Atopic dermatitis-like skin lesions with IgE hyperproduction and pruritus in KFRS4/Kyo rats

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Abstract

Background: Rats showing spontaneous atopic dermatitis (AD)-like skin lesions were observed in the Kyoto fancy rat stock 4 (KFRS4) strain breeding colony.

Objective: To establish the KFRS4 rat as a model of AD.

Methods: The clinical symptoms of AD-like skin lesions were assessed by scoring the degree of dermatitis and examining scratching behavior. The transepidermal water loss was measured to evaluate skin barrier function. Cells infiltrating the skin lesions were identified using histological and immunohistological analyses. IgE and cytokine levels were measured to examine immune status. An ointment treatment experiment was carried out to characterize dermatitis in the KFRS4 rats.

Results: Dermatitis initially appeared around 4 months of age and rapidly worsened from 6 to 8 months of age. The skin lesions accompanied scratching behavior and were predominantly observed in females. The increased transepidermal water loss indicated skin barrier dysfunction. Extensive infiltration of eosinophils, mast cells and lymphocytes was observed in the skin lesions. The plasma IgE level increased in accord with increasing severity of dermatitis. The Th2 and Th17 cytokine mRNA levels were significantly higher in the skin-draining lymph nodes than those in the

non-skin-draining lymph nodes. It was demonstrated that betamethasone improved the symptoms of dermatitis. These findings demonstrated that dermatitis in the KFRS4 rats closely resembled that seen in human AD.

Conclusion: Female KFRS4 rats have the potential to serve as an animal model of human AD.

Abbreviations

AD, atopic dermatitis

HE, hematoxylin-eosin

KFRS4, Kyoto fancy rat stock 4

LN, lymph node

TB, toluidine blue

TEWL, transepidermal water loss

TSLSS, total skin lesion severity score

1. Introduction

Atopic dermatitis (AD) is a relapsing chronic inflammatory skin disease characterized by eczematous skin lesions and intense pruritus [1]. AD affects at least 15% of children and 2–10% of adults in industrialized countries, and there is growing evidence of an increase in prevalence [2]. AD is often associated with elevated serum immunoglobulin E (IgE) levels and a personal or family history of type I allergies, allergic rhinitis and asthma.

The etiology of AD is highly complex, but several lines of evidence suggest that a genetic predisposition leads to a defective skin barrier and a dysregulated immune response [3, 4]. Environmental triggers such as food allergens, inhalable respiratory allergens, irritating substances and infectious microorganisms [5, 6] play an important role in the development of AD. Scratching induced by pruritus disrupts skin barrier functions, which also contributes to the development of AD [7]. Thus, skin barrier function, immunology, pruritus and the environment, as well as interactions among these factors, are important considerations when examining the mechanisms underlying the development of AD.

To understand the complex pathogenesis of AD in the development of effective

procedures for diagnosis, prevention and treatment, animal models of AD are essential. These models allow the in-depth investigation of pathogenesis and provide invaluable tools for diagnostic and pharmaceutical purposes. There are various mouse models of AD available, including models induced by epicutaneous sensitization, genetically engineered models and spontaneous models [8]. Among them, NC/Nga mice have been a widely used AD-like dermatitis model with an elevation in IgE levels and intense pruritus; this is primarily influenced by mite infection in specific pathogen free (SPF) conditions [9, 10]. Although mouse models are primarily used, rat models of AD are also expected to be developed because rats are widely used in biomedical and pharmaceutical research areas.

Kyoto Fancy Rat Stock 4 (KFRS4) rats have been developed from hybrids between the fancy rat and PVG/Seac inbred strain rats [11]. During inbreeding, several mutant phenotypes carried by the fancy rat are selected. As such, KFRS4 rats carry mutations that include bright coat color, hooded coat pattern, white spotting on the head and cataracts [12-15].

In the present study, we newly observed spontaneous dermatitis that accompanied scratching behavior in KFRS4 rats. This dermatitis appeared around 6

months of age and worsened progressively during the next 2 months. It seemed that there was a sex difference in incidence; almost all female rats but only some males were affected. To characterize KFRS4 rats as a model of AD, we examined the clinical symptoms, scratching behavior, histological analysis of skin lesions and immune status. In addition, we performed a treatment experiment regarding dermatitis on the KFRS4 rats using the corticosteroid betamethasone.

2. Materials and Methods

2.1. Rats

KFRS4/Kyo (NBRP#0572) and PVG/Seac (NBRP#0080) rat strains were sourced from the National BioResource Project-Rat, Kyoto University (Kyoto, Japan). Rats were bred in conventional conditions in which they were free of mites but were infected by pinworms (*Syphacia muris*). All animal experiments were approved by the Animal Research Committee of Kyoto University and were conducted according to the Regulations on Animal Experimentation of Kyoto University.

2.2. Reagents

Mouse anti-rat CD4 (clone, W3/25; 1:5000) and mouse anti-rat CD8 alpha (clone, OX-8; 1:500) were purchased from AbD Serotec (Raleigh, North Carolina, USA). Heparinoid cream (Hirudoid® Soft) and betamethasone dipropionate (Rinderon®-V Ointment 0.12%) were purchased from Maruho Co., Ltd. (Osaka, Japan) and Shionogi & Co., Ltd. (Osaka Japan), respectively.

