secrete proinflammatory cytokines, such as IL-8, granulocyte-colony stimulation factor and granulocyte macrophage-colony stimulation factor, vascular endothelial growth factor, upon stimulation with bacterial components, whereas human colonic epithelial SW480 and SW620 cells secreted proinflammatory cytokines in response to bacterial components (Uehara et al., 2001, 2005). As shown in Fig. 2, oral HSC-2, HSC-3, HO-1-u-1, tongue SAS, salivary gland HSY, pharyngeal HEp-2, esophageal TE-1, intestinal SW620, cervical HeLa, breast MCF-7, lung A549 and kidney Caki-1 cells clearly expressed protein of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2. However, HSC-3, SAS, HSY, MCF-7, A549, Caki-1, HEp-2, TE-1 and HeLa as well as oral HSC-2 and HO-1-u-1 cells, did not secrete IL-6, IL-8 and MCP-1 upon stimulation with microbial-related synthetic PAMPs (Fig. 3A–C). In contrast, intestinal HT29 and SW620 cells had the potential to produce IL-6, IL-8 and MCP-1 upon stimulation with these synthetic PAMPs (Fig. 3A–C). However, other intestinal epithelial T84 and Caco-2 cells did not secrete proinflammatory cytokines in response to these synthetic PAMPs (Fig. 3A–C).

3.3. Induction of β-defensin 2 triggered by microbial-related PAMPs in human epithelial cells

Next, we examined whether TLRs and NODs expressed in these epithelial cells actually functioned as receptors in terms of β-defensin 2 generation upon stimulation with their respective ligands. These synthetic PAMPs significantly induced β-defensin 2 generation in all the epithelial cells examined so far (Fig. 4). These epithelial cells were stained with goat IgG, followed by Alexa Fluor 488 (green) but did not show any positive fluorescence (data not shown). In accordance with the results of immunostaining assay, these epithelial cells significantly secreted β-defensin 2 into culture supernatant upon stimulation with these synthetic PAMPs (Fig. 5).

3.4. Suppression of β-defensin 2 induction upon stimulation with synthetic PAMPs in oral epithelial cells using siRNA for NF-κB

To elucidate whether NF-κB are responsible for induction of β-defensin 2 with TLR and NOD ligands, we utilized RNA interference assays targeting NF-κB mRNA. NF-κB p65 protein levels determined by flow cytometry was suppressed by *ca.* 80% using specific siRNA in oral epithelial HSC-2 cells up to 72 h (Fig. 6A). As shown in Fig. 6B, up-regulation of β-defensin 2 induced by synthetic PAMPs were significantly inhibited in NF-κB p65-silenced HSC-2 cells. These results clearly demonstrated that NF-κB is critical molecules for induction of β-defensin 2 triggered by TLR and NOD ligands.

3.5. The cell surface expression of PGRP-Iα, -Iβ and -S by oral, pharyngeal, esophageal, intestinal epithelial cells stimulated with synthetic PAMPs

We recently demonstrated that synthetic PAMPs increased the expression of PGRPs through TLRs and NODs in human oral epithelial cells (Uehara et al., 2005). We examined the possible activation of various epithelial cells in terms of PGRPs generation in response to stimulation with TLR and NOD ligands (Table 2). Consistent with a previous study, unstimulated oral HSC-2 and HO-1-u-1 epithelial cells scarcely expressed PGRP-Iα and -Iβ, whereas they definitely expressed PGRP-S. The cell surface expressions of PGRP-Iα and -Iβ in these oral epithelial cells were markedly up-regulated by stimulation with synthetic PAMPs. In contrast, the cell surface expression of PGRP-S was only slightly up-regulated by PAMPs. In

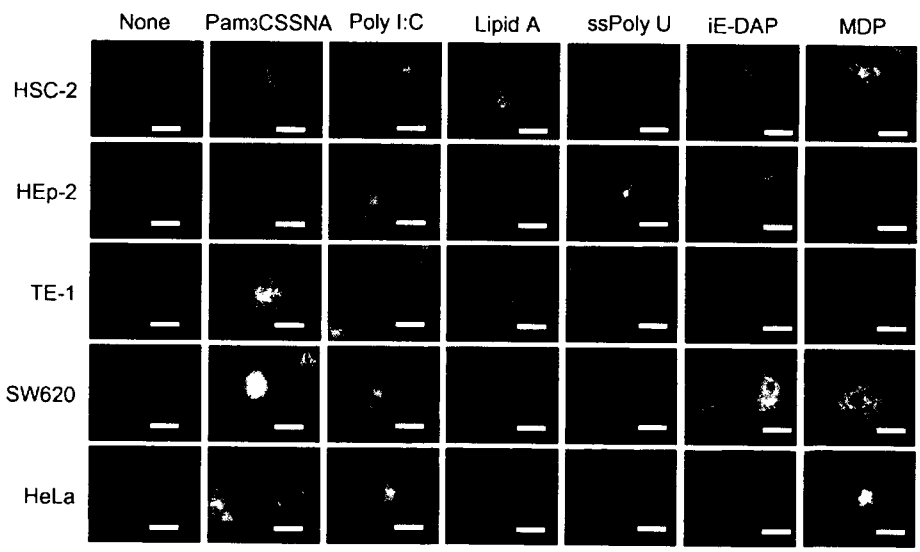

Fig. 4. The induction of β-defensin 2 in human epithelial cells upon stimulation with synthetic PAMPs. Human oral HSC-2, pharyngeal HEp-2, esophageal TE-1, intestinal SW620 and cervical HeLa epithelial cells were incubated with Pam3CSSNA (100 ng/ml), Poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPoly U (1 μg/ml), MDP (100 μg/ml), iE-DAP (100 μg/ml), respectively, for 24 h. After fixation, the cells were treated with anti-β-defensin 2 Ab or goat IgG, then visualized with Alexa Fluor 488 (green). Nuclei were visualized by staining with 4′,6-diamino-2-phenylindole (blue). The results presented are representative of three different experiments demonstrating similar results.

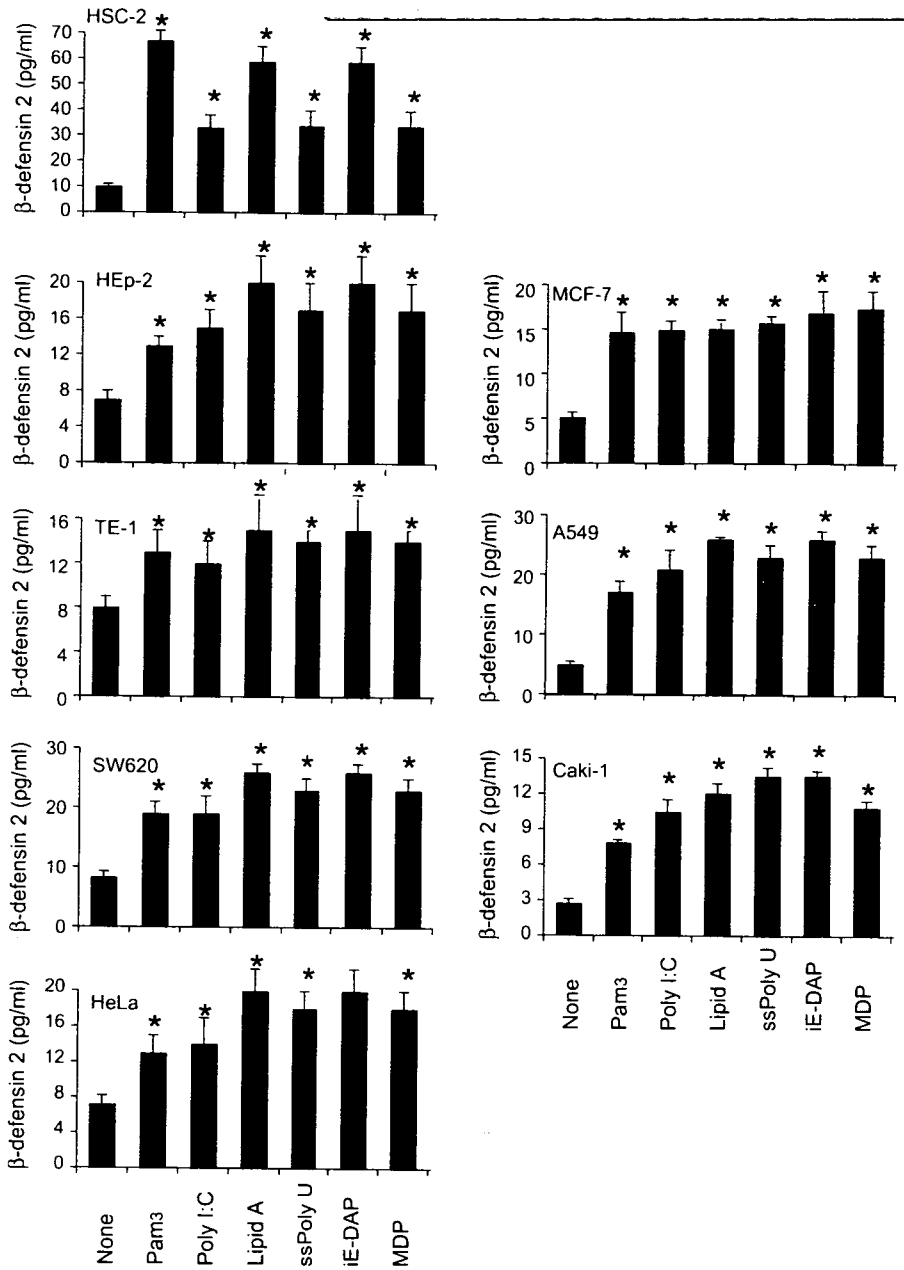

Fig. 5. Induction of β-defensin 2 in human epithelial cells upon stimulation with synthetic PAMPs. Human oral HSC-2, pharyngeal HEp-2, esophageal TE-1, intestinal SW620, cervical HeLa, breast MCF-7, lung A549 and kidney Caki-1 epithelial cells were incubated with Pam3CSSNA (100 ng/ml), Poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPoly U (1 μg/ml), MDP (100 μg/ml) and iE-DAP (100 μg/ml), respectively, for 24 h in triplicate. The levels of β-defensin 2 in the culture supernatants were determined by ELISA. Data are expressed as mean values ± S.D., and significant differences are shown. $^{*}P < 0.01$ vs. medium alone. The results presented are representative of three different experiments demonstrating similar results.

pharyngeal epithelial HEp-2 cells, unstimulated cells scarcely expressed PGRP-Iα and -Iβ, and their expression was only slightly up-regulated, even when stimulated with PAMPs. The pharyngeal epithelial cells only slightly expressed PGRP-S, and the expression was not regulated upon stimulation with PAMPs. Esophageal TE-1, intestinal SW620 and cervical HeLa cells neither expressed PGRP-Iα, -Iβ and -S, nor responded to PAMPs with the increased expression of these molecules. It must be noted that PGRP-L expression was not examined, because anti-PGRP-L mAb has not been commercially available to date.

