

Analysis of Signaling Pathways Involved in Peptidoglycan-Induced RANTES Production from Murine Langerhans Cells

Katsuhiko Matsui^a Mali Wirotasangthong^b Akemi Nishikawa^a

^aDepartment of Immunobiology, Meiji Pharmaceutical University, Tokyo, Japan; ^bDepartment of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Key Words

Atopic dermatitis • *Staphylococcus aureus* • Peptidoglycan • RANTES • p38 MAPK

Abstract

Background: Our previous study showed that percutaneous application of peptidoglycan from *Staphylococcus aureus* induced eosinophil infiltration in murine skin through production of CCL5/RANTES (regulated upon activation in normal T cells expressed and secreted) from epidermal Langerhans cells. Although it is well known that peptidoglycan is an agonist of Toll-like receptor (TLR)-2, it is unclear whether other TLR agonists are able to induce RANTES production from Langerhans cells. **Methods:** Langerhans cells were purified from murine epidermal cells by the panning method using anti-IA^d monoclonal antibody. RANTES production by Langerhans cells was investigated by RT-PCR and ELISA. Analysis of the signaling pathways responsible for RANTES production by Langerhans cells was performed by ELISA using N-acetyl-L-cysteine, SP600125, SB203580 and PD98059, which are specific inhibitors of NF- κ B activation, JNK, p38 MAPK and ERK, respectively, and was finally confirmed by Western blot analysis. **Results:** Peptidoglycan, poly(I:C), lipopolysaccharide and CpG DNA, which signal through TLR-2, TLR-3,

TLR-4 and TLR-9, respectively, were found to strongly induce RANTES production. Treatment with N-acetyl-L-cysteine inhibited all TLR agonist-induced RANTES production. However, treatment with SP600125 did not inhibit CpG DNA-induced RANTES production. Furthermore, treatment with SB203580 inhibited only peptidoglycan- and lipopolysaccharide-induced RANTES production and the inhibition was correlated with that of p38 MAPK phosphorylation. **Conclusion:** These results suggest that the signaling pathway of RANTES production from murine Langerhans cells induced by different TLR stimuli is not necessarily the same, and that inhibition of p38 MAPK may be a more specific therapeutic target for eosinophilic inflammation in patients with atopic dermatitis associated with *S. aureus* colonization.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. The chronic skin lesions of AD patients show an increased number of inflammatory cells such as mast cells, eosinophils and mononuclear cells in the dermis, together with superfi-

cial *Staphylococcus aureus* colonization [1]. *S. aureus* can be isolated from 75–96% of skin lesions of AD patients, whereas the skins of only 5–10% of healthy individuals are colonized by this organism [2, 3]. We have also found that the detection rate of *S. aureus* on lesional skin of AD patients is higher than on the nonlesional skin [3]. Furthermore, the bacterial cell count of *S. aureus* on the lesional skin of AD patients is significantly higher than that of the nonlesional skin. However, there is no significant difference between lesional and nonlesional skin in the detection rate of *S. aureus* producing superantigenic exotoxin, which was believed to be the most important pathogenic factor from *S. aureus*.

Gram-positive bacterial cell walls are composed of highly cross-linked peptidoglycan (PEG) decorated to a variable extent with teichoic acid polymers. The latter are also linked to plasma membrane phospholipids, and this lipoteichoic acid is another major cell wall component [4, 5]. Gram-positive bacteria contain no lipopolysaccharide (LPS), and lipoteichoic acid and/or PEG are thought to be major inflammatory products present in their cell walls. Therefore, we hypothesized that lipoteichoic acid and/or PEG from *S. aureus* may be pathogenic factors in AD patients [6–8]. In a previous study, we demonstrated that percutaneous application of PEG induced eosinophil migration in the dermis, like that seen in AD patients, through CCL5/RANTES (regulated upon activation in normal T cells expressed and secreted) production by epidermal Langerhans cells (LCs) [9]. Although it is well known that PEG is an agonist of Toll-like receptor (TLR)-2, it is unclear whether other TLR agonists are able to induce RANTES from murine LCs. Therefore, in this study we investigated RANTES production by murine LCs in response to different TLR stimuli and the signaling pathways involved.

