ORIGINAL ARTICLE

Peptidoglycan From *Staphylococcus aureus* Induces T_H2 Immune Response in Mice

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Abstract

Background and objective: Atopic dermatitis patients have an increased number of type 2 helper (T_H2) cells in their peripheral blood and superficial *Staphylococcus aureus* colonization. The purpose of this study was to clarify the effects of peptidoglycan (PEG) from *S aureus* on the induction of the T_H2 immune response in mice.
Methods: Mice were primed with PEG- and ovalbumin (OVA)-pulsed Langerhans cells (LCs) and given a booster OVA injection 2 days later via the hind footpad. Five days later, the cytokine response in the draining popliteal lymph nodes was investigated by reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA). IL-12 production from cultured LCs was detected by ELISA and Western blot analysis.
Results: Administration of PEG- and OVA-pulsed LCs into the hind footpads of the mice induced a T_H2-prone immune response as represented by the enhanced interleukin (IL) 4 expression in the lymph nodes. We further showed that higher levels of IL-12 p40 production by PEG-stimulated LCs relative to IL-12 p70 (p35/p40) production were associated with the induction of the T_H2 immune response. The LC-derived IL-12 p40 protein induced by PEG stimulation was detected mainly as monomeric and homodimeric IL-12 p40 subunits; other heterodimers including the IL-12 p40 subunit, such as IL-23, were undetected.
Conclusion: These results suggest that PEG may have the ability to induce the development of T_H2 cells through insufficient production of IL-12 p70 and excessive production by LCs of homodimeric IL-12 p40, a known antagonist of bioactive IL-12 p70, offering a possible explanation for the role of *S aureus* colonization in patients with atopic dermatitis.

Key words: Langerhans cells. *Staphylococcus aureus*. Peptidoglycan. T_H2. IL-12

Resumen

Antecedentes y objetivo: Los pacientes con dermatitis atópica presentan un mayor número de linfocitos T cooperadores de tipo 2 (T_H2) en la sangre periférica y una colonización por *Staphylococcus aureus* superficial. El objetivo de este estudio fue determinar los efectos del peptidoglucano (PEG) de *S. aureus* en la inducción de la respuesta inmunitaria de tipo T_H2 en ratones.
Métodos: Los ratones fueron sensibilizados con células de Langerhans (CL) pulsadas con PEG y ovoalbúmina (OVA) y, dos días después, se les administró una inyección de refuerzo de OVA en la almohadilla plantar trasera. Al cabo de días, se investigó la respuesta de las citocinas en los ganglios linfáticos poplíteos drenantes mediante reacción en cadena de la polimerasa con transcripción inversa y enzimoinmunoanálisis de adsorción (ELISA). La producción de IL-12 por parte de CL cultivadas fue detectada mediante ELISA y análisis Western Blot.
Resultados: La administración de CL pulsadas con PEG y OVA en la almohadilla plantar trasera de los ratones indujo una respuesta inmunitaria de tipo T_H2, representada por una mayor expresión de interleucina (IL) 4 en los ganglios linfáticos. Además, el estudio mostró que unos niveles más elevados de producción de p40 IL-12 por parte de CL estimuladas por PEG, respecto a la producción de IL-12 p70 (p35/p40), estaban asociados a la inducción de la respuesta inmunitaria de tipo T_H2. La proteína p40 de IL-12 obtenida de CL e inducida por estimulación con PEG se detectó principalmente como subunidades monoméricas y homodiméricas de IL-12 p40; no se detectaron otros heterodímeros de la subunidad IL-12 p40, como por ejemplo IL-23.
Conclusión: Estos resultados indican que PEG puede tener la capacidad de inducir el desarrollo de linfocitos T_H2 mediante la producción insuficiente de IL-12 p70 y la producción excesiva por parte de CL de IL-12 p40 homodimérica (un antagonista conocido de IL-12 p70 bioactiva). Estos resultados ofrecen una posible explicación de la función que desempeña la colonización por *S. aureus* en los pacientes con dermatitis atópica.

