Dexamethasone but not tacrolimus suppresses TNF-α-induced thymic stromal lymphopoietin expression in lesional keratinocytes of atopic dermatitis model

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ABSTRACT

Background: Thymic stromal lymphopoietin (TSLP) initiates the Th2-type allergic inflammation, and is thought to play an important role in the pathogenesis of atopic dermatitis (AD). TNF-α is a key cytokine which is involved in the pathophysiology of various inflammatory diseases, and the expression level is elevated in the sera and skin of patients with AD. In addition, TNF-α has been reported to induce TSLP expression in epidermal keratinocytes. Topical glucocorticoids and calcineurin inhibitors are safe and effective agents for AD, but the effects of these agents on TNF-α-induced TSLP expression are not fully understood.

Objective: To investigate whether the glucocorticosteroid dexamethasone and the calcineurin inhibitor tacrolimus could affect TSLP expression induced by TNF-α in lesional keratinocytes of AD.

Methods: The effects of topical dexamethasone and tacrolimus on TSLP expression were evaluated in an AD mouse model induced by repeated 2,4,6-trinitro-1-chlorobenzene application. Co-immunostaining for TSLP and TNF-α was performed using skin samples from AD patients and the mouse model. Normal human epidermal keratinocytes (NHEKs) were cultured with dexamethasone or tacrolimus in the presence of TNF-α to analyze TSLP expression.

Results: Topical application of dexamethasone but not tacrolimus repressed TSLP expression in the mouse model. TSLP and TNF-α showed similar distribution pattern in epidermal keratinocytes of AD lesions and the mouse model. TSLP expression was induced by TNF-α via NF-κB in a dose-dependent and an autocrine and/or paracrine manner in NHEKs, which was significantly suppressed by dexamethasone but not by tacrolimus. Similarly to TSLP expression, IL-6, TNF-α, IL-8, and IL-36γ expression induced by TNF-α were significantly suppressed by dexamethasone but not by tacrolimus in NHEKs.

Conclusion: Dexamethasone but not tacrolimus suppresses the TSLP expression induced by TNF-α in lesional keratinocytes of AD model. Our observations uncover the unreported functional difference between topical glucocorticosteroids and calcineurin inhibitors in cutaneous inflammatory diseases.

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1. Introduction

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder characterized by epidermal barrier dysfunction and a Th2 environment [1,2]. Th2 cytokines such as IL-4, IL-5, and IL-13 are generally expressed by Th2 lymphocytes, basophilis, eosinophils, and mast cells, and play roles in Th2 cell differentiation, IgE production, and eosinophils recruitment. These cytokines also affect epidermal barrier functions through signal transducer and activator of transcription 6 [3]. For example, IL-4 and IL-13 suppress the expression of filaggrin, loricrin, involucrin and desmoglein 3 in keratinocytes [3]. In addition, IL-4 down-regulates the expression of ceramide and cutaneous permeability barrier functions induced by TNF-α and IFN-γ and the recovery of cutaneous permeability barrier dysfunction in vivo [4]. Furthermore, IL-4 and IL-13 increase the expression and function of a chymotrypsin serine protease, kallikrein 7, in epidermal

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keratinocytes, which leads to excessive protease activity, and ultimately epidermal barrier dysfunction [5].

Thymic stromal lymphopoietin (TSLP) is an epithelial-derived IL-7-like cytokine and necessary to initiate or perpetuate the Th2-type allergic inflammation [6]. The levels of TSLP were significantly increased in the lesional skin of AD, and higher in acute AD than in chronic AD, indicating that TSLP is important for initiating the systemic Th2 immunity favorable for the development of allergic inflammation [7]. A recent report also showed that TSLP released from keratinocytes activates neuron to induce itch [8]. Thus, TSLP is thought to play a crucial role in the pathogenesis of AD. Besides, Volpe et al. reported that TSLP might be a therapeutic target for psoriasis [9].

On the other hand, TNF-α is a representative pro-inflammatory cytokine produced by macrophages, T-cells, B-cells, NK-cells, neutrophils, mast cells, endothelial cells, adipocytes, epidermal keratinocytes and so forth [10]. TNF-α is involved in inflammation, cell proliferation, differentiation and apoptosis, and deregulation of TNF-α production is associated with the pathogenesis of a variety of human diseases [10]. In fact, anti-TNF-α therapies have been clinically used and quite effective for various inflammatory diseases [11]. Patients with AD also have elevated TNF-α levels in the sera and skin, and TNF-α is thought to be a key regulator of inflammation of AD as well as psoriasis [12,13]. In epidermal keratinocytes, TNF-α induces the expression of TNF-α itself, IL-1α, IL-6, IL-8, IL-33, and IL-36, and these inflammatory cytokines and chemokines greatly affect the pathophysiology of inflammatory skin diseases including AD and psoriasis [14–20]. Of note, TNF-α also induces TSLP expression in human keratinocytes [21,22].

