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Cell activation by *Porphyromonas gingivalis* lipid A molecule through Toll-like receptor 4- and myeloid differentiation factor 88-dependent signaling pathway

Tomohiko Ogawa[1], Yasuyuki Asai[1], Masahito Hashimoto[1], Osamu Takeuchi[2], Tomoko Kurita[3], Yasunobu Yoshikai[4], Kensuke Miyake[5] and **Shizuo Akira[2]**

[1]Department of Oral Microbiology, Asahi University School of Dentistry, 1851 Hozumi, Hozumi-Cho, Motosu-Gun, Gifu 501-0296, Japan
[2]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
[3]Department of Microbiology, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan
[4]Division of Host Defense, Research Center for Prevention of Infectious Diseases, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan
[5]Division of Infectious Genetics, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

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Abstract

***Porphyromonas gingivalis* lipopolysaccharide (LPS) and its bioactive center, lipid A, are known to exhibit very low endotoxic activities and activate LPS-hyporesponsive C3H/HeJ mice that have a point mutation in the cytoplasmic portion of Toll-like receptor (TLR) 4, in contrast to classical enterobacterial LPS and their lipid A. In the present study, we attempted to determine which TLR mediates the response to lipid A from *P. gingivalis* strain 381. *P. gingivalis* LPS and its natural lipid A fraction induced NF-κB activation primarily in Ba/F3 cells expressing mouse TLR 2 (Ba/mTLR2), rather than in those expressing mouse TLR4 and its accessory protein MD2 (Ba/mTLR4/mMD2). Further purification of the natural lipid A fraction resulted in a significant decrease of NF-κB activation in Ba/mTLR2, although not in Ba/mTLR4/mMD2. The synthetic counterpart of *P. gingivalis* strain 381-lipid A (compound PG-381) also elicited NF-κB activation in Ba/mTLR4/mMD2, but not Ba/mTLR2. Furthermore, *P. gingivalis* purified natural lipid A and compound PG-381 lacked the ability to activate gingival fibroblasts from C3H/HeJ, TLR4 knockout (KO) and myeloid differentiation factor 88 (MyD88) KO mice. These findings demonstrate that the *P. gingivalis* lipid A molecule induces cell activation via a TLR4/MD2-MyD88-dependent pathway, and suggest the possibility that unknown bacterial components in *P. gingivalis* LPS and its lipid A may induce cell activation via TLR2.**

Introduction

Porphyromonas gingivalis is a Gram-negative, anaerobic oral black-pigmented rod with lipopolysaccharide (LPS) located on the cell surface that is frequently isolated from the periodontal pockets of patients with chronic periodontal diseases (1). We previously demonstrated that the chemical and biological properties of *P. gingivalis* LPS and its active center, lipid A, have been reported to be different from those of classical enterobacterial LPS and their lipid A (2–8). These unique properties of LPS have been also reported in *Bacteroides fragilis* and *Prevotella intermedia*, which formerly belonged to *Bacteroides* species as well as *P. gingivalis* (9–11). *P. gingivalis* lipid A exhibited a quite different phosphorylation and acylation pattern when compared with enterobacterial lipid A (2). Further, a remarkable property of *P. gingivalis* lipid A is its ability to activate cells from C3H/HeJ mice (5), which is thought to be due to its unique structure.

Correspondence to: T. Ogawa; E-mail: tomo527@dent.asahi-u.ac.jp

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To confirm this structure–activity relationship, we recently synthesized a counterpart of *P. gingivalis* strain 381-type lipid A, compound PG-381, which induced IL-6 and tumor necrosis factor (TNF)-α production in peritoneal macrophages from C3H/HeN, but not C3H/HeJ mice (12). The lack of activity observed in the synthetic lipid A compound may be attributable to either of the following two possibilities: (i) some of the structural differences between the natural product and the synthetic compound are essential for the activity or (ii) the natural lipid A fraction had not been fully purified and unknown components present in the fraction were responsible for the observed activity. However, we previously found that the natural lipid A fraction contains a trace amount of amino acids (4), thus the latter possibility is more favorable.

