



Importance of Water Content of the Stratum Corneum in Mouse Models for Contact Hypersensitivity

Takaaki Doi¹, Yoshiko Mizukawa¹, Yurie Shimoda¹, Yoshimi Yamazaki¹ and Tetsuo Shiohara¹

Although a marked rise in the prevalence of allergic diseases over the past few decades may be related to environmental factors in industrialized countries, evidence for the protective effect of humidity on the barrier function of the skin is still awaited. We asked whether an increase in the water content of stratum corneum at the site of hapten application had a strong impact on the magnitude of contact hypersensitivity (CHS). The magnitude of CHS, induced by either lipid-soluble or water-soluble hapten, was inversely correlated with the water content of stratum corneum at the hapten application site in the elicitation phase. An increase in the water content induced by exposure to high humidity for 6 hours was sufficient to ameliorate the magnitude of CHS even in mice with the genetic defect in attenuating the CHS responses, such as flaky tail mice. The reduced CHS was associated with downregulation of IL-1 α , IL-4, and IFN- γ mRNA expression. Epicutaneously applied hapten can penetrate more readily through the stratum corneum with lower water content than that with higher water content, even after tape-stripping. These findings indicate that increased levels of water in the stratum corneum serve to ameliorate the CHS beyond the genetic effects.

Journal of Investigative Dermatology (2017) 137, 151–158; doi:10.1016/j.jid.2016.08.031

INTRODUCTION

Environmental factors, especially climate variables occurring in more industrialized countries, have been increasingly recognized as having a strong impact on the rise in allergic diseases such as atopic dermatitis (AD) (Flohr and Yeo, 2011; Liu and Murphy, 2003; Silverberg et al., 2013; Yazdanbakhsh et al., 2002). Indeed, a recent multivariate analysis has clearly shown that higher humidity protects against childhood eczema (Engelbrecht et al., 2016; Sasaki et al., 2014; Silverberg et al., 2013). However, there has been a paucity of research convincingly evaluating the effects of decreased humidity on the immunostimulatory activity of allergens in various types of animal models for contact hypersensitivity (CHS) and AD.

A reduced water content of the stratum corneum (SC) associated with increased transepidermal water loss (TEWL) is a feature of AD skin and other eczematous dermatitis (Bieber, 2008; Boguniewicz and Leung, 2011; Elias, 2005; Kubo et al., 2012). In this regard, identification of loss-of-function mutation in the gene encoding epidermal structural protein filaggrin has successfully linked abnormal skin

barrier induced by filaggrin mutations with the subsequent development of AD (Palmer et al., 2006; Sandilands et al., 2007). Thus, a dogma has been established that emphasizes the role of filaggrin and SC lipid, such as ceramides (Imokawa et al., 1991; Jungersted et al., 2010), as the defensive mechanisms of the skin, while ignoring the effect of humidity on the barrier function and the subsequent development of allergic inflammation: Denda et al. demonstrated that decreased barrier function was observed in mice transferred from a humid to a dry environment, thus providing an explanation for why exacerbation of AD frequently occurs in low humidity (Ashida et al., 2001, 2003; Denda et al., 1998a, 1998b; Katagiri et al., 2003; Sato et al., 2002). An excellent review summarizing recent advances in this field are available elsewhere (Elias and Wakefield, 2014). Nevertheless, definitive evidence for a previously unrecognized role of the water content of SC in preventing inflammatory responses is still awaited.

Epicutaneous application of haptens in mice is widely used for evaluating CHS. The difference in susceptibility among different strains of mice is not completely understood, but it may have a genetic component—an absence or reduction of the CHS has been generally interpreted as showing strain-dependent defects in the molecular mechanism of hapten recognition by immune cells (Itakura et al., 2013; Nagai et al., 2000). However, in view of the fact that sensitization and elicitation primarily occur through the epidermis and that the SC is the first point of contact, the magnitude of CHS is likely to be also influenced by strain-dependent differences in the water content of SC at the site of hapten application in the mouse model.

In this study, we demonstrate that there is a high degree of variability in the water content of SC among various strains of mice analyzed. In general, high water content of the SC is

¹Department of Dermatology, Kyorin University School of Medicine, Mitaka, Tokyo, Japan

Correspondence: Yoshiko Mizukawa, Department of Dermatology, Kyorin University School of Medicine, 6-20-2, Shinkawa Mitaka, Tokyo 181-8611, Japan. E-mail: ymizu@ks.kyorin-u.ac.jp

Abbreviations: AD, atopic dermatitis; B6, C57BL/6; CHS, contact hypersensitivity; ft/ft, flaky tail; NMU, neuromedin U; OX, 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one; RH, relative humidity; SC, stratum corneum; SSH, skin surface hydration; TEWL, transepidermal water loss; TNBS, 2,4,6-trinitrobenzene sulfonic acid hydrate; TNCB, 2,4,6-trinitro-1-chlorobenzen
Received 25 March 2016; revised 9 August 2016; accepted 16 August 2016; accepted manuscript published online 17 September 2016; corrected proof published online 4 November 2016

well associated with reduced CHS. Regardless of strains of mice used and of conditions in the sensitization phase, mice display a reduced CHS when elicited at the site with high water content of SC, whereas those when elicited at the site with low water content of SC show enhanced CHS. The protective effect of high humidity on AD-like CHS characterized by a barrier abnormality is also confirmed by using flaky tail (*ft/ft*) and tape-stripped mice. We demonstrate further that the enhanced CHS at the site with low SC hydration can be attributed to the increased penetration of the hapten through the SC. These findings clearly suggest that the magnitude of CHS can only be attenuated by increasing the water content of SC at the site of hapten application during the elicitation phase, even in mice with reduced inflammatory thresholds to haptens.

RESULTS

Differences in skin surface hydration (SSH) in genetically distinct strains under different humidity conditions

Prior studies have shown that mice maintained in a dry environment become more sensitive to physical or chemical stress (Ashida et al., 2001, 2003; Denda et al., 1998a, 1998b; Katagiri et al., 2003; Sato et al., 2002). This result could be interpreted as suggesting that genetically distinct strains of mice may display different levels of CHS depending in part on their own water-holding capacity of the SC. We initially measured SSH and TEWL in the ear skin of various genetically distinct strains of mice under different environmental humidity conditions: <20% (low), 40–60% (intermediate), and >80% (high) relative humidity (RH). The SSH status was found to greatly alter depending on the humidity conditions (Figure 1a). A dramatic change in the SSH status was observed in high humidity, regardless of strains used. *Neuromedin U* (*Nmu*^{−/−}), *TCR δ*^{−/−}, and *ft/ft* mice on a C57BL/6 (B6) background displayed the lowest SSH in any humidity environment, as did BALB/c mice. In contrast, there were no dramatic changes among these strains in TEWL levels depending on the humidity conditions (Figure 1b): the highest TEWL values were observed in *ft/ft* mice regardless of the humidity conditions, indicating a profound intrinsic defect of TEWL in *ft/ft* mice. These results suggest that the baseline SSH status reflects the combined effect of environmental humidity on the SC and their intrinsic water-holding capacity, and that the skin of *Nmu*^{−/−}, *TCR δ*^{−/−}, and *ft/ft* mice has an intrinsically lower capacity to hold water from the surrounding air, comparable to that of BALB/c mice, a strain susceptible to induction of allergic inflammation.