3.6. mRNA expressions of PGRP-L, -Iα, -Iβ and -S by oral, pharyngeal, esophageal, intestinal epithelial cells stimulated with synthetic PAMPs

To examine the reasons for the low-responsiveness of pharyngeal epithelial cells and the unresponsiveness of esophageal and intestinal epithelial cells to these PAMPs in terms of PGRP expression, we further examined the mRNA expression of PGRPs by RT-PCR. As shown in Fig. 6, oral epithelial cells significantly expressed the four kinds of PGRP mRNA. Pharyngeal epithelial cells also expressed these mRNA, although the

Table 2
Expression of PGRPs on the cell surfaces upon stimulation with synthetic PAMPs in various human epithelial cells

Human cells (stimulated with)	Positive cells (%)		
	PGRP-Iα	PGRP-Iβ	PGRP-S
HSC-2 (unstimulated)	3.4	4.4	63.3
(Pam3CSSNA)	67.1*	70.8*	66.3
(Poly I:C)	48.9*	52.7*	64.0
(lipid A)	59.6*	55.7*	68.4
(ssPoly U)	52.5*	50.8*	66.1
(iE-DAP)	61.1*	71.6*	67.5
(MDP)	60.7*	58.4*	66.0
HO-1-u-1 (unstimulated)	3.0	3.5	58.8
(Pam3CSSNA)	47.0	49.0	56.4
(Poly I:C)	52.6*	55.8*	59.6
(lipid A)	59.7*	61.0*	59.2
(ssPoly U)	45.5*	56.1*	57.5
(iE-DAP)	58.1*	54.3*	57.8
(MDP)	55.3*	56.9*	56.0
HEp-2 (unstimulated)	2.9	2.4	12.2
(Pam3CSSNA)	10.3*	9.7*	14.9
(Poly I:C)	9.3*	8.6*	12.7
(lipid A)	9.1*	8.4*	14.4
(ssPoly U)	8.9*	8.2*	12.2
(iE-DAP)	9.6*	9.1*	13.2
(MDP)	9.0*	8.8*	14.0
TE-1 (unstimulated)	1.4	1.9	1.8
(Pam3CSSNA)	1.8	2.3	2.8
(Poly I:C)	1.9	2.0	2.0
(lipid A)	1.5	1.6	2.2
(ssPoly U)	1.8	1.9	2.2
(iE-DAP)	1.9	2.1	2.0
(MDP)	1.1	1.8	1.5
SW620 (unstimulated)	1.5	1.8	1.9
(Pam3CSSNA)	1.5	1.8	2.5
(Poly I:C)	1.8	1.8	2.0
(lipid A)	1.9	1.7	1.9
(ssPoly U)	1.8	2.1	2.0
(iE-DAP)	2.2	1.9	1.9
(MDP)	2.2	1.8	1.8
HeLa (unstimulated)	1.9	1.4	1.8
(Pam3CSSNA)	2.1	2.0	2.2
(Poly I:C)	1.9	2.1	1.9
(lipid A)	1.7	1.6	1.7
(ssPoly U)	1.9	1.8	1.9
(iE-DAP)	1.8	1.7	1.8
(MDP)	1.9	1.9	1.9

An asterisk indicates a significant difference vs. medium alone (unstimulated). Conc. Pam3CSSNA (100 ng/ml), Poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPoly U (1 μg/ml), MDP (100 μg/ml) and iE-DAP (100 μg/ml).

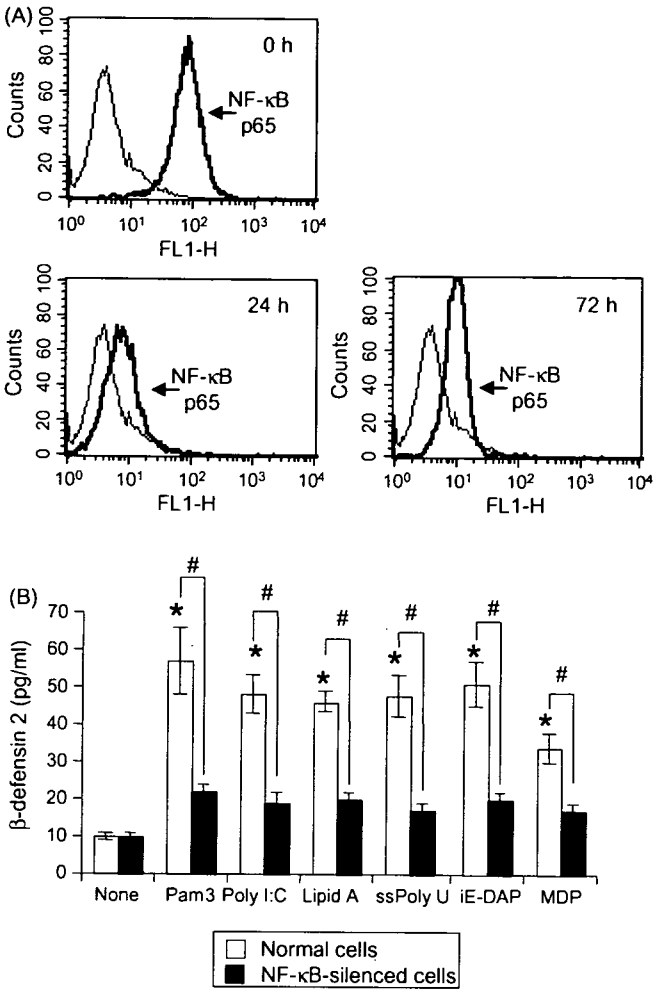

Fig. 6. siRNA for NF-κB p65 prevent up-regulation of β-defensin 2 induced by synthetic PAMPs in human oral epithelial cells. (A) Oral epithelial HSC-2 cells were transfected with siRNA for NF-κB p65. After 0, 24 and 72 h, the cells were stained with anti-NF-κB p65 Ab at 4 °C for 30 min, followed by FITC-conjugated goat anti-mouse IgG. (B) HSC-2 cells transfected with siRNA targeting NF-κB p65 for 24 h were stimulated with 100 pg/ml Pam3CSSNA, 10 μg/ml of Poly I:C, 10 ng/ml of lipid A, 1 μg/ml of ssPoly U, 100 μg/ml iE-DAP and 100 μg/ml MDP, respectively. After 24 h stimulation, the levels of β-defensin 2 in the culture supernatants were determined by ELISA. *, #Significantly different from the respective medium alone control ($P < 0.01$); #significantly different from the respective positive control ($P < 0.01$). Error bars indicated S.D. Results are representative of three different experiments.

levels of PGRP mRNA were weaker than those in oral epithelial cells (Fig. 7). Contrary to oral epithelial cells, esophageal and intestinal epithelial cells only marginally expressed the mRNA of PGRPs (Fig. 7). These results indicate that oral epithelial cells are high producers of PGRPs.

4. Discussions

Since epithelial cells are the outermost layer, and come into direct contact with bacteria in various organs, it has been speculated that various epithelial cells produce several proinflammatory cytokines in response to bacterial stimuli. However, some oral and intestinal epithelial cells have been reported not to produce proinflammatory cytokines upon stimulation with bacterial PAMPs due to the lack of TLRs expression (Abreu et al., 2001; Asai et al., 2001; Melmed et al., 2003; Naik et al., 2001; Suzuki et al., 2003). In this study, we found clear expressions of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2 in several epithelial cells using RT-PCR (Fig. 1), immunostaining (Fig. 2) and flow cytometric analyses (Table 1); nevertheless, most of the epithelial cells, except for intestinal HT29 and SW620 cells, did not secrete proinflammatory cytokines upon stimulation with PAMPs (Fig. 3) in accordance with our previous study using oral epithelial cells (Sugawara et al., 2006; Uehara et al.,

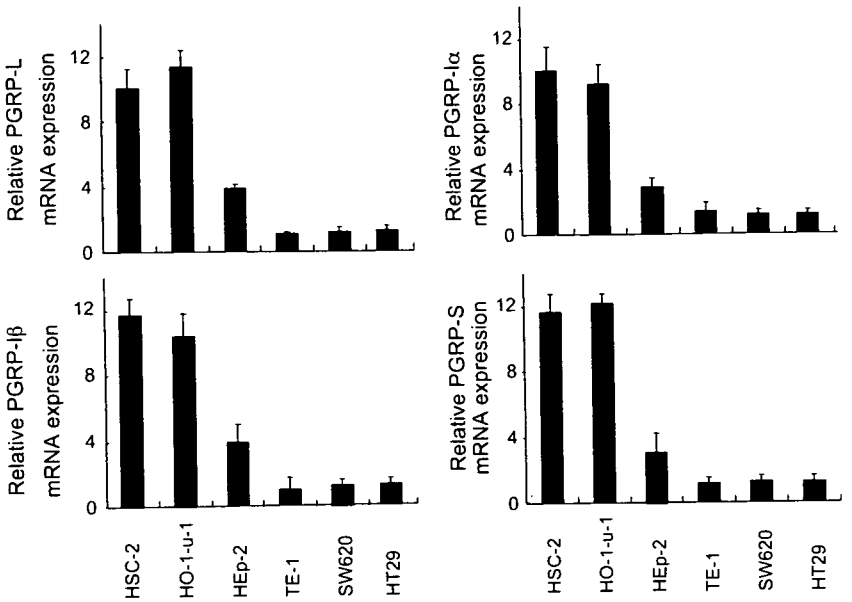

Fig. 7. PGRP mRNA expression by epithelial cells stimulated with synthetic PAMPs. Human oral HSC-2, HO-1-u-1, pharyngeal HEp-2, esophageal TE-1, intestinal SW620 and HT29 epithelial cells were cultured until reaching confluence. After the incubation, the total RNA was extracted and the mRNA expression of PGRP-L, -Iα, -Iβ and -S was analyzed by real-time PCR. Results are expressed as the relative mRNA accumulation corrected using GAPDH mRNA as an internal standard. The results presented are representative of three different experiments demonstrating similar results.

