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Nuclear factor-κB and p38 mitogen-activated protein kinase signaling pathways are critically involved in *Porphyromonas gingivalis* lipopolysaccharide induction of lipopolysaccharide-binding protein expression in human oral keratinocytes

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Keywords: human oral keratinocytes; lipopolysaccharide-binding protein; mitogen-activated protein kinase; nuclear factor-κB; *Porphyromonas gingivalis*
Accepted 25 September 2012
DOI: 10.1111/omi.12010

SUMMARY

Lipopolysaccharide (LPS) -binding protein (LBP) plays a crucial role in innate host response to bacterial challenge. *Porphyromonas gingivalis* is a keystone pathogen in periodontal disease and the shift of *P. gingivalis* LPS lipid A structure from penta-acylated (LPS_{1690}) to tetra-acylated ($LPS_{1435/1449}$) isoform may significantly contribute to periodontal pathogenesis. We recently demonstrated that LBP is expressed in human gingiva and contributes to periodontal homeostasis. Furthermore, different isoforms of *P. gingivalis* LPS differently modulate the immuno-inflammatory response, and *P. gingivalis* LPS_{1690} induces LBP expression in human oral keratinocytes (HOKs). This study further examined the signaling mechanisms of *P. gingivalis* LPS_{1690}-induced and *Escherichia coli* LPS-induced LBP expression in HOKs. Both *P. gingivalis* LPS_{1690} and *E. coli* LPS were potent inducers of LBP expression in HOKs. The former activated phosphorylation of IκBα, p65, p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), whereas the latter phosphorylated IκBα, p38 MAPK and SAPK/JNK. A nuclear translocation of NF-κB transcription factor was confirmed upon stimulation by both forms of LPS. Further blocking assay showed that *P. gingivalis* LPS_{1690} induction of LBP was through NF-κB and p38 MPAK pathways, whereas *E. coli* LPS-induced LBP expression was mediated by NF-κB, p38 MPAK and JNK pathways. This study demonstrates that NF-κB and p38 MAPK signaling pathways are involved in *P. gingivalis* LPS_{1690} induction of LBP expression in HOKs. The current findings could enhance the understanding of the molecular mechanisms of innate defense in maintenance of periodontal homeostasis.

INTRODUCTION

Lipopolysaccharide (LPS) -binding protein (LBP) has been historically dedicated to Gram-negative bacteria-induced infection (Tobias *et al.*, 1986). It is a 50-kDa polypeptide that is mainly synthesized in hepatocytes and then released as a 58- to 60-kDa glycoprotein into the bloodstream after glycosylation (Schumann *et al.*, 1990). The classical role of LBP is binding LPS to cell surface receptor and augmenting the innate host response to bacterial challenge. LBP at a high concentration exhibits an inhibitory effect on innate host response (Schumann, 2011). Notably, in addition to hepatocytes, LBP can also be expressed in other cells like respiratory type Π epithelial cells and intestinal epithelial cells (Vreugdenhil *et al.*, 1999; Dentener *et al.*, 2000). Our previous study showed for the first time that LBP could be produced by gingival epithelial cells and that its expression level in periodontally healthy subjects was significantly higher than that in patients with chronic periodontitis (Ren *et al.*, 2004, 2005a). Further study confirmed that there was a strong interplay of LBP and cytokines in periodontal heath and disease (Ren *et al.*, 2005b, 2009). These results indicate that LBP may play an important role in enhancement of innate immunity, and contribute significantly to periodontal homeostasis.

Porphyromonas gingivalis is a key periodontal pathogen (Darveau, 2010; Curtis *et al.*, 2011). It is a special, keystone species that could shape the whole oral microbial community and lead to periodontal pathogenesis (Hajishengallis *et al.*, 2011). The LPS of *P. gingivalis* is one of the crucial virulence factors and is significantly involved in the pathogenesis of periodontal disease (Jain & Darveau, 2010). *P. gingivalis* LPS differs from *Escherichia coli* LPS in structure and various biological functions (Bainbridge *et al.*, 2002; Zhang *et al.*, 2008). In addition, *P. gingivalis* LPS exhibits a great structural heterogeneity containing tetra-($LPS_{1435/1449}$) and penta-acylated (LPS_{1690}) lipid A structures (Darveau *et al.*, 2004; Lu *et al.*, 2009). Our recent studies have shown that *P. gingivalis* LPS with different lipid A structures differentially modulates the innate host response in terms of expression of human β-defensin-2 (hBD-2) and cytokines (Lu *et al.*, 2009; Herath *et al.*, 2011; Jin, 2011). Furthermore, LBP can be induced by *P. gingivalis* LPS_{1690} in human oral keratinocytes, whereas *P. gingivalis* $LPS_{1435/1449}$ does not affect the expression (Ding *et al.*, 2012). This may account for additional evidence that LBP plays an important role in periodontal health.

The immune response to microbial insult depends on both innate and adaptive immunity. Innate immunity is crucially determined by a group of pattern recognition receptors, like the Toll-like receptor (TLR) family. This family shares downstream signaling molecules. For instance, activation of TLR4 recruits the adapter molecule myeloid differentiation primary-response protein kinases 88 (MyD88) and subsequently triggers the downstream pathways like nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) cascades (Medzhitov & Janeway, 2000; Hennessy *et al.*, 2010). The NF-κB family of transcription factors includes five members – RelA (p65), RelB, c-Rel, p50 and p52 – which act as homodimers and heterodimers. The NF-κB transcription factors are sequestered in the cytoplasm by a family of inhibitors of κB, the so-called IκBs. The activation process initiated by the IκB kinase (IKK) complex leads to phosphorylation and degradation of IκB protein, and subsequently activates NF-κB (Qin *et al.*, 2005). MAPK is a family of Ser/Thr protein kinases widely conserved among eukaryotes and it is involved in many cellular programs. To date, the most extensively studied groups of mammalian MAPK are extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinases. In general, JNK and p38 kinases are more responsive to inflammatory cytokine stimulation (Roux & Blenis, 2004). However, despite this common generality, the signaling pathways initiated may be variable in different cell types in response to LPS. It has been reported that *P. gingivalis* LPS may activate cells through a different pathway from *E. coli* LPS (Bainbridge & Darveau, 2001; Darveau *et al.*, 2004).

