Cleavage of protease-activated receptors on an immortalized oral epithelial cell line by *Porphyromonas gingivalis* gingipains

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*Porphyromonas gingivalis* activates protease-activated receptors (PARs) on oral keratinocytes, resulting in downstream signalling for an innate immune response. Activation depends on *P. gingivalis* gingipains, but could be confounded by lipopolysaccharide signalling through Toll-like receptors. We therefore hypothesized that *P. gingivalis* cleaves oral keratinocyte PARs in an Arg- (Rgp) or Lys- (Kgp) gingipain-specific manner to upregulate pro-inflammatory cytokines. Immortalized human oral keratinocytes (TERT-2) were incubated with wild-type *P. gingivalis* (ATCC 33277) or strains from a panel of isogenic gingipain deletion mutants: Kgp-deficient (KDP 129); Rgp-deficient (KDP 133); or Kgp- and Rgp-deficient (KDP 136). After incubation with *P. gingivalis*, keratinocytes were probed with specific antibodies against the N-terminus of PAR-1 and PAR-2. Using flow cytometry and immunofluorescence, receptor cleavage was marked by loss of specific antibody binding to the respective PARs. TERT-2 cells constitutively expressed high levels of PAR-1 and PAR-2, and lower levels of PAR-3. *P. gingivalis* ATCC 33277 cleaved PAR-1 and PAR-2 in a dose-dependent manner, while the receptors were unaffected by the protease-negative double mutant (KDP 136) at all m.o.i. tested. The single Kgp-negative mutant preferentially cleaved PAR-1, whereas the Rgp-negative mutant cleaved PAR-2. Wild-type or Kgp-negative mutant cleavage of PAR-1 upregulated expression of IL-1α, IL-1β, IL-6 and TNF-α; the Rgp-negative mutant did not modulate these cytokines. Selective cleavage of PAR-1 on oral epithelial cells by *P. gingivalis* Rgp therefore upregulates expression of pro-inflammatory cytokines.

INTRODUCTION

*Porphyromonas gingivalis* is a Gram-negative anaerobe present in subgingival plaque and widely associated with periodontitis in adults (Chen et al., 2001; Nonnenmacher et al., 2004, 2005). Periodontal diseases and inflammation are associated with specific *P. gingivalis* virulence factors (Tatakis & Kumar, 2005), including fimbriae (Miura et al., 2005; Weinberg et al., 1997), lipopolysaccharides (LPS) (Wang et al., 2002) and gingipains (Kadowaki et al., 1994, 2003; Pathirana et al., 2007a; Potempa et al., 2000; Yoneda et al., 1990). Gingipains are cysteine proteases produced by *P. gingivalis* (Kadowaki et al., 1994; Potempa et al., 2000). Two types of gingipains have been described: arginine-specific (Arg-gingipains or Rgp) and lysine-specific (Lys-gingipains or Kgp). Rgp can be found as three variants: a 50 kDa RgpB, a 50 kDa RgpAcat and a 95 kDa HRgpA (Potempa et al., 2000). In a mouse model, Kgp and RgpB, but not RgpA, contribute to periodontal disease (Pathirana et al., 2007a), findings that were confirmed by the use of novel gingipain inhibitors (Kadowaki & Yamamoto, 2003; Kadowaki et al., 2004). On eukaryotic cell membranes, gingipains cleave the N-terminal domains to activate the protease-activated receptors (PARs), members of the seven-transmembrane superfamily of cell-surface G-protein-coupled receptors (reviewed by Coughlin, 2000; Ossovskaya & Bunnett, 2004). Four PARs are currently described: PAR-1, PAR-2, PAR-3 and PAR-4. Hence, *P. gingivalis* gingipains cleave and activate PARs on neutrophils (Lourbakos et al., 1998) and platelets (Lourbakos et al., 2001b), and Rgp activation of PAR-2 has been linked to inflammation and induction.