2001). Therefore, we (Uehara et al., 2001) and other investigators (Abreu et al., 2001; Naik et al., 2001; Suzuki et al., 2003) postulated the teleological explanation that the unresponsiveness or low-responsiveness to PAMPs in most of the epithelial cells may be related to the inability of the cells to discriminate pathogens from commensal bacteria by the mere recognization of PAMPs, and additional factors, such as cellular invasion, or other signs of pathogenicity are required to induce inflammatory responses, which in turn prevent tissue destruction due to excessive inflammatory innate immune responses to bacterial stimuli, because the epithelial cells used in this study except HeLa are constitutively interact with bacteria.

Recently, we demonstrated that oral epithelial cells are highly responsive to bacterial PAMPs through TLRs and NODs regarding the production of anti-bacterial factors, such as PGRPs and β-defensin 2 (Sugawara et al., 2006; Uehara et al., 2005). Consistent with the up-regulation of β-defensin 2 upon stimulation with microbe-related PAMPs in oral epithelial cells, various epithelial cells secrete β-defensin 2 molecules upon stimulation with respective microbial PAMPs (Figs. 4 and 5). In addition, up-regulation of β-defensin 2 upon stimulation with PAMPs in oral epithelial cells occurred via NF-κB (Fig. 6). These findings indicate that TLRs and NODs in various epithelial cells, as well as oral epithelial cells are functional, and epithelial cells might actively participate in bacterial clearance in the mucosa without an accompanying excessive inflammatory response, which might induce tissue destruction.

Contrary to the up-regulation of β-defensin 2 in the various epithelial cells so far examined upon stimulation with microbial PAMPs, the expression of PGRPs on the cell surface was not up-regulated upon stimulation with microbial PAMPs in various epithelial cells, except for oral epithelial cells (Table 2). Human PGRP-S (PGLYRP1) is present in granulocyte granules and likely participates in killing of phagocytized bacteria (Liu et al., 2001), and PGRP-L (PGLYRP2) is an *N*-acetylmuramoyl-L-alanine amidase like PGRP-SC1B in *Drosophila*, an enzyme that cleaves the stempeptide from the glycan chain of PGN and is constitutively produced in the liver and secreted into the bloodstream (Wang et al., 2003). In our previous study (Uehara et al., 2005), PGRP-Iα (PGLYRP3) and -Iβ (PGLYRP4) were more markedly up-regulated than PGRP-L or PGRP-S. Recently, Lu et al. (Lu et al., 2006) reported that PGRP-Iα and -Iβ are specifically expressed in various epithelia from the skin, eyes, salivary glands, throat, tongue, esophagus, stomach and intestine, and are bactericidal against several non-pathogenic and pathogenic bacteria. Therefore, it is of benefit for hosts that PGRPs expressions are highly up-regulated upon stimulation with bacterial cell-surface components in oral epithelial cells, which are the first cells encountered by bacteria in the oral mucosa. In fact, our results demonstrated that the expression of four kinds of PGRP mRNA on oral epithelial cells was much stronger than that on other epithelial cells (Fig. 6). Therefore, PGRPs may be critical anti-bacterial components in oral epithelial cells.

Naïve oral epithelial cells did not secrete inflammatory cytokines upon stimulation with bacterial PAMPs, including NOD1 and NOD2 ligands, although IFN-γ-primed oral epithelial cells produced inflammatory cytokines in the same condition (Uehara et al., 2002). Although proinflammatory cytokine responses of various human epithelial cells, including intestinal epithelial cells to bacterial stimuli, have not been examined so far, murine intestinal epithelial cells produce anti-bacterial factors upon stimulation with bacterial components, including NOD ligands (Kobayashi et al., 2005). There is a good possibility that naïve epithelial cells in general produce anti-bacterial factors, but not proinflammatory factors, to prevent bacterial

invasion without excessive inflammatory responses, which may lead to tissue destruction.

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Antibodies to proteinase 3 prime human monocytic cells via protease-activated receptor-2 and NF-κB for Toll-like receptor- and NOD-dependent activation

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Abstract

Anti-neutrophil cytoplasmic Abs against proteinase 3 (PR3) have been detected in relation to a wide range of inflammatory conditions, and the interaction of anti-PR3 Abs with leukocytes provokes cell activation, although how is not clear. Flow cytometric analysis revealed an increase in cell-surface CD14, Toll-like receptor (TLR)2, TLR4 and intracellular TLR3, TLR7, TLR8, TLR9, NOD1 and NOD2 expression during anti-PR3 priming in human monocytic THP-1 cells. Anti-RP3 Abs markedly promoted the release of IL-8 induced by chemically synthesized TLR and NOD ligands mimicking bacterial components: TLR2-agonistic lipopeptide (FSL-1), TLR3-agonistic poly I:C, TLR4-agonistic lipid A (LA-15-PP), TLR7/8-agonistic single stranded RNA (ssPolyU), TLR9-agonistic bacterial CpG DNA, NOD1-agonistic FK156/565 and NOD2-agonistic muramyldipeptide (MDP) in THP-1 cells and human peripheral blood mononuclear cells, although sole incubation with anti-PR3 Abs induced only a low level of IL-8. The priming response was evident after 2 h of preincubation with anti-PR3 Abs and peaked after 6 h. Priming was also observed for the production of TNF-α and monocyte chemoattractant protein-1. An RNA interference assay revealed that anti-PR3 Abs activated THP-1 cells in a PR3- and protease-activated receptor-2-dependent manner. Furthermore, the anti-PR3 Ab-mediated cell activation was significantly abolished by RNA interference targeted at PR3 mRNA and by inhibition of phospholipase C and NF-κB. These results suggest that anti-PR3 Abs prime human monocytic cells to produce cytokines upon stimulation with various bacterial components by up-regulating the TLR and NOD signaling pathway, and that these mechanisms may actively participate in the inflammatory process.

Keywords: Proteinase 3; Protease-activated receptor-2; Toll-like receptors; NODs; Human monocytic cells

1. Introduction

Anti-neutrophil cytoplasmic Abs (ANCA) were first identified in patients with necrotizing glomerulonephritis (Davies et al., 1982). ANCA is an autoantibody directed against the enzymes located in the primary granules of neutrophils and lysosomes of monocytes. An established association does exist between the occurrence of ANCA, especially those targeting proteinase 3 (PR3), and the development of active Wegener's granulomatosis (WG) (van der Woude et al., 1985). ANCA have since been detected in relation to a wide range of inflammatory, infectious and neoplastic conditions (Ardiles et al., 1997; Bartůňková et al., 2003; Hagen et al., 1993; Savige et al., 2000).

ANCA have been reported to be causally involved in the pathogenesis of various inflammatory diseases; the autoantibody titer correlates with disease activity (Tervaert et al., 1989), and ANCA directly activate a wide variety of inflammatory functions in neutrophils, such as secretion of oxygen radicals, proteases and lipid mediators, once PR3 is expressed on the leukocyte surface (Falk et al., 1990; Grimminger et al., 1996; Muller Kobold et al., 1999; Savage et al., 1992) under inflammatory conditions. Additionally, in isolated monocytes, anti-PR3 Abs stimulate the release of proinflammatory cytokines (Casselman et al., 1995;

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibodies; PR3, proteinase 3; WG, Wegener's granulomatosis; MCP-1, monocyte chemoattractant protein-1; PAR, protease-activated receptor; PRRs, pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; PGNs, peptidoglycans; TLRs, Toll-like receptors; MDP, muramyldipeptide; DAP, diaminopimelic acid; PLC, phospholipase C; FCS, fetal calf serum; PBMCs, peripheral blood mononuclear cells; TNF-α, tumor necrosis factor-α; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, short interfering RNA

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plied by Astellas Pharmaceutical Co. (Tokyo, Japan). Mouse anti-human PR3 mAb (CLB-12.8) was obtained from CLB (Amsterdam, The Netherlands). Anti-TLR2 mAb (TL2.1) (mouse IgG2a), anti-TLR3 mAb (mouse IgG2a), anti-TLR4 mAb (HTA125) (mouse IgG2a), anti-TLR7 mAb (mouse IgG2a) and anti-TLR9 mAb (mouse IgG2a) were purchased from eBioscience (San Diego). Goat anti-NOD1 Ab (L-17) was obtained from Cayman Chemical (Ann Arbor, ML, USA). Rabbit anti-NOD2 Ab was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-human PAR-1 mAb ATAP2 (mouse IgG) raised against aa 42–55 of human PAR-1, anti-human PAR-2 mAb SMA11 (mouse IgG2a) raised against aa 37–50 of human PAR-2 and rabbit anti-human PAR-3 polyclonal Ab raised against aa 1–13 of human PAR-3 were obtained from Santa Cruz Biotechnology. Isotype control mouse IgG (MOPC-2) was purchased from Sigma–Aldrich. The PLC inhibitor U73122 and its control U73343 were provided by Calbiochem-Novabiochem (La Jolla, CA, USA). All other reagents were obtained from Sigma–Aldrich, unless otherwise indicated.

2.2. Cells and cell culture

The human monocytic leukemia 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 peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood of healthy adult donors by Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation at $800 \times g$ for 20 min at room temperature. The isolated PBMCs were washed three times with PBS and suspended in RPMI 1640 medium.

2.3. Determination of cytokines in culture supernatants

The cells were collected and washed twice in PBS. The cells (10^5 per 200 μl per well) were incubated with or without stimulant in RPMI 1640 medium with 1% FCS for 24 h in 96-well culture plates. The culture supernatants were collected and the levels of IL-8, MCP-1 and TNF-α were determined with an ELISA kit (OptEIA ELISA Kits, BD Pharmingen). The concentrations of the cytokines in the supernatants were determined using the i-PLATEmanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur cytometer (BD Biosciences, Mountain View, CA, USA). THP-1 cells were cultured with or without anti-PR3 Abs for 24 h at 37 °C, then collected and washed in PBS. Cells were stained with anti-CD14 Ab, anti-TLR2 Ab, anti-TLR4 Ab, anti-PAR-1 Ab, anti-PAR-2 Ab, anti-PAR-3 Ab, anti-PR3 Ab or control IgG at 4 °C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated secondary Ab (BioSource International, Camarillo, CA, USA) at 4 °C for an additional 30 min. For TLR3, TLR7, TLR8, TLR9, NOD1 and NOD2, intracellular staining was performed. Briefly, the cells were washed with staining buffer, fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences) for 15 min at 4 °C and then incubated with anti-TLR3 Ab, anti-TLR7 Ab, anti-TLR8 Ab, anti-TLR9 Ab, anti-NOD1 Ab, anti-NOD2 Ab or control IgG for 30 min, followed by FITC-conjugated secondary Ab at 4 °C for another 30 min. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded < 2% of events positive with the isotype Ab control. Fluorescence to the right was counted as specific binding.

