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Interleukin-1 receptor-associated kinase-M in gingival epithelial cells attenuates the inflammatory response elicited by *Porphyromonas gingivalis*

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Background and Objective: Recent studies have revealed that negative regulatory molecules, including interleukin-1 receptor-associated kinase-M (IRAK-M), control the overactivation of Toll-like receptor (TLR) signaling. The role of IRAK-M in human gingival epithelial cells (HGECs), which express TLRs, remains unclear. The present study examined the role of IRAK-M on interleukin-8 and macrophage chemoattractant protein-1 (MCP-1) expression in HGECs stimulated with *Porphyromonas gingivalis* and TLR ligands.

Material and Methods: Primary HGECs and an SV40 T-antigen-immortalized HGEC line (epi 4) were stimulated with live or heat-killed *P. gingivalis*, *P. gingivalis* lipopolysaccharide or the synthetic lipopeptide PAM_3CSK_4, and subsequent expression of IRAK-M, interleukin-8 and MCP-1 was evaluated at the mRNA and protein levels. The effects of IRAK-M on interleukin-8 and MCP-1 expressions were evaluated by IRAK-M-specific RNA interference (RNAi)-based loss-of-function assay.

Results: All tested stimulants up-regulated the expression of IRAK-M in HGECs. The *P. gingivalis* lipopolysaccharide or PAM_3CSK_4 increased MCP-1 expression, whereas live *P. gingivalis* down-regulated the MCP-1 expression in HGECs. However, IRAK-M RNAi increased the expression of MCP-1 irrespective of up- or down-regulation mediated by the respective stimulants. Interleukin-8 gene expression, up-regulated by all tested stimulants, was further enhanced by IRAK-M RNAi. In contrast, IRAK-M RNAi had no effect on the interleukin-8 protein levels, irrespective of the stimulant, indicating that post-translational modification, not IRAK-M, controls interleukin-8 protein expression.

Conclusion: Interleukin-1 receptor-associated kinase-M appeared to have distinct regulatory roles on the interleukin-8 and MCP-1 produced by HGECs, further suggesting an important role for interleukin-8 in the immune reponse to periodontopathic bacteria.

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Bacterial accumulation at the dentogingival junction initiates gingival inflammation and the subsequent development of periodontitis. The innate immune response by gingival epithelial cells provides a critical first line of defense against periodontopathogens. Interestingly, stimulation of cultured gingival epithelial cells with Toll-like receptor (TLR) ligands derived from periodontopathic bacteria induces various proinflammatory cytokines (1–4) and chemokines (5,6). These proinflammatory cytokines and mediators derived from gingival epithelial cells play important roles in activating both innate and adaptive immunity (7,8). It is accepted that chronic inflammation resulting from activation of both innate and adaptive immune responses is the cause of periodontal tissue destruction (7,8). Therefore, activation of TLR signaling in the gingival epithelial cells in response to bacterial challenge is the determinant point to elicit the inflammatory responses in the context of periodontal disease.

The inflammatory response is counter-regulated by the production of endogenous anti-inflammatory mediators, including the negative regulatory molecules in TLR-signaling pathways, such as interleukin-1 receptor-associated kinase-M (IRAK-M), which plays a pivotal role in down-regulation of TLR-signaling pathways. Although IRAK-M had originally been found to be highly restricted to monocytes/macrophages (9,10), it has been reported that epithelial cells also express functionally sufficient IRAK-M. For example, it is reported that intrahepatic biliary epithelial cells (11) express IRAK-M and are involved in endotoxin tolerance. In addition, IRAK-M is involved in controlling TLR activation in the intestinal epithelium (12). However, neither the relationship between IRAK-M and the TLR-signaling pathways nor the expression and function of IRAK-M in gingival epithelial cells has been reported. Therefore, since human gingival epithelial cells (HGECs) have been reported to express various functional TLRs, such as TLR2 and TLR4 (5), we decided to investigated the role of IRAK-M in the production of the proinflammatory chemokines, specifically interleukin-8 (IL-8) and macrophage chemoattractant protein-1 (MCP-1) in response to stimulation with *Porphyromonas gingivalis* and related TLR ligands. Since induction of IL-8 and MCP-1 in response to bacterial TLR ligands results in the recruitment of neutrophils and macrophages, respectively, that can augment the inflammatory responses in the local gingival tissue, any effect on such proinflammatory chemokines by IRAK-M would suggest that it plays a distinct regulatory role in periodontal inflammation. Therefore, the present study examined the effects of IRAK-M on IL-8 and MCP-1 expressions induced by the periodontal pathogen, *P. gingivalis*, in the primary culture of HGECs and an SV40 T-antigen-immortalized HGEC line (epi 4) by employing an IRAK-M-specific RNA interference (RNAi)-based loss-of-function assay.