Palabras clave: Células de Langerhans. *Staphylococcus aureus*. Peptidoglucano. T_H2. IL-12.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesion. The majority of AD patients show increased expression of type 2 helper (T_H2) cytokines such as interleukin (IL) 4, IL-5, and IL-13 in peripheral blood mononuclear cells (PBMCs) and superficial *Staphylococcus aureus* colonization of the skin [1]. Colonization rates of 75% to 96% have been detected in the skin lesions of patients with AD, contrasting with rates of just 5% to 10% in the skin of healthy individuals [2,3]. In a previous study of patients with AD, we found a higher rate of *S aureus* colonization and a higher bacterial cell count in lesional skin compared to nonlesional skin [3]. No significant difference, however, was found in the rate of *S aureus* producing superantigenic exotoxin.

Gram-positive bacterial cell walls are composed of highly cross-linked peptidoglycan (PEG) decorated to a variable extent with teichoic acid polymers. These polymers are also linked to plasma membrane phospholipids to form lipoteichoic acid (LTA), which is another major cell wall component [4,5]. Gram-positive bacteria do not contain lipopolysaccharide (LPS), and LTA and/or PEG are thought to be the major inflammatory products in their cell walls. Therefore, we hypothesized that LTA and/or PEG from *S aureus* might play a more important pathogenic role than superantigenic exotoxins in AD. In previous studies, we found that LTA and PEG were able to induce IL-5 production by PBMCs from patients with AD [6], and that intradermal injection of LTA induced localized AD-like inflammation associated with significantly increased numbers of T_H2-type cells in the dermis of allergen-sensitized mice [7]. Furthermore, we demonstrated that percutaneous injection of PEG induced T_H2 cell infiltration in the dermis [8]. A recent study by Mandron et al [9] demonstrated that human dendritic cells (DCs) conditioned with enterotoxin B from *S aureus* (SEB) promoted T_H2 cell polarization. The authors also suggested that Toll-like receptor (TLR) 2 signaling of DCs induced by SEB might be involved in this process. Although PEG is a well-known ligand of TLR2 [10], it is unclear whether it has the ability to induce a systemic T_H2-dominant immune response such as that seen in AD patients. In the present study, therefore, we investigated the T_H1/T_H2 regulation of Langerhans cells (LCs) stimulated with PEG.

Materials and Methods

Peptidoglycan

PEG derived from *S aureus* was obtained from Fluka (Buchs SG), reconstituted in phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1 mg/mL, and sonicated for 1 hour before use.

Mice

Female specific-pathogen-free BALB/c mice were obtained from Japan SLC (Hamamatsu) and used at the age of 6 to 8 weeks. They were housed in plastic cages with sterilized paper bedding in a clean, air-conditioned room at 24°C and 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.

Purification of LCs

LCs in the epidermis were separated as described by Tada et al [11]. Briefly, mouse skin was treated with dispase (3000 U/mL; Godo Shusei) 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) (RPMI 10) for 3 hours at 37°C. The epidermis was separated from the dermis and incubated in RPMI 10 containing 0.025% deoxyribonuclease I (Sigma) for 20 minutes at room temperature. An epidermal cell suspension was obtained by vigorous pipetting of epidermal sheets. This suspension was then treated with mouse anti-mouse $I\text{-}A^d$ monoclonal antibody (clone 040-220, IgG2a) (1: 600; Meiji Dairies Co.) in RPMI 10 for 45 minutes on ice. Next, the cells were incubated in plates precoated with goat anti-mouse immunoglobulin (Ig) G polyclonal antibody (1:100, Sigma) for 45 minutes at 4°C, and adherent cells were used as LCs. $I\text{-}A^d$ positive cells were purified to around 95% purity as determined by flow cytometry.

Stimulation of LCs With Peptidoglycan and Immunization Protocol

T_H1/T_H2 regulation with PEG was investigated according to the method of Maldonado-Lópenz et al [12], with modification. Briefly, LCs were adjusted to 5×10^5 cells/mL in RPMI 10 and then incubated with 30 μg/mL ovalbumin (OVA) in the presence or absence of 10 μg/mL PEG at 37°C in a humidified atmosphere with 5% CO_2. The cells were collected after incubation for 18 hours, washed in RPMI 10, and administered at a dose of 1×10^5 cells into both hind footpads of the mice. After 2 days, 30 μg OVA was injected into both hind footpads as a booster, and draining popliteal lymph nodes were harvested 5 days later. Cytokine expression in the lymph node cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA).