2.5. RNA extraction and RT-PCR assay

Total cellular RNA was obtained using Isogen (Nippon Gene, Tokyo, Japan) and reverse transcribed using random hexamer primers and avian myeloblastosis virus reverse-transcriptase XL (Life Sciences, St. Petersburg, FL, USA). The primers used for PCR were as follows: PR3, forward, 5′-ATGGTGGGCGGGCACGAGGCG-3′ and reverse, 5′-GCGGCCAGGGACGAAAGTGCA-3′; PAR-2, forward, 5′-GCAGCCTCTCTCTCCTGCAGTGG-3′ and reverse, 5′-CTTGCATCTGCTTTACAGTGCG-3′; human glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The primers for PR3, PAR-2 and GAPDH were designed to generate fragments of 662, 1066 and 983 bp, respectively. PCR was performed for 35 cycles for 1 min at 94 °C, 1 min at 55 °C for PAR-2 or at 60 °C for PR3 and GAPDH; 1 min at 72 °C. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light.

2.6. RNA interference

Transfection for targeting endogenous PAR-2, PR3, p65 and Lamin A/C was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and short interfering RNA (siRNA) (final concentration, 200 nM), according to the manufacturer's instructions. The siRNA for PAR-2, PR3 and p65 was purchased from Santa Cruz. The siRNA for Lamin A/C was purchased from B-Bridge International (San Jose, CA, USA).

2.7. Data analysis

All experiments in this study were performed at least three times to confirm the reproducibility of the results. For most of the experiments, values are represented as the means ± S.D. of triplicate assays. The significance of differences between the two means was evaluated by one-way ANOVA using the Bonferrori or Dunnett method, and values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Treatment with anti-PR3 Abs up-regulated the expression of CD14, TLR2, TLR3, TLR4, TLR7, TLR8, TLR9, NOD1 and NOD2 in human monocytic THP-1 cells

As mentioned above, Hattar et al. (2005) reported that anti-PR3 Abs up-regulate the expression of CD14 on human primary monocytes and neutrophils, and endowed the cells with the ability to produce inflammatory cytokines at remarkable levels upon stimulation with LPS or LTA. Therefore, we examined the effects of anti-PR3 Abs on the expression of various TLRs and NODs in addition to CD14 in THP-1 cells. THP-1 cells constitutively expressed these molecules. The incubation of THP-1 cells with 1 μg/ml of the anti-PR3 Abs resulted in the up-regulated expression of TLR2 and TLR4, as well as CD14, at the cell surface (Fig. 1, filled graphs). Furthermore, intracellular levels of TLR3, TLR7, TLR8, TLR9, NOD1 and NOD2 were also clearly increased in the cells. On the other hand, equal concentrations of an isotype-matched mouse IgG were completely ineffective in this respect (Fig. 1, bold line). We (Uehara et al., 2004) recently demonstrated that proinflammatory cytokines induced production of PR3 as the membrane-bound form in human oral epithelial cells, and antibodies to PR3 activated oral epithelial cells through PAR-2. We revealed that THP-1 cells constitutively expressed PAR-1, PAR-2, PAR-3 and PR3 at their surface, and that the addition of anti-PR3 Abs also up-regulated the expression of PAR-2 and PR3 (Fig. 1). In contrast to PAR-2, anti-PR3 Abs little affected the expression of PAR-1 or PAR-3 on THP-1 cells.

3.2. Anti-PR3 Abs enhanced TLR and NOD ligand-induced secretion of proinflammatory cytokines in THP-1 cells

The above observation raised the question as to whether the up-regulated expression of TLRs and NODs evoked by anti-PR3 Abs induced amplified responses to respective ligands. We examined this in terms of the production of inflammatory cytokines upon stimulation with the respective ligands after priming with anti-PR3 Abs. Stimulation with the TLR2-agonistic FSL-1, TLR3-agonistic poly I:C and TLR9-agonistic CpG DNA significantly induced production of MCP-1, TNF-α and IL-8 and anti-PR3 Abs also weakly induced production of MCP-1 and TNF-α, whereas other TLR and NOD ligands were scarcely active in this respect (Fig. 2). When THP-1 cells were preincubated with 1 μg/ml of anti-PR3 Abs for 6 h, and subsequently challenged with the various TLR and NOD ligands for a further 18 h, a massive increase in the TLR and NOD ligand-induced production of IL-8, MCP-1 and TNF-α was observed in THP-1 cells (Fig. 2), but an isotype-matched mouse IgG was completely inactive in this respect (data not shown).

Next, an analysis of the kinetics of the anti-PR3-elicited priming response was performed. THP-1 cells were incubated with anti-PR3 Abs for various periods of time prior to being challenged with the TLR and NOD ligands. Although the magnitude of the priming reaction differed among respective ligands, similar kinetic patterns were observed (Fig. 3). Only a slight elevation in levels of IL-8 was observed in THP-1 cells after a 2 h priming period with anti-PR3 Abs, and the ratio of the response was increased until 6 h, when maximal enhancement was observed (Fig. 3). Thereafter, the priming effect decreased, although longer priming periods were still effective in this respect.

3.3. Anti-PR3 Abs promoted TLR and NOD ligand-induced secretion of proinflammatory cytokines in PBMCs

Next, we examined whether similar priming effects of anti-PR3 Abs were observed in human PBMCs. We isolated PBMCs from four healthy adult donors. Consistent with the results for THP-1 cells, anti-PR3 Abs promoted the TLR and NOD ligand-induced secretion of IL-8, MCP-1 and TNF-α in PBMCs (Fig. 4).

3.4. Priming effect of anti-PR3 Abs occurred in a PAR-2-, PR3-, PLC- and NF-κB-dependent manner

As we found that the addition of anti-PR3 Abs to oral epithelial cells in culture induced the aggregation of PR3 on the cells and the secretion of proinflammatory cytokines by the cells, we examined whether priming effects of anti-PR3 Abs on CD14, TLR and NOD expression in THP-1 cells also occurred through PAR-2 and/or PR3. To confirm the involvement of PAR-2 and/or PR3, we used siRNA to diminish the expression of PAR-2 and PR3 in THP-1 cells. The transfection of THP-1 cells with PAR-2- and PR3-specific siRNA resulted in an inhibition of PAR-2 and PR3 mRNA, but not GAPDH mRNA, from 8 h up to 72 h of culture (Fig. 5A). In transfected cells, the anti-PR3 Ab-mediated priming effect was significantly reduced by PAR-2- and PR3-specific siRNA (Fig. 5B). In common with many G protein-coupled receptors, the principal mechanism of PAR-mediated activation is through Gαq proteins, resulting in the activation of PLC. To examine whether PLC is also involved in the anti-PR3 Ab-mediated priming effect of THP-1 cells, THP-1 cells were treated with anti-PR3 Abs in the presence of the PLC inhibitor U73122 or the control compound U73343 for 24 h. As shown in Fig. 6A, the inhibition of PLC abolished the production of IL-8 induced by anti-PR3 Abs. It has been reported that agonists of PAR-2 induced the activation of NF-κB (Rahman et al., 2002; Shpacovitch et al., 2002; Uehara et al., 2004, 2005). We examined the mechanism using siRNA targeting p65 which is a component of the NF-κB pathway. The up-regulation of IL-8 secretion induced by anti-PR3 Abs was significantly inhibited in p65-silenced THP-1 cells (Fig. 6B). Lamin A/C-specific siRNA was used as a negative control in these experiments (Figs. 5 and 6). These results indicated that the secretion of cytokines induced by anti-PR3 Abs occurred through PLC and NF-κB, which are located downstream of the PAR-2 and PR3 signaling pathway.

4. Discussion

Among the ANCA, those targeting PR3 (cANCA) have a strong and specific association with WG (van der Woude et al., 1985). Besides their significance as seromarkers, a pathogenic