Differences in the magnitude of CHS under different humidity conditions

BALB/c mice develop CHS with a greater severity than do other strains such as B6. To investigate whether the baseline SSH status in these mice could have the impact on the magnitude of CHS to hapten, BALB/c, B6, *Nmu*^{−/−}, *IL-1R*^{−/−}, toll-like receptor 1^{−/−}, toll-like receptor 2^{−/−}, *TCR δ*^{−/−}, and *ft/ft* mice were sensitized and then elicited with different haptens, 2,4,6-trinitro-1-chlorobenzene (TNCB) or 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one (OX), in intermediate humidity (Figure 1c). The magnitude of CHS was higher in mice characterized by low SSH, such as *Nmu*^{−/−},

BALB/c, and *ft/ft* mice, than those characterized by high SSH, although an increased TEWL level in *ft/ft* mice was also associated with enhancement of the CHS. Total amino acid levels as natural moisturizer factor components were related neither to the SSH nor to the magnitude of CHS (Supplementary Figure S1 online). These results indicate that the magnitude of CHS is apparently inversely related to the baseline SSH status. This finding highlights the emerging view that, in addition to the inherent ability of T cells to respond to hapten, the SSH status at the site of hapten application may be one of the critical determinants of the magnitude of CHS.

Effects of high humidity during the sensitization or elicitation phase on the magnitude of CHS

To experimentally dissociate the effects of high humidity on the magnitude of ensuing CHS during the sensitization or elicitation phase, we initially sensitized BALB/c and B6 mice with TNCB and OX, respectively, under two different humidity conditions, during a critical 6-hour period (1 hour before hapten application to 6 hours after application) during the sensitization phase (Supplementary Figure S2 online). No significant reduction in ensuing CHS was observed when B6 or BALB/c mice were sensitized either in high humidity (Supplementary Figure S3a online) or with lower doses of the specific haptens (Supplementary Figure S3b). In contrast, strict dose dependency was only observed in the elicitation phase (Supplementary Figure S4 online).

We next investigated the effects of high humidity during the elicitation phase on the magnitude of CHS (Supplementary Figure S2). Whereas TNCB-sensitized BALB/c mice developed vigorous CHS in intermediate humidity, they mounted only mild CHS in high humidity, indistinguishable from those observed in TNCB-sensitized B6 mice in intermediate humidity (Figure 2a). Even in *ft/ft* mice, CHS to TNCB was significantly ameliorated in high humidity (Figure 2b). We then compared the ability of B6 and *Nmu*^{−/−} mice to mount CHS to OX under different humidity conditions (Figure 2c). Thus, TNCB-sensitized *ft/ft* mice and OX-sensitized *Nmu*^{−/−} mice, respectively, showed much lower CHS in high humidity. The SSH of the ear skin of *Nmu*^{−/−} or *ft/ft* mice in high humidity was higher than that of B6 mice in intermediate humidity. Nevertheless, mice transferred from an intermediate to a high humidity condition showed no apparent visible changes in appearance (Figure 2d). These results indicate that an increase in the SSH at the elicitation site alone suffices to ameliorate the magnitude of CHS even in otherwise susceptible strains, such as BALB/c, *Nmu*^{−/−}, and *ft/ft* mice, characterized by lower SSH and the resultant increased susceptibility to allergic inflammation.

Effects of high humidity on the CHS induced by water-soluble haptens

Because the effects of high humidity on the CHS could be limited to the CHS induced by the specific lipid-soluble haptens, we next asked whether the CHS induced by the water-soluble haptens could also be ameliorated in high humidity. To this end, BALB/c mice were sensitized with a water-soluble hapten, 2,4,6-trinitrobenzene sulfonic acid hydrate (TNBS), by applying to the tape-stripped skin.

