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Atypical clinical appearance of eosinophilic pustular folliculitis of seborrheic areas of the face

Eosinophilic pustular folliculitis is a pruritic eruption that preferentially involves the face. It is characterized by well-demarcated erythema, extending peripherally with a central clearing and pigmentation, together with sterile pustules lining the periphery. We describe five cases of eosinophilic pustular folliculitis with pruritic papules and erythema on seborrheic areas of the face, which lacked the typical features of classic eosinophilic pustular folliculitis—pustules and peripheral extension—but showed eosinophilic infiltration of the hair follicles, histologically. The eruption quickly responded to oral indomethacin except for one case that responded to tranilast and one case that was associated with acquired immunodeficiency syndrome, with recurrences in defined areas of the face. Our findings in these cases suggest that eosinophilic pustular folliculitis may vary in clinical appearance.

Key words: eosinophilic pustular folliculitis, hair follicle, indomethacin, sebaceous gland, tacrolimus

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Eosinophilic pustular folliculitis (EPF), first described by Ofuji *et al.* in 1965 [1], is characterized by crops of pruritic, follicular, sterile papulopustules in fairly defined areas. The eruption tends to extend peripherally with a central clearing and pigmentation. The duration and extent of these eruptions and the interval between relapses varies. EPF responds to oral indomethacin within a few days and, unless treated, new lesions continue to develop in defined areas over a certain period. Although the mechanism of EPF still remains uncertain, Th2-mediated immune responses and/or COX metabolites are presumed to be involved in the pathogenesis of EPF [2-4]. Here, we describe five cases of episodic papules and/or erythema associated with severe pruritus of the face, which we considered to be variants of EPF. Since these patients lacked typical clinical features of EPF—pustules and peripheral extension—they received inadequate treatment under wrong diagnoses such as eczema, acne and bacterial folliculitis.

Case reports

Case 1

A 39-year-old woman presented with a 1-month history of multiple pruritic and indurated papules on the forehead and cheeks, which were unresponsive to roxythromycin or minocycline (*figure 1A*). She was otherwise healthy. The papules cleared after 1 week with oral tranilast, 300 mg daily – a drug used for the treatment of allergic disorders, keloids and hypertrophic scars – which was administered to reduce scar formation after the skin biopsy. The histology of a skin biopsy taken from a papule on the forehead showed dense lymphocyte infiltration, with a number of eosinophils in the dermis and the upper part of the subcutis. The infiltration of eosinophils was pronounced in

the infundibular epithelium and around the hair follicles and sebaceous glands (*figures 2A-B*). Blood examination showed a normal leukocyte count of $5.9 \times 10^9/L$ (normal range, 3.1-9.7) with 8.9% eosinophils (normal range, 3-5%), and values for hemoglobin, platelet count, renal and liver function tests were all within the normal reference ranges. The diagnosis of EPF was made based on the histology, despite the absence of pustules or an annular configuration of the lesions. Oral indomethacin was initiated; however, the patient stopped taking indomethacin due to gastric pain. Tranilast was continuously administered for 4 weeks leading to a complete clearance of the eruption. In the following year, the eruption recurred episodically and responded well to tranilast within a few weeks.

Case 2

A 40-year-old man presented with a 3-month history of multiple, pruritic erythematous papules on the forehead and cheeks, which had not responded to topical steroids (*figure 1B*). He had started taking voglibose for diabetes mellitus 4 months previously but had ceased the medicine after the eruption appeared. Photo testing with 50 and 100 mJ/cm² of UVB and 10 J/cm² of UVA did not produce erythema. The histology of a skin biopsy taken from an area of erythema on the face showed infiltration of eosinophils in and around the hair follicles (*figures 2C-D*). Examination of the peripheral blood showed a normal leukocyte count of $6.7 \times 10^9/L$ with 5.5% eosinophils, and normal values for hemoglobin, platelet count, and renal and liver function tests. Human immunodeficiency virus (HIV) serology was negative. Based on the histology, the diagnosis of EPF was made; the eruption was successfully treated with oral indomethacin and topical tacrolimus. In the following years, he had periodical relapses, which responded to topical tacrolimus.

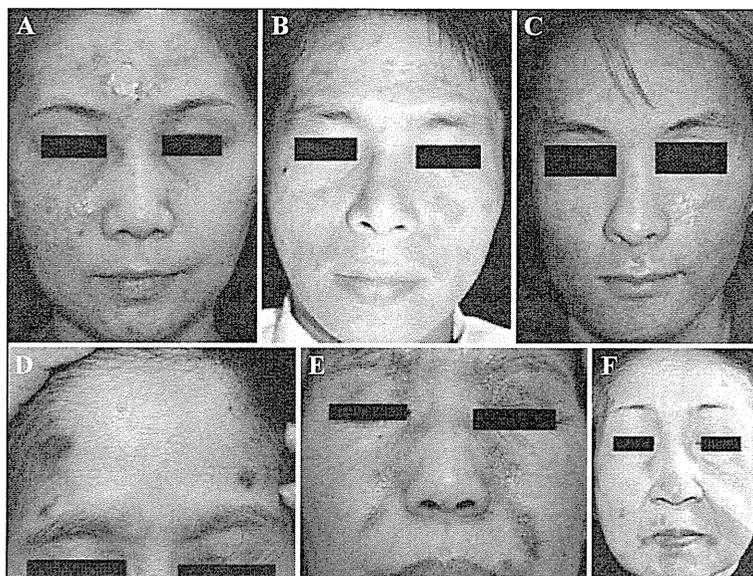


Figure 1. A) Indurated papules on the forehead and cheeks in case 1. B) Erythema on the forehead and cheeks with periorbital sparing in case 2. C) Diffuse erythema and papules with periorbital sparing on the face in case 3. D) Erythema with pigmentation on the forehead at the initial presentation in case 4 and E) exudative erythema at the time of exacerbation. F) Scattered patches of erythema on the forehead, cheeks and periorally in case 5.

Case 3

A 29-year-old man presented with a 3-month history of papules and a diffuse erythema on the face with periorbital sparing, associated with severe pruritus (*figure 1C*). The eruption was resistant to oral and topical antibiotics and corticosteroids. He was otherwise healthy. The histology of a skin biopsy showed a lymphocyte infiltration with eosinophils in the hair follicles and sebaceous glands (*figures 2E-F*). The eruption was resistant to oral indomethacin, minocycline, tranilast and topical tacrolimus. Examination of the peripheral blood showed a leukocyte count of $5.8 \times 10^9/L$ with 16.0% eosinophils, an elevated IgE level of 1900 IU/mL and normal values for hemoglobin, platelet count, renal and liver function tests. HIV serology was positive with an HIV-1 RNA level of 1.6×10^5 copies/mL, a low CD4 count of 106/ μL , and a decreased CD4/CD8 ratio of 0.18. He was diagnosed with acquired immunodeficiency syndrome (AIDS). As CD4 counts started to recover after commencement of highly active antiretroviral therapy (HAART), the eruption gradually resolved with topical tacrolimus. During a 3-year follow-up, there was no recurrence and he continued to receive HAART.

Case 4

A 56-year-old woman presented with a 2-month history of pruritic and well-demarcated erythematous patches with post-inflammatory hyperpigmentation on the forehead, associated with several necrotic papules on the arms, which had been resistant to topical steroids (*figure 1D*). She was otherwise healthy. The histology of a skin biopsy taken from a necrotic papule on the arm showed a subcorneal collection of eosinophils around a hair follicle. Minocycline

was effective for both the erythema on the face and the papules on the arms and there were no new lesions, although the pigmentation on the face persisted during the 5-month follow-up. Five months after her initial visit, an exudative erythema associated with severe pruritus suddenly appeared on the seborrheic areas of the face (*figure 1E*). The histology of a biopsy taken from the erythema showed a perivascular lymphocyte infiltrate in the dermis and a subcorneal pustule filled with a number of eosinophils around a hair follicle (*figures 2G-H*). The blood examination showed a normal leukocyte count of $5.8 \times 10^9/L$ with 7.6% eosinophils, and normal values for hemoglobin, platelet count, and renal and liver function tests. EPF was suspected from the histological picture. Oral indomethacin was initiated and her eruption resolved within 1 week. During a 2-month follow-up, she experienced several recurrences, which were successfully treated with oral indomethacin. She also experienced palmoplantar vesicles during the exacerbation, which also responded to oral indomethacin.

Case 5

A 60-year-old woman presented with a pruritic, exudative erythema on the face (*figure 1F*). She had been treated for several months at another clinic with a topical corticosteroid ointment with a putative diagnosis of acute eczema. The histology of a biopsy taken from the erythema on the face showed perivascular lymphocyte infiltration in the dermis, subcorneal pustules and spongiosis of the perifollicular epidermis containing a few eosinophils (*figures 2I-J*). The eruption subsided within 1 week after treatment with oral indomethacin and topical tacrolimus. During a 6-month follow-up, the eruption reappeared but was successfully treated with oral indomethacin and topical tacrolimus.

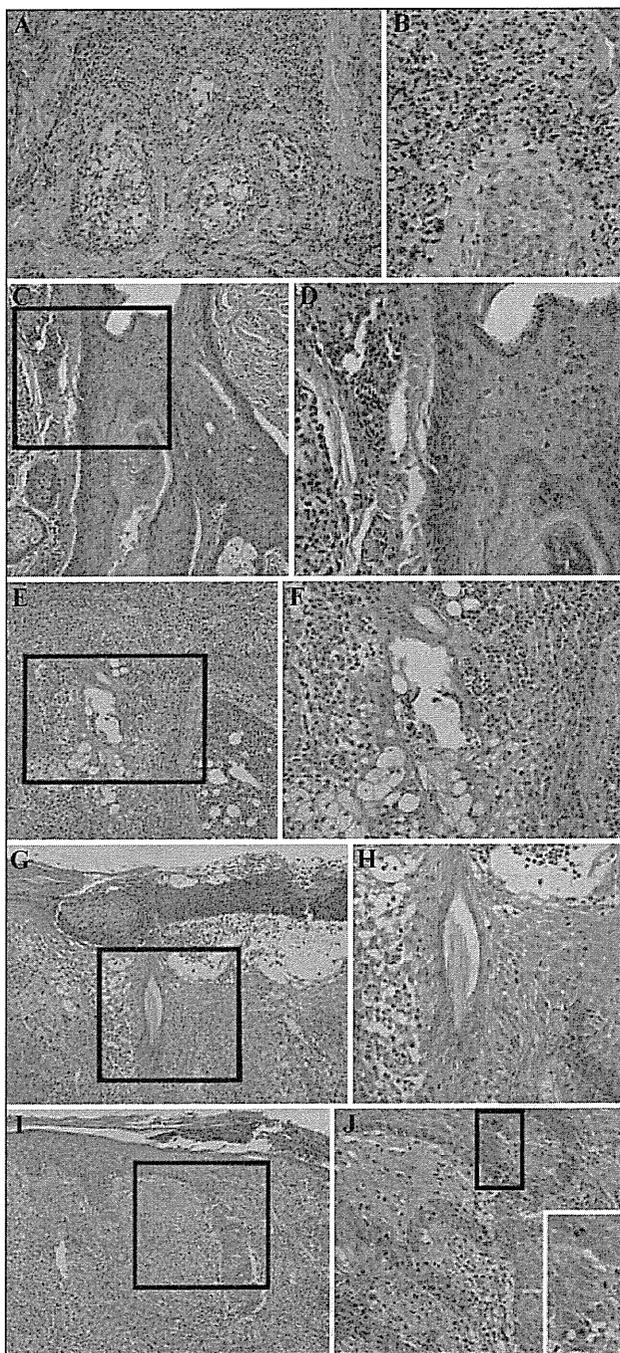


Figure 2. The histopathology of biopsies from the five cases (hematoxylin-eosin stain). **A)** A biopsied papule of case 1 shows that eosinophils infiltrated the sebaceous glands (original magnification $\times 200$) and **B)** around the hair follicle (original magnification $\times 400$). **C)** Histology of case 2 shows demodex mites in the dilated pore of a hair follicle (original magnification $\times 200$). **D)** Higher magnification of the rectangular areas in (C) shows that eosinophils infiltrated into and around the hair follicle (original magnification $\times 400$). **E)** Histology of case 3 showing the destruction of a hair follicle and sebaceous glands with infiltration of mononuclear cells and numerous eosinophils (original magnification $\times 200$). **F)** Higher magnification of the rectangular area in (E) shows eosinophils in the hair follicle (original magnification $\times 400$). **G)** The biopsy of an erythematous area of case 4 shows a subcorneal pustule arising from a hair follicle (original

Discussion

We experienced five cases of pruritic eruptions, mainly distributed on the face. Their clinical features varied, with indurated papules and/or erythema with periorbital sparing on the face (Cases 1-3), or crops of exudative erythema mimicking acute eczema (Cases 4, 5). The differential diagnosis included acne, seborrheic dermatitis and contact dermatitis. However, there was an absence of comedos and eruptions were resistant to oral antibiotics or topical corticosteroids. Although their histologies also varied, they shared the common feature of an infiltration of eosinophils around hair follicles, sebaceous glands and/or eccrine glands. Based on the histology, we diagnosed these cases as EPF. Three patients were successfully treated with oral indomethacin, one with tranilast, and the other with HAART and topical tacrolimus. Except for the case with AIDS, the patients had relapses over certain intervals. Although EPF is characterised by a well-demarcated erythema extending peripherally with a central clearing and pigmentation together with sterile pustules lining the periphery, our cases suggest that there may be variations which lack the typical features of EPF. In the original report of a case of "subcorneal pustular dermatosis" by Ofuji *et al.* in 1965 [1], they described a middle-aged woman who developed crops of small pustules on the face, trunk and upper arms; the histology showed subcorneal pustules in hair follicles filled with a number of eosinophils (in their original report, eosinophils were mistaken as neutrophils). She had eosinophilia ($1.2 \times 10^9/L$) and experienced recurrences. This case was later reconsidered and diagnosed as EPF in 1970 [5]. Cases 4 and 5 showed subcorneal pustules of hair follicles histologically, which were similar to Ofuji's original case.