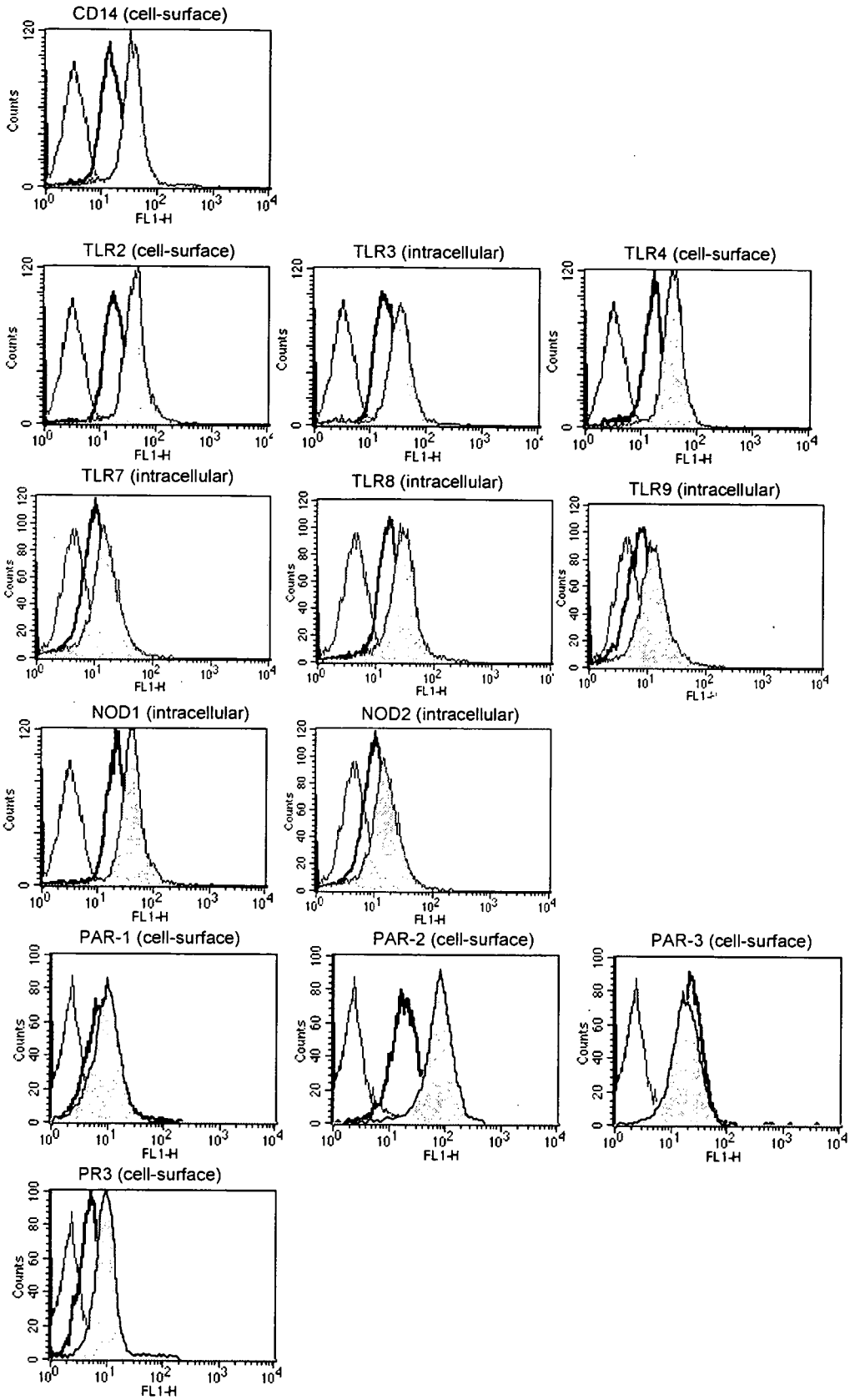

Fig. 1. Up-regulation of the expression of CD14, TLRs, NODs, PAR-2 and PR3 in monocytic cells in response to anti-PR3 Abs. THP-1 cells were stimulated with anti-PR3 Abs (1 μg/ml, filled graphs) or an isotype-matched control IgG (1 μg/ml, bold line). After 6 h of incubation, the expression of cell-surface CD14, TLR2, TLR4, PAR-1, PAR-2, PAR-3 and PR3 and intracellular TLR3, TLR7, TLR8, TLR9, NOD1 and NOD2, was assessed by flow cytometry. The thin lined curve is the staining with a control Ab. The results presented are representative of four different experiments demonstrating similar results.

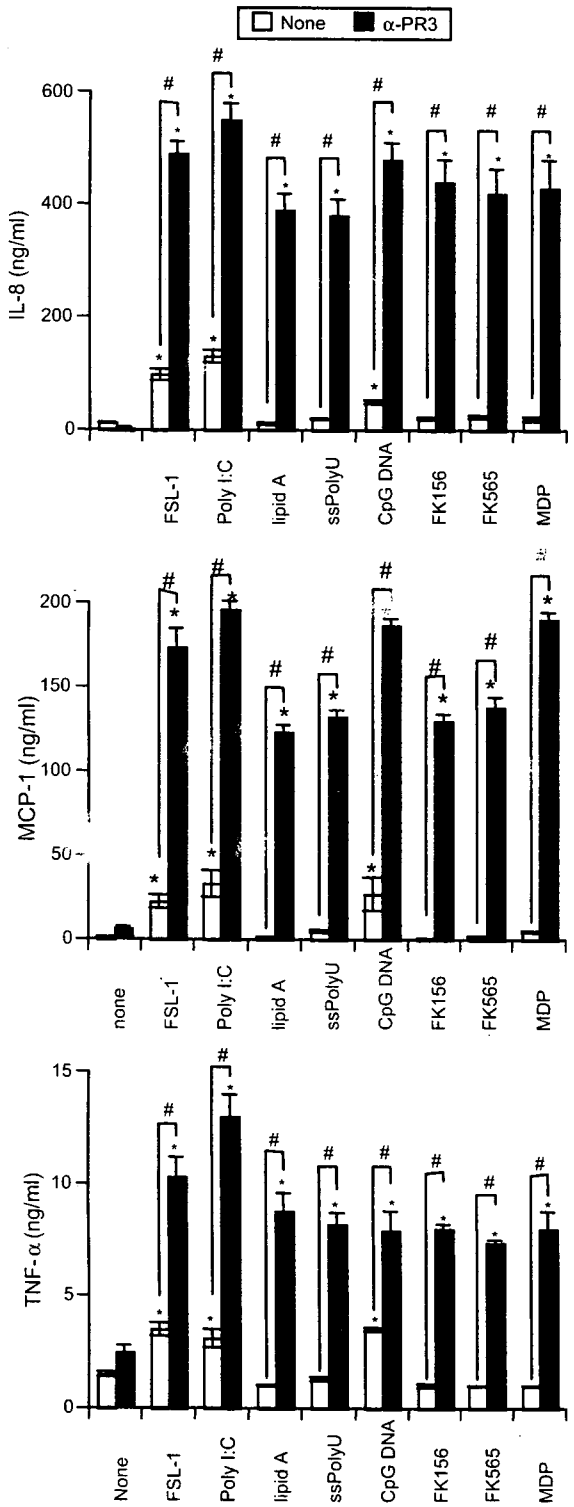

Fig. 2. Enhancement of TLR and NOD ligand-induced cytokine production in human monocytic cells preincubated with anti-PR3 Abs. THP-1 cells were preincubated for 6 h with anti-PR3 Abs (closed bar), with equal amounts of an isotype-matched IgG (sham incubation, open bar) or without antibodies (none). Subsequently, the cells were challenged with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml), FK565 (100 μg/ml) or MDP (100 μg/ml) for 18 h. TNF-α, MCP-1 and IL-8 levels in the culture supernatants were determined by ELISA, and expressed as means ± S.D. *,#Significantly different from sham-incubated THP-1 cells and from respective cultures stimulated with the respective ligands alone. The results are representative of three different experiments.

role has been proposed for these autoantibodies in relation to their capacity to activate leukocytes in vitro (Falk et al., 1990; Grimminger et al., 1996; Muller Kobold et al., 1999; Savage et al., 1992). In the present study, an alternative approach was chosen to define the priming effect of ANCA against PR3 on inflammatory leukocyte functions: human monocytic THP-1 cells were preincubated with substimulatory concentrations of anti-PR3 Abs and possible augmented cell activation by various bacterial PAMPs was examined. As mentioned above, Hattar et al. (2005) first reported that anti-PR3 Abs primed CD14-dependent human leukocytes for the enhanced production of inflammatory cytokines upon stimulation with TLR4-agonistic LPS or TLR2-agonistic lipoteichoic acid. In this study, we demonstrated that incubation with anti-PR3 Abs significantly up-regulated the expression of various PRRs (TLR2, TLR3, TLR4, TLR7, TLR8, TLR9, NOD1 and NOD2) in addition to CD14 in human monocytic THP-1 cells (Fig. 1). In this context, however, Hattar et al. (2005) found no increase in the expression of TLR2 and TLR4 by FACS analysis after treatment with anti-PR3 Abs, and analogized that anti-PR3 Abs primed CD14-dependent, but TLR2- and TLR4-independent, leukocyte activation. Although it is unclear why they did not find an increase in the expression of TLRs, the difference in staining methods may be one reason. In their study, the cells were directly stained with PE-labeled Abs targeting TLR2 or TLR4. On the other hand, in our study the cells were stained with various TLR or NOD Abs, followed by a FITC-conjugated secondary Ab. We further demonstrated that the generation of IL-8, which plays a critical pathogenic role in ANCA-associated inflammation (Cockwell et al., 1999), and other proinflammatory cytokines, such as TNF-α and MCP-1, was strongly up-regulated upon stimulation with the respective TLR and NOD ligands in the primed monocytic cells (Fig. 2). In addition, it is interesting that similar priming effects by anti-PR3 Abs were observed with human PBMCs (Fig. 4). It should be noted that we solely used chemically synthesized PAMPs, because natural bacterial cell surface preparations are inevitably contaminated with minor bioactive components that might have confused the results. Therefore, these results clearly indicated that antibodies to PR3 primed TLR2-, 3-, 4-, 7-, 8- and 9-, NOD1- and 2- and CD14-dependent cell activation in human monocytic cells. As we used a mouse monoclonal Ab against PR3 in this study, it must be mentioned that Hattar et al. (2002b) demonstrated that three commercial mouse anti-PR3 Abs (4A3, 4A5 and 12.8 [used in the present study]), as well as human anti-PR3 (PR3-ANCA) originating from pooled serum of five patients with monospecific anti-PR3 Ab-positive WG established by adsorption on a PR3 affinity column similarly and specifically bound to cell-surface PR3.

We previously reported that antibodies to PR3 aggregated cell surface PR3 on cytokine primed-human oral epithelial cells, and activated the cells in a PAR-2-, PLC- and NF-κB-dependent manner (Uehara et al., 2004). The same pathway might be involved in the priming effect of anti-PR3 Abs on the monocytic THP-1 cells, because the effect was significantly suppressed in PR3-, PAR-2- and NF-κB-silenced THP-1 cells and by an inhibitor against PLC (Figs. 5 and 6). Namely, priming by anti-