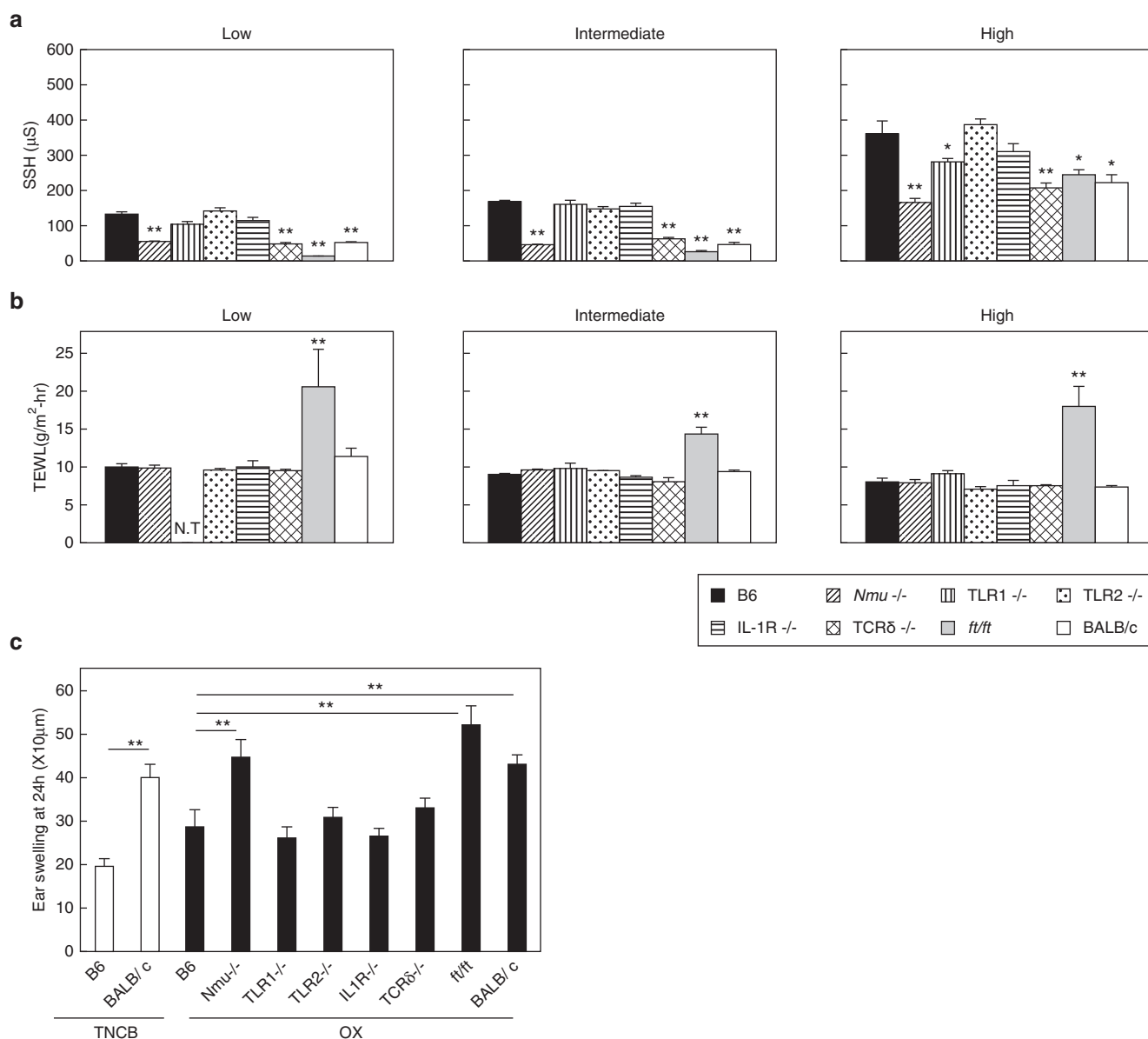


Figure 1. Mouse strain-dependent difference in SSH and TEWL levels of the ear skin in three different humidity conditions (low, intermediate, and high), and hapten-specific CHS in various genetically distinct strains of mice sensitized and elicited in intermediate humidity. (a) SSH. (b) TEWL. (c) Ear swelling. A group of mice were kept in low (<20% RH), intermediate (40–60% RH), or high (>80% RH) humidity (a, b). (c) TNCB- or OX-specific CHS in various strains. * $P < 0.05$, ** $P < 0.01$, versus B6. B6, C57BL/6; CHS, contact hypersensitivity; *ft/ft*, flaky tail; NMU, neuromedin U; N.T., not tested; OX, 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one; RH, relative humidity; SSH, skin surface hydration; TEWL, transepidermal water loss; TNCB, 2,4,6-trinitro-1-chlorobenzene.

BALB/c mice were sensitized with TNBS at a concentration of 1% when applied to the tape-stripped skin: sensitization to TNBS was not possible without the tape-stripping (Figure 3). The CHS to TNBS was also significantly ameliorated in high humidity, indicating that the beneficial effects of exposure to high humidity on the CHS are not limited to the specific lipid-soluble haptens but can be detected even in the CHS induced by water-soluble haptens. Maintaining the increased SSH status by exposure to high humidity could thus be a useful means of protecting against the elicitation of CHS in the previously sensitized mice.

The time needed to induce the increase in SSH sufficient for ameliorating CHS

To investigate whether a critical time of sustained exposure to high humidity exists, OX-sensitized B6 mice were housed in high humidity for 1, 3, 6, or 24 hours after elicitation. A profound reduction in CHS responses was only observed in high humidity for 6 hours after elicitation (Figure 4a): a small but significant reduction in CHS was also observed in high humidity for 1 to 3 hours after elicitation. The remarkable reduction in CHS was only observed in high humidity for initial 6 hours after elicitation (Figure 4b). Because no significant alteration in Langerhans

Figure 2. TNCB-specific or OX-specific CHS in BALB/c, B6, *ft/ft*, and *Nmu*^{-/-} mice elicited in high or intermediate humidity. (a) TNCB-specific CHS in BALB/c and B6 mice. (b) TNCB-specific CHS in *ft/ft* and B6 mice. (c) OX-specific CHS in *Nmu*^{-/-} and B6 mice. These mice similarly sensitized were elicited under each condition from 1 hour before elicitation to 6 hours after elicitation. In (b), mice were used predominantly at >26 weeks of age. (d) Appearance of earlobes in BALB/c mice in intermediate (left) or high (right) humidity for 6 hours. Data (a, b, c) are expressed as the mean ± SEM (n = 3–10). **P* < 0.05, ***P* < 0.01, B6, C57BL/6; CHS, contact hypersensitivity; *ft/ft*, flaky tail; NMU, neuromedin U; N.S., not significant; OX, 4-ethoxymethoxy-2-phenyl-2-oxazoline-5-one; SEM, standard error of the mean; SSH, skin surface hydration; TNCB, 2,4,6-trinitro-1-chlorobenzene.

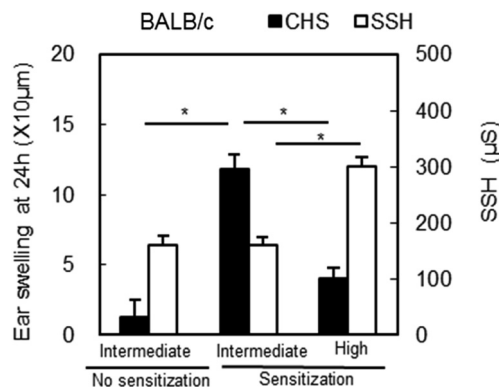
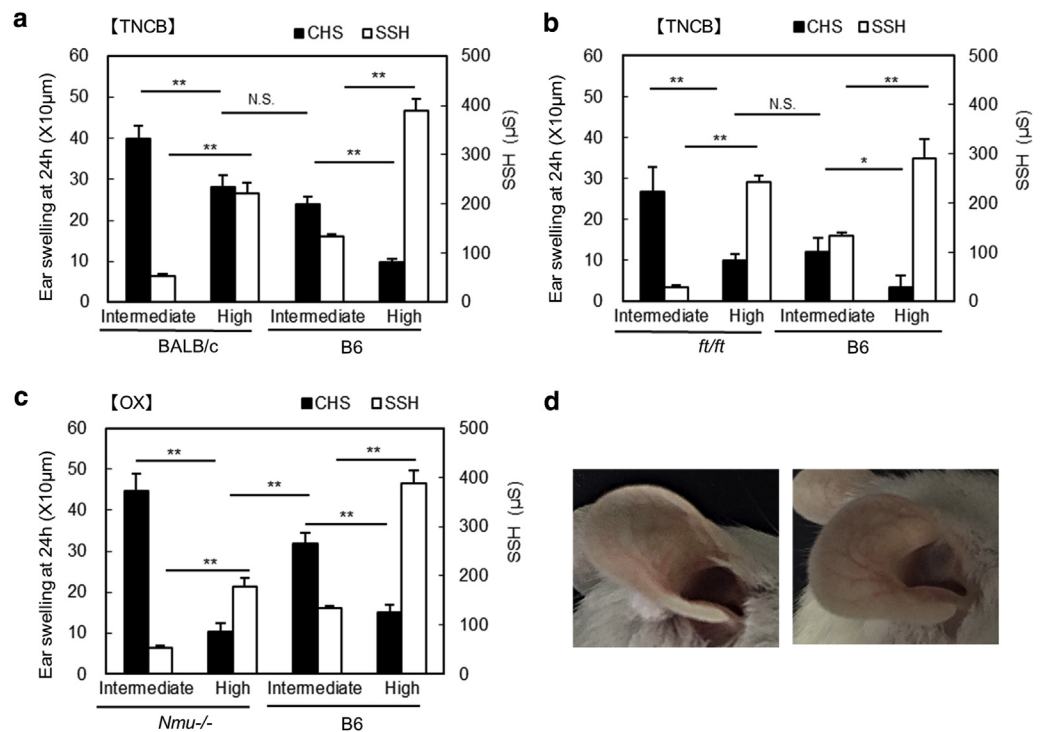


Figure 3. Water-soluble TNBS-specific CHS in BALB/c mice elicited in high or intermediate humidity. These mice were sensitized with 1% TNBS after tape-stripping on both sides of earlobes on day -7. No sensitized mice were left untreated except for tape-stripping. After tape-stripping, these mice thus sensitized were kept in each condition 1 hour before elicitation on day 0. Then, they were elicited with 1% TNBS under each condition during ensuing 6 hours. Data are expressed as the mean ± SEM (n = 3–4). **P* < 0.01, versus sensitization/intermediate. CHS, contact hypersensitivity; SEM, standard error of the mean; SSH, skin surface hydration; TNBS, 2,4,6-trinitrobenzene sulfonic acid hydrate.

cell numbers (Supplementary Table S1 online) occurred on such a short-term exposure to high humidity, these results suggest that the ameliorating effects of exposure to high humidity on CHS could be solely mediated by an increase in the SSH.