2.3. Clinical observation

The clinical severity of the skin lesions was scored in male (n = 8) and female (n = 8) KFRS4 rats according to the macroscopic diagnostic criteria, termed the total skin lesion severity score (TSLSS) [16]. Briefly, the TSLSS was designated as the sum of the individual scores, graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for the symptoms of erythema, eczema, crust, edema and erosion in six regions of the body, namely the face, head and neck, forelegs, hind legs, dorsal and ventral regions of the trunk.

2.4. Histology

For histological analysis, major organs including the skin were harvested, fixed in buffered 10% formalin, embedded in paraffin and stained with hematoxylin-eosin (HE) [17]. For examination of mast cells, skin sections were stained with toluidine blue (TB). The number of mast cells was calculated as the average from 20 fields of dermatitis (n = 4) and normal (n = 4) skin samples (original magnification, ×400). For immunohistochemistry, 10-μm frozen sections of the skin from 6-month-old KFRS4 rats were fixed in methanol:acetone (1:1) for 10 min at 4°C and washed in PBS.

Sections were treated with 10% goat serum in PBS and then incubated for 1 h at room temperature with anti-CD4 and CD8 antibodies. After incubation with the primary antibodies, sections were treated with peroxidase-conjugated secondary antibody (Histofine Simplestain MAX-PO: Nichirei, Tokyo, Japan) and signals were visualized using a DAB substrate kit (Nichirei).

2.5. Measurement of plasma IgE

Rats were anesthetized with isoflurane and blood samples were collected from the tail tip using heparinized hematocrit tubes. Total plasma IgE levels were measured using a rat IgE EIA kit (Takara Bio Inc., Otsu, Shiga, Japan).

2.6. Skin barrier function

The dorsal region of the skin was shaved in female KFRS4 (n = 6) and PVG (n = 6) rats before measurement. To evaluate the inside-to-outside barrier function, transepidermal water loss (TEWL) was measured using a Tewameter Vapo Scan (Asahi Biomed, Tokyo, Japan) at 24°C and 46% relative humidity at 5 and 9 weeks of age. TEWL measurements were carried out in triplicate for each rat.

2.7. Scratching behavior

Pruritus was evaluated in terms of the level of scratching behavior. Rats aged 4 months (n = 6), 6 months (n = 6) and 8 months (n = 6) were placed individually in plastic cages (30 cm × 30 cm × 20 cm). After an acclimation period (60 min), the number of scratching movements was counted in the morning during the 60-min observation period.

2.8. Treatment of dermatitis

Four-month-old female KFRS4 rats (n = 18) were divided into three groups. Group 1 rats were untreated (n = 6), group 2 rats were treated with heparinoid cream (n = 6) and group 3 rats were treated with betamethasone (n = 6). Ointment was applied to the skin of the head and neck three times per week. Every 4 weeks, the clinical observation of the skin, an examination of scratching behavior and measurement of plasma IgE were performed. At 7 months of age, animals were sacrificed under isoflurane anesthesia and the spleen, adrenal glands, lymph nodes (LNs) and skin were harvested for histological analysis.

2.9. Quantitative real-time polymerase chain reaction

Thirteen skin-draining LNs and 8 non skin-draining LNs (mesenteric and renal) were harvested from 3 KFRS4 rats. Messenger RNA (mRNA) was isolated using ISOGEN II (Nippon Gene Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction was performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc., Otsu, Shiga, Japan) with SYBR Premix Ex Taq II (Takara Bio Inc.). By monitoring the amplification curves of a test sample and reference samples that contained 10^1 to 10^6 molecules of the gene of interest, the number of target molecules in the test sample was analyzed. The number of target molecules was normalized to that of peptidylprolyl isomerase A (*Ppia*) as an internal control [18]. The primers used are listed in Supplementary Table 1.

2.10. Hinkelmann staining

Peripheral blood samples were collected from the tail veins of dermatitis-affected (6–8 months of age; n = 7) and non-affected (4–5 months of age; n = 6) female KFRS4 rats. Blood samples (10 μL) were stained with Hinkelmann's solution (Muto Pure Chemicals, Tokyo, Japan).

2.11. Statistical analysis

Data were analyzed using an unpaired two-tailed *t*-test. P values < 0.05 were considered significant. All results are shown as the mean ± standard deviation (SD).

3. Results

3.1. Spontaneous dermatitis in KFRS4 rats

Skin lesions initially appeared as erythema on the lips or forelegs at around 4 months of age. Until 6 months of age, mild skin lesions consisting of erythema, eczema, and crust were seen on the skin of the KFRS4 rats. With increasing age, severe edema and erosion were seen, particularly in the skin of the head and neck region and the dorsal region of the trunk (Fig. 1A). These skin lesions were observed in 100% of 8-month-old female and 50% of 8-month-old male KFRS4 rats examined. TSLSS was significantly higher in females than in the age-matched males (Fig. 1B). The frequency of scratching behavior during the 60-min observation period dramatically increased after 6 months of age in female KFRS4 rats (Fig. 1C). In contrast, no skin lesions or scratching behavior were observed in PVG rats, a parental strain of KFRS4.

