Th2 and Th17 Induce Dry Skin in a Mouse Model of Arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive destruction of cartilage and bone, with persistent chronic inflammation and pain as the main symptoms. Non-articular manifestations in the blood vessels, lungs, kidneys, and skin are observed in about 40% of patients with RA. Disruption of skin barrier function (i.e., dry skin), causing itching, is one symptom that can diminish the patient’s QOL. Itching is also associated with other diseases such as atop dermatitis and can result in injury to the stratum corneum and consequently, to the development of skin infections.

We previously reported that dry skin occurs in a mouse model of arthritis and that the mechanism involves mast cells and histamine release. Reactive oxygen species (ROS) and stress hormones have also been found to stimulate mast cells and other dry skin inducers. However, it is not known whether factors other than mast cells contribute to the induction of dry skin in arthritis mice.

Our earlier study confirmed that thymic stromal lymphopoietin (TSLP) level is increased in arthritic mice. TSLP is a master regulator of allergic reaction that is secreted by epithelial cells including keratinocytes, whereas TSLP receptor is expressed by hematopoietic cells including monocytes, mast cells, cluster of differentiation (CD)11c+ dendritic cells (DCs), T cells, and B cells. TSLP activates CD11c+ myeloid DCs, leading to the differentiation of naïve T cells into Th2 cells that produce IL-17, -21, and -22. IL-17 has been detected in skin affected by allergic dermatitis and psoriasis. The orphan nuclear receptor retinoid-related orphan receptor gamma t (RORγt) has been identified as a Th17 cell-specific transcription factor that cooperates with signal transducer and activator of transcription (STAT3) to induce production of IL-23 receptor, which further promotes Th17 cell differentiation and IL-17 transcription by enforcing RORγt expression. Moreover, IL-6-induced expression of RORγt is mediated by STAT3 activated by Janus kinase (JAK). Based on these findings, we speculated that cytokines released from Th2 and Th17 cells that are activated by DCs, induce dry skin in arthritis, and tested this hypothesis using a mouse model of RA.

MATERIALS AND METHODS

Animals Specific pathogen-free DBA/1JImmsLc mice (10 weeks old) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). These mice had previously been used as a collagen-induced arthritic mouse model. The animals were housed under a 12:12-h light/dark cycle at a constant temperature of 23 ± 2°C and 55 ± 10% relative humidity. The mice had free access to water and laboratory chow (CE-2; Oriental Yeast Co., Tokyo, Japan). After allowing the mice to breed for 1 week, the study was initiated and conducted for 5d. On the fourth day, hair on the dorsal skin was clipped and a depilatory cream (Veet Hair Removal Cream Tube Fit Sensitive; Reckitt Benckiser, Slough, U.K.) was used to remove the remaining hair to allow evaluation of dry skin. We had confirmed that the hair cream did not cause skin abnormalities in the mice. Skin and blood samples were collected on the final day of the experiment. All experiments were performed under sodium pentobarbital anaesthesia.

Experimental Design Untreated DBA/1JImmsLc mice and DBA/1JImmsLc collagen-induced arthritic mice served as the control and arthritis groups, respectively (n = 5 per group).
The mice were randomly assigned (n = 5 per group) to receive Th17 cell inhibitor (Wako Pure Chemical Industries, Ltd., Osaka, Japan; SP600125) by intraperitoneal injection (20 mg/kg resuspended in 0.1% dimethylsulfoxide) or Th2 cell inhibitor (TAIHO Pharmaceutical Co., Tokyo, Japan; IPD-1151T) by oral administration (30 mg/kg resuspended in distilled water) once a day for 4 d. SP600125 obstructs c-Jun N-terminal kinase (JNK), which is a signal transducer, and decreases Th17 cell.\(^{33}\) On the other hand, IPD-1151T suppresses cytokine release from Th2 cell.\(^{34}\) Control mice were administered the corresponding vehicles according to the same protocol. Experiments involving animals were carried out in accordance with internationally accepted standards, and were approved by the animal care committee of Suzuka University of Medical Science.

**Measurement of the Hind Limb Volume** On the final day, the hind limb volume of the mice—which reflects the rate of edema—was measured as previously described.\(^{28}\) Briefly, hind limbs were immersed in a beaker filled with distilled water and the displaced volume was measured.

**Measurement of Transepidermal Water Loss (TEWL)** On the final day, TEWL in the dorsal skin of each mouse was measured. TEWL serves as a marker of skin permeability, reflecting the barrier function of the skin; increased TEWL would indicate dry skin. Measurements were carried out using the Tewameter TM300 (Courage \& Khazaka Electronic GmbH, Cologne, Germany).\(^{29}\) Values were recorded once the reading had stabilized, typically 10 s after the probe was placed on the skin. Data are presented as the average of three independent measurements.