Mammalian Toll-like receptors (TLR) comprise a large family with extracellular leucine-rich repeats and a cytoplasmic Toll/IL-1R homology domain, and are implicated in the recognition of pathogen-associated microbial products (13,14). So far, 10 members (TLR1–10) have been reported (15–19), among which TLR4 has been shown to be a critical receptor and signal transducer for LPS, because LPS-mediated cytokine release is abrogated in LPS-hyporesponsive C3H/HeJ mice with a natural TLR4 mutation as well as in TLR4 knockout (KO) mice (20,21). To respond efficiently to LPS, TLR4 requires an accessory protein, MD2 (22). Although TLR2 has also been implicated in LPS recognition, the responses to enterobacterial LPS in TLR2 KO mice are comparable to those of wild-type mice (23). However, a recent study suggested that overexpressed TLR2 is extremely sensitive to minor contamination in commercial LPS preparations and that neither human nor murine TLR2 plays a role in LPS signaling in the absence of contaminants (24). On the other hand, TLR2 is known to be essential for the signaling of some bacterial components other than LPS, such as *Staphylococcus aureus* peptidoglycan (23,25), muramyl dipeptide (26), bacterial lipoprotein (27–30), zymosan (31), and bacterial fimbriae and their peptides (26). Further, both TLR2 and TLR4 require an adaptor protein, myeloid differentiation factor 88 (MyD88), to initiate an intracellular signaling cascade leading to the activation of NF-κB. MyD88 KO mice do not respond to ligands for either TLR2 or TLR4 (28,29), and it was previously demonstrated that *P. gingivalis* LPS activates murine macrophages through TLR2 and/or TLR4 (32,33). Therefore, we attempted to determine which TLR is used by *P. gingivalis* lipid A for signaling.

In the present study, we purified the *P. gingivalis* natural lipid A fraction on the basis of immunobiological activity, and then compared the activity between natural and synthetic lipid A in order to confirm the receptor utilized by *P. gingivalis* lipid A for cell activation.

Methods

Bacterial compounds

The natural lipid A fraction was separated from *P. gingivalis* strain 381 bacterial cells according to a previous method (2). Briefly, LPS was extracted by a hot phenol–water method and hydrolyzed by acetic acid, after which the hydrophobic products were subjected to silica gel column chromatography

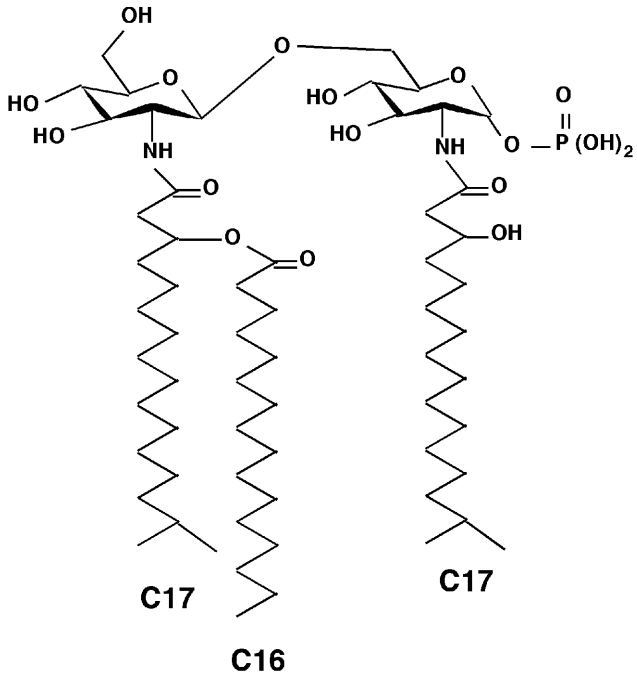

Fig. 1. Proposed chemical structure of the major lipid A component of *P. gingivalis* strain 381 (3).

to give the natural lipid A fraction. Further purification of the natural lipid A fraction was performed by TLC on a silica gel 60 plate (5721; Merck, Darmstadt, Germany) using solvent system A, which consisted of a combination of chloroform–methanol–water (65/24/4, v/v/v). Spots on the TLC were visualized using anisaldehyde-sulfuric acid and ninhydrin reagents for analysis, and ethanol for separation. Silica gel on the spot (R_f = 0.3) was scraped, and the materials were extracted using chloroform–methanol–water–triethylamine (50/50/15/1, v/v/v/v) and dried. The extract was then separated by TLC using solvent system B, which consisted of a chloroform–methanol–25% ammonium solution (65/25/5, v/v/v). Components corresponding to the major (R_f = 0.03) and minor (R_f = 0.2) spots were then extracted, and those from the major spot were used as purified natural lipid A.

P. gingivalis strain 381-type lipid A (compound PG-381) (Fig. 1) and *Escherichia coli*-type lipid A (compound 506) were chemically synthesized as described previously (12). Both bacterial and synthetic compounds were dissolved at a concentration of 2 mg/ml in a 0.1% triethylamine aqueous solution and a cell wall peptidoglycan specimen of *Staphylococcus aureus* was prepared in our laboratory, as described previously (34).