Variations of classic EPF have been reported. A subtype manifesting as discrete, erythematous, follicular papules has been described in immunosuppressed individuals, especially those with HIV infection, and is called immunosuppression-associated EPF [6]. Infancy-associated EPF also differs from classic EPF, lacking peripheral extension and an annular arrangement [6], although there is controversy as to whether it qualifies as a distinct entity or whether there is actually another disease, such as scabies or insect bites [7]. Moreover, another variant of EPF that clinically mimics palmoplantar pustulosis has been described in the literature [8-10]; some of these cases had features of classic EPF on the face, but others appeared to be distinct. The palmoplantar lesions showed a favorable response to indomethacin and the histology showed subcorneal multilocular pustules filled with numerous eosinophils. The term eosinophilic pustular "folliculitis" seems to be

Figure 2. (Continued) magnification $\times 200$). **H)** Higher magnification of the rectangular area in (G) shows a pustule containing numerous eosinophils and eosinophilic spongiosis of the infundibular epithelium (original magnification $\times 400$). **I)** Histology in case 5 shows a subcorneal pustule over a hair follicle and spongiosis of outer root sheath (original magnification $\times 200$). **J)** Higher magnification of the rectangular areas in (I) shows a few eosinophils in the perifollicular epidermis (original magnification $\times 400$). Inset shows a higher magnification of the rectangular area in (J).

inappropriate to describe this palmoplantar variant, because the palms and soles contain no follicles. Furthermore, atypical EPF with a butterfly rash-like distribution has been reported, which showed a symmetrically distributed erythema without papules or pustules [11]. In addition, characteristic features of EPF are sometimes masked by inappropriate use of systemic steroids, leading to wrong diagnoses such as drug-related reactions, eosinophilic cellulitis, and fungal infection [12]. The above-mentioned atypical cases, including our five cases, suggest that EPF may manifest as an eruption without a tendency for peripheral extension, pustules, papules or follicular involvement. Therefore, the terminology and diagnostic criteria regarding EPF should be reconsidered to include such variations. We propose the term, “episodic eosinophilic dermatosis of the face” or “episodic eosinophilic dermatosis, a palmoplantar variant” to include both classic EPF and atypical cases of EPF such as those presented here. We propose additional criteria for episodic eosinophilic dermatosis of the face; the clinical and histological characteristics are summarized in *table 1*. Recurrences over months or years are typical. Severe pruritus that is resistant to topical steroids seems to be an important clinical feature. The diagnosis should be based on the histological finding of an eosinophilic infiltrate in the hair follicles or sebaceous glands, or subcorneal eosinophilic pustules. Careful serial biopsy sectioning may be required to detect the eosinophilic infiltrate. The effectiveness of oral indomethacin may also be a diagnostic feature, because no other inflammatory condition shows such a favorable response to indomethacin. We do not separate cases with respect to the presence of immunosuppression because discrete follicular papules may not be associated with immunosuppression as shown in our case (case 1). Cases resistant to conventional therapy, including oral indomethacin and topical tacrolimus, are highly suggestive of HIV infection and should be examined for this possibility. Treatment options for EPF include oral indomethacin, dapsone, minocycline, metronidazole, topical tacrolimus, and so on [13-17]; indomethacin is used most frequently and shows apparent clinical improvement in the majority of the cases [13, 14]. The mechanism of action by which indomethacin is effective for EPF remains mostly unclear. CRTH2, a receptor of prostaglandin D2 (PGD2), is expressed on

eosinophils, basophils, and Th2 cells; the PGD2- CRTH2 pathway seems to be involved in several allergic skin diseases [18, 19]. On exposure to PGD2, eosinophils may exhibit chemotaxis [19]. In other experiments, PGD synthase (PGDS) is detected in eosinophils; PGDS-positive eosinophils are accumulated around pilosebaceous areas in EPF. Sebocytes produce eotaxin-3, an eosinophilic chemoattractant, in the presence of PGD [4]. These findings may explain that EPF shows massive eosinophilic infiltration around pilosebaceous units [4]. Indomethacin, a COX inhibitor, inhibits the prostaglandin pathway and may lead to remission of the symptoms. In conclusion, episodic eosinophilic dermatosis of the face should be considered as a differential diagnosis of pruritic eruptions on the face that are resistant to conventional anti-inflammatory therapy. ■

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Table 1. Criteria of episodic eosinophilic dermatosis of the face.

Clinical appearance
Papules, pustules, or erythema, leaving post-inflammatory pigmentation
Peripheral extension may be observed.
Distribution
On seborrheic areas of the face with periorbital sparing
Symptoms
Severe pruritus
Course
Recurrences over months or years
Favorable response to indomethacin (a dependable diagnostic tool)
Resistant to topical corticosteroids
Histological findings
An eosinophilic infiltrate in hair follicles or sebaceous glands
Subcorneal eosinophilic pustules

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Author Offprint

PGD₂ induces eotaxin-3 via PPAR_γ from sebocytes: A possible pathogenesis of eosinophilic pustular folliculitis

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Background: Eosinophilic pustular folliculitis (EPF) is a chronic intractable pruritic dermatosis characterized by massive eosinophil infiltrates involving the pilosebaceous units. Recently, EPF has been regarded as an important clinical marker of HIV infection, and its prevalence is increasing in number. The precise mechanism by which eosinophils infiltrate into the pilosebaceous units remains largely unknown. Given that indomethacin, a COX inhibitor, can be successfully used to treat patients with EPF, we can assume that COX metabolites such as prostaglandins (PGs) are involved in the etiology of EPF.

Objective: To determine the involvement of PGs in the pathogenesis of EPF.

Methods: We performed immunostaining for PG synthases in EPF skin lesions. We examined the effect of PGD₂ on induction of eotaxin, a chemoattractant for eosinophils, in human keratinocytes, fibroblasts, and sebocytes and sought to identify its responsible receptor.

Results: Hematopoietic PGD synthase was detected mainly in infiltrating inflammatory cells in EPF lesions, implying that PGD₂ was produced in the lesions. In addition, PGD₂ and its immediate metabolite 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂) induced sebocytes to produce eotaxin-3 via peroxisome proliferator-activated receptor gamma. Consistent with the above findings, eotaxin-3 expression was immunohistochemically intensified in sebaceous glands of the EPF lesions.

Conclusion: The PGD₂/PGJ₂-peroxisome proliferator-activated receptor gamma pathway induces eotaxin production from sebocytes, which may explain the massive eosinophil infiltrates observed around pilosebaceous units in EPF. (*J Allergy Clin Immunol* 2012;129:536-43.)

Key words: Prostaglandin D₂, hematopoietic prostaglandin D synthase, eotaxin-3/CCL26, sebocyte, peroxisome proliferator-activated receptor gamma

Abbreviations used

CRT _{H2} :	Chemoattractant receptor-homologous molecule expressed on T _{H2} cells
EPF:	Eosinophilic pustular folliculitis
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
H-PGDS:	Hematopoietic prostaglandin D synthase
L-PGDS:	Lipocalin-type prostaglandin D synthase
PG:	Prostaglandin
PPAR _γ :	Peroxisome proliferator-activated receptor gamma
siRNA:	Small-interfering RNA

Eosinophilic pustular folliculitis (EPF) is a chronic intractable pruritic dermatosis characterized by massive eosinophil infiltrates involving the pilosebaceous units.¹ The evidence accumulated to date indicates that T_{H2}-mediated immunologic mechanisms are involved in the pathogenesis of EPF.^{2,3} Recently, EPF has been regarded as an important clinical marker of HIV infection, and its prevalence is increasing in number.⁴ An immunohistochemical study has demonstrated the expression of intercellular adhesion molecules for inflammatory cells including eosinophils around hair follicles.⁵ Other studies have reported that IL-5 level, which induces proliferation and differentiation of eosinophils, is elevated in the blood and skin lesions of patients with EPF, but it can be decreased by treatment with IFN- γ .^{6,7} Three members of the eotaxin family—eotaxin-1/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26—are known to promote the growth and recruitment of eosinophils and skin inflammation.⁸ T_{H2} cytokines, such as IL-4, -5, and -13, enhance the production of eotaxins by skin component cells, such as lymphocytes, macrophages, endothelial cells, fibroblasts, and keratinocytes.⁹⁻¹¹ These findings suggest that the pathogenesis of EPF consists of a T_{H2}-type immune response; intriguingly, however, EPF is usually resistant to topical or systemic corticosteroids that suppress the functions of T cells. Therefore, the pathogenesis of EPF might not be explained solely by T_{H2} immunity. Since EPF can be successfully treated with indomethacin, a COX inhibitor,¹² we hypothesize that the prostaglandin (PG) family known as the prostanoids, which occur downstream of COX, might be involved in the etiology of EPF.

Prostanoids are released from cells immediately after their formation. Because they are chemically and metabolically unstable, they usually function only locally through membrane receptors on target cells.¹³ Recently, individual prostanoid receptor gene-deficient mice have been used as models to dissect the respective roles of each receptor in combination with the use of compounds that selectively bind to prostanoid receptors as agonists or antagonists.^{14,15} The prostanoids PGD₂ and PGE₂ are 2 of the major COX metabolites in the skin. PGE₂ has been reported to have an inhibitory effect on eosinophil trafficking and

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activation.¹⁶ PGD₂, on the other hand, is known to be involved in chronic allergic inflammation.¹⁷ Two types of PGD synthase (PGDS), which catalyzes the isomerization of PGH₂, a common precursor of various prostanoids that catalyze PGD₂, have been identified: one is the lipocalin-type PGDS (L-PGDS), and the other is the hematopoietic PGDS (H-PGDS).¹⁸ L-PGDS is localized in the central nervous system, the male genital organs, the heart, and melanocytes in skin.^{18,19} H-PGDS is widely distributed in the peripheral tissues and localized in antigen-presenting cells, mast cells, megakaryocytes, T_H2 lymphocytes, and dendritic cells.^{18,20-22}

The aim of this study was to verify the hypothesis that prostanoids are involved in the development of eosinophil infiltration in the pilosebaceous units of the EPF skin lesions. We found that inflammatory cells in EPF lesions were positively immunostained for H-PGDS, suggesting that PGD₂ production was increased in EPF lesions. Moreover, we found that PGD₂ increased eotaxin-3 mRNA expression in sebocytes via peroxisome proliferator-activated receptor gamma (PPAR γ) and that eotaxin-3 was detected around sebaceous glands in EPF lesions. Our data suggest that PGD₂ is involved in the pathogenesis of EPF lesions by inducing eotaxin-3 from sebocytes via PPAR γ .

METHODS

Human subjects

We obtained biopsy specimens from 5 patients with EPF, 6 patients with folliculitis, and 4 healthy subjects. Informed consent was obtained from all subjects involved in this study. The Ethics Committee of Kyoto University approved the study.

