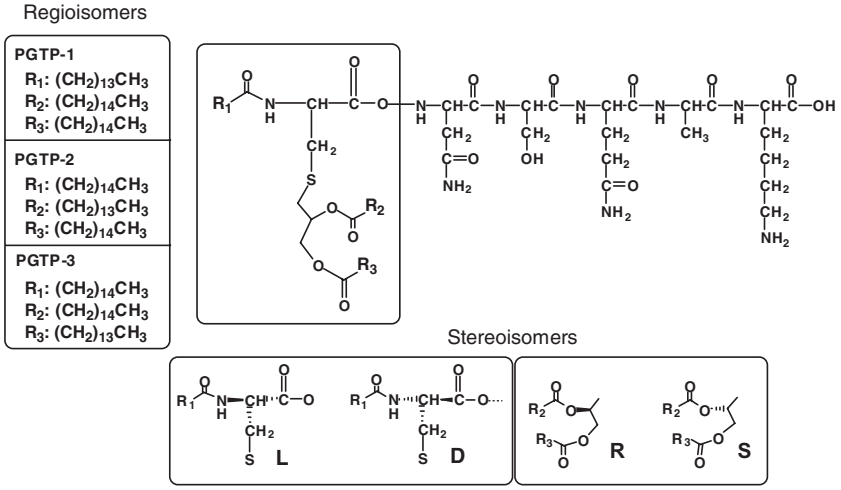

Fig. 4. Chemical structures of synthetic lipopeptide derivatives of *Porphyromonas gingivalis* used in this study.

50 ng/ml of amphotericin B at 1×10^6 cells/ml. These cells were distributed to each well of a 96-well flat-bottomed plate at 2×10^5 cells per 200 μl, after which they were incubated for 2 h at 37°C in humidified air containing 5% (v/v) CO_2. Each well was then washed twice with phosphate-buffered saline (PBS; Sigma Chemical Co.) to remove non-adherent cells and those attached to the culture plate served as peritoneal macrophages. These cells were stimulated with various doses of the test specimens for 24 h at 37°C. Following incubation, the culture supernatants were collected and analysed for secreted IL-6 using an ELISA kit (eBioscience, San Diego, CA, USA).

Statistical analysis

Cytokine production was analysed by one-way analysis of variance (ANOVA), using the Bonferroni or Dunn method, and the results are presented as the mean ± standard error of the mean (s.e.m.).

Results

NF-κB activation of Ba/F3 cells stimulated with PGTP compounds

All PGTP compounds used in the experiments were first analysed for TLR2-dependent signalling using a luciferase assay. All PGTP compounds induced NF-κB activation in Ba/mTLR2, whereas Ba/mTLR4/mMD-2 and Ba/κB induced a little response (Fig. 5). Among these PGTP compounds, PGTP-RL (Fig. 5a,e,i) and -RD (Fig. 5d,h,l) showed potent NF-κB activation at lower concentrations than PGTP-SL (Fig. 5b,f,j) and -SD (Fig. 5c,g,k), However, there were no remarkable differences between PGTP-RL and -RD, or PGTP-SL and -SD. Similarly, Pam_3CSK_4-R (Fig. 5m) and FSL-1-R (Fig. 5o) exhibited a dose-dependent NF-κB activation that was different from that of Pam_3CSK_4-S (Fig. 5n) and FSL-1-S (Fig. 5p), respectively. Further, PGTP1, PGTP2 and PGTP3 showed nearly the same NF-κB activation with each stereoisomer group. *E. coli* LPS (Fig. 5q) and compound 506 (Fig. 5r) were used as positive control stimulants of TLR4 and induced a clear NF-κB activation in only Ba/mTLR4/mMD-2.

Cytokine production by human and murine cells

NF-κB activation leads to the induction of a number of inflammatory cytokines [28]. To clarify the correlation with NF-κB activation by PGTP compounds, IL-8-producing activities were examined in human PBMC after 24 h of stimulation with various PGTP compounds (Fig. 6). The PGTP-RL and -RD compounds were found to be potent IL-8 inducers, in contrast to the PGTP-SL and -SD compounds. Similarly, Pam_3CSK_4-R and FSL-1-R showed significant IL-8 production as compared to each S counterpart. Furthermore, the production patterns of IL-6 from J774·1 cells were similar to those of IL-8 in PBMC stimulated with these test specimens (Fig. 7). However, the differences between R- and S-stereoisomers were clear with murine J774·1 cells but not human PBMC. Compound 506 also clearly exhibited IL-8 and IL-6 production.