Luciferase assay

An IL-3-dependent line, Ba/F3 (35), was transfected with p55IgκLuc, an NF-κB-dependent luciferase reporter construct, for measuring NF-κB activity with a luciferase assay (36). Cell lines stably expressing the reporter construct (Ba/κB cells) were screened by measuring spontaneous luciferase activity. Ba/κB cells were then transfected with the pEFBOS expression vector encoding mouse TLR2. The expression of

TLR2 was confirmed by intracellular staining for the flag epitope, which was attached to the C-terminus of TLR2. The established cell line expressing mTLR2 and the p55IgκLuc reporter construct (Ba/mTLR2) was used in this study. Ba/F3 cells stably expressing mouse TLR4/MD2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD2) were established as described previously (22). These cells were maintained in RPMI 1640 (Sigma, St Louis, MO) supplemented with IL-3, 50 μM 2-mercaptoethanol and 10% FBS (Sigma) at 37°C in a 5% (v/v) CO_2 atmosphere. They were inoculated onto 96-well plates at 1×10^5/100 μl of RPMI 1640 supplemented with 10% FBS, and stimulated with the indicated doses of each test specimen such as bacterial compounds and TNF-α (Dainippon Pharmaceutical, Osaka, Japan) as a control. After 4 h at 37°C, 100 μl of the Bright-Glo luciferase assay reagent (Promega, Madison, WI) was added to each well and then luminescence was quantified with a luminometer (Turner Designs luminometer model TD-20/20; Promega).

Preparation of murine gingival fibroblasts

TLR2 KO, TLR4 KO and MyD88 KO mice were generated by gene targeting and maintained as described previously (20,23,37). Gingival tissues (2–5 mg) were then obtained from the mice and the explants were cultured in α-MEM (Nikken Biomedical Kyoto, Japan) supplemented with 50 μg/ml gentamicin, 50 ng/ml amphotericin B (Sigma) and 10% FBS (12). The cells were grown and maintained in α-MEM containing 10% FBS at 37°C in a 5% (v/v) CO_2 atmosphere, and were used for the assay at the fifth and 13th passages.

Cytokine assay

Murine gingival fibroblasts were suspended at 2×10^5 cells/ml of α-MEM supplemented with 10% FCS and then the indicated doses of the test specimens were added to the cell cultures (2×10^4 cells/well). Cells were incubated at 37°C for 18 h, and then KC and IL-6 production was measured in the culture supernatants by means of a commercial ELISA kit system (Genzyme, Cambridge, MA). The assay was performed according to the manufacturer's instructions and the results were determined using a standard curve prepared for each assay.

Statistical analysis

Data were analyzed by a one-way ANOVA, using the Bonferroni or Dunn method, and the results are presented as the mean ± SEM. When an individual result is presented, it is representative of at least three independent experiments.

Results

Purification of P. gingivalis natural lipid A

Natural lipid A was purified by TLC from the *P. gingivalis* natural lipid A fraction prepared previously (2). In the TLC analysis of the natural lipid A fraction using solvent system A, we observed one anisaldehyde-sulfuric acid reagent-positive spot (R_f = 0.3) and traces of ninhydrin reagent-positive spots (Fig. 2B). An extract from the spot (R_f = 0.3) containing lipid A was then subjected to a second TLC procedure. Two spots, a major spot (R_f = 0.03), visualized by anisaldehyde-sulfuric acid reagent, and a minor spot (R_f = 0.2), visualized slightly by sulfuric acid, were observed using solvent system B (Fig. 2C). The approximate ratio of the major and minor components were 20:1 (w/w). Since the major one was confirmed to be lipid A by NMR (data not shown), it was used as purified natural lipid A.

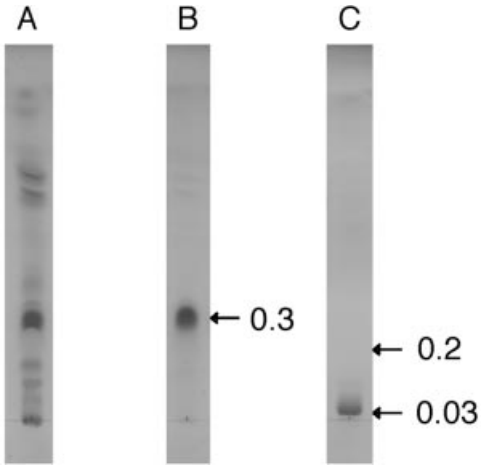

Fig. 2. TLC profiles of hydrophobic products from the weak acid hydrolysate of LPS using solvent system A (chloroform–methanol–water, 65/25/4) (A), the natural lipid A fraction using solvent system A (B) and the extract from the spot (R_f = 0.3) of lane B using solvent system B (chloroform–methanol–25% ammonia solution, 65/25/5) (C). The spots were visualized with an anisaldehyde-sulfuric acid reagent, and are represented by arrows and the R_f value.