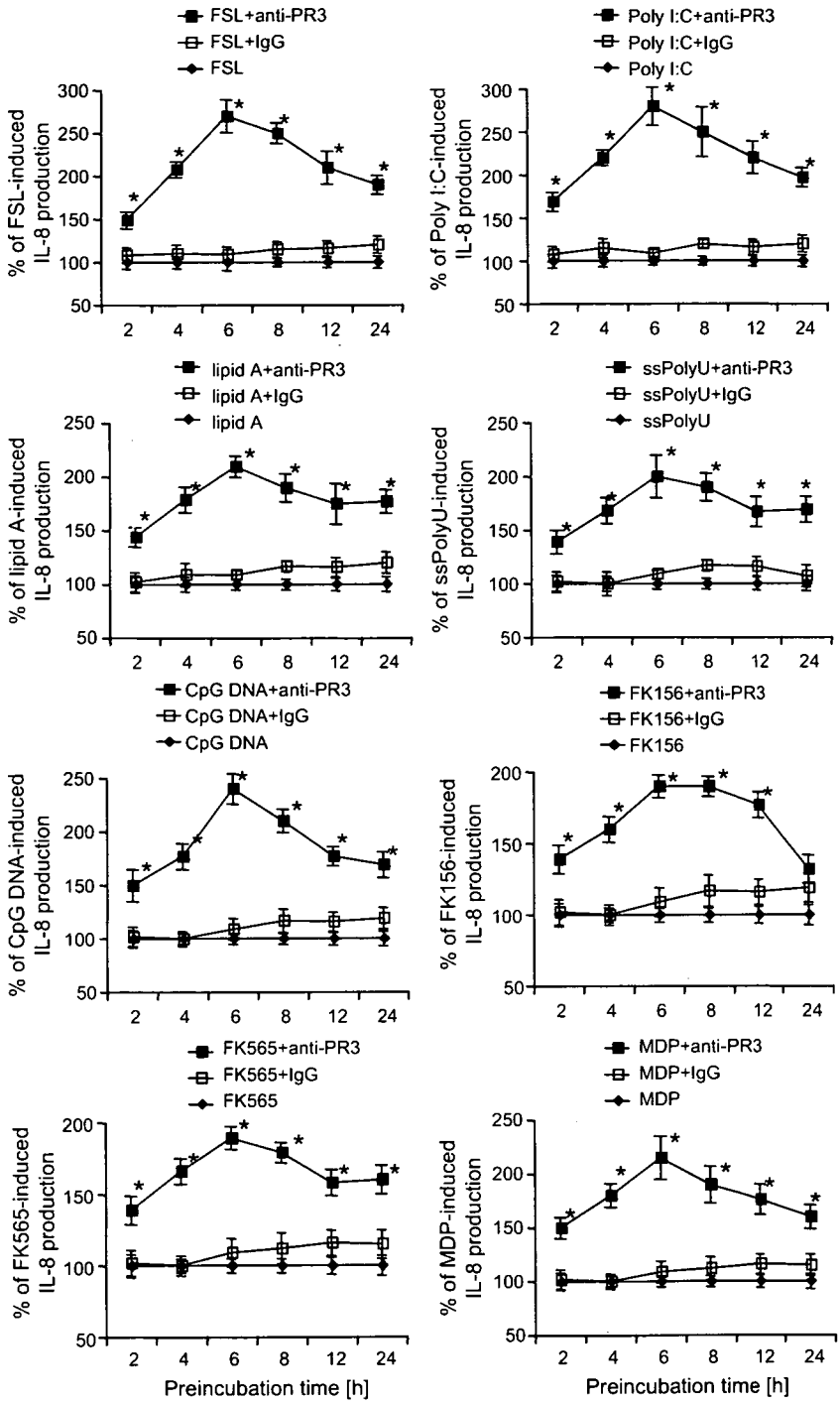

Fig. 3. Time course of the priming effects of anti-PR3 Abs for TLR and NOD ligand-induced IL-8 production. THP-1 cells were preincubated for the indicated periods with 1 μg/ml of anti-PR3 Abs, with equal amounts of an isotype-matched IgG (sham incubation) or without antibodies (none). Subsequently, the THP-1 cells were challenged with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml), FK565 (100 μg/ml) or MDP (100 μg/ml) for 18 h. The IL-8 released into the supernatant is given as a percentage of the respective TLR and NOD ligand-induced IL-8 production in sham-primed cells. Data reflect the means ± S.D. of at least four independent experiments. *Significantly different from isotype-matched IgG-incubated THP-1 cells.

PR3 Abs occurred through PLC and NF-κB, which were located downstream of the PAR-2 and PR3 signaling pathway. It should be noted that a PAR-2-agonistic peptide could not prime THP-1 cells (data not shown), although the peptide might activate the cells via the same pathway as the anti-PR3 Abs. Further studies are required to elucidate the complete mechanisms for the priming effect of anti-PR3 Abs.

It is conceivable that bacterial components exhibit powerful immuno-adjuvant activities through the activation of antigen-presenting cells via TLR and NOD pathways against various antigens, including autoantigens, which in turn might induce severe autoimmune diseases. In fact in animal models, NOD2-agonistic MDP induces autoimmune diseases such as experimental encephalomyelitis, orchitis, uveoretinitis, thy-

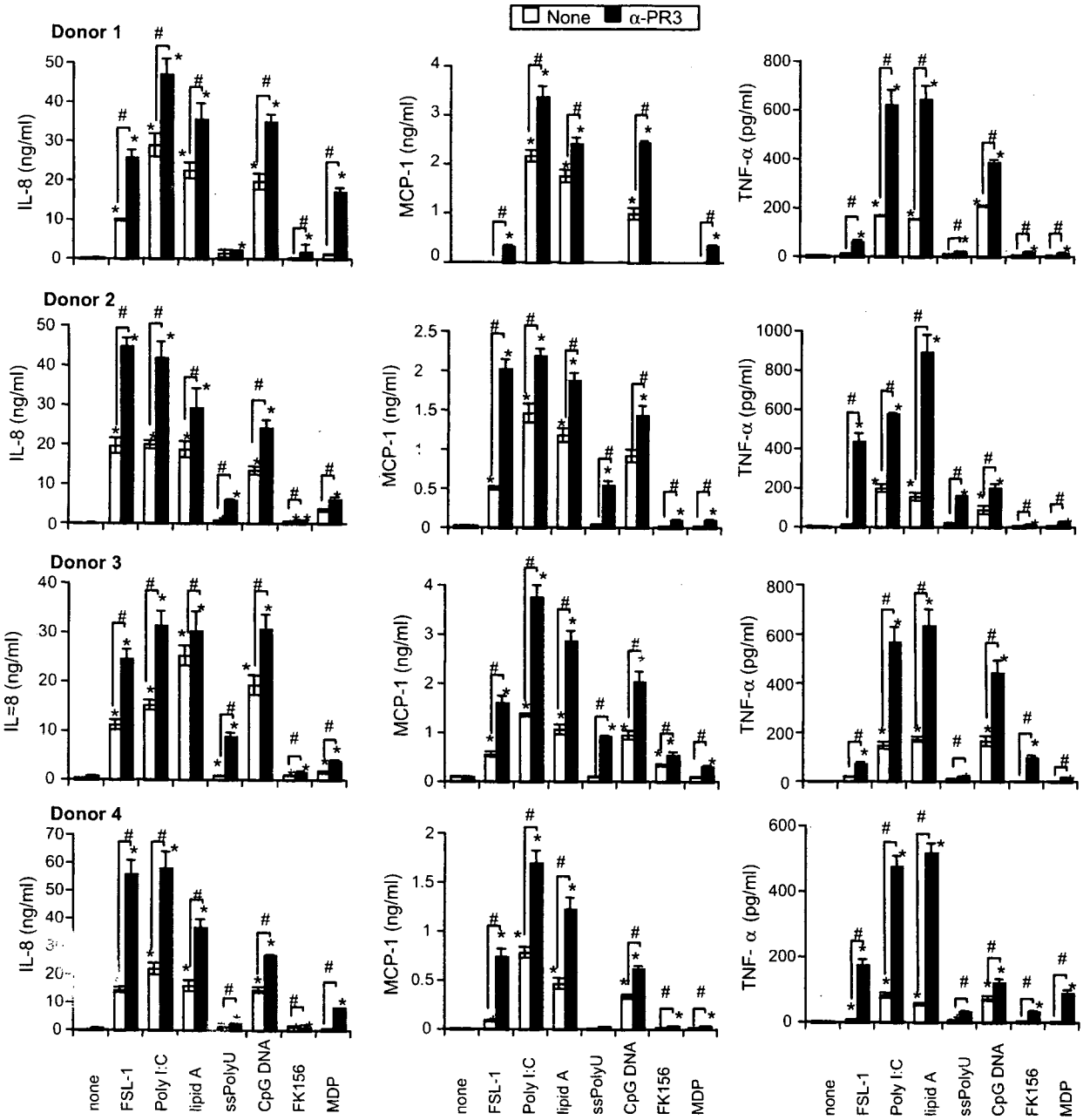

Fig. 4. Enhancement of TLR and NOD ligand-induced cytokine productions in human PBMCs preincubated with anti-PR3 Abs. PBMCs (donor 1–4) were preincubated for 6 h with anti-PR3 Abs (closed bar), with equal amounts of an isotype-matched IgG (sham incubation, open bar) or without antibodies (none). Subsequently, PBMCs were challenged with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml) or MDP (100 μg/ml) for 18 h. TNF-α, MCP-1 and IL-8 levels in the culture supernatants were determined by ELISA, and expressed as means ± S.D. *,#Significantly diffrent from sham-incubated PBMCs cells and from respective cultures stimulated with the respective ligands alone. The results are representative of three different experiments.

roiditis and polyarthritis. In contrast, the present study showed a completely different model for the possible induction of autoimmu... by bacterial components. Namely, bacterial TLR- and ...D-ligands possibly induced tissue destruction through exce... inflammatory responses by triggering the activation of AN... -primed cells. The background for the present experimental ...ionale is based on the fact that the disease activity in WG, which is paralleled by a rising ANCA titer (Tervaert et al., 1989; van der Woude et al., 1985), appears to be triggered by bacterial infections (DeRemee, 1988; Falk et al., 1990; Stegeman et al., 1994, 1996). As suggested by Condliffe et al. (1998) and Hallett and Lloyds (1995), in vivo, the response of leukocytes to bacteria depends on the state of cellular activation, varying from "dormant" via "primed" to "fully activated". Priming is a key mechanism involved in the regulation of the leukocyte-dependent host defense. Although they do not directly activate neutrophil and monocyte functions, priming agents induce a "sensitization" of the leukocytes for subsequent stimulation with naturally occurring agonists, such as bacteria-derived products. In addition to the important roles of ANCA in the regulation of inflammatory leukocyte functions, the phenomenon of leukocyte priming by ANCA may also be relevant to the pathogenesis of

