

# Thymic stromal lymphopoietin expression is increased in the horny layer of patients with atopic dermatitis

Y. Sano,<sup>\*‡</sup> K. Masuda,<sup>\*‡</sup>  
R. Tamagawa-Mineoka,<sup>\*</sup>  
H. Matsunaka,<sup>†</sup> Y. Murakami,<sup>†</sup>  
R. Yamashita,<sup>†</sup> E. Morita<sup>‡§</sup> and  
N. Katoh<sup>\*‡</sup>

<sup>\*</sup>Department of Dermatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, <sup>†</sup>Research & Development, TOKIWA Pharmaceutical Co. Ltd, Tokyo, and <sup>‡</sup>Department of Dermatology, Faculty of Medicine, Shimane University, Shimane, Japan

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Correspondence: N. Katoh, Department of Dermatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan.

E-mail: nkatoh@koto.kpu-m.ac.jp

<sup>§</sup>Y.S. and K.M. and E.M. and N. K., respectively, contributed equally to this work.

## Summary

Thymic stromal lymphopoietin (TSLP) is known for its capacity to induce CD11c<sup>+</sup> myeloid dendritic cells to promote T helper type 2 (Th2)-skewed inflammatory responses. Although increased expression of TSLP was reported in the lesional skin of limited numbers of patients with atopic dermatitis (AD), the relationships between the degree of TSLP expression in the skin and the severity of AD, epidermal barrier function and eruption type remain to be elucidated. The aim of this study was to examine the relationships between the degree of TSLP expression in the skin and the severity of AD, eruption type and epidermal barrier function using a non-invasive method in a sizeable group of the patients. Stratum corneum tissue was obtained from AD patients by tape stripping, and the stratum corneum TSLP (scTSLP) expression level was evaluated using a TSLP-specific antibody followed by image analysis. The correlations between the scTSLP intensity and the severity scoring of AD (SCORAD) index and epidermal barrier function, such as stratum corneum hydration and transepidermal water loss (TEWL), were analysed. The changes in the scTSLP level induced by the application of moisturizer were also examined. The scTSLP expression level was increased in AD patients compared with healthy subjects and was correlated with SCORAD, especially with the dry skin score, and stratum corneum hydration. Moisturizer application resulted in reduced scTSLP levels. The scTSLP level can be used as a biomarker of AD severity and particularly epidermal barrier status.

**Keywords:** atopy, CD4 T cells (Th2), skin immunology/disease

## Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin disease, which is associated frequently with decreased epidermal barrier function and T helper type 2 (Th2)-dominant immune responses to food and environmental allergens [1]. In mice, it was demonstrated that epicutaneous sensitization of tape-stripped skin with protein antigen induced Th2-dominated immune responses [2,3], suggesting that injury to the stratum corneum elicits Th2-inducible cytokine production in keratinocytes and/or dendritic cells. Thymic stromal erythropoietin (TSLP) is an interleukin (IL)-7-like cytokine that is produced preferentially by epithelial cells such as keratinocytes and airway mucosa [4]. TSLP is known for its capacity to induce CD11c<sup>+</sup> myeloid dendritic cells to promote Th2-skewed inflammatory responses [5,6]. Th2 cells induced in the presence of TSLP

do not produce IL-10, and are thus regarded as inflammatory Th2 cells [6]. More importantly, injury to the stratum corneum by tape-stripping of the skin or the application of sodium lauryl sulphate to the skin induces TSLP expression and elicits a Th2 response in humans and mice [7,8]. TSLP is highly expressed by keratinocytes in the lesional skin of AD patients [6]. However, the sample numbers were limited. Moreover, the relationships between the degree of TSLP expression in the skin and the severity of AD, epidermal barrier function and eruption type remain to be elucidated.

Recently, Morita *et al.* established a novel technique in which immunofluorescent labelling was combined with tape-stripping to allow semi-quantitative measurements of cytokine expression [9]. They demonstrated that the thymus and activation-regulated chemokine (TARC) levels in the stratum corneum were increased in the skin lesions of

AD patients and that the increases were correlated with disease severity. This method is easy to perform and is non-invasive, and is thus suitable for estimating the cytokine levels in stratum corneum tissue samples from many subjects, which would be expected to reflect their expression levels in epidermal keratinocytes [9,10].

In this study, we utilized this technique to measure the TSLP levels in the stratum corneum (scTSLP). This is the first report to test for TSLP expression in the stratum corneum in a considerable number of AD patients. We also examined whether the degree of TSLP expression in the skin correlates with disease severity, epidermal barrier function such as stratum corneum hydration and transepidermal water loss (TEWL), and/or eruption type in AD patients.