NF-κB activation of Ba/F3 cells stimulated with P. gingivalis lipid A

We examined the TLR2- and TLR4-dependent activation of *P. gingivalis* LPS and lipid A compounds in Ba/F3 cells using a luciferase assay. Compound 506 as a TLR4 ligand (Fig. 3F) and *S. aureus* peptidoglycan as a TLR2 ligand (Fig. 3G) were used as control compounds. *P. gingivalis* LPS (Fig. 3A) and its natural lipid A fraction (Fig. 3B) induced NF-κB activation in Ba/mTLR2 cells, while NF-κB activation in Ba/mTLR4/mMD2 cells was very weak. As a result of further purification of the natural lipid A fraction, the purified natural lipid A (Fig. 3C) caused a significant decrease of NF-κB activation in Ba/mTLR2 cells, though its activity in Ba/mTLR4/mMD2 cells remained. Furthermore, the *P. gingivalis* synthetic lipid A, compound PG-381, clearly induced NF-κB activation in only Ba/mTLR4/mMD2 cells (Fig. 3D); however, the activity was very weak as compared to compound 506 (Fig. 3F). On the other hand, the *P. gingivalis* lipid A minor component(s) clearly induced NF-κB activation in Ba/mTLR2 cells (Fig. 3E). Using RT-PCR, we previously found that Ba/F3 cells constitutively express TLR2 mRNA; however, TLR3, TLR4 and TLR5 mRNA were not detected by RT-PCR or Northern hybridization (22). For this reason, Ba/κB cells were thought to respond to *P. gingivalis* LPS, the natural lipid A fraction, the minor component(s) and *S. aureus* peptidoglycan in the present assay (Fig. 3A, B, E and G). Further, stimulation with TNF-α resulted in almost similar NF-κB activation in these three Ba/F3 cells (Fig. 3H).