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Abbreviations: Kgp, lysine-specific gingipain(s); PAR, protease-activated receptor; Rgp, arginine-specific gingipain(s); TLR, Toll-like receptor.
of alveolar bone loss in periodontitis (Holzhausen et al., 2005, 2006). Consistent with these findings, PAR-2-deficient mice lose less alveolar bone than wild-type mice after challenge with P. gingivalis supernatants, which appears to be caused by Rgp signalling through PAR-2 (Holzhausen et al., 2006). In KB cells (a HeLa-like carcinoma cell line), however, P. gingivalis Rgp appears to activate PAR-1 and PAR-2 and release IL-6, a potent stimulator of osteoclast differentiation and bone resorption (Lourbakos et al., 2001a). Hence, the PAR–gingipain specificity leading to release of pro-inflammatory cytokines is unclear, in part complicated by the presence of P. gingivalis LPS with concomitant activation through Toll-like receptors (TLRs) also leading to expression of pro-inflammatory cytokines (Chen et al., 2008; Diya et al., 2008; Eskan et al., 2008).

In the present study, we determined the P. gingivalis gingipain cleavage specificity for PARs on oral keratinocytes. By using an immortalized oral epithelial cell line, TERT-2, we have shown that PAR-1 and PAR-2 are differentially cleaved by P. gingivalis Rgp and Kgp to upregulate expression of pro-inflammatory cytokines.

**METHODS**

**Cell cultures.** OKF6/TERT-2 (TERT-2), an immortalized oral epithelial cell line provided by J. Rheinwald (Harvard Medical School, Cambridge, MA) (Dickson et al., 2000), was used to model primary gingival epithelial cells. TERT-2 cells were grown in 5 % CO2 at 37 °C in keratinocyte serum-free and calcium-free medium (InvitroLife Technologies) supplemented with 0.4 mM CaCl2, 25 μg ml−1 bovine pituitary extract and 0.2 ng ml−1 epidermal growth factor, as previously reported (Giacaman et al., 2007). Culture medium was changed every 3 days and cells were subcultured when they reached approximately 60% confluence.

**Bacterial strains and culture conditions.** P. gingivalis strain ATCC 33277 and a panel of isogenic deletion mutants that fail to express Kg (KDP 129; Δkgp) (Okamoto et al., 1998), Rgp (KDP 133; ΔrgpA ΔrgpB) (Nakayama et al., 1995) or both gingipains (KDP 136; Δkgp ΔrgpA ΔrgpB) (Shi et al., 1999) (kindly provided by K. Nakayama, Nagasaki University, Nagasaki, Japan) were used. P. gingivalis strains were grown anaerobically in a Coy chamber (85 % N2, 5 % CO2 and 2% H2) at 37 °C on Todd–Hewitt agar plates (Difco) or in Todd–Hewitt broth. Agar and broth were supplemented with 5 μg haemin ml−1 and 1 μg menadione ml−1 (both Sigma-Aldrich). Agar plates were also supplemented with 5 % (v/v) defibrinated sheep blood. Bacteria were grown to early stationary-phase (OD620 ~ 0.8–1.0).

Spent bacterial broth with early stationary phase P. gingivalis cells containing secreted and cell-wall-associated gingipains (Potempa et al., 2000) was added to keratinocyte cultures at 37 °C in an m.o.i. (bacteria to keratinocytes) of 10:1, 100:1 or 1000:1. In preparation, keratinocytes at 60% confluence were washed with Dulbecco’s PBS (DPBS) (Mediatech) and the medium was replaced with pre-warmed serum-free MEM (Mediatech). In the presence of inoculated P. gingivalis, TERT-2 cells were incubated at 37 °C in 5 % CO2 for 1 h. Control TERT-2 cells were inoculated with fresh sterile P. gingivalis growth medium and maintained under the same conditions. For some control experiments, TERT-2 cells were inoculated for 30 min in serum-free MEM (Mediatech) at 37 °C in 5 % CO2 with either 100 nM N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) or 100 nM Δ-thrombin (MP Biomedicals). After incubation with P. gingivalis, sterile control medium, TPCK-trypsin or thrombin, keratinocytes were washed three times with DPBS and processed for the appropriate analysis as below.