## Materials and methods

### Subjects

Forty-two patients (22 men and 20 women; median age: 26 years, range: 20–63) who were diagnosed according to the criteria established by the Japanese Dermatological Association [11] and 20 healthy controls (nine men and 11 women; median age: 31 years, range: 21–42) were included into this study. The inclusion criteria for the healthy subjects were as follows: (i) those without any inflammatory skin diseases, (ii) those whose physical findings were normal, (iii) those with no past history or family history of related allergies and (iv) those whose laboratory values were within the normal range.

All patients were treated with topical corticosteroids and oral anti-histamines. Both the treatments were ceased approximately 24 h before the examination day, and the patient's body was cleansed the day before the tape-stripping.

Written informed consent was obtained from all patients and healthy volunteers. This study was approved by the ethical committee of Kyoto Prefectural University of Medicine and Shimane University Faculty of Medicine.

### Assessment of the patients' clinical conditions

The overall severity of each patient's AD was evaluated using the severity scoring of AD (SCORAD) index [12].

### Measurement of stratum corneum hydration and TEWL

Measurements of stratum corneum hydration and TEWL were performed under standardized conditions; i.e. at a room temperature of 20–22°C and a humidity level of 40–55%. Before measurement, the subjects were given time to adapt to the room conditions but were not allowed to cover the measurement sites with their clothes. Clinically

normal areas of the extensor or flexor aspects of their forearms and their backs were used as control sites. Stratum corneum hydration was evaluated with a Skicon-200EX (I.B.S, Shizuoka, Japan). TEWL was measured using the Tewameter® TM300 (Courage and Khazaka Electronic GmbH, Köln, Germany). All the measurements were performed three times for each site, and the measured values were expressed as the median of three recordings.

### Moisturizer treatments

Twenty-four AD patients who complained primarily of dryness were asked to apply a moisturizer (Oligomarine® Lotion; Tokiwa Pharmaceutical Co. Ltd, Tokyo, Japan) twice a day, i.e. in the morning and evening, for 8 weeks. The composition of the moisturizer is shown in the Supporting information, Table S1. The moisturizer was applied to the entire body, and the cubital fossa was evaluated. Before and after the trial, the AD symptoms experienced by the patients over their entire body were evaluated using the SCORAD index, and skin manifestations were evaluated on the cubital fossa. Moreover, stratum corneum hydration and TEWL were measured, and stratum corneum tissue samples were obtained with tape stripping.

### Measurement of the scTSLP and other cytokine concentration

The tape-stripping method and measurement of the stratum corneum TSLP expression level were carried out according to the method described in a previous report by Morita *et al.* [9]. Briefly, Cellophane tape (24 mm × 50 mm, Serotape®; Nichiban, Tokyo, Japan) was applied to the cubital fossa and unaffected and affected sites on the trunk, pressed for approximately 10 s, and then removed gently. The resultant sample was kept at –20°C until further analysis. The tape was attached to an aminopropyltriethoxysilane-coated slide glass (Matsunami Glass, Osaka, Japan), and the slide glass was immersed overnight in *n*-hexane (Nacalai, Tokyo, Japan) to remove the adhesive tape spontaneously. The slide glass was then immersed in cold acetone for 10 min, and the cells were fixed on the slide glass and blocked with 1.0% bovine serum albumin. After washing with phosphate-buffered saline without calcium or magnesium, the cells were incubated overnight with an anti-TSLP antibody (R&D Systems, Minneapolis, MN, USA), anti-IL-4 and IL-13 (H-112 and H-129, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or their isotype-matched control immunoglobulin (Ig)G (Santa Cruz Biotechnology) at 4°C. The cells were then incubated with anti-sheep IgG AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature while being protected from light. After being mounted, the cells were observed under a fluorescence microscope (BZ-8100; Keyence, Tokyo, Japan). To

estimate the fluorescence intensity of stratum corneum, we set the exposure time in which TSLP-specific fluorescence was observed while no background fluorescence was detected. All the conditions, including the established exposure time and other setting-up of fluorescent microscopy, were identical throughout all the experiments. The bright-field and fluorescent images were photographed, and the fluorescence intensity and cell area were determined using Photoshop® software (Adobe Systems Incorporated, San Jose, CA, USA). The scTSLP expression level was defined as the fluorescence intensity per cell area. To estimate the fluorescence intensity by using Photoshop®, the fluorescence intensity was measured in five separate fields and then three values among them were taken by deleting maximum and minimum values to obtain mean values.

### Statistics

The data are presented as mean  $\pm$  standard error of the mean (s.e.m.) values unless indicated otherwise. For statistical analyses, Wilcoxon's signed-rank test was used for comparisons between two groups, and the Steel–Dwass test was used for comparisons among three or more groups. Spearman's rank correlation test was used to assess the correlation between the scTSLP values and scIL-4, scIL-13, SCORAD or each eruption score. The results were considered to be significant or correlated when the relevant *P*-value was less than 0.05.