Identification of the PGTP receptor

To address the possible contribution of a cell-surface receptor to PGTP signalling, we examined the responsiveness of peritoneal macrophages from wild-type, TLR1 KO, TLR2 KO and TLR6 KO mice to the PGTP1-RL, PGTP2-RL and PGTP3-RL compounds. All PGTP-RL compounds distinctly induced IL-6 production in wild-type macrophages (Fig. 8). In contrast, IL-6 production in response to PGTP-RL compounds was completely abrogated in macrophages lacking TLR2, whereas TLR1 KO and TLR6 KO macrophages showed nearly the same IL-6-producing activities as wild-type macrophages after stimulation with PGTP-RL compounds. Triacylated Pam_3CSK_4 and

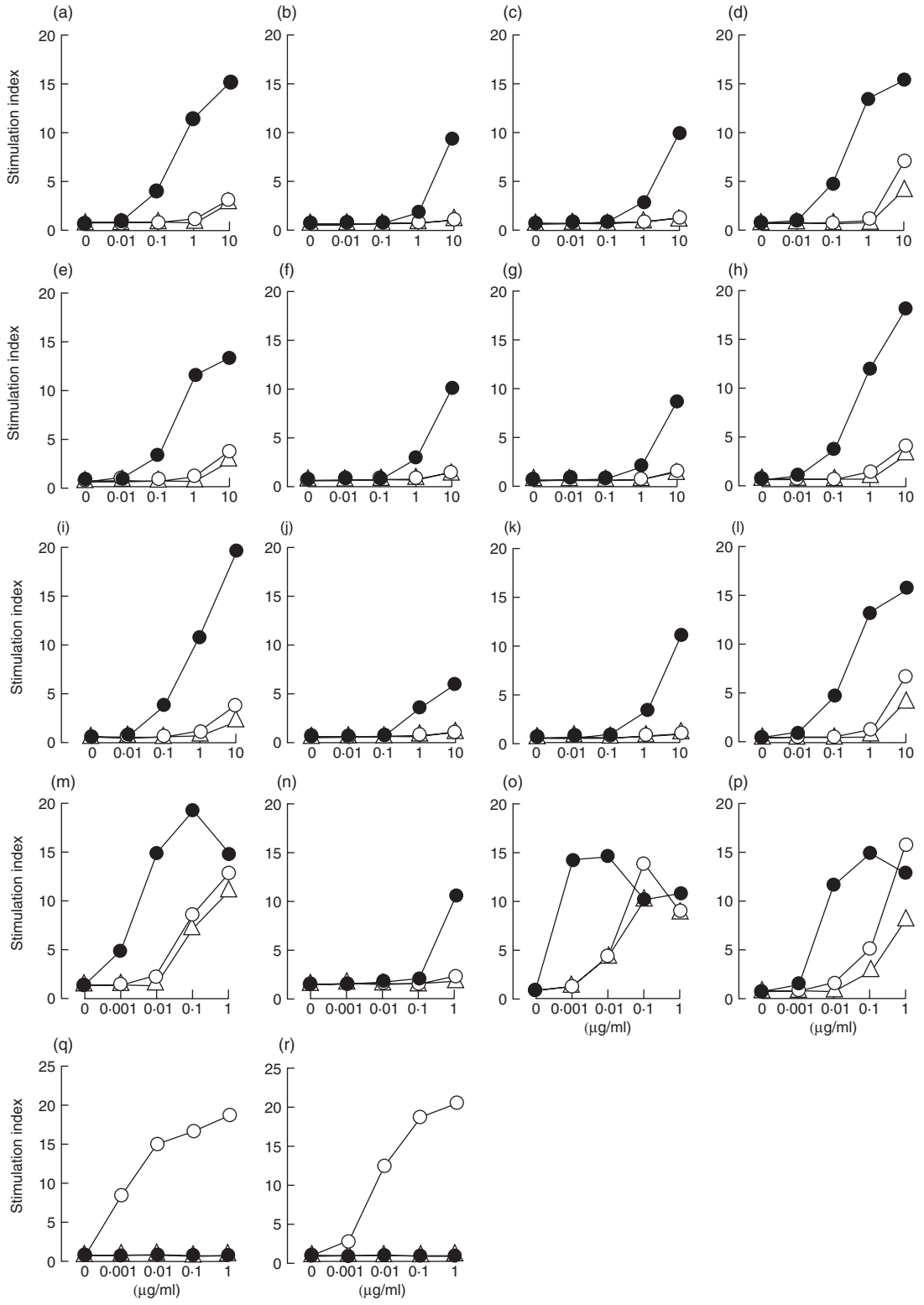

Fig. 5. TLR2-dependent nuclear factor (NF)-κB activation of Pg-LP N-terminal peptide moieties (PGTP) compounds. Ba/κB (open triangle), Ba/mTLR2 (closed circle) and Ba/mTLR4/mMD-2 (open circle) were stimulated for 4 h with the indicated doses of PGTP1-RL (a), PGTP1-SL (b), PGTP1-SD (c), PGTP1-RD (d), PGTP2-RL (e), PGTP2-SL (f), PGTP2-SD (g), PGTP2-RD (h), PGTP3-RL (i), PGTP3-SL (j), PGTP3-SD (k), PGTP3-RD (l), Pam_3CSK_4-R (m), Pam_3CSK_4-S (n), FSL-1-R (o), FSL-1-S (p), *Escherichia coli* lipopolysaccharide (LPS) (q), and compound 506 (r). NF-κB activation was determined using a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to non-stimulated activity.