Human oral keratinocytes (HOKs) are the main component of oral and gingival epithelia, which not only function as a physical barrier, but essentially act as a biological barrier. Much evidence shows that oral/gingival keratinocytes actively participate in the immune response that is related to periodontal pathogenesis (Yamamoto *et al.*, 1994; Sfakianakis *et al.*, 2002). Our recent study demonstrates that *P. gingivalis* LPS_{1690}-induced LBP expression could be through both TLR2 and TLR4, which is different from *E. coli* LPS-induced LBP expression through TLR4 alone (Ding *et al.*, 2012). However, the signaling mechanisms involved in *P. gingivalis* LPS_{1690}-induced LBP expression remain

unclear. The present study aimed to examine the specific downstream signaling pathways involved in *P. gingivalis* LPS_{1690}-induced LBP expression in HOKs, and evaluate the potential difference in the signaling mechanisms of LBP expression following *P. gingivalis* LPS_{1690} and *E. coli* LPS stimulation.

METHODS

Cell culture

The primary HOKs were obtained from ScienCell Research Laboratories (Carlsbad, CA) and used in our recent study (Ding *et al.*, 2012). In brief, HOKs were incubated in a serum-free oral keratinocyte medium containing basal medium, 1% oral keratinocyte growth factor supplement and 1% penicillin and streptomycin solution, at 37°C with 5% CO_2. The medium was changed every other day until the culture reached approximately 50% confluence, and it was then changed daily. Cells at third or fourth passage were subsequently used for the experiments.

Porphyromonas gingivalis LPS preparation

The *P. gingivalis* (ATCC 33277) LPS was prepared by an established cold $MgCl_2$-ethanol method, through digestion of whole-cell extracts with proteinase k, and successive solubilization and precipitation (Darveau & Hancock, 1983; Darveau *et al.*, 2004; Ding *et al.*, 2012). It was then purified to keep final protein contamination $< 0.1\%$. The fraction of fatty acids was analysed by gas chromatography–mass spectroscopy. The *P. gingivalis* LPS with penta-acylated (LPS_{1690}) lipid A structure was prepared and analysed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Guo *et al.*, 1997). *E. coli* LPS (0111:B4) was obtained from Sigma (St Louis, MO) and employed as a reference control.

LPS stimulation and blocking assay

In the study of LPS-activated signaling pathways, HOKs were treated with 100 ng ml^{-1} of *P. gingivalis* LPS_{1690} and *E. coli* LPS from 5 min to 4 h. For the blocking assay, cells were pretreated for 30 min with 10 μM IKK-β inhibitor (IKK 2 inhibitor IV, Merck, Darmstadt, Germany), p38 MAPK inhibitor SB202190 (Sigma) or stress-activated protein kinase (SAPK)/JNK inhibitor SP600125 (Sigma), which were dissolved in dimethyl sulfoxide (DMSO), and then stimulated with 100 ng ml^{-1} *P. gingivalis* LPS_{1690} or *E. coli* LPS for 15 min and 24 h, respectively. HOKs treated with culture medium or DMSO alone served as negative controls.

Extraction of total cellular and nuclear proteins

Total cell proteins including cytoplasmic and nuclear proteins were extracted by M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL). Nuclear protein was prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific). The concentration of protein was measured by the bicinchoninic acid protein assay (Thermo Scientific).

Western blot

Total cellular extracts were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane. Afterwards, it was blocked with Pierce Protein-free blocking buffer for 1 h at room temperature. After incubation firstly with specific primary antibodies (1 : 2000) at 4°C overnight, secondary antibodies (1 : 20,000) was then added and incubated for 1 h at room temperature with gentle agitation. The membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 min. The signals were detected on X-ray films. Except for LBP primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), all the other primary antibodies including p-IκBα (ser32), IκBα, p-p65 (ser536), p65, p-p38 MAPK (Thr180/Thr182), p-SAPK/JNK (Thr183/Thr185) and α-tubulin were obtained from Cell Signaling Technology (Danvers, MA). The secondary antibodies were obtained from Invitrogen (Carlsbad, CA). To quantify the bands obtained via Western blot analysis, ImageJ software-based analysis (http://rsb.info.nih.gov/ij/) was employed to quantify the integrated density.

Assay of transcription factor p65

An NF-κB p65 Transcription Factor Assay kit (Thermo Scientific) was used to evaluate the levels of activated p65 transcription factor in nuclear protein. Each well of the kit was coated with biotinylated-consensus DNA sequence, which binds only the active

form of p65. Briefly, 2 μg nuclear proteins was added to each well according to the manufacturer's instruction. The signals were then detected by a Chemidoc^{TM} XRS (Bio-Rad, Benicia, CA).

Statistical analysis

All the experiments were repeated in three independent assays. The results were presented as mean ± SD. If both the normality test and the test of homogeneity of variances were justified, the statistical significance was determined using one-way analysis of variance (Dunnett test or Bonferroni and least significant difference methods). Otherwise, a non-parametric test was used. A P-value < 0.05 was considered statistically significant. The analysis was undertaken by IBM SPSS Statistic 19 (SPSS Inc, IBM Corp., Armonk, NY).

RESULTS

Porphyromonas gingivalis LPS_{1690}-induced activation of NF-κB signaling pathways

To investigate the activation of NF-κB signaling pathways, HOKs were treated in the absence or presence of *P. gingivalis* LPS_{1690} and *E. coli* LPS for up to 4 h, and then protein lysates were analysed for total IκBα and the phosphorylation of IκBα (Fig. 1) and NF-κB