## Results

### scTSLP concentrations in patients with AD and healthy subjects

scTSLP immunoreactivity was visualized successfully and evaluated as described in a previous report, in which the stratum corneum level of TARC was evaluated (Supporting information, Fig. S1) [9]. No fluorescence was detected with isotype-matched control IgG. The mean fluorescence intensity was  $76.4 \pm 9.0$  ( $n = 42$ ) in the stratum corneum tissue obtained from the affected skin of the AD patients,  $24.6 \pm 4.6$  ( $n = 20$ ) in the unaffected skin of the AD patients, and  $17.4 \pm 1.1$  ( $n = 20$ ) in the healthy individuals (Fig. 1a). Positive immunofluorescence for both IL-4 and IL-13 was also observed in tape-stripped stratum corneum obtained from the patients with AD ( $n = 42$ ) (Fig. 1b). The fluorescence intensities for IL-4 and IL-13 were correlated significantly with those for TSLP (Fig. 1).

### Correlation between the scTSLP concentration and skin symptom severity

Correlations between the scTSLP intensity and the skin lesion severity scores (total score, erythema score, oedema/

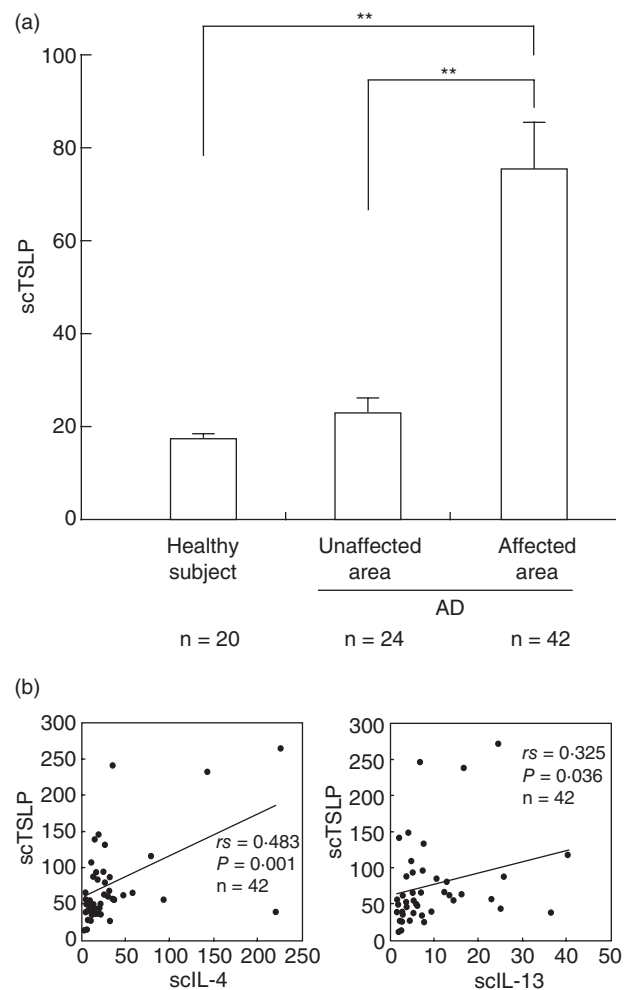


**Fig. 1.** (a) The stratum corneum (sc) thymic stromal lymphopoietin expression (TSLP) concentration of the trunk in atopic dermatitis (AD) patients and healthy individuals. In the AD patients, samples were taken from affected and non-affected areas of the trunk. Data are expressed as mean values  $\pm$  standard error of the mean. Steel–Dwass test,  $**P < 0.01$ . (b) The fluorescent intensities of sc interleukin (scIL)-4 or scIL-13 was demonstrated with scTSLP from the affected skin of the AD patients ( $n = 42$ ). Spearman's rank correlation test.

papule score, oozing/crust score, excoriation score, lichenification score and xerosis score) were evaluated in the areas of affected skin (trunk and cubital fossa) subjected to tape-stripping in the AD patients (Fig. 2). The scTSLP level was correlated significantly with the total score. Among the six types of eruption, the xerosis score ( $r = 0.400$ ,  $P = 0.009$ ) was correlated significantly with the scTSLP intensity, but was not correlated significantly with the erythema score ( $r = 0.237$ ,  $P = 0.135$ ), oozing/crust score ( $r = -0.016$ ,  $P = 0.922$ ), oedema/papule score ( $r = 0.049$ ,  $P = 0.759$ ), excoriation score ( $r = 0.001$ ,  $P = 0.995$ ) or lichenification score ( $r = -0.098$ ,  $P = 0.543$ ) (Fig. 3). The itching score was also correlated significantly with the scTSLP level ( $r = 0.358$ ,  $P = 0.027$ ).