Histology and cytokine expression during the elicitation phase under different humidity conditions

Tissue injury and leukocyte infiltration were markedly worse in B6 mice elicited in intermediate humidity (Figure 5a) than

those elicited in high humidity (Figure 5b). The numbers of infiltrating leukocytes in the dermis of OX-elicited B6 mice in high humidity were significantly lower when compared with those in intermediate humidity (Figure 5c). These findings indicate that a decrease in tissue damage and leukocyte infiltration elicited in high humidity is indeed attributable to the decrease in the SSH at the site of hapten application.

We performed real-time PCR analyses of skin lesions under different humidity conditions. As compared with the cytokine expression profiles observed in the elicitation site of B6 mice in intermediate humidity, the CHS generated in those mice in high humidity resulted in significant downregulation of IL-1 α , IL-4, and IFN- γ mRNA expression (Figure 5d). These data indicate that the CHS in high humidity in B6 mice results in the decreased expression of T helper type 1 (IFN- γ) and T helper type 2 (IL-4) cytokines, and suggest that decreased expression of those cytokines in the skin lesions elicited in high humidity may reflect the general decrease in T helper type 1 and T helper type 2 effector cell recruitment to the skin sites.

Decreased absorption of epicutaneously applied hapten through the skin with high water content of SC

We next assessed whether epicutaneously applied hapten can penetrate more readily through the SC with lower water content than that with higher water content. As shown in Figure 6a, peak absorption of TNCB occurred 0.5 hours after application of TNCB to BALB/c skin in intermediate humidity, with the penetration being decreased by 3 hours. There was a >2-fold greater penetration of hapten through the skin with lower water content compared with that with higher water content in the BALB/c skin at 0.5 hours (Figure 6a).

Similar decreased absorption of epicutaneously applied water-soluble hapten, TNBS (Figure 6b), associated with

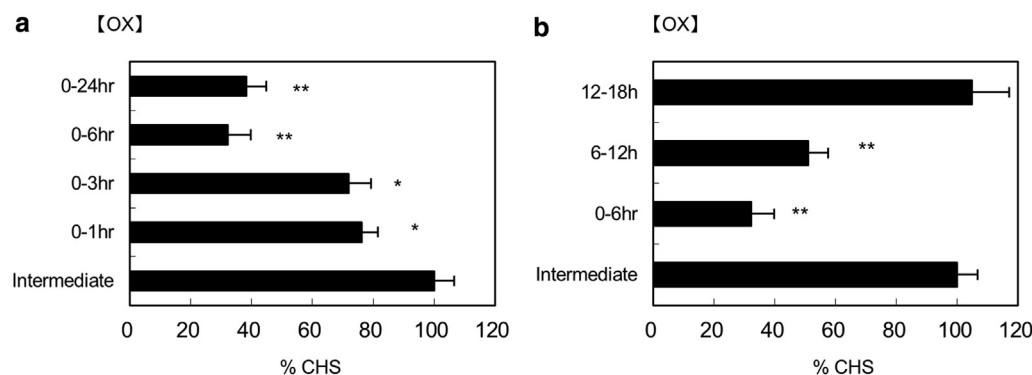


Figure 4. The time needed for inhibiting the ensuing CHS. B6 mice sensitized with OX were exposed to high humidity for the indicated period of time after elicitation ($n = 10$); they were also exposed 1 hour before elicitation. Percent CHS was calculated as follows: (CHS in high humidity during the indicated period/CHS in intermediate humidity) $\times 100$. * $P < 0.05$, ** $P < 0.01$. B6, C57BL/6; CHS, contact hypersensitivity; OX, 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one.

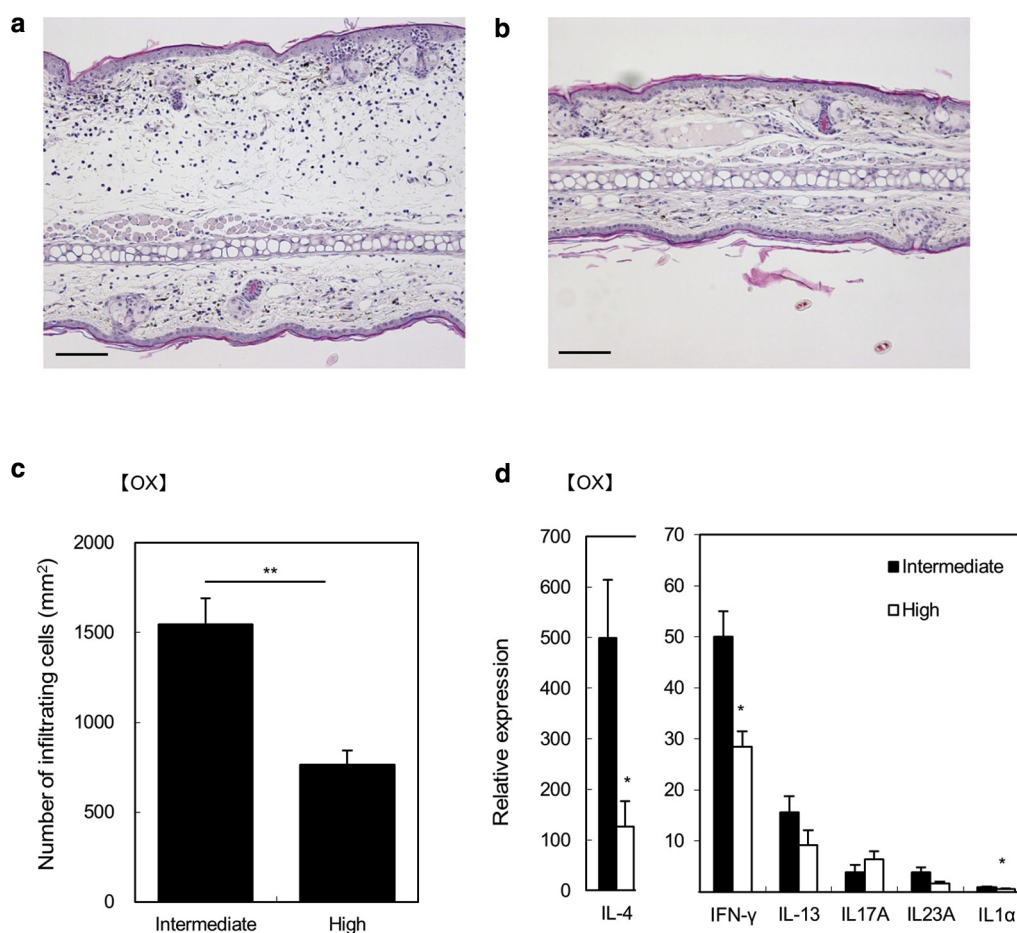


Figure 5. Histopathological findings and cytokine mRNA expression of the ears at 24 hours after elicitation with OX in intermediate or high humidity in B6 mice. (a) Mice sensitized with OX on day -7 were elicited on day 0 in intermediate humidity. (b) Mice sensitized with OX were exposed to high humidity from 1 hour before to 6 hours after elicitation. Original magnification $\times 160$, scale bar = 50 μm . (c) Numbers of histologically identifiable dermal infiltrating cells in intermediate or high humidity. (d) Mice sensitized with OX were elicited on day 0 in intermediate or high humidity. The level of mRNA expression for each cytokine is expressed as relative gene expression when the expression in the untreated B6 mice was normalized to 1.0. * $P < 0.05$, ** $P < 0.01$. B6, C57BL/6; OX, 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one.

decreased CHS to the hapten (Figure 3), was observed in high humidity. These results indicate that the skin with high water content of SC does not easily allow the penetration of epicutaneously applied hapten, either lipid soluble or water soluble.