Reverse Transcription-Polymerase Chain Reaction Assay

In order to determine the levels of mRNA expression for various cytokines, mRNA was extracted from lymph node cells or LCs using a Quick Prep Micro mRNA purification kit (GE Healthcare). Then, the 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: ß-actin (540 base pairs [bp]) 5' primer, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 3' primer, 5'-CTCTTTGATGTCACGCACGATTTC-3'; interferon (IFN) γ (405 bp) 5' primer, 5'-GCTACACACTGCATCTTGGCTTTG-3' and 3' primer, 5'-CACTCGGATGAGCTCATTGAATGC-3'; IL-4 (400bp) 5' primer, 5'-AGTTGTCATCCTGCTCTTCTTTCTC-3' and 3' primer, 5'-CGAGTAATCCATTTGCATGATGCTC-3'; IL-12 p35 (542 bp) 5' primer, 5'-TCTCTGGACCTGCCAGGTGT-3'

and 3' primer, 5'-CCTGTTGATGGTCACGACGCG-3'; IL-12 p40 (309 bp) 5' primer, 5'-AACCTCACCTGTGACACGCC-3' and 3' primer, 5'-CAAGTCCATGTTTCTTTGCACC-3'; IL-23 p19 (247 bp) 5' primer, 5'-TGCTGGATTGCAGAGCAGTAA-3' and 3' primer, 5'-AGTCCTTGTGGGTCACAACC-3'. Each PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, USA) in 25 μL of reaction mixture comprising 1.5 μL cDNA (corresponding to 16 ng mRNA starting material), 200 μM deoxynucleotide triphosphate mixture, 400 nM of each PCR primer, and 25 U/mL Ex Taq DNA polymerase (Takara). The reaction conditions were as follows: one 4-minute cycle at 94°C, 35 cycles comprising 45 seconds at 94°C, 45 seconds at 61°C, and 2 minutes at 72°C, followed by one 7-minute cycle at 72°C. The PCR products were separated on a 2% agarose gel containing ethidium bromide.

Quantification of IFN-γ and IL-4 Production by Lymph Node Cells

Lymph node cells were adjusted to 1×10^6 cells/mL in RPMI 10. The cultures (0.2 mL/well) were incubated in 96-well culture plates (Nunc) in the presence of Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen Dynal AS) at 37°C in a humidified atmosphere with 5% CO_2. The culture supernatants were collected after incubation for 48 hours, and the IFN-γ and IL-4 concentrations were measured using ELISA kits for quantification of murine IFN-γ and IL-4, respectively (R & D Systems).

Quantification of IL-12 Production by Langerhans cells

LCs were adjusted to 5×10^5 cells/mL in RPMI 10. The cultures (0.2 mL/well) were incubated in 96-well culture plates (Nunc) in the presence or absence of 10 μg/mL PEG at 37°C in a humidified atmosphere with 5% CO_2. The culture supernatants were collected after incubation for 48 hours, and IL-12 p40 and IL-12 p70 concentrations were measured using ELISA kits for the quantification of murine IL-12 (R & D Systems).

Immunoprecipitation and Western Blotting

LCs (5×10^5 cells/mL) in RPMI 10 were incubated in the presence or absence of 10 μg/mL PEG at 37°C in a humidified atmosphere with 5% CO_2. The culture supernatant was collected after incubation for 48 hours and concentrated to 1/10 volume with a Centricon 10 (Millipore). Rat anti-mouse IL-12 p40 monoclonal antibody (clone C17.8; IgG2a) (R & D systems) was cross-linked to Dynabeads protein G (Dynal Biotech) in accordance with the manufacturer's instructions. The concentrated culture supernatant was precleared with Dynabeads protein G for 30 minutes at 4°C before incubation with antibody-linked Dynabeads overnight at 4°C. The immunoprecipitated Dynabeads complexes were washed 5 times with immunoprecipitation buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 0.5% Nonidet P-40, 0.5% glucopyranoside, 1 μg/mL aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). Proteins were eluted by boiling in nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then subjected to electrophoresis (10% polyacrylamide gel) and transferred to a polyvinylidene difluoride membrane (Atto). After blocking for 1 hour at 37°C with Block Ace blocking buffer (Yukizirushi), IL-12 p40, IL-12 p35, and IL-23 p19 were detected by overnight incubation at 4°C with 0.1 μg/mL biotinylated goat anti-mouse IL-12 p40 antibody, goat anti-mouse IL-12 p70 antibody (p35 specific) and goat anti-mouse IL-23 p19 antibody (R & D Systems), respectively, in 10% Block Ace with 0.05% Tween 20. The membrane was washed 4 times for 10 minutes each time in 10% Block Ace with 0.05% Tween 20, and subsequently incubated with streptavidin-horseradish peroxidase polymer conjugate (Sigma) in 10% Block Ace with 0.05% Tween 20. After 4 more washes in in the same solution, the membrane was processed for immunodetection using an enhanced chemiluminescence detection system (GE Healthcare).

