

The Th2 cytokine, interleukin-4, abrogates the cohesion of normal stratum corneum in mice: implications for pathogenesis of atopic dermatitis

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Abstract: There is mounting evidence that Th2 cytokines adversely affect skin barrier functions and contribute to the pathogenesis of atopic dermatitis (AD). AD is also characterized by abnormal cohesion in the stratum corneum (SC). However, the contribution of Th2 cytokines to this abnormality remains unknown. This study examined the effects of IL-4, a prototypic Th2 cytokine, on the cohesion of the SC. Structural and physiological assessments revealed that repeated intradermal injections of IL-4 compromised the cohesion of the SC of normal hairless mice. Two potential mechanisms were explored to account for the altered cohesion. First, IL-4 decreased the amount of corneodesmosomes and down-regulated the expression of desmoglein 1, but not of corneodesmosin (CDSN) or loricrin expression, in murine skin and in cultured human keratinocytes (KC). IL-4 did not affect the skin surface pH, and *in situ* zymography revealed no net change in total serine protease activity in the IL-4-treated SC. Yet, IL-4

enhanced expression of kallikrein (KLK)7, while simultaneously down-regulating KLK5 and KLK14. Finally, IL-4 did not alter the expression of the lympho-epithelial Kazal-type inhibitor (LEKTI) in KC. This study suggests that IL-4 abrogates the cohesion of SC primarily by reducing epidermal differentiation.

Abbreviations: Th2, T-helper type 2; IVL, involucrin; FLG, filaggrin; LOR, loricrin; AMPs, antimicrobial proteins; IL, interleukin; Stat, signal transducer and activator of transcription; SC, stratum corneum; KLK, kallikreins; AD, atopic dermatitis; CD, corneodesmosome; CE, cornified envelope; DSG, desmoglein; DSC, desmocollin; CDSN, corneodesmosin; LEKTI, lympho-epithelial Kazal-type inhibitor; TGase, transglutaminase.

Key words: cohesion of stratum corneum – corneodesmosome – desmoglein 1 – Th2 cytokine

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Introduction

Th2 cytokines have been well known to be involved in the pathogenesis of atopic dermatitis (AD) (1,2). There is mounting evidence that Th2 cytokines adversely affect skin barrier functions, thus, even though not the initiator of AD, likely amplify disease phenotype as part of a putative ‘outside-to-inside,’ the ‘back-to-outside’ vicious cycle (3). For example, Th2 cytokines decrease expression of involucrin (IVL), loricrin (LOR), filaggrin (FLG) and antimicrobial proteins (AMPs) in human epidermis and/or in cultured human keratinocytes (KC) (4–6). The Th2 cytokine-induced decrease in expression of corneocyte proteins could compromise permeability barrier function via a failure to provide an adequate corneocyte scaffold for lamellar bilayer organization (7,8). Moreover, the decreased expression of the cathelicidin protein, LL-37, in AD (9) could also affect barrier function in AD, as this AMP is required not only for protection against invasion by microbial pathogens, but also for permeability barrier function (10). Furthermore, Th2 cytokines suppress ceramide synthesis (11, 12), a distinctive feature of AD (13), likely accounting, at least in part, for abnormal permeability barrier homeostasis in AD (11,12). Finally, interleukin-4 (IL-4) deficiency protects Stat6VT transgenic mice from the development of allergic skin inflammation, while also augmenting permeability barrier homeostasis, with a concomitant increased expression of proteins in the epidermal differentiation complex (IVL and FLG) (14). Together, these

observations suggest that Th2 cytokines, particularly IL-4, could play an important role in the pathogenesis of AD.

Yet, whether Th2 cytokines also alter the cohesion of the stratum corneum (SC), another important determinant of skin barrier function (15), is unknown. The importance of SC cohesion in AD pathogenesis is most clearly demonstrated in Netherton syndrome, a disease characterized by severe atopic abnormalities (16), in which the activity of serine proteases [kallikreins (KLKs)] is elevated, leading to profound thinning of the SC (17). Accordingly, both reduced SC cohesion and the thinning of SC also have been observed in AD (18). Although these phenomena are generally attributed to a pH-induced increase in serine protease activity (18), the possibility that Th2 cytokines are involved in altering the cohesion of the SC has not been explored.

