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Modification of allergic inflammation in murine model of rhinitis by different bacterial ligands: involvement of mast cells and dendritic cells

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Clinical and Experimental Allergy

Summary

Background It has been suggested that airway bacterial infections exacerbate allergic disorders, and bacterial components in the air affect allergic inflammation via Toll-like receptors expressed on mast cells and dendritic cells in the airway mucosa.

Objective Peptidoglycan (PGN) is a major component of the bacterial cell wall. We investigated the effect of PGN on the effector phase of allergic inflammation, in comparison with the effect of CpG-oligodeoxynucleotides (CpG), which is known to be a Th1 adjuvant.

Methods Ovalbumin (OVA)-sensitized mice were challenged intranasally with OVA alone or OVA together with PGN or CpG. Nasal allergic symptoms and eosinophilia were scored, and the OVA-specific cytokine response was examined in the cells of cervical lymph nodes and nasal mucosa. Bone marrow-derived mast cells (BMMCs) and dendritic cells (BMDCs) were stimulated with PGN or CpG *in vitro*, and the expression level of cytokines and chemokines was examined by RT-PCR. In addition, the expression level of chemokines was examined by RT-PCR in mast cells of OVA-sensitized mice challenged with OVA alone or OVA together with PGN or CpG.

Results PGN exposure exacerbated the nasal allergic symptoms and eosinophilia, whereas CpG exposure suppressed them. In addition, PGN exposure increased the OVA-specific IL-4 response in the cells, whereas CpG exposure decreased it. On the other hand, there were no significant differences in the OVA-specific IFN- γ response. PGN but not CpG induced the expression of thymus and activation-regulated chemokine (TARC) and macrophage/monocyte-derived chemokine (MDC) in both BMMCs and mast cells of mice sensitized and challenged with OVA. CpG but not PGN induced the expression of IFN- β and interferon-inducible protein-10 (IP-10) in BMDCs, and histamine did not influence this effect.

Conclusion These results demonstrate that PGN exposure exacerbates allergic inflammation mainly via mast cells, whereas CpG exposure suppresses allergic inflammation mainly via dendritic cells.

Keywords allergic rhinitis, CpG-oligodeoxynucleotides, dendritic cells, mast cells, peptidoglycan

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Introduction

According to the hygiene hypothesis, an increased microbial burden early in life is believed to be relevant to a decreased prevalence of atopic sensitization. One of the substances responsible for this effect is endotoxin, which is a major cell wall component of Gram-negative bacteria and is ubiquitous in the environment. A recent study showed an inverse association between endotoxin and the prevalence of atopic sensitization [1]. On the other

hand, it has been reported that endotoxin exposure early in life is associated with increased risk of wheezing [2], and endotoxin exposure is also known to exacerbate asthma [3, 4]. It has been proven that endotoxin affects allergic disorders via Toll-like receptor 4 (TLR4) [5].

However, endotoxin represents only a part of the bacterial components present in the total microbial burden. Peptidoglycan (PGN) is a cell wall component of not only Gram-positive but also Gram-negative bacteria, and is ubiquitous in the environment, like endotoxin. It is known

that PGN signals via TLR2 [6, 7], and that its derivatives, γ -D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), signal via nucleotide-binding oligomerization domain protein 1 (NOD1) and NOD2, respectively [8, 9]. Recently, it was reported that a major component of bacterial PGN, *N*-acetyl muramic acid, serves as a marker of microbial exposure, and ubiquitous environmental exposure to PGN is inversely associated with the prevalence of allergic disorders, supporting the hygiene hypothesis [10]. On the other hand, it was reported that TLR2 ligands such as PGN and Pam₃Cys functioned as Th2 adjuvants on immunization (sensitization) in mice, in contrast to the TLR9 ligand including CpG-oligodeoxynucleotides (CpG), which acts as a Th1 adjuvant [11, 12]. It is likely that PGN as well as endotoxin affects allergic disorders, because genetic variation in TLR2 was reported to be a major determinant of the susceptibility to allergic disorders in children of farmers [13].

Thus, it would be of interest to know how PGN, one of the major bacterial components, affects the effector phase of allergic inflammation. Studies of this question should help to clarify the relationship between bacterial infection and allergic inflammation. In the present study, we investigated the effect of PGN exposure on the effector phase of allergic inflammation, in comparison to that of CpG exposure, using a murine model of allergic rhinitis (AR) induced by ovalbumin (OVA). In addition, we examined the effects of PGN and CpG on mast cells and dendritic cells, both of which have been implicated in allergic inflammation and express TLRs [14–19].

Methods

Reagents and mice

PGN derived from *Staphylococcus aureus* was obtained from Fluka (Buchs, Switzerland). The level of lipopolysaccharide (LPS) in PGN was measured by a *Limulus* color KY test (Wako Pure Chemical Industries, Osaka, Japan), and was confirmed to be negligible (less than 10 pg of LPS per μ g of PGN) to cause significant biological effects. Phosphorothioate CpG 1826 (5'-TCCATGACGTTCTGACGTT-3') was synthesized by PROLIGO (Kyoto, Japan). LPS derived from *Escherichia coli* was obtained from Sigma (St Louis, MO, USA).

