

Peptidoglycan from *Staphylococcus aureus* Induces IL-4 Production from Murine Spleen Cells via an IL-18-Dependent Mechanism

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Key Words

Atopic dermatitis · IL-4 · IL-18 · Peptidoglycan · *Staphylococcus aureus*

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. Skin lesions of AD patients show an increased number of Th2 cells in the dermis and superficial *Staphylococcus aureus* colonization. The purpose of this study was to predict the effects of peptidoglycan (PEG) from *S. aureus* on the induction of interleukin (IL)-4 production in AD patients. **Methods:** PEG was applied to barrier-disrupted abdominal mouse skin every 5 days. Twenty days later, IL-4 production in the spleen was investigated by reverse transcription-polymerase chain reaction (RT-PCR). Spleen cells from normal mice were also treated in vitro with PEG and processed for IL-4 production by RT-PCR and enzyme-linked immunosorbent assay. **Results:** IL-4 production was significantly increased in the spleen of PEG-treated mice compared with that of PBS-applied mice. In addition, in vitro experiments demonstrated that PEG was able to induce IL-4 production in murine spleen cells. Furthermore, IL-4 production was associated with IL-18 production, but not with IL-2 production from PEG-stimulated spleen cells, and IL-4 mRNA was expressed in CD4+ lymphocytes in the spleen cells. In in vivo experiments, percutaneous treat-

ment with PEG induced mRNA expression not only for IL-4 but also for IL-18 in the spleen. **Conclusion:** These results suggest that PEG may influence the induction of a Th2-dominant cytokine response in AD patients through IL-4 production from CD4+ T cells stimulated with PEG-induced IL-18.

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Introduction

Atopic dermatitis (AD) is an inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. Acute skin lesions of AD patients show an increased number of interleukin (IL)-4-producing Th2-type cells in the dermis and superficial *Staphylococcus aureus* colonization [1]. *S. aureus* can be isolated from 75–96% of skin lesions of AD patients, whereas only 5–10% of healthy skin are colonized by this organism [2, 3]. In our previous study in AD patients, the detection rate of *S. aureus* was higher on skin lesions than on non-lesional skin [3].

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, and lipoteichoic acid and/or PEG are thought to be

major inflammatory products in their cell walls [6, 7]. Therefore, we hypothesized that lipoteichoic acid and/or PEG from *S. aureus* may be pathogenic factors in AD patients. In previous studies, percutaneous application of PEG increased the numbers of mast cells and eosinophils in murine skin, as seen in AD patients [8, 9]. In those studies, we incidentally detected IL-4 production in the spleens of PEG-treated mice [unpubl. results]. IL-4 is an important cytokine for the induction of a Th2-dominant cytokine response in AD patients. Therefore, we investigated the effects of PEG on IL-4 production by murine spleen cells.

Methods

PEG and Animals

PEG derived from *S. aureus* was obtained from Fluka (Buchs, Switzerland). PEG was reconstituted in phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1 mg/ml and sonicated for 1 h before use. Female-specific pathogen-free BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and studied at 6–8 weeks of age. They were housed in plastic cages in a clean, air-conditioned room at 24°C and allowed free access to a standard laboratory chow and water. All procedures performed on the mice were in accordance with the guidelines of the Animal Care and Use Committee of the Meiji Pharmaceutical University, Tokyo, Japan.