The SC is a self-renewing tissue in which corneocytes that are shed from the surface are replaced by newly cornified cells. This process is carefully orchestrated to provide the SC with remarkable initial cohesion, prior to progressive distal detachment of corneocytes (19). Corneodesmosomes (CDs) are essential for the cohesion of the SC (15). CDs contain at least two desmosomal cadherins, desmoglein (DSG) 1 and desmocollin (DSC) 1, as well as a unique protein, corneodesmosin (CDSN), and proteins that embed these structures into the cornified envelope (CE; for example, desmoplakin and envoplakin) (20). Therefore, CDs can be viewed as homologous junctional structures that protrude from the CE.

The persistence and degradation of CDs is regulated by the pH-dependent activity of serine proteases (KLKs) and at least one potent serine protease inhibitor, the lympho-epithelial Kazal-type inhibitor (LEKTI) (17,21,22). Eight species of KLKs have been detected in human SC, and KLK7 accounts for about 40% of total amounts of them (23). LEKTI specifically inhibits at least three of these KLKs; that is, KLK5, KLK7 and KLK14 (22). Thus, either a Th2 cytokine-mediated decline in differentiation, and/or protease/antiprotease up-regulation could account for altered SC cohesion in AD.

This study demonstrated that the Th2 cytokine, interleukin (IL)-4, adversely affected the cohesion of the SC by reducing the amount of CD. Of the two potential mechanisms that could explain this abnormality, our current results suggest that the altered SC cohesion can be attributed primarily to the inhibitory effects of IL-4 on epidermal differentiation, rather than enhanced serine protease activity.

Materials and methods

Animals

Female C57BL/6 mice, which are probably the most widely used of all inbred strains, were used at 12–48 weeks of age. All animals were housed under conventional conditions and had free access to a commercial diet and water. This study was approved by the Ethics of Animal Experimentation Committee of Oita University.

Culture of normal human keratinocytes

Normal human KC were cultured as previously reported (11). Normal human KC (DS Pharma Biomedical, Osaka, Japan) were cultured in human keratinocyte serum-free medium (DS Pharma Biomedical) at 37°C in a humidified atmosphere of 5% CO₂ in air. The cultured cells were used for all experiments when they had reached 60–70% confluence. In some experiments, the medium was changed to that containing an elevated concentration of CaCl₂ (1.8 mM) and/or human recombinant IL-4 (20 ng/ml; RD Systems, Minneapolis, MN, USA). Keratinocyte cells were incubated for 24 h and then were homogenized in Isogen[®] (Nippon Gene, Toyama, Japan), which contained phenol and acid guanidinium thiocyanate. Total RNA and proteins were isolated according to the manufacturer's protocol.

Treatment of mice with interleukin-4 and sample collections

Five consecutive daily intradermal injections of murine recombinant IL-4 (1.4 ng in 280 µl; Peprotech, Rocky Hill, NJ, USA) or vehicle (Hanks' Balanced Salt Solution: HBSS, Invitrogen, Grand Island, NY, USA) were administered via shaved abdominal skin, as described previously (24). The area of the intradermal injection of murine recombinant IL-4 or HBSS was 3.8 cm² (22 mm in diameter), which is the same area as that of surface of a D-squameTM tape (Promotool, Tokyo, Japan). D-squameTM tapes were used in the assessment of cohesion of SC as described below. The skin specimens were excised 24 h after the last intradermal injection, and their epidermal sheets were separated by treatment with 1000 IU/ml dispase (Godo Shusei, Tokyo, Japan) for approximately 1 h at 37°C. Each epidermal sheet was homogenized in protein extraction buffer (40 mM Tris-HCl, pH 7.5, 10 mM EDTA containing protease inhibitors and 0.5% Nonidet P-40). The homogenates were centrifuged; the detergent-insoluble pellet was solubilized in protein extraction buffer containing 6 M urea and 50 mM dithiothreitol and analysed by Western blotting.

Western blotting

Samples prepared from treated cultured human KC and murine epidermal sheets were separated by 7.5 or 10 % SDS-PAGE before transfer to Hybond nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Membranes were probed with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized with ECLTM Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Band intensities were quantified using NIH Image software. Rabbit polyclonal antibody against DSG 1, rabbit polyclonal antibody against CDSN and HRP-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against β-actin was purchased from Biovision Research Products (Mountain View, CA, USA). Rabbit polyclonal antibody against LOR was purchased from Covance (Emeryville, CA, USA).