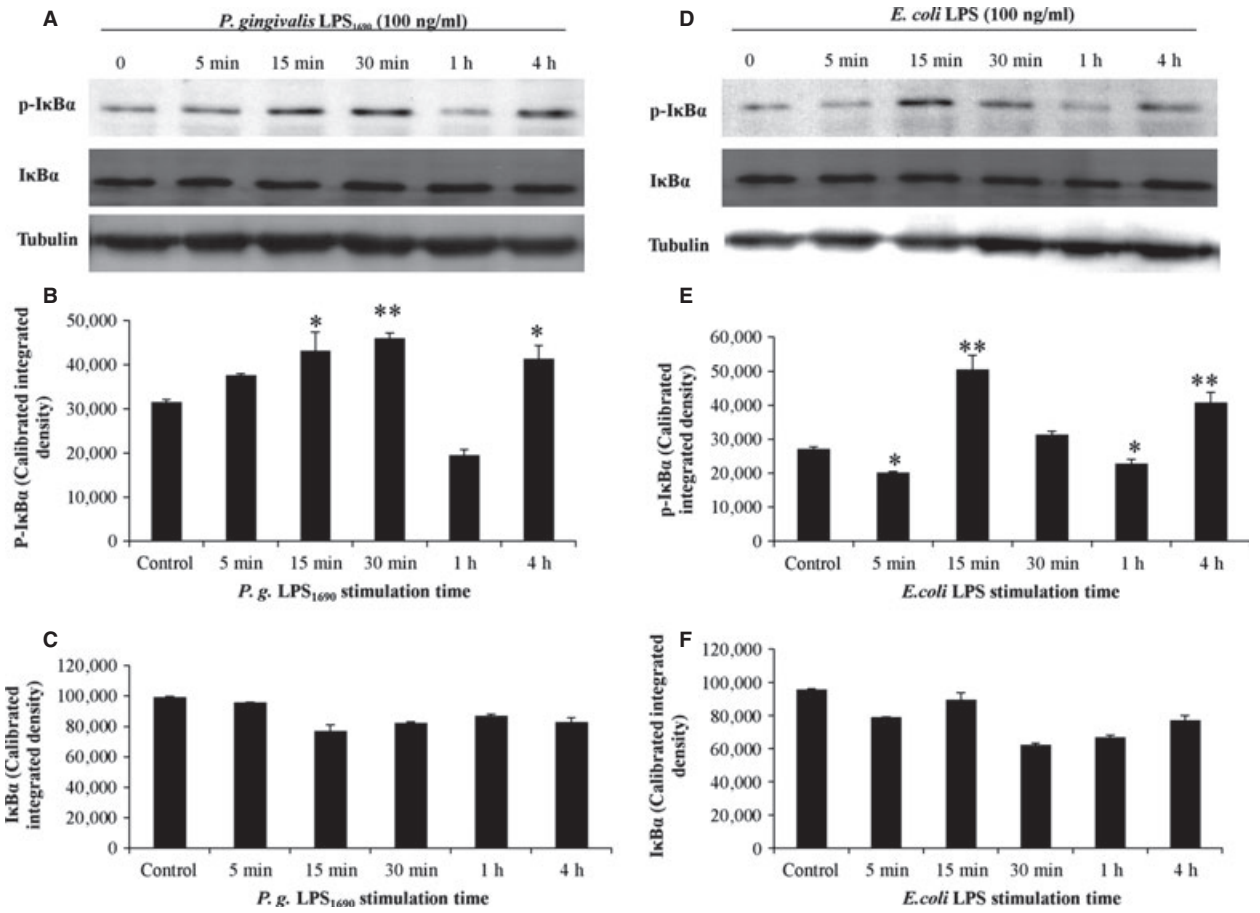

Figure 1 Human oral keratinocytes (HOKs) were treated in the absence or presence of *Porphyromonas gingivalis* penta-acylated lipopolysaccharide (LPS_{1690}) and *Escherichia coli* lipopolysaccharide (LPS) for up to 4 hours, then protein lysates were analysed for the total IκBα and the phosphorylation status of IκBα. Expression level of tubulin was used as a loading control. IκBα was phosphorylated after 15 min of *P. gingivalis* LPS_{1690} treatment, peaked at 30 min, and then decreased at 1 h. However, the signal was upregulated again at 4 h (A, B). In contrast, *E. coli* LPS-induced phosphorylation of IκBα was strongly increased at 15 min, and then decreased gradually at 30 min and 1 h. The signal was also upregulated at 4 hours (D, E). The expression of IκBα protein was decreased when the phosphorylated IκBα was increased upon both *P. gingivalis* LPS_{1690} (A, C) and *E. coli* LPS (D, F) treatments, although there was no significant difference. One representative experiment of three is shown. *$P < 0.05$, **$P < 0.001$ versus control group.

p65 (Fig. 2). Expression levels of tubulin (Fig. 1) and total NF-κB p65 (Fig. 2) were used as loading controls respectively. IκBα was phosphorylated after 15 min of *P. gingivalis* LPS_{1690} treatment, peaked at 30 min, and had decreased at 1 h. However, the signal was upregulated again at 4 h (Fig. 1A,B). In contrast, *E. coli* LPS-induced phosphorylation of IκBα significantly increased at 15 min, and then decreased gradually at 30 min and 1 h. The signal was also upregulated again at 4 h (Fig. 1D,E). The phosphorylation of IκBα protein usually involves the eventual degradation of IκBα by the proteasome. The expression level of IκBα protein was therefore investigated. It was found that the level of IκBα protein decreased with phosphorylation of IκBα upon both *P. gingivalis* LPS_{1690} (Fig. 1A,C) and *E. coli* LPS (Fig. 1D,F) stimulation, although there was no significant downregulation. Phosphorylation of NF-κB p65 was stimulated and reached the peak at 15 min after *P. gingivalis* LPS_{1690} stimulation, then gradually decreased at 30 min, 1 and 4 h (Fig. 2A,B). However, there was no significant phosphorylation of NF-κB p65 upon *E. coli* LPS stimulation during 4 h (Fig. 2C,D). These results indicate that *P. gingivalis* LPS_{1690} may activate several components of the NF-κB signaling pathways, which is to some extent different from *E. coli* LPS.

Porphyromonas gingivalis LPS_{1690}-induced translocation of NF-κB transcription factor p65

To further confirm whether LPS also induces the nuclear translocation of NF-κB, nuclear proteins were isolated and measured by transcription factor enzyme-linked immunosorbent assay kits for NF-κB p65. As shown in Fig. 3, *P. gingivalis* LPS_{1690} at 100 ng ml^{-1} induces the translocation of p65 to nucleus at 15 min, peaks at 30 min and then vanishes at 1 h (lane 2). Whereas 100 ng ml^{-1} *E. coli* LPS induces the translocation early, at 5 min, and quickly vanishes at 15 min (lane 1).

Porphyromonas gingivalis LPS_{1690}-induced activation of MAPK signaling pathways

Four distinct families of MAPKs in mammalian cells have been identified. Of them, p38 MAPK and JNK

