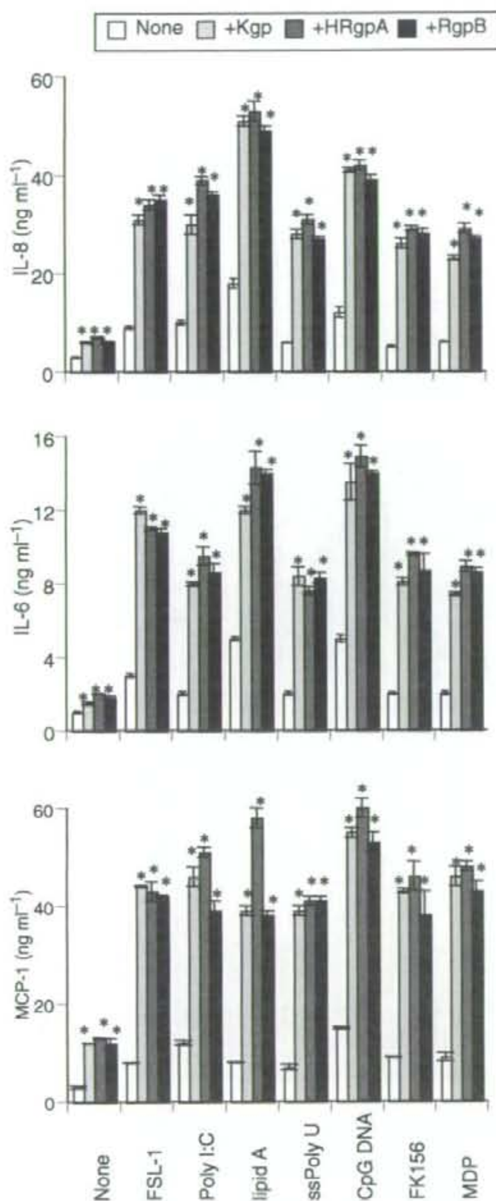

Fig. 5. Synergistic effects of gingipains and, synthetic TLR or NOD ligands on secretion of proinflammatory cytokines in cultured PBMCs. PBMCs were incubated with 200 nM of Kgp, HRgpA, or RgpB in the presence of either of FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8, IL-6 and MCP-1 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone and the respective control. Results representative of four different experiments are shown.

inflammatory cytokines from human monocytic cells via PAR-1, -2 and -3 in combination with TLR or NOD agonists. This is the first report of synergistic effects of signalling of PARs and TLR or NOD and indicates a link between the PAR system and innate immunity. The involvement of PARs in the synergistic effects were shown by the results that (i) PAR-APs mimicked the effects of gingipains (Fig. 2), (ii) the effects were completely inhibited by treating gingipains with inhibitors specific to each proteinase (Fig. 3C), and (iii) decreasing the expression of PARs by treating cells with siRNA suppressed the effects (Fig. 6B). Activation by gingipains has been shown for PAR-1, -2 and -4 (Lourbakos *et al.*, 2001a,b; Tancharoen *et al.*, 2005; Uehara *et al.*, 2005a) but no report has demonstrated PAR-3's activation. The present results clearly demonstrated that Kgp and Rgps synergistically increased the secretion of proinflammatory cytokines in combination with a TLR or NOD agonist through PAR-1, -2 and -3 (Fig. 6B). It is obvious that the effects of kgp are dependent on the cleavage of PARs by its enzymatic activity (Fig. 3C). PAR-3 is activated by cleavage at the carboxy-terminal side of the Lys residue in the tethered ligand (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001), which is consistent with the substrate specificity of Kgp (Pike *et al.*, 1994); however, PAR-1 and PAR-2 require cleavage after the Arg residue and are activated by Rgps. The result that the synergistic effects of gingipains and synthetic PAMPs on IL-8 secretion were inhibited by about 50% in all PAR-silenced THP-1 cells irrespective of the PAMPs indicates an involvement of Rgps and Kgp in the activation of PAR-1 and PAR-2 and PAR-3 respectively (Fig. 6B). Although in Figs 3 and 4 there seems to be some differences in IL-8 production between Kgp, HRgpA and RgpB, in Fig. 5 such differences appear to disappear by a combination of each gingipain with PAMPs. Therefore, the synergism between gingipains and PAMPs varies depending on the combination used, cells used and cytokines secreted. Anyway, the present study shows cross-talk between the PAR system, and TLR or NOD1/2 system, and suggests a new interaction between bacteria and the host defence system. However, previous studies have demonstrated that gingipains would rapidly and efficiently inactivate (Calkins *et al.*, 1998; Baba *et al.*, 2002; Bodet *et al.*, 2005) or be related to decreased extracellular levels of various proinflammatory cytokines, including IL-6 and IL-8 (Steffen *et al.*, 2000), even though their mRNA levels are increased after infection with wild-type *P. gingivalis* but not with its mutant deficient in gingipains (Baba *et al.*, 2002). In addition, there is accumulating evidence that gingipains are responsible for shedding and cleavage of CD14 receptors after treatment of human macrophage-like cells with the bacterium (Sugawara *et al.*, 2000; Duncan *et al.*, 2004). Taken together, we cannot rule out the possibility

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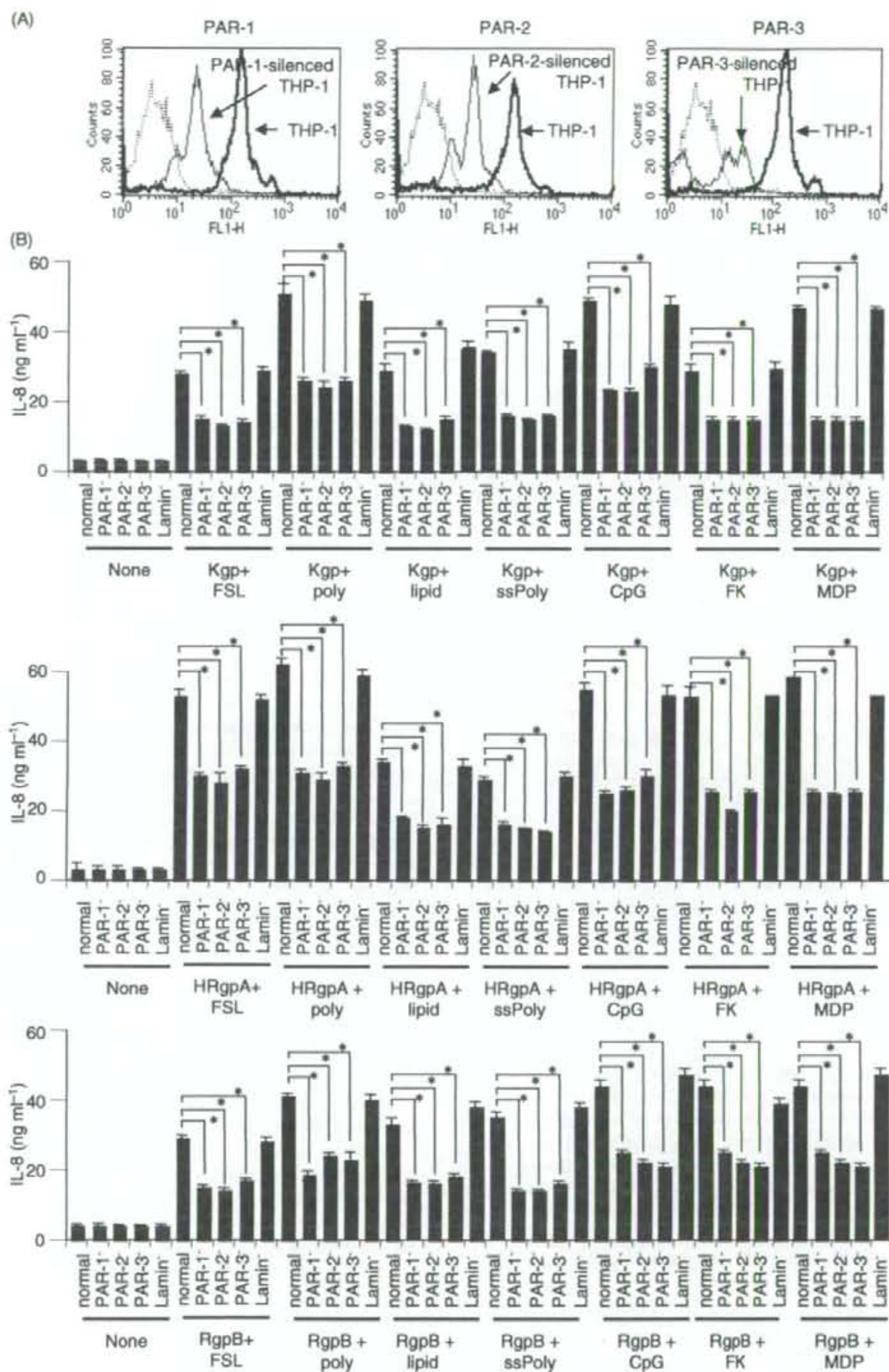

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Fig. 6. Suppression of synergistic effects of gingipains and synthetic PAMPs in THP-1 cells treated with siRNA for PAR-1, PAR-2, or PAR-3.
A. THP-1 cells were transfected with siRNA targeting the PAR-1, PAR-2 or PAR-3 gene. Lamin A/C was used as a control. After 24 h, the cells were stained with Abs specific for PAR-1, PAR-2, or PAR-3 or, control IgG at 4 C for 30 min, followed by FITC-conjugated secondary Abs, and then subjected to flow cytometry.
B. THP-1 cells transfected with siRNA targeting the PAR-1, PAR-2, PAR-3 or Lamin A/C gene for 24 h were stimulated with 200 nM of a gingipain with either FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}), or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. *$P < 0.01$ versus respective control (normal cells). Results representative of three different experiments are shown.

that gingipains may produce biologically inactive fragments of these cytokines, thereby contributing to an increased capacity of the bacterium to evade the host immune system mechanisms.

Porphyromonas gingivalis has been reported to possess TLR agonistic PAMPs: TLR4 agonist lipid A (Ogawa *et al.*, 2007), TLR2 agonist lipopeptides (Asai *et al.*, 2007), fimbriae (Asai *et al.*, 2001) and TLR9 agonist CpG DNA (Takeshita *et al.*, 1999). It should be noted, however, that TLR4-agonistic activity of *P. gingivalis* lipid A was exceptionally weak (Ogawa *et al.*, 2007). Furthermore, *P. gingivalis* PGN carries LL-DAP (Holt and Bramanti, 1991), which scarcely activated NOD1 unlike usual *meso*-DAP (Uehara *et al.*, 2006). Therefore, synergism between signalling via PARs induced by gingipains and PAMPs derived from the bacterium might be responsible for definite inflammatory responses induced by the bacterium in relation to pathogenesis of periodontitis. In addition, we may also speculate of the role of these processes in enhancement of host defence mechanisms.

Experimental procedures

Reagents

We used chemically synthesized PAMPs to avoid the influence of minor components in microbial preparations. The synthetic MDP, a NOD2 agonist, and *Escherichia coli* type lipid A (LA-15-PP), a synthetic TLR4 agonist, were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). The *Mycoplasma salivarium* type diacyl lipopeptide FSL-1, a TLR2/6 agonist, was obtained from RMC microcollections (Tübingen, Germany). Poly I:C, a TLR3 agonist, and ssPoly U, a TLR7 agonist, were purchased from Sigma-Aldrich (St Louis, MO, USA). A conventional CpG DNA, CpG DNA 1826 [TCCAT GACGTTCCTGACGTT (CpG motif is underlined)], a TLR9 agonist, was purchased from SIGMA Genosys (Tokyo, Japan). FK156 (D-lactoyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly) (Kitaura *et al.*, 1982), a NOD1 agonist, was supplied by Astellas Pharmaceutical (Tokyo, Japan). Synthetic PAR-1AP (SFLLRN), PAR-2 AP (SLIGKV) and PAR-3AP (TFRGAP) were purchased from Takara (Otsu, Japan). All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Cells and cell culture

The human monocytic leukaemia cell line THP-1, supplied by the Health Science Research Resources Bank (Osaka, Japan), was cultured in RPMI 1640 medium (Nissui Seiyaku, Osaka, Japan) with 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified CO_2 atmosphere. The THP-1 cells were maintained in a logarithmic phase of growth (2×10^5 to 2×10^6) by passage every 3–4 days.

Human PBMCs were isolated from heparinized peripheral blood of healthy adult donors by Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation at 800 *g* for 20 min at room temperature. The isolated PBMCs were washed three times with PBS and suspended in RPMI1640 medium.

