

NOD1 and NOD2 Mediate Sensing of Periodontal Pathogens

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ABSTRACT

In bacterial infection, Nucleotide-binding Oligomerization Domain (NOD) 1 and NOD2 induce innate immune responses by recognizing fragments of the bacterial component peptidoglycan (PGN). To determine the roles of these receptors in detection of periodontal pathogens, human embryo kidney cells expressing NOD1 or NOD2 were stimulated with heat-killed *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* or their soluble PGNs (sPGNs). All bacteria and their sPGNs could stimulate activation of NF- κ B. However, there were differences in NOD1- and NOD2-stimulatory activities among the species of bacteria. *P. gingivalis* showed weaker NOD1- and NOD2-stimulatory activities than those of other bacteria. These differences in activities were confirmed by production of interleukin-8 from oral epithelial cells stimulated with sPGNs. These findings indicate that both NOD1 and NOD2 might be involved in the recognition of periodontal pathogens and that the weak NOD-stimulatory property of *P. gingivalis* might be helpful for survival in the periodontal pocket.

INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by periodontopathic bacteria and is characterized by inflammation of supportive tissue surrounding teeth. Gram-negative bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* have been reported to be associated with periodontitis (Nishihara and Koseki, 2004; Slots *et al.*, 1980; Socransky *et al.*, 1998).

Peptidoglycan (PGN) is a constituent of the cell membrane of bacteria and is one of the endotoxins that induce inflammatory responses such as the production of cytokines and inflammatory mediators (Stewart-Tull, 1980; Yoshimura *et al.*, 1999). PGN is a heteropolymer built of glycan strands cross-linked through peptide chains. The peptide chains are made of two kinds of short peptides, stem peptides that are linked to the glycan chain, and a cross bridge that links between the stem peptides. The glycan of PGN consists of repeating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), but there is a diversity in the composition and sequence of both the stem peptides and the cross bridge in PGN in bacterial species (Schleifer and Kandler, 1972). A notable difference is that the third amino acid of the stem peptide is *meso*-diaminopimelic acid (*meso*-DAP) in PGN of Gram-negative bacteria in contrast to L-Lys in that of Gram-positive bacteria. The number of amino acids of stem peptides ranges from two to five (mostly tetrapeptides).

Recently, Nucleotide-binding Oligomerization Domain (NOD) 1 and NOD2 were identified as cytosolic sensors for fragments of PGN. Nod1 recognizes gamma-D-glutamyl-*meso*-DAP, which is preferentially found in Gram-negative bacteria (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a), whereas Nod2 recognizes muramyl dipeptide (MDP), which is found in PGN of virtually all bacteria (Girardin *et al.*, 2003b). NOD1 and NOD2 have been reported to be involved in the recognition of infectious bacteria, the induction of immune responses, and the elimination of

bacteria through activation of NF- κ B (Kim *et al.*, 2008; Viala *et al.*, 2004).

NOD1 and NOD2 are expressed in oral and periodontal pocket epithelium (Sugawara *et al.*, 2006). Because of increased expression of NOD1 and NOD2 at the inflammatory gingival site, NOD1 and NOD2 are thought to play a role in the development of periodontitis. However, it has been reported that some periodontal pathogens such as *P. gingivalis* and *F. nucleatum* have L.L-DAP and *meso*-Lanthionine in PGN, respectively, instead of *meso*-DAP, which is essential for recognition by NOD1 (Barnard and Holt, 1985; Vasstrand *et al.*, 1979). Since NOD1 and NOD2-stimulatory activities of muopeptides are greatly affected by the kind of third amino acid as well as by the number of amino acids of stem peptides (Girardin *et al.*, 2003c; Magalhaes *et al.*, 2005), it is not clear whether a periodontal pathogen or its PGN are potent to stimulate NOD1 and NOD2. The aim of this study was to determine the roles of NOD1 and NOD2 in detection of periodontal pathogens.

MATERIALS & METHODS

Bacteria

The bacteria used in this study were *P. gingivalis* strains ATCC 33277, W83, TDC60, TDC117, TDC275, SU63 and GAI7802, *A. actinomycetemcomitans* Y4, *F. nucleatum* ATCC 10953, *Escherichia coli* MC4100, and *Aerococcus viridans* ATCC 10400. The cells were washed with sterilized distilled water and lyophilized.

Peptidoglycan Purification

PGN purification was done according to a past report (de Jonge *et al.*, 1992). Purified Gram-negative and Gram-positive insoluble PGNs were resuspended in PBS (2 mg/mL) and treated with mutanolysin (80 U/mL, Sigma, St. Louis, MO, USA) for 24 hr at 37°C for solubilization. Mutanolysin cleaves the bond between MurNAc and GlcNAc. After digestion, each sample was boiled for 10 min and centrifuged, and the supernatant was used as soluble PGN (sPGN) stock solution.

