Possible Involvement of Type 2 Cytokines in Alloknesis in Mouse Models of Menopause and Dry Skin

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ABSTRACT

Alloknesis, an abnormal itch sensation induced by innocuous stimuli, is a key phenomenon in the vicious itch-scratch cycle in patients with atopic dermatitis. Dry skin and pruritus, including alloknesis, are major health problems in peri- and post-menopausal women. We recently reported permeability barrier dysfunction in ovariectomised (OVX) mice—a model of menopause—and found that the dysfunction was related to dry skin. However, the mechanism of the itch remains unknown. Therefore, we examined touch- and pruritogen-evoked alloknesis and epidermal innervation in OVX mice and acetone, diethyl ether, and water (AEW)-treated mice, for the experimental dry skin model. Both alloknesis and epidermal innervation were comparable in OVX and AEW mice. Neutralising antibodies against IL-4 and IL-13 inhibited alloknesis in both OVX and AEW mice as early as 30 min after intradermal administration. Comparable values close to the measurement limit of IL-4 were found in the skin of HRT and Sham mice as well as AEW and the control mice, but the levels of IL-4 were within the measurement limit in OVX mice. We could not detect mRNAs of IL-4 or IL-13 in any groups of mice. On the other hand, the number of eosinophils and basophils was increased in OVX and AEW mice. These results suggest that impaired barrier function in cooperation with type 2 cytokines derived from eosinophils and basophils in the skin or with endogenous type 2 cytokine may trigger the development of alloknesis, and thus, these cytokines could be a therapeutic target for sensitive skin.

Key words: Alloknesis, Type 2 Cytokines, Mouse Model, Menopause, Atopic Dermatitis

INTRODUCTION

Severe vulvar itching and burning, pruritus, formication, and skin irritability are common symptoms in peri-menopausal and post-menopausal women and are potentially related to dry skin.[1,2] We recently reported dysfunction of the stratum corneum and enhanced irritant dermatitis in ovariectomised (OVX) mice—a model of menopause—and found that these effects were alleviated by hormone replacement treatment (HRT) with 17ß-estradiol (E2). [3] Decreased hydration of the stratum corneum, delay in permeability barrier recovery after acute barrier disruption, and weakened integrity of the stratum corneum were found in the OVX mice. This stratum corneum dysfunction could be attributed to lower levels of epidermal differentiation molecules, including loricrin and involucrin, and lower expression of desmoglein-1, a corneodesmosome molecule. However, there is no evidence linking itching in menopause to permeability barrier dysfunction.

Alloknesis is an abnormal itch sensation induced by innocuous mechanical stimuli. It is initially experienced in normal skin surrounding the site of intradermally injected histamine.[4] Patients with atopic dermatitis experience severe itch and alloknesis as well as permeability barrier dysfunction. Artificial permeability barrier disruption also induces itch[5] and alloknesis,[6,7] possibly via epidermal innervation induced by an imbalance of nerve elongation factors and semaphorin 3A (Sema3A) in mice.[8] Thus, permeability barrier dysfunction could cause alloknesis in a mouse model of menopause similar to that occurring in human disease, as well as in a dry skin mouse model induced by treatment with acetone, diethyl ether, and water (AEW).

Nerve growth factor (NGF), amphiregulin, the nerve elongation factor artemin, and the nerve repulsion factor Sema3A play various roles in aberrant nerve elongation and sprouting in patients with atopic dermatitis [9] and mouse models of pruritus.[8] Thymic stromal lymphopoietin (TSLP) and IL-33 produced by keratinocytes, probably in association with dry

skin, [10 11] also cause chronic itch in allergic contact dermatitis and atopic dermatitis. The T-helper type 2 (Th2) cytokines IL-4 and IL-13, [12 13] as well as IL-3, also play important roles in itch and alloknesis in mice and in patients with atopic dermatitis and other pruritic skin diseases, [13 14 15 16] indicating that chronic itch is dependent on neuronal IL-4Rα and Jak1 signalling.

We aimed to investigate alloknesis in mouse models of menopause and dry skin by examining epidermal innervation and the expression of alloknesis-related molecules, as well as the effects of intracutaneous administration of neutralising antibodies against IL-4 and IL-13.