Purification and activation of gingipains

HRgpA, RgpB and Kgp were purified from *P. gingivalis* HG66 culture supernatant, as described previously (Pike *et al.*, 1994; Potempa *et al.*, 1998). The purity of each enzyme was checked by SDS-PAGE. In a 10% Tricine gel (Von Jaggow), RgpB showed a single band with an apparent molecular weight of 48 kDa and the purity was > 95% as determined by laser densitometric scanning of the gel. HRgpA was composed of four major and one minor band on SDS-PAGE and each protein band was identified as a HRgpA component by N-terminal sequence analysis (Pike *et al.*, 1994). The amount of active enzyme in each purified gingipain was determined by active site titration using FPR-cmk and Z-FK-cmk for Rgps and Kgp respectively (Potempa *et al.*, 1997). The concentration of fully activated gingipains with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the proteinases. Therefore, gingipain concentrations indicate active enzyme concentrations. The gingipains were activated in 0.2 M HEPES, 5 mM $CaCl_2$ and 10 mM cysteine, pH 8.0, at 37°C for 10 min, and then diluted with the medium or buffer. To block enzymatic activity, the activated gingipains were incubated with FPR-cmk or Z-FK-cmk for 10 min at room temperature before use.

Measurement of cytokines

The cells were collected and washed twice in PBS. They (2×10^5 cells per ml) were cultured in RPMI 1640 medium, supplemented with 10% FCS, in the presence or absence of a stimulant for 24 h in 96-well culture plates. Then, the culture supernatants were collected and the levels of IL-6, IL-8 and MCP-1 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (OptEIA ELISA, BD Pharmingen, San Diego, CA, USA). The concentrations of a cytokine in the supernatants were calculated using the LS-Platemanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

RNA interference

siRNAs (200 nM, the final concentration) for targeting the genes of PAR-1, PAR-2, PAR-3 or Lamin A/C were purchased from

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Santa Cruz Biotechnology (Santa Cruz, CA, USA) and introduced into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The viability of the transfected cells was more than 95% when assessed with the Trypan blue exclusion test, and the cells did not change morphologically after the transfection.

Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur flow cytometer and CELLQuest software (BD Biosciences, San Diego, CA, USA). Washed THP-1 cells were stained with mouse monoclonal antibodies specific for PAR-1, PAR-2 or PAR-3 (Santa Cruz), or isotype-matched control IgG at 4°C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or goat IgG (Biosource International, Camarillo, CA, USA) at 4°C for a further 30 min. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded less than 2% of the events as positive for the secondary Ab in the absence of the primary antibodies. Fluorescence to the right was counted as specific binding.

Data analysis

All experiments were performed at least three times to confirm the reproducibility of the results. Values are shown as the means ± SD from triplicate assays. The significance of differences was statistically evaluated by the one-way analysis of variance, using the Bonferroni or Dunnett method, and *P*-values less than 0.05 were considered significant.

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References

Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124:** 783–801.

Amano, A. (2007) Disruption of epithelial barrier and impairment of cellular function by *Porphyromonas gingivalis*. *Front Biosci* **12:** 3965–3974.

Asai, Y., Ohyama, Y., Gen, K., and Ogawa, T. (2001) Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. *Infect Immun* **69:** 7387–7395.

Asai, Y., Makimura, Y., and Ogawa, T. (2007) Toll-like receptor 2-mediated dendritic cell activation by a *Porphyromonas gingivalis* synthetic lipopeptide. *J Med Microbiol* **56:** 459–465.

Baba, A., Kadowaki, T., Asao, T., and Yamamoto, K. (2002) Roles for Arg- and Lys-gingipains in the disruption of cytokine responses and loss of viability of human endothelial cells by *Porphyromonas gingivalis* infection. *Biol Chem* **383:** 1223–1230.

Bodet, C., Chandad, F., and Grenier, D. (2005) Modulation of cytokine production by *Porphyromonas gingivalis* in a macrophage and epithelial cell co-culture model. *Microbes Infect* **7:** 448–456.

Calkins, C.C., Platt, K., Potempa, J., and Travis, J. (1998) Inactivation of tumor necrosis factor-α by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. Implications of immune evasion. *J Biol Chem* **273:** 6611–6614.

Chen, Z., Potempa, J., Polanowski, A., Wikstrom, M., and Travis, J. (1992) Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J Biol Chem* **267:** 18896–18901.

Coughlin, S.R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407:** 258–264.

Déry, O., Corvera, C.U., Steinhoff, M., and Bunnett, N.W. (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* **274:** C1429–C1452.

Duncan, L., Yoshioka, M., Chandad, F., and Grenier, D. (2004) Loss of lipopolysaccharide receptor CD14 from the surface of human macrophage-like cells mediated by *Porphyromonas gingivalis* outer membrane vesicles. *Microb Pathog* **36:** 319–325.

Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E. (2006) Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* **7:** 1250–1257.

Holt, S.C., and Bramanti, T.E. (1991) Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit Rev Oral Biol Med* **2:** 177–281.

Imamura, T., Pike, R.N., Potempa, J., and Travis, J. (1994) Pathogenesis of periodontitis: a major arginine-specific cysteine proteinase from *Porphyromonas gingivalis* induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. *J Clin Invest* **94:** 361–367.

Imamura, T., Potempa, J., Pike, R.N., and Travis, J. (1995a) Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. *Infect Immun* **63:** 1999–2003.

Imamura, T., Potempa, J., Pike, R.N., Moore, J.N., Barton, M.H., and Travis, J. (1995b) Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation: implications for bleeding tendency at periodontitis sites. *Infect Immun* **63:** 4877–4882.

Imamura, T., Potempa, J., Tanase, S., and Travis, J. (1997) Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*. *J Biol Chem* **272:** 16062–16067.

Jagels, M.A., Travis, J., Potempa, J., Pike, R., and Hugli, T.E. (1996) Proteolytic inactivation of the leukocyte C5a receptor by proteinases derived from *Porphyromonas gingivalis*. *Infect Immun* **64:** 1984–1991.

Kadowaki, T., Nakayama, K., Yoshimura, F., Okamoto, K., Abe, N., and Yamamoto, K. (1998) Arg-gingipain acts as a major processing enzyme for various cell surface proteins

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in *Porphyromonas gingivalis*. *J Biol Chem* **273:** 29072–29076.

Kitaura, Y., Nakaguchi, O., Takeno, H., Okada, S., Yonishi, S., Hemmi, K., *et al.* (1982) N^2-(γ-D-Glutamyl)-*meso*-2(L),2′(D)-diaminopimelic acid as the minimal prerequisite structure of FK-156: its acyl derivatives with potent immunostimulating activity. *J Med Chem* **25:** 335–337.

Lourbakos, A., Chinni, C., Thompson, P., Potempa, J., Travis, J., Mackie, E.J., and Pike, R.N. (1998) Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from *Porphyromonas gingivalis*. *FEBS Lett* **435:** 45–48.

Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M.R., Andrade-Gordon, P., Santulli, R., *et al.* (2001a) Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect Immun* **69:** 5121–5130.

Lourbakos, A., Yuan, Y., Jenkins, A.L., Travis, J., Andrade-Gordon, P., Santulli, R. *et al.* (2001b) Activation of protease-activated receptors by gingipains from *Porphyromonas gingivalis* leads to platelet aggregation: a new trait in microbial pathogenicity. *Blood* **97:** 3790–3797.

O'Brien, P.J., Molino, M., Kahn, M., and Brass, L.F. (2001) Protease activated receptors: theme and variations. *Oncogene* **20:** 1570–1581.

Ogawa, T., Asai, Y., Makimura, Y., and Tamai, R. (2007) Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. *Front Biosci* **12:** 3795–3812.

Pike, R., McGraw, W., Potempa, J., and Travis, J. (1994) Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. *J Biol Chem* **269:** 406–411.

Potempa, J., Pike, R., and Travis, J. (1995) The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. *Infect Immun* **63:** 1176–1182.

Potempa, J., Pike, R., and Travis, J. (1997) Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biol Chem* **378:** 223–230.

Potempa, J., Mikolajczyk-Pawlinska, J., Brassell, D., Nelson, D., Thøgersen, I.B., Enghild, J.J., and Travis, J. (1998) Comparative properties of two cysteine proteinases (gingipains R), the products of two related but individual genes of *Porphyromonas gingivalis*. *J Biol Chem* **273:** 21648–21657.

Scott, C.F., Whitaker, E.J., Hammond, B.F., and Colman, R.W. (1993) Purification and characterization of a potent 70-kDa thiol lysyl-proteinase (Lys-gingivain) from *Porphyromonas gingivalis* that cleaves kininogens and fibrinogen. *J Biol Chem* **268:** 7935–7942.

Steffen, M.J., Holt, S.C., and Ebersole, J.L. (2000) *Porphyromonas gingivalis* induction of mediator and cytokine secretion by human gingival fibroblasts. *Oral Microbiol Immunol* **15:** 172–180.

Sugawara, S., Nemoto, E., Tada, H., Miyake, K., Imamura, T., and Takada, H. (2000) Proteolysis of human monocyte CD14 by cysteine proteinases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. *J Immunol* **165:** 411–418.

Tada, H., Sugawara, S., Nemoto, E., Takahashi, N., Imamura, T., Potempa, J., *et al.* (2002) Proteolysis of CD14 on human gingival fibroblasts by arginine-specific cysteine proteinases from *Porphyromonas gingivalis* leading to down-regulation of lipopolysaccharide-induced interleukin-8 production. *Infect Immun* **70:** 3304–3307.

Tada, H., Sugawara, S., Nemoto, E., Imamura, T., Potempa, J., Travis, J., *et al.* (2003) Proteolysis of ICAM-1 on human oral epithelial cells by gingipains. *J Dent Res* **82:** 796–801.

Takeshita, A., Imai, K., and Hanazawa, S. (1999) CpG motifs in *Porphyromonas gingivalis* DNA stimulate interleukin-6 expression in human gingival fibroblasts. *Infect Immun* **67:** 4340–4345.

Tancharoen, S., Sarker, K.P., Imamura, T., Biswas, K.K., Matsushita, K., Tatsuyama, S., *et al.* (2005) Neuropeptide release from dental pulp cells by RgpB via proteinase-activated receptor-2 signaling. *J Immunol* **174:** 5796–5804.

Uehara, A., Sugawara, S., Muramoto, K., and Takada, H. (2002) Activation of human oral epithelial cells by neutrophil proteinase 3 through protease-activated receptor-2. *J Immunol* **169:** 4594–4603.

Uehara, A., Muramoto, K., Takada, H., and Sugawara, S. (2003) Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. *J Immunol* **170:** 5690–5696.

Uehara, A., Muramoto, K., Imamura, T., Nakayama, K., Potempa, J., Travis, J., *et al.* (2005a) Arginine-specific gingipains from *Porphyromonas gingivalis* stimulate production of hepatocyte growth factor (scatter factor) through protease-activated receptors in human gingival fibroblasts in culture. *J Immunol* **175:** 6076–6084.

Uehara, A., Yang, S., Fujimoto, Y., Fukase, K., Kusumoto, S., Shibata, K., *et al.* (2005b) Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell Microbiol* **7:** 53–61.

Uehara, A., Fujimoto, Y., Kawasaki, A., Kusumoto, S., Fukase, K., and Takada, H. (2006) *Meso*-diaminopimelic acid and *meso*-lanthionine, amino acids specific to bacterial peptidoglycans, activate human epithelial cells through NOD1. *J Immunol* **177:** 1796–1804.

Uehara, A., Iwashiro, A., Sato, T., Yokota, S., and Takada, H. (2007) Antibodies to proteinase 3 prime human monocytic cells via protease-activated receptor-2 and NF-κB for Toll-like receptor- and NOD-dependent activation. *Mol Immunol* **44:** 3552–3562.

Wingrove, J.A., DiScipio, R.G., Chen, Z., Potempa, J., Travis, J., and Hugli, T.E. (1992) Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas* (*Bacteroides*) *gingivalis*. *J Biol Chem* **267:** 18902–18907.

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Synergism between TLRs and NOD1/2 in Oral Epithelial Cells

ABSTRACT

Oral epithelium is the first barrier against oral bacteria in periodontal tissue. Oral epithelial cells constitutively express Toll-like receptors (TLRs) and NOD1/2, functional receptors which induce the production of antibacterial factors such as peptidoglycan recognition proteins (PGRPs) and β-defensin 2, but not pro-inflammatory cytokines such as interleukin (IL)-8. In this study, we hypothesized that innate immune responses in the oral epithelium are enhanced in inflamed tissue. We found that NOD1 and NOD2 agonists, in combination with TLR agonists, synergistically induced production of PGRPs and of β-defensin 2 in human oral epithelial cells *via* NF-κB. In contrast, co-stimulation with NOD1/2 and TLR ligands had no effect on the production of pro-inflammatory cytokines (IL-6, IL-8, and monocyte chemoattractant protein-1). These findings indicate that, in innate immune responses to invading microbes, a combination of signaling through TLRs and NODs leads to the synergistic activation of antibacterial responses in the oral epithelium.