Materials and Methods

PEG derived from *S. aureus* was obtained from Fluka (Buchs, SG, Switzerland). Poly(I:C) and highly purified LPS from *Escherichia coli* R515 (Re) were purchased from Sigma (St. Louis, Mo., USA) and Alexis Biochemicals (Nottingham, UK), respectively. Phosphorothioate-stabilized CpG DNA (5'-TCCATGACGTT-CCTGATGCT-3') was obtained from Qiagen (Tokyo, Japan). Female specific-pathogen-free BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and used when they were 6–8 weeks of age. They were housed in plastic cages in a clean, air-conditioned room at 24°C and were allowed free access to a standard laboratory diet and water. All procedures performed on the mice were in accordance with the guidelines of the animal care and use committee of Meiji Pharmaceutical University, Tokyo.

LCs in the epidermis were separated as described by Tada et al. [10]. LCs (at a purity of >95%) were adjusted to 1×10^6 cells/ml in RPMI 1640 medium with L-glutamine (Sigma) containing 10% fetal bovine serum (Sigma), 25 mM HEPES (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco RBL, Grand Island, N.Y., USA) (RPMI 10). The cultures (0.2 ml/well) were incubated in 96-well culture plates (Nunc, Roskilde, Denmark) in the presence of TLR agonists at 37°C in a humidified atmosphere with 5% CO₂. The culture supernatants were collected after incubation for 48 h, and the RANTES concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits for quantification of murine RANTES (R&D Systems, Minneapolis, Minn., USA). The data were expressed as means $\pm$ SD, and differences between means were analyzed using Student's *t* test with a 2-tailed test of significance. Differences at *p* < 0.05 were considered to be significant.

In order to determine the levels of RANTES mRNA expression in LCs, mRNA was extracted from each set of TLR agonist-stimulated LCs (5×10^6 cells) after 24 h of incubation using a Quick Prep Micro mRNA purification kit (GE Healthcare, Buckinghamshire, UK). Then, cDNA was synthesized from 160 ng of the mRNA using a first-strand cDNA synthesis kit (GE Healthcare). PCR was performed using the following primers: β -actin (540 bp) 5' primer, 5'-GTGGGCCGCTCTAGGCAC-CAA-3' and 3' primer, 5'-CTCTTTGATGTCACGCAC-GATTTC-3'; RANTES (321 bp) 5' primer, 5'-CATCCTCACTGCAGCCGCC-3' and 3' primer, 5'-CCAAGCTGGCTAG-GACTAGAG-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Conn., USA) with a 25-µl reaction volume containing 1.5 µl of cDNA (corresponding to 16 ng mRNA starting material), as described previously [7], and the PCR products were separated on a 2% agarose gel containing ethidium bromide. The level of expression of each TLR mRNA in fresh LCs was also analyzed, as described above. Primers used for amplification of each TLR have been described elsewhere [11, 12].

Analysis of the signaling pathway for RANTES production from LCs was performed by ELISA, as described above, using the chemical inhibitors N-acetyl-L-cysteine (Sigma), SP600125 (Calbiochem, Darmstadt, Germany), SB203580 (Calbiochem) and PD98059 (Calbiochem), which specifically inhibit nuclear factor- κ B (NF- κ B) activation, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), respectively [13, 14]. Furthermore, the LCs (1×10^6 cells/ml) stimulated with TLR agonists in 24-well culture plates were washed twice with PBS, and whole-cell lysates were prepared with extraction buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 µg/ml aprotinin and 10 µg/ml leupeptin). Cell lysates were electrophoresed by SDS-PAGE (5–20%) and transferred to nitrocellulose membranes for Western blot analysis. The membranes were treated with Block-Ace (Dainippon Sumitomo Pharma, Osaka, Japan) overnight at 4°C and probed with primary antibodies raised against phosphorylated p38 MAPK and nonphosphorylated p38 MAPK, respectively (Cell Signaling, Beverly, Mass., USA). The primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG (Dako, Carpinteria, Calif., USA) and visualized with the ECL system (GE Healthcare).

Results

The first experiments were carried out to examine the influence of different TLR stimuli on the induction of RANTES production from murine epidermal LCs. Murine LCs were treated with PEG, poly(I:C), LPS and CpG DNA, which signal through TLR-2, TLR-3, TLR-4 and TLR-9, respectively [15, 16], and the production of RANTES from LCs was confirmed by RT-PCR analysis after 24 h of incubation and by ELISA after 48 h of incubation. Figure 1 shows that all TLR agonists tested elicited RANTES mRNA expression (fig. 1a), and the expression of mRNA was correlated with the synthesis of cytokine protein (fig. 1b). Expression of TLRs by fresh LCs was also confirmed by RT-PCR to explain their capacity to respond to each TLR agonist (fig. 1c). LCs after 48 h of stimulation with TLR ligands had a cell yield of $>0.9 \times 10^6$ cells/ml with a cell viability of $>92\%$, and no significant differences were evident between nonstimulated and stimulated LCs (data not shown).