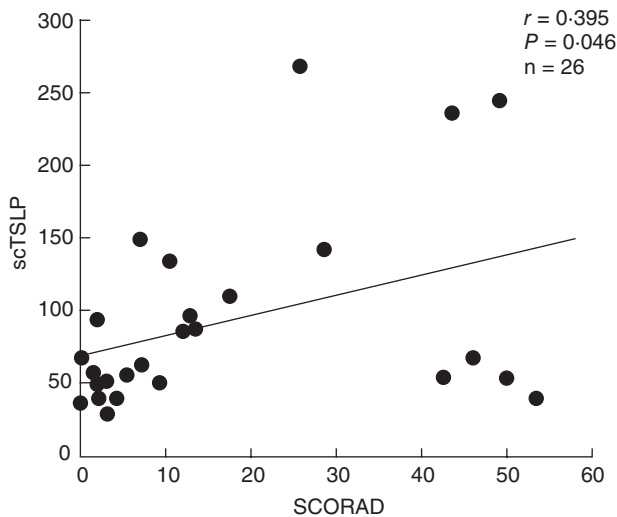


Fig. 2. Correlation between the stratum corneum (sc) TSLP concentration in the trunk and cubital fossa was assessed with the SCORAD index ( $n = 26$ ).

#### Correlation between epidermal barrier function and scTSLP level

Conductance ( $r = -0.416$ ,  $P = 0.043$ ) and TEWL ( $r = 0.365$ ,  $P = 0.022$ ) were correlated significantly with scTSLP expression in the affected area of the AD patients, further supporting the finding that xerosis was correlated with the scTSLP level (Fig. 4). The mean SCORAD index was  $14.97 \pm 3.55$  before the moisturizer application in the AD patients ( $n = 24$ ), while it was  $9.02 \pm 2.54$  after 8 weeks of moisturizer application ( $P = 0.001$ ). Skin conductance was increased significantly from  $52.2 \pm 10.5$  to  $78.6 \pm 13.4$  ( $P = 0.034$ ) by this treatment in these patients. TEWL showed a tendency to be reduced from  $18.8 \pm 3.1$  to  $13.6 \pm 2.1$  ( $P = 0.067$ ). In these AD patients, application of the moisturizer resulted in a reduction in the scTSLP level from  $88.0 \pm 12.6$  to  $64.1 \pm 6.4$  ( $n = 23$ ,  $P = 0.039$ ) (Fig. 5), although the expression levels of scTSLP did not show significant correlations with TEWL ( $r = 0.167$ ,  $P = 0.436$ ) or skin conductance ( $r = -0.128$ ,  $P = 0.552$ ) after application of the moisturizer.

#### Discussion

At first, we tried to analyse the TSLP concentration in stratum corneum tissue obtained from tape-stripped samples using enzyme-linked immunosorbent assay (ELISA). Unfortunately, the TSLP concentrations of the tape-stripped samples were below the detection limit of the ELISA. The amount of TSLP cDNA in tape-stripped samples was too small for quantitative assessment of gene expression by reverse transcription-polymerase chain reaction (RT-PCR). Thus, we combined an immunostaining technique with the tape-stripping method in this study

and demonstrated that the scTSLP expression level was increased significantly in patients with adult-type AD compared with healthy subjects. This finding is consistent with the findings of a previous report that demonstrated the crucial role of TSLP produced by keratinocytes in the pathogenesis of AD in some mouse models [13–15] and the increased expression of TSLP in the keratinocytes of AD patients [6]. This result supports our hypothesis that the scTSLP intensity probably reflects the expression of TSLP in keratinocytes in AD patients. It has been demonstrated that the serum levels of TSLP are not elevated in patients with adult-type AD [16]. Our report is the first to demonstrate the increased expression of TSLP in patients with adult AD using a non-invasive method.

In the present study, the scTSLP level was correlated significantly with the xerosis score, but not with acute-phase parameters such as the erythema, oedema/papule or oozing/crusts scores. Xerosis is regarded as a phenotype of epidermal barrier dysfunction in AD, which is regarded as a primary abnormality in the pathogenesis of AD [17]. Demehri *et al.* showed that loss of Notch signalling within keratinocytes in mice impairs their ability to execute the terminal differentiation programme, resulting a defective skin barrier [18]. It is important to note that TSLP is overproduced by these keratinocytes. Their further experiments demonstrated that the over-production of TSLP is a common and direct readout of differentiation/barrier formation defects in keratinocytes, and not a repressed target of Notch signalling. They conclude that TSLP is a reporter of keratinocyte differentiation/barrier defects, although the precise molecular mechanism of this pathway remains unanswered. AD is characterized by disrupted terminal differentiation of keratinocytes in the lesional skin [19], which contributes to defective barrier function recognized clinically as xerosis. Thus, it is suggested that impaired terminal differentiation and barrier function in the epidermis of AD patients directly induces increased production of TSLP by a thus far unknown mechanism. Another possibility is the contribution of protease/protease-activated receptor (PAR)-2 signalling in the epidermis. Desquamation is regulated by the balance between serine proteases, including kallikrein (KLK)5 and KLK7 and their inhibitors such as lymphoepithelial Kazal-type-related inhibitor (LEKTI) encoded by serine protease inhibitor Kazal-type 5 (SPINK5) [20]. Predominant expression of the serine proteases over their inhibitors leads to corneodesmosomal cleavage and subsequent desquamation, which is often recognized clinically as xerosis. In AD patients, genetic variation within the KLK7 has been demonstrated, resulting in an increase of the half-life of the KLK7 mRNA and enzymatic activity of KLK7 [21]. It has also been shown that KLK7 expression is increased in the epidermis of patients with AD, and transgenic mice over-expressing KLK7 in keratinocytes develop pathological skin changes resembling AD [22]. Conversely, several studies have reported that polymorphisms in

