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Cellular Signaling Molecules Associated with Peptidoglycan-Induced CCL3 Up-Regulation

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Abstract

Peptidoglycan (PGN) is detected in inflammatory cell-rich regions of human atheromatous plaques. The present study investigated the effects of PGN on CC chemokine ligand 3 (CCL3) expression, which is elevated in the atherosclerotic arteries, and determined cellular factors involved in PGN-mediated CCL3 up-regulation in mononuclear cells, with the goal of understanding the molecular mechanisms of inflammatory responses to bacterial pathogen-associated molecular patterns in diseased arteries. Exposure of human monocytic leukemia THP-1 cells to PGN resulted in enhanced secretion of *CCL3* and profound induction of the *CCL3* gene transcript. Both events were abrogated by oxidized 1-palmitoyl-2-arachidonosyl-sn-phosphatidylcholine, an inhibitor of Toll-like receptors 2/4. Pharmacological inhibitors such as U0126, SP6001250, Akt inhibitor IV, rapamycin, RO318220, diphenyleneiodonium chloride, and N-acetylcysteine also significantly attenuated PGN-mediated CCL3 up-regulation. However, polymyxin B, LY294002, and SB202190 did not influence CCL3 expression. We propose that PGN contributes to enhanced CCL3 expression in atherosclerotic plaques and that Toll-like receptors (TLR2), Akt, mTOR, mitogen-activated protein kinase, and reactive oxygen species are involved in that process.

Key Words: CCL3, Macrophages, Peptidoglycan

INTRODUCTION

The immigration and infiltration of monocytes and T cells into the vascular wall, which is guided by endothelial leukocyte adhesion molecules and chemokines, are prominent features in both human and experimental atherosclerotic disease (Libby, 2002; Aukrust *et al.*, 2008). Chemokines are divided into four families (CC, CXC, CX3C, XC) based on the position of the first two cysteine residues. Among them, the CC chemokine family is detected within atherosclerotic lesions, where it controls homeostasis and other activities of emigrated cells (Liehn *et al.*, 2006). CC chemokine ligand 3 (CCL3), also known as macrophage inflammatory protein-1α, is expressed at higher levels in human arteriosclerotic lesions than in normal aortic tissue (Hayes *et al.*, 1998). As CCL3 is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes (Menten *et al.*, 2002), clinical and animal studies have linked CCL3 to atherosclerosis. Enhanced CCL3 expression in ApoE −/− mice is associated with progression and stability of atherosclerotic plaques (Lutgens *et al.*, 2005). Peripheral blood mononuclear cells from patients with familial hypercholesterolemia spontaneously release high level of CCL3 (Holven *et al.*, 2003). In addition, drugs with anti-atherogenic properties, such as hydroxymethylglutaryl-CoA reductase inhibitors, down-regulate CC chemokine ligands and receptors. Simvastatin suppresses expression of the chemokines CCL2, CCL3, and CCL4, as well as the chemokine receptors CCR1, CCR2, CCR4, and CCR5, in endothelial cells and macrophages (Veillard *et al.*, 2006). Therefore, it is important to understand the regulation of CCL3 expression because of its close association with atherosclerosis.

High serum levels of cholesterol, like oxidized low density lipoprotein, play an important role in the proinflammatory process that triggers initiation of atherosclerosis (Libby, 2002). In addition, the overall cumulative infectious burden can act as an additional factor accelerating disease progression (Espinola-Klein *et al.*, 2002). Infectious pathogens including *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, and cytomegalovirus are detected in atherosclerotic lesions (Espinola-Klein *et al.*, 2002; Taniguchi *et al.*, 2003). Peptidoglycan (PGN), the major constituent of the cell wall of Gram-positive bacteria and a component of the outer membrane of Gram-negative bacteria, is a pathogen-associated molecular pattern (PAMP) that

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has been detected in human atherosclerotic lesions (Laman *et al.*, 2002). At nonmucosal sites like vasculature, PGN is recognized by Toll-like receptors (TLRs) expressed by immune cells (e.g. macrophages) (Yoshimura *et al.*, 1999), which triggers intracellular signal transduction pathways, resulting in the production of pro-inflammatory cytokines (Langer *et al.*, 2008) and chemokines (Wang *et al.*, 2000). Since PGN is presumed to be an additional proinflammatory factor in atherosclerotic lesions (Laman *et al.*, 2002), elucidation of the mechanism of action of PGN-induced inflammatory responses will broaden current knowledge of the roles of bacterial PAMPs in atherogenesis.