Signaling through TLRs occurs by sequential recruitment of the adaptor molecule MyD88 and the serine/threonine kinase IRAK, and subsequently activates MAPKs and NF- κ B [17]. There are at least 3 distinct MAPK signaling pathways in mammals, including ERK, JNK and p38 MAPK [18]. Next, therefore, we examined whether the signaling pathway for RANTES production from LCs is identical among all TLR stimuli by using specific inhibitors of NF- κ B activation, JNK, p38 MAPK and ERK, respectively. As shown in figure 2, treatment with a chemical inhibitor of NF- κ B, N-acetyl-L-cysteine, completely inhibited RANTES production induced by all TLR stimuli. Treatment with the JNK inhibitor SP600125 strongly inhibited PEG-, poly(I:C)- and LPS-induced RANTES production, whereas the inhibitory effect on CpG DNA-induced RANTES production was not significant. Furthermore, an inhibitor of p38 MAPK, SB203580, markedly inhibited PEG- and LPS-induced RANTES production, whereas the inhibitory effect on poly(I:C)- and CpG DNA-induced RANTES production was not significant. Interestingly, our results indicated that the ERK pathway in PEG- and LPS-stimulated LCs negatively regulated RANTES production, because LC treatment with an ERK inhibitor, PD98059, induced up-regulation of RANTES production. None of the inhibitors had any effect on RANTES production from nonstimulated LCs or the cell yield of nonstimulated and stimulated LCs, and inhibition of RANTES production from LCs was also observed after simultaneous treatment with inhibitors and TLR ligands (data not shown).