Semiquantitative RT-PCR

The expression of DSG 1, CDSN, KLK5, KLK7, KLK14, LEKTI and transglutaminase (TGase) 1 mRNA in normal human epidermal KC was examined by semiquantitative RT-PCR, which was performed as described previously (11). Fifty nanogram of RNA was reverse-transcribed using random hexamers (Takara Shuzo, Shiga, Japan) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resultant cDNA was amplified by PCR in the presence of 1 mCi ³²P-dCTP. PCR was performed for the appropriate number of cycles (1 min at 94°C; then 1 min at 58°C for DSG 1, CDSN, KLK5, KLK7, KLK14, LEKTI, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), or at 60°C for TGase 1; and 1 min at 72°C) within the linear ranges of amplification: 20 cycles for DSG 1, CDSN and KLK7; 22 cycles for KLK5, KLK14, LEKTI and G3PDH; and 26 cycles for TGase 1. Aliquots (1 ml) of each sample after the amplification were analysed by electrophoresis on a 5% acrylamide/Tris-borate-EDTA gel, and the products were visualized by autoradiography. DNA was quantified by a phosphor image analysis (BAS2000; Fuji Medical Systems, Stanford, Connecticut). The levels of mRNA for each molecule were normalized by reference to levels of mRNA for G3PDH. The primers for amplification of cDNA are listed in the Table S1.

Assessment of the integrity of the stratum corneum

The cohesion of SC was assessed as previously described (25). The skin surface was cleaned with a single ethanol wipe. D-SquameTM tapes (Promotool) then were placed sequentially onto the test areas for 2 s each using the disc applicator according to the manufacturer's instructions (Promotool), removed with forceps and stored at 4°C in glass scintillation vials. Sequential tape strippings were performed on the abdomens of the mice 24 h after the last application of IL-4 or HBSS. The amount of protein removed per D-SquameTM strip was measured as previously described (26). The protein content per stripping was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Lyophilized, bovine gamma globulin (Bio-Rad Laboratories) was used as the standard in all assays, because it correlated best with human SC. Each tape was incubated with 1 ml of 1 M NaOH for 1 h at 37°C in an incubator shaker and then was neutralized thereafter with 1 ml of 1 M HCl in the scintillation vials. Subsequently, 15 µl of this solution was incubated in 135 µl distilled water plus 150 µl of the Bio-Rad protein dye for 5 min. The absorption was measured at 595 nm. An empty D-SquameTM

tape, as well as distilled water incubated with the Bio-Rad dye, served as negative control. The amount of calculated protein was then normalized to the skin surface area (mg/cm^2).

Electron microscopy

Skin biopsies from the mice were fixed in Karnovsky's fixative overnight and postfixed with either 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously (27). Ultrathin sections were examined using an electron microscope (Zeiss 10A; Carl Zeiss, Thornwood, NY, USA), operated at 60 kV, or electron microscope (JEM-1200EX II; JEOL, Tokyo, Japan) operated at 80 kV.

Quantitative morphology with electron microscopy

The measurement of the number of layers of SC and the quantification of amount of CDs were performed using electron microscopic images that were taken by an investigator blinded to the source of the specimen (i.e. HBSS or IL-4). The length of CDs was measured at random from the first and second cell layers of the lower SC. The ratio of the total length of intact CDs to the total length of CE was determined by planimetry, as described previously (25).

Serine protease activity

Serine protease activity was assessed in freshly obtained skin samples by *in situ* zymography, as previously described (25). Five-micrometre frozen sections were incubated with BODIPY-F10-casain (Molecular Probes, Eugene, OR, USA) for 2 h at 37°C. The sections were washed 3× with 1% Tween 20 solution, counterstained with propidium iodide for 1 min. The sections then were examined with a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan).

Results

IL-4 reduces SC cohesion in normal mice, which correlates with a reduction in CDs

Quantitative electron microscopy revealed that the amount of CDs declined in IL-4-treated mice (Fig. 1), which correlated with the decreased number of layers of SC (Fig. 2a,b). Consistent with the observed decrease in the number of layers of SC, less corneocyte protein tended to be removed per first and second sequential tape stripping, and about 20% more corneocyte protein was removed per third and fourth sequential tape stripping from mice treated with IL-4 than from comparably stripped controls (Fig. 2c). Together, these results show that exogenous IL-4 alters SC cohesion, which correlates with a reduced number of layers of SC, likely due to a decrease in the amount of CDs.