METHODS

Mice, ovariectomy, and HRT

Female C57BL/6 mice (B6) were purchased from Japan SLC (Shizuoka, Japan) at seven weeks of age. All mice were housed in groups of five to six per cage (21.5×32×13.5 cm^3) under conventional conditions on a 12 h light/dark cycle, with food and water *ad libitum*. The mice were randomly assigned to either a sham procedure or bilateral ovariectomy at the age of eight weeks. Ovaries were removed through a midline skin incision, followed by flank incisions of the peritoneum, and the skin was closed with metallic clips. HRT was performed immediately after ovariectomy by the implantation of a subcutaneous, slow-releasing E2 tablet (Innovative Research of American, Sarasota, FL) primed to release a total amount of 0.025 mg of E2 for 60 d, to maintain the serum concentration of E2 at physiologically maximal levels.[3] The experiments were approved and performed according to the guidelines of the Animal Care and Use Committee of Dokkyo University School of Medicine.

Dry skin model

A mixture of acetone and diethyl ether (1:1) was applied to a circumscribed area at the nape for 15 s, immediately followed by the application of distilled water for 30 s, once a day for 5 d (AEW treatment). Control mice were treated in the same manner but with the application of water for 45 s.[7]

Alloknesis test

Alloknesis was evaluated four weeks after ovariectomy/HRT. Areas of around 2 cm diameter were shaved on the necks and backs of mice using a razor under systemic anaesthesia 4 d before the test. The mice were allowed to acclimate to a cage for 60 min before testing. Mechanical stimuli were delivered five times within 1 min to the nape of the neck using von

Frey filaments providing various degrees of force (Muromachi Kikai, Tokyo, Japan). The force was gradually increased from 0.008 *g* to 4.0 *g*. Each round of mechanical stimuli was delivered in greater than 5 s intervals. The presence or absence of a scratch bout was recorded. A scratch bout was defined as the lifting of the hind limb toward the injection site and the placing of the limb back on the floor.

Pruritogen-evoked alloknesis test

Pruritogen-evoked alloknesis was assessed using a previously reported method with minor modifications. [7] Following a 10 μL intradermal injection of histamine (Sigma-Aldrich, St. Louis, MO, USA; 271 nmol dissolved in saline), chloroquine (Sigma-Aldrich, 193 nmol dissolved in saline), or serotonin (Sigma-Aldrich, 23.5 nmol dissolved in saline) at the shaved nape, mice received five separate innocuous mechanical stimuli using a von Frey filament (0.008 g bending force, Muromachi Kikai) at the injection site 35 min after the intradermal injection of pruritogen. The presence or absence of scratching bouts was recorded after each stimulus. These innocuous stimuli were repeated six times at 5 min intervals (30 stimulations in total). The alloknesis score was the total number of positive responses during the 30 min observation period, starting 35 min after pruritogen injection. Detailed information can be found in the Supplementary Materials and Methods.

mRNA expression analysis using reverse transcription polymerase chain reaction (RT-PCR)

Dorsal skin was collected from the B6 mice four weeks after ovariectomy/HRT for mRNA expression analysis using RT-PCR. Detailed information can be found in the Supplementary Materials and Methods. The primers are listed in online Table S1.

Immunohistochemical staining for epidermal innervation and infiltrating cells

For immunostaining with PGP9.5, skin samples were fixed using 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4 °C. Sections of 30 μm thickness (CM1850UV; Leica Microsystems, Wetzlar, Germany) were stained with primary polyclonal antibodies against PGP9.5 (Ab27053, Abcam, Cambridge, UK) and anti-rabbit secondary antibodies (Ab15077, Abcam), and signals were evaluated under a fluorescence microscope (BZ-X810; KEYENCE, Japan). Images were acquired from 12 skin sections from each animal at 20× magnification (four mice per group). Eosinophils were detected based on their specific morphology by staining with haematoxylin and eosin; mast cells were detected by positive metachromatic staining using toluidine blue; basophils were detected by positive immunostaining with anti-mMCP-8 antibody (clone TUG8; BioLegend, San Diego, CA, USA). Detailed information can be found in the Supplementary Materials and Methods.