Female BALB/c mice (Japan SLC, Shizuoka, Japan) raised and maintained under specific-pathogen-free conditions, were used at 7–8 weeks of age. All the experimental procedures performed on mice were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Kyoto, Japan.

Sensitization and challenge of mice with ovalbumin

Mice were sensitized intraperitoneally with 250 μ L of phosphate-buffered saline (PBS) containing 10 μ g of OVA

(grade V; Sigma) adsorbed to 2 mg of alum (Pierce Chemical Company, Rockford, IL, USA) on day 1 and day 8. Mice were challenged intranasally with 10 μ L of PBS containing 100 μ g of OVA for 7 consecutive days from day 15 to day 21. In order to investigate the effect of PGN, CpG or LPS, mice of some groups were administered OVA together with PGN (1, 10, or 100 μ g), CpG (0.1, 1, or 10 μ g) or LPS (0.1, 1, or 10 μ g) intranasally on days 18, 19, and 20. In some experiments, mice were administered OVA together with PGN (100 μ g) or CpG (10 μ g) on days 16, 17, and 18. Mice of the control group were injected intraperitoneally with alum alone on days 1 and 8, and challenged intranasally with PBS.

Evaluation of nasal allergic symptoms

The severity of AR was determined by the frequency of sneezing and nose-scratching during 10 min after the last intranasal challenge with OVA on day 21, as described previously [20].

Histological examination

The nasal tissues were removed 1 day after the last intranasal challenge and fixed in 10% neutral formalin and decalcified. Coronal nasal sections were stained with haematoxylin and eosin, and the number of eosinophils in the submucosal region of the nasal septum was counted in four high power fields (HPFs; 10×40) and expressed as cells/HPF, as described previously [21].

Ovalbumin-specific cytokine production

One day after the last intranasal challenge, cervical lymph nodes were removed aseptically. Then, the nasal cells were released from nasal tissues by treatment with collagenase (Liberase Blendzyme 3; Roche Diagnostics, Indianapolis, IN, USA), and mononuclear cells were isolated using a stepwise Percoll gradient as described by Asanuma *et al.* [22]. Cells were suspended at 5×10^6 cells/mL (cervical lymph nodes) or 2×10^6 cells/mL (nasal mononuclear cells) in RPMI culture medium: RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FBS (Gibco-BRL), 5 μ g/mL gentamicin (Wako Chemicals USA, Richmond, VA, USA) and 5×10^{-5} M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan). Cells were stimulated with 100 μ g/mL of OVA for 72 h at 37 °C in 5% CO₂. The culture supernatants were collected and the level of IFN- γ was determined by ELISA as described previously [23]. The level of IL-4 was determined by using the OptEIA mouse IL-4 ELISA set (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol.

Detection of ovalbumin-specific interleukin-4-producing CD4⁺ T cells

Cells of cervical lymph nodes were stimulated with 100 µg/mL of OVA for 20 h. After an additional incubation for 8 h in the presence of monensin (Pharmingen) as an inhibitor of intracellular protein transport, cells were collected and stained with PE-Cy5-conjugated rat anti-mouse CD4 monoclonal antibody (Pharmingen). Next, cells were fixed and permeabilized using a commercial Cytofix/Cytoperm kit (Pharmingen), and intracellular IL-4 was stained with PE-conjugated rat anti-mouse IL-4 monoclonal antibody (Pharmingen). PE-conjugated rat IgG₁ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an isotype control. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences), and the results were expressed as the number of IL-4-producing CD4⁺ T cells per 10⁶ cells.

Generation of bone marrow-derived mast cells and dendritic cells

Bone marrow cells were prepared from the femur of female BALB/c mice. Bone marrow-derived mast cells (BMMCs) were induced as described previously [14–16]. In brief, bone marrow cells were cultured at 1 × 10⁶ cells/mL in RPMI culture medium in the presence of 10% WEHI-3-conditioned medium as a source of IL-3. Non-adherent cells were transferred to fresh RPMI culture medium once a week, and after 3–4 weeks of culture, more than 95% of the cells became identifiable as mast cells, as described previously [16], and were used for experiments. Bone marrow-derived dendritic cells (BMDCs) were induced as described previously [24]. In brief, bone marrow cells were cultured in RPMI culture medium in the presence of 20 ng/mL of GM-CSF (Pepro-Tech, London, UK), and after 10 days of culture, the cells were harvested for experiments.

Expression of Toll-like receptor 2 and 9 in bone marrow-derived mast cells and dendritic cells

The expression of TLR2 and TLR9 mRNAs in BMMCs and BMDCs was examined by RT-PCR. Splenic CD4⁺ T cells were used as a negative control. Total RNA was extracted with NucleoSpin RNA II (Macherey Nagel, Duren, Germany) and cDNA was reverse transcribed by using a random primer (Invitrogen, Carlsbad, CA, USA) as described previously [23]. PCR was performed by using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The following primer sets were used [23, 25, 26]: TLR2 sense, 5'-CTTCCTGGTTCCTGCTCGTTCTC-3'; TLR2 anti-sense, 5'-CAAGAACAAAGAAAATGAGTCAAG-3'; TLR9 sense, 5'-CTACAACAGCCAGCCCTTTA3'; TLR9 anti-sense, 5'-GCTGAGGTTGACCTCTTTCA-3'; β-actin sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; β-actin anti-sense, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'.