3.2. Skin barrier function in KFRS4 rats

Because skin barrier dysfunction is a common characteristic of AD [7, 19], we measured the TEWL, an established indicator of barrier function. The TEWL of KFRS4

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rats was significantly higher than that of PVG rats both at 5 and 9 weeks of age (Fig. 1D) when no dermatitis was observed clinically and histologically. This finding indicates a defect in the skin barrier function of KFRS4 rats before the appearance of dermatitis.

3.3. Histological examination of skin lesions in female KFRS4 rats

The histology of early skin lesions in KFRS4 rats showed epidermal hyperplasia with mild spongiosis, parakeratosis, and infiltration of lymphocytes in the epidermis and superficial dermis (Fig. 2A). As the lesions progressed, there was extensive infiltration of eosinophils and lymphocytes in the dermis. A skin ulcer that was covered by crusts formed by fibrin exudate and necrotic tissue debris was often observed (Fig. 2B). In addition, mast cells that were detected using TB staining increased in number in the dermis of dermatitis-affected skin as compared with non-dermatitis skin (Fig. 2C). The infiltration of both $CD4^+$ and $CD8^+$ T cells was observed in the dermatitis-affected skin (Fig. 2D). The number of $CD4^+$ cells was significantly increased in the dermatitis region as compared with the non-dermatitis region (78.3 ± 15.6 vs 0.33 ± 0.50; $P < 0.01$). In addition, the number of $CD8^+$ cells was

significantly increased (18.2 ± 4.68 vs 0.50 ± 0.53; P < 0.01; Fig. 2D). None of these changes were observed in PVG rats.

3.4. Immune status of KFRS4 rats in the steady state

To elucidate the immune status of female KFRS4 rats, we measured the levels of total IgE, because the increased severity of AD is reported to be correlated with elevated IgE levels [16, 20]. IgE levels were significantly higher in female KFRS4 rats than in age-matched male KFRS4 rats from the onset (5 months of age) to the exacerbation phase of dermatitis (7 months of age) (Fig. 3A). To examine the immune status of KFRS4 rats showing severe dermatitis, we measured the Th1 (interferon (IFN)-γ), Th2 (interleukin (IL)-4), Th17 (IL-17A, IL-22 and IL-23), and Treg (IL-10), plasma cell (tumor necrosis factor [TNF]α) cytokine mRNA levels of the skin-draining mandibular and axillary LNs of the affected rats, and compared them with those of the non-skin-draining LNs. The mRNA levels of IL-22, IL-23, IL-4 and TNFα were significantly higher in the skin-draining LNs than in the non-skin-draining LNs (Fig. 3B). Furthermore, we examined the number and proportion of eosinophils in the peripheral blood of KFRS4 rats; this was because an increase in the number of

eosinophils has been found in most patients with AD and correlates with disease activity [21]. Eosinophils increased significantly in number in dermatitis-affected KFRS4 rats as compared with non-affected rats (Fig. 3C). The proportion of eosinophils in leukocytes was also significantly increased ($2.7 \pm 1.1\%$ in non-affected rats vs $12.4 \pm 4.0\%$ in dermatitis-affected rats; $P < 0.01$).

These findings suggest that Th2, Th17 cells and eosinophils, similar to those in human AD patients [22], play an important role in the pathogenesis of dermatitis in KFRS4 rats.

3.5. Suppression of dermatitis by steroids

Because topical steroid treatment is commonly used for AD, we carried out ointment treatment experiments in female KFRS4 rats. Heparinoid cream was used as a control. When compared with untreated and heparinoid-treated rats, the rats treated with betamethasone ointment showed a significant improvement in TSLSS (Fig. 4A) and scratching behavior (Fig. 4B). The IgE level in betamethasone-treated rats tended to be lower than in non-treated or heparinoid-treated rats, but was not significantly different among them.

Histological examination showed that marked skin inflammation and ulceration were evident in the non-treated or heparinoid cream-treated rats but were virtually absent in the betamethasone-treated rats (Fig. 4C). These findings indicate that betamethasone is effective in treating dermatitis in KFRS4 rats, which is also observed in patients with AD.

4. Discussion

KFRS4 rats showed spontaneous dermatitis that accompanied scratching behavior, elevation of plasma IgE, and Th2 and Th17 cytokine mRNAs. The histology of the skin lesion was characterized by the infiltration of lymphocytes, eosinophils and mast cells. The increased TEWL indicated skin barrier dysfunction in KFRS4 rats. In addition, the treatment with betamethasone ointment had marked effects on suppression of dermatitis. These clinical, immunological and histological features of KFRS4 rat dermatitis closely resemble those seen in human AD. Thus, KFRS4 rats are considered to be a good model of AD.