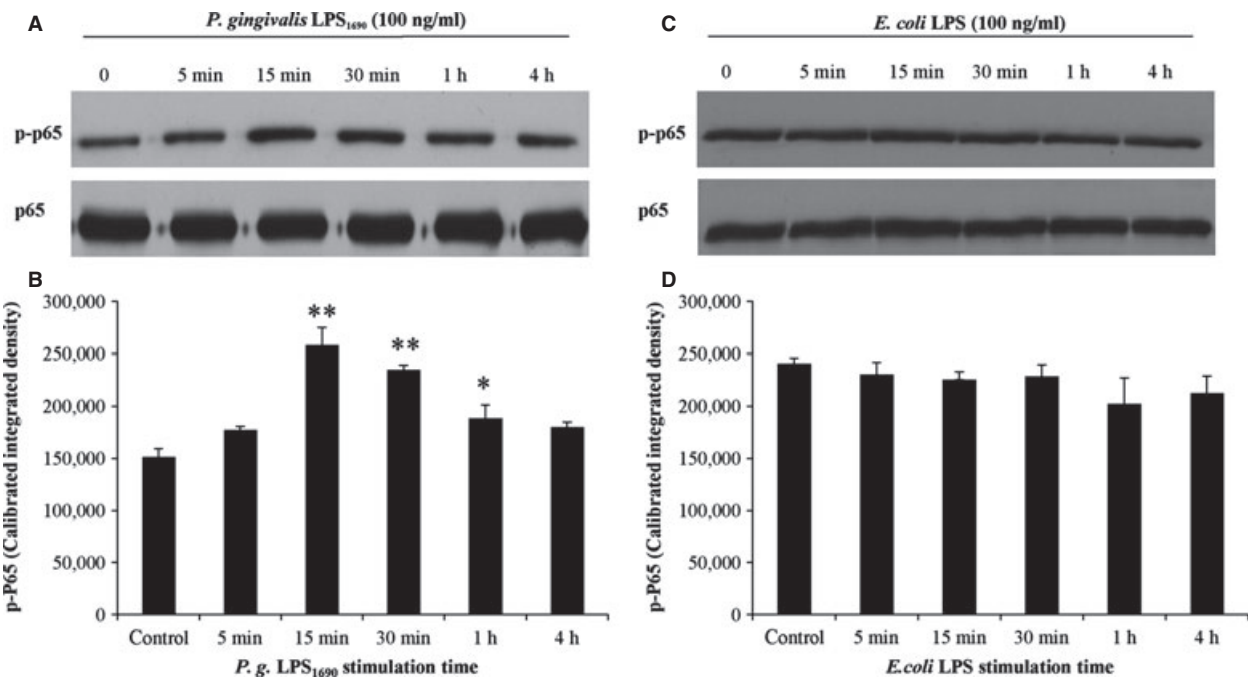

Figure 2 Protein lysates of stimulated human oral keratinocytes (HOKs) were analysed for the phosphorylation status of nuclear factor-κB (NF-κB) p65. Expression level of total NF-κB p65 was used as a loading control. Phosphorylation of NF-κB p65 was stimulated and reached the peak at 15 min after *Porphyromonas gingivalis* penta-acylated lipopolysaccharide (LPS_{1690}) treatment, then gradually decreased at 30 min, 1 h and 4 h (A, B). However, there was no stimulation of phosphorylated NF-κB p65 upon *Escherichia coli* LPS treatment for 4 h (C, D). One representative experiment of three is shown. $*P < 0.05$, $**P < 0.001$ versus control group.

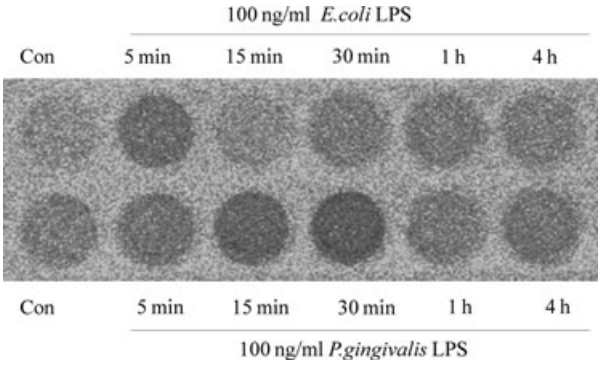

Figure 3 Nuclear factor-κB (NF-κB) p65 DNA-binding is increased upon *Porphyromonas gingivalis* penta-acylated lipopolysaccharide (LPS_{1690}) and *Escherichia coli* LPS stimulation. Human oral keratinocytes (HOKs) were stimulated with LPS (100 ng ml^{-1}) for 5 min to 4 h. Nuclear proteins were extracted for the NF-κB p65 transcription factor assay. Lane 2, *P. gingivalis* LPS_{1690} at 100 ng ml^{-1} induces the translocation of p65 to the nucleus at 15 min, but disappears at 1 h. Lane 1, 100 ng ml^{-1} *E. coli* LPS induces the translocation early at 5 min, and disappears at 15 min. One representative experiment of three is shown.

are related to inflammation (Chen & Thorner, 2007). Protein lysates of simulated HOKs were therefore analysed for the phosphorylation of p38 MAPK and SAPK/JNK (Fig. 4). Tubulin was used as a loading control. Phosphorylated p38 MAPK was quickly and strongly increased at 5 min after *P. gingivalis* LPS_{1690} stimulation, and the intensity of the band gradually decreased over time, although phosphorylation was still detectable from 15 min to 4 h (Fig. 4A,B). Phosphorylated SAPK/JNK protein was also strongly increased at 5 min, after *P. gingivalis* LPS_{1690} stimulation, and then declined over time (Fig. 4A,C). The *E. coli* LPS-induced phosphorylation of p38 MAPK and SAPK/JNK was similar to the above pattern (Fig. 4D–F). These results indicate that both *P. gingivalis* LPS_{1690} and *E. coli* LPS activate p38 MAPK and JNK signaling pathways.

Involvement of NF-κB pathway in *P. gingivalis* LPS_{1690} induction of LBP

To further elucidate the potential involvement of the NF-κB pathway in LPS-induced LBP expression, the HOKs were pretreated with IKK-β inhibitor for 30 min, and then incubated with medium or *P. gingivalis* LPS_{1690} and *E. coli* LPS at 100 ng ml^{-1}. Then protein lysates were analysed for the phosphorylation of IκBα and LBP expression. Expression level of tubulin was used as a loading control (Fig. 5). The blocking efficiency was confirmed by evaluating the phosphorylation of IκBα, which was shown to be significantly blocked by 10 μM IKK-β inhibitor (Fig. 5A,B). Subsequent observation showed that both *P. gingivalis* LPS_{1690} and *E. coli* LPS-induced LBP expressions were significantly down-regulated (Fig. 5A,C). When DMSO was used as a control separately it showed no effect on the testing results (data are not shown). The above results indicate that both *P. gingivalis* LPS_{1690} and *E. coli* LPS induction of LBP involves the NF-κB pathway.