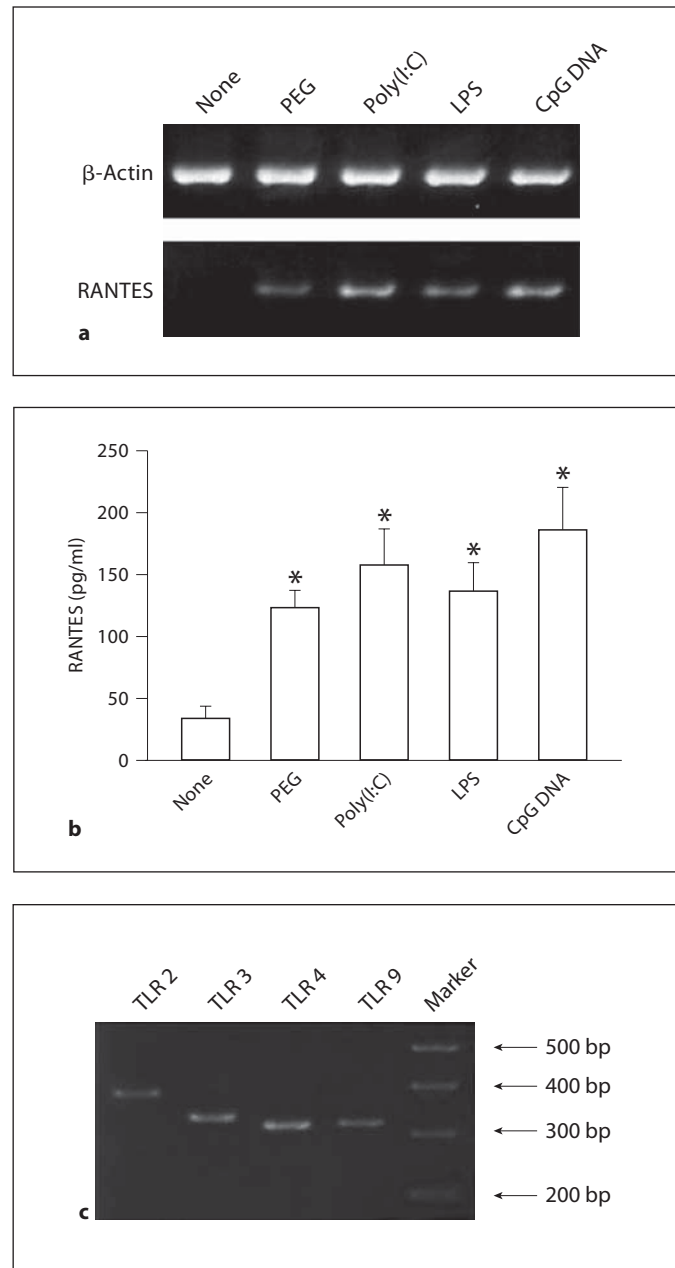


Fig. 1. Effects of TLR ligands on RANTES production from epidermal LCs. LCs (1×10^6 /ml) from mouse epidermis were incubated with 10 μ g/ml PEG, 10 μ g/ml poly(I:C), 1 μ g/ml LPS and 1 μ M CpG DNA for 24–48 h. **a** After 24 h of incubation, cytoplasmic mRNA was extracted from LCs (5×10^5 cells), reverse-transcribed, and amplified by PCR using β -actin and RANTES primer sets. The data shown are representative results of 4 independent experiments. **b** After 48 h of incubation, culture supernatants were assayed for RANTES production using ELISA. The results are expressed as means $\pm$ SD ($n = 5$). **c** Cytoplasmic mRNA from fresh LCs (5×10^5 cells) was also reverse-transcribed and amplified by PCR using each TLR primer set. The data shown are representative results of 4 independent experiments. * $p < 0.01$ versus the nontreatment group.

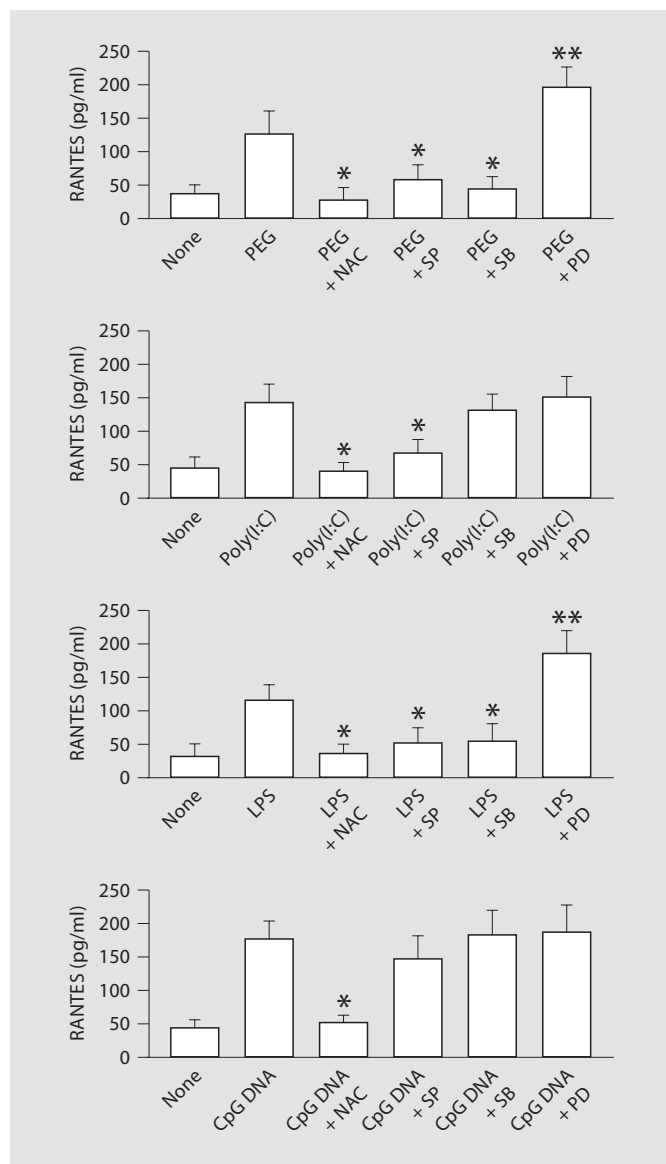


Fig. 2. Inhibition of RANTES production by chemical inhibitors of signaling pathways. LCs (1×10^6 /ml) from mouse epidermis were pretreated with 5 mM N-acetyl-L-cysteine (NAC), 2.5 μ M SP600125 (SP), 250 nM SB203580 (SB) and 25 μ M PD98059 (PD) for 30 min, and then the cells were incubated with 10 μ g/ml PEG, 10 μ g/ml poly(I:C), 1 μ g/ml LPS or 1 μ M CpG DNA. After 48 h of incubation, the culture supernatants were assayed for RANTES production using ELISA. The results are expressed as means $\pm$ SD (n = 5). * $p < 0.01$; ** $p < 0.05$ versus TLR ligand stimuli.