Topical glucocorticosteroids and calcineurin inhibitors are commonly used as clinical agents for AD [23–25]. However, the effects of these agents on TNF-α-induced TSLP expression in human epidermal keratinocytes have not been studied well. Here we report that topical application of the glucocorticosteroid dexamethasone but not the calcineurin inhibitor tacrolimus suppressed TSLP expression in a mouse model of AD induced by repeated hapten (TCNB) application, and that TNF-α-induced TSLP expression in cultured epidermal keratinocytes is suppressed by the dexamethasone but not by tacrolimus.

2. Materials and methods

2.1. Mice

Male BALB/c mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and were used at the age of 8 weeks. All animal experiments were approved by the Animal Care and Use Committee of Okayama University.

2.2. Contact-sensitizing agent and sensitization

2,4,6-Trinitro-1-chlorobenzene (TCNB) was obtained from Tokyo Kasei Co. (Tokyo, Japan), dissolved in acetone to 1% solutions, and used for sensitization and elicitation. Mice were sensitized by an epicutaneous application of 20 μl of 1% TCNB to the right ear 7 days before the first elicitation (day 0), and then 20 μl of 1% TCNB was repeatedly applied to the sensitized ear every two days from day 0 to day 24 [26]. White petrolatum, 0.12% dexamethasone or 0.1% tacrolimus ointment was topically used twice a day from day 25 to day 29, and the ear thickness was measured every day. On day 29, the mice were euthanized and the ears were collected for hematoxylin–eosin staining, immunofluorescence staining, real-time PCR, and ELISA analyses.

2.3. Skin samples

This study was approved by the Ethics Committee of Okayama University (No. 1538). Human skin samples were collected from patients with AD and from normal healthy volunteers at Okayama University Hospital. For all procedures, informed consent was obtained. After the injection of local anesthesia, 3 mm punch biopsies were taken from the untreated lesional skin of individuals with AD and from normal healthy volunteers. Formalin-fixed, paraffin-embedded skin samples were cut into 4-μm sections and used for the immunofluorescence studies.

2.4. Immunofluorescence staining

For immunofluorescence staining, the sections of human skin samples were incubated at 4 °C overnight with mouse monoclonal anti-human TNF-α antibody (Abcam, Cambridge, MA) and rabbit anti-human TSLP antibody (Abcam). After washing three times, Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) and Alexa Fluor 594 goat anti-mouse IgG antibody (Molecular Probes) were applied for 1 h at room temperature. Fluorescence signals were observed by confocal laser scanning microscopy (LSM510; Zeiss, Jena, Germany) at the Central Research Laboratory, Okayama University Medical School.

The sections of murine skin samples were incubated at 4°C overnight with goat polyclonal anti-murine TNF-α antibody (LifeSpan Biosciences, Seattle, WA) and rabbit polyclonal anti-murine TSLP antibody (LifeSpan Biosciences). After washing three times, Alexa Fluor 488 donkey anti-goat IgG antibody (Molecular Probes) and Alexa Fluor 594 donkey anti-rabbit IgG antibody (Molecular Probes) were applied for 1 h at room temperature. Images were obtained using KEYENCE fluorescence microscope BZ-X700 (Keyence, Osaka, Japan).

2.5. Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Cascade Biologics/Invitrogen (Portland, OR; catalogue number C-001-5C), and grown in serum-free Epilife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM Ca²⁺ and 1 × Epilife Defined Growth Supplement (EDGS; Cascade Biologics/Invitrogen) at 37°C under standard tissue culture conditions. Cultures were maintained for up to eight passages in this media with the addition of 100 U/ml penicillin and 50 μg/ml streptomycin (Chemicon, Temecula, CA) in 24-well flat bottom plates (Corning Incorporated Life Sciences, Lowell, MA). Cells were stimulated with TNF-α (0.1–50 ng/ml, eBioscience, Chemicon), glucocorticosteroid dexamethasone (10⁻⁷, 10⁻⁶ and 10⁻⁵ M, Sigma, St. Louis, MO), and a calcineurin inhibitor tacrolimus (10⁻⁷, 10⁻⁶ and 10⁻⁵ M, FK506, Sigma) for 24 h. After stimulation, total cell media were stored at −20°C until analysis. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) after supernatant collection. RNA was stored at −80°C until use.