Involvement of p38 MAPK pathway in *P. gingivalis* LPS_{1690} induction of LBP

To further elucidate the potential involvement of p38 MAPK and JNK pathways in LPS-induced LBP expression, the HOKs were pretreated with 10 μM p38 MAPK inhibitor SB202190 and JNK inhibitor SP600125, respectively, for 30 min. Then protein lysates were analysed for LBP expression and the phosphorylation of p38 MAPK and SAPK/JNK. The phosphorylation of p38 MAPK (Fig. 6A,B) and SAPK/JNK (Fig. 7A,B) was significantly blocked by SB202190 and SP600125, respectively. In addition, both *P. gingivalis* LPS_{1690}-induced and *E. coli* LPS-induced LBP expression was significantly downregulated by p38 MAPK inhibitor (Fig. 6A,C). However, JNK inhibitor could not significantly downregulate *P. gingivalis* LPS_{1690}-induced LBP expression. In contrast, *E. coli* LPS-induced LBP expression could be inhibited by JNK inhibitor (Fig. 7A,C). The DMSO was used as a control separately, and it exhibited no effect on the testing results (data are not shown). These data indicate that *P. gingivalis* LPS_{1690} induction of LBP involves the p38 MAPK pathway, but not the JNK pathway. This is different from *E. coli* LPS induction of LBP, which involves both p38 MAPK and JNK pathways.

DISCUSSION

The present study suggests that *P. gingivalis* LPS_{1690} and *E. coli* LPS are potent inducers of LBP expression in HOKs. The *P. gingivalis* LPS_{1690} induction of LBP involves activation of both NF-κB and p38 MAPK signaling pathways, which to some extent are different from *E. coli* LPS induction of LBP via NF-κB, p38

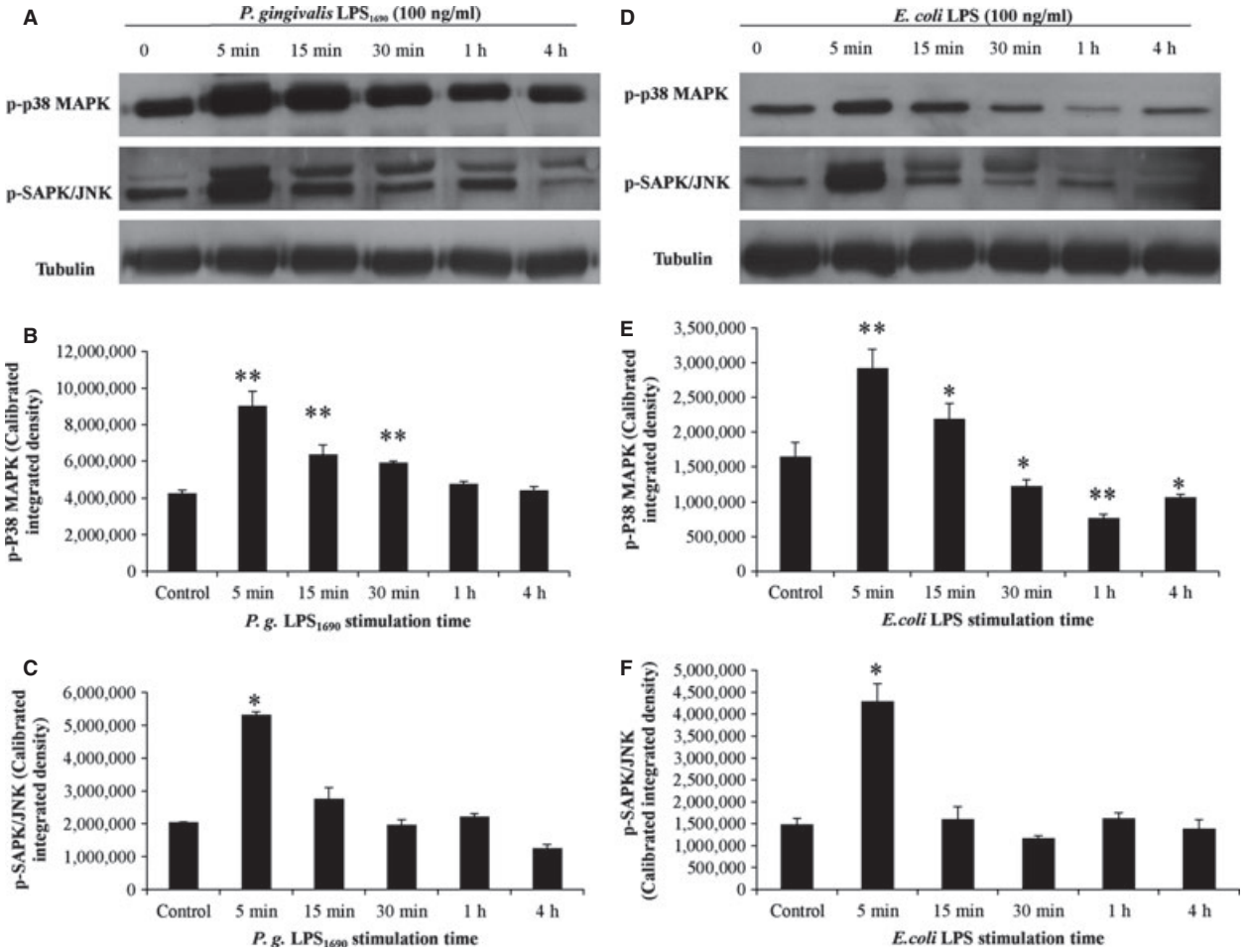

Figure 4 Protein lysates of stimulated human oral keratinocytes (HOKs) were analysed for the phosphorylation status of p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). Expression level of tubulin was used as a loading control. Phosphorylated p38 MAPK protein was quickly and strongly increased at 5 min after *Porphyromonas gingivalis* penta-acylated lipopolysaccharide (LPS_{1690}) stimulation, and the intensity of the band gradually decreased over time, although phosphorylation was still detectable at 15 min to 4 h (A, B). Phosphorylated SAPK/JNK protein was also strongly increased at 5 min, after *P. gingivalis* LPS_{1690} stimulation, and then declined over time (A, C). The *Escherichia coli* LPS-induced phosphorylation of p38 MAPK and SAPK/JNK was similar to the above pattern (D–F). One representative experiment of three is shown. $^{**}P < 0.001$ versus control group.