IL-4 does not alter net serine protease activity or LEKTI expression

To assess the basis for the IL-4-induced decrease in CD, we first examined whether IL-4 alters the expression of key desquamators KLKs and/or LEKTI in cultured normal human KC. Levels of mRNAs of KLK5 and KLK14, which were both up-regulated by increased concentrations of calcium, instead were down-regulated when IL-4 was added to the medium in KC (Fig. 3a,c). Yet, levels of KLK7 mRNA appeared to modestly increase with an increase in the concentration of calcium ($P = 0.08$), and the increase was augmented by exogenous IL-4 (Fig. 3b). Levels of mRNAs of LEKTI were not influenced by either increased calcium or by IL-4 (Fig. 3d). There were no differences in the skin surface pH, an important regulator of the serine protease activity, in IL-4- versus vehicle-treated mice (Fig. S1). Finally, *in situ* zymography showed

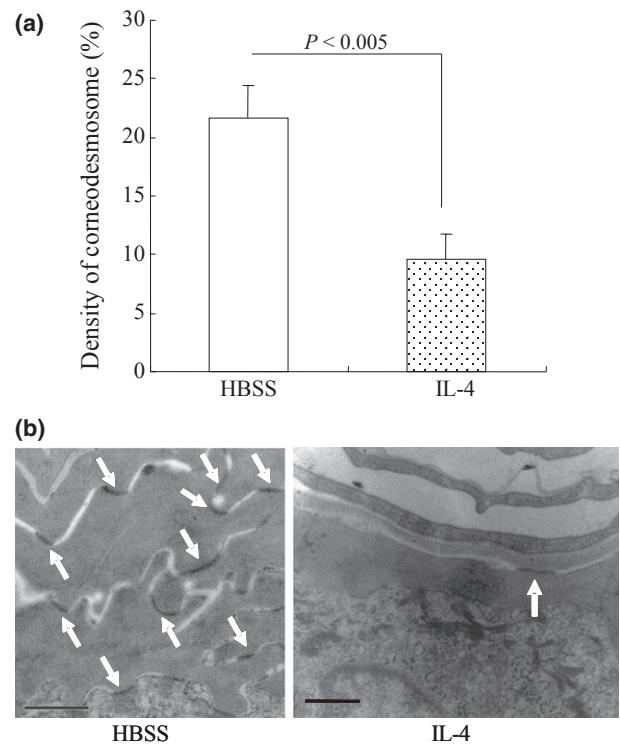


Figure 1. Intradermal administration of IL-4 reduced the amount of corneodesmosomes (CDs). Biopsies were performed after five consecutive daily intradermal injections of murine recombinant IL-4 or Hanks' balanced salt solution (HBSS). The amount of CDs (a) was measured as described in the Material and methods. Representative images for analysis of the stratum corneum (b) are shown. Arrows indicate CDs. (a) $n = 10$ (one mouse each; similar results were obtained in a second independent experiment). Bars in (b), 0.5 μm .

that IL-4 altered neither net serine protease activity nor the localization of serine proteases in the SC (Fig. 3e). Because net KLK activity did not change, no further assessment of the activities of individual KLK was carried out.

IL-4 down-regulates the expression of DSG 1 and other epidermal differentiation-linked proteins

We next examined whether IL-4 alters the expression of DSG1 and CDSN, which are the essential structural proteins of CDs. As expected (28), an increase in exogenous calcium stimulates expression of mRNA and protein of DSG 1 and of mRNA of TGase 1, two representative epidermal differentiation markers, in KC (Figs 4a,c and S2a). Yet, the expected calcium-induced increase in DSG1 and TGase 1 was inhibited by exogenous IL-4 (Figs 4a,c and S2a). In contrast, the expression of mRNA and protein of CDSN was not influenced either by an increase in calcium concentration or by treatment with exogenous IL-4 (Fig. 4b,d). Consistent with these *in vitro* results, Western blotting revealed reduced expression of DSG1 and LOR, but not CDSN, in the epidermis of IL-4-treated mice in comparison with the controls (Figs 4e,f and S2b).

Discussion

This study clearly demonstrates that IL-4 compromises SC cohesion, a key abnormality of likely pathogenic importance in AD (15). The abrogation of SC cohesion was accompanied by a reduction in the amount of CDs. Although the amount of CDs is known to be regulated by a balance of serine proteases (KLKs)