Measurement of NF- κ B Activation

Human embryo kidney (HEK) 293T cells were kindly provided by D. Golenbock (University of Massachusetts Medical School). The cells were seeded into 96-well plates and incubated in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). Then the cells were transfected for 24 hr with pUNO/vector, pUNO/hNod1 or pUNO/hNod2 plasmid (Invivogen, San Diego, CA, USA) plus NF- κ B-*firefly luciferase* and *renilla luciferase* reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activities were measured at 12 hr after stimulation with bacterial samples. The activity of firefly

luciferase was normalized to that of renilla luciferase. Each value was divided by the value for unstimulated cells that were transfected with pUNO/vector and represented as an NF- κ B fold activation. A-iE-DAP (AnaSpec, San Jose, CA, USA) and MDP (Bachem AG, Bubendorf, Switzerland) were used as ligands for NOD1 and NOD2, respectively. *Drosophila* S2* cells stably transfected with *dipteracin-luciferase* reporter plasmids were maintained in Schneider's drosophila medium (Gibco) supplemented with 10% FBS and 800 μ g/mL of G418 (Kaneko *et al.*, 2005). After differentiation into macrophage-like cells by incubating with 1 μ M 20-hydroxyecdysone for 24 hr, the cells were stimulated with bacterial samples for 4 hr and luciferase activities were measured.

Measurement of Interleukin-8 Production

Human oral epithelial cell line HSC-2 was provided by the RIKEN CELL BANK (Tsukuba, Japan) and the cells were maintained in MEM (Gibco) supplemented with 10% FBS. After treatment with 1,000 units/mL of interferon (IFN) - γ (Sigma) for 3 days, the cells were stimulated with sPGN samples in a serum-starved condition (no FBS) to increase the uptake of bacterial samples into the cells. The concentration of FBS in the medium was increased to 10% by adding FBS at 2 hr after stimulation, and the cells were further incubated for 22 hr. The interleukin-8 (IL-8) levels in culture medium were measured by an enzyme-linked immunosorbent assay (DuoSet Kit, R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

The data were analyzed with one-factor ANOVA and Fisher's PLSD test using StatView software (HULINKS, Tokyo, Japan). Probability level of $P < 0.05$ was considered to be significant.

RESULTS

We performed overexpression assays in HEK293T cells to explore functions of NODs because these cells are highly transfectable and lack most of the endogenous Toll-like receptors (TLRs) that mediate bacterial recognition and activation of NF- κ B. MDP could not stimulate NF- κ B activation in HEK/vector cells, but A-iE-DAP weakly stimulated the cells due to the expression of endogenous NOD1 (Fig. 1A and data not shown). However, strong NF- κ B activation was observed in HEK/NOD1 and HEK/NOD2 cells after stimulation with A-iE-DAP and MDP, respectively. These results indicated that expressed NOD1 and NOD2 functioned in the HEK293T cells and that this overexpression system is useful for monitoring cell response specific for the activation of NOD1 or NOD2.

Next, the cells were stimulated with heat-killed periodontopathic bacteria. *A.*

actinomycetemcomitans, *F. nucleatum* and *E. coli* weakly but significantly induced activation of NF- κ B in HEK/vector cells, but *P. gingivalis* and *A. viridans* failed to stimulate the cells (Fig. 1B). *A. actinomycetemcomitans*, *F. nucleatum* and *E. coli* strongly stimulated HEK/NOD1 cells in a dose-dependent manner, and significant activations were observed at concentrations of 10-100 μ g/mL (Fig. 1C). *P. gingivalis* also stimulated HEK/NOD1 cells, but its activity was much weaker than the activities of the other three Gram-negative bacteria. Gram-positive *A. viridans* could not activate NOD1. In the case of HEK/NOD2 cells, *A. actinomycetemcomitans* and *E. coli* activated NOD2 in a dose-dependent manner and those activities were statistically significant at concentrations of 10-100 μ g/mL. In contrast, *P. gingivalis*, *F. nucleatum*, *A. viridans* could not activate NOD2 significantly even at the maximum concentration of 100 μ g/mL (Fig. 1D).