DISCUSSION

The importance of the water content of SC for barrier function relevant to cutaneous inflammation has been suggested both

by the worsening of hapten-induced CHS when mice are exposed to low humidity (10% RH) for 2 days (Sato et al., 2002) and by the increased production of IL-1 and increased numbers of mast cells in such an environment (Ashida et al., 2001, 2003). These findings highlight the emerging view that the genetic susceptibility to haptens in CHS may reside not only in differential responses of T cells and antigen-presenting cells, but also within epidermal

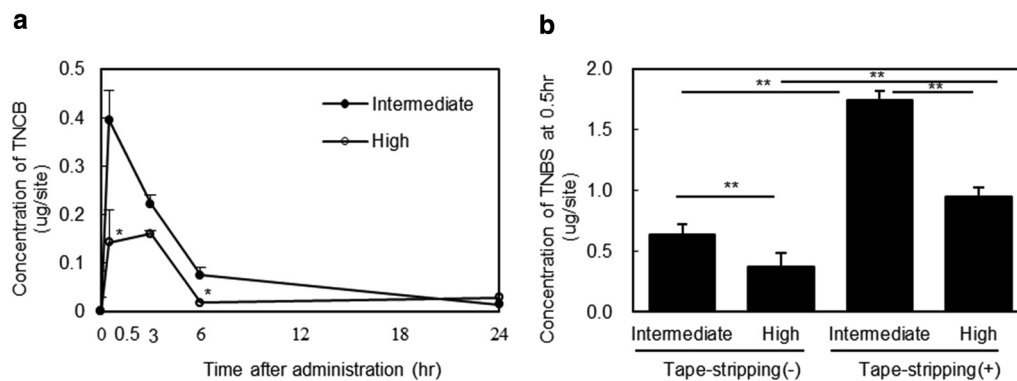


Figure 6. Concentrations of TNCB or TNBS in the skin after a single percutaneous application of TNCB or TNBS to the ear of BALB/c mice kept in intermediate or high humidity. (a) Concentration of TNCB 24h after a single application of TNCB. (b) Concentration of TNBS at 0.5hr after a single application of TNBS. Before TNBS application, the ears of mice were tape-stripped (b). A group of mice were kept in intermediate humidity, whereas another group of mice were kept in high humidity. Data are expressed as the mean $\pm$ SEM (n = 5). * $P < 0.05$, ** $P < 0.01$. SEM, standard error of the mean; TNBS, 2,4,6-trinitrobenzene sulfonic acid hydrate; TNCB, 2,4,6-trinitro-1-chlorobenzene.

barrier function as evidenced by the water content of SC. The failure of B6 mice to mount vigorous CHS to TNCB may reside in part in their relatively high water content of the SC. If so, one might expect that even BALB/c, *Nmu*^{-/-}, and *ft/ft* mice, which respond vigorously to the hapten in normal intermediate humidity, may display reduced CHS when elicited in high humidity. As expected, our results clearly show that the magnitude of CHS in these otherwise susceptible strains of mice was greatly downregulated through an increase in the water content of SC only by exposing them to high humidity only for 6 hours. This is supported by the inverse relationship between the magnitude of CHS and the baseline SSH in genetically distinct strains we analyzed. Surprisingly, such a short-term exposure (1 hour in Figure 4) to high humidity was sufficient to ameliorate the vigorous CHS. Consistent with this, our absorption experiments clearly indicate that the penetration of epicutaneously applied haptens is concentrated in the first 0.5 hours after hapten application (Figure 6b) and that the skin characterized by the reduced water content of SC is more conducive to epicutaneously applied hapten. In this regard, the “golden” period for ameliorating CHS is likely to be the first 0.5 hours after hapten application. Such a short-term exposure was unlikely to be sufficient to induce an increase in SC lipid and the other natural moisturizer factor.

Recent studies have demonstrated that abrupt changes from high to low humidity cause deterioration in epidermal barrier function, as evidenced by a marked increase in TEWL and an increase in Langerhans cell number and epidermal IL-1 levels (Ashida et al., 2001; Hosoi et al., 2000). The findings may make it likely that the reduced CHS observed in mice maintained in high humidity for 1–6 hours was due to the effect of an abrupt switch from high to normal humidity. We would argue, however, against this possibility for the following reasons: first, we did not observe any increase in IL-1 levels (data not shown) and Langerhans cell numbers 1–6 hours after a switch from high to normal humidity (Supplementary Table S1), and the treatment period of 1–6 hours was too short for these effects to occur in the environmental conditions used in this study. Second, the same magnitude of the decrease in CHS was also observed in mice

maintained in high humidity during the ensuing 24-hour period without a switch to intermediate humidity; there was no significant difference in the CHS between a group of mice maintained in high humidity for 0–6 hours and those for 0–24 hours (Figure 4a).

Consistent with the protective effect of high humidity, recent studies have shown that a decrease in RH in urban areas has accelerated globally over the past 20 years (Ziska et al., 2003). In view of the effect of low humidity on skin barrier function and the subsequent CHS, the accelerated decrease in RH, particularly in an urban setting, is more serious than global warming. Repeated cutaneous exposure to corticosteroids (Sheu and Chang, 1991), soap, and other chemicals including many haptens (Kitagaki et al., 1995, 1997, 1999, 2014), together with decreased humidity, would serve to decrease the water content of SC, thereby predisposing otherwise resistant individuals to develop allergic diseases. Our findings suggest that humidification of indoor air, particularly in places where exposure to allergens at high risk, may help decrease the likelihood of developing allergic diseases.

An increase in the water content of SC at the site of hapten application, either lipid-soluble or water-soluble hapten, had a strong impact on the magnitude of CHS only during the elicitation phase but not the sensitization phase. Consistent with this, strict dose-response dependencies on the magnitude of CHS responses were only found in the elicitation phase. For instance, doses two times lower (0.5%) than the optimal dose developed reduced CHS even in sensitized mice (Supplementary Figure S4), while such strict dose-response dependencies were not observed in the sensitization phase (Supplementary Figure S3). Thus, the concentration of the hapten penetrated into the skin during the elicitation phase is the most important limiting factor responsible for the determination of the magnitude of hapten-induced CHS. These results indicate that increasing SC hydration at the site of hapten application only during the elicitation phase would serve to ameliorate CHS by reducing the penetration of the hapten into the skin. In this regard, long-term use of moisturizers is a key component of the daily management of chronic inflammatory conditions such as AD,

because they can restore disrupted barrier function by increasing SC hydration (Caussin et al., 2009; Horimukai et al., 2014; Levi et al., 2010). In view of our finding that not only an acute form of CHS (Figure 2) but also CHS in AD-like barrier-disrupted mice with impaired water-holding capacity, such as *fl/fl* and tape-stripped mice (Figures 2b and 3), can significantly be improved only by exposing them to high humidity, increasing SC hydration at the site of allergen entry would be the most efficient method of therapeutic intervention in the treatment of allergic cutaneous inflammation. This is an invaluable clue—it would be better than any drug we have in that there is no adverse effect.