The microbiological environments where animals are bred play important roles in the development of dermatitis. NC/Nga mice develop dermatitis when bred in conventional conditions or under specific pathogen free conditions with an infection of house dust mites [9]. *Staphylococcus aureus* colonization on the skin drives inflammation in spontaneous or genetically modified rodent models of AD [23, 24]. KFRS4 rats are bred without mites but with infection by pinworms (*Syphacia muris*), which are known to influence allergic reactions in mice [25]. Thus, pinworms may be involved in the development of dermatitis in KFRS4 rats through modulation of the

immune system or the generation of allergens. The development of pinworm-free

KFRS4 rats in specific pathogen free conditions would be required to address the

microbiological environment issue.

Although no sex differences have been observed in human AD patients [26, 27]

or mouse models of AD [9, 16, 28], we found a clear sex difference in the dermatitis of

KFRS4 rats. Female KFRS4 rats developed much more severe dermatitis as compared

with age-matched males. Because male and female KFRS4 rats are kept in identical

environments, female sex apparently predisposes KFRS4 rats to AD-like skin lesions.

There are sex differences in immune responses, especially from the perspective of

collagen vascular diseases, and the immune response plays a pivotal role in the

development of AD [7, 29, 30]. Thus, it is possible to consider that the sex difference

may influence the development of AD in certain conditions. The sex difference in the

immune response is attributed not only to differences in sex hormones, such as estrogen,

testosterone and progesterone, but also to X chromosome gene contributions [30]. The

X chromosome gene contributions would appear as X-linked diseases in which males

show the severe phenotype and females are relatively unaffected. Thus, it is likely that

sex hormones may contribute to the higher incidence of dermatitis found in female

KFRS4 rats. To this end, developing ovariectomy or orchiectomy in KFRS4 rats would be needed.

We carried out an ointment treatment experiment in KFRS4 rats and found that steroid (betamethasone) was effective in suppressing dermatitis. This finding suggested that KFRS4 rats are suitable for the evaluation of new drugs or treatments for AD. Owing to 100% prevalence of dermatitis, female KFRS4 rats could be considered as providing an efficient bioassay system. Indeed, in KFRS4 rats, skin barrier dysfunction occurred before the appearance of dermatitis. Daily application of moisturizer during the neonatal period reduces the risk of AD [31]. Thus, it would be interesting to examine the preventive effect of moisturizer on dermatitis in KFRS4 rats before the appearance of dermatitis.

In summary, KFRS4 rats spontaneously developed dermatitis that accompanied an elevation of IgE and scratching behavior. The dermatitis was evidently suppressed by topical application of betamethasone. In addition, increased TEWL was observed before the onset of dermatitis, which indicates that there was skin barrier dysfunction in KFRS4 rats. Taken together, KFRS4 rats have the potential to serve as an animal model of human AD that would be useful for diagnostic and pharmaceutical purposes, and

could provide insights into the possible mechanisms associated with human AD.

Acknowledgements

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Figure legends

Fig. 1. Spontaneous dermatitis in Kyoto Fancy Rat Stock 4 rats. (A) Clinical photographs of 5-, 6- and 7-month-old Kyoto Fancy Rat Stock 4 (KFRS4) rats. (B) Total skin lesion severity scores (TSLSS) for male and female KFRS4 rats. Females showed significantly higher scores than age-matched males after 6 months of age. (C) Scratching behavior of female KFRS4 rats. (D) Total epidermal water loss (TEWL) through the dorsal skin of 5- and 8-week-old female PVG and KFRS4 rats. *: $P < 0.05$; **: $P < 0.01$.

Fig. 2. Histological observations of the skin lesions of KFRS4 rats. (A, B) HE-stained sections of the dermatitis region in 7-month-old KFRS4 rats. In the eczematous lesions (A; original magnification ×200), epidermal hyperplasia with mild spongiosis and infiltration of lymphocytes in the epidermis and superficial dermis were observed. In the progressed lesion (B; original magnification ×40), severe infiltrations of lymphocytes and eosinophils (inset; original magnification ×400) in the dermis and ulcers were observed. (C) TB-stained section of the dermatitis region of KFRS4 rats

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(left, original ×400) and the number of mast cells per field under 400-fold magnification (right). (D) Immunohistochemical staining of CD4 and CD8 in the skin of 6-month-old KFRS4 rats. Both CD4$^+$ and CD8$^+$ lymphocytes (arrows) were observed in the skin lesions but not in the normal skin of KFRS4 rats (left, original magnification ×400). The number of CD4$^+$ and CD8$^+$ cells per field under 400-fold magnification (right). **: $P < 0.01$.

Fig. 3. Immunological status of KFRS4 rats. (A) Total plasma IgE levels in male (open) and female (closed) KFRS4 rats. Females showed significantly higher IgE levels than age-matched males. (B) Expression levels of cytokines in the lymph nodes (LNs) of the dermatitis-affected female KFRS4 rats. (C) Number of eosinophils in the peripheral blood of the dermatitis-affected and non-affected KFRS4 rats. *: $P < 0.05$; **: $P < 0.01$.