KEY WORDS: peptidoglycan recognition proteins (PGRPs), Toll-like receptors (TLRs), NOD1/2, oral epithelial cells, β-defensin 2.

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INTRODUCTION

The immune system provides protection against a wide variety of pathogens. Immunity can be divided into two major categories: innate and adaptive. In the innate immune system, defenses against invasive pathogens are initiated when pathogen-associated molecular patterns are recognized by the pattern recognition molecules of hosts. In mammals, these pathogen-associated molecular patterns are recognized specifically by their respective Toll-like receptors (TLRs): peptidoglycans and lipopeptides mainly by TLR2, double-stranded RNA by TLR3, lipopolysaccharide (LPS) by TLR4, single-stranded RNA by TLR7, and bacterial CpG DNA by TLR9 (Akira *et al.*, 2006). Furthermore, it has been reported that 2 active entities of peptidoglycans, desmuramylpeptides containing diaminopimelic acid and muramylpeptides, were recognized by intracellular receptors NOD1 and NOD2, respectively (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a,b; Inohara *et al.*, 2003; Uehara *et al.*, 2006).

Previously, we reported that oral epithelial cells constitutively expressed TLR2, TLR4, NOD1, and NOD2, and that stimulation with ligands for these receptors induced production of antimicrobial peptides such as peptidoglycan recognition proteins (PGRPs) (Uehara *et al.*, 2005b) and β-defensin 2 (Sugawara *et al.*, 2006), but not pro-inflammatory cytokines (Uehara *et al.*, 2005b; Sugawara *et al.*, 2006). In addition, we found the clear expression of TLR2, TLR3, TLR4, TLR7, NOD1, and NOD2 in tongue, salivary gland, pharyngeal, esophageal, intestinal, cervical, breast, lung, and kidney epithelial cells, as well as in oral epithelial cells, and these cells secrete β-defensin 2, but not pro-inflammatory cytokines in response to TLR and NOD agonists (Uehara *et al.*, 2007). Concerning innate immune responses in human monocytic cells, the NOD2-agonistic muramyldipeptide and NOD1-agonistic diaminopimelic acid-containing peptides, in combination with the TLR2-agonistic lipopeptide, TLR4-agonistic lipid A, and TLR9-agonistic CpG DNA, synergistically induced production of IL-8 (Uehara *et al.*, 2005a). In this study, we examined the possible synergistic effects of NOD1/2 and TLR agonists in terms of the production of antimicrobial peptides in oral epithelial cells. We used only chemically synthesized components, because natural microbial preparations are inevitably contaminated with minor bioactive components that might confuse the results.

MATERIALS & METHODS

Reagents

Synthetic muramyldipeptide (MurNAc-L-Ala-D-isoGln) and an *Escherichia coli*-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Double-stranded Poly I:C was obtained from Sigma-Aldrich (St. Louis, MO, USA). Single-stranded Poly U was provided by

Invivogen (San Diego, CA, USA). A conventional CpG DNA, CpG DNA 1826 (TCCATGACGTTCCTG ACGTT), was purchased from SIGMA Genosys (Tokyo, Japan). A synthetic *Mycoplasma*-type diacyl lipopeptide FSL-1 (S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-GDPKHPKSF) was obtained from EMC Microcollections (Tübingen, Germany). A synthetic desmuramylpeptide, a peptidoglycan fragment containing diaminopimelic acid, FK156 (D-lactoyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly), was supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Non-enzymatic cell dissociation solution was obtained from Sigma-Aldrich. All other reagents were from Sigma-Aldrich, unless otherwise indicated.

Cells and Cell Culture

The human oral epithelial cell line HSC-2 was obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University. HSC-2 cells were grown in RPMI 1640 medium with 10% heat-inactivated FCS. Human gingival epithelial cells were prepared from explants of normal human gingival tissue, with informed consent from the donors. The experimental procedure was approved by the Ethical Review Board of Tohoku University Graduate School of Dentistry.

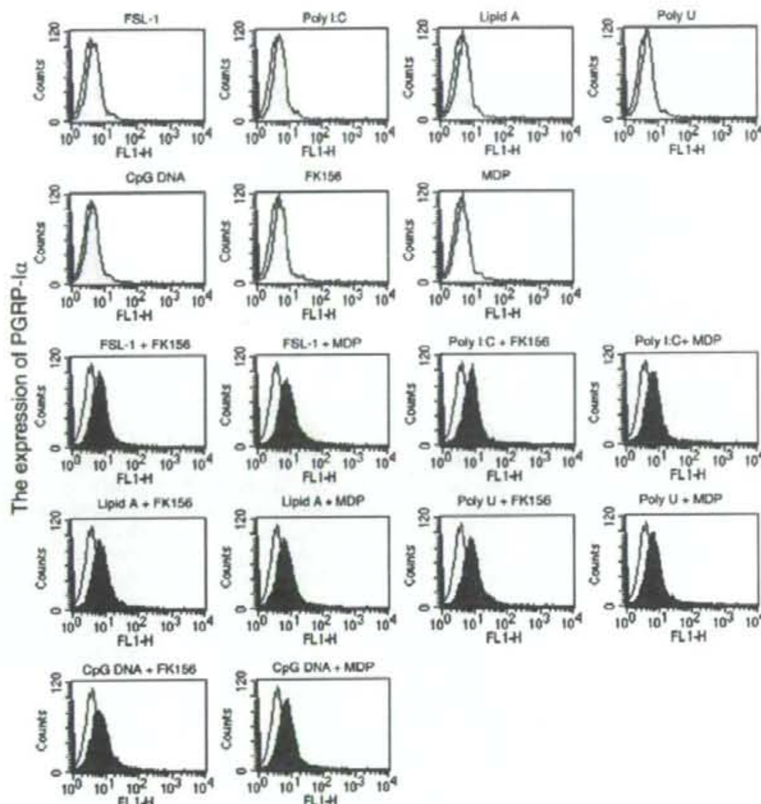

Figure 1. Synergistic effects of NOD1 and NOD2 agonists in combination with synthetic TLR agonists for induction of PGRP-Iα in oral epithelial cells. Oral epithelial HSC-2 cells were stimulated with FK156 (100 μg/mL) or muramyldipeptide (MDP) (100 μg/mL) plus FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), Poly U (10 μg/mL), or CpG DNA (10 nM) for 24 hrs in triplicate. The expression of PGRP-Iα was assessed by flow cytometry. The unshaded curve represents medium alone. The results presented are representative of 3 different experiments.

Flow Cytometry

Flow cytometric analyses were performed by means of a FACSCalibur cytometer (BD Biosciences, Mountain View, CA, USA). The cells were collected by non-enzymatic cell dissociation solution and washed in PBS. They were stained with anti-PGRP-Iα (mouse IgG), anti-PGRP-Iβ (mouse IgG), or anti-PGRP-S (mouse IgG) antibodies (Imgenex, San Diego, CA), or control IgG at 4°C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA, USA) at 4°C for an additional 30 min. It must be noted that PGRP-L expression was not examined, because no anti-PGRP-L antibodies are yet available commercially.

Cytokine Measurements

To investigate the production of inflammatory cytokines by oral epithelial cells, we collected the supernatant from each culture. The production of cytokines (IL-6, IL-8, and MCP-1) was measured with OptEIA ELISA kits (PharMingen, San Diego, CA, USA). The level of β-defensin 2 was measured with an ELISA Development Kit (PeproTEch EC, London, UK). The concentrations of the cytokines in the supernatants were determined with the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

RNA Interference

Transfections for targeting endogenous NF-κB p65 and Lamin A/C were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and short-interfering (si) RNA (final concentration, 200 nM) for 24 hrs at 37°C, according to the manufacturer's instructions. The viability of the cells after transfection was more than 95%, as assessed by a 0.2% trypan blue exclusion test, and the morphology was not changed by the transfection. The siRNA for NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the siRNA for Lamin A/C, from B-Bridge International (Mountain View, CA, USA).

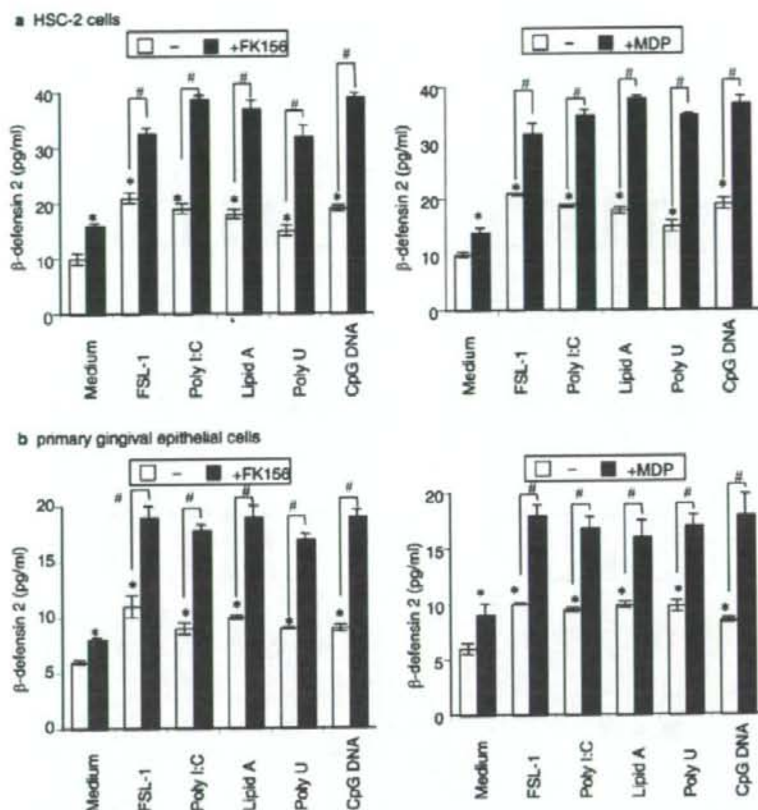

Figure 2. Synergistic effects of NOD1 and NOD2 agonists in combination with synthetic TLR agonists for induction of β-defensin 2 secretion in oral epithelial cells. Oral epithelial HSC-2 cells **(a)** and primary gingival epithelial cells **(b)** were stimulated with FK156 (100 μg/mL) or muramyldipeptide (MDP) (100 μg/mL) plus FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), Poly U (10 μg/mL), or CpG DNA (10 nM) for 24 hrs in triplicate. Concentrations of β-defensin 2 in the culture supernatants were determined by ELISA, and expressed as means ± SD. *,#Marked values differed significantly from those obtained with medium alone or from respective cultures stimulated with the indicated ligands, respectively. The results presented are representative of 3 different experiments.

RESULTS

Production of PGRPs in Human Oral Epithelial Cells Stimulated with NOD1-agonistic FK156 and NOD2-agonistic Muramyldipeptide in Combination with Various TLR Ligands

Chemically synthesized TLR and NOD ligands—FSL-1 (TLR2 agonist), Poly I:C (TLR3 agonist), lipid A (TLR4 agonist), Poly U (TLR7 agonist), CpG DNA (TLR9 agonist), FK156 (NOD1 agonist), and muramyldipeptide (NOD2 agonist)—slightly increased the expression of PGRP-Iα, -Iβ, and -S in human oral epithelial cells (Fig. 1 and APPENDIX). To elucidate the possible synergistic effects of NODs with various TLR agonists, we examined the production of PGRPs in human oral epithelial cells stimulated with NOD1-agonistic FK156 in combination with various TLR ligands. Clear synergistic effects were observed between FK156 and 5 synthetic TLR ligands. NOD2-agonistic muramyldipeptide in combination with TLR agonists also synergistically up-regulated the production of PGRPs (Fig. 1 and APPENDIX).

NOD1-agonistic FK156 and NOD2-agonistic Muramyldipeptide in Combination with Chemically Synthesized TLR Agonists Synergistically Induced the Production of β-defensin 2 in Human Oral Epithelial Cells

Next, we examined the synergistic effects of NOD and TLR ligands in terms of the production of β-defensin 2 by ELISA. Consistent with the results regarding PGRP production, FK156 and muramyldipeptide, in combination with TLR agonists, synergistically induced the production of β-defensin 2 in oral epithelial cells (Fig. 2a). Primary gingival epithelial cells also showed synergistic production of β-defensin 2 upon stimulation with NOD and TLR ligands (Fig. 2b).