Material and methods

Bacterial strains and culture

Porphyromonas gingivalis strain 381 was cultured in modified Gifu anaerobic medium (GAM broth, Nissui, Tokyo, Japan) in an anaerobic jar (Becton Dickinson Microbiology System, Cockeysville, MD, USA) in the presence of AnaeroPack™ (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) for 48 h at 37°C. Bacterial suspensions were prepared in phosphate-buffered saline (PBS) without Mg^{2+}/Ca^{2+} using established growth curves and spectrophotometric analysis. Where appropriate, bacteria were heat-killed at 60°C for 60 min before addition to the cell culture.

Reagents and antibodies

The synthetic lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine·3HCl (PAM_3CSK_4) was purchased from EMC Microcollections GmbH (Tübingen, Germany). Lipopolysaccharide (LPS) from *P. gingivalis* 381 was kindly provided by H. Kumada and T. Umemoto (Department of Microbiology, Kanagawa Dental University, Yokosuka, Japan). Rabbit anti-human IRAK-M (Chemicon International, Temecula, CA, USA), mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, UK) and ECL Plus Western Blotting Reagent Pack (Amersham Biosciences, Buckinghamshire, UK) were used for Western blotting. The primers and probes for real-time PCR for GAPDH were purchased from Applied Biosystems (Foster City, CA, USA).

Cell preparation and culture

An SV40 T-antigen-immortalized gingival epithelial cell line, epi 4, was kindly provided by Dr Shinya Murakami (Osaka University Graduate School of Dentistry, Osaka, Japan) and maintained in Humedia-KG2 (Kurabo, Osaka, Japan) supplemented with 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 0.4% (v/v) bovine pituitary extract, 0.1 ng/mL human epidermal growth factor, 50 μg/mL gentamicin and 50 ng/mL amphotericin B, as described previously (13).

With the approval of the Institutional Review Board of Niigata University and upon obtaining written informed consent, human gingival epithelial cells (HGECs) were prepared from clinically normal gingival tissue obtained following the extraction of a non-infected third molar. The tissues were treated overnight with Dulbecco's modified minimal essential medium (DMEM) containing 0.025% trypsin and 0.01% EDTA at 4°C. After washing with PBS and subsequent chopping into small pieces, the tissues were suspended in Epilife, containing supplement S7 and penicillin–streptomycin–amphotericin B solution (all from Cascade Biologics, Portland, OR, USA), and were seeded into 12-well plates (TPP, Trasadingen, Switzerland). The tissues were removed when the cells started to grow and were maintained until the cells reached confluence.

The monocytic cell line THP-1 was maintained in 25 mM Hepes-buffered RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin.

Human embryonic kidney cells (293T cells) were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

For the stimulation experiments, epi 4 cells were seeded into a 24-well culture plate (TPP) at a concentration of 2×10^5 cells/mL in the medium in each well. After 24 h of incubation, the attached cells were washed extensively with Epilife and co-cultured with live *P. gingivalis* at a multiplicity of infection of 50, 1 μg/mL of *P. gingivalis* LPS or 1 μg/mL of PAM_3CSK_4 in Epilife containing Supplement S7 for 12 (gene expression) or 24 h (protein synthesis).

Gene expression analysis

Total RNA was isolated using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then treated with RNase-free Dnase I (Invitrogen). The RNA was then reverse-transcribed into cDNA using a random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (Invitrogen).

Polymerase chain reaction amplification of cDNA was performed using oligonucleotide primers specific for IRAK-M (forward, 5′-ACACTTCCG GTCCCACCTAGA-3′; reverse, 5′-CA GAGAAATTCCGAGGGCA-3′) and β-actin (forward, 5′-GCGAGAAGAT GACCCAGATCATGTT-3′; reverse, 5′-GCTTCTCCTTAATGTCACGCA CGAT-3′). Polymerase chain reaction amplification was performed with Taq DNA polymerase (Promega, Madison, WI, USA) using a DNA thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems). The amplification cycle profile was as follows: denaturation at 94°C for 2 min; annealing at 59°C for 45 s; extension at 72°C for 30 s. After 30 cycles of amplification, each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