CD86 expression in bone marrow-derived dendritic cells stimulated with peptidoglycan or CpG

BMDCs (1 × 10⁶ cells/mL) were stimulated for 24 h with 10 µg/mL of PGN or 1 µg/mL of CpG. Cells were stained with FITC-conjugated rat anti-mouse CD86 monoclonal antibody (eBioscience, San Diego, CA). FITC-conjugated rat IgG_{2a} antibody (Santa Cruz Biotechnology) was used as an isotype control. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences).

Cytokine and chemokine responses in bone marrow-derived mast cells and dendritic cells stimulated with peptidoglycan or CpG

BMMCs (1 × 10⁶ cells/mL) were stimulated for 5 h with 10 µg/mL of PGN or 1 µg/mL of CpG, and BMDCs (1 × 10⁶ cells/mL) were stimulated for 6 h with 10 µg/mL of PGN or 1 µg/mL of CpG in the presence or absence of 10⁻⁵ M histamine (Sigma), according to previous reports [12, 14, 15, 27, 28] and our preliminary experiments. The expression level of cytokines and chemokines was determined by RT-PCR. The following primer sets were used [23, 29–31]: IL-4 sense, 5'-CGAAGAACACCACAGAGAGTGAGCT-3'; IL-4 anti-sense, 5'-GACTCATTCATGGTGCAGCTTATCG-3'; IL-5 sense, 5'-CTCTAGTAAGCCACTTCTA-3'; IL-5 anti-sense, 5'-TGATACCTGAATAACATCCC-3'; IL-6 sense, 5'-TGGAGTCACAGAAGGAGTGGCTAAG-3'; IL-6 anti-sense, 5'-TCTGACCACAGTGAGGAATGTCCAC-3'; thymus and activation-regulated chemokine (TARC) sense, 5'-CCTGGCTGCTCTGCTCTGGGGAC-3'; TARC anti-sense, 5'-GGCATCCCTGGAACACTCCACTG-3'; macrophage/monocyte-derived chemokine (MDC) sense, 5'-GCCCTCTGGTCATTAGACACCTG-3'; MDC anti-sense, 5'-TCGTTGGCAAGGCTCTTGCTG-3'; IFN-β sense, 5'-TCCGAGCAGAGATCTTCAGGAAC-3'; IFN-β anti-sense, 5'-TCTGAGGCATCAACTGACAGGTC-3'; interferon-inducible protein 10 (IP-10) sense, 5'-GAAATCATCCCTGCGAGCCTATCC-3'; anti-sense, 5'-GC AATTAGGACTAGCCATCCACTGGG-3'.

In some experiments, BMDCs (1 × 10⁶ cells/mL) were stimulated for 24 h with 10 µg/mL of PGN or 1 µg/mL of CpG in the presence or absence of 10⁻⁵ M histamine. The culture supernatants were collected and the level of IL-12p70 was measured by ELISA using a combination of non-labelled and biotin-labelled rat anti-mouse IL-12p70 monoclonal antibodies (Endogen, Woburn, MA, USA) as described previously [23]. The level of IL-6 was estimated by using the OptEIA mouse IL-6 ELISA set (Pharmingen) according to the manufacturer's protocol.

Enrichment of mast cells from nasal mucosa

OVA-sensitized mice were challenged intranasally with OVA on days 15, 16, and 17. On day 18, mice were

challenged with OVA alone or together with 100 µg of PGN or 10 µg of CpG. Five hours later, nasal tissues were collected and treated with collagenase. CD117⁺ cells were enriched from the resultant nasal cells by using the BD IMag Cell Separation System (BD Bioscience) and a combination of biotin-conjugated rat anti-mouse CD117 monoclonal antibody (eBioscience) and streptavidin particles plus – DM (BD Bioscience). CD117⁺ cells from nasal mucosa were used for RT-PCR analysis.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of the values obtained. *p* < 0.05 was considered statistically significant.

Results

Effect of exposure to peptidoglycan or CpG on effector phase of allergic rhinitis

PGN and endotoxin are among the major bacterial components. These bacterial components seem to affect allergic inflammation via TLRs [5, 13]. Moreover, recent studies showed that PGN functions as a Th2 adjuvant on immunization (sensitization) [11, 12]. These findings prompted us to investigate whether PGN affects the effector phase of allergic inflammation. For this purpose, we investigated the effect of PGN exposure in OVA-sensitized mice, using a murine model of AR. In addition, we compared the effect of PGN with that of CpG, which was reported to suppress the effector phase of allergic inflammation [32, 33].