**Blood Analysis** On the final day of the experiment, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Nacalai Tesque, Kyoto, Japan) and blood samples were collected by cardiac puncture. Plasma was separated from blood samples by centrifugation at 3000 \(\times\) g for 10 min at 4°C, and the supernatant was used for protein concentration analysis. Plasma levels of IL-6 and -17, TSLP, and TNF-\(\alpha\) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (IL-6: BioLegend, San Diego, CA, U.S.A.; IL-17, TSLP, and TNF-\(\alpha\): R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s instructions. Optical density was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

**Histopathology** Dorsal skin tissue samples were isolated and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd.). Fixed tissue specimens were embedded in Tissue Tek OCT Compound (Sakura Finetek, Tokyo, Japan) and frozen. The tissue blocks were cut into 5-\(\mu\)m-thick sections that were stained with hematoxylin and eosin (H&E) for histopathological analysis of the tissue, according to conventional procedures. H&E stain the nucleus and cytoplasm, hence enabling histopathological determination of tissue condition. We evaluated the H&E-stained skin tissue under a microscope. To determine overall skin thickness, 10 regions where the skin appeared flat in acquired images were randomly selected and the length from the outer layer of epidermis to the border of subcutis was measured, and average value calculated. Additionally, skin specimens were stained with toluidine blue to visualize mast cells. We microscopically evaluated the skin tissue stained by toluidine blue following conventional procedures. The mast cells were quantified by counting their number per mm\(^2\) field in 10 randomly selected regions. Moreover, the tissue sections were washed with PBS and incubated overnight at 4°C with a primary antibody against the DC marker CD11c (1:50; Becton Dickinson and Company, NJ, U.S.A.). After washing in PBS and incubating at room temperature for 2 h with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (IgG)
(1:30; Dako Cytomation, Glostrup, Denmark), the number of DCs was quantified under a fluorescence microscopy using ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.).

**Western Blotting** Dorsal skin samples were homogenized in lysis buffer (Kurabo, Osaka, Japan) and then centrifuged at 8000×g for 10 min. The supernatant from each sample was collected and stored at −80°C until analysis. After thawing, equal amounts of protein (5 μg/lane) were loaded onto a 4–12% Bis-Tris Bolt gel (Life Technologies, Carlsbad, CA, U.S.A.) and electrophoretically separated at 200 V for 20 min. The proteins were then transferred using an iBlot Western blotting system (Life Technologies) to a nitrocellulose membrane that was blocked overnight at 4°C with 5% skim milk, and incubated at 25°C for 1 h with primary antibodies against CD11c (1:1000; Becton Dickinson), RORγt (1:1000; Bio-Byot, Cambridgeshire, U.K.), GATA3 (1:1000; Cell Signaling Technology, Danvers, MA, U.S.A.), and β-actin (1:5000; Sigma-Aldrich, St. Louis, MO, U.S.A.). The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the signal was detected with ImmunoStar Zeta (Wako Pure Chemical Industries, Ltd.) and a lumino-image analyzer (LAS-4000; FUJIFILM, Greenwood, SC, U.S.A.). Protein bands were analyzed by densitometry, and the signal intensity was normalized to that of β-actin.

**Statistical Analysis** Data are presented as the mean ± standard deviation. The Student’s t-test or Tukey’s test was used to evaluate the differences between groups, with p < 0.05 indicating statistical significance.

**RESULTS**

The chemical treatments had no effect on control (non-arthritic) mice (data not shown).

**DC Abundance in the Skin** Since DCs activate Th2 and Th17 cells that produce IL-6 or -17, or TNF-α, CD11c+ DCs in the skin of control and arthritic mice were detected by immunohistochemistry and subsequently quantified. The number of DCs was higher in arthritic mice as compared to control mice (Fig. 1A). This was confirmed by Western blot analysis of skin tissue lysates from the two groups (Fig. 1B).

**GATA3 and RORγt Expression in the Skin** The expression of GATA3 and RORγt—which are transcription factors expressed by Th2 and Th17 cells, respectively—was increased in arthritic mice as compared to control mice, as determined by Western blotting (Fig. 2).

**Comparison of Hind Limb Volumes** Since edema is a major symptom of arthritis, we evaluated the level of edema in each group by measuring hind limb volume. We found that the volume was greater in arthritic mice than in control mice; however, the difference between the two groups was abolished by treatment with Th2 or Th17 cell inhibitor (Fig. 3).