**Flow cytometry.** After incubation with P. gingivalis or controls, TERT-2 cells were washed three times with DPBS and detached from the flask by gentle scraping. Since trypsin activates PARs (Lourbakos et al., 2001a), this enzyme was not used to detach the cells. Cells were counted, fixed for 10 min at 4 °C in 4 % (v/v) paraformaldehyde and reacted with optimized dilutions of primary antibodies against PAR-1, −2 and −3. The antibodies used were murine monoclonal antibodies against PAR-1 (ATAP) (Santa Cruz Biotechnology) or PAR-2 (Zymed), or rabbit polyclonal antibody against PAR-3 (Santa Cruz Biotechnology). Primary antibodies and cells were incubated for 30 min at 4 °C, washed three times with PBS supplemented with 2 % (v/v) FBS, and washed cells were then incubated in the dark for 30 min with a 1:100 dilution of goat anti-murine IgG secondary antibody conjugated with phycoerythrin (Jackson ImmunoResearch Laboratories) to detect PAR-1 or PAR-2, or with goat anti-rabbit IgG secondary antibody conjugated with FITC (Sigma) for PAR-3. Murine monoclonal antibodies IgG1 and IgG2a (BD Biosciences and BD Pharmingen) were used as isotype controls for PAR-1 and PAR-2, respectively. For PAR-3 detection, rabbit polyclonal IgG was used as isotype control. Fluorescent signals were monitored with a FACScan (BD Biosciences) using CelleQuest software. Each antibody was analysed and reactivity was confirmed in at least three independent experiments.

**Immunofluorescence microscopy.** TERT-2 cells (2 × 105) were grown as described above and seeded on coverslips in 12-well plates. Cells (60% confluent) were infected with P. gingivalis for 1 h at various m.o.i. After incubation with P. gingivalis, cells were washed three times with DPBS, fixed in 4 % (v/v) paraformaldehyde, and blocked with 3 % (v/v) BSA for 30 min at room temperature to minimize non-specific antibody binding. To detect PARs, TERT-2 cells were incubated with 80 μl of a 1:100 dilution of murine monoclonal antibody against PAR-1 (ATAP) or PAR-2 (SAM-11) (Santa Cruz Biotechnology) for 1 h in the dark. Murine IgG1 and IgG2a antibodies were used as isotype controls for PAR-1 and PAR-2, respectively. Cells were washed five times with DPBS and then incubated in the dark for one additional hour with Alexa Fluor 568-conjugated goat anti-murine IgG Antibody (Molecular Probes) and 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) at 1:2000 and 1:300 dilutions, respectively. Coverslips were washed five times, mounted on glass slides, and cells were observed using a Nikon Eclipse E800 fluorescence microscope. All experiments were performed in triplicate.

**Quantitative PCR.** Since incubation with P. gingivalis for longer than 6 h causes cells to detach from the flask (Nisapakultorn et al., 2001), keratinocytes were incubated in TERT medium with P. gingivalis for 3 h, as previously described (Giacaman et al., 2007), washed five times with DPBS and then incubation continued for the times indicated. At times up to 48 h after initial exposure to P. gingivalis, TERT-2 cells were harvested. Total RNA was extracted using an Rneasy Plus Mini kit (Qiagen) according to the manufacturer’s instructions, quantified using a 2100 Bioanalyzer (Agilent Technologies), and 500 ng was used as template to synthesize cDNA using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then diluted 1:5 with RNase/DNase-free water and 1 μl (5 ng) was used as template to amplify cDNA using the Superscript III First Strand Synthesis System for RT-PCR. (Invitrogen). The cDNA was then diluted 1:5 with RNase/DNase-free water and 1 μl (5 ng) was used as template to amplify cDNA using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then diluted 1:5 with RNase/DNase-free water and 1 μl (5 ng) was used as template to amplify cDNA using the Superscript III First Strand Synthesis System for RT-PCR. Quantitative PCR was performed on each sample in triplicate using an ABI7900 HT (Applied Biosystems). Data were analysed using SDS 2.1 software (Applied Biosystems), normalizing all genes to human β-actin (SuperArray Bioscience). RTPCR primers (SuperArray Bioscience) were used to detect specific mRNAs for the cytokines IL-1α, IL-1β, IL-6, TNF-α, IL-8 and IFN-γ. At each time...
point, expression of the selected mRNAs in cells incubated with P. gingivalis was calculated relative to the housekeeping gene β-actin (ΔCt) for each sample, and then expressed relative to untreated cells at the same time point using the 2−ΔΔCt method (Livak & Schmittgen, 2001).

**Statistical analyses.** Statistical analyses were performed by Student's t test for paired values using GraphPad software (GraphPad Software). Differences were considered significant at P<0.05.