Touch-evoked alloknesis with neutralising antibodies against IL-4 and IL-13 or intradermal administration of IL-4 or IL-13

After examining alloknesis, 0.01 μg of anti-IL-4 (Ab9728; Abcam) and anti-IL-13 antibodies (Ab109815; Abcam), which is equivalent to 3 times the ND_{50} for 7.5 ng/mL IL-4 and 5 times the ND_{50} for 10.0 ng/mL IL-13, or isotype IgG as control in 10 μL of phosphate buffered saline (PBS) was intradermally injected into the neck skin. Touch-evoked alloknesis was repeatedly evaluated 30 min, 5 h, 24 h, and/or 48 h after intradermal administration of the antibodies or isotype control IgG. Similarly, touch-evoked alloknesis was evaluated before and after intradermal injection of 0.1, 1, and 10 pg IL-4 (PeproTech, Cranbury, NJ, USA) in 50 μL of PBS or 1, 10, and 100 pg IL-13 in 50 μL of PBS.

Enzyme-linked immunosorbent assay (ELISA) for IL-4 and IL-13 in the skin

Harvested skin samples were frozen in liquid nitrogen and stored at -80 ºC until analysis. The skin was placed in 100 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and a cocktail of proteinase inhibitors. The extract was evaluated for murine IL-4 or IL-13 using an ELISA assay kit (Abcam). The measurement limit concentrations, determined as the average values from the blank wells +/- 3 SD, were 5 pg/mL for IL-4 and 10 pg/mL for IL-13. According to the manufacturer, the measurement sensitivity was 1 pg/mL for IL-4 and 1.3 pg/mL for IL-13.

Statistical analysis

All experiments were repeated at least three times. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California USA, www.graphpad.com). Data were analysed using one-way analysis of variance with Tukey's test to compare more than two groups. Two-tailed unpaired *t* tests or paired *t* tests were performed as appropriate. Error bars indicate mean ± standard deviation. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Operated OVX mice with or without HRT and sham operated mice (day 0) were evaluated for touch- and pruritogen-evoked alloknesis, mRNA levels of alloknesis-related molecules, infiltrating cells, and epidermal innervation in the skin 4 d after shaving the hair (day 28) at the posterior areas of the necks.

Touch-evoked alloknesis in a mouse model of menopause

The number of scratch responses was significantly higher in OVX mice than in Sham mice and HRT mice at 0.07 *g* ($P < 0.05$), 0.16 *g*, and 0.4 *g* ($P < 0.01$) of von Frey stimulation (Figure 1). However, the number of scratch responses was comparable between HRT and Sham mice.

Pruritogen-evoked alloknesis in a mouse model of menopause

In all cases of pretreatment with the pruritogens: histamine, serotonin, and chloroquine, the number of scratch bouts in response to mechanical stimuli with a von Frey filament at 0.008 *g* was higher in OVX mice than in HRT and Sham mice (Figure 1b). HRT mice showed significantly fewer scratch responses than Sham mice in all cases of pretreatment with histamine, serotonin, and chloroquine.

Epidermal innervation

The observation, in our previous study, of alloknesis in combination with permeability barrier dysfunction in OVX mice prompted us to examine epidermal innervation, since a mouse model of dry skin induced by treatment with AEW also showed alloknesis. PGP9.5 immunostaining revealed obvious epidermal innervation in the OVX mice comparable with that in the AEW-treated mice (Figure 1c, d). Sham and HRT mice, and control mice for the

AEW mice did not show epidermal innervation (Figure 1c), and mRNA levels of NGF and Sema3A were comparable among the OVX, Sham, and HRT mice (Figure 1e).

Expression of alloknesis-related molecules and Merkel cells in the skin

To thoroughly characterise alloknesis and itch, we measured the mRNA levels of the following alloknesis-related molecules identified in previous reports: IL-4 and IL-13, [12 13] IL-31, [17] artemin, [18 19 20] 11ß-hydroxysteroid dehydrogenase (11ß-HSD), [20] TSLP,[10] piezo-type mechanosensitive ion channel component 2 (Piezo2), [21] and aryl hydrocarbon receptor.[19]. Representative results are shown in Figure S1. We could not detect IL-4, IL-13, or IL-31 mRNA in the skin of any of the mice (data not shown). Levels of the other molecules were comparable among the OVX, Sham, and HRT mice, although the TSLP mRNA levels in the OVX mice were lower than those in the HRT mice. We could not find Merkel cells by immunostaining with CK20 in the skin samples derived from mice of any of the groups (data not shown).