Histologic examination

Paraffin-embedded sections were stained with hematoxylin-eosin and immunostained with H-PGDS, a monoclonal mouse antihuman antibody (dilution 1:500), L-PGDS, a polyclonal rabbit antihuman antibody (dilution 1:1000) (both were established at the Osaka Bioscience Institute, Osaka, Japan), and eotaxin-3/CCL26, a polyclonal goat antihuman antibody (dilution 1:100, R&D Systems, Minneapolis, Minn). As negative controls for H-PGDS and L-PGDS antibodies, we used isotype-matched control antibody and rabbit serum, respectively. Antigen retrieval was achieved by pepsin treatment for L-PGDS and preincubation with proteinase K for eotaxin-3. Nonspecific binding was blocked by addition of 10% goat serum for 30 minutes at room temperature. Afterward, sections were incubated for 1 hour at room temperature with the primary antibody followed by incubation with a species-specific biotinylated immunoglobulin (Vector, Burlingame, Calif) for 30 minutes at room temperature. Thereafter, they were incubated for 30 minutes with the avidine-biotin-peroxidase complex kit (Vector) and visualized with 3,3'-diaminobenzidine. They were lightly counterstained with hematoxylin. The number of immunoreactive cells per high power field was enumerated at 3 locations (original magnification $\times 200$) per sample, and data were expressed as the number of H-PGDS- and L-PGDS-positive cells per high power field.

Preparation of human eosinophils and flow cytometry

Peripheral blood was obtained from 3 patients with EPF and 3 healthy donors. Polynuclear cells were separated by centrifugation of whole blood over Mono-Poly Resolving Medium (DS Pharma Biomedical, Osaka, Japan), followed by removal of remaining red cells by ACK lysing buffer (Lonza Walkersville, Inc, Walkersville, Md). They were stained with the antibodies against surface markers of eosinophils: antihuman CCR3-phycoerythrin (dilution 1:100, R&D Systems) and antihuman CD16-fluorescein isothiocyanate (dilution 1:100, Becton Drive Biosciences,

Franklin Lakes, NJ). Eosinophils were identified with CCR3 positive and CD16 negative by flow cytometric analysis. With the use of an IntraStain kit (Becton Drive Biosciences), intracellular H-PGDS was detected by staining with polyclonal rabbit antihuman H-PGDS antibody (dilution 1:50, Cayman Biochemical) followed by antirabbit Alexa Fluor 647 (dilution 1:200, Life Technologies, Tokyo, Japan). The expression of H-PGDS was analyzed for mean fluorescence intensity.

For purification of eosinophils, the peripheral blood of patients with mild allergic rhinitis was collected by negative selection by using Eosinophil Isolation Kit (Miltenyl Biotec, Bergisch Gladbach, Germany). Both the purity and the viability of eosinophils were confirmed to exceed 95%.

Cell culture

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were grown in Humedia-KG2 medium (Kurabo) with human epidermal growth factor (0.1 ng/mL), insulin (10 μ g/mL), hydrocortisone (0.5 μ g/mL), gentamicin (50 μ g/mL), amphotericin B (50 ng/mL), and bovine brain pituitary extract (0.4%, v/v). Primary skin fibroblasts were isolated by standard methods²³ from healthy human skin and were cultured grown in Dulbecco modified Eagle medium (Gibco, Karlsruhe, Germany) with 10% FBS (Gibco).

The immortalized human sebaceous gland cell lines SZ95 (a kind gift from Dr Christos C. Zouboulis) were cultured in sebomed basal medium (Biochrom AG, Berlin, Germany) with 10% FBS and recombinant human epidermal growth factor (Sigma Chemical, St Louis, Mo).

As for normal human epidermal keratinocytes and fibroblasts, the cells grew to 80% to 90% confluent and were starved for 3 hours, followed by treatment with PGD₂ (10 μ M) (Cayman Biochemical) for 24 hours at 37°C in 5% CO₂.

Agonists used were the DP agonist BW245c (Cayman Biochemical), the chemoattractant-homologous receptor expressed on T_H2 cells (CRT_H2) agonist 15-keto-PGD₂ (DK-PGD₂) (Cayman Biochemical), and the PPAR γ agonist 15-deoxy- Δ 12,14-PGJ₂ (15d-PGJ₂) (Cayman Biochemical). Antagonists used were the DP antagonist BWA868c (Cayman Biochemical), the CRT_H2 antagonist CAY10471 (Cayman Biochemical), and the PPAR γ antagonist GW9662 (Cayman Biochemical). Sebocytes were starved for 3 hours and treated with PGD₂ (1-20 μ M), BW245c (1-10 μ M), DK-PGD₂ (1-10 μ M), and 15d-PGJ₂ (1-7 μ M) for 21 hours at the confluency of 30% to 40%. For treatment with antagonists, BWA868c (1-10 μ M), CAY10471 (1-10 μ M), and GW9662 (1-3 μ M) (Cayman Biochemical) were preadded at 30 minutes.

SZ95 cells were transfected with PPAR γ small-interfering RNA (siRNA) or nontargeting siRNA (Dharmacon, Lafayette, Colo) at 20% confluence by using Lipofectamine 2000 (Life Technologies). At 48 hours after transfection, the cells were starved for 3 hours and treated with or without PGD₂ (7.5 μ M) for an additional 21 hours.

For detection of PGD₂, purified eosinophils (1×10^6 cells per well) were incubated in 50 μ L of RPMI 1640 with 10% FBS in the presence and absence of 10^{-6} mol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Mo) and 10^{-5} mol/L calcium ionophore A23187 (Sigma-Aldrich). The concentration of PGD₂ in the supernatant was detected by the use of PGD₂-MOX Enzyme Immunoassay Kit (Cayman Biochemical).

Quantitative RT-PCR

Total RNA was isolated with RNeasy kits and digested with DNase I (Qiagen, Hilden, Germany). The cDNA was reverse transcribed from total RNA samples by using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR was performed by using Light Cycler 480 SYBR Green I Master (Roche, Mannheim, Germany) and the Light Cycler real-time PCR apparatus (Roche) according to the manufacturer's instructions. The primers used for PCR had the following sequences: eotaxin-1, 5'-CTC CGCAGCACTTCTGTGGC-3' (forward) and 5'-GGTCGGCACAGATATCCTTG-3' (reverse); eotaxin-2, 5'-GCCTTCTGTTCTGGGTGTC-3' (forward) and 5'-CCTCCTGAGTCTCCACCTTG-3' (reverse); eotaxin-3, 5'-CCTCCTGAGTCTCCACCTTG-3' (forward) and 5'-AAGGGGCTTGT

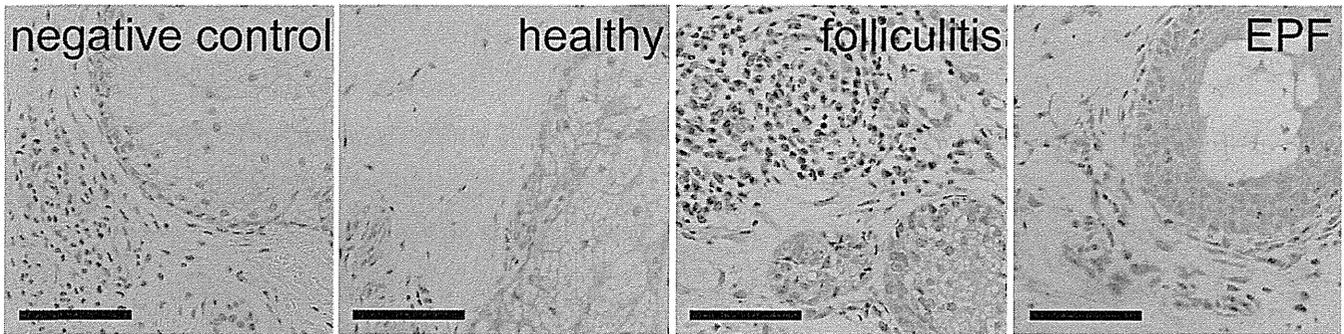
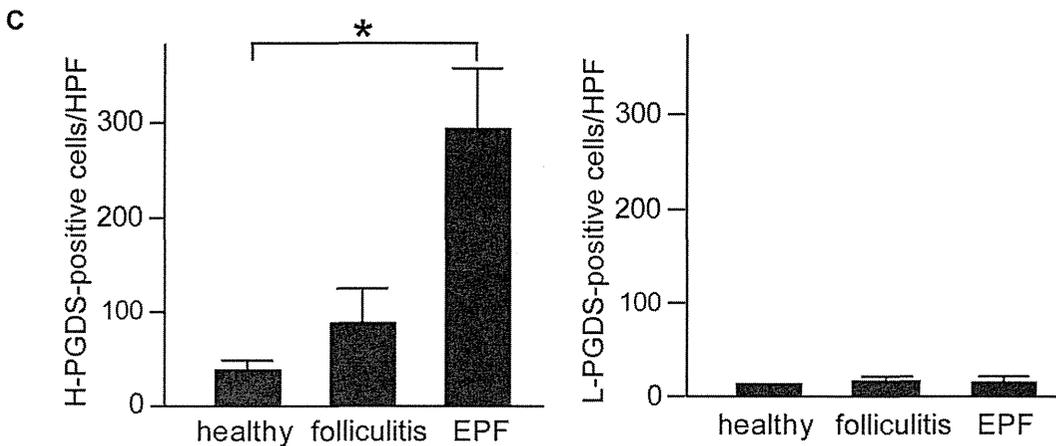
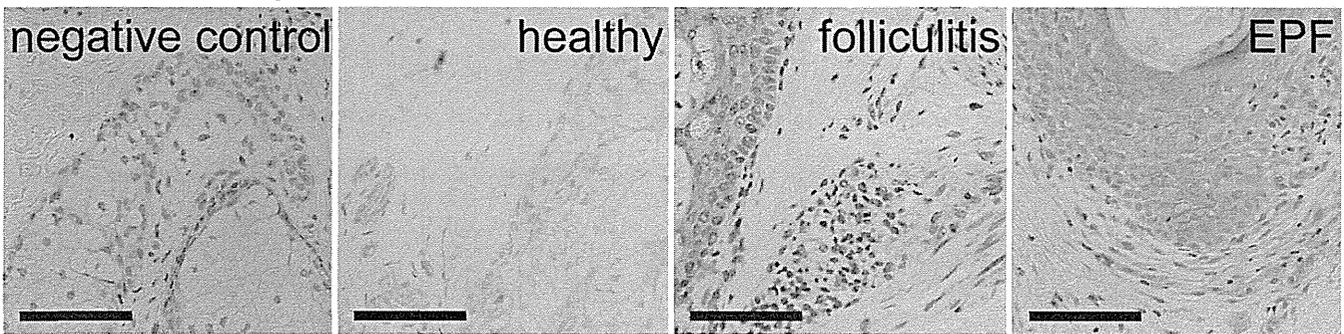
A immunostaining for H-PGDS**B** immunostaining for L-PGDS

FIG 1. Immunohistochemistry of PGDS. Skin specimens taken from healthy subjects ($n = 4$), patients with folliculitis ($n = 6$), and patients with EPF ($n = 5$) were immunostained for H-PGDS (**A**) and L-PGDS (**B**) and respective negative controls. The infiltrating inflammatory cells around the pilosebaceous gland in EPF were stained with anti-H-PGDS antibody. **C**, The numbers of H-PGDS- and L-PGDS-positive cells were counted. Bar = 100 μm . * $P < .05$. HPF, High power field.

GGCTGATT-3' (reverse); PPAR γ , 5'-ACAGACAAATCACCATTTCGT-3' (forward) and 5'-CTCTTTGCTCTGCTCCTG-3' (reverse); and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-AATGTCACCGTTGTC CAGTTG-3' (forward) and 5'-GTGGCTGGGGCTCTACTTC-3' (reverse). The results were normalized to those of the housekeeping GAPDH mRNA.

Statistical analysis

Unless otherwise indicated, data are presented as means \pm SD and are a representative of 3 independent experiments. P values were calculated with the 2-tailed Student t test. P values less than .05 are considered to be significantly different between the indicated groups and are shown as asterisk in the figures.

RESULTS**Increased H-PGDS expression in EPF lesions**

To verify PGDS expression in EPF lesions, we performed immunostaining with anti-H-PGDS and anti-L-PGDS antibodies. We found that the infiltrating inflammatory cells around pilosebaceous units were strongly positive for H-PGDS in lesions from patients with EPF, but not in healthy subjects (Fig 1, *A*). There were a few cells stained for L-PGDS (Fig 1, *B*). The number of H-PGDS-positive cells was significantly greater in EPF skin lesions than in normal healthy skin samples or in folliculitis lesions (Fig 1, *C*).