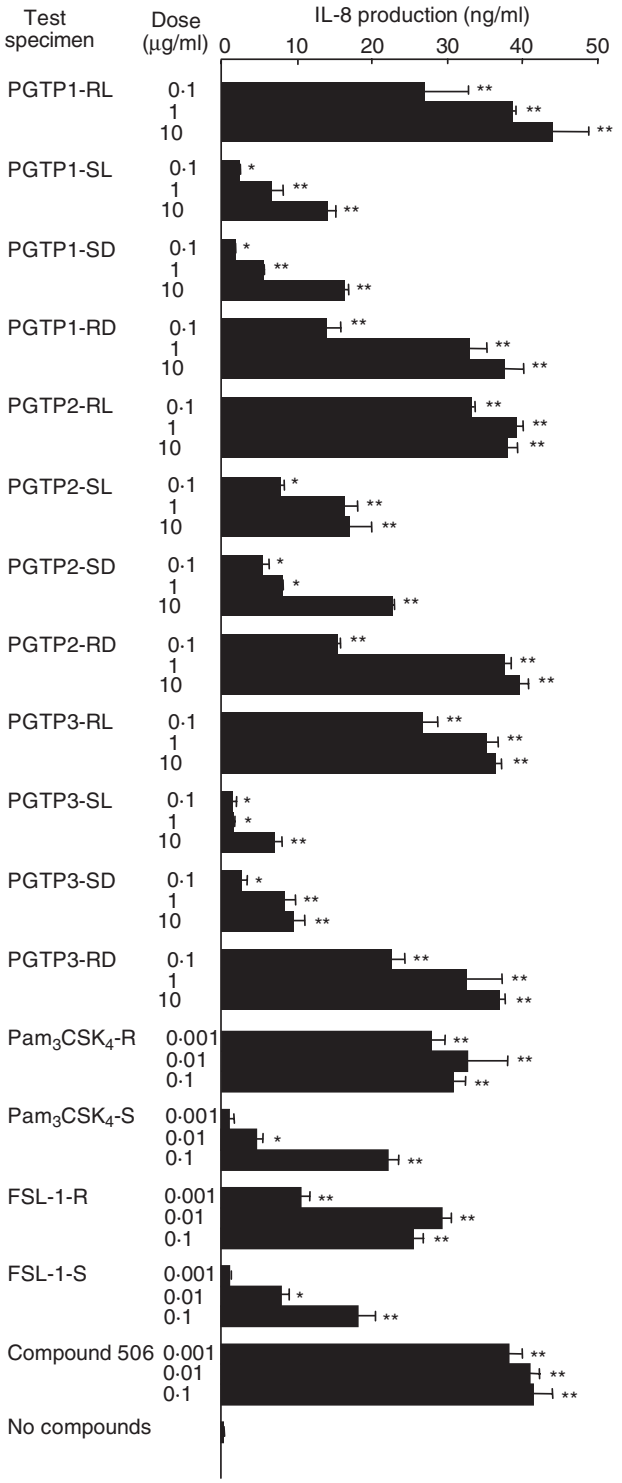

Fig. 6. Interleukin (IL)-8 production by human peripheral blood mononuclear cells (PBMC) in response to stimulation by Pg-LP N-terminal peptide moieties (PGTP) compounds. The cells were cultured at 37°C for 24 h with the indicated doses of the test specimens. Following incubation, the supernatants were collected and IL-8 production was determined by enzyme-linked immuosorbent assay (ELISA). Experiments were performed at least four times, and representative results are presented. Each assay was performed in triplicate and the data are expressed as the mean ± s.e.m. Statistically significant difference from the mean value of each test specimen against no compounds (**$P < 0{\cdot}01$, *$P < 0{\cdot}05$) was evaluated by analysis of variance and the Bonferroni or Dunn method.

diacylated MALP-2 showed a requirement for TLR1 and TLR6, respectively, for their signalling. These findings are consistent with the current assumption that the requirement for TLR1 or TLR6 is based on the acylation pattern of lipopeptide [21,22]. *E. coli* LPS exhibited almost the same IL-6 production with all types of macrophages used these experiments.

Discussion

P. gingivalis is generally recognized as a major aetiological agent of periodontal diseases [1,2], and its LPS, one of its most examined virulence factors, participates in the development of periodontal destructive lesions [17]. However, we recently reported that the principal cell-activating molecule was a triacylated lipoprotein contaminated in LPS and its lipid A preparations [3,23], which suggested that nearly all the previous studies regarding the biological activities of *P. gingivalis* LPS and its lipid A preparations reported those activities