Then we stimulated HEK/NOD1 and HEK/NOD2 cells with sPGNs. Before the experiment, we confirmed biological activities of purified sPGNs by measuring the expression of antimicrobial

peptide gene *dipteracin* in *Drosophila* S2* cells by a luciferase reporter assay. *Diptericin* is induced only by Gram-negative bacterial PGN stimulation in S2* cells (Kaneko *et al.*, 2004). All stock solutions of sPGNs obtained from Gram-negative bacteria showed almost the same activity against S2* cells at the dilution of 1/1000 (Fig. 2A). *A. viridans* sPGN could not induce the expression of *dipteracin*. As was found in experiments using heat-killed bacteria, HEK/NOD1 cells were strongly activated by stimulation with sPGNs from *A. actinomycetemcomitans*, *F. nucleatum* and *E. coli* (Fig. 2B). The sPGN from *P. gingivalis* could activate NOD1 significantly, but its activity level was 10-100-times lower than those of sPGNs from other Gram-negative bacteria. The sPGN from *A. viridans* could not activate NOD1. In contrast, all sPGNs activated NOD2 (Fig. 2C). *A. actinomycetemcomitans* and *A. viridans* showed the strongest NOD2-stimulatory activities, followed by *F. nucleatum* and *E. coli*, and *P. gingivalis* showed the weakest activity. The sPGNs from *F. nucleatum* and *P. gingivalis* could activate NOD2 only at the dilution of 1/100.

Both NOD1- and NOD2-stimulatory activities of *P. gingivalis* were weaker than those of other periodontal pathogens. Therefore, we investigated whether these weak activities were species-specific for *P. gingivalis*. HEK/NOD1 and HEK/NOD2 cells were stimulated with heat-killed *P. gingivalis* strains W83, TDC60, TDC117, TDC275, SU63 and GAI7802 in addition to ATCC 33277. Although all *P. gingivalis* strains exhibited weaker NOD1-stimulatory activity than the activity of *E. coli*, strains W83 and GAI7802 significantly activated NOD1 at the concentration of 10 µg/mL (Fig. 3A). Strains W83 and GAI7802 could activate NOD2 as strongly as *E. coli* could, but other strains did not show significant activity until the concentration was increased to 100 µg/mL (Fig. 3B).

To further address the role of NODs in induction of the inflammatory cytokine IL-8 in periodontal cells, HSC-2 cells were stimulated with sPGN. Before the stimulation, HSC-2 cells

were pretreated with IFN- γ for 3 days. IFN- γ treatment significantly increased the response to A-iE-DAP (Fig.4A). The sPGNs from *E. coli*, *A. actinomycetemcomitans* and *F. nucleatum* stimulated HSC-2 cells to induce IL-8 more strongly than did those from *P. gingivalis* and *A. viridans* (Fig. 4B).

DISCUSSION

PGN of *A. actinomycetemcomitans* has been reported to contain *meso*-DAP, which is typically observed in most Gram-negative bacteria such as *E. coli* (Barnard and Holt, 1985; Schleifer and Kandler, 1972). PGNS of *P. gingivalis* and *F. nucleatum* have been reported to contain L,L-DAP and *meso*-Lanthionine, respectively, instead of *meso*-DAP (Barnard and Holt, 1985; Vasstrand *et al.*, 1979). *A. viridans* has L-Lys-type PGN, which is typical for Gram-positive bacteria (Schleifer and Kandler, 1972). In the present study, there were differences in NOD1-stimulatory activities among the species of bacteria. *A. actinomycetemcomitans* showed the strongest activity, the same as that of *E. coli*, followed by *F. nucleatum*, and *P. gingivalis* showed the weakest activity among these Gram-negative bacteria. *A. viridans* did not activate NOD1. These results are consistent with the results of a previous study using synthetic muramyl tripeptides (MTP) for stimulation of HEK cells expressing NOD1 (Girardin *et al.*, 2003c). It was shown in that study that NOD1-stimulatory activity of L,L-DAP containing MTP (MTP_{L,L-DAP}) was significantly weaker than that of MTP_{*meso*-DAP} or MTP_{*meso*-Lanthionine}. Thus, it is likely that the NOD1-stimulatory activities of periodontal pathogens are greatly influenced by the kind of third amino acid of the stem peptide in their PGN.

All PGNS contain an MDP structure in the muopeptide; however, the NOD2-stimulatory activities differed among bacterial strains. *A. actinomycetemcomitans* showed the strongest activity, similar to that of *A. viridians*, followed by *E. coli* and *F. nucleatum*, and *P. gingivalis* showed the weakest activity. Girardin *et al.* reported that MTP_{L-Lys} could activate HEK cells expressing NOD2 as strongly as MDP could, but MTP_{*meso*-DAP}, MTP_{*meso*-Lanthionine} and MTP_{L,L-DAP} could not activate these cells (Girardin *et al.*, 2003c). These results suggested that MDP but not MTP is critical for NOD2-stimulatory activities in PGNS of Gram-negative bacteria. A study on

stem peptides in PGN of *E. coli* demonstrated that most of the stem peptides were tripeptides or tetrapeptides and that NOD2-stimulatory dipeptides accounted for only 2.1% of all stem peptides (Glauner *et al.*, 1988). The percentage of dipeptides in mucopeptides was reported to differ depending on the species or strains of bacteria (Antignac *et al.*, 2003). *E. coli* and *A. actinomycetemcomitans* might contain large amounts of dipeptides compared to *P. gingivalis*. The strong NOD2-stimulatory activities of *P. gingivalis* strains W83 and GAI7802 might be explained by this possibility. *P. gingivalis* has been classified into virulent and avirulent strains based on its ability to form necrotic abscesses in an animal model (Grenier and Mayrand, 1987). Inoculation of strain W83 in mice induced abscesses, secondary lesions, sepsis and death of animals with higher frequency than strain ATCC 33277 did (Neiders *et al.*, 1989). In the present study, strain W83 exhibited stronger NOD1- and NOD2-stimulatory activities than those of strain ATCC 33277. Further experiments may be necessary to demonstrate the relationship between NODs-stimulatory activities and the heterogeneity of virulence among *P. gingivalis* strains.