First, we investigated how the exposure to PGN, CpG, or LPS on days 18–20 influenced the effector phase of rhinitis. Allergic symptoms such as sneezing and nose-scratching developed in mice sensitized and challenged with OVA (OVA/OVA). PGN exposure exacerbated these symptoms in a dose-dependent manner, whereas exposure to 10 µg of CpG relieved these symptoms (Fig. 1a). On the other hand, administration of 10 µg of LPS resulted in an exacerbation of allergic symptoms (Fig. 1a). In addition, a larger number of eosinophils infiltrated into the nasal mucosa of PGN-treated mice, whereas a significantly smaller number of eosinophils infiltrated in CpG-treated mice compared with OVA/OVA mice (Figs 1b–f). A larger number of eosinophils infiltrated in mice treated with 10 µg of LPS than in OVA/OVA mice (Fig. 1b). The results indicate that the effect of PGN was not due to contaminating LPS in the PGN preparation used here. Next, we investigated whether the exposure to PGN or CpG at an earlier stage, on days 16–18, influenced the effector phase of rhinitis. PGN exposure resulted in increases of both the frequency of allergic symptoms and the number of eosinophils infiltrating into the nasal mucosa, whereas CpG

exposure showed an opposite effect (Figs 1g and h). Taken together, these results indicate that PGN showed an ability to exacerbate the effector phase of AR at local sites, whereas CpG showed an ability to suppress it. On the other hand, neither PGN nor CpG exposure affected the production of anti-OVA IgE antibody in the serum (data not shown).

Ovalbumin-specific cytokine response in cells of cervical lymph nodes and nasal mucosa

Based on the above results, it was likely that PGN and CpG affected the OVA-specific immune response at local sites. We next examined the effect of exposure to PGN or CpG on the OVA-specific cytokine response in cells of cervical lymph nodes and nasal mucosa. IFN-γ was produced by the cells of cervical lymph nodes but not nasal mononuclear cells of OVA/OVA mice in response to OVA, and the OVA-specific IFN-γ response was not affected significantly by exposure to PGN or CpG (Fig. 2a). In contrast, a significant level of IL-4 was produced by both types of cells of OVA/OVA mice in response to OVA. The OVA-specific IL-4 response was increased significantly in both types of cells of PGN-treated mice, compared with the response of the cells of OVA/OVA mice, whereas the response was decreased significantly in the cells of CpG-treated mice (Fig. 2b). These results indicate that PGN and CpG affected the induction of the antigen-specific Th2 response, but not the antigen-specific Th1 response, at local sites.

Because neither PGN nor CpG exposure affected the OVA-specific cytokine response in spleen cells (data not shown), it was likely that the difference of the antigen-specific Th2 response at local sites was due to the difference of the number of antigen-specific Th2 cells recruited to local sites. Therefore, we examined the effect of exposure to PGN or CpG on the number of OVA-specific IL-4-producing CD4⁺ T cells in cervical lymph nodes. Flow cytometric analysis revealed that there were a significantly larger number of OVA-specific IL-4-producing CD4⁺ T cells in PGN-treated mice compared with OVA/OVA mice, whereas there were a smaller number of these cells in CpG-treated mice (Fig. 3). These results indicate that PGN treatment increased the number of antigen-specific Th2 cells at the sites of allergic inflammation, resulting in the increase of the antigen-specific Th2 response at the sites, whereas CpG treatment had the opposite effect without enhancing the antigen-specific Th1 immune response at the sites.

Effects of peptidoglycan and CpG on mast cells

Mast cells are not only effector cells of allergic (anaphylactic) reactions but also important cells for the initiation of innate and acquired immune responses against