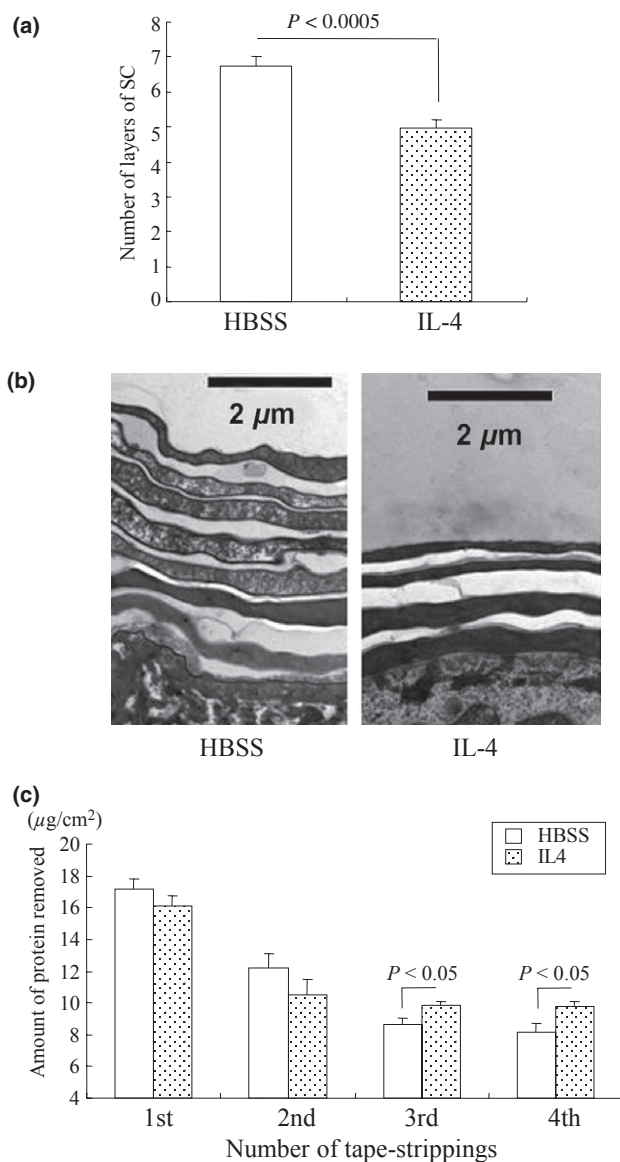


Figure 2. Intradermal administration of IL-4 reduced the cohesion of stratum corneum (SC). Biopsies and physiological assessments were performed after five consecutive daily intradermal injections of murine recombinant IL-4 or Hanks' balanced salt solution (HBSS). The numbers of layers of SC (a), representative images for analysis of SC (b) and the amount of protein in the removed SC after each tape stripping (c) are shown. (a) $n = 15$ (three mice) for HBSS and $n = 44$ (four mice) for IL-4, (c) $n = 4$ (four mice each).

and antiprotease activity in the SC (17,21), *in situ* zymographic studies showed that there was little or no difference in net serine protease activity in IL-4- versus vehicle-treated skin. The KLK5 and KLK14 mRNA levels were down-regulated by IL-4. However, mRNA levels of KLK7 were up-regulated by IL-4, as demonstrated recently (29). While these results clearly demonstrate that expression of KLKs is influenced by IL-4, the mechanism for these effects remains unexplored. In contrast, the expression of LEKTI mRNA was not affected either by an increase in the concentration of calcium in the medium or by exogenous IL-4. Thus, expression of LEKTI may not be influenced by the degree of epidermal differ-