FK156 and Muramyldipeptide in Combination with Chemically Synthesized TLR Agonists Did Not Induce the Production of Pro-inflammatory Cytokines in Human Oral Epithelial Cells

Oral epithelial cells did not secrete pro-inflammatory cytokines upon stimulation with TLR and NOD ligands (Uehara *et al.*, 2005b, 2007). Contrary to PGRPs and β-defensin 2, co-stimulation with NOD1/2 and TLR ligands had no effect on the production of pro-inflammatory cytokines (IL-6, IL-8, and monocyte chemoattractant protein-1) (Fig. 3).

Specific Suppression by siRNA Targeting NF-κB against Synergistic β-defensin 2 Production Induced by a Combination of FK156 or Muramyldipeptide in Combination with Synthetic TLR Agonists

To clarify the signaling pathway of the synergistic effects of NOD1 and NOD2 agonists with TLR agonists, we utilized RNA interference assays, targeting NF-κB p65 mRNA. NF-κB protein levels determined by flow cytometry were suppressed by *ca.* 80% with specific siRNA in oral epithelial HSC-2 cells up to 72 hrs (Uehara *et al.*, 2007). The synergistic effects induced by FK156 or muramyldipeptide and TLR ligands were almost completely diminished in NF-κB-silenced cells, but not

in Lamin siRNA-silenced cells (Fig. 4).

DISCUSSION

In this study, we clearly demonstrated that NOD1 and NOD2 agonists exhibited synergistic effects with TLR2, TLR3, TLR4, TLR7, and TLR9 agonists to induce the production of antimicrobial peptides such as PGRPs and β-defensin 2 in human oral epithelial cells. We also demonstrated that NF-κB is involved in the synergistic effects of NOD1 and NOD2 agonists in combination with TLR agonists.

It has been shown that muramyldipeptide can act synergistically with LPS to induce production of inflammatory cytokines (Yang *et al.*, 2001; Takada *et al.*, 2002; Traub *et al.*, 2004, 2006; Tada *et al.*, 2005; Takada and Uehara, 2006). As well as NOD2-agonistic muramyldipeptide, NOD1-agonistic iE-DAP was also reported to induce TNF-α and IL-6 secretion, and, when combined with LPS, generated a greater response than that generated by LPS alone (Chamaillard *et al.*, 2003). Our studies with the human myelomonocytic cell line THP-1 revealed that a NOD1-agonistic FK156 or FK565 induced IL-8 production by itself and in synergy with TLR2, TLR4, and TLR9 ligands (Uehara *et al.*, 2005a). Furthermore, NOD1- and NOD2-agonists are reported to be able to stimulate the release of cytokines and chemokines in synergy with LPS in human monocytes and dendritic cells (Fritz *et al.*, 2005; van Heel *et al.*, 2005). This is the first report that NOD1 and 2 have synergistic effects with TLR2-, TLR3-, TLR7-, and TLR9-agonists, as well as TLR4-agonistic lipid A (LPS), to induce production of an antimicrobial peptide in human epithelial cells.

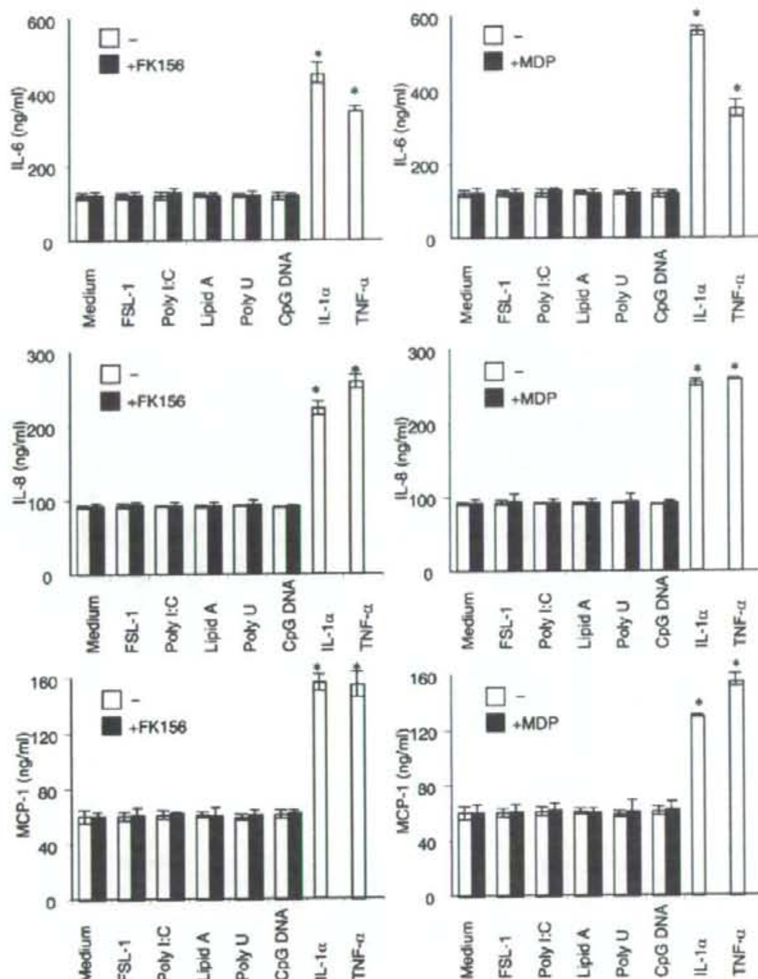

Figure 3. Stimulation with NOD1 and NOD2 agonists in combination with synthetic TLR agonists did not induce the secretion of pro-inflammatory cytokines in oral epithelial cells. Oral epithelial cells were stimulated with FK156 (100 μg/mL) or muramyldipeptide (MDP) (100 μg/mL) plus FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), Poly U (10 μg/mL), or CpG DNA (10 nM) for 24 hrs in triplicate. IL-1α (10 ng/mL) and TNF-α (10 ng/mL) were used as positive controls. Concentrations of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in the culture supernatants were determined by ELISA, and expressed as means ± SD. *$P < 0.01$ *vs.* medium alone. The results presented are representative of 4 different experiments.

It is reasonable for oral epithelial cells to produce antimicrobial factors synergistically, without the accompanying inflammatory cytokines, upon stimulation with microbial components. Therefore, pattern recognition molecules on oral epithelial cells are functional, and oral epithelial cells actively participate in bacterial clearance without accompanying inflammatory responses in the oral mucosa.

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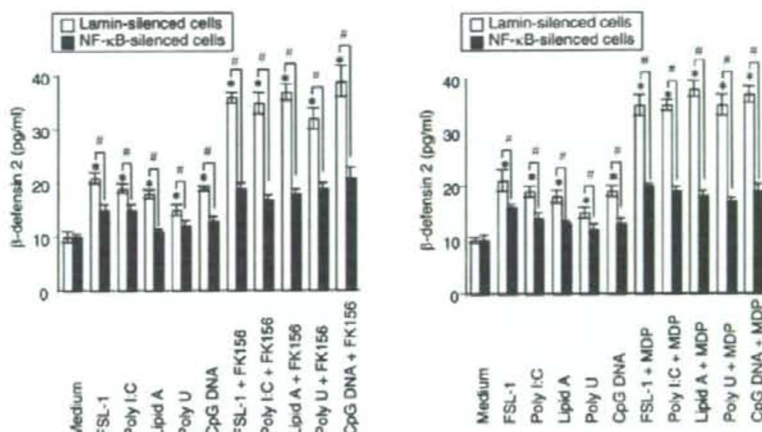

Figure 4. Specific suppression of synergistic effects of NOD1 and NOD2 agonists in combination with TLR ligands in oral epithelial cells with siRNA for NF-κB p65. Oral epithelial cells were transfected with NF-κB- or Lamin-specific siRNA. After 24 hrs, the transfected cells were stimulated with FK156 (100 μg/mL) or muramyldipeptide (MDP) (100 μg/mL) plus FSL-1 (1 nM), Poly I:C (10 μg/mL), lipid A (10 ng/mL), Poly U (1 μg/mL), or CpG DNA (1 μM) for an additional 24 hrs in triplicate. Concentrations of β-defensin 2 in the culture supernatants were determined by ELISA, and expressed as means ± SD. *,#Marked values differed significantly from those obtained with medium alone or from respective cultures stimulated with the indicated ligands, respectively. The results presented are representative of 3 different experiments.

REFERENCES

Akira S, Uematsu S, Takeuchi O (2006). Pathogen recognition and innate immunity. *Cell* 124:783-801.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, *et al.* (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 4:702-707.

Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreulx D, Caroff M, *et al.* (2005). Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* 35:2459-2470.

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jéhanno M, Viala J, *et al.* (2003a). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 300:1584-1587.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, *et al.* (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278:8869-8872.

Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, *et al.* (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 278:5509-5512.

Sugawara Y, Uehara A, Fujimoto Y, Kusumoto S, Fukase K, Shibata K, *et al.* (2006). Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. *J Dent Res* 85:524-529.

Tada H, Aiba S, Shibata K, Ohteki T, Takada H (2005). Synergistic effect of Nod1 and Nod2 agonists with Toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun* 73:7967-7976.

Takada H, Uehara A (2006). Enhancement of TLR-mediated innate immune responses by peptidoglycans through NOD signaling. *Curr Pharm Des* 12:4163-4172.

Takada H, Yokoyama S, Yang S (2002). Enhancement of endotoxin activity by muramyldipeptide. *J Endotoxin Res* 8:337-342.

Traub S, Kubasch N, Morath S, Kresse M, Hartung T, Schmidt RR, *et al.* (2004). Structural requirements of synthetic muropeptides to synergize with lipopolysaccharide in cytokine induction. *J Biol Chem* 279:8694-8700.

Traub S, von Aulock S, Hartung T, Hermann C (2006). MDP and other muropeptides—direct and synergistic effects on the immune system. *J Endotoxin Res* 12:69-85.

Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K, *et al.* (2005a). Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell Microbiol* 7:53-61.

Uehara A, Sugawara Y, Kurata S, Fujimoto Y, Fukase K, Kusumoto S, *et al.* (2005b). Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via Toll-like receptors, NOD1 and NOD2 in human oral epithelial cells. *Cell Microbiol* 7:675-686.

Uehara A, Fujimoto Y, Kawasaki A, Kusumoto S, Fukase K, Takada H (2006). *Meso*-diaminopimelic acid and *meso*-lanthionine, amino acids specific to bacterial peptidoglycans, activate human epithelial cells through NOD1. *J Immunol* 177:1796-1804.

Uehara A, Fujimoto Y, Fukase K, Takada H (2007). Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol Immunol* 44:3100-3111.

van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BM, Mengin-Lecreulx D, *et al.* (2005). Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol* 35:2471-2476.

Yang S, Tamai R, Akashi S, Takeuchi O, Akira S, Sugawara S, *et al.* (2001). Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect Immun* 69:2045-2053.

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Antibodies to Proteinase 3 Prime Human Oral, Lung, and Kidney Epithelial Cells To Secrete Proinflammatory Cytokines upon Stimulation with Agonists to Various Toll-Like Receptors, NOD1, and NOD2▽

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Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies, the detection of which in serum can be used in the diagnosis of Wegener's granulomatosis (WG). Proteinase 3 (PR3) is a major target antigen of ANCA in WG patients, and the interaction of PR3 ANCA with leukocytes causes a debilitating autoimmune disease. The first signs and symptoms in WG patients are observed in the oral cavity, lungs, and kidneys. Human epithelial cells generally do not secrete proinflammatory cytokines upon stimulation with pathogen-associated molecular patterns (PAMPs). In this study, anti-PR3 antibodies (Abs) and PR3 ANCA-containing sera from WG patients endowed human oral, lung, and kidney epithelial cells with responsiveness to PAMPs in terms of the production of proinflammatory cytokines, such as interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1, and tumor necrosis factor alpha. Protease-activated receptor-2 (PAR-2) agonist peptides mimicked the priming effects of PR3 ANCA against PAMPs. Furthermore, the anti-PR3 Ab-mediated cell activation was significantly abolished by RNA interference targeting PAR-2 and NF-κB. This is the first report of priming effects of anti-PR3 Abs (PR3 ANCA) on epithelial cells. The results suggest that anti-PR3 Abs (PR3 ANCA) prime human epithelial cells to produce cytokines upon stimulation with various PAMPs, and these mechanisms may be involved in severe chronic inflammation in WG.