In the present study, we investigated whether PGN affected the secretion of CC chemokines involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes. PGN significantly enhanced the secretion of CCL3 by THP-1 cells. Moreover, cellular molecules involved in PGN-mediated CCL3 expression were identified; TLR2, Akt, mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPKs), and reactive oxygen species (ROS) were shown to be involved in PGN-mediated CCL3 up-regulation.

MATERIALS AND METHODS

Cell culture and materials

The THP-1 human acute monocytic leukemia cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained as suggested by ATCC. PGN isolated from *Staphylococcus aureus*, polymyxin B and oxidized 1-palmitoyl-2-arachidonosyl-sn-phosphatidylcholine (OxPAPC) were purchased from InvivoGen (San Diego, CA). Endotoxin-free bovine serum albumin (BSA), RO318220, GF109203X, LY294002, diphenyleneiodonium chloride (DPI), N-acetylcysteine (NAC), rapamycin, and SP600125 were purchased from Sigma-Aldrich (St. Louis, MO). U0126, SB202190, and Akt inhibitor IV (Akti IV) were purchased from Cell Signaling Technology (Danvers, MA). For inhibition experiments, THP-1 cells were treated for 1 h with indicated chemicals prior to stimulation for 9 h with PGN (1 μg/ml). CCL3 released into the medium was measured by an enzyme linked immunosorbent assay (ELISA) and CCL3 gene transcript was amplified by reverse transcription-polymerase chain reaction (RT-PCR).

CCL3 ELISA

The amount of secreted CCL3 was determined using commercially available ELISA kits, according to the manufacturer's instructions (BD Biosciences, San Diego, CA). THP-1 cells incubated with 0.1% BSA in RPMI medium 1640 overnight were exposed to PGN and the cell culture medium was collected. The medium and standards for CCL3 were added to a microtiter plate precoated with monoclonal antibody against CCL3. After incubation for 2 h, the plate was washed and incubated with an enzyme-conjugated polyclonal antibody specific for CCL3. The substrate was added after several plate washes, and color intensity was measured. The amount of CCL3 present in the medium was determined from a standard curve. Data are expressed as mean ± SD.

RT-PCR

THP-1 cells incubated with 0.1% BSA in RPMI medium 1640 overnight were exposed to PGN. Total RNAs were extracted from cells and reverse-transcribed for 1 h at 42°C with Moloney murine leukemia virus reverse transcriptase. PCR amplification was performed for 25 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) in the presence of primers. The primers for CCL3 were 5-TTTCTGGACCCACTCCTCAC-3 (forward) and 5-GGCTCTCTGCAACCAGTTCT-3 (reverse), and the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5-AAGCTCTGCGTGACTGTCCT-3 (forward) and 5-GCTTGCTTCTTTTGGTTTGG-3 (reverse). PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Statistical analysis (1-way ANOVA) was performed using PRISM, version 5.0 (GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

RESULTS

Up-regulation of CCL3 expression by PGN at messenger and protein levels

In order to investigate the effects of PGN on CCL3 expression in macrophages, we examined the level of *CCL3* gene transcript by RT-PCR (Fig. 1A, B). The *CCL3* gene transcript

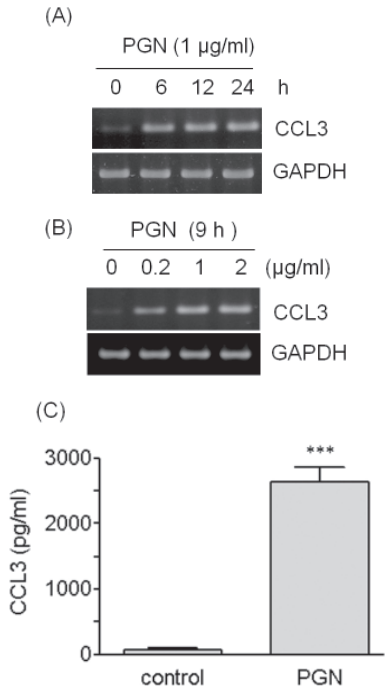

Fig. 1. Expression of CCL3 at the messenger and protein levels in response to PGN. THP-1 cells (1×10^6 cells/ml) were incubated for indicated time periods with 1 μg/ml PGN or for 9 h with the indicated amount of PGN (A, B). *CCL3* gene transcripts were amplified by RT-PCR. THP-1 cells (1×10^6 cells/ml) were stimulated for 9 h with or without (control) PGN (1 μg/ml). (C) The amount of CCL3 released into the medium was measured by ELISA. ***$p<0.001$ vs. control.