Fig. 4. Effects of ointments on dermatitis in KFRS4 rats. (A) TSLSS of ointment-treated and non-treated KFRS4 rats (left) and clinical photographs of 7-month-old rats (right). Note that topical application of betamethasone ointment

greatly suppressed the dermatitis. (B) Scratching behavior in ointment-treated and non-treated KFRS4 rats. (C) HE-stained sections of the skin on which ointment was applied (original magnification, $\times 40$). All results are shown as means $\pm$ SD (n = 6). *$P <$ 0.05.

Author contributions

TK and TS designed the research. MY, DT, AY, AN, YH and KY performed the experiments. MT, MK and HH performed the pathological examination. TK, HH and MT wrote the paper. KK, HH and TS revised the manuscript.

Figure 1

Figure 2

Figure 3

A

Total skin lesion severity score

- □ non-treated
- ▨ Heparinoid
- ■ Betamethasone

Age (months): 4, 5, 6, 7

non-treated   Heparinoid   Betamethasone

B

Number of scratches (10-min observation)

- □ non-treated
- ▨ Heparinoid
- ■ Betamethasone

Age (months): 4, 5, 6, 7

C

non-treated   Heparinoid   Betamethasone
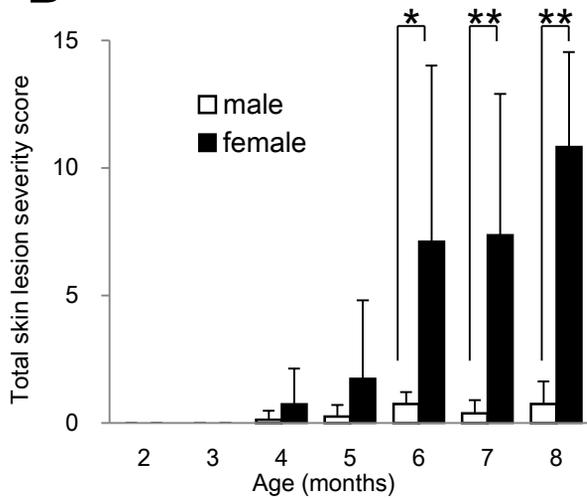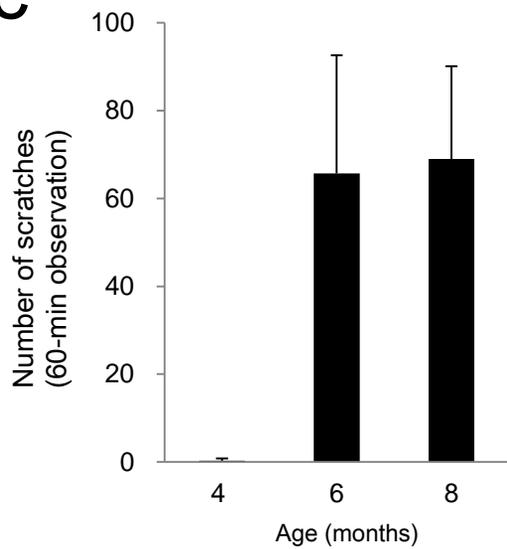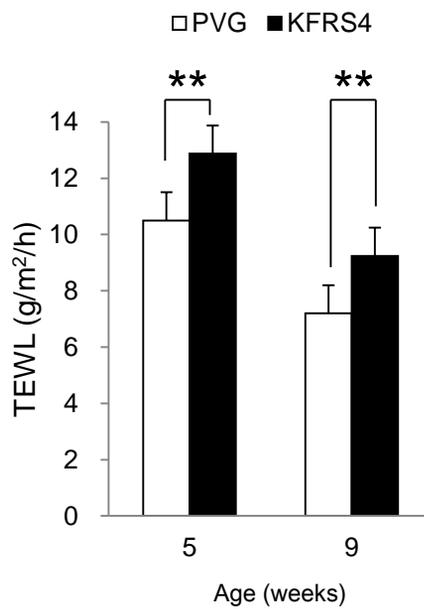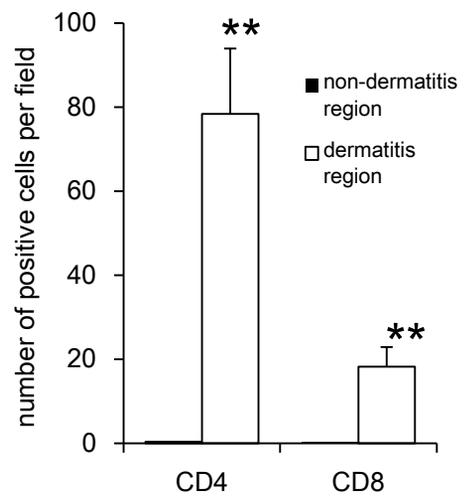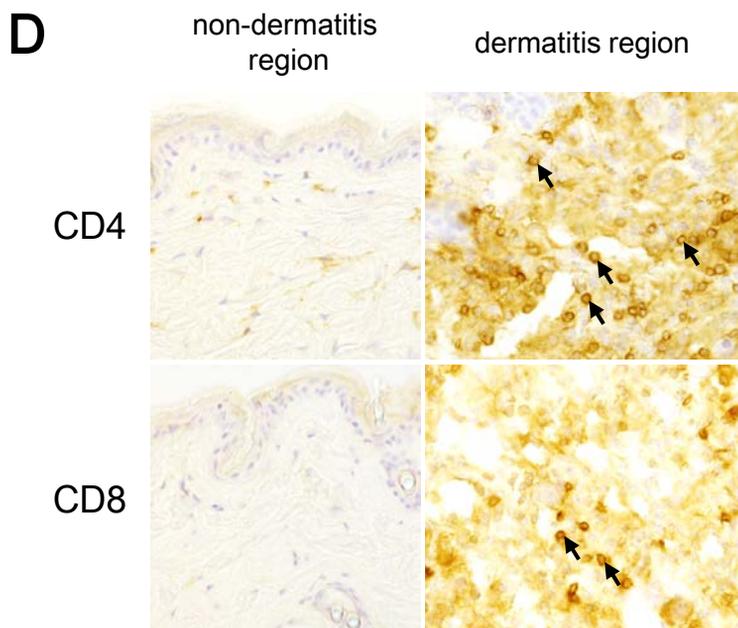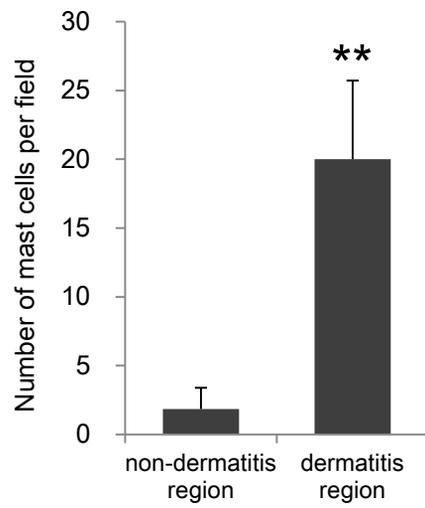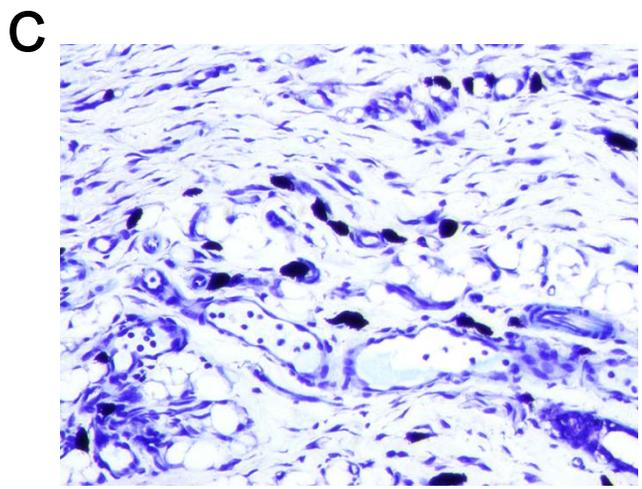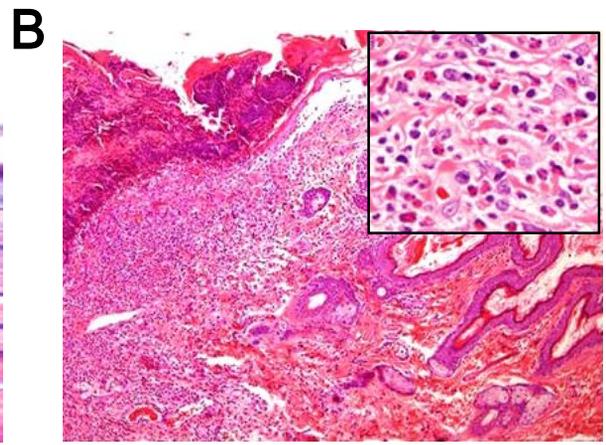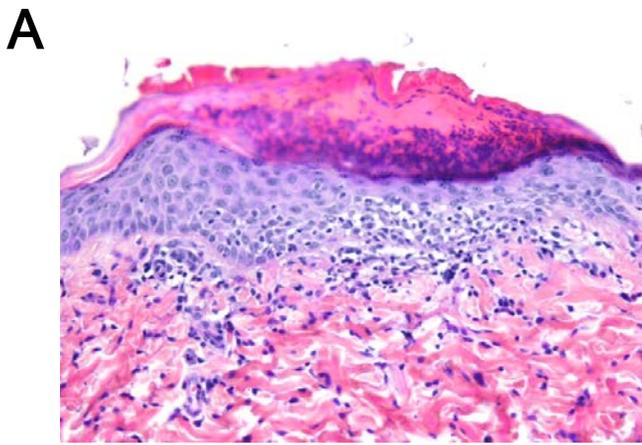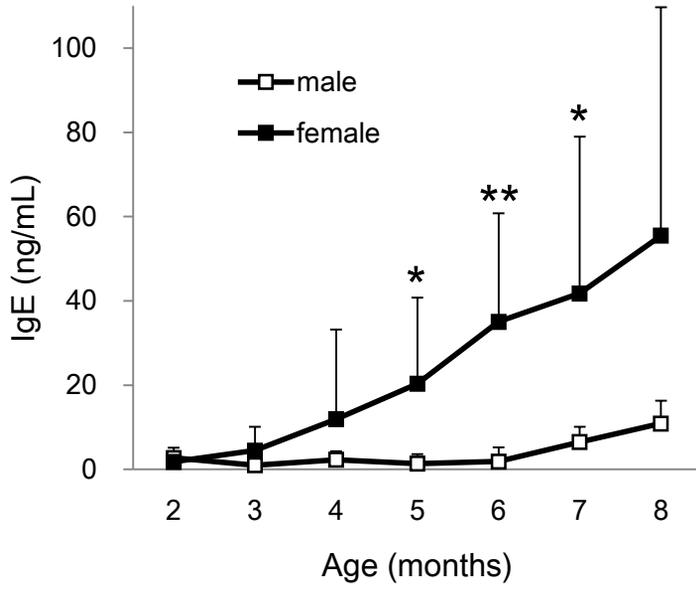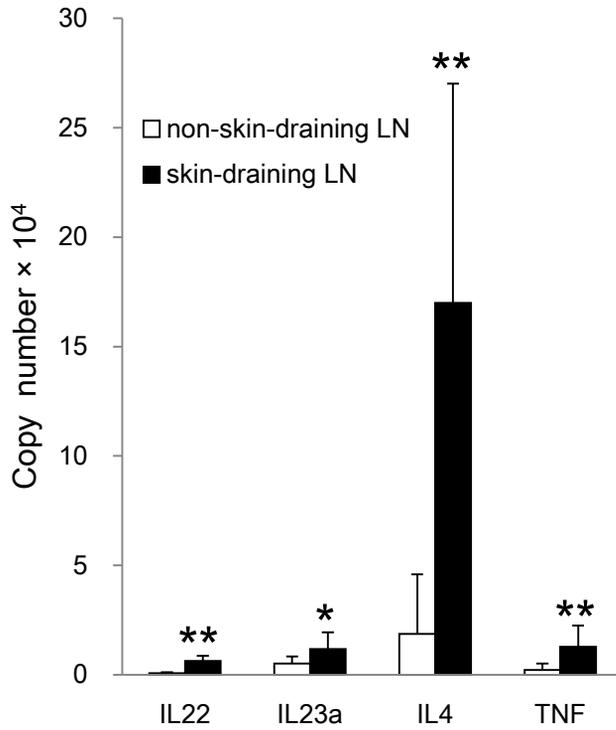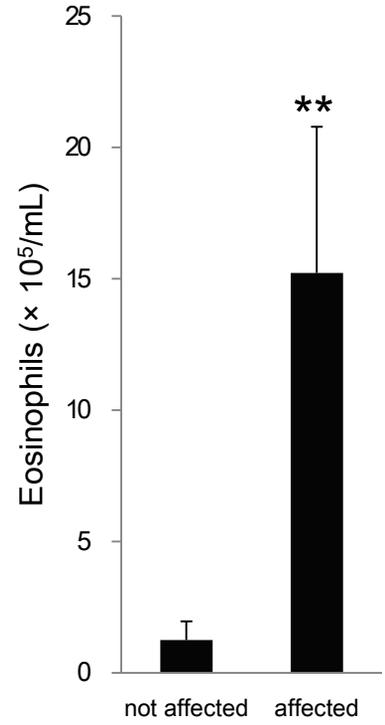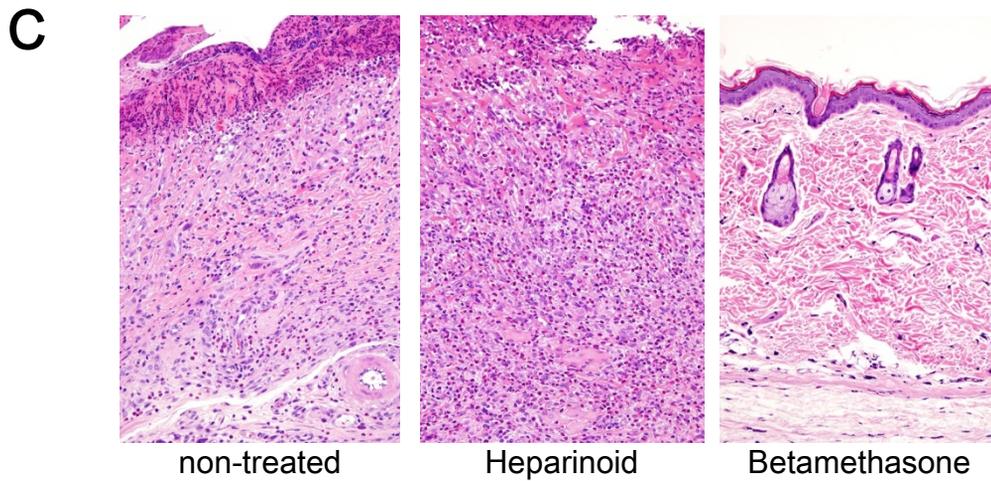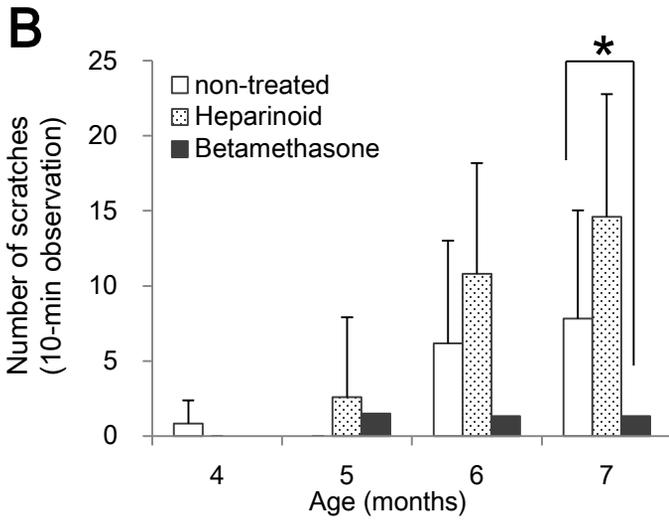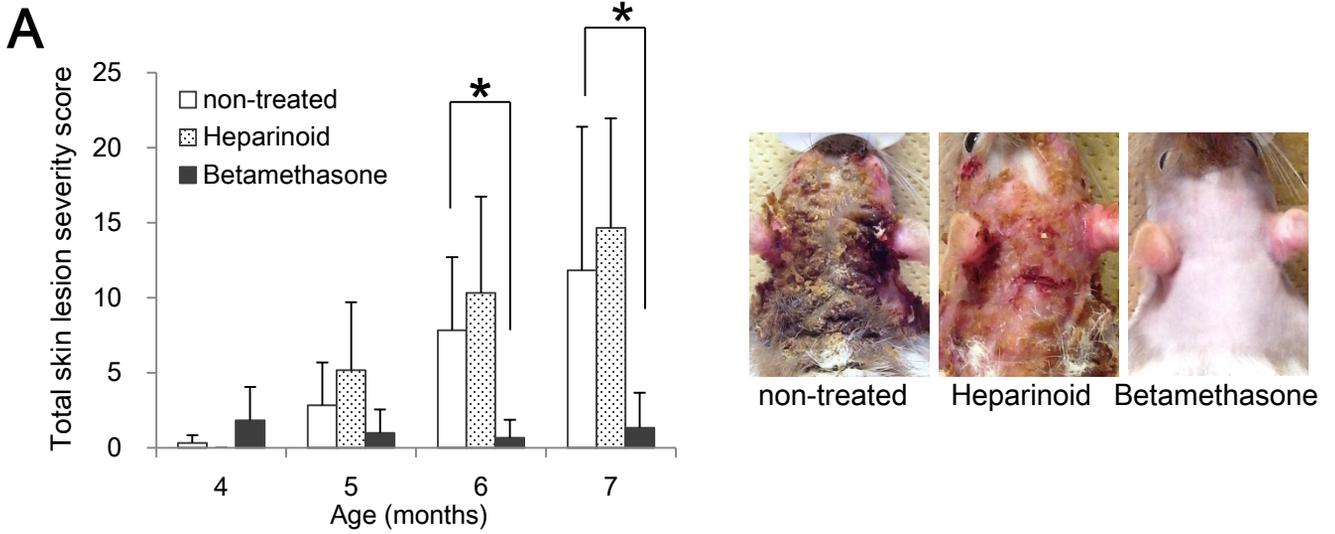
Figure 4