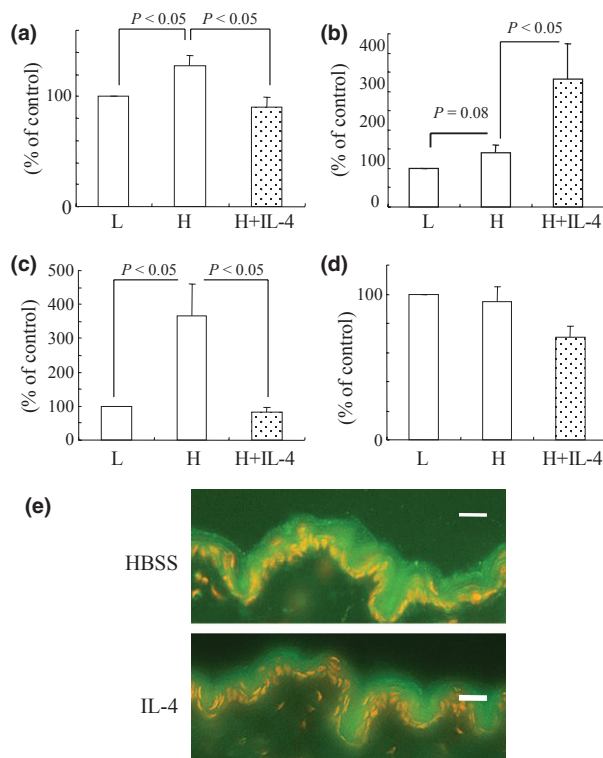


Figure 3. Effects of IL-4 on the expression of kallikreins (KLKs) and lympho-epithelial Kazal-type inhibitor (LEKTI) mRNA and on serine protease activity in murine stratum corneum. Effects of IL-4 on transcripts levels of KLKs (KLK5 (a), KLK7 (b), KLK14 (c) and LEKTI (d) in cultured normal human keratinocytes (KC) were examined by semiquantitative RT-PCR as described in the Material and methods. Normal human KC were cultured in medium with low (L) or high concentration (H), and incubation with IL-4 was performed in normal human KC exposed to a high concentration of calcium (H+IL-4); $n = 6$. Skin samples were collected after five consecutive daily intradermal injections of murine recombinant IL-4 or Hanks' balanced salt solution (HBSS) for assessment of serine protease activity by *in situ* zymography (e) as described in the Material and methods. Green stain represents serine protease activity, and red counterstain represents nuclear staining with propidium iodide. Mag bar = 20 μm .

entiation to the same extent as DSG1 or KLKs (30), although expression of LEKTI might be influenced under the condition that further promotes epidermal differentiation (31). These results on the effects of IL-4 on the expression of mRNAs for the KLKs and LEKTI could account for the lack of a net change in serine protease activity. Despite the fact that changes in the protein expression do not always correlate with alterations in enzyme activity, we did not pursue changes in the activities of individual KLK, as bulk enzyme activity was not altered. Moreover, there were no significant differences in skin surface pH, which could independently regulate serine protease activity (25). These results suggest that the abrogation of SC cohesion, induced by treatment with IL-4, is likely not ascribable to a dysregulation of KLK or anti-KLK activities.

The expression of DSG 1, a representative structural protein of CDs, in cultured human KC was up-regulated with increases in the concentration of calcium in the medium, as previously demonstrated (28), a phenomenon that was inhibited by exogenous IL-4 at both the mRNA and protein level. Interestingly, the expression of another attachment-related molecule, DSG 3, a representative structural component of desmosomes in the nucle-

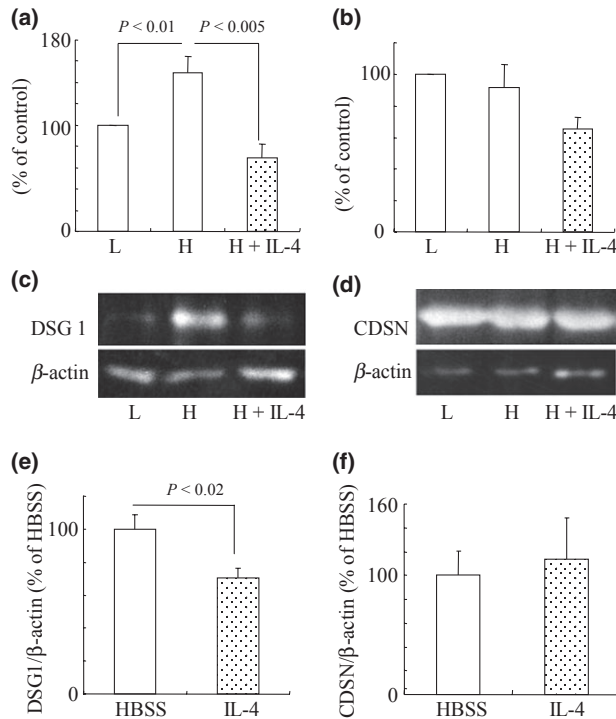


Figure 4. IL-4 down-regulated expression of desmoglein (DSG) 1 but not corneodesmosin (CDSN). Expression of DSG 1 (DSG1; a, c, e) and CDSN (b, d, f) in cultured normal human keratinocytes (KC) (a, b, c, d) and in murine epidermis (e, f), treated with IL-4 or vehicle (Hanks' balanced salt solution: HBSS), was examined by semiquantitative RT-PCR (a, b) and Western blotting (c, d, e, f) as described in the Material and methods. Normal human KC were cultured in the medium with either low (L) or high concentrations (H), and incubation with IL-4 was performed in normal human KC exposed to high concentration of calcium (H+IL-4). (a, b) $n = 6$; (e, f) $n = 10$ (10 mice each).