Antineutrophil cytoplasmic antibodies (ANCA) form a heterogeneous group of antibodies (Abs) that target antigens present mostly in azurophilic granules of polymorphonuclear leukocytes. ANCA were first discovered in the 1970s, when cytoplasmic fluorescence was observed during investigations of anti-nuclear Ab in human granulocytes by indirect fluorescence (33). In the 1980s, the spectrum of diseases associated with ANCA became clearer, and vasculitis (7, 14) was identified as a common sign of these diseases. Proteinase 3 (PR3) (21) and myeloperoxidase (MPO) (9) have been identified as two of the main targets of ANCA. PR3 ANCA have been reported to be causally involved in the pathogenesis of Wegener's granulomatosis (WG); the auto-Ab titer correlates with disease activity (27, 32). Anti-PR3 Abs (PR3 ANCA) directly activate a wide variety of inflammatory functions in leukocytes, such as the secretion of cytokines (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], IL-6, IL-8, and monocyte chemoattractant protein-1 [MCP-1]), oxygen radicals, proteases, and lipid mediators, once PR3 is expressed on the surface under inflammatory conditions (4, 10, 13, 15, 22, 23, 25, 26). Anti-PR3 Abs provoke a marked release of cytokines from human monocytes, with the early appearance of TNF-α and IL-1β and the delayed release of IL-6, IL-8, and thromboxane A_2 (15). In addition, anti-PR3 Abs induce the release of MCP-1 from human mononuclear cells (23). Hattar et al. (16) reported that PR3 was detected in human renal tubular epithelial cells treated with TNF-α and that primed cells respond to anti-PR3 Abs with the activation of a phosphoinositide-related signal transduction pathway. Recently, Bartůňková et al. (3) reported that the interaction of PR3 ANCA with TNF-α-primed mononuclear cells stimulates the release of IL-8 via cross-linking between Fc gamma receptors and PR3 expressed on the monocyte cell surfaces. Hattar et al. (17) demonstrated a priming effect of PR3 ANCA for the activation of isolated monocytes and neutrophils by bacterial cell surface components such as lipopolysaccharide (LPS) and lipoteichoic acid. Although the incubation of monocytes and neutrophils with ANCA alone resulted in only a low level of IL-8 release, preincubation with ANCA resulted in a markedly enhanced release of IL-8 upon stimulation with LPS. Recently, we revealed that a murine anti-human PR3 monoclonal Ab primes human monocytic THP-1 cells for enhanced activation upon stimulation with various microbial components (30). These results indicated that PR3 ANCA specifically prime leukocytes, and the resulting enhanced responsiveness to bacterial components may contribute to the development and maintenance of inflammatory lesions of WG.

Previously, we revealed that proinflammatory cytokines, such as IL-1α, alpha interferon (IFN-α), IFN-β, and IFN-γ, induce the production of PR3 in membrane-bound and se-

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cretory forms in human oral epithelial cells and that the addition of anti-PR3 Abs to cytokine-primed oral epithelial cells in culture induces the aggregation of PR3, followed by the activation of protease-activated receptor-2 (PAR-2), which results in remarkable secretion of IL-8 and MCP-1 (31). PAR family members are G protein-coupled receptors characterized by a proteolytic cleavage of the N terminus that exposes tethered ligands and autoactivates the receptor function (6, 8, 24). There are four members of this family. PAR-2 is activated by trypsin and mast cell tryptase, as well as coagulation factors VIIa and Xa. Because PARs are expressed on a wide variety of cell types, including neutrophils, they are believed to play important roles in several pathophysiological processes, including growth, development, inflammation, tissue repair, and pain.

The innate immune system recognizes microorganisms through a series of pattern recognition receptors that have been highly conserved during evolution and are specific for common motifs found in microorganisms but not in eukaryotes, designated pathogen-associated molecular patterns (PAMPs) (1, 18, 20). Representative microbial PAMPs are the lipid A moiety of LPS, lipopeptides, peptidoglycans (PGNs), and viral double-stranded and single-stranded RNAs. Akira et al. demonstrated that these PAMPs are recognized specifically by the respective Toll-like receptor (TLR) (2). In addition, some NOD-like receptor family members were demonstrated previously to be intracellular receptors for partial structures of PGNs; NOD1 and NOD2 recognize a diaminopimelic acid (DAP)-containing peptide moiety (5, 11, 29) and a muramyl dipeptide (MDP) moiety (12, 19), respectively.

The first signs and symptoms in WG patients are in the oral, lung, and kidney epithelia. Human epithelial cells, including those of the oral cavity, lungs, and kidneys, generally do not secrete proinflammatory cytokines upon stimulation with PAMPs (28). In this study, we examined whether anti-PR3 Abs (PR3 ANCA) were capable of priming human oral, lung, and kidney epithelial cells. If they are, the mechanism may be involved in the pathogenesis of ANCA-related inflammatory diseases represented by WG.

MATERIALS AND METHODS

Reagents. Synthetic MDP (MurNAc-L-Ala-D-isoGln) and an *Escherichia coli*-type lipid A (LA-15-PP) were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). Double-stranded poly(I-C) was obtained from Sigma-Aldrich (St. Louis, MO). Single-stranded poly(U) was purchased from Invivogen (San Diego, CA). A conventional CpG DNA, CpG DNA 1826 (TCC ATGACGTTCCTGACGTT [the CpG motif is underlined]), was provided by Sigma-Genosys (Tokyo, Japan). A synthetic *Mycoplasma*-type diacyl lipopeptide, FSL-1 {S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-cysteinyl-GDPKHPKSF}, was purchased from EMC Microcollections (Tübingen, Germany). Two synthetic desmuramylpeptides—DAP-containing PGN fragment FK156 (D-lactoyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly) and the FK156 derivative FK565 (heptanoyl-γ-D-Glu-*meso*-DAP-D-Ala)—were supplied by Astellas Pharmaceutical Co. (Tokyo, Japan). Recombinant human IL-1α and TNF-α were provided by Dainippon Pharmaceutical Co. (Osaka, Japan). A PAR-2 agonist peptide (PAR-2AP; SLIGKV) was synthesized by Takara (Otsu, Japan). Nonenzymatic cell dissociation solution (CDS) was obtained from Sigma-Aldrich. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Serum samples. ANCA-containing sera were obtained from four patients with WG at Tohoku University Hospital, Sendai, Japan. The samples were immediately purified by centrifugation, aliquoted, and frozen at −70°C until being used. PR3 ANCA and MPO ANCA titers were reconfirmed by the enzyme immunoassay method by BML Co. (Sendai, Japan). All of the ANCA-containing sera were PR3 ANCA-positive and MPO ANCA-negative specimens. Normal serum from a healthy adult donor was used as a control.

Cells and cell culture. The human oral epithelial cell line HSC-2, the human lung epithelial cell line A549, and the human kidney epithelial cell line Caki-1 were obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum, with a change of medium every 3 days. To avoid cell surface markers, we used Sigma's CDS. CDS contains no protein and allows the dislodging of cells without enzymatic modification or the adsorption of foreign proteins.

Cytokine measurements. To investigate the production of inflammatory cytokines by epithelial cells, we collected the supernatant from each culture. The production of cytokines (IL-6, IL-8, MCP-1, and TNF-α) was measured using OptEIA ELISA kits (PharMingen, San Diego, CA). The concentrations of the cytokines in the supernatants were determined using the LS-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

RNA interference. Transfections for targeting endogenous PAR-2, NF-κB p65, and lamin A/C were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and short interfering RNA (siRNA; final concentration, 200 nM) for 24 h at 37°C, according to Invitrogens instructions. The viability of the cell cultures after transfection was more than 95%, as assessed by a 0.2% trypan blue exclusion test, and the cells' morphological character was unchanged after transfection. siRNAs for PAR-2 and NF-κB p65 were purchased from Santa Cruz Biotechnology, and the siRNA for lamin A/C was purchased from B-Bridge International.

RESULTS

Human oral, lung, and kidney epithelial cells do not secrete proinflammatory cytokines upon stimulation with PAMPs. We previously reported that various human epithelial cells do not secrete proinflammatory cytokines (28). As shown in Fig. 1, human oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells did not secrete IL-8 upon stimulation with the synthetic PAMPs FSL-1 (a TLR2 agonist), poly(I-C) (a TLR3 agonist), lipid A (a TLR4 agonist), poly(U) (a TLR7 agonist), CpG DNA (a TLR9 agonist), FK156 and FK565 (NOD1 agonists), and MDP (a NOD2 agonist). In contrast, human epithelial cells secreted IL-8 in response to TNF-α and IL-1α as positive controls.

Treatment with anti-PR3 Abs primed human oral, lung, and kidney epithelial cells to secrete IL-6, IL-8, MCP-1, and TNF-α upon stimulation with PAMPs. We demonstrated previously that anti-PR3 Abs enhance TLR and NOD agonist PAMP-induced secretion of proinflammatory cytokines by human monocytic THP-1 cells and human peripheral blood mononuclear cells (30). In the present study, we examined the production of inflammatory cytokines in human epithelial cells upon stimulation with PAMPs after priming with anti-PR3 Abs. When human oral, lung, and kidney epithelial cells were preincubated with 1 μg of anti-PR3 Abs/ml for 6 h and subsequently challenged with the various TLR and NOD agonist PAMPs for a further 18 h, massive production of IL-8 was observed, whereas stimulation with the anti-PR3 Abs by themselves had scarcely any effect (Fig. 2). Priming effects were also observed for the production of IL-6, MCP-1, and TNF-α (Fig. 3).

PR3 ANCA-containing sera primed human oral, lung, and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs. To further demonstrate the possible immunopathological properties of ANCA from WG patients, we examined whether the ANCA-containing sera were capable of priming human epithelial cells similarly to murine anti-PR3 Abs. We

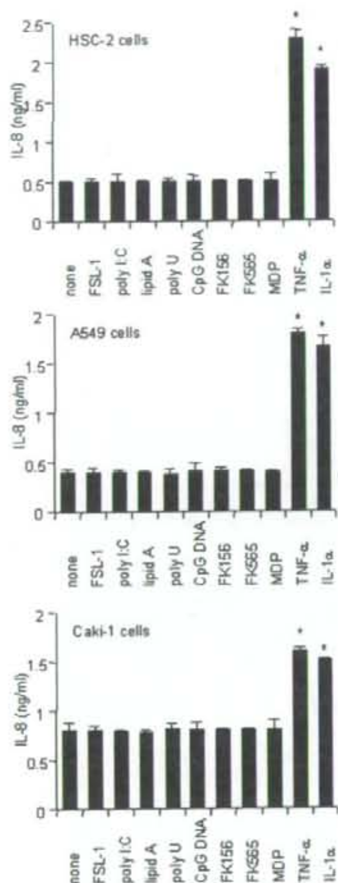

FIG. 1. Human oral, lung, and kidney epithelial cells did not secrete IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), MDP (100 μg/ml), TNF-α (10 ng/ml), or IL-1α (10 ng/ml) for 24 h in triplicate. Human TNF-α and IL-1α were used as positive controls. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± standard deviations (SD). *, $P < 0.01$ versus results for cells stimulated with medium alone. The results presented are representative of three different experiments demonstrating similar results.

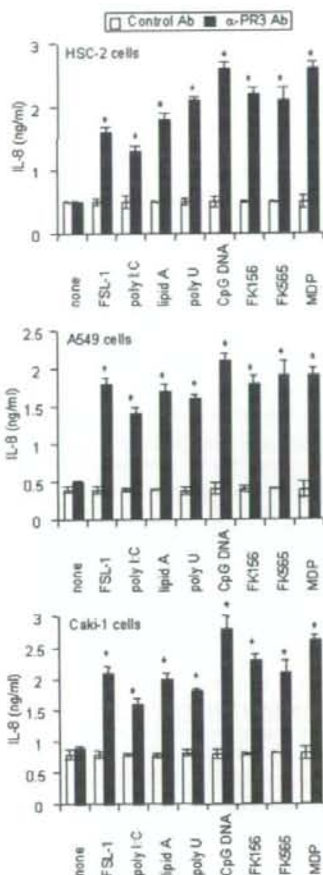

FIG. 2. Human oral, lung, and kidney epithelial cells preincubated with anti-PR3 Abs secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with anti-PR3 Abs (1 μg/ml) or with an equal amount of an isotype-matched immunoglobulin G (IgG) Ab. Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± SD. *, significantly different from results for cells in the respective cultures incubated with control IgG ($P < 0.01$). The results presented are representative of three different experiments demonstrating similar results.

obtained sera from patients with WG at Tohoku University Hospital and carried out experiments. The titers of PR3 ANCA and MPO ANCA in sera were determined. All sera from four patients were PR3 ANCA positive (titer, >3.0 U/ml), whereas none was MPO ANCA positive (titer, <1.3 U/ml). In this study, representative PR3 ANCA-positive sera from a WG patient (sample S9-32 from patient S9) were compared with normal serum. We examined the production of IL-8 upon stimulation with TLR and NOD agonist PAMPs. IL-8 production was significantly induced when human oral, lung, and kidney epithelial cells were incubated with the patients' sera compared with the production in cells incubated with normal serum (Fig. 4). These results clearly indicated that PR3 ANCA in the sera from WG patients exerted a priming effect similar to that of murine monoclonal anti-human PR3 Abs in vitro.