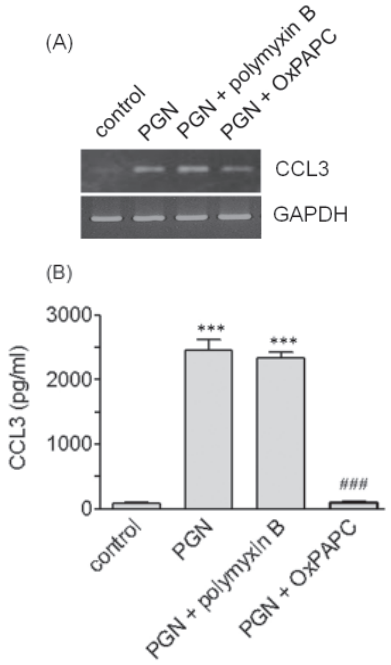

Fig. 2. Effects of OxPAPC and polymyxin B on PGN-mediated CCL3 up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after treatment for 1 h with OxPAPC (30 μg/ml) or polymyxin B (10 mg/ml). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, $^{###}p$<0.001 vs. PGN.

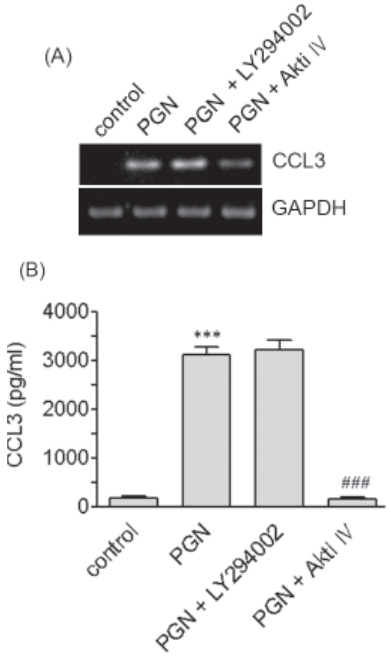

Fig. 3. Effects of LY294002 and Akti IV on PGN-mediated CCL3 up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after pretreatment with LY294002 and Akti IV (10 μM each). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, $^{###}p$<0.001 vs. PGN.

from THP-1 cells was barely detected in the absence of PGN. However, PGN significantly induced transcription of CCL3 as early as 6 h post-treatment and persisted up to 24 h post-treatment. The PGN-mediated induction of *CCL3* gene transcript occurred in the presence of as low as 0.2 μg/ml of PGN. We investigated whether PGN had any effect on the release of the chemokine (Fig. 1C). ELISA revealed that THP-1 cells secreted a low amount of CCL3 in the absence of PGN (70 pg/ml), while CCL3 release was remarkably increased in response to PGN (2,630 pg/ml). We examined if TLRs mediated CCL3 expression in response to PGN using OxPAPC, a TLR2/4 inhibitor (Fig. 2). Treatment with OxPAPC inhibited PGN-mediated expression of CCL3 at the mRNA and protein levels. PGN-mediated secretion of CCL3 was almost completely blocked and reduced to that of the control cells in the presence of OxPAPC, and PGN-induced expression of CCL3 transcripts was also abrogated in the presence of OxPAPC. Since it is conceivable that PGN preparations could be contaminated with lipopolysaccharide (LPS), which increases the secretion of pro-inflammatory cytokines and chemokines, we examined whether LPS contributed to PGN-mediated CCL3 up-regulation using polymyxin B, a potent inhibitor of LPS. Polymyxin B did not attenuate PGN-mediated CCL3 secretion or expression of its transcript (Fig. 2).