For real-time PCR, primers and probe specific for GAPDH were purchased from Applied Biosystems. Primers and probes specific for IRAK-M (forward, 5′-GGCCTGGATTCAT GTCTCTCA-3′; reverse, 5′-GCCTGC CAAACAGAAGAGCTT-3′; probe, 5′-CCTCCCTGCCCTCGGAATTTC TCTG-3′), Src homology 2 domain-containing inositol phosphatase (SHIP; forward, 5′-TGGTGTGTCAG TCTTATGGCAGTA-3′; reverse, 5′-C CGGGACCGTTCTTGGA-3′; probe, 5′-ACATCATGACGAGTGACCACA GCCCTG-3′) and suppressor of cytokine signaling-1 (SOCS-1; forward, 5′-CCCTGGTTGTTGTAGCAGCTT-3′; reverse, 5′-CAACCCCTGGTTTGTGC AA-3′; probe, 5′-ACCTGAACTC GCACCTCCTACCTCTTCATG- 3′) were designed using Primer Express version 2.0 software (Applied Biosystems). Reactions were conducted in a 25 μL reaction mixture in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using TaqMan Gene Expression Assays (Applied Biosystems) containing 900 nM of the primer and 250 nM of the probe and incubated for 10 min at 95°C, followed by 40 cycles of a two-step amplification procedure composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. ABI PRISM SDS 2.0 software (Applied Biosystems) was used to analyze the standards and to carry out the quantifications. The relative quantity of each mRNA was normalized to the relative quantity of GAPDH mRNA.

Western blotting

The cultured cells were washed with ice-cold PBS twice, and the protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) supplemented with a Halt Protease Inhibitor Cocktail Kit (Pierce Biotechnology) and Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology) according to the manufacturer's instructions. Cell debris was pelletted by centrifugation at 12000*g* for 10 min at 4°C. The protein concentration in the supernatant was determined using a Pierce protein assay kit (Pierce Biotechnology) according to the manufacturer's instructions.

Twelve micrograms of each sample were solubilized by SDS sample buffer, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Co., Bedford, MA, USA), Western blotted with each antibody, and determined with ECL (GE Healthcare, Amersham Place, UK). To reprobe for GAPDH, the membranes were washed three times with wash buffer (Tris-buffered saline containing 0.1% Tween-20 and 0.5% skim milk) and Western blotted with anti-GAPDH antibody, as described above.

The membrane was exposed to X-ray film (Fuji RX-U, Fuji Film Co., Minamiashigara, Kanagawa, Japan), processed and photographed. The intensity of the signal was quantified using Scion Image 4.02 (National Institute of Health, Bethesda, MD, USA). The intensity of each molecule was expressed after normalization to the GAPDH intensity.

Transfection of small interfering RNA (siRNA)

Stealth RNAi against IRAK-M and Stealth RNAi-negative control were purchased from Invitrogen. The sequence of the sense siRNA was 5′-CCUUGGCACAUUCGAAUCGGU AUAU-3′ and the sequence of the antisense siRNA was 5′-AUAUACCGAU UCGAAUGUGCCAAGG-3′. The sequence for the Stealth RNAi-negative control has not been published. The epi 4 cells were transfected with 10 nM siRNA using Lipofectamine RNAi MAX (Invitrogen), according to the manufacturer's instructions, and they were treated 24 h post-transfection. Specific gene silencing was confirmed by real-time PCR and Western blotting.

Cytokine assay

The levels of IL-8 and MCP-1 in the supernatants of epi 4 culture were determined by using commercially available ELISA kits (BioSource, Camarillo, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All experiments were performed in triplicate wells for each set of conditions and repeated at least twice. Results are expressed as the means ± SEM. Data were analyzed by Student's

paired *t*-test. A probability value of < 0.05 was considered statistically significant.

Results

Expression of IRAK-M in HGECs

By gene expression analysis, it was confirmed that both HGECs and epi 4 cells expressed IRAK-M equally, although the level of expression was much lower than that found in THP-1 cells. The gene expression of IRAK-M by HGECs and epi 4 cells was further validated by Western blotting (Fig. 1). Since these findings suggested that epi 4 cells retain the characteristics of normal HGECs, subsequent experiments were thus carried out using epi 4 cells.

Up-regulation of IRAK-M in epi 4 cells by *P. gingivalis* and TLR ligands

The gene expression of IRAK-M was up-regulated by live *P. gingivalis*, the LPS preparation and PAM_3CSK_4. The effect was most prominent in cells stimulated by live *P. gingivalis*, followed by PAM_3CSK_4- and LPS-stimulated cultures (Fig. 2). Since we demonstrated IRAK-M to be expressed in HGECs and since it is known to function as a negative regulatory signal molecule in TLR-signaling pathways, its up-regulation, especially with live *P. gingivalis* stimulation, suggests that IRANK-M may participate in the down-regulatory mechanism of the TLR-signaling pathways activated by *P. gingivalis*.