HSC-2 cells responded well to A-iE-DAP to produce IL-8 after IFN- γ treatment. The sPGNs from *A. actinomycetemcomitans* and *E. coli* induced IL-8 production in HSC-2 cells. *P. gingivalis* consistently induced a weaker response, but it should be noted that the differences between *P. gingivalis* and the other bacteria in IL-8 expression was not as great as the differences in NF- κ B activation.

Muramidase treatment was carried out to obtain sPGNs. Both NOD1 and NOD2-stimulatory activities of insoluble PGNs were increased after treatment with muramidase (data not shown). Lysozyme confers muramidase activity in saliva and plays a role in bacterial killing by destroying the bacterial cell wall. Therefore, lysozyme might facilitate recognition of bacteria by NOD1 and NOD2 in addition to bactericidal functions.

P. gingivalis has been reported to invade host epithelial cells as well as *A. actinomycetemcomitans* and *F. nucleatum* (Han *et al.*, 2000; Jandik *et al.*, 2008; Lamont *et al.*, 1995; Meyer *et al.*, 1991). A previous study revealed that intracellular recognition by NOD1 and NOD2 is critical for clearance of the intracellular bacterium *Listeria monocytogenes* (Kobayashi *et al.*, 2005). In the present study, we demonstrated that NOD1 and NOD2-stimulatory activities of *P. gingivalis* were weaker than those of other periodontal pathogens. In addition, *P. gingivalis* LPS has a unique structure of lipid A different from enterobacterial LPS (Ogawa, 1993) and acts as an antagonist for human lipid A receptor, TLR4 (Yoshimura *et al.*, 2002). These weak immunogenic properties of *P. gingivalis* might be one of the strategies of this bacterium for escape from innate immunity and survival in the periodontal pocket (Darveau *et al.*, 1998).

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REFERENCES

Antignac A, Rousselle JC, Namane A, Labigne A, Taha MK, Boneca IG (2003). Detailed structural analysis of the peptidoglycan of the human pathogen *Neisseria meningitidis*. *J Biol Chem* 278:31521-31528.

Barnard MR, Holt SC (1985). Isolation and characterization of the peptidoglycans from selected gram-positive and gram-negative periodontal pathogens. *Can J Microbiol* 31:154-160.

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.

Darveau RP, Belton CM, Reife RA, Lamont RJ (1998). Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. *Infect Immun* 66:1660-1665.

de Jonge BL, Chang YS, Gage D, Tomasz A (1992). Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. *J Biol Chem* 267:11248-11254.

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno 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.

Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, *et al.* (2003c). Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem* 278:41702-41708.

Glauner B, Holtje JV, Schwarz U (1988). The composition of the murein of *Escherichia coli*. *J Biol Chem* 263:10088-10095.

Grenier D, Mayrand D (1987). Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. *J Clin Microbiol* 25:738-740.

Han YW, Shi W, Huang GT, Kinder Haake S, Park NH, Kuramitsu H, *et al.* (2000). Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. *Infect Immun* 68:3140-3146.

Jandik KA, Belanger M, Low SL, Dorn BR, Yang MC, Progulsk-Fox A (2008). Invasive differences among *Porphyromonas gingivalis* strains from healthy and diseased periodontal sites. *J Periodontal Res* 43:524-530.

Kaneko T, Goldman WE, Mellroth P, Steiner H, Fukase K, Kusumoto S, *et al.* (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the

Drosophila IMD pathway. *Immunity* 20:637-649.

Kaneko T, Golenbock D, Silverman N (2005). Peptidoglycan recognition by the *Drosophila* Imd pathway. *J Endotoxin Res* 11:383-389.

Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Nunez G (2008). The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* 28:246-257.

Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, *et al.* (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307:731-734.

Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A (1995). *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 63:3878-3885.

Magalhaes JG, Philpott DJ, Nahori MA, Jehanno M, Fritz J, Le Bourhis L, *et al.* (2005). Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin. *EMBO Rep* 6:1201-1207.

Meyer DH, Sreenivasan PK, Fives-Taylor PM (1991). Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. *Infect Immun* 59:2719-2726.

Neiders ME, Chen PB, Suido H, Reynolds HS, Zambon JJ, Shlossman M, *et al.* (1989).