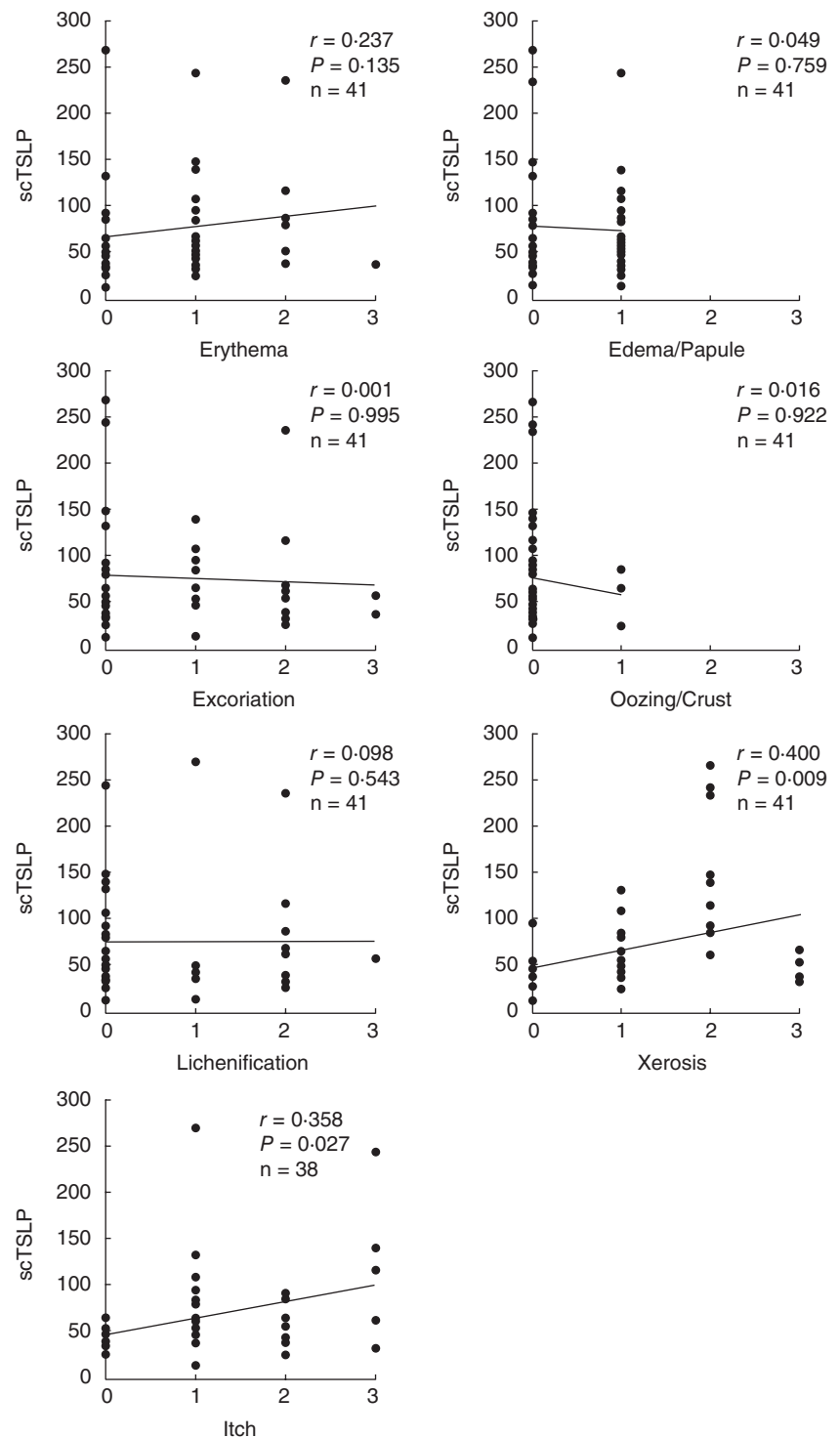


**Fig. 3.** Correlation between the stratum corneum (sc) thymic stromal lymphopoietin expression (TSLP) concentration and the skin symptom severity score of affected skin subjected to tape-stripping in atopic dermatitis patients. The results obtained from the trunk and cubital fossa are presented together.