Supplementary Table 1 Primers used in the study

Symbol	Gene name	GenBank acc	Forward	Forward start	Reverse	Reverse start	Product size
Ifng	interferon gamma	NM_138880.2	AGGCCATCAGCAACAACATAAGTG	266	GACAGCTTTGTGCTGGATCTGTG	405	140
Il10	interleukin 10	NM_012854.2	CAGACCCACATGCTCCGAGA	110	CAAGGCTTGGCAACCCAAGTA	250	141
Il17a	interleukin 17A	NM_001106897.1	CTGATCAGGACGAGCGACCA	40	ACTGTAGCCTCCAGGTTCAGTAGCA	126	87
Il22	interleukin 22	NM_001191988.1	GATGCTCTGCCCATCAACTCC	94	CCCGATGAGCCTGACATCTG	228	135
Il23a	Interleukin 23, alpha subunit p19	NM_130410.2	TCCCGGAATCTCTGCACACT	416	CAGACCTTGGCGGATCCTT	601	186
Il4	interleukin 4	NM_201270.1	TGCACCGAGATGTTTGTACCAGA	169	TTGCGAAGCACCCTGGAAG	260	92
Ppia	peptidylprolyl isomerase A (cyclophilin A	NM_017101.1	GGCAAATGCTGGACCAAACAC	342	AAACGCTCCATGGCTTCCAC	476	135
Tnf	tumor necrosis factor	NM_012675.2	AACTCGAGTGACAAGCCCGTAG	400	GTACCACCAGTTGGTTGTCTTTGA	532	133