Alloknesis was inhibited by neutralising antibodies against IL-4 and IL-13

We hypothesised that the release of small amounts of Th2 cytokines due to mild inflammation could cause alloknesis in OVX mice, because we could not detect specific mRNA expression of the alloknesis-related molecules. Intradermal administration of neutralising antibodies against IL-4 and IL-13 30 min before stimulation with von Frey filaments completely inhibited touch-evoked alloknesis in the OVX mice (Figure 2a, Figure S2). Intradermal administration of these antibodies also diminished touch-evoked alloknesis in the AEW mice (Figure 2b). The suppressive effects of the neutralising antibodies were also confirmed for at least 48 h after administration of the antibodies in both dry skin models (Figure 2a, b). Following individual administration, each neutralising antibody exerted inhibitory effects on

touch-evoked alloknesis in both dry skin models when assessed using several weights and different time points; however, the degree of inhibition was partial when compared with combined use of these antibodies (Figure 2c, d). Neutralizing antibodies against IL-13 showed inhibitory effect in AEW mice, although without statistical significance (Figure 2d).

Skin IL-4 and IL-13 levels in both mice models of dry skin

The levels of IL-4 in the skin were low and close to the measurement limit. The mean values of IL-4 were comparable in Sham and HRT mice (4.2±1.7 (SD) pg/mL and 5.3±2.2 pg/mL), while those in OVX mice were under the measurement limit. AEW and control mice treated with water showed comparable levels of IL-4 in the skin (6.8±2.0 pg/mL and 6.1±2.0 pg/mL). The levels of IL-13 in the skin were under measurement limit in mice of all groups.

Alloknesis was not fully induced by intradermal administration of IL-4 or IL-13 in naïve mice

We next examined whether IL-4 or IL-13 could induce touch-evoked alloknesis in naïve mice. We did not observe spontaneous scratching 2 h after administration of IL-4 or IL-13. Touch-evoked alloknesis was induced 30 min after intradermal administration of 1.0 pg and 10 pg IL-4 using 0.04 g and 0.07 g von Frey filaments in a dose-dependent manner, while the same dose of IL-13 was insufficient to induce alloknesis (Figure 3a, b). Administration of 100 pg IL-13 induced alloknesis 30 min after injection (Figure 3b). We also observed increased scratching following stimulation with von Frey filament 5 h after intradermal administration of IL-4 and IL-13, although without statistical significance, but not at later time points (Figure 3a, b). IL-4 and IL-13 were used at these doses because doses higher than these could not be detected in the skin of the dry skin model mice.

Increased numbers of basophils and eosinophils in OVX and AEW mice

To investigate our hypothesis, we evaluated the differential cell counts of infiltrating cells in the skin (Figure 4). The numbers of basophils and eosinophils in OVX mice were greater than those in Sham and HRT mice; this was also the case in the AEW mice compared with the control mice. The numbers of mast cells were comparable among the OVX, Sham, and HRT mice, but higher in the AEW mice.

DISCUSSION

In this study, we explored the functional changes and mechanisms related to some of the clinical symptoms of menopause, such as skin irritation and pruritus, using the OVX mouse model of menopause. Three surprising observations in the OVX mice were the presence of touch- and pruritogen-evoked alloknesis, epidermal innervation, and inhibition of touch-evoked alloknesis following administration of neutralising antibodies against IL-4 and IL-13. All results in the OVX mice were comparable with those in the AEW mice. Thus, based on our results, we propose that permeability barrier dysfunction in OVX mice plays a critical role in disrupting the stability of the skin.

Dysfunction of the permeability barrier was observed at four weeks and enhanced at six weeks after ovariectomy in the Hos:HR-1 mice without hair.[3] The barrier function of B6 mice, which are widely used to study alloknesis, after shaving hair was more vulnerable and sensitive than that of the Hos:HR-1 mice after ovariectomy.[3] Hence, we used B6 mice four weeks after ovariectomy in this study. There were no differences in skin appearance and no spontaneous scratching 4 d after shaving hair among the OVX, Sham, or HRT mice in this study; thus, there were no indications of the induction of alloknesis and epidermal innervation.