Heterogeneity of virulence among strains of *Bacteroides gingivalis*. *J Periodontal Res* 24:192-

198.

Nishihara T, Koseki T (2004). Microbial etiology of periodontitis. *Periodontol 2000* 36:14-26.

Ogawa T (1993). Chemical structure of lipid A from *Porphyromonas (Bacteroides) gingivalis* lipopolysaccharide. *FEBS Lett* 332:197-201.

Schleifer KH, Kandler O (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36:407-477.

Slots J, Reynolds HS, Genco RJ (1980). *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. *Infect Immun* 29:1013-1020.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134-144.

Stewart-Tull DE (1980). The immunological activities of bacterial peptidoglycans. *Annu Rev Microbiol* 34:311-340.

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.

Vasstrand EN, Hofstad T, Endresen C, Jensen HB (1979). Demonstration of lanthionine as a natural constituent of the peptidoglycan of *Fusobacterium nucleatum*. *Infect Immun* 25:775-780.

Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, *et al.* (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* *cag* pathogenicity island. *Nat Immunol* 5:1166-1174.

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D (1999). Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 163:1-5.

Yoshimura A, Kaneko T, Kato Y, Golenbock DT, Hara Y (2002). Lipopolysaccharides from periodontopathic bacteria *Porphyromonas gingivalis* and *Capnocytophaga ochracea* are antagonists for human toll-like receptor 4. *Infect Immun* 70:218-225.

Figure Legends

Fig. 1. Activation of NOD1 and NOD2 by stimulation with periodontopathic bacteria samples. HEK293T cells were transfected for 24 hr with pUNO/vector, pUNO/hNod1 or pUNO/hNod2 plasmid together with NF- κ B-*firefly luciferase* and *renilla luciferase* reporter plasmid. After 24 hr, the cells were stimulated with 1 μ g/mL of A-iE-DAP, 1 μ g/mL of MDP or heat-killed bacteria. Luciferase activities were measured 12 hr after stimulation and expressed as means \pm SD of triplicate experiments. The results are representative of 3 different experiments. *P. g.*, *P. gingivalis* ATCC 33277; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*; *A. v.*, *A. viridans*. $\dagger\dagger P < 0.01$ vs. control vector; $*P < 0.05$, $**P < 0.01$ vs. (-); $\#P < 0.05$, $##P < 0.01$ vs. *P. g.*

Fig. 2. Activations of NOD1 and NOD2 by sPGNs from periodontopathic bacteria. (A) *Diptericin* expression in *Drosophila* S2* cells by stimulation with purified sPGNs from periodontopathic bacteria. S2* cells stably transfected with *diptericin-luciferase* reporter plasmid were seeded into 96-well plates and differentiated into macrophage-like cells by incubating with 1 μ M 20-hydroxyecdysone for 24 hr. Then the cells were stimulated with sPGN samples and the luciferase activities were measured 4 hr after stimulation and expressed as means \pm SD of triplicate experiments. The results are representative of 3 different experiments. HEK293T cells were transfected for 24 hr with pUNO/hNod1 (B) or pUNO/hNod2 plasmid (C) together with NF- κ B-*firefly luciferase* and *renilla luciferase* reporter plasmid. After 24 hr, the cells were stimulated with sPGN samples. Then luciferase activities were measured 12 hr after stimulation and expressed as means \pm SD of triplicate experiments. The results are representative of 3 different experiments. *P. g.*, *P. gingivalis* ATCC 33277; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*; *A. v.*, *A. viridans*. $*P < 0.05$, $**P < 0.01$ vs. (-); $\#P < 0.05$, $##P < 0.01$ vs. *P. g.*

Fig. 3. Activations of NOD1 and NOD2 by strains of *P. gingivalis*. HEK293T cells were transfected for 24 hr with pUNO/hNod1 (A) or pUNO/hNod2 plasmid (B) together with NF- κ B-*firefly luciferase* and *renilla luciferase* reporter plasmid for 24 hr and the cells were stimulated with heat-killed *P. gingivalis* strains: W83, TDC60, TDC117, TDC275, SU63, GAI7802 and ATCC 33277. Luciferase activities were measured 12 hr after stimulation and expressed as means \pm SD of triplicate experiments. The results are representative of 3 different experiments. * P < 0.05, ** P < 0.01 vs. (-)