ated layers of the epidermis (but not CDs), is also suppressed by IL-4 (32). The inhibitory effect of IL-4 on the expression of DSG1 and DSG3 appears to reflect global inhibitory influences of IL-4 on epidermal differentiation. In fact, IL-4 suppresses the expression of several epidermal differentiation-related molecules (i.e. IVL, LOR and FLG) in KC (4,5). We found that the inhibitory effect of IL-4 on expression of DSG 1 in cultured human KC was paralleled by diminished expression of TGase 1. In addition, IL-4 also stimulates keratinocyte proliferation (33). The observation that the expression of CDSN, another structural protein of CDs, is not affected either by increases in the calcium concentration or by exogenous IL-4 suggests that an increase in the concentration of calcium alone may not suffice to up-regulate its expression. In fact, CDSN is exclusively expressed in highly differentiated KC (30). Additional treatments that further promote keratinocyte differentiation (31) might be required to up-regulate the expression of CDSN. The lack of a significant inhibitory effect by IL-4 on the expression of CDSN in this study suggests that more efficient and stronger epidermal proliferation-inducing stimulation than IL-4 might be required to affect the expression of CDSN. Consistent with the results from the *in vitro* experiments using cultured normal human KC, the expression of DSG1 and LOR, one of the

epidermal differentiation-related molecules, was reduced in the epidermis of IL-4-treated mice in comparison with controls in parallel with the reduction in the amount of CDs, although expression of CDSN was again not affected by treatment with IL-4. CDSN is a product of lamellar body secretion (20), and this process also may not be influenced by IL-4. Taken together, the present study demonstrates that IL-4 likely compromises the cohesion of the SC, likely via the inhibitory effects of IL-4 on epidermal differentiation, which, in turn, would account for reductions in amount of CD. Slowdown of the SC formation due to abnormal differentiation of epidermis also might be involved in the decreased number of layers of SC.

This study also suggests that normal epidermal differentiation might be essential to constitute normal CDs and SC cohesion, and, inversely, even a tiny abrogation of epidermal differentiation could be harmful to SC cohesion. Some abrogation of the CE due to abnormal epidermal differentiation might also result from IL-4 exposure, as CDs can be viewed as junctional structures that are part of the CE. In fact, one of CE's most important structural proteins, LOR (7,8), was also reduced by IL-4 treatment in this study. According to previous reports (4,5), other structural proteins of CE, such as IVL and FLG, might be also reduced by IL-4, although expression of them was not examined in this study. Thus, altered SC cohesion can be attributed to both decreased CDs and compromised CEs.

Both abnormal SC cohesion and thinning of the SC occur in patients with AD (18). While these changes have been attributed to an increased serine protease activity (15,18), the negative effects of Th2 cytokines on CDs could also contribute to the pathogenesis of barrier abnormalities in AD. Accordingly, reduced epidermal differentiation has been demonstrated in AD (34), which can now be attributed, at least in part, to the negative effect of Th2 cytokines on reduction of epidermal differentiation-related molecules, such as IVL, FLG and LOR (4,5). The present study supports again that reduction in Th2-related inflammation could be one of the therapeutic strategies to restore the permeability barrier homeostasis, leading to improve and/or prevent emergence of AD.

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Author contributions

Yutaka Hatano designed and performed the experiments, analysed the data and wrote the manuscript. Yasuko Adachi and Takashi Sakai contributed to the experiments using cultured normal human KC. Rieko Kurahashi and Kazumoto Katagiri contributed partially to the animal experiments. Peter Elias and Debra Crumrine contributed to the ultrastructural analysis. Peter Elias also gave excellent scientific suggestion and contributed to writing the manuscript. Sakuhei Fujiwara contributed to the design of the experiments.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

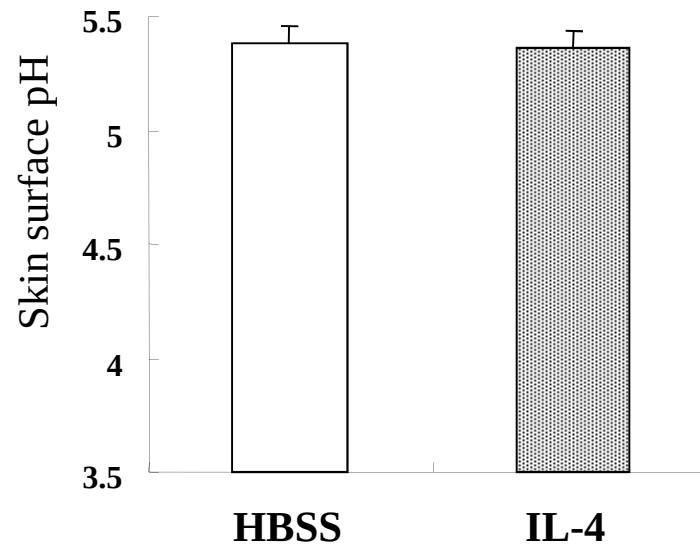
Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of IL-4 on pH of skin surface.