Epidermal innervation has been repeatedly reported in mice with permeability barrier dysfunction, including AEW mice, as evidenced by a decreased ratio of the levels of Sema3A to nerve elongation factors, including NGF.[8,20,22] Both OVX and AEW mice in this study showed epidermal innervation and comparable levels of Sema3A and NGF mRNAs. Additionally, alloknesis was found in OVX mice and AEW mice in this study. However, whether the nature of the permeability barrier dysfunction was the same in the OVX and AEW mice remains unclear, as the former mouse model was established over four to six weeks and the latter within 5 d. The mechanism of induction of alloknesis in mice with barrier

dysfunction has not been elucidated. Epidermal innervation may be one possible mechanism; however, it is not a direct explanation for alloknesis, as human patients with prurigo nodularis, an intense pruritic skin disease, show no epidermal innervation.[23,24] We measured the mRNA levels of the specific molecules related to alloknesis. We could not find any specific mRNA for these molecules in the OVX mice.

Oetjen and colleagues[13] reported that IL-4 rapidly sensitises subsets of neurons to a variety of pruritogens and significantly amplifies scratching behaviour in response to low doses of known pruritogens, such as histamine. They speculated that type 2 cytokines may act as master regulators of chronic itch by intensifying itch responses to multiple pruritogens present in inflamed skin, although intradermal administration of IL-4 did not directly induce scratching. In contrast, Campion and colleagues[12] showed that intradermal administration of IL-4 and IL-13 evokes scratching behaviour in mice, and attributed the difference in their results from those of Oetjen[13] to the different doses of the type 2 cytokines used in their experiments. Although the neutralising antibodies against IL-4 and IL-13 inhibited the development of alloknesis in the OVX and AEW mice, the mRNA of IL-4, IL-13, and IL-31 could not be detected in the OVX mice. In addition, we detected a small amount of IL-4, close to the measurement limit, in Sham and HRT mice as well as AEW and its control mice but not in OVX mice, which showed alloknesis. We also examined whether intradermal administration of IL-4 or IL-13 could induce alloknesis directly and found that 1 and 10 pg IL-4 and 100 pg IL-13 induced alloknesis in a dose-dependent manner 30 min after administration, but not later. These results suggest that Th2 cytokines play a role in, but are not fully responsible for, the induction of alloknesis. Other factors derived from dry skin are required to induce a full blown alloknesis. Two possibilities explain the presence of Th2 cytokines in the skin of dry skin models. First, our detection of IL-4 in the skin might be a false-positive result due to technical issues, as the detected levels were very close to the

measurement limit. When considering this possibility, the presence of small amounts of type 2 cytokines, which we could not detect and presumably were derived from basophils and eosinophils, could play a key role in the induction of alloknesis. Second, we detected IL-4 in the skin of AEW, Sham, and HRT mice by ELISA. However, the levels were close to the measurement limit and comparable between AEW and control mice. In addition, we did not detect IL-4 in the skin of OVX mice. Collectively, these results indicate that "endogenous IL-4", as shown by increased barrier function in the absence of IL-4 in IL-4-deficient mice[25] and which we could not detect, could play a crucial role in alloknesis induction. This concept can be adopted for human pruritic diseases, such as uremic pruritus and chronic idiopathic pruritus, in which type 2 cytokines are not relevant and patients are successfully treated with dupilumab, an anti-IL-4Rα1 antibody. [26] We used an extremely low amount of IL-4 (1 and 10 pg) and IL-13 (100 pg) to induce alloknesis compared with those used by Campion (1 μg IL-4 and/or IL-13) [12] to induce spontaneous scratching because we could not detect a large amount of these cytokines the skin. We had reported that a very small amount of IL-4, 0.01 pg/50 μL, impaired the recovery of the permeability barrier function after acute disruption.[27] Thus, the small amounts of type 2 cytokines could be important to understanding the pathology associated with barrier dysfunction and alloknesis and play a crucial role in the vicious cycle between scratching and permeability barrier dysfunction in skin diseases associated with pruritus and dry skin, such as atopic dermatitis.