after being induced by *P. gingivalis* lipoprotein. Thus far, various evidences of the correlations between the structures and biological activities of bacterial triacylated lipopeptides have been clarified [29,30]; however, there is no known report regarding a synthetic *P. gingivalis* lipopeptide. An odd-numbered carbon chain in the acyl group is a unique characteristic of *P. gingivalis* lipopeptide compared with other reported synthetic lipopeptide derivatives [23]. The numbers of odd-numbered carbon chains of fatty acid are known to be much fewer than those of even-numbered chains, because of the mechanism of the biosynthesis pathway [31]. Therefore, we considered that synthesis of *P. gingivalis* lipopeptide would help to elucidate the mechanism involved with the development and progression of periodontal diseases.

We investigated the relationships between the structures and biological activities of 12 different *P. gingivalis* triacylated lipopeptide derivatives, which consisted of four combinations of stereoisomers and three types of acyl group regioisomers, in order to examine the influence of stereoconfiguration on cell activation (Fig. 4). Stereoconfiguration plays an important role in signal transduction representative of muramyl dipeptide (MDP), the minimal essential structural unit responsible for the immunoadjuvant activity of peptidoglycan. The cytotoxic activity of L-isoglutaminyl stereoisomer of MDP was demonstrated to be much weaker than that of its natural products [32]. It was also suggested that the natural lipoprotein had an *R* configuration [33]. In the present results, the PGTP-RL and -RD compounds showed stronger TLR2-dependent NF-κB activation and cytokine production than the PGTP-SL and -SD compounds