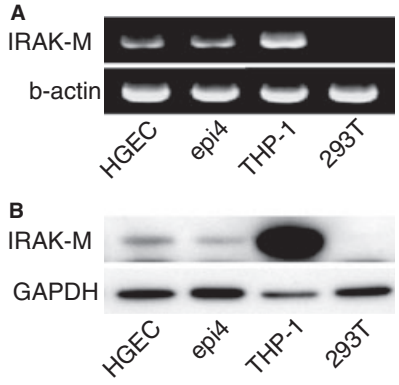

Fig. 1. Expression of interleukin-1 receptor-associated kinase-M (IRAK-M) mRNA (A) and protein (B) in different cell types. The PCR products after 30 cycles were analyzed. The size of PCR products is indicated. β-Actin expression was used as a control. For Western blotting, cell lysates were separated by SDS–PAGE and immunoblotted with anti-IRAK-M antibody. Expression of IRAK-M was confirmed at either the mRNA or the protein level.

Specific suppression of IRAK-M by siRNA and the effect of this suppression on gene expressions of IL-8 and MCP-1 elicited by the tested subtances

To confirm the specific suppression of IRAK-M by siRNA, specific siRNA and negative control siRNA were transfected into epi 4 cells at a concentration of 10 nM, and the expression of IRAK-M was measured at 24 h. As shown in Fig. 3, Western blotting demonstrated a knock-down effect of approximately 60–70%.

Once knockdown of IRAK-M had been achieved, the effect of IRAK-M suppression on IL-8 and MCP-1 mRNA expression could be determined using the test substances, live *P. gingivalis* and *P. gingivalis* LPS (Fig. 4). In control siRNA transfection experiments, the stimulatory effect of the test substances was more prominent for gene expression of IL-8 than for MCP-1. In IRAK-M-specific siRNA-transfected epi 4 cells, the mRNAs for IL-8 and MCP-1 were both up-regulated, and the degree of gene expression of IL-8 and MCP-1 was similar. However, the profiles of gene expression by the different stimulants were quite distinct, even though the overall stimulatory effect was lower in MCP-1. That is, although live *P. gingivalis* suppressed gene expression of MCP-1, siRNA transfection could rescue this suppression. Moreover, while live *P. gingivalis* showed higher stimulatory effect on gene expression of IL-8 than *P. gingivalis* LPS or PAM_3CSK_4 (TLR2 ligand; Fig. 4A), *P. gingivalis* LPS induced the highest gene expression of MCP-1 among the three stimuli tested (Fig. 4B), indicating that multiple different TLR-activation pathways may be involved

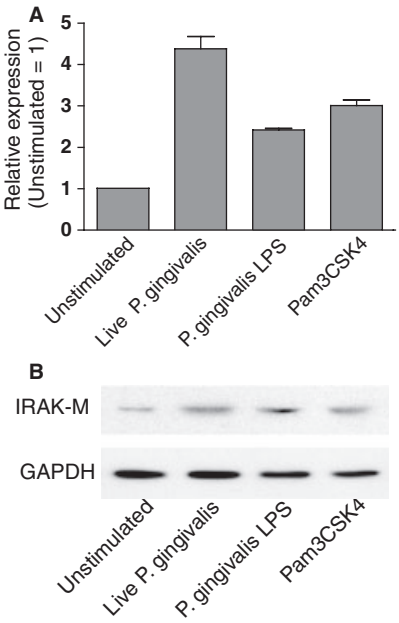

Fig. 2. Up-regulation of IRAK-M in epi 4 cells by *P. gingivalis* and TLR ligands. (A) After stimulation with the indicated stimulants for 12 h, total RNA was extracted from the cells, and gene expression of IRAK-M was analyzed by real-time PCR. Data are shown as mRNA expression relative to the expression without stimulation. Results are shown as the means + SEM of three independent experiments. There is a significant difference in the IRAK-M levels either between the unstimulated and stimulated cultures, or between each treatment group ($p < 0.05$). (B) The epi 4 cells were unstimulated or stimulated with the indicated stimulants for 24 h. Cell lysates were separated by SDS–PAGE, and immunoblotted with anti-IRAK-M antibody. Results are representative of four independent experiments.

in the gene expression of IL-8 and MCP-1. In particular, the gene expression of MCP-1 induced by *P. gingivalis* LPS may be up-regulated by TLR2-related signaling pathways, whereas other TLRs rather than TLR2 appeared to play a pivotal role in induction of gene expression of IL-8 induced by live *P. gingivalis*. Taken together, these findings lead us to the conclusion that, irrespective of the different TLR-activation pathways that elicit gene expression of IL-8 or MCP-1 in HGECs by the stimulation with *P. gingivalis*, IRAK-M seemed to be able to down-regulate the signaling