In addition, the differential role of IL-4 or IL-13 in alloknesis was evaluated using the relevant neutralising antibodies. Neither of the neutralising antibodies, when used individually, caused complete inhibition when compared with their combined use. Neutralizing antibodies against IL-4 inhibited alloknesis in the AEW mice whereas those against IL-13 showed a inhibitory effect on alloknesis in OVX mice. The difference in experimental procedure, 4–6 weeks for OVX mice and 5 days for AEW mice, may have led to

these differential effects. Based on our results, we infer that a large dose of IL-13 is required to induce alloknesis in naïve mice. Moreover, IL-4 and IL-13 may play key roles during the acute and chronic phases of skin pathology, respectively. However, the role of these cytokine in the dry skin models cannot be concluded since our interpretations are based on a small number of experiments using polyclonal antibodies, whose binding sites on their respective cytokines for inducing the antagonising effect are unknown.

Dry skin and associated proinflammatory cytokines, such as IL-1α, TNF-α, and IL-6, combined with permeability barrier dysfunction, induce inflammation due to type 2 cytokines and lead to alloknesis. [28] Besides, basophils and eosinophils, both of which are increased in number in OVX and AEW mice, as well as type 2 cytokines, could be a source of pruritogens. These cell types also play a key role in irritant contact dermatitis in which basophils are recruited rapidly and subsequently attract eosinophils in the skin. [29] Basophils are involved in the pathology of various pruritic skin diseases [14] and are major early producers of IL-4 and IL-13, which are critical for triggering and maintaining allergic responses.[29,30] Mast cells could be another source of type 2 cytokines. The number of mast cells was increased in the AEW mice but not in the OVX mice. Miyamoto and colleagues showed no infiltrating cells, but a comparable number of mast cells in the dermis of AEW and control mice.[5] These researchers also showed that the number of spontaneous scratching bouts after AEW treatment was comparable between mast-cell-deficient mice and normal mice. The numbers of basophils and eosinophils were small, but higher in the OVX and AEW mice in this study. Special attention and specific antibodies would be required to detect basophils.

HRT abrogates epidermal innervation, alloknesis, and inflammatory cell infiltration in OVX mice. Oestrogen has a potent anti-inflammatory function; we have previously shown its suppressive effect on irritant dermatitis in OVX mice.[3] However, the protective effects of E2 on skin barrier function could also contribute to its anti-inflammatory effects. E2 protects the

oesophageal epithelium from IL-13-induced barrier dysfunction and remodelling by inhibiting IL-13-induced tyrosine kinase 2, phosphorylation of signal transducer and activator of transcription 6, and EoE-dysregulated gene expression via oestrogen receptor 2.[31] Epidermal undifferentiation in OVX mice, which is reversed by E2,[3] could be associated with the increased production of inflammatory cytokines by keratinocytes, as observed after acute barrier disruption.[28] Thus, oestrogen may work on multiple pathological processes to restore homeostasis in skin affected by barrier disruption and inflammation.

This study began with exploring the mechanism of pruritus in menopause. Pruritus is a common problem in menopausal women and is most severe in lesions of the genitals, a major target of E2.[1] As shown in this study, type 2 cytokines in relation with permeability barrier dysfunction could lead to the itch observed in menopausal women. Ageing, another factor exacerbating damage to the skin barrier,[2] could be involved in the barrier dysfunction-type 2 cytokine-itch axis. Although more older patients present with atopic dermatitis, their clinical symptoms are not severe.[32] Thus, older people have suppressive pathology as a compensatory effect, in spite of permeability barrier dysfunction. An additional underlying mechanism is responsible for the development of atopic dermatitis, as evidenced by the observation that, although ichthyosis vulgaris exhibits the same barrier dysfunction as atopic dermatitis, it is not always accompanied by refractory itch and dermatitis.[33,34] Our study findings have inherent limitations in their application to humans since our results are solely based on the mouse models. Further, to validate our findings, it is necessary to confirm the role of small amounts of type 2 cytokines using type 2 cytokines or their receptor-deficient murine models of dry skin in future studies.

In summary, epidermal innervation and alloknesis in relation to small amounts of type 2 cytokines that are derived from mild inflammatory cells or endogenous were observed in mouse models of menopause, as well as in artificial dry skin. This is the first report to show

the involvement of type 2 cytokines in two mouse models of permeability barrier dysfunction with alloknesis. It is not clear whether both models share the same type of barrier dysfunction or how these models reflect dry skin-based diseases in humans. However, low amounts of type 2 cytokines should be taken into consideration for the therapy of skin diseases involving dry skin, such as atopic dermatitis.