Roles Akt and mTOR in PGN-induced CCL3 expression

PGN activates Akt pathway as it enhances Akt phosphorylation (Lee *et al.*, 2011). To investigate if Akt might participate in PGN-mediated CCL3 expression, we used two inhibitors, LY294002 and Akti IV (Fig. 3). LY294002, a morpholine derivative of quercetin, is a potent reversible inhibitor of phosphoinositide 3-kinases (PI3Ks), an Akt activator. Akti IV is an inhibitor of Akt protein kinase. Akti IV significantly affected CCL3 expression at the mRNA and protein levels. Akti IV markedly attenuated PGN-mediated induction of *CCL3* gene expression and completely blocked CCL3 secretion. LY294002 did not affect PGN-induced gene transcription and secretion of CCL3. Akt exerts its biological effects through the activation of mTOR (Hahn-Windgassen *et al.*, 2005). Therefore, we investigated whether mTOR participated in PGN-mediated CCL3 expression using rapamycin, an inhibitor of mTOR (Fig. 4). Rapamycin affected CCL3 expression. PGN-mediated CCL3 secretion was significantly reduced in the presence of rapamycin with attenuation of PGN-induced *CCL3* gene transcription.

Roles of PKC in PGN-induced CCL3 expression

We have reported that PGN activates PKC in THP-1 cells (Lee *et al.*, 2011). To assess the roles of PKC in PGN-mediated CCL3 up-regulation, we used two different PKC inhibitors, GF109203X and RO318220. Of the two inhibitors, RO318220 not only remarkably attenuated PGN-induced *CCL3* gene transcription but also almost completely blocked PGN-mediated CCL3 secretion. GF109203X, however, only slightly attenuated PGN-mediated CCL3 up-regulation at the mRNA and protein levels (Fig. 5).

Roles of MAPKs and ROS in PGN-induced CCL3 expression

PGN enhances phosphorylation of extracellular signal-regulated kinase (ERK), p38 MAPK, and c-jun N-terminal kinase (JNK) (Lee *et al.*, 2011). To assess the roles of MAPKs in PGN-induced CCL3 up-regulation, we used the following MAPK inhibitors: SB202190 (p38 MAPK inhibitor), SP600125

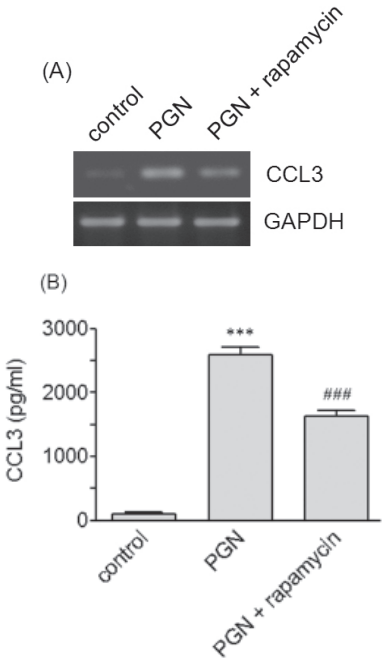

Fig. 4. Effects of rapamycin on PGN-mediated *CCL3* up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after pretreatment with rapamycin (100 nM). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, ###p<0.001 vs. PGN.

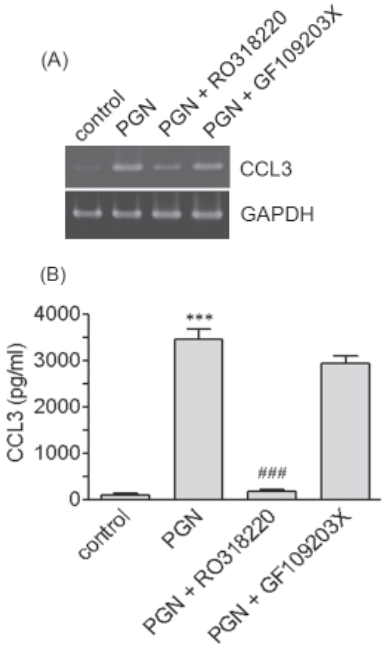

Fig. 5. Effects of PKC inhibitors on PGN-mediated CCL3 up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after pretreatment with RO318220 (1 μM) or GF109203X (3 μM). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, ###p<0.001 vs. PGN.