**RESULTS**

Expression of PAR-1, PAR-2 and PAR-3 on oral epithelial cells

To learn whether TERT-2 cells constitutively express surface PARs, resting, untreated keratinocytes were analysed by flow cytometry for PAR-1, PAR-2 and PAR-3. TERT-2 cells expressed surface PAR-1, -2 and -3 (Fig. 1). PAR-1 and PAR-2 were more frequently expressed at detectable levels than PAR-3 (P<0.001).

Thrombin and trypsin effects on PAR cleavage

Since thrombin and trypsin cleave PARs (Hansen et al., 2004), we treated TERT-2 cells to confirm cleavage of PAR-1 and PAR-2. Compared to levels on untreated cells, PAR-1 was significantly cleaved (60% reduction) by thrombin (Fig. 2A, E), but PAR-2 was unaffected (Fig. 2B, I). In contrast, trypsin cleaved both PAR-1 (Fig. 2A, F) and PAR-2 (Fig. 2B, J) to barely detectable levels. Although PAR-3 was detected at low levels (Fig. 1), thrombin and trypsin apparently did not cleave this receptor (data not shown). For all subsequent experiments, thrombin and trypsin were used as positive controls for PAR-1, and PAR-1 and PAR-2 cleavage, respectively.

**P. gingivalis cleavage of PAR-1 and PAR-2 receptors on oral epithelial cells**

TERT-2 cells were incubated for 1 h with P. gingivalis ATCC 33277 in spent medium at various m.o.i. to verify cleavage of PARs. P. gingivalis cleaved PAR-1 (Fig. 3A) and PAR-2 (Fig. 3B) in a dose-dependent manner. PAR-1 (Fig. 3A) was cleaved more effectively than PAR-2 at each m.o.i. tested (Fig. 3B) and PAR-2 cleavage was only detectable at m.o.i. greater than 100:1 (Fig. 3B). At m.o.i. 100:1 and 1000:1, P. gingivalis reduced PAR-1 signals to levels comparable to trypsin treatment and similar to untreated cells incubated with the isotype control antibody (Fig. 3A). Cleavage of the PARs by P. gingivalis was confirmed by immunofluorescence microscopy (Fig. 3C-L).

Effect of gingipains on PAR cleavage

To determine whether gingipains produced by intact cells of P. gingivalis were responsible for PAR cleavage, keratinocytes were incubated with an isogenic mutant strain, KDP 136, which fails to express either gingipain. Unlike the wild-type, KDP 136 was unable to cleave either PAR-1 or PAR-2 at any m.o.i. tested up to 1000:1 (data not shown). To investigate the role of each P. gingivalis cysteine protease on PAR cleavage, TERT-2 cells were incubated with a Lys- (KDP 129) or Arg-gingipain-deletion mutant (KDP 133), or with the wild-type ATCC 33277. P. gingivalis KDP 129 cleaved PAR-1 (Fig. 4A), but not PAR-2 (Fig. 4B), suggesting that cleavage of PAR-1 was Arg-gingipain specific. When oral keratinocytes were incubated with KDP 133, which does not express the Arg-gingipains (Fig. 4C, D), the percentage of PAR-1 positive cells was only slightly reduced (Fig. 4C) whereas the percentage of PAR-2 positive cells was reduced to levels similar to the wild-type strain (Fig. 4D). This suggested that PAR-2 cleavage was largely Lys-gingipain-dependent. Since PAR-2 was significantly cleaved by the wild-type, cleaved only moderately by the Rgp-deficient mutant and was unaffected by the double mutant, the two proteases are suggested to act synergistically to cleave PAR-2.

Gingipain-mediated regulation of pro-inflammatory cytokines

To determine which gingipain upregulates expression of pro-inflammatory cytokines, oral keratinocytes were incubated with P. gingivalis ATCC 33277 or the gingipain mutant strains. IL-1α, IL-1β, IL-6, TNF-α, IL-8 and IFN-γ expression was analysed by real-time PCR. By 6 h, P. gingivalis induced significant increases in the expression of pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α (Fig. 5A-D), but not IL-8 and IFN-γ (data not shown), by TERT-2 cells. Furthermore, upregulation of IL-1β, IL-6, IL-1α and TNF-α appeared to be gingipain-dependent. While ΔKgp (KDP 129) and the wild-type induced expression of a similar cytokine profile, the ΔRgp (KDP 133) and the double mutant KDP 136 were unable to upregulate the...
pro-inflammatory cytokines. These findings suggest that Arg-gingipains selectively cleave PAR-1 to upregulate pro-inflammatory cytokines in oral keratinocytes.