Figure S2. Effects of IL-4 on expression of transglutaminase-1 in cultured normal human Keratinocytes and of loricrin in the epidermis.

Table S1. Primers used for RT-PCR.

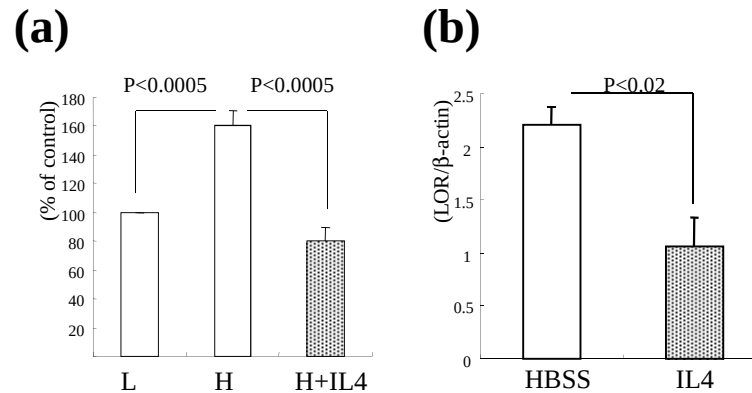
Supplementary Figure 1: Effect of IL-4 on pH of skin surface



Skin surface pH was measured using SKIN-pH-METER 905, (Courage & Khazaka, Cologne, Germany), which was performed after five consecutive daily intradermal injections of murine recombinant IL-4 or Hanks' balanced salt solution (HBSS) as described in the Material and Methods. n=24 (6 mice).

Supplementary Figure 2:

Effects of IL-4 on expression of transglutaminase-1 in cultured normal human Keratinocytes and of loricrin in the epidermis



The effects of IL-4 on transcripts levels of transglutaminase 1 (a) in cultured normal human keratinocytes and on protein expression of loricrin (LOR, b) in epidermis treated with IL-4 or vehicle (Hanks' balanced salt solution: HBSS) were examined by semi-quantitative RT-PCR and western blotting, respectively, as described in the Material and Methods. Normal human keratinocytes were cultured in the medium with low (L) or high concentration (H) and incubation with IL-4 was performed in normal human keratinocytes exposed to high concentration of calcium (H+IL-4). n=6 (a), n=5 (b).

Supplementary Table

Supplementary Table 1. Primers used for RT-PCR

Gene product	Primer	Sequence
Desmoglein 1	Upstream	5"-AATGGCTACATTTGCAGGACA-3"
	Downstream	5"-ATCTCGGTCAGAGCCTCTTACA-3"
CDSN	Upstream	5"-TCCCCCAATCACCTCTGTAG-3"
	Downstream	5"-CAACCTTGGGGTAGTGGAGA-3"
KLK5	Upstream	5"-TGATCACAGCCTTGCTTCTG-3"
	Downstream	5"-TCCTCGCACCTTTTCTGACT-3"
KLK7	Upstream	5"-ATGGCAAGATCCCTTCTCCT-3"
	Downstream	5"-GGCAGCCTGACTTTCTTCAC-3"
KLK14	Upstream	5"-ACGCACCCCAACTACAACCTC-3"
	Downstream	5"-CGTTTCCTCAATCCAGCTTC-3"
LEKTI	Upstream	5"-TGGATCAGAATCAGGGAAGG-3"
	Downstream	5"-TCACTGCACTCATCCTTTGC-3"
Transglutaminase 1	Upstream	5"-AATCCTCTGATCGCATCACC-3"
	Downstream	5"-AGCTAAGCAGGATCTCCACG-3"
G3PDH	Upstream	5"-CCCATCACCATCTTCCAG-3"
	Downstream	5"-CCTGCTTCACCACCACCTTCT-3"

CDSN: corneodesmosin; KLK: kallikreins; LEKTI: lympho-epithelial Kazal type inhibitor;
G3PDH: glyceraldehyde-3-phosphate dehydrogenase