Treatment of Mice and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Percutaneous application of PEG to mice was performed as described previously [8]. In order to determine IL-4 and IL-18 mRNA expression in the spleens of PEG-applied mice at 20 days, spleen cells were prepared in RPMI 1640 with L-glutamine (Sigma, St. Louis, Mo., USA) containing 10% fetal calf serum (Sigma), 25 mM Hepes (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, N.Y., USA; RPMI 10), and total RNA was extracted from 1×10^7 spleen cells by the single-step method with TRI Reagent (Molecular Research Center, Cincinnati, Ohio, USA). Then, cDNA was synthesized from 4 µg total RNA using a first-strand cDNA synthesis kit (GE Healthcare, Little Chalfont, UK). PCR was performed using the following primers: β -actin (540 bp): 5' primer, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 3' primer, 5'-CTCTTTGATGTACACGACGATTTC-3'; IL-4 (400 bp): 5' primer, 5'-AGTTGTCATCTGCTCTTCTTCTC-3' and 3' primer, 5'-CGAGTAATC-CATTTGCATGATGCTC-3', and IL-18 (434 bp): 5' primer, 5'-ACTGTACAACCGCAGTAATACGG-3' and 3' primer, 5'-AGTGAACATTACAGATTTATCCC-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Conn., USA) in a 25-µl reaction volume containing 1.5 µl of cDNA (corresponding to 400 ng total RNA starting material) as described previously [8], and the PCR products were separated on 2% agarose gel containing ethidium bromide.

Treatment of Cultured Spleen Cells, RT-PCR and Enzyme-Linked Immunosorbent Assay (ELISA)

For in vitro experiments, spleen cells from normal mice were adjusted to 2×10^6 cells/ml in RPMI 10. The cultures (5 ml/well) were incubated in 6-well culture plates (Sumilon, Tokyo, Japan) in the presence or absence of 10 µg/ml PEG at 37°C in a humidified atmosphere with 5% CO₂. In some experiments, the spleen cells were incubated with 10 µg/ml anti-mouse IL-2 neutralizing monoclonal antibody (R & D Systems, Minneapolis, Minn., USA) or 10 µg/ml anti-mouse IL-18 neutralizing monoclonal antibody (MBL, Nagoya, Japan) in the presence of 10 µg/ml PEG. In order to determine IL-4 and IL-18 mRNA expression in the PEG-stimulated spleen cells, total RNA was extracted after incubation for 24 or 48 h and processed for RT-PCR as described above. The culture supernatants were also collected after incubation for 48 or 96 h, and IL-4 and IL-18 concentrations were measured using ELISA kits for quantification of murine IL-4 (BioSource, Camarillo, Calif., USA) and murine IL-18 (MBL). Furthermore, to clarify whether IL-4 production in the PEG-stimulated spleen cells originated from T cells, CD4+ and CD8+ lymphocytes were separated, respectively, from spleen cells stimulated with PEG for 48 h using SpinSep (StemCell Technologies, Vancouver, B.C., Canada). Next, mRNA was extracted from 1×10^6 separated lymphocytes (>95% purity) using a Quick Prep Micro mRNA purification kit (GE Healthcare). Then, cDNA was synthesized from 160 ng of the mRNA using a first-strand cDNA synthesis kit (GE Healthcare). PCR was run in a 25-µl reaction volume containing 1.5 µl of cDNA (corresponding to 16 ng of mRNA starting material) as described above.

Statistics

Data were expressed as means ($\pm$ SD) and differences between means were analyzed using Student's t test with a two-tailed test of significance. Differences at $p < 0.05$ were considered to be significant.

Results

The first experiments were carried out to examine the induction of IL-4 production in the spleen after percutaneous application of PEG to mice. Mice were treated with 100 µg of PEG on a 10-cm² area of barrier-disrupted abdominal skin every 5 days, and 20 days later the spleen cells were processed for detection of IL-4 by RT-PCR. As shown in figure 1a, IL-4 mRNA was detected in the spleen cells of PEG-applied mice but not in those of PBS-applied mice. Subsequently, in order to determine whether IL-4 is produced in the spleen cells stimulated in vitro with PEG, cytokine mRNA expression in the spleen cells was confirmed by RT-PCR 48 h after PEG stimulation. Figure 1b shows that IL-4 mRNA was detected in the spleen cells stimulated with PEG. Next, using ELISA, we examined whether IL-4 mRNA expression in the spleen cells stimulated with PEG was correlated with the synthesis of cytokine protein. IL-4 ELISA was carried out using cul-