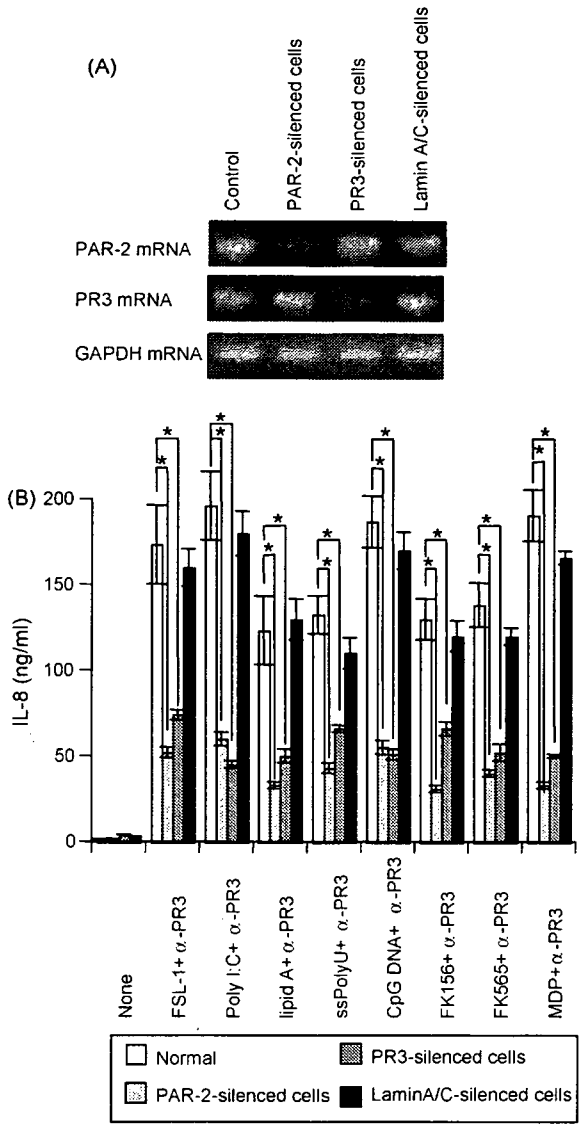

Fig. 5. Involvement of PAR-2 and endogenous PR3 in anti-PR3 Ab-induced cellular activation. (A) THP-1 cells were transfected with PAR-2-, PR3- or Lamin A/C-specific siRNA. After transfection for 24 h, the expression of PAR-2, PR3 and GAPDH mRNA was analyzed by RT-PCR. (B) THP-1 cells were transfected with PAR-2-, PR3- or Lamin A/C-specific siRNA for 24 h, and preincubated for 6 h with anti-PR3 Abs, with equal amounts of an isotype-matched IgG (sham incubation) or without antibodies (none). Subsequently, the cells were challenged with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml), FK565 (100 μg/ml) or MDP (100 μg/ml) for 18 h. The IL-8 levels in the culture supernatants were determined by ELISA and expressed as means ± S.D. *Significantly diffrent from the respective controls. The results are representative of three different experiments.

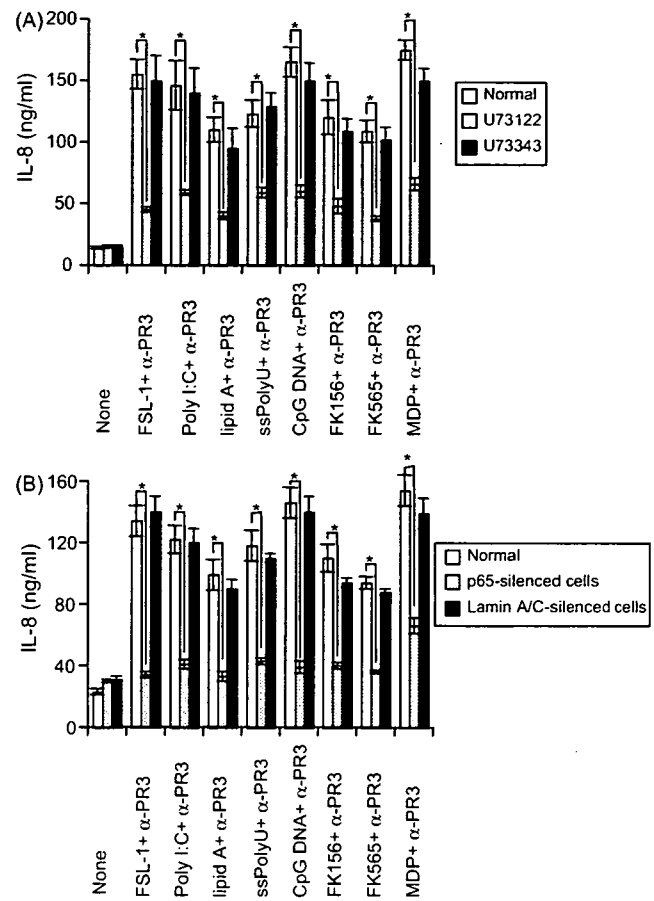

Fig. 6. Involvement of PLC and NF-κB in anti-PR3 Ab-induced cellular activation. (A) THP-1 cells were incubated with or without 10 μM U73122 or control U73343 for 30 min. Then, the cells were incubated for 6 h with anti-PR3 Abs, with equal amounts of an isotype-matched IgG (sham incubation) or without antibodies (none). This was followed by stimulation with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml), FK565 (100 μg/ml) or MDP (100 μg/ml) for 18 h. (B) THP-1 cells cultured with NF-κB p65- or Lamin A/C-specific siRNA for 24 h were incubated for 24 h with anti-PR3 Abs, with equal amounts of an isotype-matched IgG (sham incubation) or without antibodies (none), then stimulated with FSL-1 (1 nM), poly I:C (10 μg/ml), lipid A (10 ng/ml), ssPolyU (10 μg/ml), CpG DNA (1 μM), FK156 (100 μg/ml), FK565 (100 μg/ml) or MDP (100 μg/ml) for 24 h. The IL-8 levels in the culture supernatants were determined by ELISA and expressed as means ± S.D. *Significantly diffrent from the respective controls. The results are representative of four different experiments.

inflammation. Indeed, in active WG, neutrophils and monocytes display a phenotype attributable to a state of cellular preactivation with the enhanced surface expression of activation marker such as CD11b and CD64 (Muller Kobold et al., 1998, 1999).

In conclusion, anti-PR3 Abs (ANCA), being only weak direct activators of monocytes and neutrophils to release cytokines per se, exert a major priming effect on these leukocytes, enhancing their responsiveness to secondary stimulation with bacterial PAMPs. Up-regulation of various PRRs, including TLRs and NODs, acting as respective PAMPs, was characterized as one mechanism underlying the anti-PR3-elicited priming response. Such cooperation between anti-PR3 Abs and bacterial PAMPs may well trigger exacerbations of disease activity during infections and contribute to the persistence of inflammatory lesions, which might be a novel model for the pathogenesis of autoimmune diseases.

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Molecular cloning and functional characterization of porcine nucleotide-binding oligomerization domain-2 (NOD2)

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Abstract

The nucleotide-oligomerization domain (NOD) 2 is an important molecule involved in host defense. In this study, we report the cloning and characterization of porcine NOD2 (poNOD2) cDNA. The open reading frame of poNOD2 contains 3042 bp which encode 1013 amino acid residues. The putative poNOD2 protein shares higher level of homology with human counterpart (81.6% amino acid identity) than the mouse protein (76.6% amino acid identity). In order to determine the function of poNOD2, we established human embryonic kidney (HEK) 293 cells transfected to express poNOD2 cDNA. We found that poNOD2 was expressed not only in the cytoplasm but also in the inner side of the plasma membrane of HEK293 cells. HEK293 cells expressing poNOD2 responded to muramyl dipeptide (MDP) by activation of the nuclear factor kappa B (NF-κB). Quantitative real-time PCR revealed that poNOD2 mRNA was expressed by a number of tissues isolated from adult and newborn swine such as esophagus, duodenum, jejunum, ileum, ileal Peyer's patches (Pps), colon, spleen, and mesenteric lymph nodes (MLNs). In the newborn swine, the expression of poNOD2 mRNA was detected at higher levels in MLNs and spleen as compared to other tissues. In the adult swine, the highest expression was observed in ileal Pps. Furthermore, Toll-like receptor (TLR) and NOD2 ligands as well as immunobiotic lactic acid bacteria (LAB) enhanced the expression of NOD2 in gut-associated lymphoid tissues (GALT) in adult and newborn swine. Our results implicate NOD2 as an important immunoregulator in the swine intestinal immunity.

Keywords: NOD2; cDNA cloning; Swine; GALT; Immunobiotics

1. Introduction

Innate immunity provides the first line of defense against pathogenic organisms while maintaining immunological homeostasis. Monocytes and dendritic cells along with other innate immune cells express germ-line-encoded pattern recognition receptors that recognize structurally conserved-microbial components, namely pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). Many such molecular patterns bind to Toll-like receptors (TLRs) and activate a variety of transcription factors such as nuclear factor kappa B (NF-κB), crucial for the production of T-helper (Th) 1- and/or Th2-cytokines by immune cells (Akira et al., 2006).

During the host-microbe interaction, host molecules other than TLRs also recognize and respond to PAMPs. For instance, some members of the NACHT (domain present in NAIP, CIITA, HET-E and TP1)-LRR (leucine-rich repeat) family (the NLR family) which includes nucleotide-oligomerization domain (NOD) proteins, play a crucial role in recognizing PAMPs (Meylan et al., 2006). NOD has been reported to contain three distinct domains: a carboxy-terminal LRR domain, which is involved in ligand recognition; a central NOD (also known

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as NACHT domain), which facilitates self-oligomerization and has ATPase activity, and an amino-terminal caspase-recruitment domain (CARD) or pyrin domain that is involved in protein–protein interaction (Strober et al., 2006). NOD1 and NOD2 recognize low-molecular peptidoglycan (PGN) fragments (γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively). They mediate activation of mitogen-activated protein kinases (MAPKs) and NF-κB through a CARD-dependent recruitment of RIP2 (Inohara et al., 2003; Viala et al., 2004).

In recent years, there has been a growing interest in the swine immune system because of its potential as a model for the study of human immune system with lower cost (Shimosato et al., 2005a; Tohno et al., 2005b). In addition, in the digestive system, swine gastrointestinal tract shows many structural aspects more similar to the human system as compared to rodent systems. However, the swine immune system and its relationship to the pattern recognition receptors remain poorly understood. Although we have previously cloned several porcine TLR family members and analyzed the specificity of their ligands (Shimosato et al., 2003, 2005a; Tohno et al., 2005a, 2007a), the cellular and molecular mechanisms underlying NOD recognition of PAMPs in swine immune system require elucidation.

In the current study, we cloned and sequenced porcine NOD2 (poNOD2) cDNA isolated from ileal Peyer's patches (Pps) of the adult swine. We established a cell line over expressing poNOD2 and investigated its function. In addition, we examined the expression of poNOD2 in diverse tissues from adult and newborn swine and analyzed the capacity of PAMPs and immunobiotic lactic acid bacteria (LAB) to regulate the expression of poNOD2 in gut-associated lymphoid tissues (GALT) of adult and newborn swine.