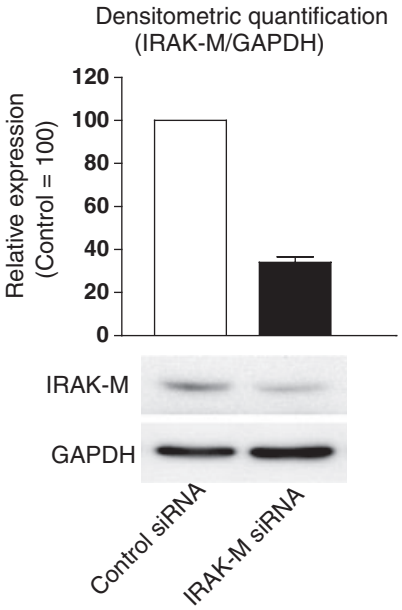

Fig. 3. Specific suppression of IRAK-M by siRNA transfection. Negative control siRNA and IRAK-M-specific siRNA were transfected into epi 4 cells at a concentration of 10 nM, and the expression of IRAK-M was analyzed by Western blotting at 24 h. The top panel shows the result of the densitometric analysis with Western blotting ($n = 3$; means + SEM).

pathways activated by all the different TLRs.

Effect of IRAK-M-specific siRNA transfection on IL-8 and MCP-1 protein production in stimulated HGECs

We have shown that IRAK-M is up-regulated in epi 4 cells, which retain the characteristics of normal HGECs, by *P. gingivalis* and TLR ligands. In IRAK-M-specific siRNA-transfected epi 4 cells, we further showed that the mRNAs for IL-8 and MCP-1 were both up-regulated and that the degree of gene expression IL-8 and MCP-1 was similar. In terms of protein expression, MCP-1 content in the culture supernatant was up-regulated by the siRNA-induced down-regulation of IRAK-M when stimulated with either *P. gingivalis* LPS or PAM_3CSK_4 (Fig. 5). However, in the culture of epi-4 cells stimulated with live *P. gingivalis*, the protein expression level of MCP-1 was much lower than in the

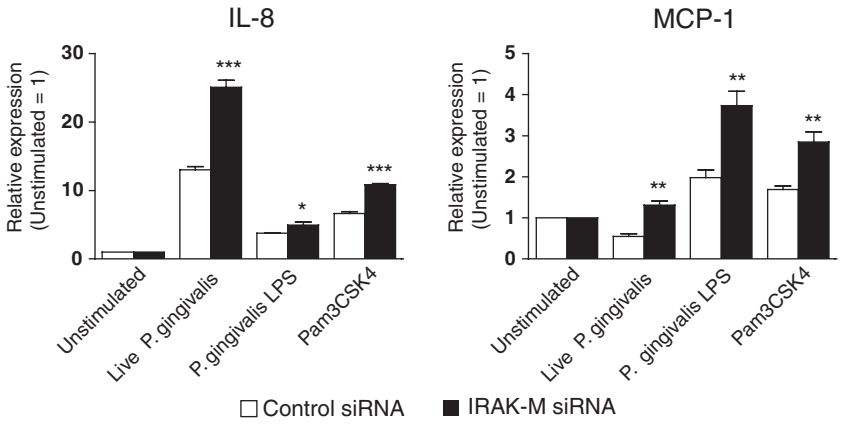

Fig. 4. Effects of specific suppression of IRAK-M by siRNA transfection on the expression of IL-8 and MCP-1 mRNA. The epi 4 cells were transfected with negative control siRNA or IRAK-M-specific siRNA and then were either unstimulated or stimulated with the indicated stimulants for 12 h. Total RNA was extracted from the cells and the expression of human IL-8 and MCP-1 mRNA was analyzed by real-time PCR. Results are shown as the means + SEM of three independent experiments. The significant differences are shown ($*p < 0.05$, $**p < 0.005$, $***p < 0.0001$).