Fig. 4. Production of IL-8 in oral epithelial cells. (A) HSC-2 cells were stimulated with A-iE-DAP or MDP in a serum-starved condition after treatment with or without IFN- γ . At 2 hr after stimulation, the cell culture medium was supplemented with 10% FBS by adding FBS and further incubated for 22 hr. The levels of IL-8 in culture medium were measured. (B) IFN- γ -primed HSC-2 cells were stimulated with sPGNs in a serum-starved condition. At 2 hr after stimulation, the cell culture medium was supplemented with 10% FBS by adding FBS and further incubated for 22 hr. The levels of IL-8 in culture medium were measured. Data are expressed as means \pm SD of triplicate experiments. The results are representative of 3 different experiments. *P. g.*, *P. gingivalis* ATCC 33277; *A. a.*, *A. actinomycetemcomitans*; *F. n.*, *F. nucleatum*; *E. c.*, *E. coli*; *A. v.*, *A. viridans*. * P < 0.05, ** P < 0.01 vs. (-); # P < 0.05, ## P < 0.01 vs. *P. g.*

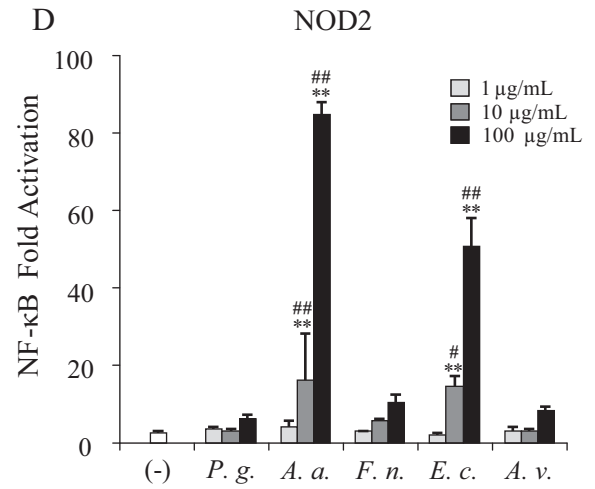
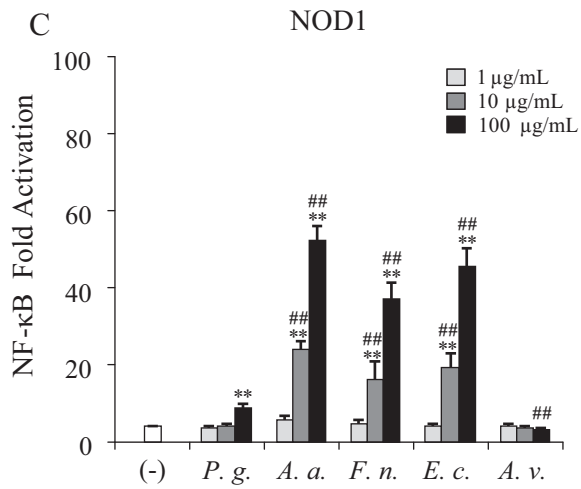
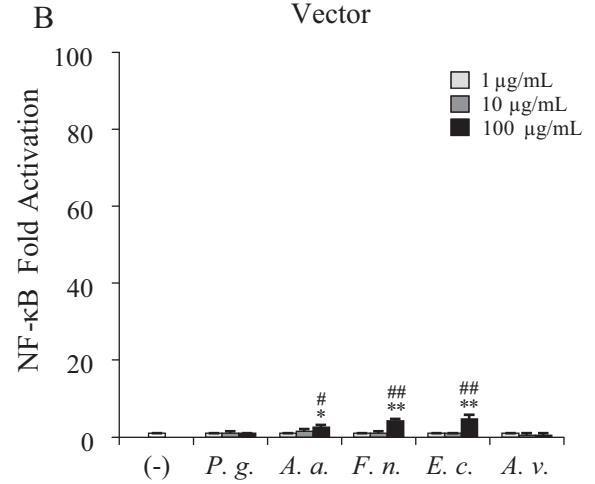
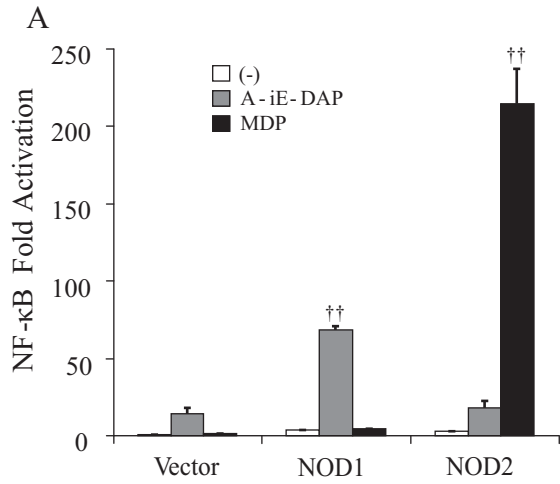