**Histopathological Analysis of Skin** We measured dorsal skin TEWL in each group of mice as a measure of skin dryness. Arthritic mice had higher TEWL than control mice, but

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**Fig. 3. Effect of Th Cell Inhibitor Treatment on Hind Limb Volume in Arthritic Mice**

Data are presented as mean ± standard deviation (n = 5). *p < 0.05 (Tukey’s test).

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**Fig. 4. Effect of Th Cell Inhibitor Treatment on the Skin Condition of Arthritic Mice**

(A) Measurement of TEWL from the dorsal skin of mice on the day after depilation. (B) H&E staining of skin tissue sections. Scale bar = 100μm. Overall skin thickness was estimated based on the average length from the outer layer of the epidermis to the border of the subcutis in 10 randomly selected fields for each sample. Data are presented as mean ± standard deviation (n = 5). *p < 0.05 (Tukey’s test). (Color figure can be accessed in the online version.)
this was alleviated by treatment with Th2 or Th17 cell inhibitor (Fig. 4A). Additionally, hematoxylin and eosin staining of skin tissue samples revealed that the arthritic mice had thicker skin than control mice, although this difference was abolished by treatment with Th17 cell inhibitor. However, Th2 cell inhibitor treatment resulted in thicker skin in the control as compared to arthritic mice (Fig. 4B).

**Plasma TSLP Levels** We previously reported that TSLP, a master regulator of immune response, was upregulated in arthritic mice.\(^{10}\) We therefore measured plasma TSLP concentration by ELISA and found that the level was higher in arthritic mice than in control mice, and was unaltered by treatment with Th2 or Th17 cell inhibitor (Fig. 5).

**Mast Cell Abundance in the Skin** Our previous study had suggested that mast cell activation induces skin dryness;\(^{9}\) we therefore quantified the number of mast cells in skin specimens stained with toluidine blue. The number of mast cells was higher in arthritic mice relative to the control group (Fig. 6). Treatment with Th17 cell inhibitor abolished the difference between the two groups, whereas Th2 cell inhibitor had no effect.

**DISCUSSION** The results of this study demonstrate that the number of DCs was increased in the skin of arthritic mice as compared to control mice. Additionally, the numbers of GATA3\(^+\) Th2, ROR\( \gamma \)\(^t\) Th17, and mast cells were higher in the arthritic mice, which also exhibited increased TEWL, skin thickness, and plasma levels of TSLP and IL-6 and -17.

DCs are immunocompetent cells that are involved in antigen presentation and induce differentiation and proliferation of T and B cells. In this study, we examined the role of Th2 and Th17 cells—which are thought to differentiate from DCs—in the induction of dry skin associated with arthritis. The up-regulation of GATA3 and ROR\( \gamma \)\(^t\) expression in skin samples from arthritic mice, relative to that in control mice, indicate that Th2 and Th17 cells contribute to the induction of dry skin in this model. This was confirmed by treating the mice with an inhibitor of cytokine release from Th2 cells or a c-Jun N-terminal kinase inhibitor that suppresses Th17 cells. We have thus confirmed that these inhibitors have a specific cell suppressing effect (Fig. S1).