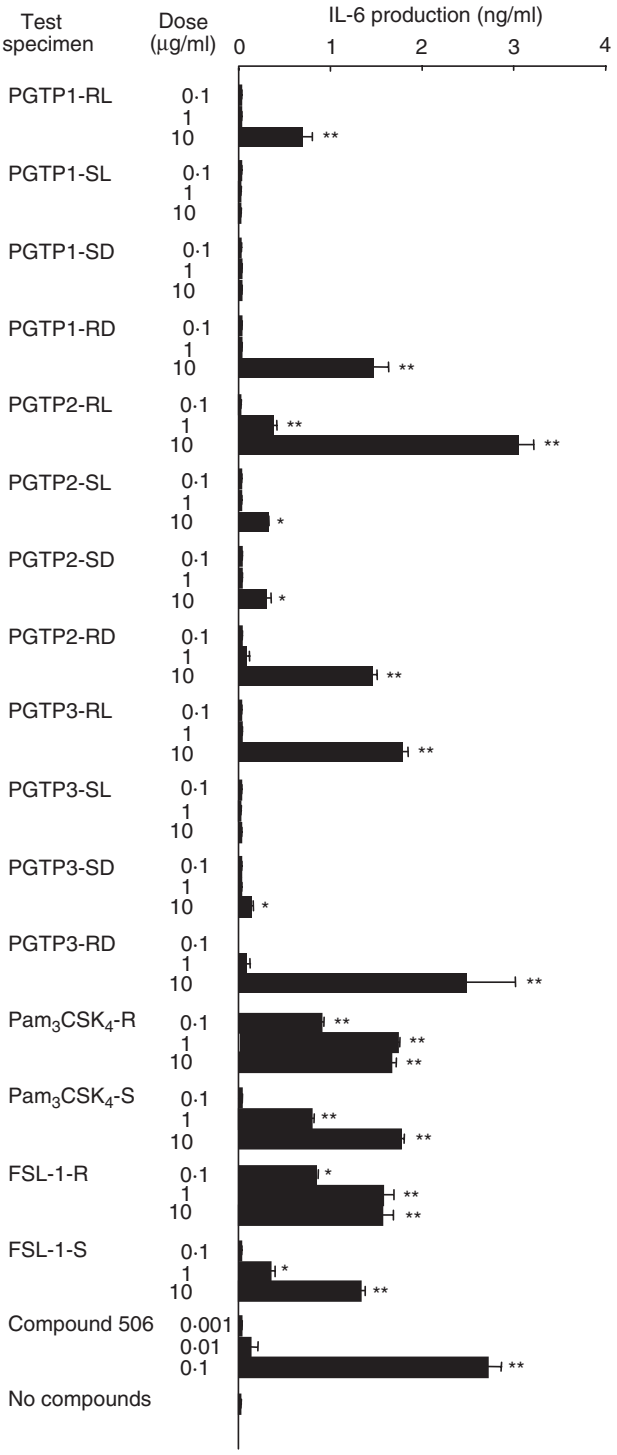

Fig. 7. Interleukin (IL)-6 production by J774·1 cells in response to stimulation by Pg-LP N-terminal peptide moieties (PGTP) compounds. The cells were cultured at 37°C for 24 h with the indicated doses of the test specimens. Following incubation, the supernatants were collected and IL-6 production was determined by enzyme-linked immuosorbent assay (ELISA). Experiments were performed at least three times, and representative results are presented. Each assay was performed in triplicate and the data are expressed as the mean ± s.e.m. Statistically significant difference from the mean value of each test specimen against no compounds (**$P < 0{\cdot}01$, *$P < 0{\cdot}05$) was evaluated by analysis of variance and the Bonferroni or Dunn method.

(Figs 5–7). Similarly, Pam_3CSK_4-R and FSL-1-R exhibited more potent activities as compared with Pam_3CSK_4-S and FSL-1-S. Therefore, we concluded that the cell activating capacities differed between the glyceryl stereoisomers (*R* and *S*), whereas there were no differences between the cysteinyl stereoisomers (L and D). Our results also suggest that the acyl group in the glycerol residue, but not the N-acyl group, plays a crucial role in TLR2 recognition.

We also observed differences between the *R*- and *S*-configurations in regard to their cell activating capacities with human and murine cells. There were distinct differences in cell activation shown regarding IL-6 production by J774·1 cells as compared to IL-8 production by human PBMC (Figs 6 and 7). Those differences between human and murine cells were speculated to be attributable to the fact that the PGTP compounds originated from a human pathogenic bacterium, *P. gingivalis*, therefore human cells are likely to be tolerant to the *S*-configuration, which scarcely exists in nature. In fact, species-specific differences in cellular pattern recognition have been reported for certain LPS variants and Taxol, suggesting variant uses of TLR4 and MD-2 [34–36]. Furthermore, it was shown that human but not murine TLR2 could distinguish between tri-palmitoylated and tri-lauroylated peptides, indicating that species-specific TLR2 recognition may exist [37]. Therefore, our results indicate that murine TLR2 may recognize the *R*- and *S*-configurations of lipopeptide compounds more strictly than human TLR2.