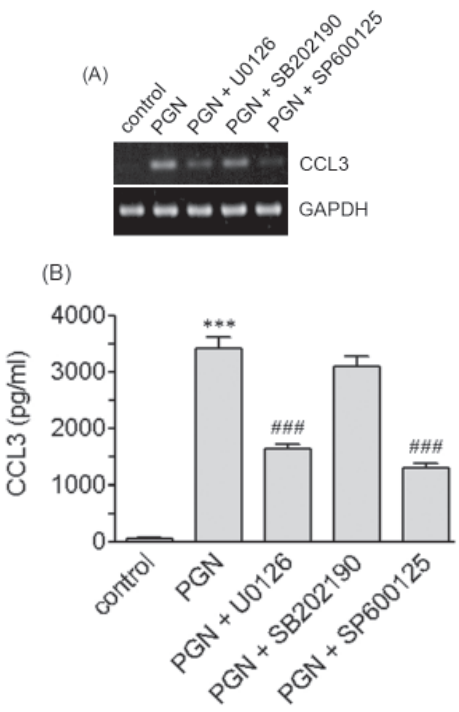

Fig. 6. Effects of inhibitors of MAPKs on PGN-mediated CCL3 up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after pretreatment with indicated MAPKs inhibitors (10 μM each). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, ###p<0.001 vs. PGN.

(JNK inhibitor), and U0126 (ERK inhibitor) (Fig. 6). U0126 and SP600125 significantly blocked PGN-mediated CCL3 secretion as well as profoundly inhibiting PGN-induced *CCL3* gene transcription. SB202190 slightly attenuated PG-induced *CCL3* gene transcription without influencing secretion of the chemokine. We also investigated whether ROS influenced CCL3 expression using NAC and DPI (Fig. 7). DPI significantly inhibited PGN-mediated CCL3 secretion as well as attenuating PGN-induced *CCL3* gene transcription. NAC affected CCL3 expression at the protein level as NAC significantly inhibited PGN-mediated CCL3 secretion, with little influence on CCL3 transcription.

DISCUSSION

The present study demonstrates that PGN, a bacterial component present in atherosclerotic lesions, up-regulates CCL3 expression both at the mRNA and protein levels in human macrophage THP-1 cells. This finding is consistent with the reported induction of CCL2, CCL3, CCL4, and CXCL8 expression by PGN and LPS in human blood monocytes (Wang *et al.*, 2000). It is possible that LPS contamination occurring during PGN preparation contributes to, or may be responsible for, the production of chemokines through activation of TLR4. To specifically investigate whether LPS contributed to PGN-mediated CCL3 expression, we used polymyxin B, which abrogates the biological effects of LPS by binding to it (Cardoso *et al.*, 2007). Polymyxin B had no influence on PGN-mediated

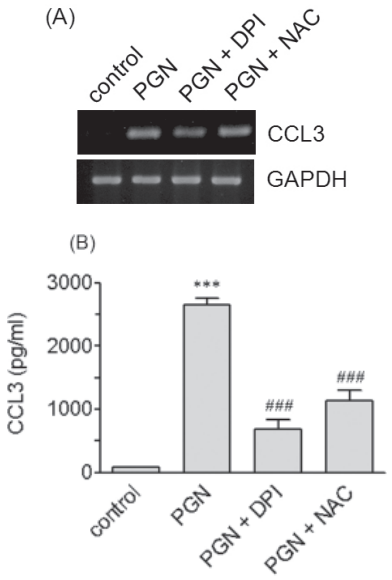

Fig. 7. Effects of ROS quenchers on PGN-mediated CCL3 up-regulation. THP-1 cells were stimulated for 9 h with or without PGN (1 μg/ml) after pretreatment with DPI (10 μM) or NAC (5 mM). *CCL3* gene transcript was amplified (A), and amount of CCL3 released into the medium was measured (B). ***p<0.001 vs. control, ###p<0.001 vs. PGN.

CCL3 expression at both the mRNA and protein levels, which indicates that the up-regulation of CCL3 observed in this study was due to induction by PGN.