MAPK and JNK pathways. LBP has been implicated in participating in a number of inflammatory diseases like sepsis (Mierzchala *et al.*, 2011). Although it has been extensively studied in various bacteria-induced inflammatory diseases in humans, the study of its roles in the local immune system is limited. Periodontal pathogenesis is characterized by bacterial LPS activation of series proinflammatory cytokines and mediators from various host cells that may mediate uncontrolled immuno-inflammatory responses and lead to tissue destruction and tooth loss. As far as we know, we are the first group to report the local expression of LBP in human gingiva (Ren *et al.*, 2004). Subsequently, further study shows a strong interplay of LBP with cytokines in diseased periodontal pocket tissues and healthy gingival tissues (Ren *et al.*, 2005b, 2009). Moreover, our recent study indicates that LBP is differentially regulated by different isoforms of *P. gingivalis* LPS, and its expression can be upregulated significantly by *P. gingivalis* LPS_{1690}, but not by *P. gingivalis* $LPS_{1435/1449}$ (Ding *et al.*, 2012). A recent study supports the theory that *P. gingivalis* LPS may use its lipid A structural content to modulate the innate host response in various microenvironmental conditions like different levels of hemin (Al-Qutub *et al.*, 2006). The *in vitro* study

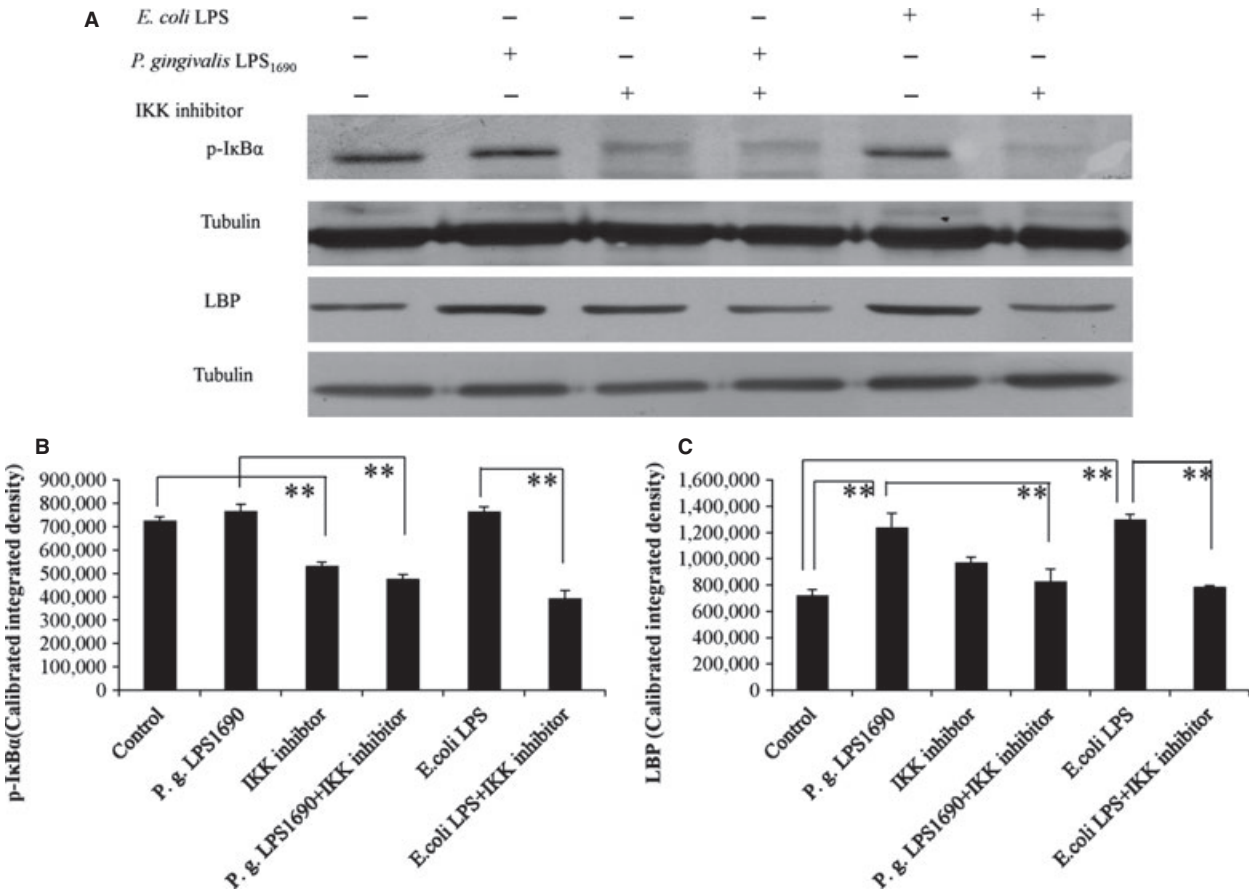

Figure 5 Human oral keratinocytes (HOKs) were pretreated for 30 min with IκB kinase (IKK-β) inhibitor that targets nuclear factor-κB (NF-κB), and then was incubated with medium or *Porphyromonas gingivalis* penta-acylated lipopolyaccharide (LPS_{1690}) and *Escherichia coli* LPS at 100 ng ml^{-1}. Then protein lysates were analysed for LPS-binding protein (LBP) expression and the phosphorylation status of IκBα. Expression level of tubulin was used as a loading control. After 24 h, it was found that the phosphorylation status of IκBα was significantly blocked by 10 μM IKK-β inhibitor (A, B). Subsequently, both *P. gingivalis* LPS_{1690} and *E. coli* LPS-induced LBP expression was significantly downregulated (A, C). Dimethyl sulfoxide was used as a control separately and it showed no effect on the testing results (data are not shown). One representative experiment of three is shown. $^{**}P < 0.001$.

shows that at a low hemin concentration, *P. gingivalis* produces predominantly the form of LPS with a penta-acylated lipid A structure; whereas it expresses mainly the isoform with tetra-acylated lipid A structures at an increased concentration of hemin (Al-Qutub *et al.*, 2006). From a clinical point of view, the hemin levels at tooth site may increase with the severity of periodontal inflammation. It is therefore conceivable that the interaction of host with *P. gingivalis* varies with different periodontal conditions, through regulation of series innate defense molecules that have been reported by our group including E-selectin, cytokines, hBDs and LBP (Reife *et al.*, 2006; Lu *et al.*, 2009; Herath *et al.*, 2011; Jin, 2011; Ding *et al.*, 2012). The gingival epithelial cells are the first set of host cells to come in contact with microbes and they are in a unique position to function as an early signaling system linking to the underlying cells. Given the importance of LBP in innate host defense and its potential involvement in periodontal disease, we have been particularly interested in the signaling mechanisms of LBP expression induced by *P. gingivalis* LPS_{1690} in human oral epithelia cells.