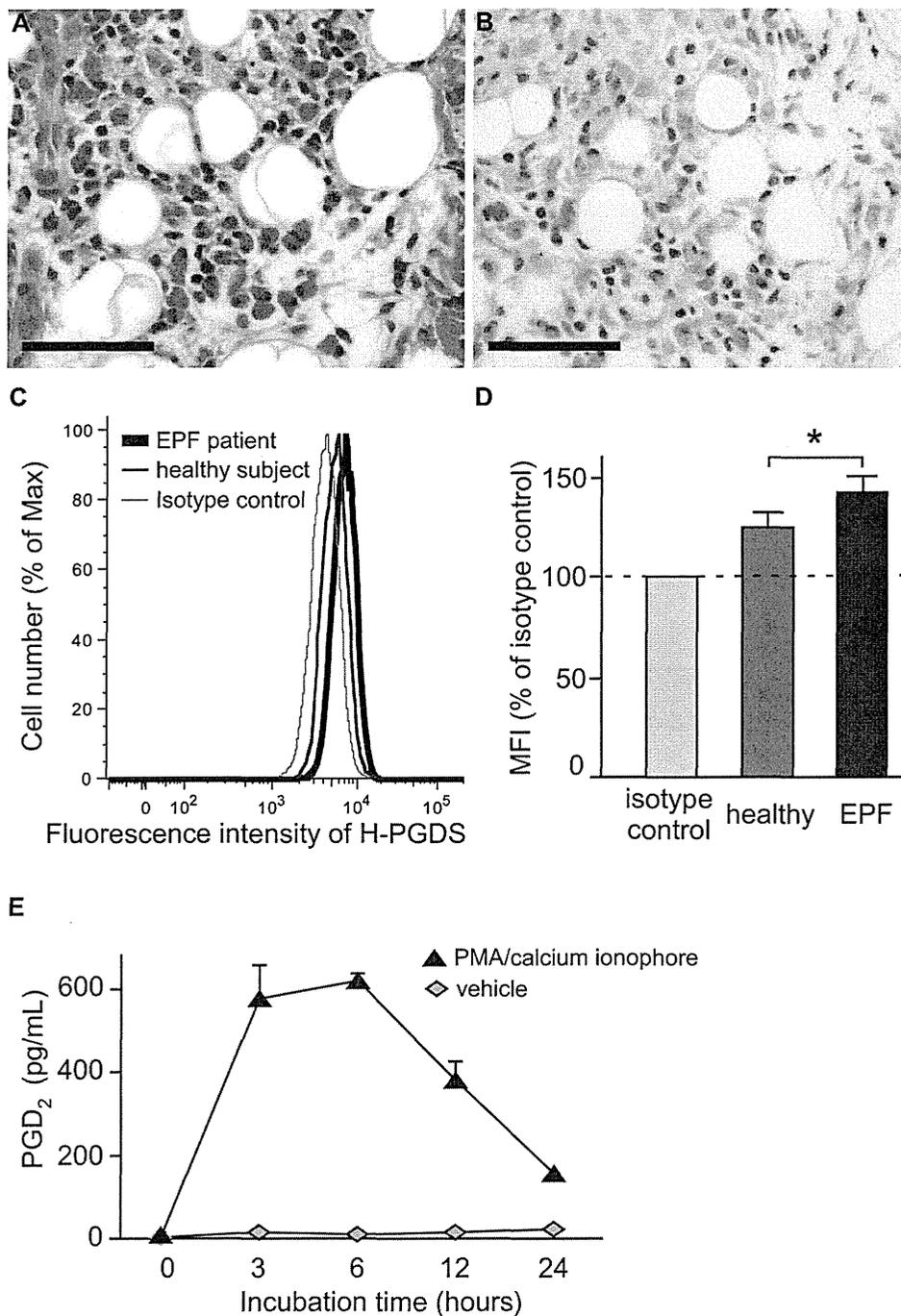


FIG 2. H-PGDS expression and PGD₂ production in eosinophils. Skin specimens of patients with EPF were stained with hematoxylin-eosin (A) and anti-H-PGDS antibody (B). Bar = 100 μ m. H-PGDS expression in eosinophils was determined by flow cytometry. C, Representative flow cytometry results. D, The MFI of isotype control was set as 100%, and the MFI of H-PGDS was calculated accordingly (n = 3). *P < .05. E, PGD₂ levels in eosinophil culture supernatants with or without phorbol 12-myristate 13-acetate and calcium ionophore. MFI, Mean fluorescence intensity.

H-PGDS expression and PGD₂ production in eosinophils

Numerous infiltrating eosinophils were stained with anti-H-PGDS antibody (Fig 2, A and B), suggesting that eosinophils may express H-PGDS. In fact, flow cytometric analysis showed that H-PGDS was detected in eosinophils, and its expression level was higher in patients with EPF than in healthy

subjects (Fig 2, C and D). In addition, we examined the production of PGD₂ from the supernatant of eosinophil culture at 0, 3, 6, 12, and 24 hours after incubation with or without phorbol 12-myristate 13-acetate and calcium ionophore. We found that a significant amount of PGD₂ was produced by eosinophils activated with phorbol 12-myristate 13-acetate and calcium ionophore (Fig 2, E).

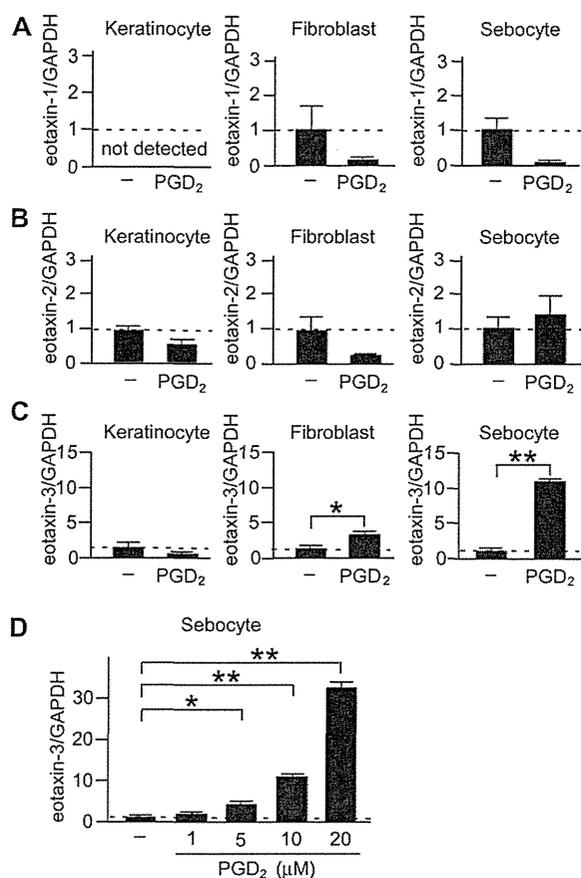


FIG 3. Effect of PGD₂ on eotaxin mRNA expression in human keratinocytes, fibroblasts, and the sebaceous gland cell line SZ95. Cells were incubated with PGD₂ (A-C; 10 μM). The mRNA expression levels of eotaxin-1 (Fig 3, A), eotaxin-2 (Fig 3, B), and eotaxin-3 (Fig 3, C and D) were evaluated by means of quantitative RT-PCR and normalized according to that of GAPDH. Data are shown as arbitrary units where the value for an unstimulated sample is set at 1 (n = 4). *P < .05, **P < .01.

PGD₂ increased eotaxin-3 mRNA expression in human sebocytes

We next asked whether PGD₂ could affect the production of chemokines for eosinophil migration. Since the diagnostic hallmark of EPF is the accumulation of eosinophils around the pilosebaceous units, we focused on sebocytes, the cells that constitute the pilosebaceous glands, as well as on keratinocytes and fibroblasts. We found that PGD₂ did not affect the expression of eotaxin-1 or -2 in human keratinocytes, fibroblasts, or sebocytes (Fig 3, A and B). It did induce eotaxin-3 expression, only slightly in fibroblasts but markedly in the human sebaceous gland cell line SZ95 (Fig 3, C). Moreover, we observed that PGD₂ increased eotaxin-3 mRNA expression in sebocytes in a dose-dependent manner (Fig 3, D). These findings suggest that PGD₂ induces eotaxin-3 production abundantly in sebocytes and that sebocytes might play a key role in eosinophil trafficking to the pilosebaceous units in EPF.

Dispensable role of the DP1 and CRT_{H2} receptors in PGD₂-induced eotaxin-3 expression

Two receptors for PGD₂ have been identified: one is DP1, and the other is CRTH2, also known as DP2, both of which are G

protein-coupled receptors.^{24,25} We next undertook to determine which receptor mediates eotaxin-3 upregulation by PGD₂. Neither the DP1 agonist BW245c nor the CRT_{H2} agonist DK-PGD₂ induced eotaxin-3 in the human sebaceous gland cell lines SZ95 (Fig 4, A). In addition, eotaxin-3 upregulation induced by PGD₂ was not suppressed by either the DP1 antagonist BW868c or the CRT_{H2} antagonist CAY10471 (Fig 4, B).

Involvement of PPAR_γ in PGD₂-induced eotaxin-3 expression in human sebocytes

PGD₂ spontaneously converts into the cyclopentenone PGs of the J series, such as PGJ₂, Δ12-PGJ₂,¹² and 15d-PGJ₂.²⁶ We found that 15d-PGJ₂ dose dependently increased eotaxin-3 expression in sebocytes (Fig 5, A). PGJ₂ elicits its function through PPAR_γ, and the PPAR_γ antagonist GW9662 suppressed 15d-PGJ₂-induced eotaxin-3 increase in a dose-dependent manner (Fig 5, B). We also observed that PGD₂-induced eotaxin-3 increase was suppressed by GW9662 in a dose-dependent manner (Fig 5, C). In addition, we examined the effect of PPAR_γ knockdown by RNA interference in order to confirm the role of PPAR_γ in PGD₂-induced eotaxin-3 expression. We observed that PPAR_γ mRNA expression was inhibited by PPAR_γ siRNA and that PGD₂-induced eotaxin-3 increase in sebocytes was suppressed by siRNA knockdown of PPAR_γ (Fig 5, D). In addition, we compared PPAR_γ expression among keratinocytes, fibroblasts, and sebocytes and found that it was higher in sebocytes than in keratinocytes and fibroblasts (Fig 5, E). These data suggest that PGD₂ induces eotaxin-3 expression in sebocytes, through PPAR_γ, which is highly expressed in sebocytes. Consistently, eotaxin-3 expression tended to be greater in sebocytes of EPF lesions than in those of normal skin samples (Fig 5, F).

DISCUSSION

In our current study, H-PGDS was detected in eosinophils by means of flow cytometric analysis, and these H-PGDS-positive cells were accumulated around the pilosebaceous areas in EPF, implying that PGD₂ is abundantly produced in this condition. In addition, eotaxin-3, which is produced by sebocytes via PPAR_γ upon stimulation by PGD₂, was highly expressed in the sebaceous glands in EPF lesions, likewise implying an abundance of PGD₂ in EPF. These findings may provide an explanation of the massive eosinophil infiltration that occurs around the pilosebaceous units in EPF.

Since indomethacin is generally effective against EPF, COX metabolites are presumed to be involved in the pathomechanism of EPF. Among these COX metabolites, the prostanoid PGD₂ has previously been reported to directly attract inflammatory cells such as T_{H2} cells, eosinophils, and basophils and to be involved in chronic allergic inflammation.^{17,27} This partly explains how the prostanoids are involved in the mechanism of EPF. Yet it remains unclear how eosinophils infiltrate the pilosebaceous units. In this study, we found that PGD₂ induces eotaxin-3 upregulation in sebocytes. PGD₂ enhances eotaxin-3 expression even in fibroblasts. Since the dermal papilla is a discrete population of specialized fibroblasts, PGD₂ may indirectly attract eosinophils via eotaxin produced by sebocytes and dermal papilla cells.