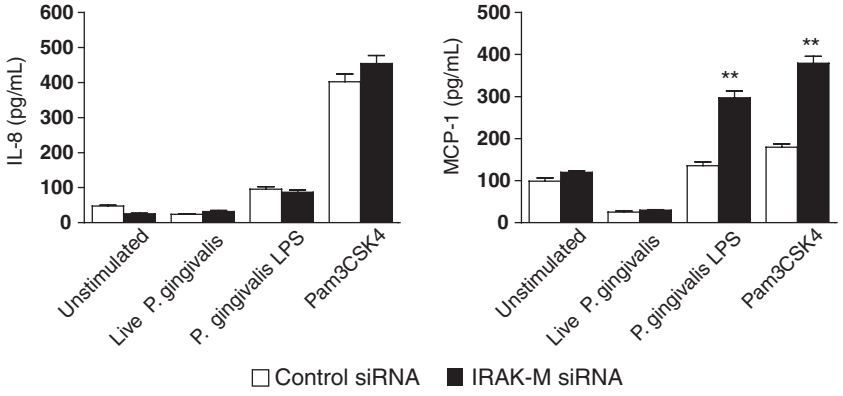

Fig. 5. Effects of specific suppression of IRAK-M by siRNA transfection on the production of IL-8 and MCP-1. The epi 4 cells were transfected with negative control siRNA or IRAK-M-specific siRNA, and then either unstimulated or stimulated with the indicated stimulants for 24 h. Interleukin-8 and MCP-1 in the culture supernatants were analyzed by ELISA. Results are shown as the means + SD of three independent experiments. The significant differences between the IRAK-M-specific siRNA-transfected cultures and control siRNA-transfected cultures in each stimulation condition are indicated ($**p < 0.005$).

untreated control culture (Fig. 5), even in the IRAK-M-specific siRNA transfected culture, in which mRNA expression was up-regulated so as to be comparable to that of the unstimulated culture (Fig. 4). Furthermore, in spite of upregulation of the gene expression of IL-8 in the IRAK-M-specific siRNA-transfected culture (Fig. 4), a knock-down effect mediated by IRAK-M-specific siRNA was not observed for IL-8 protein production (Fig. 5). Furthermore, in contrast to the robust stimulatory effect of live *P. gingivalis* on gene expression of IL-8 in HGECs (Fig. 4), the level of IL-8 protein detected in the culture supernatant was as low as that found in the unstimulated culture (Fig. 5). These results may be summarized as follows. IRAK-M can suppress the gene expressions of IL-8 and MCP-1 via modulation of TLR-mediated signaling pathways, whereas the expressions of IL-8 and MCP-1

proteins were more affected by post-translational modification than by IRAK-M-mediated suppression of TLR-signaling pathway activation.

Effect of heat treatment of *P. gingivalis* on IL-8 expression in HGECs

The role of virulent factors produced from live *P. gingivalis* on both gene and protein expression of IL-8 by epi 4 cells was then analyzed (Fig. 6). As shown in Fig. 6A, both the live and heat-killed bacteria significantly up-regulated the gene expression of IL-8, with a higher stimulatory effect being elicited by the live bacteria. However, the IL-8 protein level of the live *P. gingivalis*-stimulated culture was much lower compared with the heat-killed *P. gingivalis*-stimulated culture, suggesting that virulent factors produced from live *P. gingivalis* could, at least in part, be attributed to the degradation of the IL-8 produced by HGECs (Fig. 6B). These results suggested that the post-translational modifications that affected the production of IL-8 and MCP-1 proteins (Fig. 5) may be derived from the virulent factors produced from live *P. gingivalis*.

Expression of SOCS-1 and SHIP by *P. gingivalis* and TLR ligands

Both SOCS-1 and SHIP were constitutively expressed in epi 4 cells. In contrast to inducible IRAK-M expression in response to bacterial stimulation (Fig. 2), no apparent changes in the gene expression of SOCS-1 or SHIP were found for any of the tested bacterial stimuli, except for SHIP expression with PAM_3CSK_4 stimulation, where apparent down-regulation was observed (Fig. 7). Compared with the results of IRAK-M induction (Fig. 2) and IRAK-M siRNA (Figs 3 and 4), these data (Fig. 7) suggested to us that the negative regulation of IL-8 and MCP-1 mRNA expression elicited by live *P. gingivalis* or *P. gingivalis* LPS is derived from the stimulation-dependent induction of IRAK-M, but little or not at all by the constitutively expressed SOCS-1 or SHIP, the major negative regulators for inflammatory intracellular signals.

Discussion

The present study demonstrated that gene expressions of IL-8 and MCP-1 were significantly down-regulated by an up-regulation of IRAK-M when HGECs were stimulated via TLRs. Interleukin-8 and MCP-1 are the prominent chemokines produced by HGECs, and their mRNA expressions were induced by TLR-mediated signaling (Fig. 4). The IL-8 secreted from HGECs mediates neutrophil transepithelial migration and accumulation at sites of infection (14). In addition to serving as a physical barrier, the gingival epithelial cell secretion of IL-8 is an important defense mechanism against periodontopathic bacteria. Likewise, the MCP-1 derived from gingival epithelial cells induces the accumulation of monocytes and macrophages in the underlying connective tissue so as to stimulate an adaptive immune response. Therefore, the up-regulation of IRAK-M in gingival epithelial cells may be a self-protective mechanism against tissue destruction caused by overreaction of inflammatory responses elicited by stimulation with *P. gingivalis*. However, a novel theory that *P. gingivalis* proactively manipulates the host response is also supported by a recent report that *P. gingivalis* evades TLR-mediated immunity dependent on the exploitation of chemokine receptors, such as CXCR4 (15). Thus, *P. gingivalis* appears to employ distinct mechanisms for escaping innate immune surveillance by suppression of functionalities of innate immune phagocytes, such as neutrophils and macrophages, by manipulating the expression of IRAK-M, which can suppress TRL-signal-mediated IL-8 and MCP-1 expression in HGECs.