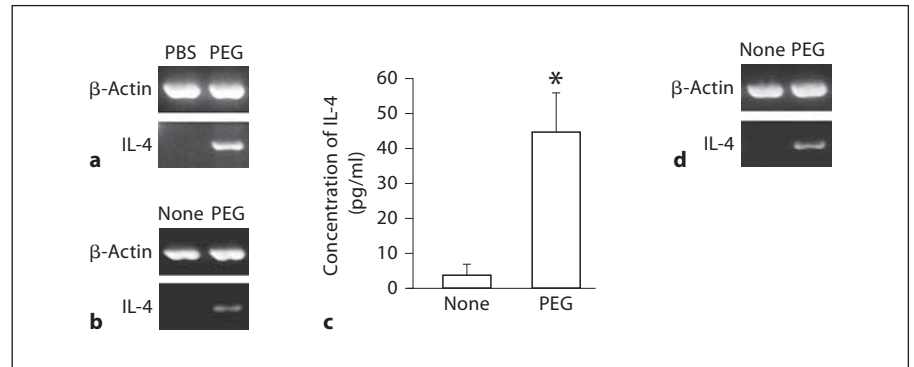


Fig. 1. Effect of PEG on IL-4 production from murine spleen cells. **a** A 10-cm² area of barrier-disrupted abdominal mouse skin was treated with 100 μ l PBS or 100 μ l PEG solution (1 mg/ml) every 5 days, and 20 days after the first application, total RNA was extracted from the spleen cells of each mouse, reverse-transcribed and amplified by PCR using β -actin and IL-4 primer sets. **b** Spleen cells from normal mice (2×10^6 /ml) were cultured with or without 10 μ g/ml PEG, and total RNA was extracted at 48 h, reverse-transcribed and amplified by PCR using β -actin and IL-4 primer

sets. **c** Culture supernatants of the spleen cells (2×10^6 /ml) were also collected 96 h after PEG (10 μ g/ml) stimulation, and IL-4 concentration was measured by ELISA. Means $\pm$ SD ($n = 5$). * $p < 0.01$ vs. no treatment. **d** CD4⁺ lymphocytes were purified from the spleen cells (2×10^6 /ml) 48 h after PEG (10 μ g/ml) stimulation, and mRNA was extracted, reverse-transcribed and amplified by PCR using β -actin and IL-4 primer sets. **a, b, d** The data shown are representative of four independent experiments.

ture supernatants of spleen cells stimulated with PEG for 96 h. Figure 1c shows that IL-4 production from spleen cells was significantly increased 96 h after PEG stimulation. In addition, since IL-4 mRNA was expressed in purified CD4⁺ lymphocytes (fig. 1d) but not in CD8⁺ lymphocytes (data not shown), we hypothesized that the IL-4 production originated from CD4⁺ T cells.

Since it is well known that stimulation of naïve T cells with IL-2 and IL-18 induces IL-4 production from CD4⁺ T cells in an allergen-independent manner [10, 11], we examined whether or not IL-4 production from the PEG-stimulated spleen cells was associated with IL-2 and/or IL-18 production. As shown in fig. 2a, IL-4 mRNA expression induced in the spleen cells by PEG stimulation in vitro was inhibited by anti-IL-18 antibody but not by anti-IL-2 antibody. Furthermore, IL-4 mRNA expression correlated with IL-4 protein synthesis in the culture supernatants (fig. 2b), suggesting that the PEG-induced IL-4 production from splenic CD4⁺ T cells was associated with IL-18 production in the spleen cells.