SPINK5 are associated with AD [23,24]. The increase in serine protease activity activates PAR-2 on keratinocytes of stratum granulosum [25]. More importantly, KLK5 has been demonstrated to activate PAR-2 directly, which induces increased expression of TSLP in mice [26]. Taken together, the accumulated evidence suggests that TSLP is over-expressed in barrier-perturbed skin, which may

contribute to Th2-skewed immune responses in patients with AD.

In our study, the expression levels of scTSLP in unaffected areas of AD patients were not significantly different from those in healthy subjects. It seems to be contradictory to our hypothesis that reduced barrier function would result in scTSLP expression, because it is widely known that

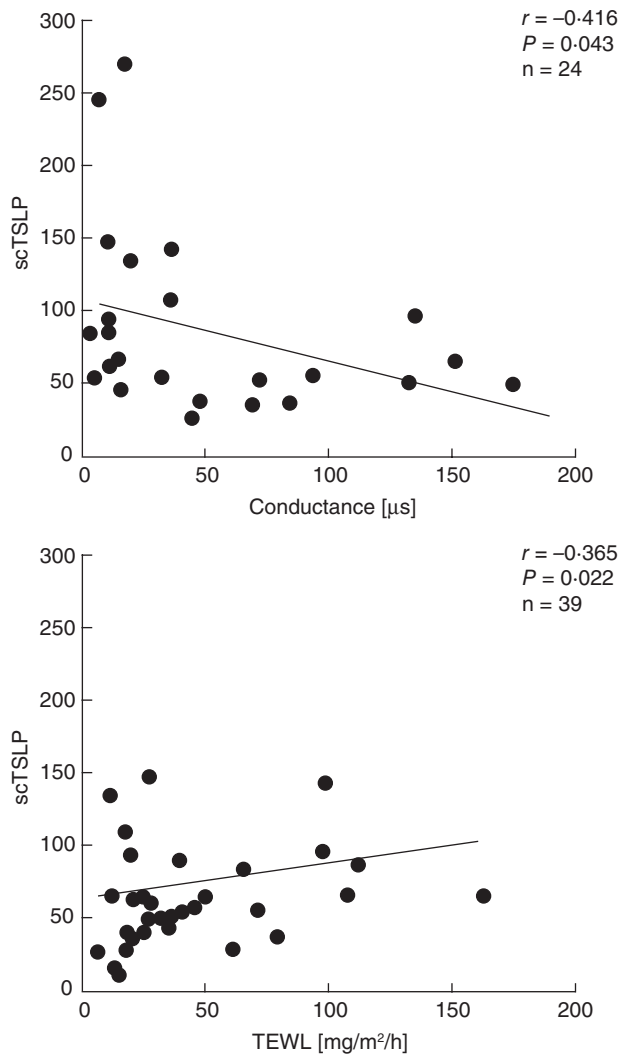


**Fig. 4.** Correlations between the stratum corneum (sc) thymic stromal lymphopoietin expression (TSLP) concentration and conductance ( $n = 24$ ) and transepidermal water loss (TEWL) ( $n = 39$ ) were assessed in the normal skin of atopic dermatitis (AD) patients.

impaired skin barrier exists even in clinically unaffected areas of AD compared to those of healthy subjects. One explanation of this contradiction is that the epidermal barrier function of the unaffected area might not be impaired enough to up-regulate TSLP expression, because it has been demonstrated that the barrier function of non-lesional skin of AD patients is less impaired when compared with those of lesional skin [27]. Alternatively, there might be some bias in sampling in this study, because many more AD patients with mild severity participated than severe AD patients. In addition, as it is difficult to find unaffected areas on the trunk in patients with severe AD, samples of the unaffected areas were obtained mainly from patients with mild AD. In this study, SCORAD showed a significant correlation with TEWL and an opposite correlation with skin hydration in unaffected areas (see Supporting information,

Fig. S2). It is suggested that the barrier function of mild AD patients would be less impaired than severe patients, which may thus result in low scTSLP expression in unaffected areas observed in our study. We believe that more uniform sampling in patients' severity might bring a significant increase in mean intensity of scTSLP in unaffected areas; indeed, the expression levels of scTSLP in unaffected areas of patients whose SCORAD were greater than 10 were increased significantly when compared with those in healthy skin ( $43.7 \pm 9.3$ ,  $n = 9$  versus  $17.4 \pm 1.1$ ,  $n = 20$ ,  $P = 0.023$ ).

In this study, we showed that the application of a moisturizer resulted in reduced scTSLP levels. Prevention management including the application of moisture-rich skin-care products and the avoidance of skin scrubbing during bathing from infancy could reduce the expression of epidermal TSLP and thereby prevent the development of atopic disorders. Indeed, a recent pilot study revealed that prevention management with emollient from birth reduced the prevalence of AD in infants who were considered to be at high risk of developing AD [28].