Among TLRs, TLR2 appears to be characteristic in its necessity of cooperation with other TLRs, such as TLR1 and TLR6. To address the TLR1- and TLR6-dependencies of PGTP-recognition by TLR2, we examined the responsiveness of peritoneal macrophages from wild-type, TLR1 KO, TLR2 KO, and TLR6 KO mice to PGTP-RL compounds, and found that the PGTP-RL compounds activated host cells in a TLR2-dependent and TLR1-/TLR6-independent manner (Fig. 8). It was also recently demonstrated that a diacylated MALP2 elongated analogue, MALP2-SK4, was necessary for TLR2 but not TLR6 recognition [38] and triacylated lipopeptides with short-length ester-bound fatty acids activated cells through TLR2 but not TLR1 [39], suggesting that both lipid and N-terminal peptide moieties in PGTP compounds contribute to that recognition in a TLR1-/TLR6-independent manner. In conclusion, the present results demonstrated that *P. gingivalis* synthetic lipopeptide derivatives induced cell activation in a TLR2- but not TLR1-/TLR6-dependent manner.

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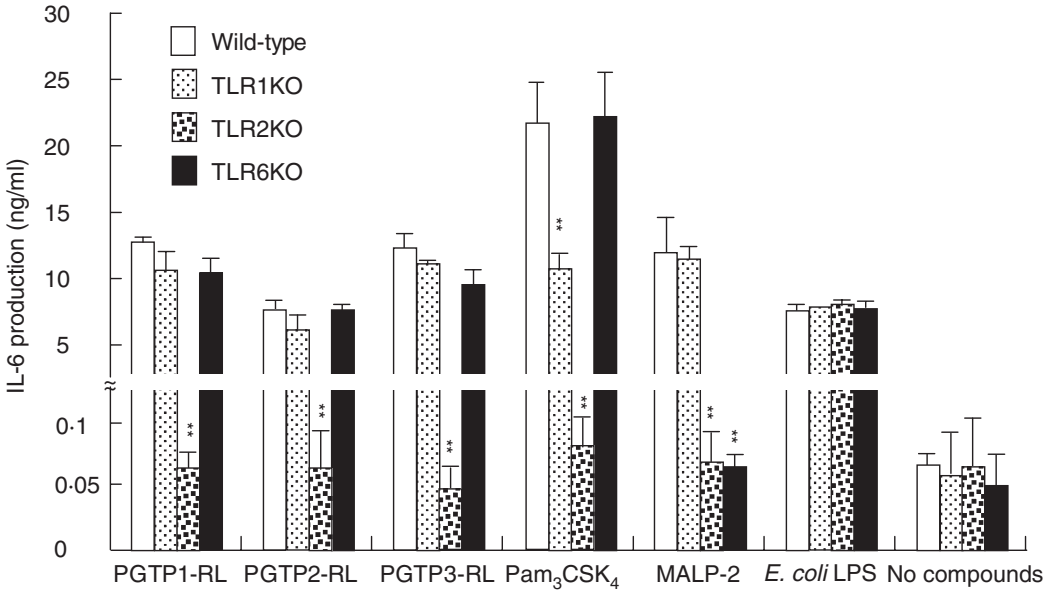

Fig. 8. IL-6 production by peritoneal exudated macrophages from wild-type, TLR1 KO, TLR2 KO, and TLR6 knock-out mice in response to stimulation by Pg-LP N-terminal peptide moieties (PGTP)–RL compounds. The cells were cultured at 37°C for 24 h with 1 μg/ml of PGTP compounds and 0·01 μg/ml of *Escherichia coli* lipopolysaccharide (LPS), Pam_3CSK_4, and MALP-2. Following incubation, the supernatants were collected and IL-6 production was determined by enzyme-linked immuosorbent assay (ELISA). Experiments were performed at least three times, and representative results are presented. Each assay was performed in triplicate and the data are expressed as the mean ± s.e.m. Statistically significant difference from the mean value of each test specimen against that of wild-type mice ($^{**}P < 0{\cdot}01$) was evaluated by analysis of variance and the Bonferroni or Dunn method.

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