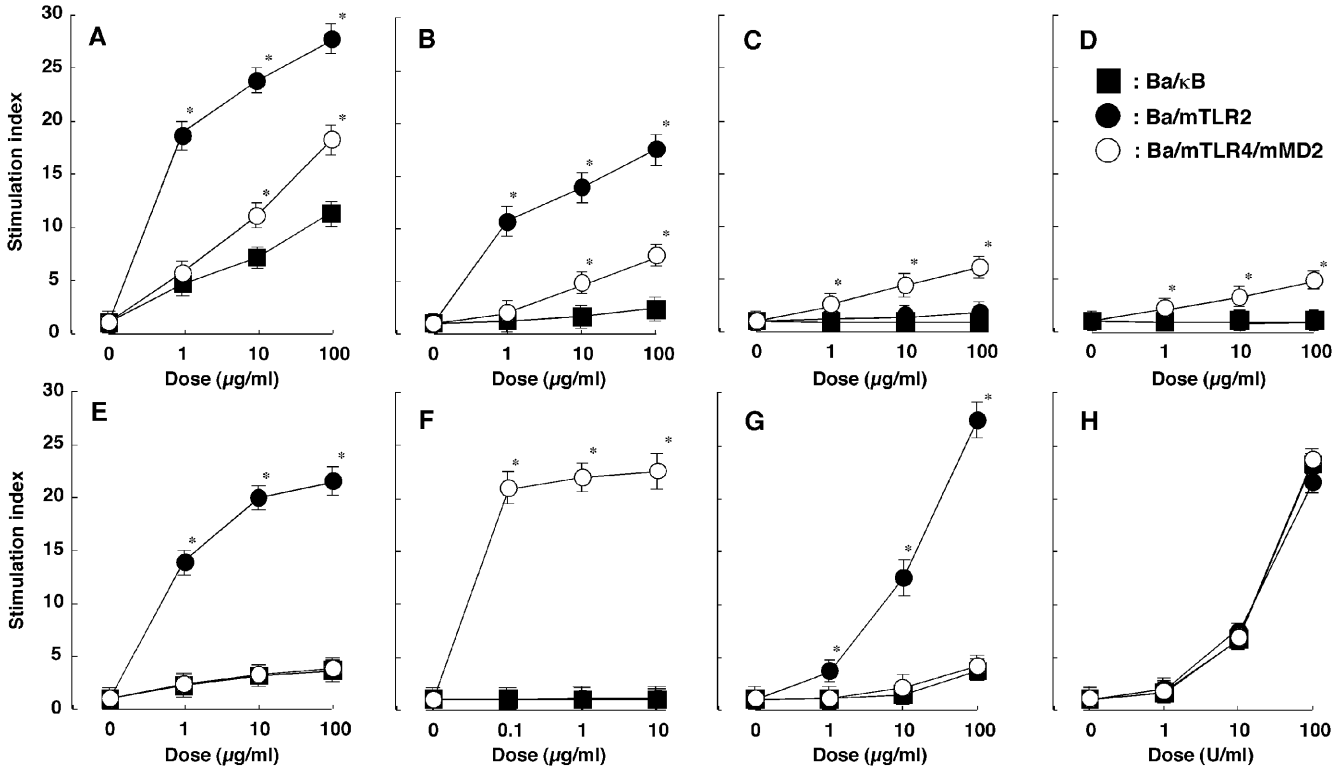

Fig. 3. NF-κB activation in Ba/F3 cells stimulated with *P. gingivalis* LPS (A), *P. gingivalis* natural lipid A fraction (B), *P. gingivalis* purified natural lipid A (C), compound PG-381 (D), *P. gingivalis* lipid A minor component(s) (E), compound 506 (F), *S. aureus* peptidoglycan (G) and TNF-α (H). Ba/κB, Ba/mTLR2 and Ba/mTLR4/mMD2 cells were stimulated with the indicated doses of each test specimen for 4 h, and luciferase activities were measured. Results are shown as relative luciferase activity, which is a ratio of stimulated activity to non-stimulated activity, in each cell line. Each assay was done in triplicate and the data are expressed as the means ± SEM. Significant differences were seen compared to Ba/κB cells in each dose of test specimen ($P < 0.01$).

Cytokine production from murine gingival fibroblasts in response to lipid A specimens

We established fibroblasts from gingival tissues derived from C3H/HeN and C3H/HeJ mice, and examined the production of KC, a chemokine, and IL-6 using murine gingival fibroblasts after stimulation with *P. gingivalis* purified natural lipid A, and synthetic compounds PG-381 and 506. *P. gingivalis* purified natural lipid A (Fig. 4A) and compound PG-381 (Fig. 4B) induced a weaker KC production in C3H/HeN gingival fibroblasts as compared with compound 506 (Fig. 4C). Among these three lipid A, *P. gingivalis* purified natural lipid A alone resulted in no or only marginal KC production in C3H/HeJ gingival fibroblasts (Fig. 4A). *S. aureus* peptidoglycan exhibited KC-producing activities in both C3H/HeN and C3H/HeJ gingival fibroblasts (Fig. 4D). Moreover, IL-6 production in gingival fibroblasts from C3H/HeN and C3H/HeJ mice coincided with KC production (Fig. 4E–H) (12). We next examined KC production in gingival fibroblasts from wild-type, TLR2 KO, TLR4 KO and MyD88 KO mice. *P. gingivalis* purified natural lipid A induced KC production in gingival fibroblasts from wild-type and TLR2 KO mice, while KC production in those from TLR4 KO mice was seen scarcely (Fig. 5A). Compounds PG-381 as well as 506 definitely exhibited KC-producing activities in wild-type and TLR2 KO gingival fibroblasts, although not in those from TLR4 KO mice (Fig. 5B and C). On the other hand, *S. aureus* peptidoglycan induced KC production in gingival fibroblasts from TLR4 KO but not TLR2 KO mice (Fig. 5D). However, there was no KC production by any of these specimens in MyD88 KO gingival fibroblasts (Fig. 5A–D). Further, the production of IL-6 was similar to KC in the murine gingival fibroblasts stimulated with these test specimens (Fig. 5E–H). These results demonstrated that *P. gingivalis* synthetic lipid A activates gingival fibroblasts through a TLR4- and MyD88-dependent pathway.

Discussion

P. gingivalis LPS and its lipid A have been reported to exhibit very low endotoxic activities, and activate LPS-hyporesponsive C3H/HeJ mice in a manner different from other LPS and their lipid A (3,5,6,38,39). It was also shown that further purification removed the contaminating proteins from *P. gingivalis* 381 LPS preparations, which was different from those derived from *Enterobacteriaceae*, while the potency to induce cytokine production in C3H/HeJ peritoneal macrophages remained (11). Recently, Hirschfeld *et al.* showed that a purified preparation of *P. gingivalis* LPS could activate murine macrophages through TLR2, but not TLR4 (32). We also demonstrated previously that TNF-α-producing activity induced by *P. gingivalis* LPS remained in TLR4 KO mice (33).