Next, to verify the activity of an inhibitor that blocks the activation of p38 MAPK, LCs were treated with SB203580, and Western blotting was performed to detect the phosphorylation of p38 MAPK. As shown in figure 3, PEG and LPS stimulation up-regulated the phosphoryla-

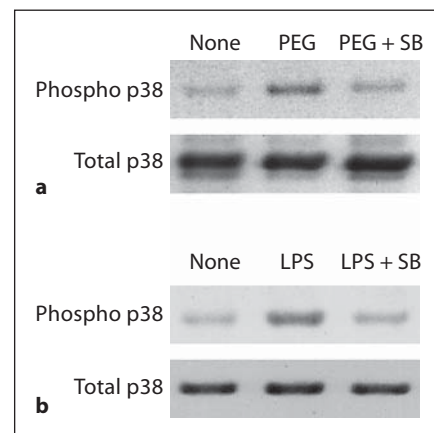


Fig. 3. Phosphorylation of p38 MAPK in PEG- (a) and LPS-stimulated (b) LCs and the influence of SB203580 (SB) on the phosphorylation. LCs were pretreated with 250 nM SB203580 for 30 min and subsequently stimulated with 10 μ g/ml PEG or 1 μ g/ml LPS for 30 min. The phosphorylation of p38 MAPK in the LCs was detected by Western blotting. Membranes were incubated with antibodies raised against the phosphorylated form of p38 MAPK. The membranes were then reprobated with antibodies against total p38 MAPK as loading controls. Blots are representative of results obtained in 3 separate experiments.

tion of p38 MAPK, and the p38 MAPK inhibitor SB203580 inhibited both of these increases in phosphorylation, suggesting that RANTES production induced by PEG and LPS stimulation was associated with activation of the p38 MAPK pathway. However, poly(I:C) and CpG DNA stimulation did not increase the phosphorylation of p38 MAPK, and SB203580 had no influence on the phosphorylation in nonstimulated and stimulated LCs (data not shown).

Discussion

RANTES has been reported to elicit significant migration of eosinophils [19]. Furthermore, the amount of RANTES is increased in the lesional skin of AD patients, suggesting that localized RANTES production may be associated with eosinophil infiltration in AD [20]. Since the skin of most AD patients shows superficial *S. aureus* colonization and barrier disruption due to reduced ceramide levels [21], PEG from *S. aureus* would be expected to penetrate the skin and may play a critical role in perpetuating skin tissue inflammation through eosinophil infiltration [9]. Pathogens are controlled by both the innate and adaptive arms of the immune system. In

mammals, many receptors are involved in the recognition of infectious agents. Among them, TLRs have been identified as a major family of receptors in the recognition of pathogen-associated molecular patterns [16]. Our results clearly demonstrated that, although all TLR stimuli were able to induce RANTES production from LCs, stimulation with PEG and LPS, but not poly(I:C) and CpG DNA, did so in a p38 MAPK activation-dependent manner. Furthermore, inhibition of the ERK pathway in PEG- and LPS-stimulated LCs augmented RANTES production. These results indicated that the underlying mechanism responsible for the down-regulation of RANTES production was hyperactivation of the ERK pathway in LCs by PEG and LPS. Although the mechanism responsible for this kind of negative regulation of RANTES production by the ERK pathway was not clarified in the present study, a similar phenomenon has been observed in IL-12 p40 production from LPS-stimulated macrophages [22]. Studies of this mechanism are currently underway. Fujita et al. [23] have reported that murine LCs do not produce RANTES in response to LPS. Since the levels of RANTES production from non-stimulated LCs in their study were very high compared with our data, excessive stimulation of LCs by the cul-

ture media they used may have resulted in nonresponsiveness to LPS. In addition, a recent study has demonstrated that muramyl dipeptide as a Nod-2 agonist induces RANTES production from murine macrophages [24]. Since PEG from *S. aureus* contains muramyl dipeptide as a minimal bioactive structure, TLR-2 stimuli by PEG and Nod-2 stimuli by muramyl dipeptide may exert a synergistic effect for induction of RANTES production from LCs. In any event, the above findings suggest that the signaling pathway of RANTES production from murine epidermal LCs by different TLR stimuli is not necessarily the same, and that pathway-specific regulation might occur in MAPKs of each cell. In particular, it seems that the involvement of p38 MAPK in RANTES production is specific to stimulation with bacterial cell wall components such as PEG and LPS. Although our data showed that murine LCs expressed TLR-4 and TLR-9, these receptors are absent from human LCs [25]. Therefore, PEG as a TLR-2 agonist would be more important in the context of RANTES production and its possible trigger in the human system. Inhibition of p38 MAPK in LCs may be a potential therapeutic target for eosinophilic inflammation associated with *S. aureus* colonization in AD patients.

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