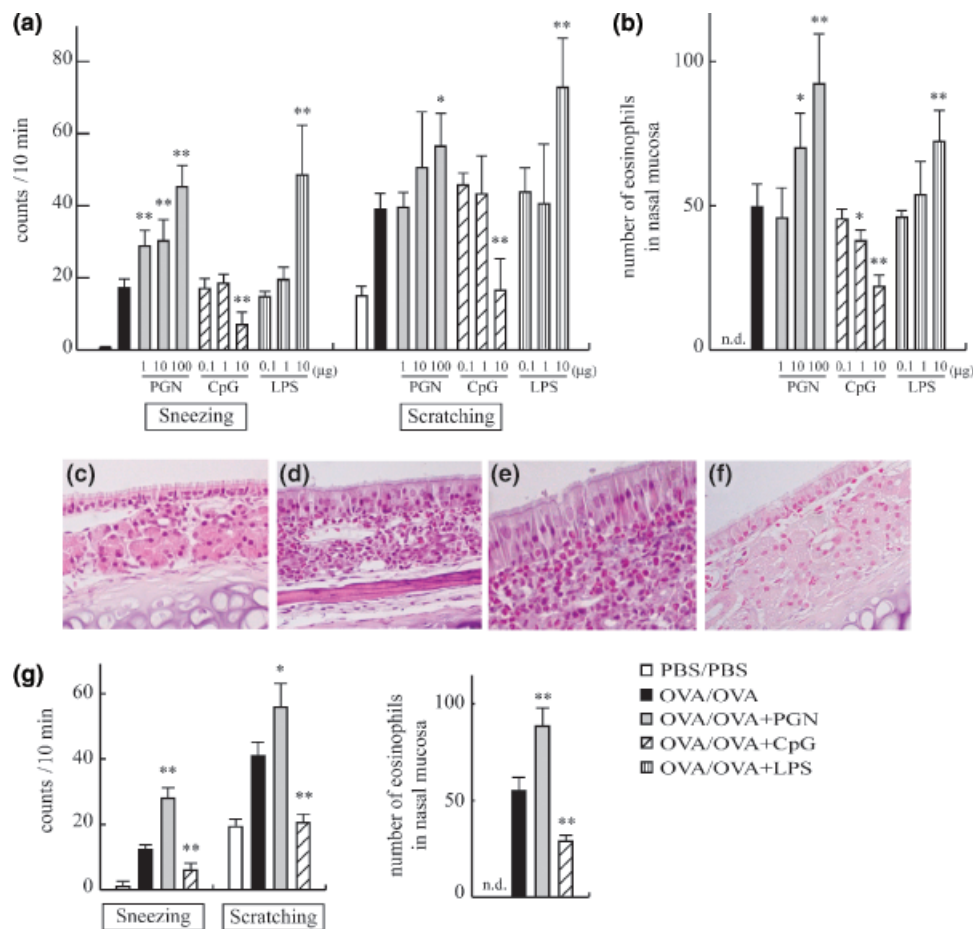


Fig. 1. Nasal allergic symptoms and eosinophil infiltration in nasal mucosa. (a–f) Ovalbumin (OVA)-sensitized mice were challenged intranasally with OVA on days 15–21. Mice of some groups were administered OVA together with peptidoglycan (PbG), CpG or LPS on days 18–20. The frequency of sneezing and nose-scratching was counted during 10 min after the last intranasal challenge with OVA (a). One day after the last intranasal challenge, mice were sacrificed. Coronal sections of nasal tissues were stained with haematoxylin and eosin (H&E). The number of eosinophils infiltrating into the nasal septum was counted (b). Typical nasal sections ($\times 400$) are shown. (c) PBS/PBS, (d) OVA/OVA, (e) OVA/OVA+PbG (100 µg), (f) OVA/OVA+CpG (10 µg). Data are representative of three independent experiments. (g, h) OVA-sensitized mice were challenged intranasally with OVA on days 15–21. Mice of some groups were administered OVA together with 100 µg of PbG or 10 µg of CpG on days 16–18. The nasal symptoms (g) and eosinophil infiltration (h) were examined. Data are representative of two independent experiments. Four to five mice were used per group and data are the mean ($\pm$ SD). * $p < 0.05$, ** $p < 0.01$ by Student's t test, compared with OVA/OVA.

pathogens [14]. Mast cell-dependent reactions are mediated by a number of cytokines, chemokines and chemical mediators. It seems that bacterial components affect allergic responses via TLRs expressed on mast cells in the airway mucosa [14, 15, 17]. In the present study, we examined the effects of PbG and CpG on BMMCs that we had confirmed by RT-PCR to express both TLR2 and TLR9 *in vitro* (Fig. 4a). The expression of IL-4 and IL-5 was induced in BMMCs stimulated with PbG but not CpG, although the level of IL-6 expression induced by PbG was similar to that induced by CpG (Fig. 4b), and IL-12p35 expression was not induced in BMMCs (data not shown). Moreover, the expression of TARC and MDC was induced in BMMCs stimulated with PbG but not CpG (Fig. 4b). These results indicate that treatment with PbG but not CpG induced the expression of Th2 cytokines and chemokines in BMMCs. Next, we examined whether PbG

exposure actually showed such an effect *in vivo* on mast cells of mice sensitized and challenged with OVA. Marked expression of TARC and MDC was detected only in the mast cells of PbG-treated mice (Fig. 4c). However, we were not able to detect the expression of IL-4 or IL-5 in the mast cells of mice of any of the groups. This may be due to the very small number of mast cells obtained from mice. Based on these results, it seems that PbG enhanced the Th2 immune response at the sites of allergic inflammation via mast cells, resulting in the exacerbation of allergic responses.

Effects of peptidoglycan and CpG on dendritic cells

Immature dendritic cells in the airway mucosa capture antigens and then undergo a maturation process. In the mature state, dendritic cells serve as the most potent

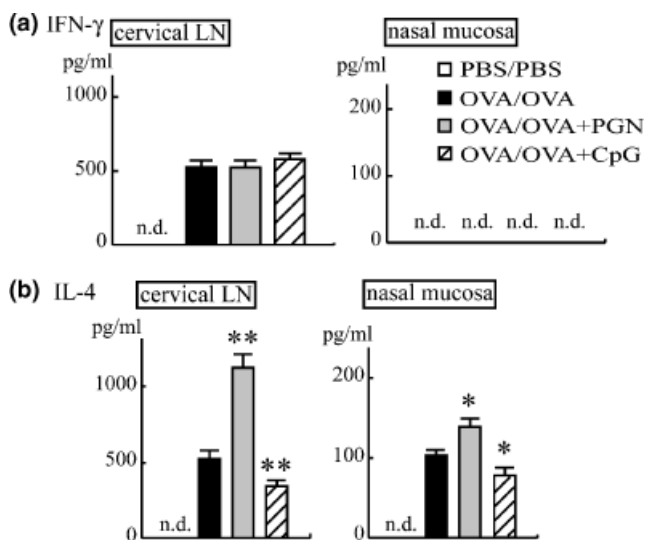


Fig. 2. Ovalbumin (OVA)-specific cytokine production. OVA-sensitized mice were challenged intranasally with OVA on days 15–21. Mice of some groups were administered OVA together with 100 μ g of PGN or 10 μ g of CpG on days 18–20. Cervical lymph node cells and nasal mononuclear cells were prepared and stimulated with 100 μ g/mL of OVA for 72 h. IFN- γ (a) and IL-4 (b) in the culture supernatants were measured by ELISA. Data are representative of three independent experiments, and are the mean $\pm$ SD. n.d., not detected; * p < 0.05, ** p < 0.01 by Student's *t*-test, compared with OVA/OVA.