Treatment with PAR-2AP primed human oral, lung, and kidney epithelial cells to secrete IL-8 upon stimulation with PAMPs. As we found that anti-PR3 Abs primed human monocytic cells via PAR-2 (30), we examined whether the priming effects of PAR-2AP also occurred in human epithelial cells.

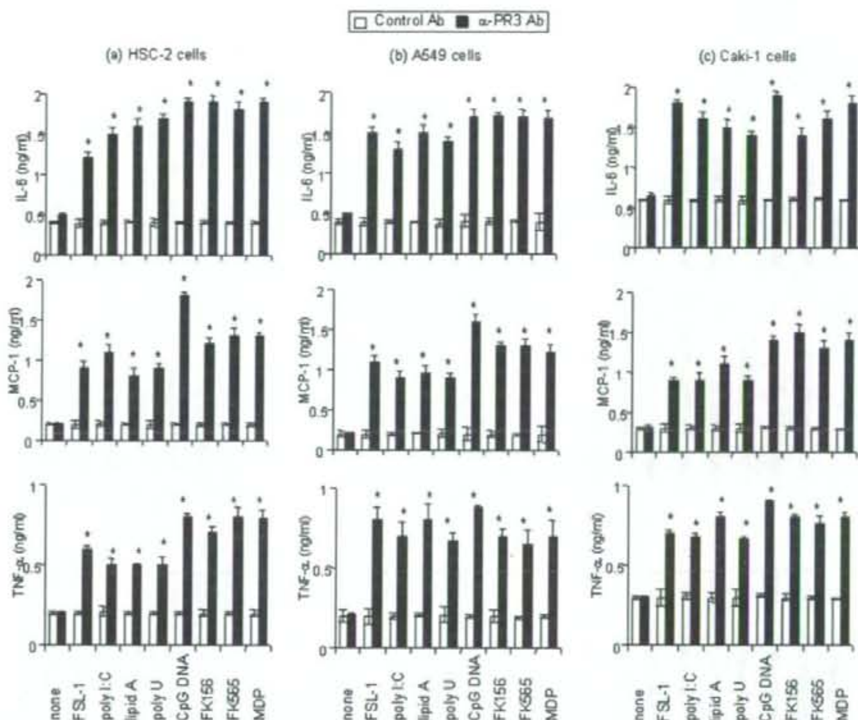

FIG. 3. Human oral, lung, and kidney epithelial cells preincubated with anti-PR3 Abs secreted IL-6, MCP-1, and TNF-α in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with anti-PR3 Abs (1 μg/ml) or with an equal amount of an isotype-matched IgG Ab. Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-6, MCP-1, and TNF-α in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± SD. *, significantly different from results for cells in the respective cultures incubated with control IgG ($P < 0.01$). The results presented are representative of three different experiments demonstrating similar results.

Clear priming effects of PAR-2AP on IL-8 production by epithelial cells in response to TLR and NOD agonist PAMPs were observed (Fig. 5).

The priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner. To clarify the signaling pathway of the priming effects of anti-PR3 Abs upon stimulation with TLR and NOD agonist PAMPs, we utilized RNA interference assays targeting PAR-2 and NF-κB p65. PAR-2 and NF-κB p65 protein levels determined by flow cytometry were suppressed by ca. 80% by specific siRNAs in the cells for up to 72 h (28, 30). As shown in Fig. 6, the priming effects induced by anti-PR3 Abs were almost completely inhibited in cells in which PAR-2 and NF-κB were suppressed but not in cells in which lamin was suppressed. These results demonstrated that the priming effects of anti-PR3 Abs occurred in a PAR-2- and NF-κB-dependent manner.

DISCUSSION

Among ANCA, those targeting PR3 (PR3 ANCA) have a strong and specific association with WG (27, 32). Besides their significance as seromarkers, a pathogenic role has been proposed for these auto-Abs in relation to their capacity to activate leukocytes in vitro (4, 10, 13, 15, 22, 23, 25, 26). We previously reported that incubation with anti-PR3 Abs significantly upregulates the production of proinflammatory cytokines upon stimulation with various PAMPs (30). The first signs and symptoms in WG patients are in the oral cavity, lungs, and kidneys. In the present study, an alternative approach was chosen to define the priming effects of PR3 ANCA from WG patients on human oral, lung, and kidney epithelial cells; the epithelial cells were preincubated with substimulatory concentrations of human PR3 ANCA or murine anti-PR3 Abs, and the possible activation of the cells by various TLR and NOD agonist PAMPs was examined. Without preincubation, the epithelial cells generally did not secrete proinflammatory cytokines (Fig. 1). Surprisingly, preincubation with murine anti-PR3 Abs primed them to secrete proinflammatory cytokines (Fig. 2 and 3). In addition, similar to murine anti-PR3 Abs, PR3 ANCA-containing sera from WG patients, but not control serum, primed the cells to secrete proinflammatory cytokines (Fig. 4). These results indicated that PR3 ANCA in the sera from WG patients exerted a clear priming effect similar to that of murine anti-PR3 Abs in vitro and strengthen our

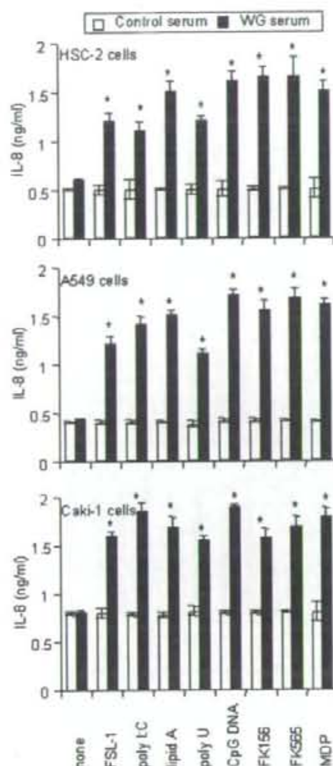

FIG. 4. Human oral, lung, and kidney epithelial cells preincubated with PR3 ANCA-containing sera secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with 1:100 dilutions of PR3 ANCA-containing sera from WG patients or with equal amounts of normal serum. Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± SD. *, significantly different from results for cells in the respective cultures incubated with normal serum ($P < 0.01$). The results presented are representative of three different experiments demonstrating similar results.

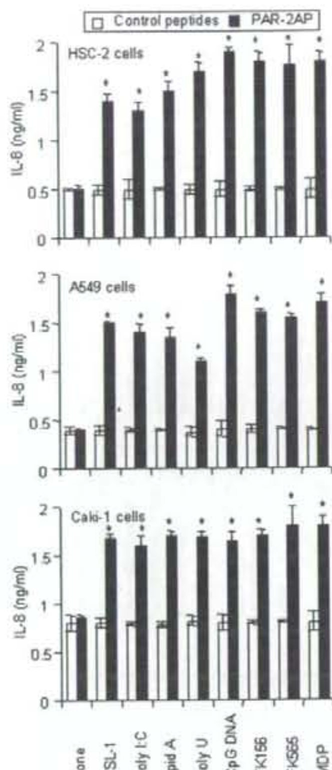

FIG. 5. Human oral, lung, and kidney epithelial cells preincubated with PAR-2AP secreted IL-8 in response to synthetic PAMPs. Oral epithelial HSC-2, lung epithelial A549, and kidney epithelial Caki-1 cells were preincubated for 6 h with PAR-2AP (10 nM) or with an equal amount of control peptides. Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined by ELISAs. Data are expressed as mean values ± SD. *, significantly different from results for cells in the respective cultures incubated with control peptides ($P < 0.01$). The results presented are representative of three different experiments demonstrating similar results.

conclusion and the relevance of our study to the human situation.

Concerning signaling pathways, anti-PR3 Abs activated human cells via PAR-2 and NF-κB in a TLR- and NOD-dependent manner (30, 31). PAR-2AP had a priming effect with potency similar to that of anti-PR3 Abs (Fig. 5), and the effects of anti-PR3 Abs also occurred through PAR-2 and NF-κB in oral, lung, and kidney epithelial cells (Fig. 6). We used solely chemically synthesized PAMPs, because natural microbial components are inevitably contaminated with minor bioactive components that might have affected the results. Therefore, these results clearly indicated that anti-PR3 Abs primed human epithelial cells for TLR- and NOD-dependent cell activation.

It is conceivable that microbial components (PAMPs) exhibit powerful immunoadjuvant activities against various antigens, including autoantigens, through TLR and NOD pathways, which in turn may induce severe autoimmune diseases. In the important roles of PR3 ANCA in the regulation of inflammatory leukocyte functions, the ANCA, being only weak direct activators of monocytes and neutrophils to release cytokines per se, exert a definite priming effect on these leukocytes, enhancing their responsiveness to secondary stimulation with PAMPs (30). In this study, we first reported that oral, lung, and kidney epithelial cells were primed to secrete proinflammatory cytokines by anti-PR3 Abs upon stimulation with PAMPs whereas these epithelial cells normally did not produce proinflammatory cytokines in response to PAMPs. The first signs and symptoms in WG patients are severe inflammation in the

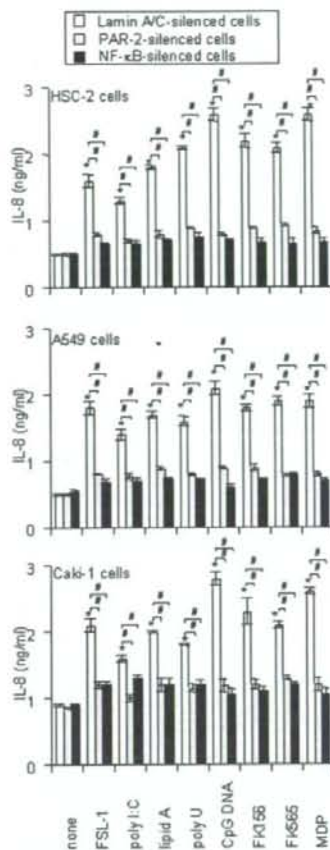

FIG. 6. The priming effect of anti-PR3 Abs occurred in a PAR-2- and NF-κB p65-dependent manner. Oral epithelial HSC-2, lung A549, and kidney Caki-1 cells were transfected with PAR-2-, NF-κB p65-, or lamin A/C-specific siRNA. After 24 h, the transfected cells were preincubated for 6 h with anti-PR3 Abs (1 μg/ml). Subsequently, the cells were stimulated with FSL-1 (1 nM), poly(I-C) (10 μg/ml), lipid A (10 ng/ml), poly(U) (10 μg/ml), CpG DNA (10 nM), FK156 (100 μg/ml), FK565 (100 μg/ml), or MDP (100 μg/ml) for 24 h in triplicate. The levels of IL-8 in the culture supernatants were determined by an ELISA. Data are expressed as mean values ± SD. * and #, values differed significantly from those obtained with medium alone and from those obtained for respective cultures stimulated with the indicated ligands, respectively ($P < 0.01$). The results presented are representative of three different experiments demonstrating similar results.

oral cavity, lungs, and kidneys. Such cooperation between PR3 ANCA and PAMPs may well trigger exacerbations of WG during infections and contribute to the persistence of inflammatory lesions, which may be a novel model for the pathogenesis of WG.