Even though it is evident that PGN induces expression of CCL3 chemokine, cellular molecules involved in CCL3 expression have not yet been clarified. In the present study, we tried to identify the cellular factors that contribute to PGN-mediated CCL3 expression. Because PGN is a bacterial PAMP recognized by TLR2 (Kawai and Akira, 2006), we investigated receptor-mediated CCL3 expression using OxPAPC, an inhibitor of TLR2/4. OxPAPC blocked the secretion of the chemokine and transcription of its gene. The complete inhibition of PGN-mediated CCL3 expression by OxPAPC, but not by polymyxin B, indicates that TLR2 is responsible for PGN-induced CCL3 expression.

We previously reported the enhanced Akt phosphorylation in response to PGN (Lee *et al.*, 2011). Since this indicates PGN-mediated activation of the kinase, we presently investigated if Akt and PI3K were involved in PGN-mediated CCL3 expression. Akt inhibition completely blocked the secretion of CCL3 and also inhibited transcription of its gene, which indicated that Akt activity is required for PGN-mediated CCL3 expression. In contrast, PI3K inhibition with LY294002 did not attenuate CCL3 expression, suggesting that PI3K is unnecessary for CCL3 expression in response to PGN. We assumed the possibility that the concentration of LY294002 employed in this study was not sufficient for blockage of PI3K. Further investigation with wortmannin, which inhibits PI3K irreversibly, or high doses of LY294002 will help to clarify the roles of PI3K in CCL3 expression in response to PGN. Akt achieves its biological effects through protein targets including mTOR (Manning and Cantley, 2007). We investigated if mTOR was involved in PGN-mediated CCL3 expression. Rapamycin inhibition of mTOR resulted in significant reduction of CCL3 secretion as well as attenuation of *CCL3* gene transcription. These results indicate that rapamycin inhibits PGN-mediated CCL3 expression at the mRNA and protein levels. Taken together, these results indicate that Akt/mTOR pathway plays a crucial role in PGN-mediated CCL3 up-regulation.

PGN elevates phosphorylation of MAPKs, and MAPKs mediate chemokine production in TLR2, 4, and 9 signaling, which indicates the involvement of the kinases in CCL3 expression (Thobe *et al.*, 2007; Lee *et al.*, 2011). Appropriately, we investigated if MAPKs played roles in the expression of the chemokine. Selective inhibition of ERK and JNK, but not of p38 MAPK, resulted in significant attenuation of CCL3 expression. These results indicate that activation of MAPKs, particularly ERK and JNK, is required for PGN-mediated CCL3 expression. Since PGN causes increased ROS production in human blood leukocytes (Saetre *et al.*, 2000), DPI and NAC were used to investigate the involvement of ROS in CCL3 expression. DPI is an inhibitor of NADPH oxidase, which produces ROS. Thus, DPI inhibits ROS formation (Miesel *et al.*, 1995). NAC, a thiol compound that can act as a cysteine source for the repletion of intracellular glutathione, acts as a direct scavenger of ROS (Spagnuolo *et al.*, 2006). Presently, DPI and NAC significantly attenuated PGN-mediated secretion of CCL3 by THP-1 cells, which suggests that ROS have a role in CCL3 expression. Of the two ROS quenchers, DPI markedly attenuated CCL3 expression at the messenger level. These results indicate that ROS actively participate in PGN-mediated CCL3 up-regulation.

We assumed that PKC would play roles in PGN-mediated CCL3 expression based on the knowledge that THP-1 cells express PKC-βI, -βII, -λ, -η, and -θ subtypes and that PKC is able to activate Akt in a TLR2-dependent fashion (Asehnoune *et al.*, 2005). Appropriately, we investigated the roles of PKC in PGN-mediated CCL3 expression using PKC inhibitors. RO318220 attenuated CCL3 expression, but GF109203X did not. These two inhibitors are bisindolylmaleimide derivatives of staurosporine and inhibit mixed isoforms of PKC. RO318220 inhibits Akt, c-Raf, MAPKK-1, and p42 MAPK in addition to PKC, but GF109203X does not (Sipma *et al.*, 1996). We found that activities of Akt and p44/42 MAPK are required for CCL3 expression. Therefore, it seems plausible to think that the additional inhibitory activity of other enzymes by RO318220 may lead to the difference between the two inhibitors in their effects on CCL3 expression. We think that drawing a conclusion on the roles of PKC should be reconsidered when experiments are performed with RO318220 or GF109203 alone. Further studies are needed to clarify whether PKC plays roles in PGN-mediated CCL3 expression.

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