Our previous study demonstrated that IRAK-M not only plays a regulatory role in the TLR-signaling-mediated inflammatory response induced by *P. gingivalis*, but may also serve as an escape mechanism of *P. gingivalis* from immune surveillance and may thus be involved in the chronic infection of periodontal tissues by this bacterium (16). Although it was reported that IRAK-M is restricted to monocytes/macrophages (10), recent reports have shown that epithelial cells in the liver and intestine possess this negative regulatory factor (11,12). Since gingival epithelial cells exist in direct contact with a biofilm of bacteria in the oral cavity, it is possible that this

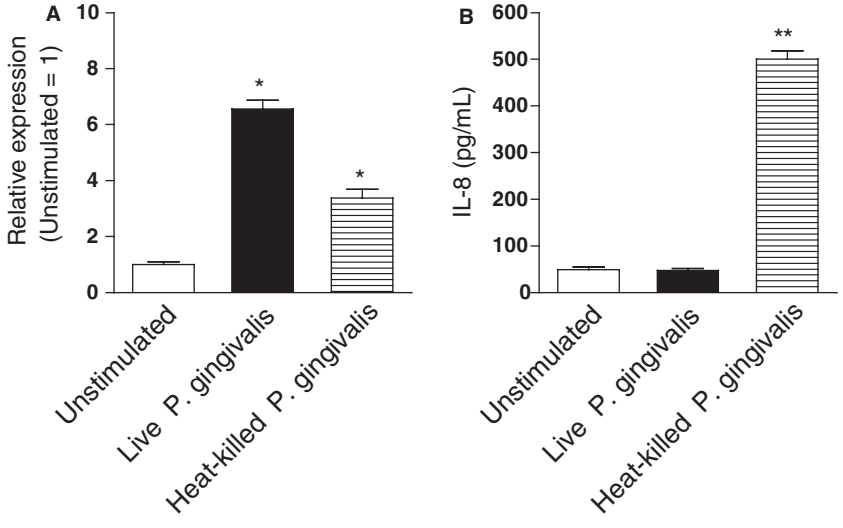

Fig. 6. Effects of heat-killed *P. gingivalis* on IL-8 expression in HGECs. The epi 4 cells were stimulated with either live or heat-killed *P. gingivalis* at an multiplicity of infection of 50. (A) Total RNA was extracted at 12 h and the gene expression was analyzed by real-time PCR. (B) Culture supernatants were obtained at 24 h and the protein levels were measured by ELISA. The significant differences between the control and stimulated cultures are shown (*$p < 0.05$, **$p < 0.005$).

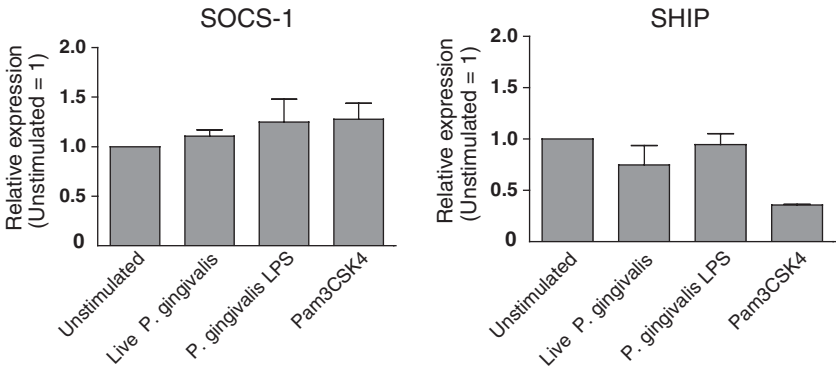

Fig. 7. Effect of treatment with *P. gingivalis* or TLR ligands on SOCS-1 and SHIP expression. After treatment with the indicated stimulants for 12 h, total RNA was extracted from the cells, and gene expression patterns of SOCS-1 and SHIP were analyzed by real-time PCR. Data are expressed as mRNA expression relative to the expression without treatment. Results are shown as the means + SEM of three independent experiments. There is no significant difference in the SOCS-1 and SHIP levels either between the unstimulated and stimulated cultures, or within each treatment group. ($p > 0.05$).

regulatory mechanism is also involved in excess inflammation, which is detrimental to the host, and that *P. gingivalis* exploits this molecule to survive at the dento-gingival junction.