antigen-presenting cells, though they exhibit down-regulation of the mechanisms of antigen capture [34]. Bacterial components affect the maturation of dendritic cells via TLRs, resulting in the modulation of cytokine and chemokine production [18, 19]. Moreover, it is well known that histamine is released by degranulation from mast cells at the site where dendritic cells capture antigens, and histamine affects the function of dendritic cells [27, 28]. Therefore, we examined the effects of PGN and CpG on the maturation of TLR2- and TLR9-expressing BMDCs used in the present study (Fig. 5a). CD86 expression was enhanced in BMDCs stimulated with PGN or CpG (Fig. 5b), indicating that BMDCs became more mature after *in vitro* stimulation with these bacterial TLR ligands, as reported previously [18, 19]. Next, we examined the effects of PGN and CpG on the induction of cytokines and chemokines from BMDCs in the presence or absence of histamine. CpG but not PGN induced the production of IL-12p70 by BMDCs, and the presence of histamine significantly suppressed this CpG-induced IL-12p70 production (Fig. 5c). On the other hand, PGN as well as CpG induced the production of IL-6 by BMDCs, and histamine did not suppress IL-6 production significantly (Fig. 5c).

CpG induced IFN- β expression in BMDCs, and IFN- β induced IP-10 expression through an autocrine loop on dendritic cells, as reported previously [34, 35]. This effect was not affected by histamine (Fig. 5d). On the other hand, PGN did not induce either IFN- β or IP-10 expression in

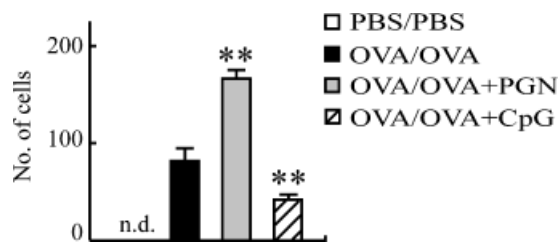


Fig. 3. Ovalbumin (OVA)-specific IL-4-producing CD4⁺ T cells in cervical lymph nodes. OVA-sensitized mice were challenged intranasally with OVA on days 15–21. Mice of some groups were administered OVA together with 100 μ g of peptidoglycan (PGN) or 10 μ g of CpG on days 18–20. Cervical lymph node cells were prepared and stained as described in Methods for flow cytometric analysis. Data are presented as IL-4-producing CD4⁺ T cells per 10⁶ cells of cervical lymph nodes. Data are representative of three independent experiments, and are the mean $\pm$ SD. n.d., not detected; ** p < 0.01 by Student's *t*-test, compared with OVA/OVA.

BMDCs in the presence or absence of histamine (Fig. 5d). These results clearly indicate that CpG but not PGN induced IP-10 (a Th1 chemokine) via the induction of IFN- β even in the presence of histamine.

Discussion

Like non-bacterial allergens, bacterial components in inhaled air have the ability to induce various immune responses in the airway mucosa. Most of the components derived from the ubiquitous airway bacteria belong to pathogen-associated molecular patterns (PAMPs), which induce signalling via TLRs. According to the hygiene hypothesis, exposure to these bacterial components early in life results in decreased atopic sensitization [1, 36]. However, airway bacterial components such as endotoxin were reported to exacerbate allergic disorders such as asthma [3, 4]. Endotoxin, which is one of the most extensively investigated substances, represents only a part of the total microbial burden. On the other hand, PGN is a major component of the cell wall of virtually all bacterial species. Thus, the total amount of PGN in the environment may reflect the greater part of the total microbial burden more closely than that of endotoxin [10]. In addition, it was reported that blood cells from farmers' children expressed a significantly higher amount of TLR2, but not TLR4, than those from non-farmers' children [37]. Furthermore, PGN was reported to function as a Th2 adjuvant on immunization (sensitization) in mice [11, 12]. Another TLR ligand, CpG, has been shown to function as a Th1 adjuvant and to suppress the effector phase of allergic inflammation [11, 12, 32, 33].