Statistical Analysis

The data were expressed as means (SD), and differences between means were analyzed using a 2-tailed *t* test. Differences at $P<.05$ were considered to be statistically significant.

Results

Effects of PEG on T_H2 Development

LCs were purified from murine epidermis and pulsed with OVA for 18 hours in the presence or absence of PEG. The OVA-pulsed LCs were injected into the hind footpads of mice, and the mice were boosted with an OVA injection into the hind footpads 2 days later. Lymph node cells were harvested 5 days after the OVA injection, and the expressions of IFN-γ and IL-4 mRNA were confirmed by RT-PCR. The data in Figure 1A indicate that PEG-stimulated LCs induced a T_H2-prone immune response, as shown by the enhanced expression of IL-4 mRNA. However, there was no difference in IFN-γ mRNA expression between mice injected with LCs stimulated with PEG and those injected with nonstimulated LCs. Subsequently, the T lymphocytes in the lymph node cells obtained were stimulated through cell surface CD3/CD28 molecules, and the IFN-γ and IL-4 concentrations in the culture supernatants were determined by ELISA. As shown in Figure 1B, the PEG-stimulated LCs induced a T_H2-prone immune response, as shown by an enhanced production of IL-4. These findings indicate that LCs stimulated with PEG induce the development of T_H2-type cells.

Production of IL-12 from LCs by PEG Stimulation

There is evidence that the maturation of T helper cell precursors into biased T_H1 or T_H2 populations is strongly influenced by cytokines in the local environment [13]. In particular, IL-12 appears as the dominant cytokine driving the differentiation of T_H1 lymphocytes both in vitro and in vivo [14,15]. We found that PEG stimulation of LCs induced significant levels of IL-12 p70 and IL-12 p40 (Figure 2). However, IL-12 p70 production was much lower than IL-12

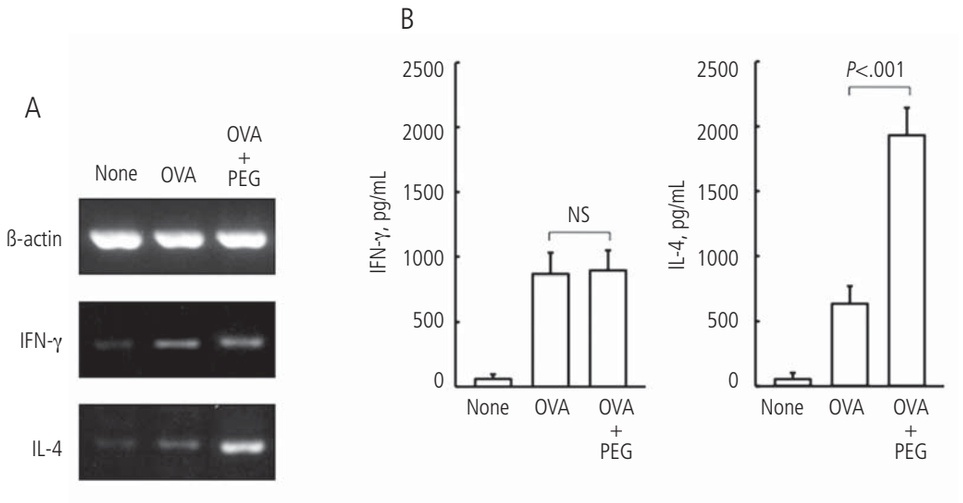

Figure 1. Effect of peptidoglycan (PEG) on T helper type 2–prone immune response in mice. The mice were primed with PEG- and ovalbumin (OVA)-pulsed Langerhans cells and given a booster injection of OVA 2 days later via the hind footpad. A, Draining popliteal lymph nodes were harvested 5 days after the OVA injection, and cytoplasmic mRNA was then extracted from lymph node cells, reverse-transcribed, and amplified by polymerase chain reaction using ß-actin, interferon (IFN) γ and interleukin (IL) 4 primer sets. The data shown are the representative results of 5 independent experiments. B, Lymph node cells were harvested 5 days after the OVA injection and stimulated through cell surface CD3/CD28 molecules for 48 hours; IFN-γ and IL-4 concentrations in the culture supernatants were determined by enzyme-linked immunosorbent assay. The results are expressed as means (SD) (n=6).