The underlying mechanism of controlling EPF by indomethacin has been reported to be attributed to the downregulation of CRT_{H2} expression.²⁸ This is an intriguing observation; however,

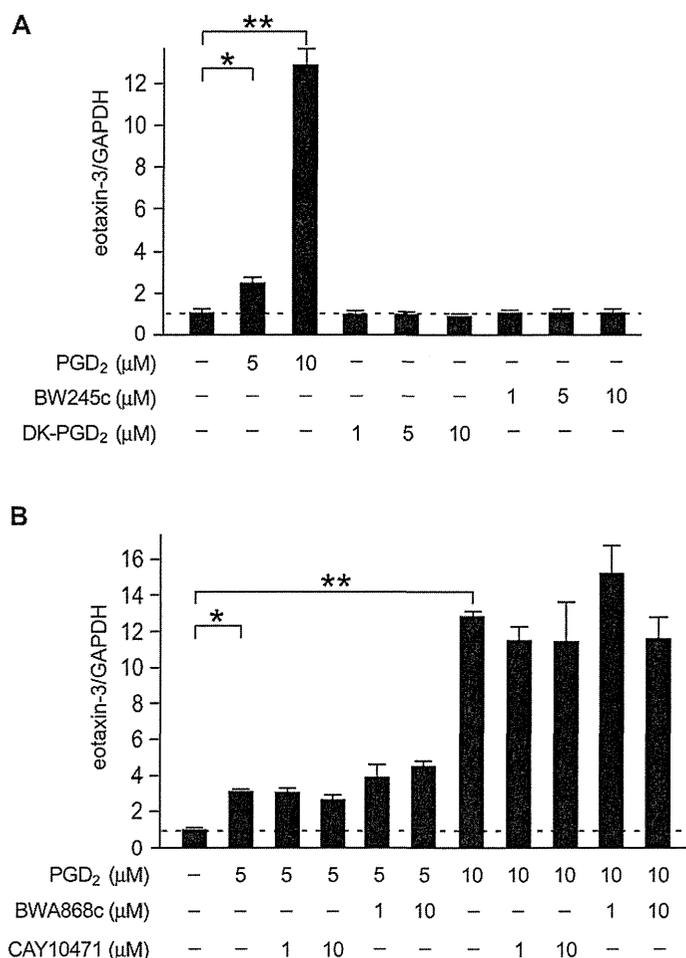


FIG 4. Role of DP1 and CRT_{H2} in eotaxin-3 mRNA expression in human sebocytes. SZ95 cells were incubated with PGD₂ in the presence or absence of the DP1 agonist BW245c and the CRT_{H2} agonist DK-PGD₂ (A) or the DP1 antagonist BWA868c and the CRT_{H2} antagonist CAY10471 (B). Eotaxin mRNA levels were evaluated by quantitative RT-PCR, and data are shown as arbitrary units where the value for an unstimulated sample is set at 1 (n = 4). *P < .05, **P < .01.

it remains unclear how indomethacin is specifically effective against EPF. Only a few reports have addressed the efficacy of indomethacin on the other eosinophil-infiltrating skin disorders.^{29,30} Our findings indicate that H-PGDS is expressed in peripheral eosinophils of patients with EPF, whereas it is only marginally expressed in those of healthy subjects; nevertheless, it remains uncertain how this difference between patients with EPF and healthy subjects arises. This unique expression profile of H-PGDS in EPF may explain the initiation and/or maintenance of the disease. H-PGDS expression is evident in T cells, and it has recently been reported that CCR8+ T_{H2} cells are essential to attract eosinophils to the skin.³¹ We detected some T-cell infiltration around the pilosebaceous units in EPF (data not shown), suggesting that CCR8+ T cells and eosinophils jointly initiate and maintain eosinophil infiltration into EPF skin lesions.

It has been demonstrated that sebocytes are capable of producing the neutrophil chemoattractant CXCL8, which may play a role in the pathogenesis of acne,³² but it remains unknown whether and how sebocytes produce eosinophil chemoattractants. Here we have demonstrated for the first time that eotaxin-3

mRNA expression in sebocytes was enhanced by incubation with PGD₂ and 15d-PGJ₂ and mediated by PPAR γ but not by DP1 or CRT_{H2}. It has been reported that 15d-PGJ₂ binds to PPAR γ where it promotes adipocyte differentiation^{33,34} and that PPAR γ is detected in sebocytes where it is involved in lipid synthesis.³⁵⁻³⁷ In our study, larger quantities of PPAR γ were detected in sebocytes than in keratinocytes or fibroblasts. Therefore, sebocytes may play an important role in attracting eosinophils into the skin under certain conditions.

Conclusions

We found that H-PGDS is expressed in EPF lesions and that PGD₂ and its metabolite 15d-PGJ₂ induce marked upregulation of eotaxin-3 via PPAR γ in sebocytes. These results may explain how EPF shows a massive eosinophil infiltration around pilosebaceous units.

Clinical implications: Inhibition of the PGD₂/PGJ₂-PPAR γ pathway may be a therapeutic target for EPF and other diseases involving eosinophil infiltration.

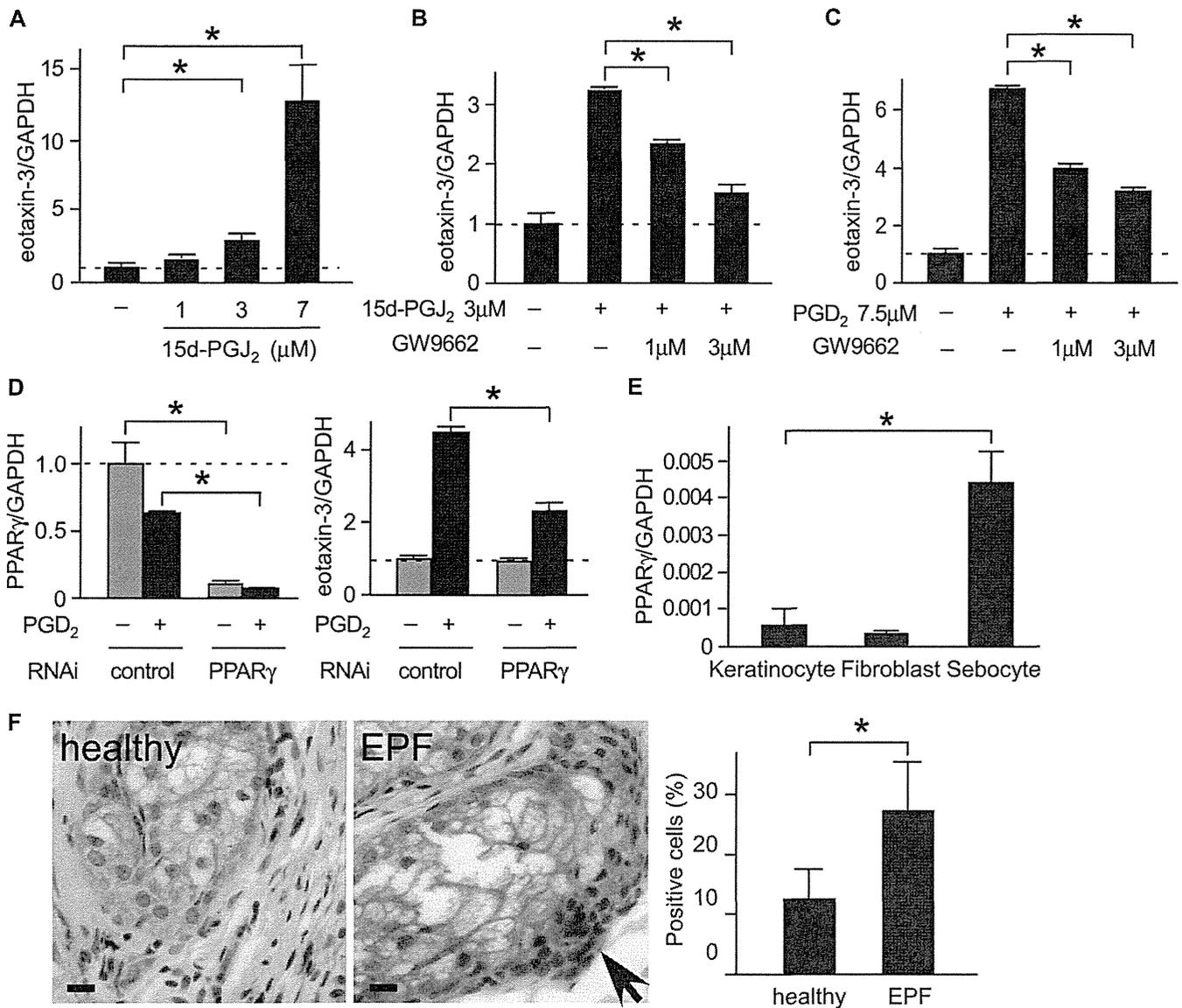


FIG 5. Involvement of PPAR γ in eotaxin-3 mRNA expression in sebocytes. SZ95 cells were incubated with 15d-PGJ₂ (A and B) or PGD₂ (C) in the presence or absence of the PPAR γ antagonist GW9662 (Fig 5, B and C). Eotaxin mRNA levels were evaluated by means of quantitative RT-PCR. D, The effect of transfection with PPAR γ siRNA on mRNA expressions for PPAR γ and eotaxin-3. E, PPAR γ mRNA expression in normal human epidermal keratinocytes, fibroblasts, and SZ95 cells. F, Immunostaining for eotaxin-3. Sebocytes strongly positive for eotaxin-3 are indicated by an arrow (right panel). The percentage positive for eotaxin-3 among sebocytes was examined (n = 3 each, right panel). Bar = 10 μ m. *P < .05.

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Langerhans cells are critical in epicutaneous sensitization with protein antigen via thymic stromal lymphopoietin receptor signaling

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Background: The clarification of cutaneous dendritic cell subset and the role of thymic stromal lymphopoietin (TSLP) signaling in epicutaneous sensitization with protein antigens, as in the development of atopic dermatitis, is a crucial issue.

Objectives: Because TSLP is highly expressed in the vicinity of Langerhans cells (LCs), we sought to clarify our hypothesis that LCs play an essential role in epicutaneous sensitization with protein antigens through TSLP signaling.

Methods: By using Langerin-diphtheria toxin receptor knock-in mice and human Langerin-diphtheria toxin A transgenic mice, we prepared mice deficient in LCs. We also prepared mice deficient in TSLP receptors in LCs by using TSLP receptor-deficient mice with bone marrow chimeric technique. We applied these mice to an ovalbumin (OVA)-induced epicutaneous sensitization model.

Results: Upon the epicutaneous application of OVA, conditional LC depletion attenuated the development of clinical manifestations as well as serum OVA-specific IgE increase, OVA-specific T-cell proliferation, and IL-4 mRNA expression in the draining lymph nodes. Consistently, even in the steady state, permanent LC depletion resulted in decreased serum IgE levels, suggesting that LCs mediate the T_H2 local environment. In addition, mice deficient in TSLP receptors on LCs abrogated the induction of OVA-specific IgE levels upon epicutaneous OVA sensitization.

Conclusion: LCs initiate epicutaneous sensitization with protein antigens and induce T_H2-type immune responses via TSLP signaling. (*J Allergy Clin Immunol* 2012;129:1048-55.)

Key words: Langerhans cell, TSLP, TSLP receptor, epicutaneous sensitization, protein antigen

Skin plays an important immunologic role by eliciting a wide variety of immune responses to foreign antigens.¹ Atopic dermatitis (AD) is a pruritic chronic retractable inflammatory skin disease that is induced by the complex interaction between susceptibility genes encoding skin barrier components and stimulation by protein antigens.^{2,3} Patients with AD exhibit compromised barrier function that leads to the activation of keratinocytes and immune cells, which favors a T_H2 bias. A wide array of cytokines and chemokines interact to yield symptoms that are characteristic of AD. For example, thymus and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) both attract T_H2 cells through CC chemokine receptor 4,⁴ levels of which correlate well with the severity of AD.⁵ Elevation in serum IgE levels is also frequently found in patients with AD, sometimes concomitant with food allergy, allergic rhinitis, and asthma.³ Yet it remains unknown how the elevation in serum IgE levels on epicutaneous sensitization with protein antigens is induced in the pathogenesis of AD.

Upon protein antigen exposure, dendritic cells (DCs) acquire antigens and stimulate the proliferation of T cells to induce distinct T helper cell responses to external pathogens.⁶ Therefore, it has been suggested that DCs initiate AD in humans⁷; however, it remains unclear which cutaneous DC subset initiates epicutaneous sensitization to protein antigens. In the mouse skin, there are at least 3 subsets of DCs: Langerhans cells (LCs) in the epidermis and Langerin-positive and Langerin-negative DCs in the dermis (Langerin⁺ dermal DCs and Langerin⁻ dermal DCs, respectively).⁸⁻¹⁰ It has been reported that the application of large molecules is localized above the size-selective barrier, tight junction (TJ), and that activated LCs extend their dendrites through the TJ to take up antigens.¹¹ Therefore, it can be hypothesized that it is not dermal DCs but rather LCs that initiate epicutaneous sensitization with protein antigens, as in the development of AD.

In humans, polymorphisms in the gene encoding the cytokine thymic stromal lymphopoietin (TSLP) are associated with the development of multiple allergic disorders through the TSLP receptor (TSLPR), which is expressed in several cell types, such as DCs, T cells, B cells, basophils, and eosinophils.^{12,13} Thus, TSLP seems to be a critical regulator of T_H2 cytokine-associated inflammatory diseases.