In the present study using a unique model of AR, we compared the effects of PGN and CpG on the effector phase of allergic response. Our results demonstrated for the first time that PGN exposure exacerbates allergic inflammation at the effector phase. In contrast, CpG

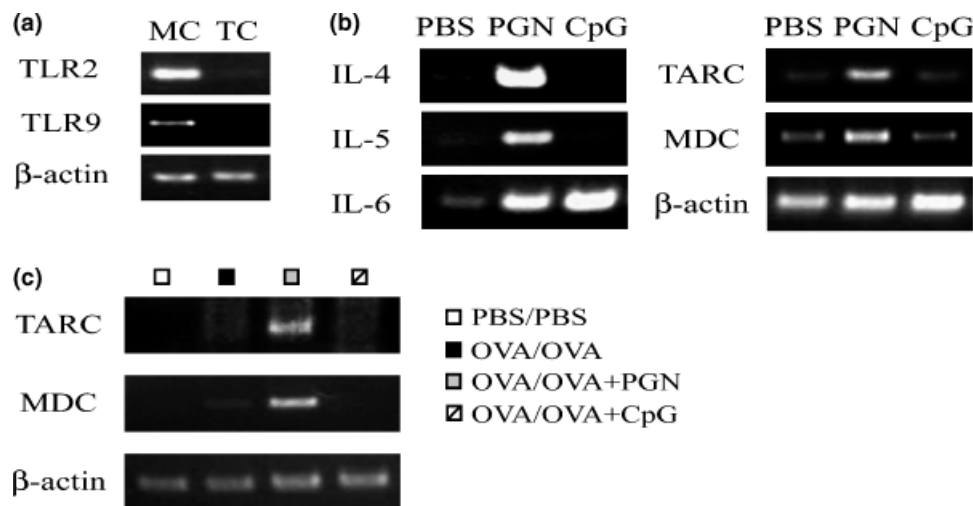


Fig. 4. RT-PCR analysis in mast cells. (a) The expression of TLR2 and TLR9 mRNAs in bone marrow-derived mast cells (BMMCs) was analyzed by RT-PCR. MC; BMMCs. TC; splenic CD4⁺ T cells (negative control). (b) BMMCs were stimulated with PGN (10 μ g/mL) or CpG (1 μ g/mL). The expression level of IL-4, IL-5, IL-6, TARC and MDC was analyzed by RT-PCR. (c) Ovalbumin (OVA)-sensitized mice were challenged intranasally with OVA on days 15–17. On day 18, mice were challenged with OVA alone or OVA together with 100 μ g of PGN or 10 μ g of CpG. Five hours later, the expression level of TARC and MDC was analyzed by RT-PCR in CD117⁺ cells enriched from nasal mucosa. Data are representative of three independent experiments.

exposure suppressed it, as described previously [32, 33]. In our murine model, neither PGN nor CpG exposure affected the systemic responses, including the production of anti-OVA IgE antibody in the serum and the OVA-specific cytokine response in spleen cells, but both PGN and CpG induced various local responses in cervical lymph nodes and nasal mucosa. In PGN-treated mice, an increase of antigen-specific Th2 cells resulted in an increase of antigen-specific Th2 response at local sites, whereas a decrease of the Th2 cells resulted in a decrease of the Th2 response in CpG-treated mice. These findings indicate that the recruitment of Th2 cells to local sites was affected differentially by PGN and CpG.

Mast cells and dendritic cells, both of which express TLRs, produce a number of cytokines and chemokines in response to bacterial ligands, and these mediators modify allergic inflammation [14–19]. IL-4 mediates the induction of vascular cell adhesion molecule-1 on the vascular endothelium, the promotion of eosinophil transmigration across endothelium, and the inhibition of eosinophil apoptosis at local sites, in addition to the induction of the IgE isotype switch in B cells and the differentiation of Th2 cells [38]. IL-5, which is secreted at local sites, regulates eosinophil trafficking, as does eotaxin [39]. It seems that the enhanced production of Th2 cytokines (IL-4 and IL-5) at local sites exacerbated eosinophilia at the nasal mucosa of PGN-treated mice. In addition, it is possible that Th2 cytokines induced in mast cells assisted the exacerbation of eosinophilia in concert with Th2 cytokines from antigen-specific Th2 cells, as suggested by the *in vitro* results.

CCR4 is selectively expressed on Th2 cells, and its ligands (TARC and MDC) are the major attractants in the selective recruitment of Th2 cells through vascular en-

dothelial cells into tissues [40, 41]. Thus, Th2 chemokines (TARC and MDC), which were induced in mast cells stimulated with PGN but not CpG, recruited a larger number of Th2 cells to the local sites. In addition, Matsui *et al.* reported that percutaneous application of PGN resulted in the infiltration of mast cells into the skin [42]. In our murine model, there were more mast cells in the nasal mucosa of PGN-treated mice compared with OVA/OVA mice, although the difference was not significant (data not shown). It is certain that PGN has the ability to exacerbate the effector phase of allergic inflammation via mast cells. Based on this finding, it appears reasonable to think that airway allergic inflammation is exacerbated by airway infection by Gram-positive bacteria such as *Streptococcus pneumoniae*, one of the commensal bacteria in the upper respiratory tract. Allergic inflammation is closely associated with bacterial infection, as suggested by the finding that allergic inflammation enhanced bacterial sinusitis caused by *S. pneumoniae* [43]. Regarding the effect of TLR ligands on mast cell degranulation, TLR2-mediated degranulation remains controversial. Supajatura *et al.* reported that TLR2 ligands induced mast cell degranulation [14], whereas other reports showed that TLR2 ligands failed to do so [44]. On the other hand, other TLR ligands have been reported not to affect mast cell degranulation [16, 44].