p40 production. In other words, the ability to induce a T_H2-prone immune response by PEG stimulation of LCs was associated mainly with IL-12 p40 production. A bioactive IL-12, IL-12 p70, is a heterodimer of p35 and p40 proteins. As shown in Figure 3, insufficient IL-12 p70 production by PEG stimulation was associated with low levels of expression of IL-12 p35 mRNA. Furthermore, the enhancement of IL-12 p40 production by PEG stimulation was associated with the levels of expression of IL-12 p40 mRNA. When PCR was performed using the same cDNA as that used in the experiment shown in Figure 1A and IL-12 primer sets, the enhancement of IL-12 p40 mRNA expression and low levels of IL-12 p35 mRNA expression, similar to that seen in Figure 3, were observed in the mice injected with PEG-stimulated LCs (data not shown).

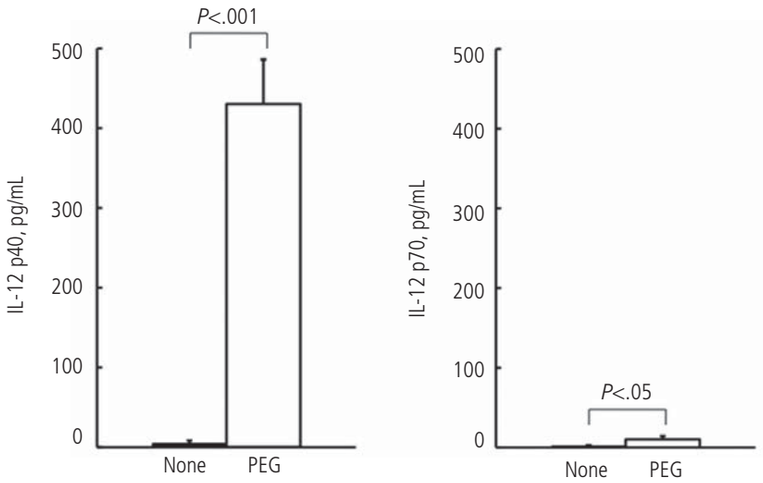

Figure 2. Effects of peptidoglycan (PEG) on interleukin (IL) 12 production from Langerhans cells (LCs). LCs (5×10^5/mL) from mouse epidermis were incubated with or without 10 µg/mL PEG for 48 hours. Supernatants were assayed for IL-12 p40 and IL-12 p70 production using enzyme-linked immunosorbent assay. The results are expressed as means (SD) (n=6).

Production of IL-12 p40 Homodimers From LCs by PEG Stimulation

It is well-known that the IL-12 family member, IL-23, is a disulfide-linked heterodimer of p19 and p40 subunits [16]. The p19 subunit is unique to IL-23 while the p40 subunit is shared with IL-12. We therefore analyzed the expression of p19 using RT-PCR. As suggested by Figure 4, we believed that PEG-stimulation of LCs might induce IL-23 p19 production and that the enhancement of IL-12 p40 production by PEG might therefore be associated with that of IL-23 production. Therefore, we tested for the presence of IL-23 and IL-12 p40 in the culture supernatant of PEG-stimulated LCs. Nonreducing SDS-PAGE and subsequent Western blotting with anti-IL-12 p40 antibody demonstrated prominently migrating bands of approximately 96 kDa and 40 kDa corresponding to the IL-12 p40 homodimer and monomer, respectively, but no bands corresponding to IL-23 (approximately 66 kDa) were observed. Western blotting with anti-IL-12 p70 antibody

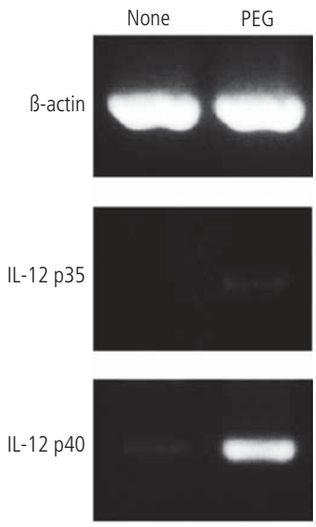

Figure 3. Effects of peptidoglycan (PEG) on interleukin (IL) 12 mRNA expression in Langerhans cells (LCs). LCs (5×10^5/mL) from mouse epidermis were incubated with or without 10 µg/mL PEG for 18 hours. Cytoplasmic mRNA was extracted from LCs, reverse-transcribed and amplified by polymerase chain reaction using ß-actin, IL-12 p35, and IL-12 p40 primer sets. The data shown are the representative results of 4 independent experiments.