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REFERENCES

1. **Aderem, A., and R. J. Ulevitch.** 2000. Toll-like receptors in the induction of the innate immune response. Nature **406:**782–787.
2. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783–801.
3. **Bartůňková, J., V. Tesař, and A. Šedivá.** 2003. Diagnostic and pathogenetic role of antineutrophil cytoplasmic autoantibodies. Clin. Immunol. **106:** 73–82.
4. **Casselman, B. L., K. S. Kilgore, B. F. Miller, and J. S. Warren.** 1995. Antibodies to neutrophil cytoplasmic antigens induce monocyte chemoattractant protein-1 secretion from human monocytes. J. Lab. Clin. Med. **126:**495–502.
5. **Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nuñez, and N. Inohara.** 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. **4:**702–707.
6. **Coughlin, S. R.** 2000. Thrombin signalling and protease-activated receptors. Nature **407:**258–264.
7. **Davies, D. J., J. E. Moran, J. F. Niall, and G. B. Ryan.** 1982. Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology? Br. Med. J. (clinical research ed.) **285:**606.
8. **Déry, O., C. U. Corvera, M. Steinhoff, and N. W. Bunnett.** 1998. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am. J. Physiol. **274:**C1429–C1452.
9. **Falk, R. J., and J. C. Jennette.** 1988. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N. Engl. J. Med. **318:**1651–1657.
10. **Falk, R. J., R. S. Terrell, L. A. Charles, and J. C. Jennette.** 1990. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. Proc. Natl. Acad. Sci. USA **87:**4115–4119.
11. **Girardin, S. E., I. G. Boneca, L. A. M. Carneiro, A. Antignac, M. Jéhanno, J. Viala, K. Tedin, M.-K. Taha, A. Labigne, U. Zähringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti, and D. J. Philpott.** 2003. Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Science **300:**1584–1587.
12. **Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem. **278:**8869–8872.
13. **Grimminger, F., K. Hattar, C. Papavassilis, B. Temmesfeld, E. Csernok, W. L. Gross, W. Seeger, and U. Sibelius.** 1996. Neutrophil activation by anti-proteinase 3 antibodies in Wegener's granulomatosis: role of exogenous arachidonic acid and leukotriene B4 generation. J. Exp. Med. **184:**1567–1572.
14. **Hall, J. B., B. M. Wadham, C. J. Wood, V. Ashton, and W. R. Adam.** 1984. Vasculitis and glomerulonephritis: a subgroup with an antineutrophil cytoplasmic antibody. Aust. N. Z. J. Med. **14:**277–278.
15. **Hattar, K., A. Bickenbach, E. Csernok, S. Rosseau, U. Grandel, W. Seeger, F. Grimminger, and U. Sibelius.** 2002. Wegener's granulomatosis: antiproteinase 3 antibodies induce monocyte cytokine and prostanoid release—role of autocrine cell activation. J. Leukoc. Biol. **71:**996–1004.
16. **Hattar, K., U. Grandel, A. Bickenbach, A. Schwarting, W.-J. Mayet, J. Bux, S. Jessen, C. Fischer, W. Seeger, F. Grimminger, and U. Sibelius.** 2002. Interaction of antibodies to proteinase 3 (classic anti-neutrophil cytoplasmic antibody) with human renal tubular epithelial cells: impact on signaling events and inflammatory mediator generation. J. Immunol. **168:**3057–3064.
17. **Hattar, K., S. van Bürck, A. Bickenbach, U. Grandel, U. Maus, J. Lohmeyer, E. Csernok, T. Hartung, W. Seeger, F. Grimminger, and U. Sibelius.** 2005. Anti-proteinase 3 antibodies (c-ANCA) prime CD14-dependent leukocyte activation. J. Leukoc. Biol. **78:**992–1000.
18. **Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, Jr., and R. A. B. Ezekowitz.** 1999. Phylogenetic perspectives in innate immunity. Science **284:**1313–1318.
19. **Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S. J. Foster, A. P. Moran, J. L. Fernandez-Luna, and G. Nuñez.** 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J. Biol. Chem. **278:**5509–5512.
20. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. Annu. Rev. Immunol. **20:**197–216.
21. **Jenne, D. E., J. Tschopp, J. Lüdemann, B. Utecht, and W. L. Gross.** 1990. Wegener's autoantigen decoded. Nature **346:**520.
22. **Muller Kobold, A. C., Y. M. van der Geld, P. C. Limburg, J. W. C. Tervaert, and C. G. M. Kallenberg.** 1999. Pathophysiology of ANCA-associated glomerulonephritis. Nephrol. Dial. Transplant. **14:**1366–1375.
23. **Novo, E., E. G.-M. Gregor, S. Nava, and L. Perini.** 1997. A possible defective estimation of antineutrophil cytoplasmic antibodies in systemic lupus ery-

thematosus due to the coexistence of periodontitis: preliminary observations. P. R. Health Sci. J. **16:**369–373.
24. **O'Brien, P. J., M. Molino, M. Kahn, and L. F. Brass.** 2001. Protease activated receptors: theme and variations. Oncogene **20:**1570–1581.
25. **Ralston, D. R., C. B. Marsh, M. P. Lowe, and M. D. Wewers.** 1997. Antineutrophil cytoplasmic antibodies induce monocyte IL-8 release. Role of surface proteinase-3, α1-antitrypsin, and Fcγ receptors. J. Clin. Investig. **100:**1416–1424.
26. **Savage, C. O. S., B. E. Pottinger, G. Gaskin, C. D. Pusey, and J. D. Pearson.** 1992. Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. Am. J. Pathol. **141:**335–342.
27. **Tervaert, J. W., F. J. van der Woude, A. S. Fauci, J. L. Ambrus, J. Velosa, W. F. Keane, S. Meijer, M. van der Giessen, G. K. van der Hem, T. H. The, et al.** 1989. Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch. Intern. Med. **149:**2461–2465.
28. **Uehara, A., Y. Fujimoto, K. Fukase, and H. Takada.** 2007. Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. Mol. Immunol. **44:**3100–3111.
29. **Uehara, A., Y. Fujimoto, A. Kawasaki, S. Kusumoto, K. Fukase, and H. Takada.** 2006. *Meso*-diaminopimelic acid and *meso*-lanthionine, amino acids specific to bacterial peptidoglycans, activate human epithelial cells through NOD1. J. Immunol. **177:**1796–1804.
30. **Uehara, A., A. Iwashiro, T. Sato, S. Yokota, and H. Takada.** 2007. Antibodies to proteinase 3 prime human monocytic cells via protease-activated receptor-2 and NF-κB for Toll-like receptor- and NOD-dependent activation. Mol. Immunol. **44:**3552–3562.
31. **Uehara, A., Y. Sugawara, T. Sasano, H. Takada, and S. Sugawara.** 2004. Proinflammatory cytokines induce proteinase 3 as membrane-bound and secretory forms in human oral epithelial cells and antibodies to proteinase 3 activate the cells through protease-activated receptor-2. J. Immunol. **173:** 4179–4189.
32. **van der Woude, F. J., N. Rasmussen, S. Lobatto, A. Wiik, H. Permin, L. A. van Es, M. van der Giessen, G. K. van der Hem, and T. H. The.** 1985. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet **1:**425–429.
33. **Wiik, A., E. Jensen, and J. Friis.** 1974. Granulocyte-specific antinuclear factors in synovial fluids and sera from patients with rheumatoid arthritis. Ann. Rheum. Dis. **33:**515–522.

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Autophagic control of listeria through intracellular innate immune recognition in drosophila

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Autophagy, an evolutionally conserved homeostatic process for catabolizing cytoplasmic components, has been linked to the elimination of intracellular pathogens during mammalian innate immune responses. However, the mechanisms underlying cytoplasmic infection-induced autophagy and the function of autophagy in host survival after infection with intracellular pathogens remain unknown. Here we report that in drosophila, recognition of diaminopimelic acid–type peptidoglycan by the pattern-recognition receptor PGRP-LE was crucial for the induction of autophagy and that autophagy prevented the intracellular growth of *Listeria monocytogenes* and promoted host survival after this infection. Autophagy induction occurred independently of the Toll and IMD innate signaling pathways. Our findings define a pathway leading from the intracellular pattern-recognition receptors to the induction of autophagy to host defense.

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The innate immune system is a powerful and evolutionally well conserved barrier to infectious pathogens. In drosophila, which rely almost entirely on innate immunity to fight microbial infection, members of the peptidoglycan-recognition protein (PGRP) family act as microbe sensors[1,2]. These receptors are found in the hemolymph, on immune cell surfaces and in the immune cells, and they recognize bacterial peptidoglycans (PGNs) and activate immune signaling pathways such as the Toll and IMD pathways. These pathways control the production of antimicrobial peptides (AMPs) through NF-κB transcription factors[1,2]. PGRP-LE, a drosophila PGRP family member present both in the hemolymph and inside immune cells[3], binds to diaminopimelic acid (DAP)-type PGN[4] and is sufficient for the induction of genes encoding AMPs after immune stimulation with tracheal cytotoxin (TCT), a monomeric DAP-type PGN[5]. AMPs are effective against the growth of bacteria or fungi in the hemolymph[6] and perhaps in phagosomal compartments as well, but cytosolic pathogens escape these humoral defenses. Mammalian cells also express intracellular receptors that detect intracellular pathogens or their products[7]. For example, the receptors Nod1 and Nod2 sense substructures from bacterial PGN in the cell cytoplasm and activate innate immune signaling pathways[8,9]. CARD9 is an adaptor protein that associates with Nod2 and elicits innate immune responses critical for protecting mice from *Listeria monocytogenes* infection[10]. The immune responses activated by these receptors to defend against intracellular bacterial growth are not apparent, however, in either mammals or insects.

Autophagy is a highly conserved cellular mechanism in which cytoplasmic components are sequestered into double-membrane structures called autophagosomes and are eventually degraded in lysosomes[11]. Autophagy is involved in diverse functions, including the removal of damaged organelles, protein turnover, the supply of nutrients in nutrient-deprived conditions, and cell survival and death[12]. In mammalian cells, autophagy is also involved in innate immune defenses against invading pathogens, such as group A streptococcus, *Shigella flexneri*, *Mycobacterium tuberculosis* and *Toxoplasma gondii*[13–17]. Group A streptococcus is an extracellular bacteria that can also invade the host cell cytoplasm, where it is rapidly sequestered into autophagosomes associated with microtubule-associated protein 1 light chain 3 (LC3; also called Atg8), which results in its degradation in the vacuole after fusion with lysosomes[13]. *M. tuberculosis* resides in phagosomes, where it interferes with phagosomal maturation and decreases the acidification of the bacteria-containing vacuole. The activation of *M. tuberculosis*–infected macrophages by interferon-γ induces autophagy; this autophagy is induced by a mechanism dependent on the immunity-related GTPase family member IRGM (also called p47 GTPase) and inhibits bacterial survival[14,18]. Although such reports have suggested that some intracellular pathogens induce autophagy and are controlled by autophagy in cultured cells, they have shown only limited effects on pathogen survival. Moreover, the mechanism(s) and sensor(s) mediating the induction of these autophagic antibacterial responses remain to be elucidated.

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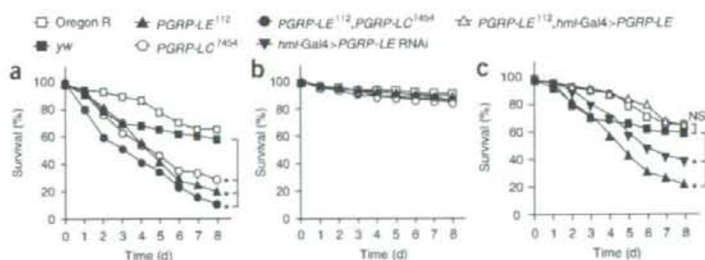

Figure 1 PGRP-LE in hemocytes is essential for resistance to *L. monocytogenes* infection *in vivo*. (**a**,**b**) Survival of wild-type (Oregon R), *yw*, *PGRP-LE*^{112}, *PGRP-LC*^{7454} and *PGRP-LE*^{112},*PGRP-LC*^{7454} flies after injection of wild-type *L. monocytogenes* (**a**) or Δ*hly L. monocytogenes* (**b**) at 28 °C. (**c**) Survival of wild-type, *yw* and *PGRP-LE*^{112} flies, as well as flies treated with RNAi targeting *PGRP-LE* with the *hml*-Gal4 driver (*hml*-Gal4 > *PGRP-LE* RNAi) and *PGRP-LE*^{112} flies with *hml*-Gal4–driven PGRP-LE expression (*PGRP-LE*^{112},*hml*-Gal4 > *PGRP-LE*), after injection of wild-type *L. monocytogenes* at 28 °C. NS, not significant; *, $P < 0.01$ (Wilcoxon-Mann-Whitney test): $P = 0.0006$, 0.0043 or 0.0043, for *yw* versus *PGRP-LE*^{112}, *PGRP-LC*^{7454} or *PGRP-LE*^{112},*PGRP-LC*^{7454}, respectively (**a**); $P = 0.0095$, for *yw* versus RNAi targeting *PGRP-LE* (**c**). Data represent the average of four independent experiments with over 30 flies of each genotype examined at the same time.

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Studies also suggest that Toll-like receptor (TLR) signaling can promote autophagy. TLR4 stimulation promotes the localization of mycobacterial phagosomes together with autophagosomes[19], but this is unlikely to be relevant during infection because *M. tuberculosis* does not express any TLR4 ligands. Many other TLR ligands also induce autophagy in cultured macrophage cell lines or mouse primary macrophages[20]. It is not yet apparent, however, if and how *M. tuberculosis* infection induces autophagy. Moreover, it is not yet known if autophagy is important in protecting the whole animal from lethal infection by *M. tuberculosis*, group A streptococcus or other intracellular infections. The idea of a link between the autophagy pathway and TLR signaling is further supported by a report that phagocytosis of TLR agonist–coated beads promotes phagosome maturation by recruiting elements of the autophagy pathway to the phagosome[21]. Localized TLR signaling in the phagosome is therefore probably important for the recruitment of autophagic components.