As with other epithelial cells, it was found that HGECs constitutively express IRAK-M mRNA (Fig. 2). Most importantly, stimulation of HGECs with bacteria or bacterial products up-regulated the expression of IRAK-M mRNA (Fig. 2). The effect of stimulants on IRAK-M mRNA expression was most prominent in the case of live *P. gingivalis*, followed by PAM_3CSK_4 and *P. gingivalis* LPS. This could result in part from the weak expression of TLR4 in HGECs (5) as well as ligand–receptor interactions. Since live *P. gingivalis* contains diverse TLR2 ligands, including FimA, peptidoglycan and atypical LPS molecules (17,18), these molecules may have a synergistic effect by inducing stronger TLR signaling than a single treatment with PAM_3CSK_4. The weak stimulatory effect of *P. gingivalis* LPS has already been reported, although the precise mechanisms for this weak activity have not been elucidated (16,19).

In addition to up-regulation of IRAK-M expression, it is possible that *P. gingivalis* is capable of disrupting the innate immune response in HGECs by causing dysfunction of IL-8. Although it has already been reported that *P. gingivalis* inhibits IL-8 expression and secretion by gingival epithelial cells (20), the precise mechanisms remain to be elucidated. The low level of IL-8 protein expression in the live *P. gingivalis*-stimulated HGEC culture (Fig. 5) may be mediated by the degradation of IL-8 protein by live *P. gingivalis*-derived proteinases, particularly gingipains (21–25), since an increased level of IL-8 was found in the heat-killed *P. gingivalis*-stimulated culture (Fig. 6B). However, despite the up-regulation of IL-8 mRNA expression in IRAK-M-specific siRNA-transfected HGECs stimulated with *P. gingivalis* LPS and PAM_3CSK_4, both of which have no proteinase activity, the effect of IRAK-M knock-down was negligible in terms of IL-8 levels in the culture supernatants compared with the control siRNA-transfected culture. Therefore, IRAK-M may not be directly involved in the regulation of the gene expression of IL-8, and there may be as yet unidentified negative regulatory mechanisms at the post-transcriptional level. Another possible mechanism responsible for this low level of IL-8 could be the Ser B protein, a haloacid dehalogenase family serine phosphatase. This protein is reported to antagonize IL-8 accumulation following stimulation of HGECs with *Fusobacterium nucleatum* (26).

In contrast to IRAK-M expression, the expressions of SOCS-1 and SHIP, other regulatory molecules in the TLR signaling pathway (27–29), were not affected by most of the treatments, suggesting that the down-regulation of TLR-pathway-mediated gene expressions of IL-8 and MCP-1 elicited by live *P. gingivalis* or *P. gingivalis* LPS is derived from the stimulation-dependent induction of IRAK-M, but little or not at all by the constitutively expressed SOCS-1 or SHIP. Suppressor of cytokine signaling-1 is a member of a family of proteins that regulates cytokine signaling pathways via inhibition of key tyrosine phosphorylation events on cytokine receptors and signaling molecules, such as janus kinase family members (30). Expression of SOCS-1 is promptly induced in macrophages upon LPS stimulation and inhibits LPS-induced nuclear factor-κB and signal transducers and activators of transcription-1 activation (27,28). Using *SHIP* gene knockout mice, it was revealed that SHIP is a phosphatase involved in the dephosphorylation of phosphoinositide 3-kinase (PI3K) and is involved in the negative regulation of LPS-induced inflammatory responses (29). Another report showed that SHIP transcripts in blood leukocytes were elevated shortly (1–2 h) after LPS administration, dropped below pre-LPS levels at 4–8 h and returned to baseline levels at 24 h (31). These studies suggest that SOCS-1 and SHIP play pivotal roles in LPS-induced inflammatory responses. Nevertheless, in the experimental conditions of the present study, which used the primary culture of HGECs and epi 4 cells, the results indicated that SOCS-1 and SHIP may not be involved in the regulation of the innate immune response to *P. gingivalis* in HGECs.

The present study, therefore, demonstrated that gingival epithelial cells increase the level of IRAK-M expression, which plays a more prominent role than other negative regulators, such as SOCS-1 and SHIP, in lowering the expression of mRNA for proinflammatory chemokines, such as IL-8 and MCP-1, in response to simulation with *P. gingivalis* and related TLR ligands. However, it appears that elevated IRAK-M expression induced by live *P. gingivalis* is more directly associated with down-regulation of MCP-1 protein production in HGECs than IL-8

protein, the expression of which seemed to be affected at a post-translational level. In summary, the results suggested that *P. gingivalis*-mediated induction of IRAK-M has a regulatory role in the production of proinflammatory chemokines in HGECs.

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