At present, we cannot determine from our data the mechanism by which the skin with higher water content is much less permeable to either lipid-soluble or water-soluble haptens. One possible explanation could be that the penetration of lipid-soluble haptens would occur less efficiently via extracellular route of SC with higher water content, than water-soluble haptens. However, this explanation does not appear to provide the mechanism for why the SC with higher water content is also less permeable to water-soluble haptens. Another explanation is that ambient humidity may be “sensed” by free nerve endings or keratinocytes themselves, thereby regulating the absorption of haptens. The ability to “sense” ambient humidity is an important task for mammals to maintain homeostasis and ensure survival (Filingeri, 2015) under various humidity conditions, although humidity sensors (i.e., hygroreceptor) have not been demonstrated in animal skin. In view of our finding that water-soluble haptens can also penetrate much less efficiently the tape-stripped skin under high-humidity conditions (Figure 6b), “humidity sensors” in the epidermis would remain operative even in barrier-disrupted skin.

Our data support the hypothesis that one potential factor that could influence the development of CHS is the water-holding capacity of the SC at the hapten application site. Nevertheless, the genetic defect in the water-holding capacity of the SC could only be corrected by exposing such deficient mice to high humidity for 1–6 hours during the elicitation phase. By just supplying the SC having genetic defects with water, even allergic inflammation in patients with AD genetically predisposed to a defective skin barrier could be prevented. We therefore should establish the reality of biological effects in the physical presence of water in the SC.

MATERIALS AND METHODS

Detailed information on experimental methods is provided in the [Supplementary Materials and Methods](#) online. All procedures used in this study were reviewed and approved by the Animal Research Committee of Kyorin University.

Mice

Male B6 and BALB/c mice were obtained from Charles River Laboratory Japan (Yokohama, Japan). *Nmu*^{−/−} mice were established as described previously (Hanada et al., 2004; Moriyama et al., 2005), and were backcrossed six generations onto the B6 background. The details of other genetically distinct strains and their maintenance are described in [Supplementary Materials and Methods](#).

Sensitization and elicitation procedure

Mice were sensitized by an epicutaneous application of 20 μ l of 1% TNCB, OX, and TNBS (prior tape-stripping needed for TNBS sensitization) solution to the left ear on day −7 and then the right ears of the sensitized mice were elicited by 20 μ l of each hapten on day 0, as described previously (Kitagaki et al., 1995, 1997, 1999, 2014). Mice were maintained at different humidity conditions, <20%, 40–60%, >80% RH, respectively, and the details are described in [Supplementary Materials and Methods](#).

Measurement of concentrations of TNCB or TNBS in the skin after its epicutaneous application

A validated analytic assay consisting of HPLC was used to determine concentrations of TNCB or TNBS in the skin samples.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Drs. Y. Hatano and T. Sakai (Department of Dermatology, Oita University) for the gift of the *fl/fl* mice. And we are grateful to Professor M. Kojima (Molecular Genetics, Institute of Life Science, Kurume University) for the gift of the *Nmu*^{−/−} mice. We wish to thank Professor M. Ohyama (Department of Dermatology, Kyorin University School of Medicine) for his support. This work was supported by grants from the Ministry of Education, Sports, Science, and Culture of Japan (TS and YM) and from the Ministry of Health, Labor, and Welfare of Japan (TS).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.08.031>.

REFERENCES

- Ashida Y, Denda M. Dry environment increases mast cell number and histamine content in dermis in hairless mice. *Br J Dermatol* 2003;149:240–7.
- Ashida Y, Ogo M, Denda M. Epidermal interleukin-1 α generation is amplified at low humidity: implications for the pathogenesis of inflammatory dermatoses. *Br J Dermatol* 2001;144:238–43.
- Bieber T. Atopic dermatitis. *N Engl J Med* 2008;358:1483–94.
- Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 2011;242:233–46.
- Caussin J, Rozema E, Gooris GS, Wiechers JW, Pavel S, Bouwstra JA. Hydrophilic and lipophilic moisturizers have similar penetration profiles but different effects on SC water distribution in vivo. *Exp Dermatol* 2009;18:954–61.
- Denda M, Sato J, Masuda Y, Tsuchiya T, Koyama J, Kuramoto M, et al. Exposure to a dry environment enhances epidermal permeability barrier function. *J Invest Dermatol* 1998a;111:858–63.
- Denda M, Sato J, Tsuchiya T, Elias PM, Feingold KR. Low humidity stimulates epidermal DNA synthesis and amplifies the hyperproliferative response to barrier disruption: implication for seasonal exacerbations of inflammatory dermatoses. *J Invest Dermatol* 1998b;111:873–8.
- Elias PM. Stratum corneum defensive functions: an integrated view. *J Invest Dermatol* 2005;125:183–200.
- Elias PM, Wakefield JS. Mechanisms of abnormal lamellar body secretion and the dysfunctional skin barrier in patients with atopic dermatitis. *J Allergy Clin Immunol* 2014;134:781–91.
- Engelbrecht KA, Johansen JD, Kezic S, Linneberg A, Thyssen JP. The effect of environmental humidity and temperature on skin barrier function and dermatitis. *J Eur Acad Dermatol Venereol* 2016;30:223–49.
- Filingeri D. Humidity sensation, cockroaches, worms and humans: are common sensory mechanisms for hygrosensation shared across species? *J Neurophysiol* 2015;114:763–7.
- Flohr C, Yeo L. Atopic dermatitis and the hygiene hypothesis. *Curr Probl Dermatol* 2011;41:1–34.
- Hanada R, Teranishi H, Pearson JT, Kurokawa M, Hosoda H, Fukushima N, et al. Neuromedin U has a novel anorexigenic effect independent of the leptin signaling pathway. *Nat Med* 2004;10:1067–73.