**DISCUSSION**

Oral keratinocytes express pro-inflammatory cytokines in response to indigenous pathogens such as *P. gingivalis*. As we have reported (Giacaman et al., 2007), *P. gingivalis* Rgp upregulates CCR5 through PAR-1 and -2 signalling. Hence, the PARs enhance the responses of keratinocytes to cytokines. In addition to selective upregulation of chemo-
cell line, TERT-2, obtained from oral mucosa (Dickson et al., 2000). Immortalized cells behave more consistently in vitro than primary cells from different donors. Unlike HeLa cells, TERT-2 cells retain the phenotype of the original oral keratinocytes. Using indirect approaches, P. gingivalis gingipains have been shown to activate PARs. Downstream signalling responses reported include calcium mobilization (Holzhausen et al., 2006; Lourbakos et al., 1998) and cytokine production (Holzhausen et al., 2006; Lourbakos et al., 2001a; Tancharoen et al., 2005). We now provide direct evidence of PAR receptor cleavage by arg- and lys-gingipains using flow cytometry and immunofluorescence. Binding of specific antibody indicates that the extracellular domain of a PAR is intact and loss of binding shows that the domain has been cleaved. Consistent with previous reports (Lourbakos et al., 2001a), resting, untreated keratinocytes show high expression of PAR-1 and PAR-2 and weak expression of PAR-3 (Fig. 1). PAR-4 has not been described in the oral epithelium (Lourbakos et al., 2001a). After incubation with thrombin, trypsin or P. gingivalis cells, ectodomain-specific PAR-1 and -2 antibodies fail to bind, indicating receptor cleavage.

Like the wild-type strain, the gingipain-deletion mutants are assumed to express LPS, although we cannot rule out alternative variants. Since the TLRs are intact and functional on the oral keratinocytes (Giacaman et al., 2007), differences in keratinocyte responses to gingipain mutants are unlikely to be attributable to differential signalling through the TLRs. In our system, P. gingivalis cleaves PAR-1 (Fig. 3A and E–G) and PAR-2 (Fig. 3B and J–L). A P. gingivalis mutant strain that expresses neither
gingipain fails to cleave PARs on oral keratinocytes, suggesting cleavage by gingipains. Since \textit{P. gingivalis} Rgp and Kgp cannot individually simulate PAR-2 cleavage induced by the wild-type (Fig. 4B, D), the two gingipains might act synergistically to cleave and activate PAR-2. Yet, \textit{P. gingivalis} KDP 129 (ΔKgp) alone cleaves PAR-1 at an m.o.i. similar to \textit{P. gingivalis} ATCC 33277 (Fig. 4A). Rgp is therefore suggested to cleave PAR-1 more effectively than PAR-2. Kgp has also been reported to be abnormally processed in an Rgp mutant (Kadowaki et al., 1998). Furthermore, only Rgp possesses the specificity to cleave the PAR-2-tethered ligand between Arg\(^{34}\) and Ser\(^{35}\) (Uehara et al., 2005). Abnormal processing of Kgp could explain why neither the Rgp mutant nor the double gingipain mutant could simulate the PAR-2 cleavage activity of the wild-type strain. Alternatively, the cleavage of PAR-2 could result from digestion by either of the gingipains acting synergistically or with other \textit{P. gingivalis} proteases. Activation and downstream signalling may depend on either Kgp or Rgp, which may explain the attenuated effect of the mutant strains on PAR-2 cleavage.