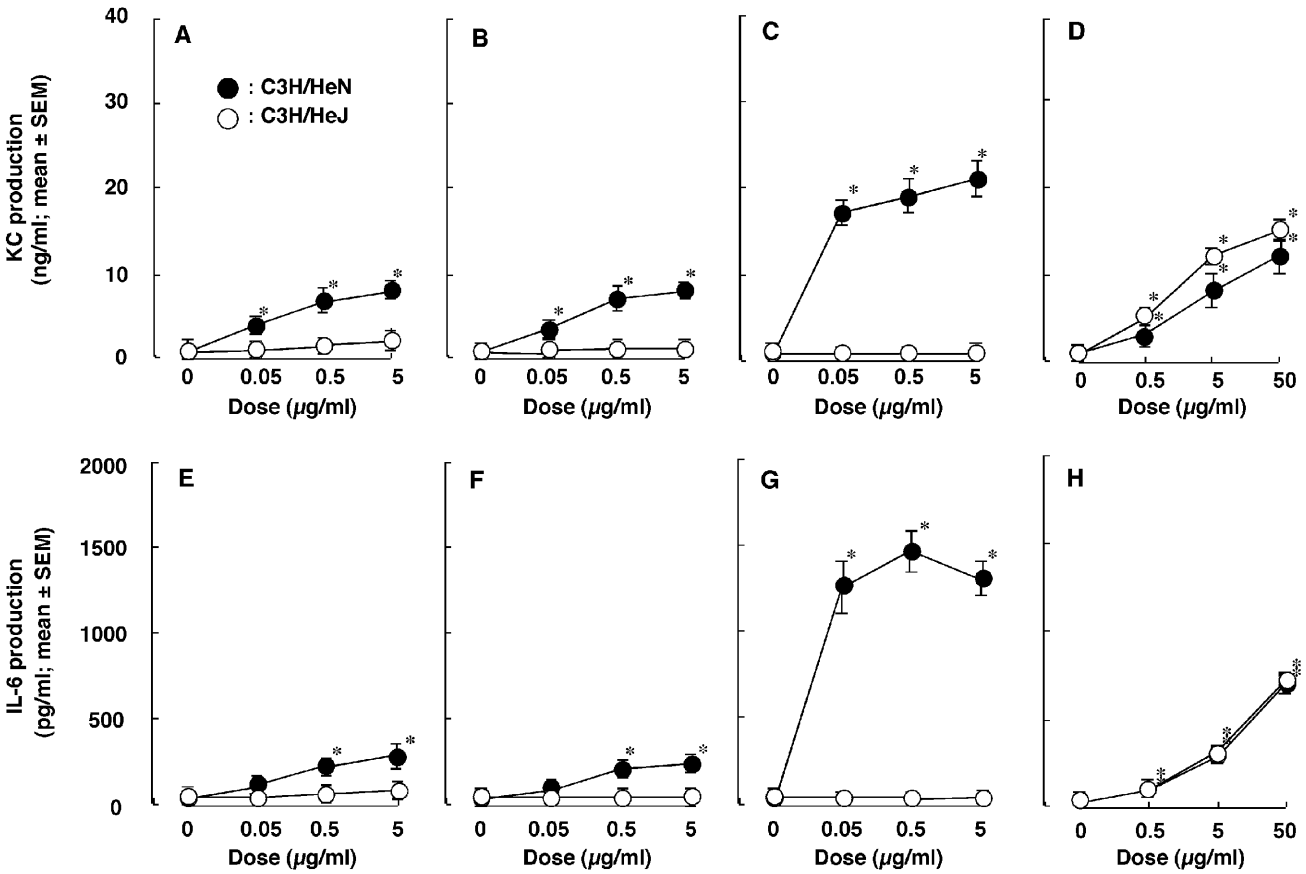

Fig. 4. KC and IL-6 production in gingival fibroblasts from C3H/HeN and C3H/HeJ mice in response to stimulation by *P. gingivalis* purified natural lipid A (A and E), compound PG-381 (B and F), compound 506 (C and G) and *S. aureus* peptidoglycan (D and H). Cells were cultured at 37°C for 18 h in α-MEM with the indicated doses of each test specimen. After incubation, the supernatants were collected, and KC (A–D) and IL-6 (E–H) production determined by ELISA. Experiments were performed at least 3 times and representative results are presented. Each assay was done in triplicate, and the data are expressed as the means ± SEM. Significant differences were seen between groups with and without the test specimens ($P < 0.01$).

In the present study, *P. gingivalis* LPS induced NF-κB activation in Ba/mTLR2 cells and the same weakly in Ba/mTLR4/mMD2 cells (Fig. 3A). Lipid A is known to be responsible for the activity of LPS and it has been demonstrated that the natural lipid A derived from *P. gingivalis* activates C3H/HeJ peritoneal macrophages (5,39). These activities of *P. gingivalis* LPS and its lipid A have been assumed to be attributable to the unusual structure of *P. gingivalis* lipid A (2,40). To confirm this assumption, we recently compared cell-stimulating activities between a *P. gingivalis* natural lipid A fraction and its synthetic counterpart, compound PG-381 (12). The natural lipid A fraction demonstrated activity in peritoneal macrophages from both C3H/HeN and C3H/HeJ mice, whereas compound PG-381 only showed activity in the C3H/HeN mice. In the present study, we also found that the natural lipid A fraction induced NF-κB activation in Ba/mTLR2 cells and the same weakly in Ba/mTLR4/mMD2 cells (Fig. 3B), whereas the synthetic compound induced NF-κB activation in only Ba/mTLR4/mMD2 cells (Fig. 3D). We previously found that an amino acid analysis of *P. gingivalis* natural lipid A fraction resulted in contamination, which was calculated to be 0.32% (w/w) protein (7), and that the contaminant(s) may have an association with the activation of C3H/HeJ mice. Manthey and Vogel also noted that LPS-associated proteins were necessary for activation in C3H/HeJ mice (41). Thus, the discrepancy may have occurred because the *P. gingivalis* natural lipid A fraction had not been fully purified and unknown components present in the fraction could have been responsible for the observed activity.