2.6. siRNA transfection

NHEKs were transfected with the siRNA control or siRNA specific for NF-κB p65 (Cell Signaling Technology, Beverly, MA) using LipoFectamine RNAiMAX (Invitrogen). After 24 h of transfection, cells were stimulated with TNF-α for 24 h.

2.7. Neutralization of TNF-α

NHEKs were stimulated with TNF-α (50 ng/ml) for 15 min. For TNF-α signaling inhibition, cells were washed with the media three times and then incubated with human TNF-α neutralizing
rabbit antibody (5 μg/ml, Cell Signaling Technology) or control IgG for 24 h.

### 2.8. Quantitative real-time PCR

Total RNA from cultured keratinocytes or murine skin samples were extracted using TRizol (Invitrogen) and reverse-transcribed using iScript (Bio-Rad, Hercules, CA). TaqMan Gene Expression Assay (Applied Biosystems ABI, Foster City, CA) was used to analyze the expressions of human TSLP (assay ID: Hs00263639_m1), IL6 (assay ID: Hs00985639_m1), TNF (assay ID: Hs00174128_m1), IL8 (assay ID: Hs00174103_m1), IL36G (assay ID: Hs00219742_m1), and murine Tslp (assay ID: Mm01157588_m1) as described in the manufacturer's instructions (User Bulletin #2 by Applied Biosystems). GAPDH mRNA was detected by probe: VIC-CATCATGACAATTGTA-MGB; primers: 5'CTTAGCACCCCTGCCAAG-3'; and 5'TGTCATGAGCTTCCACG-3, and was used as an internal control to validate RNA for each sample. Each mRNA expression was calculated as the relative expression to GAPDH mRNA.

### 2.9. ELISA

Human TSLP protein was measured by ELISA. For the determination of TSLP protein in NHEK-cultured media, 96-well EIA plates (Corning) were coated with mouse monoclonal anti-human TSLP antibody (eBioscience, San Diego, CA) at 20–25°C overnight. After washing with PBS containing 0.05% Tween 20 (washing buffer), the wells were blocked with PBS containing 1% BSA for 1 h at 20–25°C. Following incubation with the cultured...

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media or recombinant TSLP (eBioscience) as standards overnight, biotinylated rabbit polyclonal anti-human TSLP antibody (eBioscience) was used as a detection antibody. Streptavidin-conjugated HRP (R & D Systems, Minneapolis, MN) and 3,3',5,5'-tetramethylbenzidine substrate (BD Biosciences, San Jose, CA) were used for colorimetric quantification, and the reactions were stopped by 0.2 M sulfuric acid (Sigma). The absorbance at 450 nm was monitored with SpectraMax Plus 384 (Molecular Devices

Fig. 2. TSLP and TNF-α show similar distribution pattern in the AD epidermis.
(a) The localization of TSLP (green) and TNF-α (red) in AD lesional skin was visualized by immunofluorescence. Data are representative of three samples. Scale bar = 200 μm.
(b) The localization of TSLP (red) and TNF-α (green) in the lesional skin of AD mouse model was visualized by immunofluorescence. Data are representative of three samples. Scale bar = 50 μm.
Corps., Sunnyvale, CA), and the concentrations in the samples were calibrated from the standard curve of recombinant TSLP using SoftMax Pro 4.6 (Molecular Devices Corp.).

To detect murine TSLP, protein were murine skin samples were extracted with Pierce RIPA buffer (Thermo Scientific, Rockford, IL) with proteinase inhibitor mixture (complete EDTA-free; Roche, Indianapolis, IN). Protein concentration of the extract was determined by DC Protein Assay (Bio-Rad, Hercules, CA). Mouse TSLP ELISA Max Deluxe Sets (BioLegend, San Diego, CA) were used and the amount of TSLP per mg of protein were calculated.

2.10. Statistical analysis

Student’s t-test was used for statistical analysis, and a value of p < 0.05 was considered significant.

3. Results

3.1. Topical dexamethasone but not tacrolimus suppresses TSLP expression in an AD mouse model induced by repeated hapten application

To confirm the clinical effect of dexamethasone and tacrolimus on atopic dermatitis, we studied whether the topical application of dexamethasone or tacrolimus would affect lesional TSLP expression in an AD mouse model induced by repeated TNCB application. As shown in Fig. 1a, repeated dexamethasone or tacrolimus FK506 application to the sensitized ear significantly reduced ear-swelling compared with vehicle (white petrolatum). Histological analysis revealed improvement of epidermal hyperplasia and decreased dermal cell infiltration in both dexamethasone and tacrolimus groups (Fig. 1b). However, real-time PCR and ELISA analyses showed that dexamethasone but not tacrolimus significantly decreased TSLP mRNA and protein expression compared with vehicle (Fig. 1c and d).