Nuclear factor-κB is a key transcription factor central to a number of immuno-inflammatory responses (Cario *et al.*, 2000). Although there are no *in vitro* studies on the involvement of NF-κB in LBP expression in human oral epithelia, similar observations have been made in other research areas. It has been found that the pathogenic, but not commensal, bacteria use

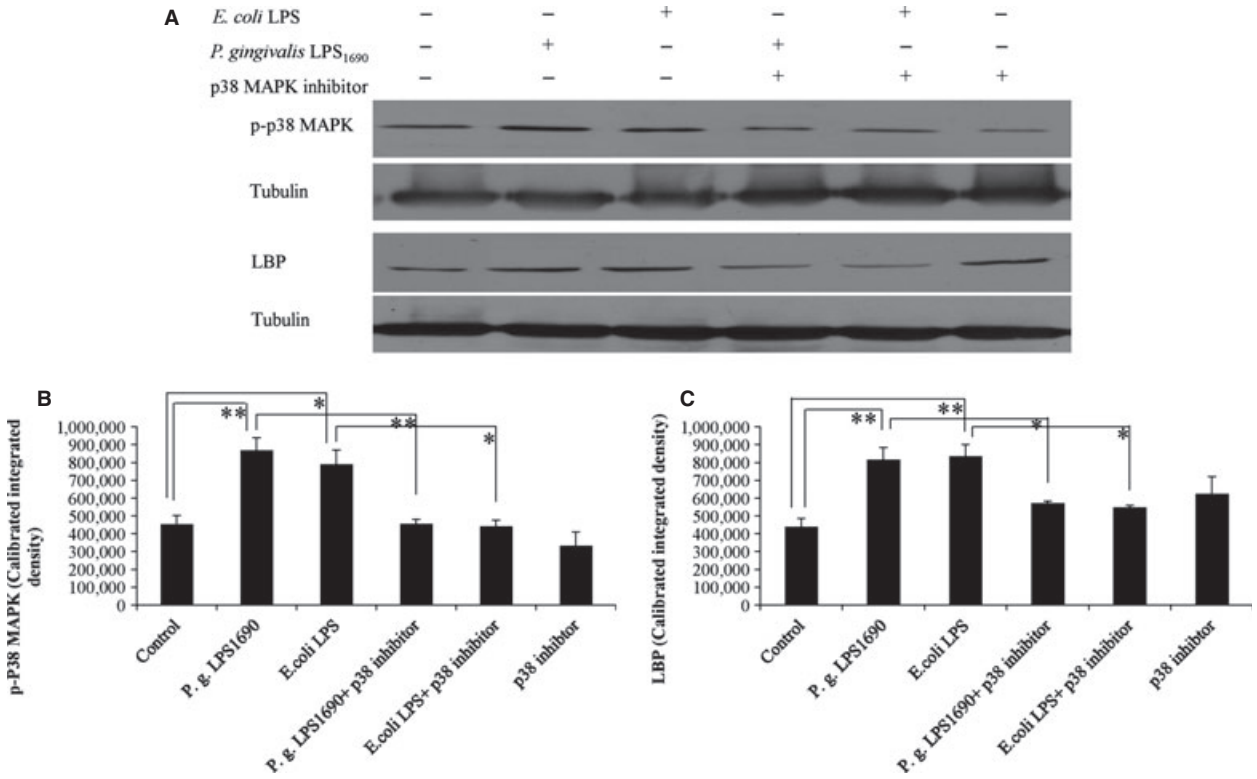

Figure 6 Human oral keratinocytes (HOKs) were pretreated for 30 min with p38 mitogen-activated protein kinase (MAPK) inhibitor SB202190. Then protein lysates were analysed for lipopolysaccharide (LPS) -binding protein (LBP) expression and the phosphorylation status of p38 MAPK. Expression level of tubulin was used as a loading control. It was found that the phosphorylation status of p38 MAPK (A, B) was significantly blocked by SB202190. Moreover, after the stimulation of *Porphyromonas gingivalis* penta-acylated LPS (LPS_{1690}) and *Escherichia coli* LPS at 100 ng ml^{-1} for 24 h, the above two LPS-induced LBP expressions were significantly downregulated by p38 MAPK inhibitor (A, C). Dimethyl sulfoxide was used as a control separately and it showed no effect on the testing results (data are not shown). One representative experiment of three is shown. $*P < 0.05$, $**P < 0.001$.

NF-κB signaling for induction of hBD-2 in oral epithelial cells (Krisanaprakornkit *et al.*, 2002; Chung & Dale, 2008). Our present study found that *P. gingivalis* LPS_{1690} activated the phosphorylation of both IκBα and p65 transcription factors, which was to some extent different from *E. coli* LPS. It is important to note that *P. gingivalis* LPS_{1690} induction of IκBα phosphorylation was slightly delayed with reference to *E. coli* LPS, and the nuclear translocation of transcription factor p65 by *P. gingivalis* LPS_{1690} stimulation was also delayed compared with *E. coli* LPS stimulation. This observed delay in NF-κB activation may be due to differential utilization of MyD88-dependent and MyD88-independent mechanisms by *P. gingivalis* LPS_{1690} and *E. coli* LPS. As previous studies have shown that TLR4 signaling of NF-κB activation consists of at least MyD88-dependent and Toll/interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM)-dependent pathways (Kawai *et al.*, 2001; Takeda & Akira, 2004). However, the latter activates NF-κB signaling pathway in a time-delayed fashion through internalization of TLR4 compared with the former (Kawai *et al.*, 1999; Wang *et al.*, 2012). It could therefore be speculated that the TRIF/TRAM-dependent pathway may be highly involved in *P. gingivalis* LPS_{1690} induction of LBP expression with reference to *E. coli* LPS. Further study is required to confirm this. Next, blocking assay by IKK-β inhibitor confirmed the involvement of NF-κB in regulation of LBP expression. The NF-κB pathway is closely involved in the expression of proinflammatory cytokines and other innate defense mediators like LBP and hBD-2 (Krisanaprakornkit *et al.*, 2002; Hayden & Ghosh, 2012). Taken together, it seems that host may orchestrate a complex regulation mechanism through sharing signaling pathways in response