Therefore, the effect of PEG on IL-18 production was next examined. As shown in figure 3a, IL-18 mRNA was weakly expressed in the spleen cells of PBS-applied mice, and the expression was augmented in the PEG-applied mice. Subsequently, in order to determine whether IL-18 is produced from the spleen cells by in vitro PEG stimulation, IL-18 mRNA expression in the spleen cells was confirmed by RT-PCR analysis 24 h after PEG stimula-

tion (fig. 3b). In addition, using ELISA, we examined whether the expression of IL-18 mRNA in the PEG-stimulated spleen cells correlated with cytokine protein synthesis. IL-18 ELISA was carried out using culture supernatants of spleen cells stimulated with PEG for 48 h. Figure 3c shows that IL-18 production from spleen cells was significantly increased 48 h after PEG stimulation.

Discussion

The purpose of the present study was to determine the effects of PEG on IL-4 production in AD patients. Th2 cytokines, including IL-4, IL-5 and IL-13, can trigger allergic disorders. Although it is known that IL-4 is critical for the polarization of naïve CD4⁺ T cells to a Th2 phenotype, Yoshimoto et al. [10] and Hoshino et al. [11] have demonstrated that IL-18 stimulates CD4⁺ T cells pretreated with IL-2, inducing IL-4 production without antigen stimulation. Furthermore, they have confirmed that when naïve CD4⁺ T cells are stimulated with IL-18 in the presence of IL-2 plus an antigen, they differentiate into antigen-specific Th2 cells through IL-18-induced IL-4 production. Since the addition of anti-IL-4 antibody to this system inhibits the differentiation of naïve CD4⁺ T cells into Th2 cells, IL-4 production from T cells stimulated with IL-18 is critical for the induction of Th2 cells. Indeed, in the sera of AD patients, IL-18 levels were high-

Fig. 2. Neutralization of IL-4 production from PEG-stimulated spleen cells. Murine spleen cells ($2 \times 10^6/\text{ml}$) were stimulated with $10 \mu\text{g/ml}$ PEG for 48 or 96 h in the presence or absence of anti-IL-2 antibody or anti-IL-18 antibody. **a** Total RNA was extracted at 48 h, reverse-transcribed and amplified by PCR using β -actin and IL-4 primer sets. The data shown are representative of four independent experiments. **b** Culture supernatants were collected at 96 h, and IL-4 concentration was measured by ELISA. Means $\pm$ SD ($n = 5$). * $p < 0.01$ vs. no treatment; ** $p < 0.001$ vs. PEG stimulation.

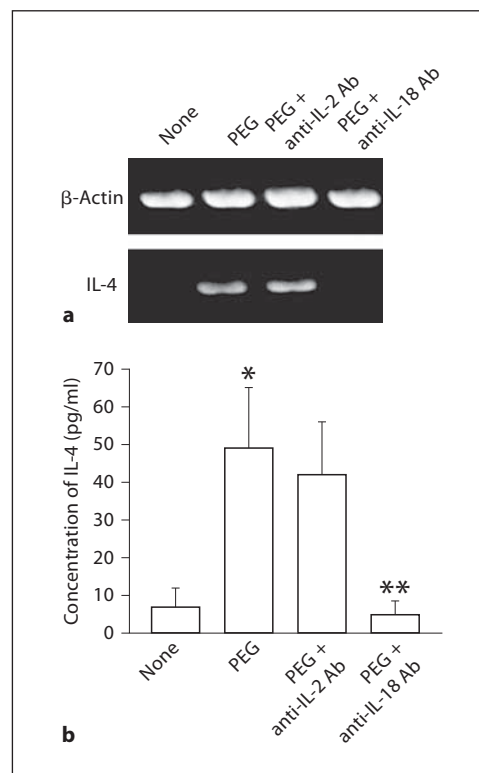
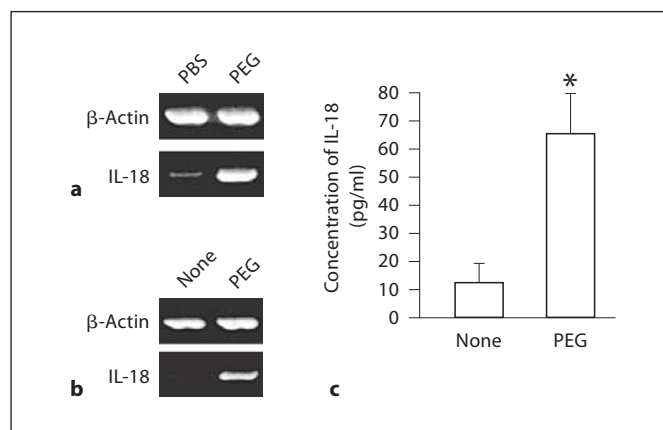