CXCR3 is selectively expressed on Th1 cells, and its ligand IP-10 selectively attracts Th1 cells [45, 46]. Since the antigen-specific Th1 response was not enhanced at the local sites of CpG-treated mice, it is likely that IP-10 did not contribute to the suppression of allergic inflammation in our murine model of rhinitis. This may be due to the low number of antigen-specific Th1 cells induced in our

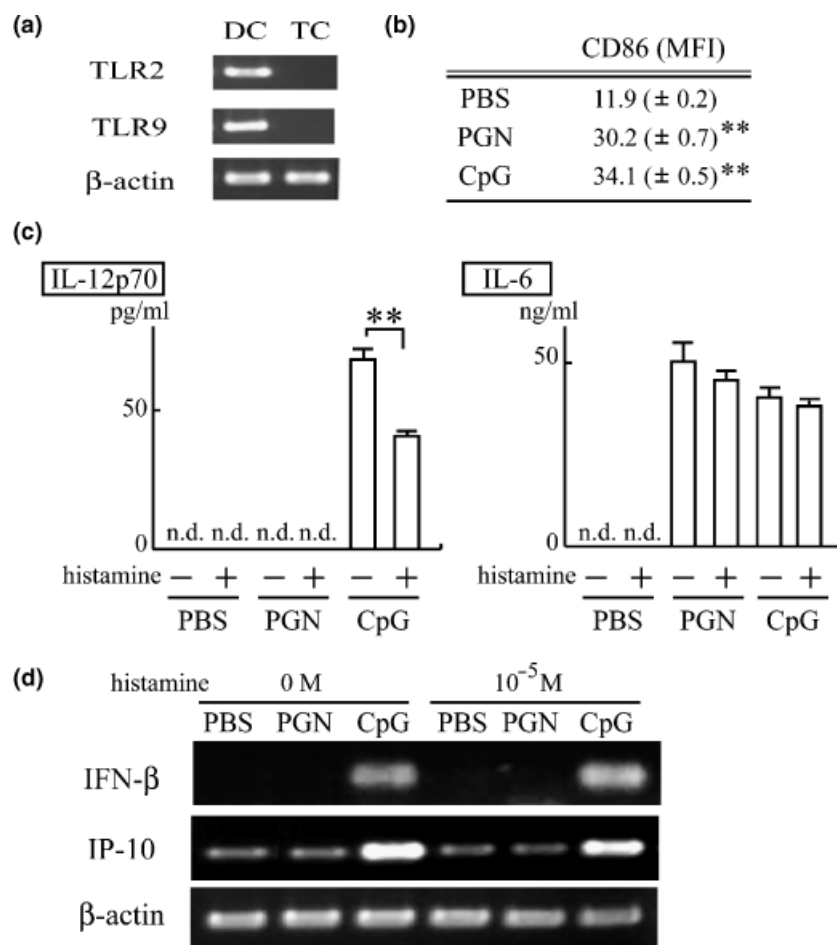


Fig. 5. *In vitro* analysis of bone marrow-derived dendritic cells (BMDCs). (a) The expression of TLR2 and TLR9 mRNAs in BMDCs was analyzed by RT-PCR. DC; BMDCs. TC; splenic CD4⁺ T cells (negative control). (b) The level of CD86 expression in BMDCs stimulated with PGN (10 μ g/mL) or CpG (1 μ g/mL) was determined by flow cytometric analysis. The results were expressed as mean fluorescence intensity (MFI) ($\pm$ SD). ** p < 0.01 by Student's *t*-test, compared with PBS. (c) BMDCs were stimulated with PGN (10 μ g/mL) or CpG (1 μ g/mL) in the presence or absence of histamine. IL12-p70 and IL-6 in the culture supernatants were measured by ELISA. (d) The expression level of IFN- β and IP-10 was analyzed by RT-PCR. Data are representative of three independent experiments, and are the mean $\pm$ SD. n.d., not detected; ** p < 0.01 by Student's *t*-test.

murine model (data not shown). On the other hand, IFN- β is a highly pleiotropic cytokine with many immunomodulatory effects. It is known that IFN- β plays an important role in the immunoregulation of Th1-mediated diseases such as multiple sclerosis [47]. Matheu *et al.* [48] recently reported that IFN- β played an important role in the immunoregulation of allergic responses. In addition, it was reported that IFN- β had the ability to inhibit Th2-mediated eosinophilia [49]. Based on these findings, it is probable that CpG has the ability to inhibit the antigen-specific Th2 response at local sites via the induction of IFN- β in dendritic cells. Regarding the effect of IFN- β on mast cells, it was reported that IFN- β did not affect histamine release from mucosal mast cells [50].

It is not still clear whether CpG in inhaled air actually contributes to the inhibition of Th2 immune response, because there are still no reports demonstrating that airway bacterial DNA, like CpG, directly modifies allergic

inflammation. CpG is regarded as one of the potential therapeutic tools against allergic inflammation at present.

In summary, bacterial ligands in the air differentially modify the effector phase of AR in mice. The present study demonstrated that the different effects of the ligands are dependent on the different responses of mast cells and dendritic cells in airway mucosa to these ligands. It is probable that bacterial infection exacerbates allergic inflammation via some PAMPs, such as PGN and endotoxin. On the other hand, other PAMPs, such as CpG, are expected to be useful as therapeutic agents against allergic inflammation.

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