Fig. 1 Okugawa *et al.*

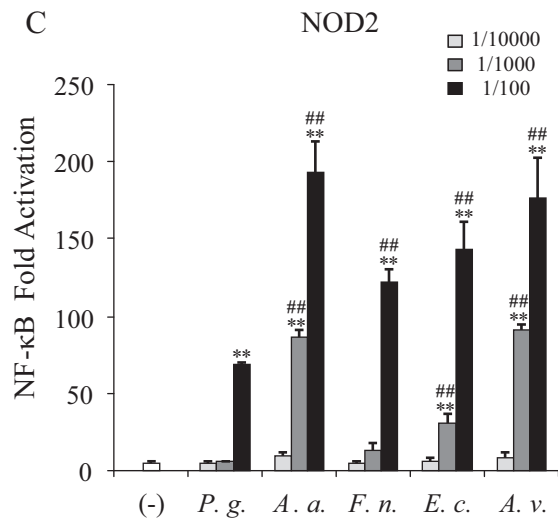
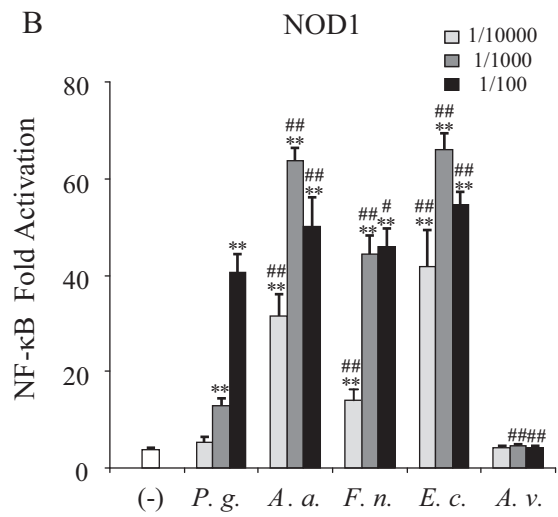
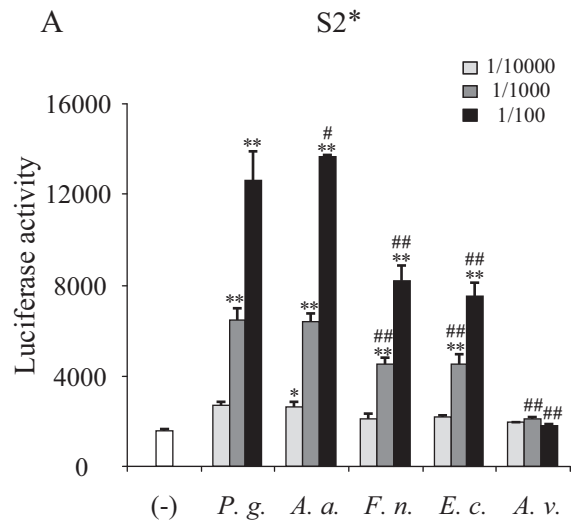