3.2. TSLP is similarly located with TNF-α in the AD epidermis

The representative inflammatory cytokine TNF-α has been reported to induce TSLP expression in keratinocytes [21,22], and also thought to be a key regulator of inflammation in AD lesions [27–29]. Therefore, we focused on TSLP and TNF-α expression in epidermal keratinocytes of AD lesions. Co-immunostaining revealed that TSLP and TNF-α showed similar distribution pattern in the AD epidermis (Fig. 2a). The same finding was also observed in the lesions of AD mouse model (Fig. 2b). These results suggest the possibility that TNF-α induces TSLP expression in an autocrine and/or paracrine manner in keratinocytes of AD lesions.

![Fig. 3. TNF-α increases TSLP expression via NF-κB in an autocrine and/or paracrine manner in NHEKs.](image-url)

(a) NHEKs were stimulated with TNF-α (0.1, 0.5, 1, and 5 ng/ml) for 24 h and the relative TSLP mRNA abundance was analyzed by qPCR. (b) TSLP protein in the cultured media was measured by ELISA. (c) NHEKs with knockdown of the p65 subunit of NF-κB were stimulated with TNF-α (50 ng/ml). The protein level of TSLP was measured by ELISA. (d) NHEKs were stimulated with TNF-α (50 ng/ml) for 15 min, washed with the media, and incubated with neutralizing anti-TNF-α antibody (5 μg/ml) for 24 h. W: washing with the media; N: neutralizing anti-TNF-α antibody; C: control IgG. **p < 0.01.

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3.3. TNF-α induces TSLP expression through nuclear factor-kappa B in an autocrine and/or paracrine manner in NHEKs

In vitro, real time-quantitative PCR (qPCR) and ELISA analyses showed that TNF-α increased both TSLP mRNA and protein in NHEKs in a dose-dependent manner as previously reported [21,22] (Fig. 3a and b). TSLP expression induction by IL-1β and TNF-α in airway epithelial cells was reported to be controlled by NF-κB [30], and consistent with this previous report, knockdown of the p65 subunit of NF-κB significantly suppressed TNF-α induced TSLP expression in NHEKs in the present study (Fig. 3c). In addition, under the stimulation with TNF-α, washing with the media significantly suppressed TSLP expression, but the addition of TNF-α neutralizing antibody after washing further decreased the expression in NHEKs (Fig. 3d). This result suggests that TNF-α could induce TSLP expression in an autocrine and/or paracrine manner in NHEKs.

3.4. Dexamethasone but not tacrolimus suppresses TSLP induction by TNF-α in NHEKs

Next we studied whether the TSLP expression induced by TNF-α in NHEKs could be suppressed by dexamethasone and tacrolimus. The glucocorticosteroid dexamethasone suppressed both the TSLP mRNA and protein expression induced by TNF-α in NHEKs in a dose-dependent manner (Fig. 4a and b). However, unlike dexamethasone, the calcineurin inhibitor tacrolimus did not suppress the induction (Fig. 4c and d). We also investigated whether some inflammatory cytokines and chemokines were suppressed by these agents in NHEKs. IL6, TNF, IL8, and IL36G mRNA expression were all suppressed by dexamethasone but not by tacrolimus, which was the same pattern as that of TSLP mRNA expression (Fig. 5a–h).

4. Discussion

Recent studies have revealed that TSLP expression is induced by TNF-α, synthetic double-stranded RNA poly (I:C), FSL-1, flagellin, kallikrein 5, and UVB in epidermal keratinocytes [6,21,31,32]. In AD, TNF-α is released by infiltrating mast cells, T-helper lymphocytes and epidermal keratinocytes [33]. Patients with AD exhibit significant elevations of TNF-α in the sera and skin [12,13], and TNF-α is thought to be a key regulator of inflammation of AD. We observed that TSLP and TNF-α showed similar distribution pattern in the lesional keratinocytes of AD patients and AD mouse model by immunostaining. In addition, we confirmed that TNF-α induced TSLP expression in a dose-dependent manner via NF-κB in NHEKs at both the mRNA and protein level as previously reported [30].

![Figure 4](http://dx.doi.org/10.1016/j.jdermsci.2015.06.016)
Fig. 5. Dexamethasone but not tacrolimus suppresses TNF-α, IL-6, IL-8 and IL-36γ induction by TNF-α in NHEKs. NHEKs were stimulated with TNF-α (50 ng/ml) and dexamethasone (10⁻⁷, 10⁻⁶ and 10⁻⁵ M) or tacrolimus (10⁻⁷, 10⁻⁶ and 10⁻⁵ M) for 24 h. The relative IL6, TNF, IL8 and IL36G mRNA abundance was analyzed by qPCR (a–h). D: dexamethasone; F: FK506.
Furthermore, we demonstrated that TNF-α induced TSLP expression in an autocrine and/or paracrine manner. These results suggest that the induction by TNF-α in lesional keratinocytes might be one of the mechanisms underlying the TSLP increase in AD lesions.