Recently, it has been reported that basophils induce T_H2 through TSLPR.¹³ On the other hand, it is also known that skin

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Abbreviations used

AD:	Atopic dermatitis
BM:	Bone marrow
BMC:	Bone marrow chimeric
CFSE:	Carboxyfluorescein succinimidyl ester
DCs:	Dendritic cells
DTA:	Diphtheria toxin subunit A
DTR:	Diphtheria toxin receptor
eGFP:	Enhanced green fluorescent protein
FITC:	Fluorescein isothiocyanate
LCs:	Langerhans cells
LN:	Lymph node
OVA:	Ovalbumin
TSLP:	Thymic stromal lymphopoietin
TSLPR:	TSLP receptor
TJ:	Tight junction
WT:	Wild type

DCs elicit a T_H2 response in the presence of mechanical injury by inducing cutaneous TSLP¹⁴ and that LCs are critical in the development of skin lesions induced by the topical application of vitamin D₃ analogues through TSLP signaling.¹⁵ However, these skin inflammation models are induced in an antigen-independent manner; therefore, it is important to address the degree to which TSLP is essential in T_H2 shifting and to identify the cells that are essential for TSLP signaling transduction upon epicutaneous sensitization, which is relevant to inflammatory skin diseases, such as AD. This will lead to the understanding of the underlying mechanism and to developing new therapeutic targets for inflammatory skin diseases.

It is known that TSLP activates human epidermal LCs and DCs *in vitro*¹⁶⁻¹⁸ and that TSLP is highly expressed in the epidermis of the lesional skin of patients with AD. Since LCs are localized in the epidermis, we hypothesized that LCs initiate epicutaneous sensitization through TSLP signaling. By applying an LC ablation system, we found that LCs are crucial for T_H2 induction and IgE production upon epicutaneous protein exposure through TSLP signaling.

METHODS

Animals and bone marrow chimera

C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). OT-II T-cell receptor transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Langerin-diphtheria toxin subunit A (DTA) mice were generated by Dr Daniel Kaplan,¹⁹ and Langerin-enhanced green fluorescent protein (eGFP)-diphtheria toxin receptor (DTR) knock-in mice were kindly provided by Dr Bernard Mallissen (CIML, Institut National de la Santé et de la Recherche Médicale, Marseille, France).

TSLPR^{-/-} mice (BALB/c or B6 background) were generated by Dr Steven Ziegler.²⁰ Seven- to 12-week-old female mice bred in specific pathogen-free facilities at Kyoto University were used for all experiments.

For LC depletion specifically, Langerin-eGFP-DTR mice were used. Intraperitoneal injection of 1 μg of DT (Sigma-Aldrich, St Louis, Mo) in 500 μL of PBS depleted Langerin⁺ DC subsets, including LCs and Langerin⁺ dermal DCs. Langerin⁺ dermal DCs in the dermis recover 1 week after DT injection, but LCs remain undetectable for 4 weeks after depletion.²¹ Since only LCs are depleted between 1 and 3 weeks after DT injection, we can evaluate the role of LCs in epicutaneous sensitization by applying ovalbumin (OVA) between 1 and 3 weeks after DT injection. Therefore, we injected DT 7 days before epicutaneous sensitization.

Control mice were intraperitoneally injected with 500 μL of PBS on the same day.

To generate bone marrow (BM) chimeric (BMC) mice, 6-week-old mice were irradiated (9 Gy) and transplanted with BM cells (1 × 10⁷ cells per recipient). All experimental procedures were approved by the institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Epicutaneous sensitization

Mice were anesthetized with diethylether (Nacalai Tesque, Kyoto, Japan) and then shaved with an electric razor (THRIVE Co Ltd, Osaka, Japan). A single skin site on each mouse was tape-stripped at least 5 times with adhesive cellophane tape (Nichiban, Tokyo, Japan). One hundred microgram of OVA in 100 μL of normal saline or placebo was placed on patch-test tape (Torii Pharmaceutical Co, Ltd, Tokyo, Japan). Each mouse had a total of three 2-day exposures to the patch, separated by 1-day intervals. Mice were euthanized at the end of the third cycle of sensitization (day 9).

Antigen-specific T-cell proliferation

To assess the OVA-specific T-cell priming capacity of cutaneous LCs, 100 μL of normal saline with or without 100 μg of OVA was placed on the shaved and tape-stripped mouse back skin. CD4 T cells were isolated from OT-II mice by using magnetic bead separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled with 8 μM of carboxyfluorescein succinimidyl ester (CFSE). Forty-eight hours after epicutaneous sensitization, 5 × 10⁶ CFSE-labeled OT-II T cells were transferred to naive mice via the tail vein. An additional 48 hours later, skin draining brachial lymph nodes (LNs) were collected and analyzed by means of flow cytometry.

Statistical analysis

Unless otherwise indicated, data are presented as means ± SDs and each data point is representative of 3 independent experiments. *P* values were calculated according to the 2-tailed Student *t* test.

A complete description of the methods, and any associated references, is available in this article's Online Repository at www.jacionline.org.

RESULTS

LC depletion impaired the development of OVA-induced allergic skin dermatitis model

To assess the role of LCs in epicutaneous sensitization with protein antigens and induction of IgE, we applied OVA to mice epicutaneously.²² In this model, we observed a rise in OVA-specific serum IgE and IgG1 levels, both of which are induced in a T_H2-dependent manner, as well as the development of dermatitis characterized by the infiltration of CD3⁺ T cells, eosinophils, and neutrophils and local expression of mRNA for the cytokines IL-4, IL-5, and IFN-γ.²² These findings exhibited characteristics of allergic skin inflammation such as AD. To evaluate the roles of LCs, we used knock-in mice expressing eGFP and DTR under the control of the Langerin gene, called Langerin-eGFP-DTR mice.²³

In the OVA-induced allergic skin dermatitis model, LC-depleted mice showed milder clinical manifestations than did LC-non-depleted mice (Fig 1, A, left panel). Histology of the patched skin area showed pronounced lymphocyte infiltration and edema in the dermis of sensitized LC-non-depleted mice, which was less apparent in sensitized LC-depleted mice (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). The histologic score of LC-depleted mice was also lower than that of LC-non-depleted mice (Fig 1, A, right

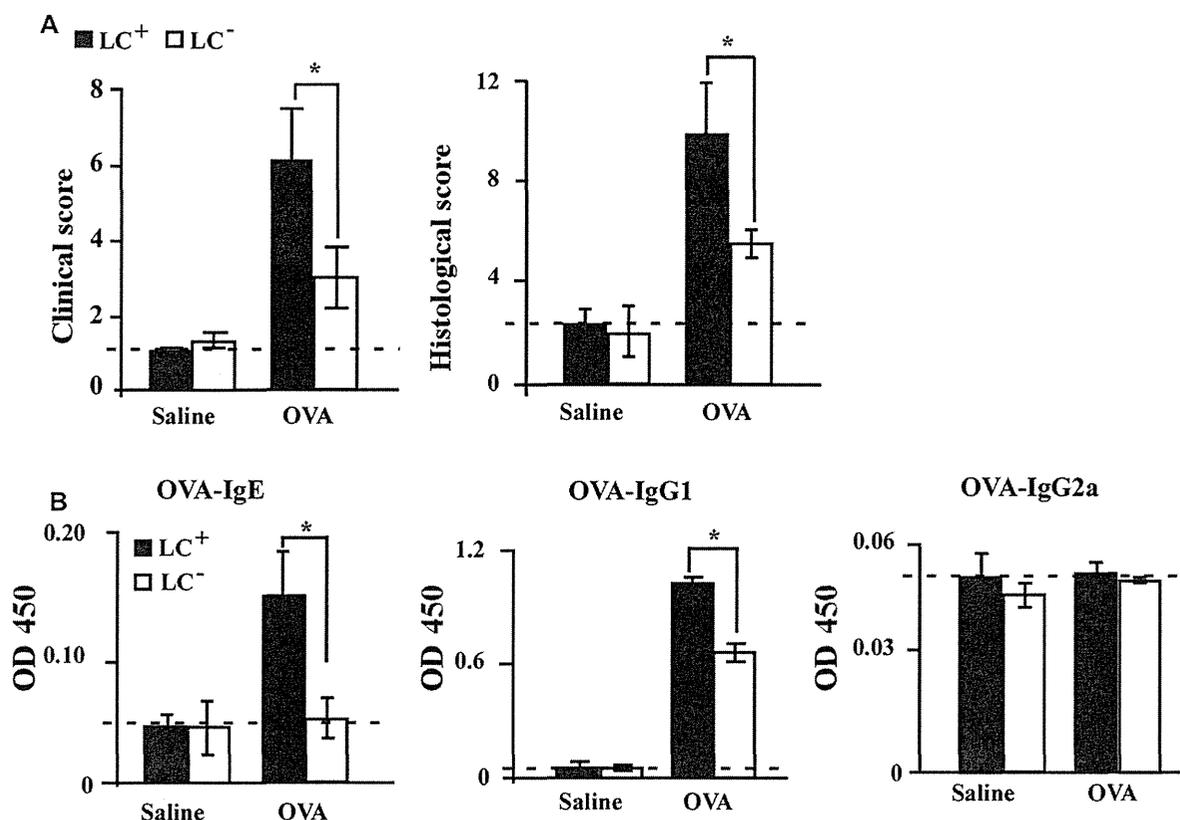


FIG 1. LCs are crucial for epicutaneous sensitization with OVA. **A**, Total clinical severity scores (*left panel*) and total histology scores (*right panel*) of LC-non-depleted (LC⁺) and LC-depleted (LC⁻) mice (n = 5 mice per group). **B**, Serum OVA-specific antibodies as determined by ELISA. OD values for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. *P < .05.

panel). In addition, serum OVA-specific IgE and IgG1 levels in LC-depleted mice were significantly lower than those in wild-type (WT) mice (Fig 1, B). On the other hand, the T_H1-dependent immunoglobulin IgG2a was not induced by the application of OVA (Fig 1, B). These data suggest that LCs are involved in the development of OVA-induced AD-like skin inflammation and induction of IgE.

Impaired T-cell proliferation and T_H2 induction by LC depletion

Priming of antigen-specific T_H2 cells and proliferation is an important step in the development of this model. To assess the T-cell priming capacity of cutaneous LCs upon protein allergen exposure, LC-depleted and LC-non-depleted mice were percutaneously sensitized with OVA on the back and transferred with CFSE-labeled OT-II T cells, which express an OVA-specific T-cell antigen receptor. Next, single-cell suspensions prepared from the skin-draining brachial LNs were analyzed by means of flow cytometry to evaluate T-cell division by LCs in the draining LNs. LC-depleted mice showed impaired T-cell division after OVA sensitization compared with LC-non-depleted mice, suggesting that LCs stimulate T-cell proliferation, at least to some degree, in this model (Fig 2, A and B).

To evaluate the role of LCs in T-cell priming, we examined the mRNA expression of T_H2 cytokine IL-4 and T_H1 cytokine IFN- γ in draining LNs after OVA sensitization. The IL-4 mRNA

expression level of draining LNs was significantly decreased in LC-depleted mice, while the IFN- γ mRNA expression level was significantly higher in LC-depleted mice than in LC-non-depleted mice (Fig 2, C). These results suggest that LCs are crucial for stimulating T-cell proliferation to a certain extent and T_H2 induction pronouncedly in skin-draining LNs in this model.

LCs are responsible for initiating epicutaneous sensitization to protein antigens

It has been reported that LCs are dispensable for initiating contact hypersensitivity to haptens, which may cause a discrepancy in our findings on the necessity of LCs for protein antigen sensitization.^{21,24} To evaluate the extent of skin penetration by protein antigens and haptens, we patched fluorescein isothiocyanate (FITC)-conjugated OVA or painted FITC on the back skin of B6 mice, and performed immunohistochemical analysis. FITC-conjugated OVA retained above the TJ was indicated by staining with anti-claudin-1 antibody (see Fig E2, left panel, in this article's Online Repository at www.jacionline.org). On the other hand, when we painted FITC on the skin of the mouse back skin, it readily penetrated into the dermis where dermal DCs locate (Fig E2, right panel).