In our study, the itch score was also correlated significantly with scTSLP expression levels that might have contributed significantly to the positive correlation between scTSLP and SCORAD, because in this study none of the inflammatory items within SCORAD showed a significant correlation with scTSLP. The correlation is interesting at all events, as scratching the skin due to itch may lead to an increase in TSLP expression because mechanical injury of the stratum corneum induces TSLP production [7,8]. In addition, PAR-2 activation is likely to be involved in AD pruritus. Schmelz and colleagues demonstrated positive

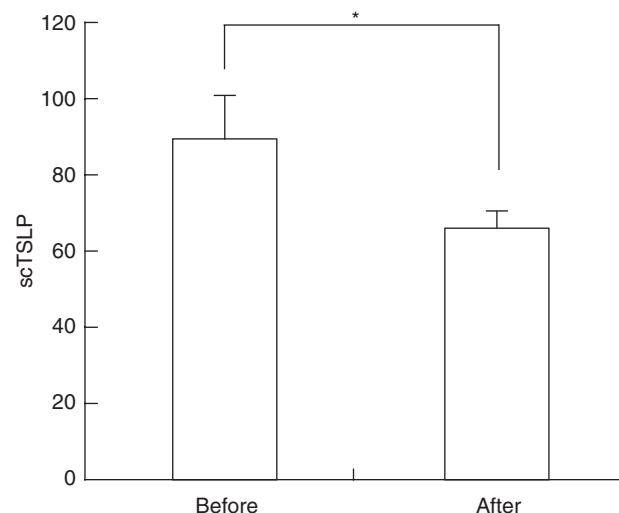


**Fig. 5.** The stratum corneum (sc) thymic stromal lymphopoietin expression (TSLP) concentration in the cubital fossa before and after the application of moisturizer for 8 weeks in atopic dermatitis (AD) patients ( $n = 23$ ). Data are shown as mean values  $\pm$  standard error of the mean. Wilcoxon's signed-rank test,  $*P < 0.05$ .

immunoreactivity for PAR-2 in keratinocytes, blood vessels, certain inflammatory cells and nerve fibre-like structures in lesional skin of patients with AD [29]. Moreover, intracutaneous injection of endogenous PAR-2 agonists intralesionally provoked enhanced and prolonged itch [29]. Thus, activated PAR-2 in the lesional skin of AD may explain the positive relationship between itch and increased levels of scTSLP.

In conclusion, we detected elevated levels of scTSLP in AD patients, especially in association with dry skin, using a combination of a non-invasive immunostaining technique and the tape-stripping method. The scTSLP intensity might be a promising biomarker of AD. It might also be useful for estimating the effects of various factors including bathing habits, air conditioning and the use of moisturizing skincare products on skin dryness.

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## Disclosure

H.M., Y.M and R.Y. are employees of Tokiwa Pharmaceutical Co. Ltd. None of the other authors have any conflicts of interest to declare.

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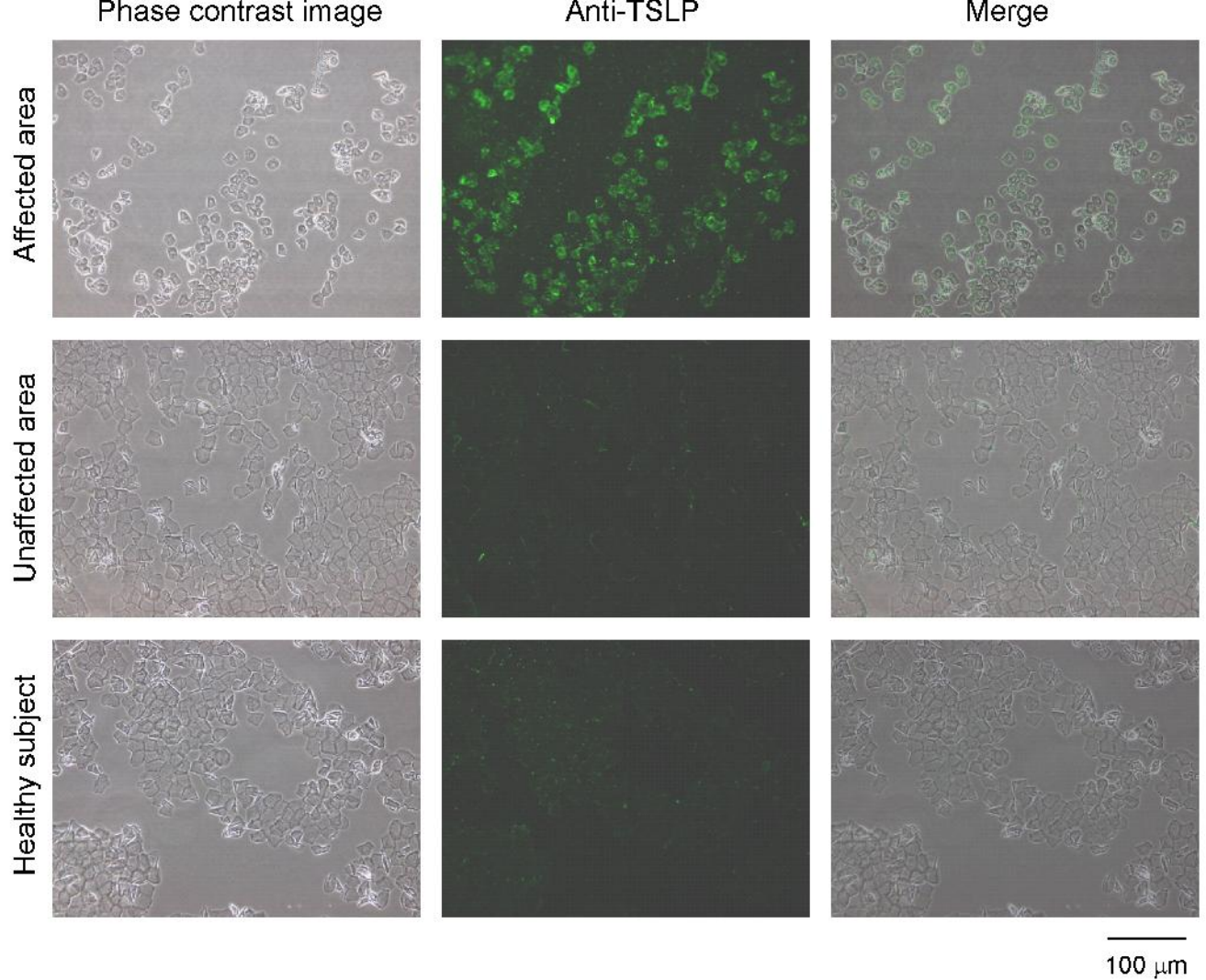
### Supporting information