Fig. 3. Effect of PEG on IL-18 production from murine spleen cells. **a** A 10-cm^2 area of barrier-disrupted abdominal mouse skin was treated with $100 \mu\text{l}$ PBS or $100 \mu\text{l}$ PEG solution (1 mg/ml) every 5 days, and 20 days after the first application total RNA was extracted from the spleen cells of each mouse, reverse-transcribed and amplified by PCR using β -actin and IL-18 primer sets. **b** Spleen cells from normal mice ($2 \times 10^6/\text{ml}$) were cultured with or without $10 \mu\text{g/ml}$ PEG, and total RNA was extracted at 24 h, reverse-transcribed and amplified by PCR using β -actin and IL-18 primer sets. **a, b** The data shown are representative of four independent experiments. **c** Culture supernatants of the spleen cells ($2 \times 10^6/\text{ml}$) were also collected 48 h after PEG ($10 \mu\text{g/ml}$) stimulation, and IL-18 concentration was measured by ELISA. Means $\pm$ SD ($n = 5$). * $p < 0.01$ vs. no treatment.



er than in those from healthy donors [12]. In acute lesions of AD patients, Th2 cytokines are predominantly expressed in localized T cells [1]. The underlying causes for the initial accumulation of Th2 cells in the cutaneous lesions are unknown. Since it was anticipated that IL-4 might be a candidate molecule responsible for this induction, we investigated whether IL-4 production was induced by PEG, and if so, whether the IL-4 production was associated with IL-18 production. Our results demonstrated that percutaneous application of PEG induced

IL-4 mRNA expression in the spleen, suggesting that percutaneous invasion of PEG may induce a Th2-type cytokine response through IL-4 production in an allergen-independent manner. In vitro experiments using normal spleen cells demonstrated that PEG induced IL-4 production from CD4⁺ T cells and that the IL-4 production was associated with IL-18 production in the spleen cells. The IL-4 production observed in the present study did not require IL-2, while IL-2 protein was also detected in the culture supernatant of spleen cells 48 h after PEG stimu-

lation (data not shown). Hoshino et al. [11] have suggested that treatment with IL-2 may upregulate IL-18 receptor expression on resting lymphocytes. Therefore, PEG stimulation may indirectly upregulate functional IL-18 receptor expression on naïve CD4⁺ T cells through another cytokine in an IL-2-independent manner. Although the present study did not reveal the mechanism by which excessive IL-18 is produced, or its source in spleen cells, PEG may stimulate splenic macrophages or dendritic cells as IL-18-producing cells through Toll-like receptor 2, resulting in IL-18 production [13]. Studies to test this hypothesis are currently underway.

Previous reports have not explained the role of *S. aureus* in the induction of the Th2-type cytokine response in AD patients. Our present results suggest that *S. aureus* colonization may play a critical role in Th2-type skin tissue inflammation through IL-18 production induced by a bacterial cell wall component such as PEG. As the skins of most AD patients show superficial *S. aureus* colonization and barrier disruption due to reduced ceramide levels [14], PEG would be expected to penetrate their skin. Therefore, irrespective of clinical signs of superinfection, antimicrobial treatment should be considered as a new therapeutic strategy at least in a subgroup of AD patients.

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