LCs are critical for IgE production

To further assess the role of LCs in IgE production, we used gene-targeted Langerin-DTA mice, which constitutively lack LCs

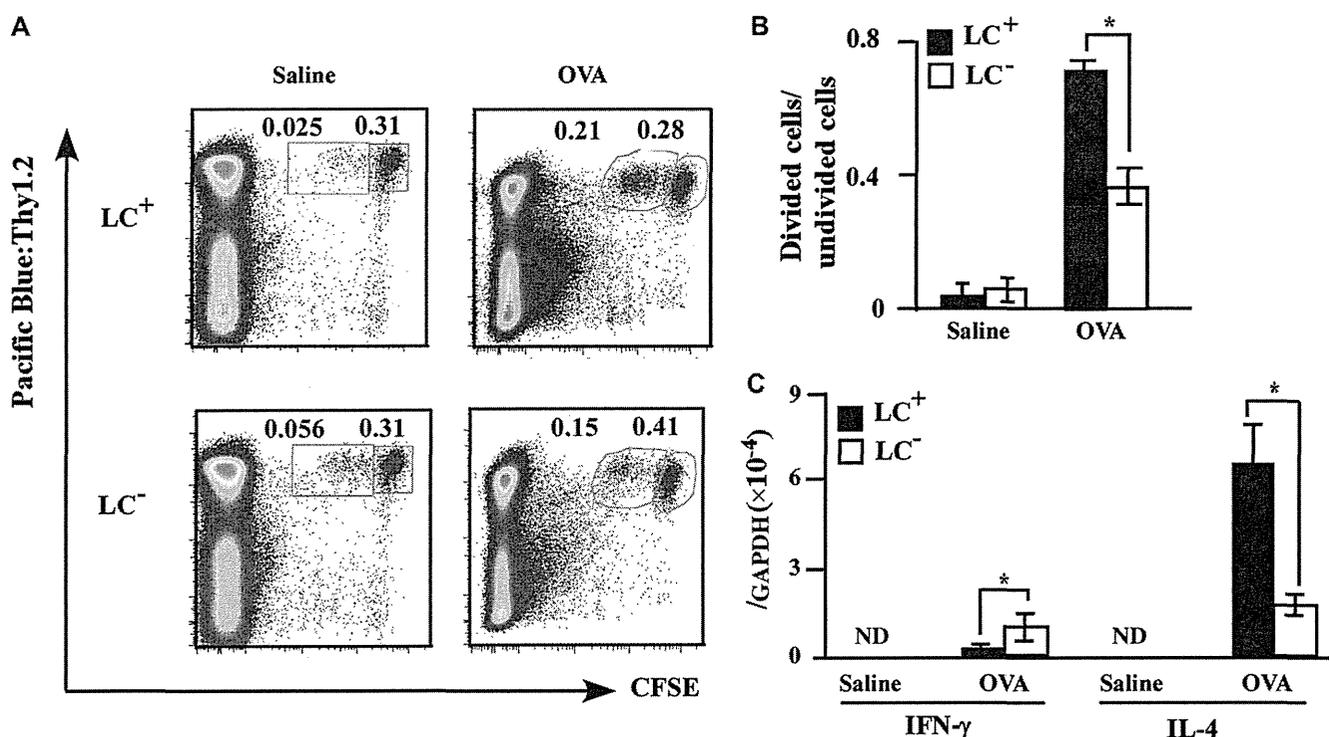


FIG 2. LCs are critical for antigen-specific T-cell proliferation. Mice in the presence or absence of LCs (LC⁺ and LC⁻, respectively) were treated with OVA and transplanted with CFSE-labeled OT-II T cells (n = 5 mice per group). Skin-draining LNs were analyzed for OVA-specific T-cell proliferation (A and B) and mRNA expression levels for IFN-γ and IL-4 (C). Boxes in (A) demarcate divided cells (left) and undivided cells (right). *P < .05. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ND, not detected.

throughout life.¹⁹ WT and Langerin-DTA mice were bred under super pathogen free conditions for 6 to 10 weeks, and serum IgE levels were measured by means of ELISA. On the FVB background, the serum IgE level was lower in Langerin-DTA mice than in WT controls (Fig 3, A, left panel), while no significant difference was seen on the C57BL/6 (B6) background (Fig 3, A, right panel). We also found that the expression level of IgE on peritoneal mast cells was decreased in LC-deficient mice in both the FVB and B6 backgrounds (Fig 3, B). Preincubation of mast cells with IgE *in vitro* did not change the data, arguing that surface expression of FcεRI on mast cells was decreased in LC-deficient mice, which is an indicator of lower serum IgE level. Therefore, the above data strongly suggest that LCs are crucial for IgE production, which is consistent with the findings in the OVA-induced skin inflammation model (Figs 1 and 2).

TSLP receptor on LCs is upregulated by protein antigen exposure

It has been reported that TSLP is involved in the exacerbation of mouse T_H2-mediated allergic inflammation through the direct stimulation of T_H2 effector cells.²⁵ However, it remains unknown which cells initiate T_H2 induction via TSLP signaling under the epicutaneous sensitization of protein antigens. TSLP is highly expressed in the skin lesions of human AD,^{17,18,26,27} and the major cells in proximity to keratinocytes are LCs; therefore, we evaluated the effect of TSLPR expression on LCs. We found that LCs expressed TSLPR, but the expression level was low under the steady state. On the other hand, the expression level of TSLPR

on LCs was pronouncedly enhanced by the topical application of OVA (Fig 4).

Establishment of BMC mice deficient in TSLPR on LCs

Next, we sought to clarify the significance of TSLP in epicutaneous sensitization with protein antigens and to identify responsible cells mediating TSLP signaling. Since cells ensuring epidermal LC renewal are radioresistant, LCs and their derivatives found in skin-draining LNs are of host origin.²⁸ We irradiated B6 mice and B6 background TSLPR-deficient (TSLPR^{-/-}) mice, and then transferred BM cells from B6 mice into the irradiated mice. TSLPR is expressed on not only LCs but also T cells, B cells, basophils, eosinophils, and dermal DCs. Of note, LCs are radioresistant while T cells, B cells, basophils, eosinophils, and dermal DCs are radiosensitive. When mice were irradiated and transplanted with BM cells, more than 95% of the blood cells in the recipient mice had been replaced with donor-derived cells within 2 months after the transfer, whereas almost 100% of LCs were derived from the host, unlike the vast majority of dermal DCs that were donor-derived at this point (Fig 5, A). Therefore, given that TSLPR^{-/-} mice were reconstituted with BM cells from B6 mice, these mice were deficient in TSLPR on LCs, but other BM-derived cells expressing TSLPR were present. Accordingly, by using a hematopoietic BMC system, we generated mice in which TSLPRs were lacking in LCs (LC-TSLPR^{-/-} BMC mice) (see Fig E3 in this article's Online Repository at www.jacionline.org).

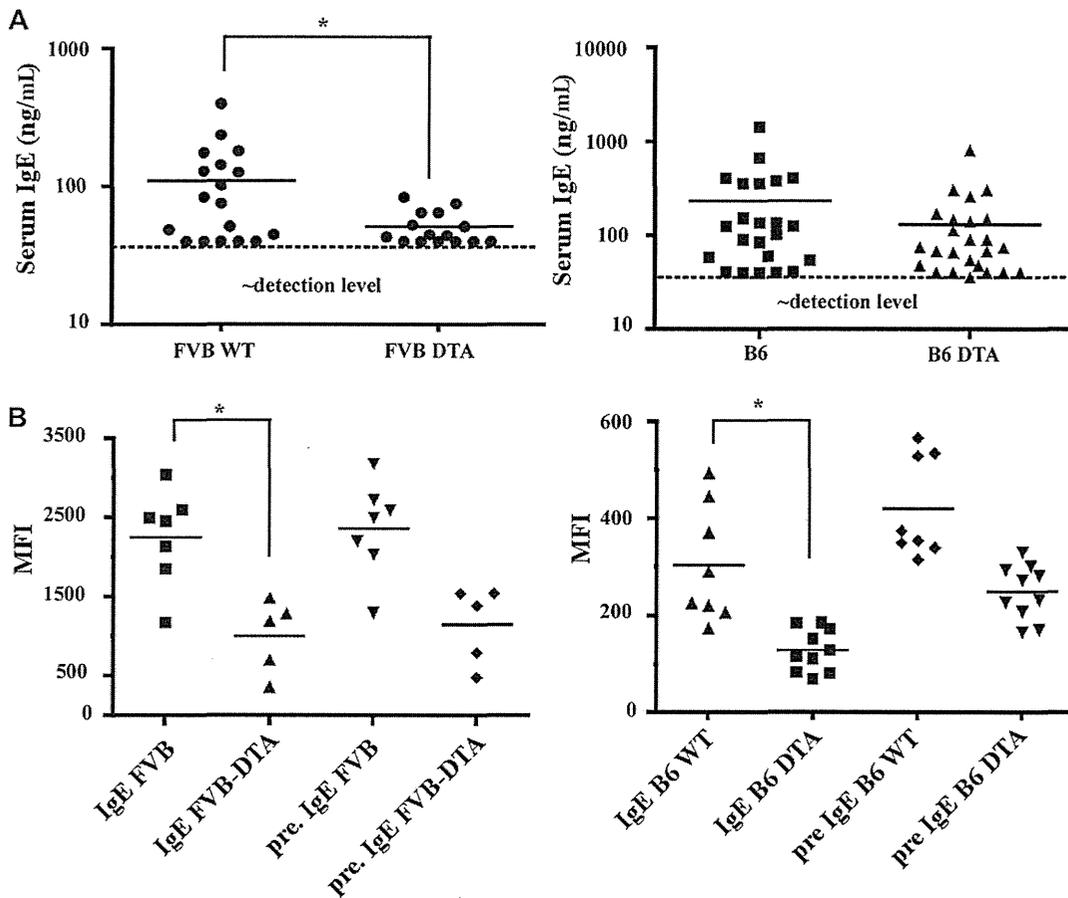


FIG 3. LCs are essential for IgE production. The serum IgE levels (A) and IgE expression levels (B) on peritoneal mast cells (indicated by MFI) of WT and Langerin-DTA mice on FVB (left panel) and B6 (right panel) backgrounds. Mast cells were also preincubated with IgE (labeled with pre-IgE) *in vitro* before the measurement of IgE expression (Fig 3, B). Each symbol represents an individual animal. **P* < .05. MFI, Mean fluorescence intensity.

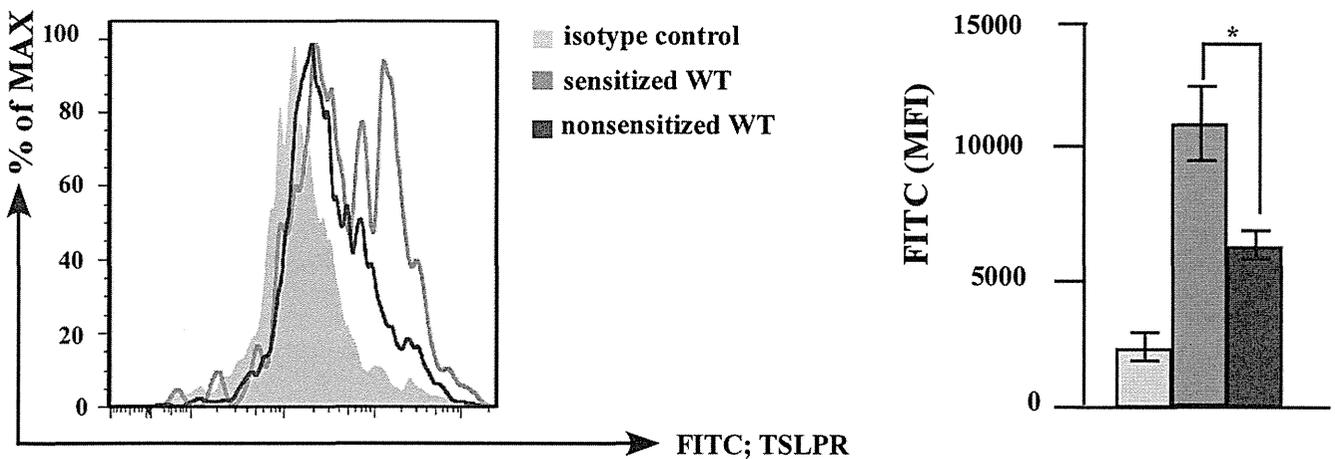


FIG 4. TSLPR on LCs is a responsible target of TSLP upon epicutaneous OVA sensitization. Epidermal cell suspensions from B6 (WT) mice with (sensitized) or without (nonsensitized) epidermal application of OVA were stained with TSLPR antibody. TSLPR expressions of MHC class II⁺ CD11c⁺ LCs was analyzed by flow cytometry (left, histogram; right, average \pm SD of MFI). *n* = 3 per group. **P* < .05. MFI, Mean fluorescence intensity.