In this study, we further purified the natural lipid A fraction based on immunobiological activity. After the first TLC separation (Fig. 2B), the extract from the spot ($R_f = 0.3$) still induced NF-κB activation in Ba/mTLR2 cells (data not shown), indicating that several compounds may have been present at the same R_f value in this solvent system. We then separated the extract using a basic solvent system to give purified natural lipid A. In the solvent system, an acidic component moved slowly rather than a non-acidic component. Since *P. gingivalis* lipid A was acidic, it was able to be separated from non-acidic contaminants (Fig. 2C). The purified natural lipid A exhibited a significant decrease of NF-κB activation in Ba/mTLR2 cell, although its activity in Ba/mTLR4/mMD2 cells remained (Fig. 3C). Although a total exclusion of Ba/mTLR2 cell activation could not be attained, the ability of Ba/mTLR2 cell

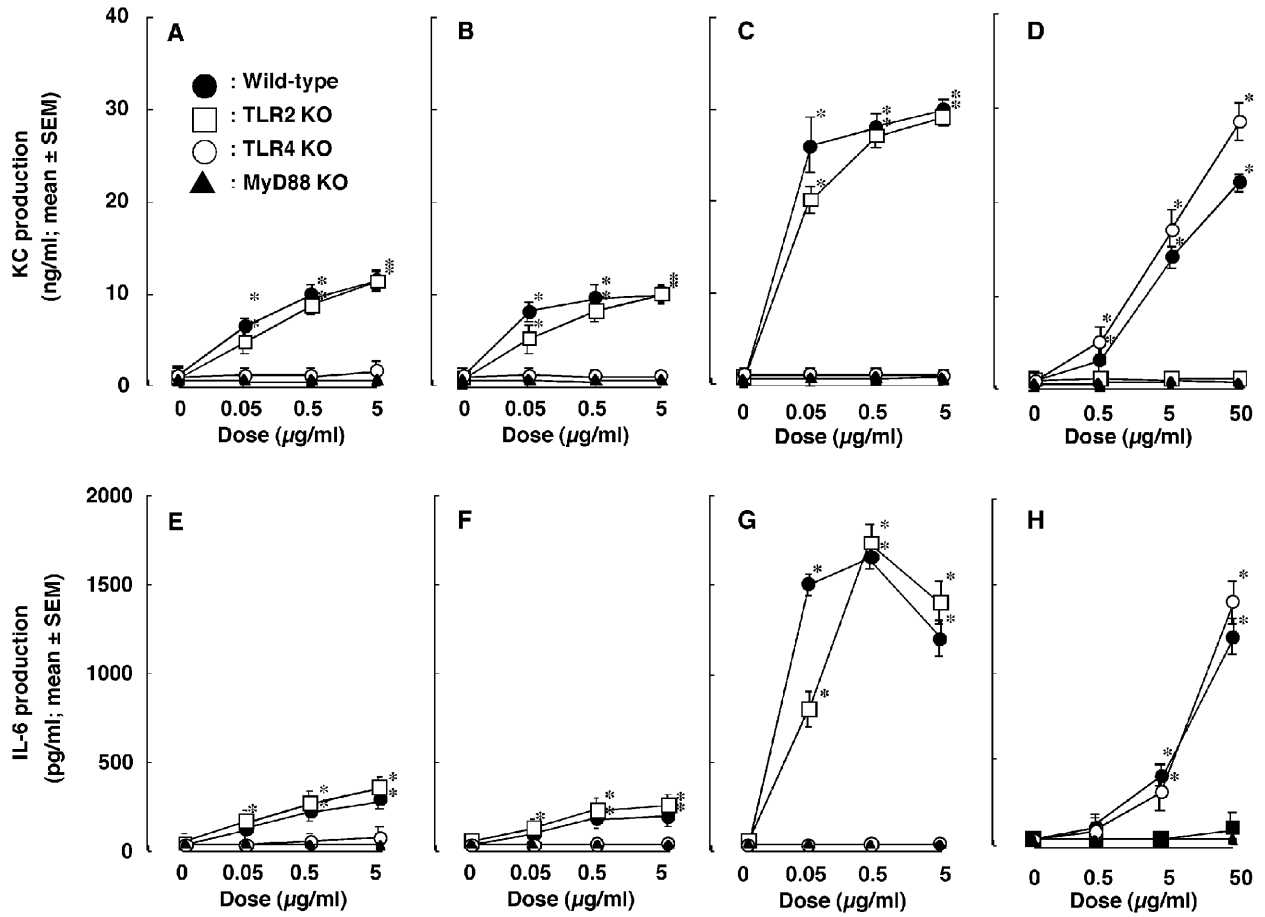

Fig. 5. KC and IL-6 production in gingival fibroblasts from wild-type, TLR2 KO, TLR4 KO and MyD88 KO mice in response to stimulation by *P. gingivalis* purified natural lipid A (A and E), compound PG-381 (B and F), compound 506 (C and G) and *S. aureus* peptidoglycan (D and H). Cells were cultured at 37°C for 18 h in α-MEM with the indicated doses of each test specimen. After incubation, the supernatants were collected, and KC (A–D) and IL-6 (E–H) production determined by ELISA. Experiments were performed at least 3 times and representative results are presented. Each assay was done in triplicate and the data are expressed as the means ± SEM. Significant differences were seen between groups with and without the test specimens ($P < 0.01$).