Glucocorticoids bind to the glucocorticoid receptor (GR) in the cytoplasm, and form a steroid–receptor complex [34]. This complex then binds as a homodimer to the glucocorticoid responsive element in target genes to regulate their transcription [34]. In addition, glucocorticoids also indirectly regulate transcription by blocking other transcription factors, including NF-κB [34]. The promoter region of human TSLP gene has the negative glucocorticoid response element, and several groups have reported the negative effects of glucocorticoid on TSLP expression in vitro and vivo [35,36].

For example, a glucocorticoid, fluorocinolone acetonide, has been reported to repress TSLP expression in an AD mouse model induced by a vitamin D3 analogue [36]. In vitro, dexamethasone but not tacrolimus suppressed the TSLP expression induced by double-stranded RNA poly (I:C) in keratinocytes [35]. In the present study, we demonstrated the suppressive effect of dexamethasone on TSLP expression in another AD mouse model which is induced by repeated hapten application. We also showed that TNF-α-induced TSLP expression is greatly down-regulated by dexamethasone in NHEKs. Our data strengthen the evidence that glucocorticoids suppress TSLP expression in lesions of AD.

On the other hand, the calcineurin inhibitor tacrolimus is a complex macrocyclic compound that binds to the intracellular protein macrophlin-12 and functions as a macrolactam immunomodulator, thereby inhibiting the activity of the phosphorylase enzyme calcineurin [24]. Since not only T-lymphocytes but also epidermal keratinocytes express immunoferrin, calcineurin and NFAT, tacrolimus as well as dexamethasone have the possibility to regulate TSLP expression by direct action on epidermal keratinocytes [37,38]. However, in fact, the effects of calcineurin inhibitors on TSLP expression in keratinocytes are controversial [8,35]. In vitro, calcineurin inhibitors have been shown not to suppress the double-stranded RNA-induced release of TSLP in cultured human keratinocytes [35]. On the other hand, TSLP secretion by PAR2 activation in human keratinocytes is reported to be dependent on NFAT, which is inhibited by the calcineurin inhibitor cyclosporine [8]. We revealed that tacrolimus did not decrease both the mRNA and protein expression of TSLP in another AD mouse model induced by repeated TNCB application. Similarly, tacrolimus did not suppress both the TSLP mRNA and TSLP protein expression induced by TNF-α in NHEKs. These findings suggest that the effect of calcineurin inhibitors on TSLP expression in keratinocytes might be dependent on the induction mechanism.

We also focused on the effects of these agents on other inflammatory cytokines and a chemokine, IL-6, TNF-α, IL-8, and IL-36γ, in NHEKs. IL-6 is another representative cytokine which is associated with the pathogenesis of autoimmune and inflammatory diseases, and its expression is also induced by TNF-α in keratinocytes [18,39]. As described above, TNF-α is a key cytokine which is involved in the pathophysiology of various inflammatory diseases, and its expression is induced by TNF-α itself in an autocrine manner in keratinocytes [19]. On the other hand, IL-8 is a chemokine which is inducible by TNF-α in keratinocytes, and mediates neutrophils recruitment [20]. Lastly, IL-36γ is one of the IL-1–like IL-36 cytokine family which is inducible by TNF-α in keratinocytes, and thought to have pro-inflammatory functions and to be involved in the pathogenesis of psoriasis [15]. In NHEKs, the expression of these cytokines and the chemokine were all suppressed by dexamethasone but not by tacrolimus, which was the same pattern as that of TSLP expression. Our findings indicate that dexamethasone is capable of inhibiting the production of keratinocyte-derived key cytokines and chemokines for atopic dermatitis and psoriasis.

In conclusion, we have reported that dexamethasone but not tacrolimus suppresses the TSLP expression induced by TNF-α in lesional keratinocytes of AD model. Our observations uncover the unreported functional difference between topical glucocorticosteroids and calcineurin inhibitors in cutaneous inflammatory diseases. Our results also suggest that topical agents which are able to block TNF-α signaling without the risks of systemic and local side effects induced by glucocorticosteroids could be a good therapeutic medication for atopic dermatitis as well as psoriasis.

Conflict of interest

The authors declare that there are no conflict of interest.

References


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