Essential target of TSLP is TSLPR on LCs in OVA-induced allergic skin dermatitis model

In the context of OVA-induced AD-like skin inflammation, LC-TSLPR^{-/-} BMC mice showed milder clinical and histologic

findings than did TSLPR^{+/+} BMC mice, but these findings were nearly comparable with those of TSLPR^{-/-} BMC mice (Fig 5, B; see Fig E4 in this article's Online Repository at www.jacionline.org). Consistently, OVA-specific IgE levels in

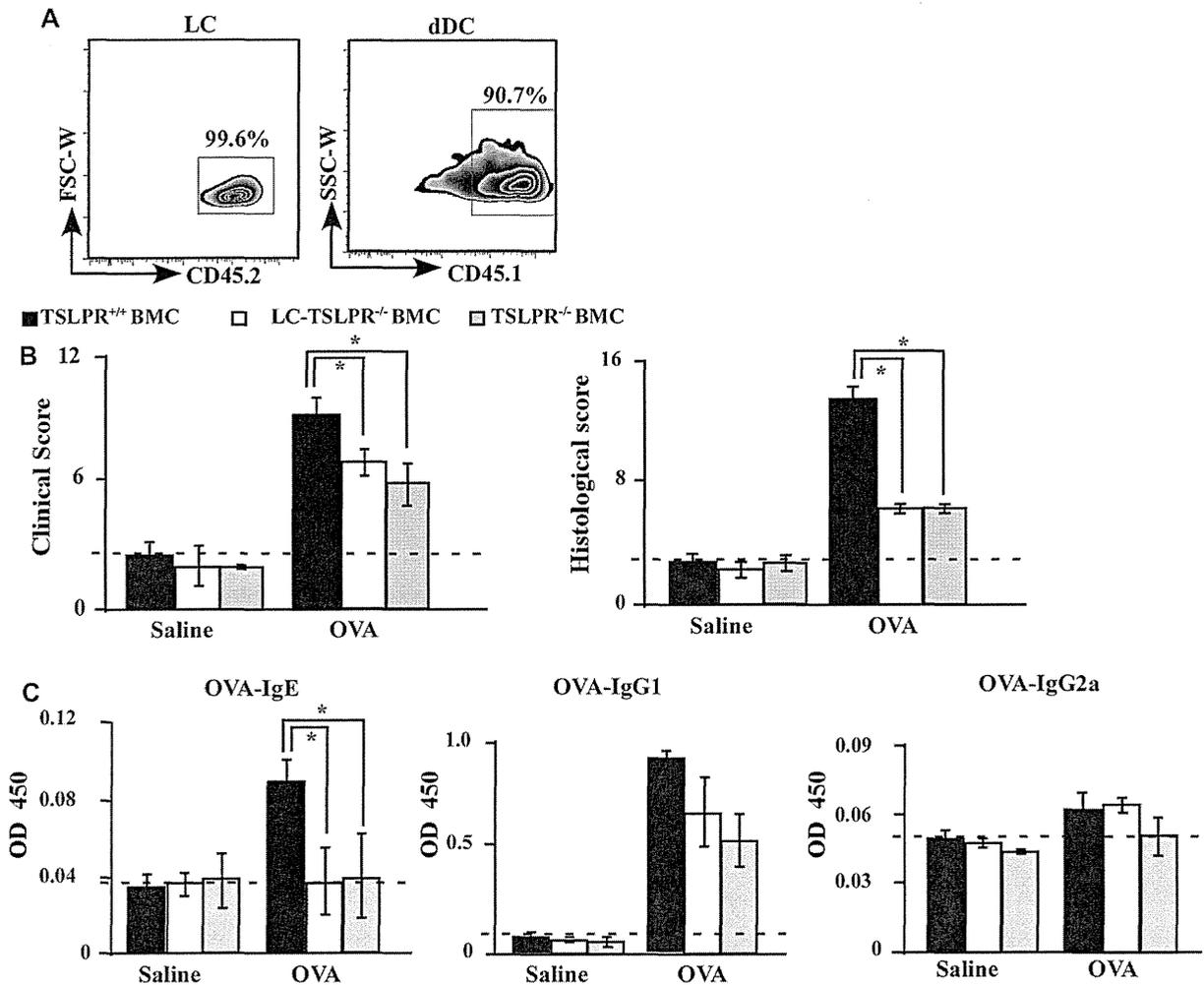


FIG 5. An essential target of TSLP for IgE induction is TSLPR on LCs. **A**, B6 (Ly45.2) mice were irradiated and transplanted with BM cells from B6 (Ly45.1) mice. The epidermis and the dermis of BMC mice were separated, and single-cell suspensions were stained and analyzed by flow cytometry. **B**, Total clinical severity scores (left panel) and histology scores (right panel) of TSLPR^{+/+} BMC, LC-TSLPR^{-/-} BMC, and TSLPR^{-/-} BMC mice (n = 5 mice per group). **C**, Serum OVA-specific antibodies as determined by ELISA. OD values for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. *P < .05.

the serum after OVA challenge were significantly lower in LC-TSLPR^{-/-} BMC mice than in TSLPR^{+/+} BMC mice (Fig 5, C). These data indicate that LCs play an important role in epicutaneous sensitization upon protein antigens in accord with IgE induction through TSLP-TSLPR signaling.

TSLPR on LCs are dispensable for antigen-specific T-cell proliferation but vital for T_H2 induction

The above results suggest that LCs stimulate T cells to differentiate into T_H2, resulting in IgE induction. To clarify this issue, we assessed the T-cell proliferation and differentiation capacity of LCs in the presence or absence of TSLPR. We transferred CFSE-labeled OT-II T cells into mice topically treated with OVA, and dividing cells in the draining LNs were measured by means of flow cytometry (Fig 6, A). The ratio of dividing OT-II CD4⁺ T cells to undivided OT-II CD4⁺ T cells was comparable among LC-TSLPR^{-/-} BMC, TSLPR^{+/+} BMC, and TSLPR^{-/-} BMC mice (Fig 6, B). In addition, IFN- γ mRNA level in the

draining LNs 96 hours after OVA application was similar among these 3 groups (Fig 6, C). On the other hand, the IL-4 mRNA expression level in skin-draining LNs was significantly lower in LC-TSLPR^{-/-} BMC mice than in the other 2 groups (Fig 6, C). These results indicate that TSLPRs on LCs are dispensable for antigen-specific T-cell proliferation but vital for inducing T_H2 differentiation.

TSLP promotes the expression of OX40L and the production of T_H2 chemokines by DCs

We next sought to elucidate the mechanism underlying T_H2 induction of LCs via TSLP-TSLPR signaling. Modulation of costimulatory molecule expression was among the candidates, as it has been demonstrated that the interaction between membrane OX40L on DCs and OX40 on naive T cells results in the induction of IL-4 production by T cells in humans,²⁶ and that treating mice with OX40L-blocking antibodies substantially inhibit the T_H2 immune responses induced by TSLP in the lung and the skin.²⁹

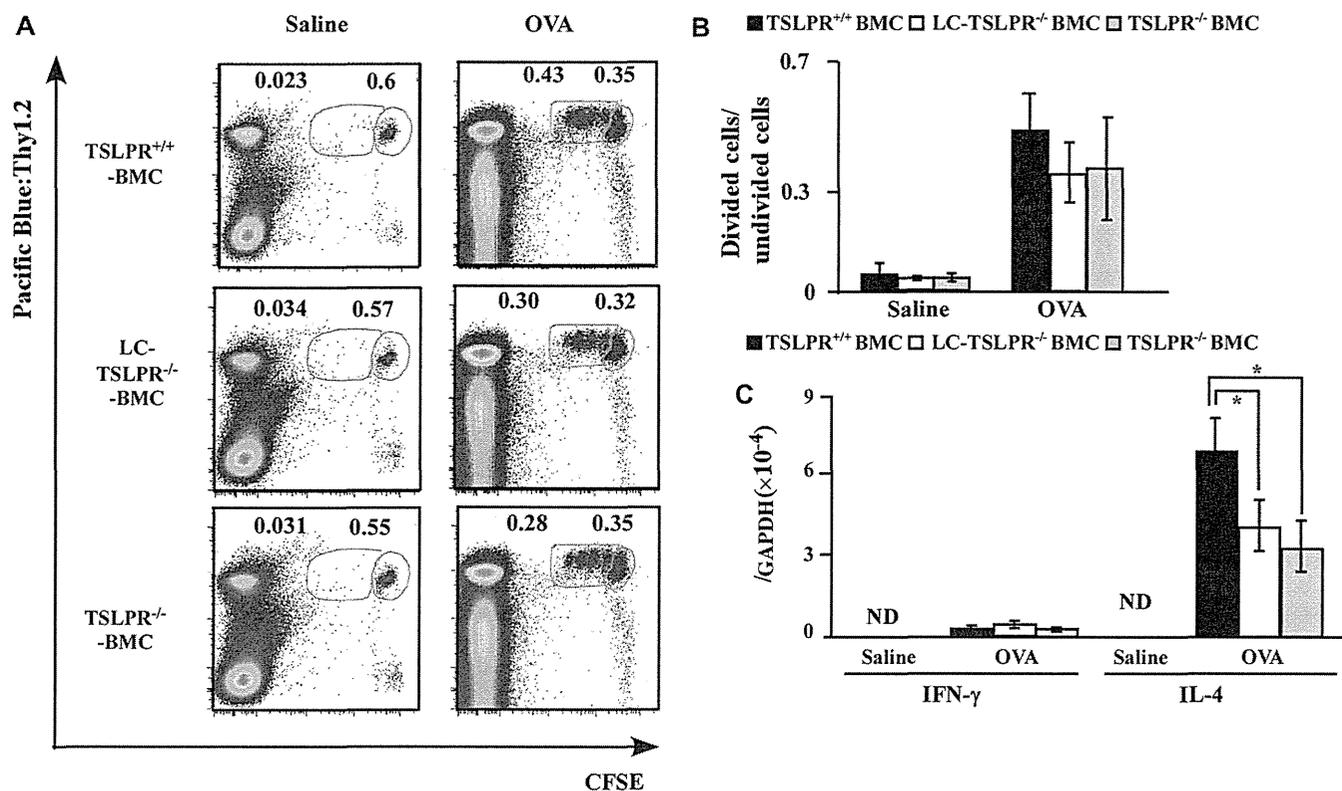


FIG 6. TSLPR on LCs are vital for T_H2 induction. TSLPR^{+/+} BMC, LC-TSLPR^{-/-} BMC, and TSLPR^{-/-} BMC mice were treated with OVA or saline and transplanted with CFSE-labeled OT-II T cells. Skin-draining LNs were analyzed for OVA-specific T-cell proliferation (A and B) and cytokine mRNA expression levels for IFN- γ and IL-4 (C). Boxes in (A) demarcate divided cells (left) and undivided cells (right). $n = 5$ mice per group. * $P < .05$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ND, not detected.

Therefore, it is important to evaluate the expression levels of costimulatory molecules on LCs in OVA-sensitized skin by means of flow cytometry. TSLPR^{-/-} (BALB/c background) and WT control BALB/c mice were percutaneously sensitized with OVA. Seventy-two hours later, epidermal cell suspensions were prepared and stained with anti-OX40L, CD80, and CD40 antibodies. The mean fluorescence intensity of OX40L expressed by LCs from OVA-sensitized TSLPR^{-/-} mice was significantly lower than that in WT control mice. On the other hand, expression levels of CD40 and CD80 on LCs were comparable between WT control and TSLPR^{-/-} mice (see Fig E5, A, in this article's Online Repository at www.jacionline.org).

It is known that serum levels of CCL17 and CCL22 correlate with the severity of AD.⁵ We incubated BM-derived DCs from BALB/c mice with recombinant mouse TSLP and found that TSLP induced DCs to express CCL17 and CCL22 mRNA (Fig E5, B), while the expression level of the T_H1 chemokine CXCL10 was suppressed by TSLP (Fig E5, C). These results suggest that TSLP instructs cutaneous DCs to create a T_H2 -permissive microenvironment by modulating the expression levels of chemokines.

DISCUSSION

In this study, we have demonstrated that LCs are the essential cutaneous DC subset in the induction of IgE upon epicutaneous sensitization with protein antigens. We also found that TSLPR expression on LCs is enhanced upon protein antigen exposure to

the skin and that LCs play an important role in this process through TSLP-TSLPR signaling. In addition, we have demonstrated that TSLP stimulation causes LCs to express OX40L as shown previously in human studies and that BM-derived DCs induce T_H2 chemokines while suppressing T_H1 chemokines, which may shift the immune environment to a T_H2 milieu.

While a previous report suggests the significance of LCs in the induction of T_H2 immune responses in humans,³⁰ other studies have reported that dermal DCs, but not LCs, are essential for murine epicutaneous sensitization with haptens, as in contact hypersensitivity that is mediated by T_H1 .^{19,21,31,32} In our study, we have demonstrated that LCs seem to be indispensable for T_H2 induction upon protein antigen sensitization. Therefore, dermal DCs and LCs may play an important role for T_H1 - and T_H2 -type immune reactions, respectively.

While protein antigens remain above the TJ, haptens can readily penetrate into the dermis as shown in Fig E2; therefore, LCs may not be essential for sensitization to haptens as reported previously.^{21,24} Upon protein antigen exposure to the skin, however, LCs are vital in the induction of antigen-specific IgE. It is still an intriguing issue how clinical and histologic scores, T-cell proliferation, and IL-4 production were only partially suppressed by the deficiency of LCs. These results suggest that other antigen-presenting cells, such as dermal DCs, might be able to induce antigen-specific T-cell proliferation in the draining LNs and that other T_H2 -inducing cells, such as basophils and mast cells, may contribute to produce IL-4 in the draining LNs. These issues need to be answered in the future.