activation by a *P. gingivalis* natural lipid A fraction was shown to be separable. Furthermore, the purified natural lipid A induced cytokine production primarily in gingival fibroblasts from C3H/HeN, wild-type and TLR2 KO mice, while cytokine production in those from C3H/HeJ and TLR4 KO mice was very weak (Figs 4A and E, and 5A and E). The remaining activity in Ba/mTLR2 cells and gingival fibroblasts from C3H/HeJ and TLR4 KO mice by the purified natural lipid A may have been caused by a trace amount of impurities that could not be removed by the system of purification used. Our results proved that *P. gingivalis* lipid A, with an unusual structure, possesses weak but similar immunobiological activities to those of classical enterococcal lipid A. Furthermore, since the magnitude of NF-κB activation in Ba/mTLR4/mMD2 cells was unchanged through all of the purification processes employed (Fig. 3A–C), the lipid A part was assumed to be responsible for all the activities related to TLR4-MD2 in *P. gingivalis* LPS.

The activity related to TLR2 in the natural lipid A fraction was concentrated in the minor component(s) (Fig. 3E). Thus all the TLR2 ligand components in the natural lipid A fraction may be non-acidic judging from the mobility on TLC in the basic solvent. The natural lipid A fraction contained the purified lipid A and the minor component(s) in the approximate ratio of 20:1. The TLR2 ligand activity of the minor component(s) was theoretically expected to be ~20 times higher than that of the natural lipid A fraction. Although the exact value of the activity coefficient was difficult to compare, it seemed reasonable that the minor components exerted stronger TLR2 ligand activities than did the natural lipid A fraction and the maximum stimulation was seen at concentrations ranging from 10 to 100 μg/ml (Fig. 3E). In addition to the component(s), some minor spots (Fig. 2A) that were observed in the TLC profile of the hydrophobic products from the weak acid hydrolysate of *P. gingivalis* LPS induced NF-κB activation in Ba/mTLR2 cells (data not shown). Since their chemical structure has not yet been determined, it is possible that the trace spots were minor lipid A with a heterogeneous number of fatty acids, phosphate, and ethanolamine (40). However, the conclusion seems to be unlikely, since there is no known evidence that a structurally defined synthetic lipid A induced cell activation through TLR2. Thus it is necessary to define the chemical structure of the minor components and confirm their activity by analysis of

their chemically synthetic compounds. These results suggest that cell surface molecules from *P. gingivalis* contain TLR2- and TLR4/MD2-binding components.

We next demonstrated that compound PG-381, similar to compound 506, elicited cytokine production in gingival fibroblasts from C3H/HeN but not C3H/HeJ mice (Fig. 4B, C, F and G). TLR4 KO gingival fibroblasts also showed no response to these compounds (Fig. 5B, C, F and G). These findings indicate that TLR4 has a role in the recognition and signaling of the lipid A portion of LPS from various Gram-negative bacteria, including *P. gingivalis*. Furthermore, neither KC nor IL-6 production was induced in MyD88 KO gingival fibroblasts stimulated with *P. gingivalis* synthetic lipid A (Fig. 5B and F), demonstrating that *P. gingivalis* synthetic lipid A-induced cytokine production is mediated through a TLR4- and MyD88-dependent pathway, as in the case of *E. coli*-type synthetic lipid A. In contrast, stimulation with *S. aureus* peptidoglycan resulted in cytokine production in TLR4 KO as well as C3H/HeJ gingival fibroblasts (Fig. 5D and H).

The natural lipid A of *P. gingivalis*, different from enterobacterial and other types of lipid A, has been shown to stimulate LPS-hyporesponsive mice. In the present study, we purified *P. gingivalis* natural lipid A by further separation and the synthetic compound, similar to other bacterial lipid A, revealed cell responsiveness via the TLR4–MD2 complex. The present results with the *P. gingivalis* natural lipid A fraction further strengthens the possibility that contamination by only a trace amount of molecule(s), such as lipoprotein, lipopeptide and other unknown components, in *P. gingivalis* natural lipid A may induce cell activation via TLR2.

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Abbreviations

KO	knockout
LPS	lipopolysaccharide
MyD88	myeloid differentiation factor 88
TLR	Toll-like receptor
TNF	tumor necrosis factor

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