The cytoplasmic sensors that recognize bacteria invading the cytoplasm and trigger autophagy remain to be identified. Here we demonstrate that in drosophila, PGRP-LE recognized cytosolic *L. monocytogenes* and was essential for inducing autophagy, which inhibits intracellular growth of the bacteria and is necessary for host survival after *L. monocytogenes* infection. In addition, the induction of autophagy after detection of the bacteria through PGRP-LE was independent of the Toll and IMD pathways, which suggests the existence of a distinct innate immune pathway responsible for the induction of autophagy by a cytoplasmic sensor.

RESULTS

PGRP-LE in resistance to *L. monocytogenes* infection

In drosophila, PGRP-LE functions as an intracellular receptor for TCT, a monomeric DAP-type PGN[5]. That finding led us to determine whether PGRP-LE is essential for the recognition of intracellular pathogens. We first analyzed the resistance of the PGRP-LE-null mutant *PGRP-LE*^{112} to infection by *L. monocytogenes*, an intracellular Gram-positive bacteria that is pathogenic to mammals and drosophila and that expresses DAP-containing PGN, which is potentially recognized by PGRP-LE[4]. As indicated by survival experiments, the *PGRP-LE*^{112} mutant was hypersusceptible to low-dose *L. monocytogenes* infection relative to wild-type flies (Oregon R) or *yw* flies (wild-type control flies with the same genetic background as the *PGRP-LE*^{112} mutant; **Fig. 1a**). Consistent with a published report demonstrating that IMD pathway mutants are susceptible to *L. monocytogenes* infection[22], *PGRP-LC*^{7454} mutants (which have very low expression of PGRP-LC, membrane-associated cell surface sensor required for the activation of the IMD pathway) were also susceptible to *L. monocytogenes* infection. The survival rate of *PGRP-LE*^{112},*PGRP-LC*^{7454} double mutants after *L. monocytogenes* infection was similar to that of the *PGRP-LE*^{112} and *PGRP-LC*^{7454} single mutants, which suggested that PGRP-LE and PGRP-LC do not have redundant functions in producing resistance to *L. monocytogenes*. *L. monocytogenes* requires listeriolysin O to lyse the host phagocytic vacuole and access the cytosol[23]. The Δ*hly* strain of *L. monocytogenes*, with deletion of the gene encoding listeriolysin O, can enter host cells by phagocytosis but is incapable of entering the cytoplasm[24]. *PGRP-LE*^{112} mutants were not susceptible to this strain, consistent with the idea that cytosolic recognition requires PGRP-LE (**Fig. 1b**).

L. monocytogenes infects phagocytic cells such as macrophages in mammals and hemocytes in drosophila[22,23]. PGRP-LE is expressed in hemocytes[3]. *PGRP-LE*^{112} mutants that expressed PGRP-LE specifically in hemocytes with the hemocyte-specific 'driver' *hemolectin*-Gal4 (*hml*-Gal4)[25] had a susceptibility to *L. monocytogenes* similar to that of wild-type flies (**Fig. 1c**), which demonstrated that *PGRP-LE* expression in hemocytes is important for resistance against *L. monocytogenes* infection. Consistent with that finding, RNA-mediated interference (RNAi) targeting *PGRP-LE* with the *hml*-Gal4 driver induced susceptibility to *L. monocytogenes* infection (**Fig. 1c**). In these conditions, *hml*-Gal4–driven RNAi efficiently decreased *PGRP-LE* expression in the hemocytes (**Supplementary Fig. 1** online) but did not decrease *PGRP-LE* expression in the fat body or gut (data not shown).

Next we determined if PGRP-LE was involved in suppressing bacterial growth *in vivo*. In wild-type flies, the growth of *L. monocytogenes* was relatively suppressed in both the humoral and the cellular fractions, whereas in the *PGRP-LE*^{112} mutant flies, *L. monocytogenes* growth in the cellular fraction began immediately after infection, and bacterial growth in the humoral fraction began to increase 6 h later (**Supplementary Fig. 2** online). In contrast to growth in the *PGRP-LE*^{112} mutant, bacterial growth in the humoral fraction of the *PGRP-LC*^{7454} mutant began to increase immediately after infection, but bacterial growth in the cellular fraction of the *PGRP-LC*^{7454} mutant was indistinguishable from that in wild-type flies (**Supplementary Fig. 2**). These data suggest that PGRP-LE is essential for suppressing the growth of *L. monocytogenes* in cells *in vivo*, whereas PGRP-LC protects the extracellular environment from these microbes.

PGRP-LE and autophagy suppress *L. monocytogenes* growth

To directly demonstrate the requirement for PGRP-LE in limiting the intracellular growth of *L. monocytogenes*, we cultured hemocytes from drosophila larvae *ex vivo*, infected them for 1 h with *L. monocytogenes*

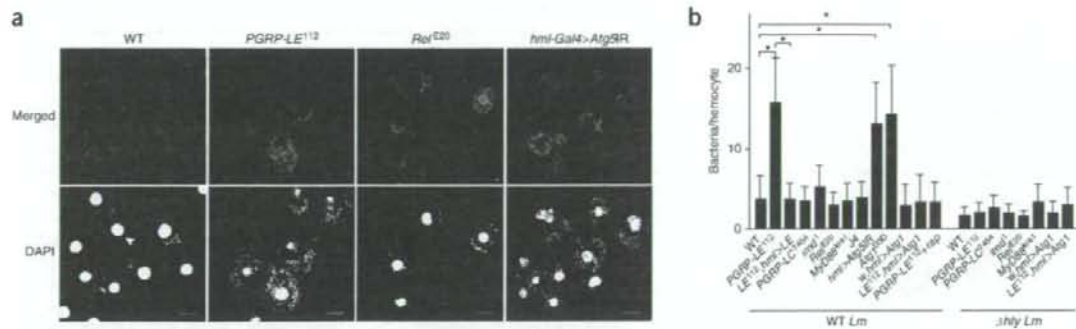

Figure 2 PGRP-LE and autophagy, but not the Toll and IMD pathways, are needed to suppress the intracellular growth of *L. monocytogenes* in hemocytes. **(a)** Microscopy of hemocytes from third instar larvae cultured *ex vivo* and infected with wild-type *L. monocytogenes*. Hemocyte nuclei (filled arrowhead) and DNA of *L. monocytogenes* (open arrowhead) are visualized by DAPI staining (blue or white); the actin cytoskeleton is stained with rhodamine-labeled phalloidin (red). *hml*-Gal4 > *Atg5*IR, expression of an inverted repeat sequence of *Atg5* to induce RNAi against *Atg5*. Scale bars, 10 μm. **(b)** Intracellular wild-type *L. monocytogenes* (WT *Lm*) or Δ*hly L. monocytogenes* (Δ*hly Lm*) counted manually in DAPI-stained infected hemocytes. LE^{112},*hml*> *LE*, *hml*-Gal4–driven expression of *PGRP-LE* in *PGRP-LE*^{112} hemocytes; *imd*^{1}, *imd*; *Rel*^{E20}, *Relish*^{E20}; *hml*> *Atg5*IR, inverted repeat sequence described in **a**; *w*, *hml*> *Atg1*, *hml*-Gal4–driven expression of *Atg1* in *w* hemocytes; LE^{112},*hml*> *Atg1*, *hml*-Gal4–driven expression of *Atg1* in *PGRP-LE*^{112} hemocytes; *PGRP-LE*^{112}+*rap*, *PGRP-LE*^{112} hemocytes plus rapamycin. *, $P < 0.001$, compared with wild type (*t*-test). Data are representative of four experiments (**a**) or at least three independent experiments (**b**; error bars, s.d. of triplicate measurements).

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and then incubated them for 5 h in gentamicin-containing medium to kill the extracellular bacteria. We visualized infection of hemocytes by *L. monocytogenes* by staining with the DNA-intercalating dye DAPI (4,6-diamido-2-phenylinodole; **Fig. 2a**) and quantified the bacteria per cell (**Fig. 2b**). Wild-type but not Δ*hly L. monocytogenes* produced an actin 'comet' in hemocytes (**Supplementary Fig. 3** online), which suggested that wild-type *L. monocytogenes* invaded the cytoplasm of the *ex vivo*–cultured hemocytes. That is consistent with a published report that *L. monocytogenes* associates with actin in hemocytes derived from infected larvae[22]. The number of wild-type *L. monocytogenes* in the *PGRP-LE*^{112} mutant hemocytes was significantly greater than that in wild-type hemocytes (**Fig. 2**). The number of Δ*hly L. monocytogenes* growing in *PGRP-LE*^{112} hemocytes was similar to that in wild-type hemocytes (**Fig. 2b**). These results suggest that PGRP-LE recognizes *L. monocytogenes* only after it enters the hemocyte cytoplasm and that the phagocytic activity required for entry of the bacteria was not affected in the *PGRP-LE*^{112} mutant. PGRP-LE expression induced by the *hml*-Gal4 driver in *PGRP-LE*^{112} hemocytes restored the growth suppression of the wild-type bacteria (**Fig. 2b**). These results indicate that PGRP-LE acts in a cell-autonomous way in suppressing the cytosolic growth of the bacteria. In contrast to *PRGP-LE*^{112} mutant hemocytes, those from strains with mutant Imd, the critical IMD pathway adaptor, or Relish, the key NF-κB transcription factor of the pathway, did not show impaired control of intracellular listeria growth (**Fig. 2a,b**). The control of intracellular bacterial growth was also not affected in hemocytes from larvae of *PGRP-LC*^{7454} flies, *MyD88*^{kra1} flies (mutant adaptor of the Toll pathway) or J4-homozygous mutant flies (lacking both *Dif* and *dorsal* (*dl*), which encode key transcription factors in the Toll pathway; **Fig. 2b**). These results demonstrated that neither the two classical innate immune signaling pathways nor the cell surface receptor for DAP-type PGN were needed to control the intracellular growth of *L. monocytogenes*; in contrast, the intracellular PGN receptor PGRP-LE was crucial. These results indicate that some mechanism other than AMP production is essential for defending against cytosolic *L. monocytogenes*.

Studies have reported that autophagy functions in innate immune responses against intracellular pathogens in mammalian cells[13–18]. As AMPs do not seem necessary to control intracellular *L. monocytogenes*, we investigated whether autophagy is required for the inhibition of *L. monocytogenes* growth in hemocytes by expressing an RNAi transgene targeting drosophila *Atg5* (CG1643), which encodes a factor essential for autophagy[26,27], with an *hml*-Gal4 driver; this efficiently decreased *Atg5* expression in hemocytes (**Supplementary Fig. 1**). *Ex vivo*–cultured hemocytes treated with *Atg5* RNAi had more bacteria (**Fig. 2**). The number of bacteria was similarly higher in hemocytes from the *Atg1*$^{\Delta3D}$ mutant (**Fig. 2b**), in which starvation-induced autophagy is completely abolished[26]. Consistent with a report showing that autophagy limits the intracellular growth of *L. monocytogenes* in mouse embryonic fibroblast cells[28], our results indicate an essential function for autophagy in the defense against *L. monocytogenes* infection. Moreover, the induction of autophagy by rapamycin treatment[29] or by forced expression of *Atg1* (ref. 30) with the *hml*-Gal4 driver decreased the number of bacteria in *PGRP-LE*^{112} cells (**Fig. 2b**), which suggests that *PGRP-LE* acts 'upstream' of autophagy in the resistance to *L. monocytogenes* infection.

We confirmed the involvement of autophagy in hemocytes during innate immune responses against *L. monocytogenes in vivo* by examining survival rates of flies in which the RNAi transgene targeting *Atg5* was expressed in hemocytes. These '*Atg5* RNAi' flies were susceptible to infection with wild-type *L. monocytogenes*, similar to *PGRP-LE*^{112} mutants, whereas they were totally resistant to the Δ*hly* strain of *L. monocytogenes* (**Fig. 3a,b**). In contrast, the susceptibility of the *Atg5* RNAi line and *PGRP-LE*^{112} mutants to *Erwinia carotovora*, an extracellular Gram-negative bacteria, was not different from that of the control flies (**Fig. 3c**). The *PGRP-LC*^{7454} mutant was more susceptible to *E. carotovora*. This is the first demonstration to our knowledge that autophagy is crucial for host survival after infection with intracellular pathogens.

Drosophila S2 cells are macrophage-like cells and are an excellent model for studying intracellular infection, especially *L. monocytogenes* infection[31,32]. S2 cells had no detectable expression of PGRP-LE