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AUTHOR CONTRIBUTIONS

Nao Ichimasu: Conceptualisation, Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Visualisation, Writing - original draft; **Yue Chen:** Conceptualisation, Data curation, Investigation, Methodology, Resources; **Keisuke Kobayashi:** Methodology, Resources**; So Suzuki:** Methodology**,** Resources**; Sakiko Chikazawa:** Methodology, Resources**; Sakiko Shimura:** Data curation, Methodology, Project administration, Software, Validation, Visualisation**; Kazumoto Katagiri:** Conceptualisation, Funding acquisition, Methodology, Project administration, Writing-review & editing, Supervision, Validation

CONFLICTS OF INTEREST

The authors state no conflict of interest.

Data Availability Statement

Research data are not shared.

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FIGURE LEGENDS

Figure 1. Enhanced light mechanical and chemical stimuli-evoked itch and epidermal innervation in a mouse model of menopause. (a) Number of scratch responses after von Frey stimulation with various forces in three groups of B6 mice subjected to sham operation (Sham), ovariectomy (OVX), or hormone replacement treatment (HRT) (n = 25). (b) Sum of alloknesis scores evoked by histamine, serotonin, and chloroquine. Stimulation was applied five times near the intradermal injection site using a 0.008 *g* von Frey filament at 5 min intervals from 35 to 60 min after pruritogen injection (n = 20–23). (c) Skin sections from sham-operated (Sham), ovariectomized (OVX), hormone replacement treatment (HRT), or AEW (acetone-diethyl ether, and water)-treated-mice were immunostained with an antibody for PGP9.5 to visualize nerve fibres. Control mice were used as the negative control (data not shown). These are representative photos of each individual experiment. Dotted lines indicate the dermo-epidermal junction. Scale bar = 50 μm. (d) Total epidermal nerve fibre density (ENFD) was quantified in Sham, OVX, HRT, and AEW mice (n = 4). (e) Levels of mRNA for semaphorin 3A (Sema3A) and nerve growth factor (NGF) normalized to β-actin mRNA levels, and their ratio in Sham, OVX, and HRT mice. Data are from at least two independent experiments. Significant differences between groups are indicated by *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Figure 2. Alloknesis was inhibited by intradermal injection of anti-IL-4 and anti-IL-13 antibodies into the skin of the necks of mice. Touch-evoked alloknesis was evaluated using 0.07 *g,* 0.16 *g,* and 0.4 *g* von Frey filaments at indicated time points after intradermal administration of neutralizing antibodies for IL4 (aIL4) and IL-13 (aIL13) in mice subjected to (a) sham operation (Sham), ovariectomy (OVX), or hormone replacement therapy (HRT) and in mice treated with (b) water and AEW (acetone-diethyl ether, and water) (n = 16). (c, d)

Differential effects of neutralizing antibodies for IL4 (aIL4) and IL-13 (aIL13) on induction of alloknesis in OVX and AEW mice. Data are from at least two independent experiments. Significant differences between groups are indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 3. Induction of alloknesis by IL-4 or IL-13 in naïve mice. Touch-evoked alloknesis was evaluated using 0.04 and 0.07 *g* von Frey filaments at indicated time points after intradermal administration of indicated doses of IL-4 (a) and IL-13 (b). Data are from at least two independent experiments. Significant differences between groups are indicated by *P < 0.05 and **P < 0.01.

Figure 4. Differential cell counts in the skin. Infiltrating cells in the skin were evaluated as follows: eosinophils were identified based on specific morphology with haematoxylin and eosin staining; mast cells, by positive metachromatic staining with toluidine blue; and basophils, by positive immunostaining with an anti-mMCP-8 antibody. Inflammatory cells were counted in five fields (40× objective). Skin samples were harvested (a) 4 d after shaving hair at four weeks in the sham-operation (Sham), ovariectomized (OVX), and hormone replacement therapy (HRT) groups of mice (n = 17–19) and (b) 24 h after AEW (acetone, diethyl ether, and water) and water (n = 16) treatment. Black arrowheads indicate eosinophils. Scale bar = 50 μm. *P <0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.