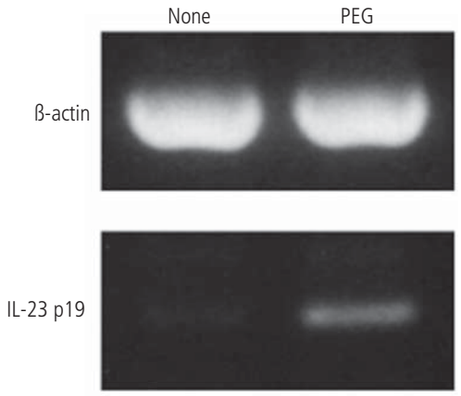

Figure 4. Effects of peptidoglycan (PEG) on IL-23 p19 mRNA expression in Langerhans cells (LCs). LCs (5×10^5/mL) from mouse epidermis were incubated with or without 10 µg/mL PEG for 18 hours. Cytoplasmic mRNA was extracted from LCs, reverse-transcribed, and amplified by PCR using ß-actin and IL-23 p19 primer sets. The data shown are the representative results of 4 independent experiments.

(p35- specific) and anti-IL-23 p19 antibody also did not reveal any noticeable bands (data not shown). These findings suggest that the majority of IL-12 p40 existed as the p40 monomer and homodimer, and that IL-12 p70 (p35/p40) and IL-23 (p19/p40) were below the detection limit in this experiment (Figure 5).

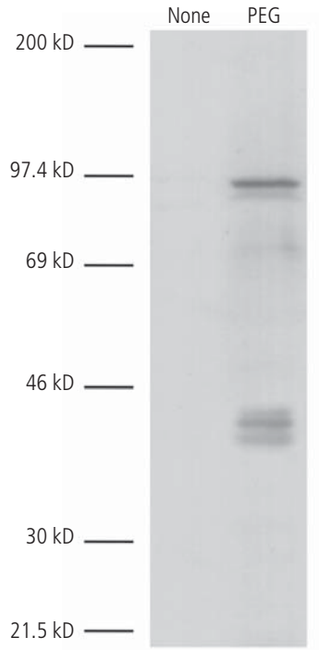

Figure 5. Western blot analysis of interleukin (IL)-12 p40 in the culture supernatant of peptidoglycan (PEG)-stimulated Langerhans cells (LCs). LCs (5×10^5/mL) from mouse epidermis were incubated with or without 10 µg/mL PEG for 48 hours. IL-12 p40 in the supernatants was concentrated, immunoprecipitated, separated by nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis, and analyzed by Western blotting, as described in the Materials and Methods section. The data shown are the representative results of 4 independent experiments.

Discussion

A high incidence of chronic skin colonization by *S aureus* is a prominent feature of AD. Although many attempts have been made to characterize the role of *S aureus* in the skin of AD patients, most studies have focused on the specific role of staphylococcal exotoxins [17]. However, half of *S aureus* strains isolated from AD patients have been found to be incapable of producing superantigens [3,18,19], and therefore the roles of these strains in the skin lesions of AD patients are not fully understood. In a previous study, we demonstrated that the intradermal injection of a cell wall component of *S aureus*, LTA, was able to induce localized AD-like inflammation, which was characterized by infiltration of eosinophils, mast cells, and mononuclear cells into the dermis [7]. It might therefore be concluded that *S aureus* strains that do not produce toxins acting as superantigens might also be capable of causing the inflammation of AD lesions.