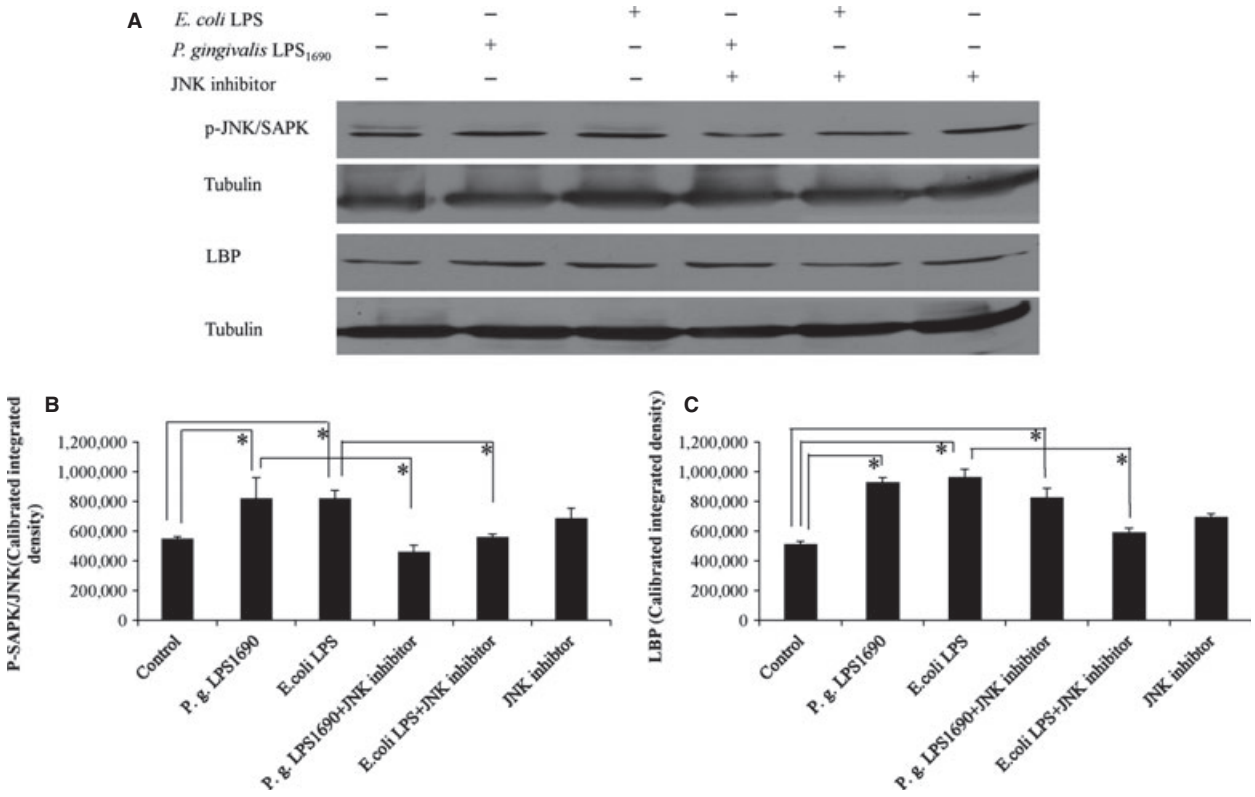

Figure 7 Human oral keratinocytes (HOKs) were pretreated for 30 min with c-Jun N-terminal kinase (JNK) inhibitor SP600125. Then protein lysates were analysed for lipopolysaccharide (LPS) -binding protein (LBP) expression and the phosphorylation status of stress-activated protein kinase (SAPK)/JNK. Expression level of tubulin was used as a loading control. It was found that the phosphorylation status of SAPK/JNK (A, B) was significantly blocked by SP600125. However, there was no significant change of *Porphyromonas gingivalis* penta-acylated LPS (LPS_{1690}) -induced LBP expression by JNK inhibitor. In contrast, the *Escherichia coli* LPS-induced LBP expression was inhibited by JNK inhibitor (A, C). Dimethyl sulfoxide was used as a control separately and it showed no effect on the testing results (data are not shown). One representative experiment of three is shown. $*P < 0.05$.

to microbial challenge, thereby contributing to the maintenance of tissue homeostasis.

The MAPK pathway is another important pathway central to LPS-induced inflammatory response. Our results showed that *P. gingivalis* LPS_{1690} could activate both p38 and JNK pathways. A blocking assay using specific inhibitors of p38 MAPK (SB202190) and SAPK/JNK (SP600125) (Karahashi *et al.*, 2000; Marchant *et al.*, 2010) confirmed that p38 MAPK is involved in *P. gingivalis* LPS_{1690}-induced LBP expression. Previous studies suggest that LPS derived from various bacterial species differentially activate host cells through different signaling pathways (Darveau *et al.*, 2002; Zhang *et al.*, 2008). For instance, *P. gingivalis* LPS activated the JNK pathway to secret cytokines in THP-1 cells, whereas *E. coli* LPS used the NF-κB, p38 MAPK and JNK pathways (Zhang *et al.*, 2008). It is currently unknown whether and to what extent the underlying intracellular signaling for LBP expression in human gingival/oral epithelia would be affected by different forms of LPS. The present study found that both *P. gingivalis* LPS_{1690} and *E. coli* LPS could activate HOKs to express LBP, through shared NF-κB and p38 MAPK signaling pathways but the JNK pathway was only involved in *E. coli* LPS-induction of LBP. Interestingly, our recent study showed that TLR4 was involved in induction of LBP expression in HOKs by both *P. gingivalis* LPS_{1690} and *E. coli* LPS, and additionally TLR2 could be activated by *P. gingivalis* LPS_{1690} as well (Ding *et al.*, 2012). The difference in structure and biological functions between *P. gingivalis* LPS and *E. coli* LPS may contribute to the discrepancy observed. It is noted that *P. gingivalis* LPS lacks heptose in its structure and is less potent in classic endotoxin assays with reference to enterobacterial LPS (Ogawa *et al.*, 2000; Wang &

Ohura, 2002). Recognition of *P. gingivalis* LPS by TLR2 may also account for the different MAPK activation profile in LBP induction with reference to *E. coli* LPS. Further study is needed to clarify this point.

Taken together with previous studies, our results here suggest that NF-κB and p38 MAPK signaling pathways are significantly involved in *P. gingivalis* LPS_{1690}-induced LBP expression in HOKs. It remains unknown how the host orchestrates to regulate the complicated innate host response for maintaining tissue homeostasis through ligand–receptor interaction and shared signaling pathways. Further study is needed to identify the specific regulatory complex in relevant signaling pathways that determine LBP expression in parallel with analysing the expression of other immuno-inflammatory molecules.

Within the limitations of the present study, the current findings demonstrate that NF-κB and p38 MAPK signaling pathways are critically involved in *P. gingivalis* LPS_{1690} induction of LBP expression in HOKs. This study could enhance the understanding of the molecular mechanisms of innate defense in the maintenance of periodontal homeostasis.

ACKNOWLEDGEMENTS

This study was supported by the Hong Kong Research Grants Council (HKU766909M, HKU 768411M and the Modern Dental Laboratory/HKU Endowment Fund to LJJ).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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