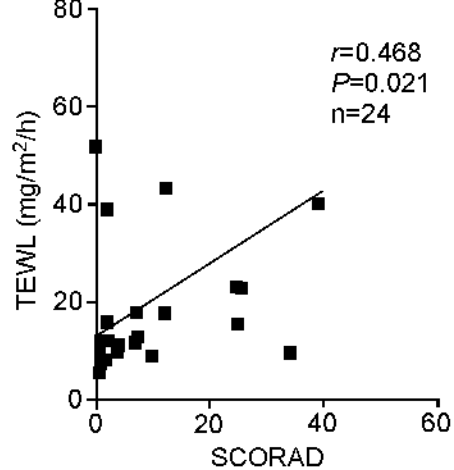
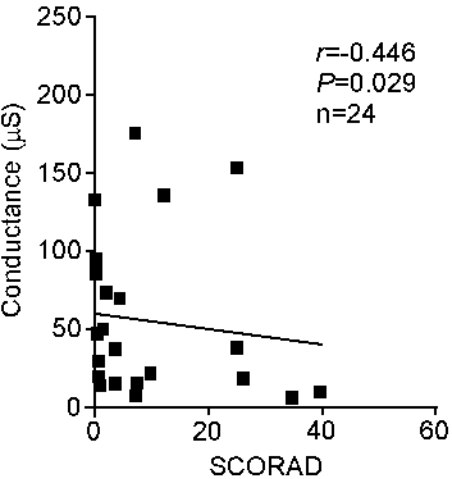
Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Immunoreactive thymic stromal lymphopoietin expression (TSLP) in the stratum corneum was visualized using a TSLP-specific antibody in conjunction with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Representative result obtained from a patient with atopic dermatitis and a healthy subject are shown.

**Fig. S2.** Correlation between severity scoring of atopic dermatitis (SCORAD) index and conductance or transepidermal water loss (TEWL) in unaffected area ( $n = 24$ ).

**Table S1.** Oligomarine® lotion contains water, butylene glycol, glycerin, seawater, dipotassium glycyrrhizate, citric acid, sodium hydroxide and methyl paraben.





Supplementary Table:

OLIGOMARINE<sup>®</sup> LOTION contains water, butylene glycol, glycerin, seawater, dipotassium glycyrrhizate, citric acid, sodium hydroxide, and methyl paraben.

Ionic composition of OLIGOMARINE<sup>®</sup>

| Ion              | OLIGOMARINE <sup>®</sup><br>[mg/L] | SEAWATER | OLIGOMARINE <sup>®</sup><br>/ SEAWATER |
|------------------|------------------------------------|----------|----------------------------------------|
| Mg <sup>2+</sup> | 72000                              | 1320     | 54.5                                   |
| K <sup>+</sup>   | 14000                              | 420      | 33.3                                   |
| Zn <sup>2+</sup> | 1.67                               | 0.0050   | 334.0                                  |
| Se <sup>4+</sup> | 2.45                               | 0.00045  | 5444.4                                 |
| Na <sup>+</sup>  | 9800                               | 11100    | 0.9                                    |