TSLP level is increased in the arthritic mice.\(^{10}\) TSLP is stimulated by ROS and not only activates mast cells, but also stimulates myeloid DCs, resulting in the differentiation of naïve T cells into Th2 cells through upregulation of the
In conclusion, the results of our study demonstrate that induction of dry skin in arthritis involves activated DCs that stimulate Th2 and Th17, which act through distinct mechanisms. Specifically, skin dryness caused by Th2 cells occurs through inflammatory cytokines such as IL-6 and TNF-α, reactive oxygen species are produced from these immune cells. Therefore, we considered that hind limb edema did not change, since Th2 and Th17 cell inhibitors could not suppress the articular destruction factors (neutrophils, macrophages, and ROS). These results suggest that dry skin in arthritis is induced by secondary factors (e.g., Th2- and Th1-related cytokines) that are upregulated in this disease. We also found that plasma levels of IL-6 and -17 and TNF-α were elevated in the arthritic mice; however, this trend was reversed by treatment with Th2 cell inhibitor. In case of Th17 cell inhibitor administration, plasma levels of IL-17 were decreased whereas those of IL-6 and TNF-α remained unchanged. To explain the decrease of IL-17, eomesdermin (Eomes) was considered to be involved. Eomes is inhibited through the c-jun transcription factor by JNK, which is a phosphorylated enzyme; as a result of which Th17 cells increase. Since SP600125, used in our study, inhibits JNK, Eomes is considered to have increased and Th17 cells to have decreased. Th17 cells mainly secrete IL-17 while IL-6 is important for the induction of naïve T cells into Th17 by DCs. The levels of cytokines other than of IL-17 were unaffected by Th17 inhibition, since IL-6 and TNF-α are mainly secreted by Th2 and mast cells, respectively. On the other hand, when the levels of IL-6 and -17 and TNF-α secreted by Th2 cells were reduced by administration of Th2 cell inhibitor, dry skin was alleviated. It is likely that the amount of IL-6 and TNF-α decreased owing to the suppression of cytokine release from Th2 cell by IPD-1151T (e.g., IL-4, -5, -6). We propose that the decrease in IL-6 suppresses IL-17 by blocking the DC-induced differentiation of naïve T cells into Th17 cells. However, the extent to which IL-17 is downregulated is likely negligible since IL-6 from other sources, such as mast cells, compensates for IL-17. In addition, arthritic mice treated with Th17, had fewer mast cells in the skin than the untreated ones. Since IL-17 secreted by Th17 promotes the function of mast cell progenitors, we speculate that inhibition of Th17 cells reduced mast cell counts in the skin. Moreover, thickness of the skin differed for the treatment with Th2 or Th17 cell inhibitor. There could be a relationship between skin thickness and the number of mast cells. In this study, we observed increased mast cells and increased thickness of skin in arthritic mice. In the arthritic mice treated with Th2 cell inhibitor, since there was no alteration in the expression of mast cells, and the thickness of skin did not change. On the other hand, when treated with Th17 cell inhibitor, since the number of mast cells decreased, thickness of the skin was thought to have decreased as well.

In this study, however, we have not attempted using both inhibitors as a combination treatment. When both Th2 and Th17 cell inhibitors are used, further amelioration of dry skin is expected, owing to the inhibition of excess secretion of IL-6 and TNF-α, and also inhibition of increase in mast cell number at the same time. Further studies would be required to validate this phenomenon.

CONCLUSION

OX40L/CCL17 gene. In addition, Th17 cells are activated by DCs and implicated in diseases associated with increased TEWL. In the present study, plasma level of TSLP was higher in arthritic mice as compared to control mice (Fig. 5). However, administration of Th2 or Th17 cell inhibitor abolished the difference between the two groups, suggesting that Th2 and Th17 act downstream of TSLP.

TEWL is an indicator of skin dryness, with higher values reflecting water loss from the epidermis. Various factors can contribute to an increase in TEWL. Our previous study on the skin of an arthritic mice revealed that the increase in mast cell number and TEWL is parallel; the mast cells are associated with dry skin. Moreover, owing to the blockage of histamine release from mast cell, the increase in TEWL was inhibited. Based on these considerations, mast cell and TEWL were assumed to be closely associated. In the context of arthritis, Th2 cells activate GATA3 and also the release of TNF-α and IL-6, which induce joint destruction and chronic inflammation, and may reduce skin collagen levels, leading to an increase in TEWL. In typical diseases associated with increased TEWL, the transcription of IL-17 and differentiation of Th17 cells may reduce skin collagen levels, leading to an increase in TEWL. In arthritic mice as compared to control mice (Fig. 5). However, administration of Th2 or Th17 cell inhibitor abolished the difference between the two groups, suggesting that Th2 and Th17 act downstream of TSLP.

To confirm the role of Th2 and Th17 in the development of dry skin, mice were administered a Th2 or Th17 cell inhibitor, which reduced TEWL and improved dry skin. However, hind limb edema was not ameliorated. The arthritis mouse model, used in this study, produces an autoantibody by recognizing type II collagen of a different kind as an antigen; edema occurs when the autoantibody attacks the articular cartilage that uses type II collagen as a formation component. This phenomenon involves neutrophils and macrophages, and

Fig. 7. Effect of Th Cell Inhibitor Treatment on Plasma Inflammatory Cytokine Levels in Arthritic Mice and Control Mice

IL-6 and -17 and TNF-α were detected by ELISA. Data are presented as mean ± standard deviation (n = 5). *p < 0.05 (Tukey’s test).
whereas that due to Th17 cells is induced by IL-17 and mast cells. Thus, Th2, Th17, and mast cells interact to induce dry skin in arthritis (Fig. 8). These findings provide a basis for the future development of therapeutics to alleviate dry skin in patients with RA and other diseases and thereby improve their QOL.

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Conflict of Interest The authors declared no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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