How selective cleavage of PAR-1 or -2 contributes to differential downstream signalling may be less clear. Although PAR-2 appears relatively insensitive to cleavage by Rgp (Fig. 4B), Rgp activation and downstream signalling appears to occur through PAR-2 (Holzhausen et al., 2005, 2006; Lourbakos et al., 1998, 2001a; Tancharoen et al., 2005). Hence, the PARs may interact functionally, resulting in pathway cross-talk at some level of signalling, since activation of PAR-1 can co-activate PAR-2 (Osovskaya & Bunnett, 2004). In primary endothelial cells, specific PAR-1 antagonists fail to completely suppress PAR-1 activation by thrombin. When PAR-2 is also blocked, however, PAR-1 signalling by thrombin is totally abrogated, suggesting transactivation of PAR-2 by cleaved PAR-1 (O’Brien et al., 2000). Conditions in these experiments were similar to our studies. Thus, functional interactions are likely to occur between PAR-1 and PAR-2 signalling pathways.

The release of pro-inflammatory cytokines by keratinocytes represents an innate immune response to pathogens and their virulence factors. Consistent with previous reports (Lourbakos et al., 2001a; Sandros et al., 2000; Uehara et al., 2008a), we show here that the \textit{P. gingivalis} virulence factor Rgp, but not Kgp, selectively induces the expression of pro-inflammatory cytokine mRNA by oral epithelial cells. Cytokine regulation was only verified at the mRNA level. Further studies appear necessary to also show regulation at
Indeed, the gingipains appear to activate PAR-1 to selectively signal the secretion of certain cytokines. Although IL-6 was specifically induced by Rgp, IL-8 was unaffected by either gingipain. A recent report (Uehara et al., 2008b) using the human oral carcinoma cell line HSC-2 suggests that IL-8 is differentially regulated by the purified gingipains. In HSC-2 cells, IL-8 appears to be downregulated by Kgp and HRgpA, while Rgp seems to be upregulated (Uehara et al., 2008b). The immortalized keratinocytes in our study originated from the normal mucosa of the floor of the mouth. Differences in IL-8 responses could be explained by phenotypic differences in the cells but may also reflect differences in the concentrations of specific gingipains associated with freshly harvested *P. gingivalis* and when used as purified proteins in optimized conditions.

The target cell phenotype and co-expressed virulence factors can affect the apparent selectivity of gingipains in upregulating cytokines. For example, *P. gingivalis* Rgp upregulates IL-8 expression in gingival fibroblasts (Oidomori et al., 2001), but not in oral epithelial cells as we show here. Furthermore, *P. gingivalis* gingipains are generally co-presented with LPS, which can synergize or simulate PAR-mediated transcriptional responses in oral keratinocytes (Giacaman et al., 2007). Since all of the *P. gingivalis* mutants used in our study express LPS, differential regulation of pro-inflammatory cytokines argues in favour of gingipain-associated cytokine induction signalled by selective PAR cleavage, rather than from synergistic LPS-mediated activation of TLRs. We cannot, however, rule out that the LPS on the gingipain-mutant strains and the resulting responses in the oral keratinocytes may be altered, given that LPS–gingipain complex formation may mask functional domains of LPS (Takii et al., 2005).

In this complex environment, *P. gingivalis* gingipains have been implicated in the pathogenesis of periodontal disease. *P. gingivalis*-containing dental plaque resides proximal to sulcular gingival keratinocytes, suggesting that activation of PARs and upregulation of pro-inflammatory cytokines could contribute to acute and chronic inflammation in periodontitis. Locally released cytokines can also circulate in the blood (Nagatomo et al., 2007), and *P. gingivalis* gingipains have been associated with platelet aggregation (Lourbakos et al., 2001b), a key event in thrombosis. In mice, systemic inflammation is promoted by gingipain activation of PAR-2 (Holzhausen et al., 2005, 2006). *P. gingivalis* invasion and systemic dissemination of gingipains could therefore link periodontal disease and cardiovascular disease.

**Fig. 5.** Induction of pro-inflammatory cytokine mRNAs by *P. gingivalis* and gingipain mutants. TERT-2 cells were incubated at m.o.i. 100:1 with *P. gingivalis* ATCC 33277, KDP 129, KDP 133 or KDP 136 in spent bacterial culture medium for 3 h, washed and maintained in fresh medium for the indicated total times. At indicated times, cells were harvested and RNA extracted to perform real-time PCR for IL-1α (A), IL-1β (B), IL-6 (C) and TNF-α (D). β-Actin was used as a housekeeping gene. Graphs show expression of each molecule relative to uninfected TERT-2 cells harvested at the same indicated time. *P*<0.05, when compared to time 0.
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