Fig. 2 Okugawa *et al.*

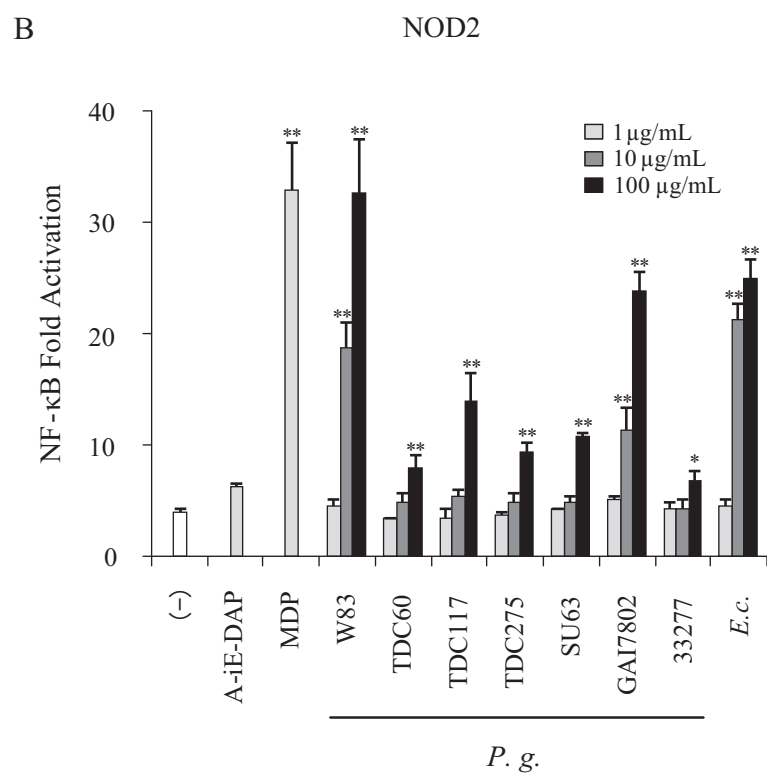
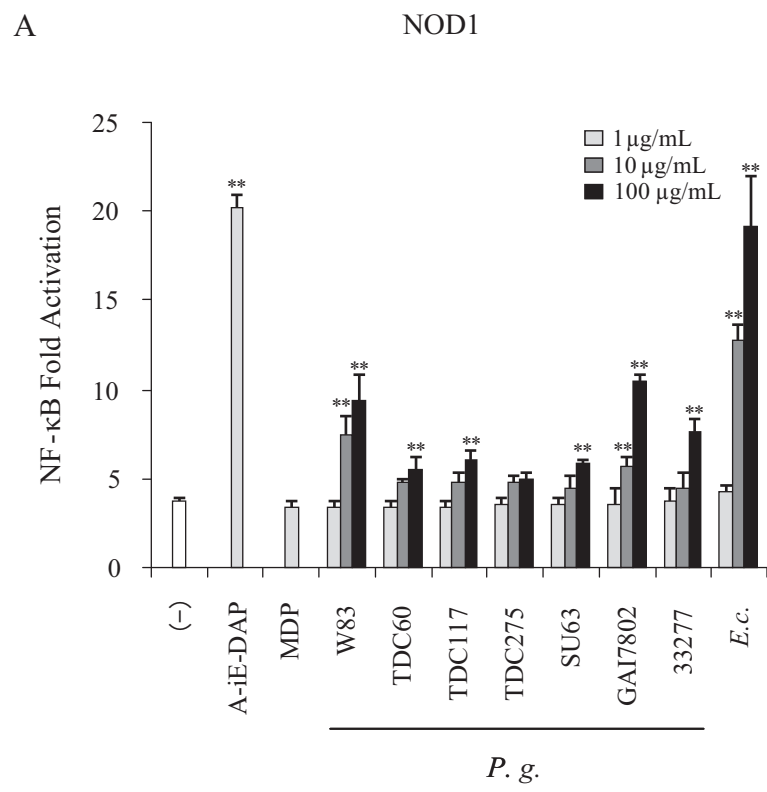
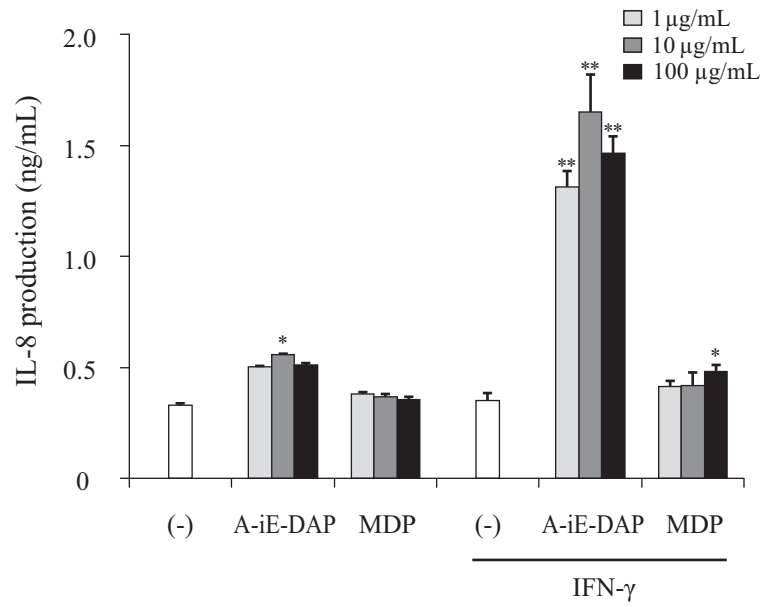


Fig. 3 Okugawa *et al.*

A



B

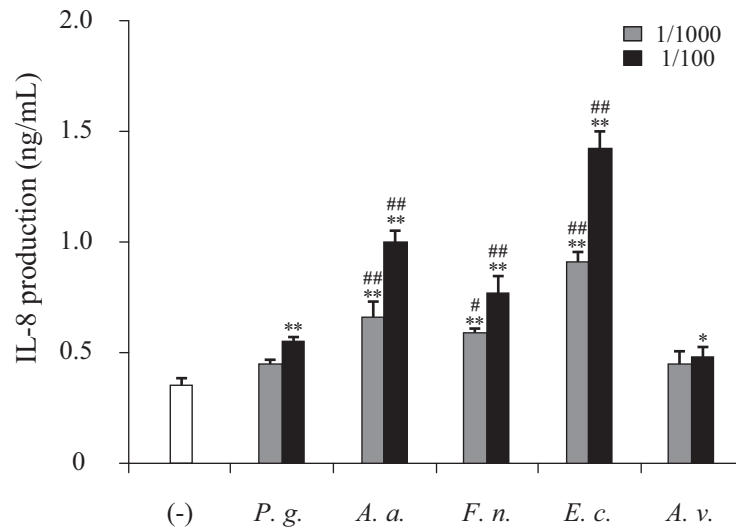


Fig. 4 Okugawa *et al.*