- Horimukai K, Morita K, Naria M, Kondo M, Kitazawa H, Nozaki M, et al. Application of moisturizer to neonates prevents development of atopic dermatitis. *J Allergy Clin Immunol* 2014;134:824–30.
- Hosoi J, Hariya T, Denda M, Tsuchiya T. Regulation of the cutaneous allergic reaction by humidity. *Contact Dermatitis* 2000;42:81–4.
- Imokawa G, Abe A, Jin K, Higaki Y, Kawashima M, Hidano A. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol* 1991;96:523–6.
- Itakura A, Ikutani M, Takatsu K, Kikuchi Y. Interleukin-5 plays a key role in mouse strain-dependent susceptibility to contact hypersensitivity through its effects on initiator B cells. *Int Arch Allergy Immunol* 2013;161:98–106.
- Jungersted JM, Scheer H, Mempel M, Baurecht H, Chfuentes L, Høgh JK, et al. Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 2010;65:911–8.
- Katagiri C, Sato J, Nomura J, Denda M. Changes in environmental humidity affect the water-holding property of the stratum corneum and its free amino acid content, and the expression of filaggrin in the epidermis of hairless mice. *J Dermatol Sci* 2003;31:29–35.
- Kitagaki H, Fujisawa S, Watanabe K, Hayakawa K, Shiohara T. Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. *J Invest Dermatol* 1995;105:749–55.
- Kitagaki H, Ono N, Hayakawa K, Kitazawa T, Watanabe K, Shiohara T. Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. *J Immunol* 1997;159:2484–91.
- Kitagaki H, Kimishima M, Teraki Y, Hayakawa J, Hayakawa K, Fujisawa S, et al. Distinct in vivo and in vitro cytokine profiles of draining lymph node cells in acute and chronic phases of contact hypersensitivity: importance of a type 2 cytokine-rich cutaneous milieu for the development of an early-type response in the chronic phase. *J Immunol* 1999;163:1265–73.
- Kitagaki H, Hiyama H, Kitazawa T, Shiohara T. Psychological stress with long-standing allergic dermatitis causes psychodermatological conditions in mice. *J Invest Dermatol* 2014;134:1561–9.
- Kubo A, Nagao K, Amagai M. Epidermal barrier dysfunction and cutaneous sensitization in atopic diseases. *J Clin Invest* 2012;122:440–7.
- Levi K, Kwan A, Rhines AS, Gorcea M, Moore DJ, Dauskardt RH. Emollient molecule effects on the drying stresses in human stratum corneum. *Br J Dermatol* 2010;163:695–703.
- Liu AH, Murphy JR. Hygiene hypothesis: fact or fiction? *J Allergy Clin Immunol* 2003;111:471–8.
- Moriyama M, Sato T, Inoue H, Fukuyama S, Teranishi H, Kangawa K, et al. The neuropeptide neuromedin U promotes inflammation by direct activation of mast cells. *J Exp Med* 2005;202:217–24.
- Nagai H, Ueda Y, Ochi T, Hirano Y, Tanaka H, Inagaki N, et al. Different role of IL-4 in the onset of hapten-induced contact hypersensitivity in BALB/c and C57BL/6 mice. *Br J Pharmacol* 2000;129:299–306.
- Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;38:441–6.
- Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 2007;39:650–4.
- Sasaki T, Furusyo N, Shiohama A, Takeuchi S, Nakahara T, Uchi H, et al. Filaggrin loss-of-function mutations are not a predisposing factor for atopic dermatitis in an Ishigaki Island under subtropical climate. *J Dermatol Sci* 2014;76:10–5.
- Sato J, Denda M, Chang S, Elias PM, Feingold KR. Abrupt decreases in environmental humidity induce abnormalities in permeability barrier homeostasis. *J Invest Dermatol* 2002;119:900–4.
- Sheu HM, Chang CH. Alterations in water content of the stratum corneum following long-term topical corticosteroids. *J Formos Med Assoc* 1991;90:664–9.
- Silverberg JL, Hanifin J, Simpson EL. Climatic factors are associated with childhood eczema prevalence in the United States. *J Invest Dermatol* 2013;133:1752–9.
- Yazdanbakhsh M, Kreamsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* 2002;296:490–4.
- Ziska LH, Gebhard DE, Frenz DA, Faulkner S, Singer BD, Straka JG. Cities as harbingers of climate change: common ragweed, urbanization, and public health. *J Allergy Clin Immunol* 2003;111:290–5.

Supplemental information

Importance of Water Content of the Stratum Corneum in Mouse Models for Contact Hypersensitivity

Takaaki Doi¹, Yoshiko Mizukawa, Yurie Komatsu, Yoshimi Yamazaki and Tetsuo Shiohara

¹ Department of Dermatology, Kyorin University School of Medicine, Mitaka, Tokyo, Japan.

Figure S1. Total amino acids in various strains of mice.

SC samples were obtained by tape-stripping with adhesive tape from five mice per strain. *p<.01.

Figure S2. Experimental protocol for investigating the effects of high humidity during the sensitization phase or the elicitation phase of the CHS.

Figure S3. TNCB- or OX- specific CHS in BALB/c or B6 mice sensitized in high or intermediate humidity condition and at different concentrations of TNCB or OX, respectively, in the sensitization phase. (a) BALB/c and B6 mice were sensitized by a single epicutaneous application of 1% TNCB (left) or 1 % OX (right) in intermediate or high humidity on day-7. (b) BALB/c and B6 mice were sensitized with 0.5 % or 1 % TNCB or OX in intermediate humidity on day -7. They were elicited with the same hapten in intermediate humidity on day 0 (a and b). Data (a and b) are expressed as the mean $\pm$ SEM (n=5). N.S. Not significant.

Figure S4. Differences in the magnitude of TNCB- or OX-specific CHS depending on the concentrations of the hapten in the elicitation phase in B6 mice. Mice sensitized with 1% TNCB or OX on day -7 were elicited by 0.25, 0.5, or 1% TNCB or OX in intermediate humidity on day 0. * $p < .05$.

Supplemental materials and methods

Mice

TLR1^{-/-} and TLR2^{-/-} mice were obtained from Oriental Bio Service (Kyoto, Japan). IL-1R^{-/-} and *ft/ft* mice were obtained from Jackson Laboratory. *Nmu*^{-/-}, TLR1^{-/-}, TLR2^{-/-}, IL-1R^{-/-}, TCR δ ^{-/-}, and *ft/ft* mice are all C57BL/6 (B6) background. At > twenty weeks of age, *ft/ft* mice developed scaly eczematous skin lesions around the neck and periauricular areas, as previously described (Oyoshi *et al.*, 2009; Scharcshmidt *et al.*, 2009).

The mice were allowed to acclimate in the animal facility for a minimum of five days before the experiments and were used predominantly at seven to twenty weeks of age: in some experiments, they were used at > twenty-six weeks of age (Figure 2b). All mice were maintained on a standard diet and water *ad libitum* at the Kyorin University animal facility under a controlled room temperature (23–25 °C) and humidity (40–60%).

Reagents

2, 4, 6-trinitro-1-chrolobenzen (TNCB) and 2,4,6-trinitrobenzene sulfonic acid hydrate (TNBS) were obtained from Tokyo Kasei Co. (Tokyo, Japan); 4-ethoxymetholero-2-phenyl-2-oxazoline-5-one (OX) was obtained from Sigma-Aldrich

Co. TNCB and OX were dissolved in acetone to 1% solutions, and water-soluble hapten, TNBS, was dissolved in ethanol to 1% solutions. These haptens were used for sensitization and elicitation, as described previously (Kitagaki *et al.*, 1987; 1995; 1999; 2014). PE-conjugated rat anti-mouse I-A/I-E mAb (clone M5/114.15.2; eBioscience) was used for immunohistochemistry of epidermal sheets. Methyl 4-Hydroxybenzoate was used as the internal standard for analytic assay in skin concentrations of TNCB.

Sensitization and elicitation procedure

For barrier disruption, both sides of the earlobes were tape-stripped seven times with adhesive cellophane tape (Nichiban, Tokyo, Japan), as described previously (Nishijima *et al.*, 1997). Ear thickness was measured with a dial thickness gauge (Mitsutoyo Corp., Tokyo, Japan) at various time points. Hapten-specific CHS reaction was assessed by measuring ear swelling at 24 h.

Unless otherwise stated, mice were housed in controlled room temperature (23-25°C) and RH (40-60%) under a 12:12-h light-dark cycle. In some experiments, the effect of changes in skin surface hydration (SSH) on the CHS was examined by transferring mice from intermediate humidity to high humidity-controlled (>80%RH) room during the indicated period.