2. Materials and methods

2.1. Experimental tissues

Experimental tissues (esophagus, duodenum, jejunum, ileum, ileal Pps, colon, spleen and mesenteric lymph nodes (MLNs)) were obtained from newborn LWD swine (genotype 1/2 Duroc, 1/4 Landrace, 1/4 Large White; Hiruzu Co., Ltd., Miyagi, Japan) before taking colostrum. Intestinal tissues and GALT from newborn swine were prepared as described previously (Tohno et al., 2006). Adult swine experimental tissues were excised from LWD swine (age 1-year-old; Hiruzu Co., Ltd.). The swine used were clinically healthy and free of infectious diseases. All procedures were conducted in accordance with the Guidelines for Animal Experimentation of Tohoku University, Japan.

2.2. Cloning of poNOD2

Total RNA was isolated from adult ileal Pps using an RNeasy Protect Starter Kit (Qiagen, Tokyo, Japan) as previously described (Shimosato et al., 2003). Primers were synthesized with the sequences deduced from alignment of conserved sequences of human (accession No. NM_022162) and mouse NOD2 (accession No. NM_145857) (Table 1). These primers were used to synthesize the first-strand cDNA by THERMOSCRIPT reverse transcriptase (Invitrogen, Carlsbad, CA, USA) from the total RNA. The PCR products were obtained and subcloned into the vector pGEM-T easy DNA (Promega, Madison, WI, USA). The 5′- and 3′-flanking regions of poNOD2 was determined with a 5′ and 3′ RACE system for rapid amplification of cDNA ends (Invitrogen). DNA sequencing was performed by the dideoxy chain termination method using a Model 4000 L DNA sequencer (Li-Cor, Lincoln, NE, USA). GENETYX-SV/RC Ver.13.0.6 software (GENETYX Co., Tokyo, Japan) was used to analyze nucleotide and deduced amino acid (aa) sequences. SMART architecture research program (http://smart.embl-heidelberg.de/) (Schultz et al., 2000) was used for the identification and annotation of protein domains.

2.3. Phylogenetic analysis

The amino acid sequences of poNOD2, and other reported NOD1 and NOD2 retrieved from the EMBL and GenBank databases were compared. The accession numbers used in this analysis are, NOD1: mouse (NM_172729), human (NM_006092), chimpanzee (XM_001165432), rhesus monkey (XM_001085719); NOD2: porcine (AB195466), mouse (NM_145857), human (NM_022162), chimpanzee (AH013759), cotton-top tamarin (AH013757), white-handed gibbon (AH013758), bovine (NM_001002889), american bison (AH013660), zebu cattle (AH013659), fugu rubripes (NM_001042448), purple urchin (XM_001175745). Multiple sequence alignments were generated using Clustal W (Thompson et al., 1994). A phylogenic tree of NOD1 and NOD2 based on amino acid sequences was generated by the MEGA 3.0 program (Saitou and Nei, 1987) and distances were analyzed using the neighbor joining method. Reliability of the tree was assessed by, performing 1000 bootstraps.

2.4. Polyclonal antibody (Ab) to poNOD2

The poNOD2 protein was analyzed using the GENETYX-SV/RC Ver.13.0.6 software to predict secondary structure, hydrophobicity, and antigenicity. Based on this analysis, 14 amino acid residues (NFSPEETEQLSQRD; aa 995–1008) were chosen for antigen peptide synthesis. Synthetic polypeptides added C at N-terminus were emulsified at a 1:1 ratio in Freund's Complete Adjuvant. The anti-poNOD2 polyclonal Ab was generated by immunizing Japanese white rabbits with synthetic polypeptides at a dose of 0.3 mg/rabbit. After two boosters at monthly intervals, rabbits were bled, and the anti-sera was collected and purified by epitope affinity chromatography. Finally, high Ab titers for poNOD2 were identified by a direct enzyme-linked immunosorbent assay (data not shown).

2.5. Cells

Human embryonic kidney cell line, HEK293 was obtained from the TKG cell bank (Institute of Development, Aging

Table 1
Primer sequences used in this study

	Sense primer	Antisense primer	Reference
Porcine NOD2 gene cloning primer			
Porcine NOD2 (100–71)	TTTCAGGCACAGAGGAGCCA	TTCCAGCAGCCAGGAAGTTC	
Porcine NOD2 (262–02)	CTTCTGGA CA CCGTCTGGA A	TGTAGAAGGAAGGCAGCCAA	
Porcine NOD2 (541–254)	GCAAGAAGGCTGCTTGATCT	ATTCTTGAGCAGGTTGCCCT	
Porcine NOD2 (1116–553)	CCGTGTCCTGTTAACCTTTG	AGGATCAGCAGGTACATGTC	
Porcine NOD2 (1468–974)	TGGATGGTGTCCARATGCCA	CTGCTTTTCTTGCAGCCTGA	
Porcine NOD2 (1816–624)	TTCCAGTGCTTCTTTGCCGC	GCCAAGAAGTTCCTCTTGCA	
Porcine NOD2 (2584–834)	TCCATGGCTAAGCTCCTTGC	TTCTCCTCCAGGCAGAGTTC	
Porcine NOD2 (2828–117)	GTGCTCAAGCCTTAGCATTG	CAAGAGTCTGGTGTCCCTG	
Porcine NOD2 (5'RACE, cDNA synthesized)		ACGTACCCTTACTCCAGACA	
Porcine NOD2 (5'RACE, 1st nested PCR)	5'RACE abridged anchor primer	ACTCCAGACAGTGTCCAGAA	
Porcine NOD2 (5'RACE, 2nd nested PCR)	Abridged universal amplification primer	CAGTCCAGAATGCTCTCGAA	
Porcine NOD2 (3'RACE, cDNA synthesized)		Oligo-d(T)17 Adapter Primer	
Porcine NOD2 (3'RACE, 1st nested PCR)	ATCCTGGAAGTCTGGCTCCGAGGAA	Abridged Universal Amplification Primer	
Porcine NOD2 (3'RACE, 2st nested PCR)	TCTCCAGAGGAGACTGAGCAGCTCA	Abridged Universal Amplification Primer	
RT-PCR			
Human β-actin	GGATGCAGAAGGAGATCACTG	CGATCCACACGGAGTACTTG	Shimosato et al., 2005a
Porcine NOD2	CCTGCCTTTTGAAGATGCTG	CTAGGCAAAGATTCTCTGCC	In this study
Real-time PCR			
Porcine β-actin	CATCACCATCGGCAACGA	GCGTAGAGGTCCTTCCTGATGT	Tohno et al.,2006
Porcine NOD2	GAGCGCATCCTCTTAACTTTCG	ACGCTCGTGATCCGTGAAC	In this study

5'RACE abridged anchor primer, Abridged Universal Amplification Primer and Oligo-d(T)17Adaptor Primer were purchased from Invitrogen.

and Cancer, Tohoku University, Sendai, Japan) and was maintained in complete Dulbecco's modified Eagle's medium (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Sigma), 50 mg/mL penicillin/streptomycin, 2 mM L-glutamine, 10 mM 2-[4-(2-hydroxyethyl)-1-piperadinyl] ethansulfonic acid, 0.11 mg/mL sodium pyruvate, and 0.5 mM 2-mercaptoethanol (Sigma).

2.6. Transfection

HEK293 cells were transfected with plasmids according to the procedures described in our previous report (Tohno et al., 2005a, 2007b). Briefly, the cells (4×10^5/well) were plated in six-well plates 24 h prior to transfection. The cells were transfected with the pcDNA3 vector (Invitrogen) encoding C-terminally FLAG-tagged poNOD2 (1 μg/well) using Lipofectamine™ 2000 (Invitrogen). The poNOD2-expressing transfectants were selected with G418 (Invitrogen) and the expression of poNOD2 was confirmed by intracellular staining with anti-FLAG M2 monoclonal Ab (Sigma). Transfectants expressing poNOD2-FLAG were designated HEK293^{poNOD2}. To construct non-tagged transfectants, the cells were transiently transfected with the pcDNA vector encording non-tagged poNOD2. HEK293 cells transfected with control plasmid vector were designated HEK293^{Cont}.

2.7. poNOD2 expression analysis

First, cDNA was synthesized from total RNA prepared from the HEK293 cells using TRIZOL reagent (Invitrogen) and amplified using rTaq polymerase (TaKaRa Bio Inc., Otsu, Japan) as previously described (Shimosato et al., 2004). The primers used for reverse transcription-polymerase chain reaction (RT-PCR) are listed in Table 1. The expression of β-actin was used as a control. PCR products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining.

For protein expression, flow cytometry was conducted as described (Tohno et al., 2005a). Briefly, HEK293^{poNOD2} cells were washed and stained for C-terminal FLAG-poNOD2 using anti-FLAG or anti-poNOD2. After washing with phosphate buffered saline (PBS) containing 2% FCS, the cells were treated with phycoerythrin (PE)-conjugated anti-mouse IgG (Sigma) or Alexa488-conjugated anti-rabbit IgG (Molecular Probes). PE or Alexa488 signal was determined by flow cytometry using a FACSCalibur™ (Becton Dickinson, Tokyo, Japan). Permeabilized cells were stained in a staining buffer containing 0.1% saponin (Sigma) (Tohno et al., 2006). Extracellular staining was also conducted using a staining buffer without saponin. The negative controls were HEK293^{Cont} or HEK293^{poNOD2} stained with the secondary Ab only. Both negative controls showed low levels of background fluorescence.

Confocal laser scanning microscopy was performed (Tohno et al., 2005a) by growing cells on glass coverslips followed by wash with PBS, and fixation with 2% paraformaldehyde containing 0.1% Triton X-100 at 4 °C for 30 min (Wang et al., 2003). For cell surface staining, the cells were fixed with 2% paraformaldehyde at 4 °C for 30 min (Wang et al., 2003). After three washes with PBS, the cells were stained with anti-FLAG M2 monoclonal Ab followed by Alexa 647-conjugated anti-mouse IgG (Molecular Probes). The non-tagged poNOD2-expressing transfectants were stained with anti-poNOD2 polyclonal Ab followed by Alexa 488-conjugated anti-rabbit IgG. All cells were protected from direct light exposure, and were washed three times with PBS and then stained with SYTOX orange (Molecular