The number of T_H2 cells is markedly increased in peripheral blood and acute skin lesions of AD patients. Therefore, it is understood that the development of T_H2 cells is etiologically important in AD. LCs are bone marrow-derived MHC class II positive antigen-presenting cells (APCs) located in the epidermis and mucosa. They belong to a DC lineage and are

crucial for primary and secondary T cell–dependent immune responses [20]. DCs are crucial in determining the outcome of antigen encounter, integrating signals derived from the antigen, its inflammatory context, and the host environment into a form that can be read by naïve T cells in lymphoid tissues and by effector T cells in peripheral tissues. LCs are capable of secreting IL-12 [21], which is produced predominantly by DCs and macrophages [22,23]. IL-12 p70 is a heterodimeric cytokine consisting of 2 disulfide-linked subunits, p35 and p40 [24]. It is known to exert multiple effects on the activation of T cells, and it increases the production of cytokines such as IFN-γ, thereby serving as a powerful mediator for T_H1-type differentiation of T helper cells both in vitro and in vivo [25]. Monomers of the p40 and p35 subunits do not have IL-12 activity, but the homodimer of p40 has been shown to bind to the IL-12 receptor and work as an antagonist of IL-12 p70 [26-28].

In this study, we investigated the capacity of mouse LCs to induce a T_H2-prone immune response. It has been proposed that T_H2-type immune responses play a key pathogenetic role in AD, and this is supported by the presence of blood eosinophilia and enhanced serum IgE levels in the majority of AD patients [29]. However, the immunoregulatory mechanism that induces T_H2 development in AD is still unknown. In the present study, the levels of IL-12 p70 induced by PEG stimulation were markedly lower than those of IL-12 p40. This result is consistent with a previous report by Re et al [10] in which PEG stimulation of human DCs failed to induce IL-12 p70 but resulted in the release of IL-12 p40. Recent studies using mouse models have shown that activation of DCs with distinct TLR agonists appears to modulate their adaptive immune responses. For example, activation of TLR2 by Pam3Cys or *Porphyromonas gingivalis* lipopolysaccharide induced a prominent T_H2-biased immune response [30-32] and the T_H2 response was associated with a failure of IL-12 p70 production in DCs. DCs activated by a superantigenic toxin, SEB, were also able to drive polarization of naïve allogenic T cells into the T_H2 subset in vitro [9]. Furthermore, the development of T_H2 cells was associated with DC activation through TLR2 signaling and an absence of IL-12 p70 production. Therefore, it seems that the downregulation of IL-12 p70 production from APCs is important for T_H2 cell development. The ELISA system for IL-12 p40 used in the present study was developed for the detection of IL-12 p40 homodimers. The system, however, shows approximately 53% and 52% cross-reactivity with IL-23 (p19/p40) and IL-12 p40 (monomer), respectively. Therefore, the IL-12 proteins from PEG-stimulated LCs were immunoprecipitated with anti-mouse IL-12 p40 monoclonal antibody and characterized by Western blot analysis. Under nonreducing conditions, the purified IL-12 p40 subunit migrated mainly at the 96-kDa and 40-kDa positions corresponding to the IL-12 p40 homodimer and monomer, respectively. Our results, therefore, suggest that the IL-12 p40 homodimer from PEG-stimulated LCs may play a critical role in T_H2 development in addition to insufficient IL-12 p70 production. In fact, increased expression of IL-12 p40 protein has been observed in LCs in chronic AD skin lesions [33].

Since PEG is a well-known TLR2 agonist [10], signaling through TLR2 on LCs would be associated with the development of T_H2 cells. In fact, the results of RT-PCR using a TLR2 primer set predicted the existence of TLR2 in LCs (data not shown), and it has already been confirmed that the PEG used in this study is a specific stimulator of TLR2 [34]. The density of *S aureus* in the skin lesions of AD patients exceeds 1×10^7 organisms/cm^2, which is equivalent to 1 to 10 μg PEG/cm^2 of skin. Therefore, the concentration of PEG used for in vitro stimulation in this study (10 μg/mL) is a realistic concentration in vivo [35], and sustained *S aureus* colonization might elicit induction of a T_H2-prone immune response by epidermal LCs.

Previous studies have not explained the role of *S aureus* in the development of T_H2 cells in AD patients. The present results suggest that skin colonization by *S aureus* may play a critical role in perpetuating skin tissue inflammation through the development of T_H2 cells induced by PEG, which are common cell components of superantigenic exotoxin-producing and -nonproducing *S aureus* strains. As the skin of most AD patients shows superficial *S aureus* colonization and barrier disruption due to reduced levels of ceramide [36], PEG would be expected to penetrate continuously into the skin. Therefore, antimicrobial treatment in a subgroup of AD patients, irrespective of whether or not they show clinical signs of superinfection, may be considered a new therapeutic strategy for AD.

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