Measurement of SSH, TEWL and total amino acid levels

The SSH status in the ear skin was measured electrically according to the method described by Tagami *et al.* (Tagami *et al.*, 1980) with a skin surface hygrometer (Skicon-200, IBS, Inc., Hamamatsu, Japan). TEWL was measured with the VapoMeter (Delfin Technologies, Kuopio, Finland). The probe consisted of a

cylindrical chamber 4.5 mm in diameter. Measurements were recorded when TEWL readings stabilized, usually about 20 seconds after the probe had been placed on the ear skin. The protocols of measurement of amino acids included minor modifications of published methods (Katagiri *et al.*, 2003; Sakai *et al.*, 2003). Samples of SC were collected by tape-stripping with adhesive tape, performed six times of a 2.5x3.8cm area of mouse dorsal skin. Free amino acids were analyzed on an L-8800 amino acid analyzer (Hitachi, Tokyo, Japan).

Measurement of concentrations of TNCB or TNBS in the skin after its epicutaneous application

After hapten application to the ear (20 μ l of 1% TNCB), the mice were divided into two groups: one group was kept in intermediate humidity (40-60%) until skin biopsy. The other was kept in high humidity (>80%) until biopsy. Samples were obtained at 0.5, 3, 6 h and 24 h after application, and residual TNCB on the skin surface was removed at least three times with 80% ethanol solution using a cotton wool pad. The biopsy specimens, 5 mm in diameter, were obtained from the same area of the ear of the mouse. To homogenize the tissue, 2 ml of acetonitrile solution containing 1% hydrochloric solution and 100 μ l of internal standards, methyl 4-Hydroxybenzoate, was added and cut into thin sections, which were transferred into a test tube. Homogenized tissues were mixed well with a vortex mixer and centrifuged at 3,000 xg for 10 minutes. After the supernatant was obtained and evaporated to dryness in a Turbo Vap at 40°C, the final residue was reconstituted with 150 μ l of methanol and 100 μ l of 0.1% phosphoric acid solution. This solution was injected onto the HPLC system. Chromatograms were developed with a 254 nm UV detector and a Symmetry Shield

RP18 Column (4.6x150 mm; Waters), a mobile phase of 60/40 (v/v) methanol/0.1% phosphoric acid solution, and a flow rate of 1 ml/min.

To measure the concentration of TNBS, 1h before hapten application (20 μ l of 1% TNBS) to the ear, tape-stripping with adhesive tape was performed six times. For 6h after TNBS application, the mice were kept in intermediate or high humidity. Samples obtained at 0.5h after TNBS application were used for our comparative analysis, because our previous studies demonstrated that peak absorption occurred at 0.5 h.

Histopathology

Skin samples were obtained at 24 h after each elicitation, at which time mice were killed under diethyl ether anesthesia, and the ears were then amputated. A central strip of the ear were fixed with formalin and embedded in paraffin. Five- μ m-thick sections were cut and stained with hematoxylin and eosin. The number of dermal infiltrating cells was enumerated by counting each cell type in the sections of the ears using Olympus microscope with a x20 objective and a x10 eyepiece in a blind fashion; 10 random fields were counted per section.

Epidermal sheets were prepared, as described previously (Nishijima *et al.*, 1997; Nagao *et al.*, 2012). B6 mice were housed in intermediate or high humidity conditions. Skin samples were removed from ears before and 6h after a shift from high to intermediate humidity, respectively. Epidermal sheets were separated from dermis with 0.5M ammonium thiocyanate. Then, the sheets were fixed in acetone, and stained with anti-mouse I-A/I-E. The number of LC was enumerated in three random visual fields at high magnification in three different sections and were expressed as cells/mm².

Isolation of total RNA and real-time RT-PCR

Skin samples were removed from ears 24 h after elicitation. Total RNA was extracted using the RNeasy fibrous tissue mini-kit (Qiagen, Ltd., Crawley, UK). Total RNA was reverse transcribed to cDNA as per directions using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA). Real-time quantitative reverse transcription-polymerase chain reaction (PCR) was performed using the TaqMan system (Applied Biosystems) on the ABI Prism 7700 Sequence Detector (Applied Biosystems). The TaqMan Gene Expression Assay kit [IL-1 α , IL-4, IL-17A, IL-23A, IFN- γ], and TaqMan Rodent glyceraldehydes-3-phosphate dehydrogenase (GAPDH) control reagents and probe set were obtained from applied Biosystems (Foster City, CA). Relative expression of real-time PCR products was determined using the $\Delta\Delta C_t$ technique (Livak and Schmittgen., 2001). Each set of samples was normalized by briefly using the difference in threshold cycle (C_t) between the target gene and housekeeping gene (GAPDH): $\Delta C_t = C_{t\text{target gene}} - C_{t\text{GAPDH}}$. Relative mRNA levels were calculated by the expression $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{t\text{sample}} - \Delta C_{t\text{calibrator}}$.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM. Differences between groups were analyzed using Mann-Whitney U-test or Student's t test or Dunnett's test for comparison between each group. Statistical significance was indicated by p values <0.05 .

Supplemental reference

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time

quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method. *Methods*. 2001; 25 : 402-8.

Nishijima T, Tokura Y, Imokawa G, Seo N, Furukawa F, Takigawa M. Altered permeability and disordered cutaneous immunoregulatory function in mice with acute barrier disruption. *J Invest Dermatol* 1997; 109: 175-82.

Oyoshi MK, Murphy GF, Geha RS. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immuno* 2009; 124: 485-93.

Sakai S, Endo Y, Ozawa N, Sugawara T, Kusaka A, Sayo T, et al. Characteristics of the epidermis and stratum corneum of hairless mice with experimentally induced diabetes mellitus. *J Invest Dermatol* 2003; 120: 79-85.

Scharschmidt TC, Man MQ, Hatano Y, Crumrine D, Gunathilake R, Sundberg JP, et al. Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. *J Allergy Clin Immunol* 2009; 124: 495-506.

Tagami H, Ohi M, Iwatsuki K, Kanamaru Y, Yamada M, Ichijo B. Evaluation of the skin surface hydration in vivo by electrical measurement. *J Invest Dermatol* 1980; 75: 500-7.

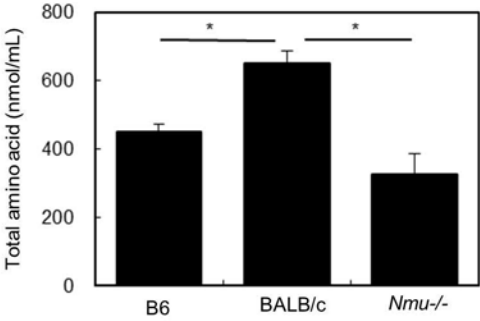
Table S1 Number of IA⁺ LC before and 6h after a switch from high to intermediate humidity

Treatment	Before ^a	After ^a
IA ⁺ cells (/cm ²) ^{b,c}	66.8 ± 4.7	66.2 ± 1.7

a Skin samples were removed from ears of B6 mice before and 6h after a switch from high to intermediate humidity.

b The number of IA⁺ LC was enumerated in three random visual fields at high magnification in three different sections and were expressed as mean positive cells/mm². ± SEM (n=3).

c There is no statistical significant difference between them.



Study design

