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**Mucocutaneous Inflammation in the Ikaros Family Zinc Finger 1 (*IKZF1*) - keratin 5
specific transgenic mice**

(Mucocutaneous Inflammation in *IKZF1* transgenic mice)

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Keywords

Ikaros Family Zinc Finger 1 (*IKZF1*), epithelial cells, mucocutaneous inflammation

Contribution statement

Experiment: MU, HN, NN, KS, KM, YH, ETM, NY

Analysis: MU, HN, NN, ETM

Write manuscript: MU, JH

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conflicts of interest: none

Abstract

Background: Our genome-wide association study documented an association between cold medicine related Stevens-Johnson syndrome / toxic epidermal necrolysis (CM-SJS/TEN) and Ikaros Family Zinc Finger 1 (*IKZF1*). Few studies examined biological and pathological functions of *IKZF1* in mucosal immunity. We hypothesized that *IKZF1* contributes to the mucocutaneous inflammation.

Methods: Human skin and conjunctival tissues were obtained for immunohistological studies. Primary human conjunctival epithelial cells (PHCjECs) and adult human epidermal keratinocytes (HEKa) also used for gene expression analysis. We also generated K5-Ikzf1-EGFP transgenic mice (Ikzf1 Tg) by introducing the Ik1 isoform into cells expressing keratin 5, which is expressed in epithelial tissues such as the epidermis and conjunctiva and then examined them histologically and investigated gene expression of the epidermis. Moreover, Ikzf1 Tg were induced allergic contact dermatitis.

Results: We found that human epidermis and conjunctival epithelium expressed IKZF1, and in PHCjECs and HEKa, the expression of IKZF1 mRNA was up-regulated by stimulation with polyI:C, a TLR3 ligand. In *Ikzf1* Tg, we observed dermatitis and mucosal inflammation including the ocular surface. In contact dermatitis model, inflammatory infiltrates in the skin of *Ikzf1* Tg were significantly increased compared with wild type. Microarray analysis showed that *Lcn2*, *Adh7*, *Epgn*, *Ifi202b*, *Cdo1*, *Gpr37*, *Duoxa1*, *Tnfrsf4*, and *Enpp5* genes were significantly up-regulated in the epidermis of *Ikzf1* Tg compared with wild-type.

Conclusion: Our findings support the hypothesis that Ikaros might participate in mucocutaneous inflammation.

Introduction

Ikaros is a transcription factor that regulates numerous biological events. IKZF1 is upregulated in granulocytes, B cells, CD4- and CD8-T cells, and in natural killer (NK) cells; it is downregulated in erythroblasts, megakaryocytes, and monocytes (1). Ikaros-null mice lack B-lineage cells, NK cells, peripheral lymph node- and fetal T-cells (2-4). Mice with the mutated Ikaros gene (*Ikzf1*) develop thymic lymphoma with high penetrance (1, 5). Thus, Ikaros family members regulate important cell-fate decisions in the development of the adaptive immune system (6).

While many studies addressed the genetic association of Ikaros family members with hematopoiesis and hematopoietic abnormalities, few addressed their biological and pathological functions in mucosal immunity. In a genome-wide association study (GWAS), *IKZF1* was established as a new susceptibility gene for systemic lupus erythematosus (SLE) (7).

In our earlier GWAS, we found an association between Ikaros Family Zinc Finger 1 (*IKZF1*) and cold medicine related Stevens-Johnson syndrome / toxic epidermal necrolysis (CM-SJS/TEN) with SOC (Severe ocular complications) in Japanese patients and also identified an association between *IKZF1* single nucleotide polymorphisms (SNPs) and CM-SJS/TEN with SOC not only in Japanese, but also in Korean and Indian populations (8). Interestingly, the ratio of Ik2:Ik1, splicing variants of *IKZF1* mRNA isoforms, was significantly associated with *IKZF1* SNPs (8). The relative quantity of Ik2, the dominant-negative form, was significantly lower in individuals with the susceptible genotype of *IKZF1* SNPs, suggesting that in CM-SJS/TEN with SOC presenting as mucocutaneous inflammation, the function of Ikaros, the protein of *IKZF1*, might be enhanced (8).

In the mucosal interface, multiple communications occur with their external environments. Not simply as a barrier, but the mucosal interface is also crucial in coordinating the ensuing immune response to control the inflammation driven by internal signals. We have reported that epithelium might contribute to the pathobiology of CM-SJS/TEN with SOC (9), because EP3, was dominantly expressed on the epithelium such as ocular surface epithelium (10), airway epithelium (11) and epidermis (12), and EP3 on the epithelium negative regulated inflammation such as ocular surface inflammation (13), airway inflammation (11) and dermatitis (12). Furthermore, we also reported that TLR3, was strongly expressed on the epithelium such as ocular surface epithelial cells (9, 14, 15) and keratinocytes (16), and regulated ocular surface inflammation (17) and dermatitis (16).

Thus, we produced K5-Ikzf1-EGFP transgenic mice (Ikzf1 Tg) by introducing the Ik1 isoform into cells expressing keratin 5, which is expressed in epithelial tissues such as the epidermis and conjunctiva. We found that polyI:C, a TLR3 ligand, upregulated *IKZF1* expressed in epidermal keratinocytes and conjunctival epithelial cells. In Ikzf1Tg mice mucocutaneous inflammation was exacerbated, indicating that *IKZF1* plays a critical role in

maintaining mucocutaneous homeostasis. Thus, *IKZF1* may participate in the aggravation of the mucocutaneous inflammation of CM-SJS/TEN with SOC.

Materials and Methods

Primary human conjunctival epithelium

This study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients after a detailed explanation of the purpose of the research and the experimental protocols.

To harvest human conjunctival epithelium, conjunctival tissues obtained at conjunctivochalasis surgery were immersed overnight in 1.0 U ml⁻¹ purified dispase (Roche Diagnostic Ltd., Basel, Switzerland) at 4°C. For immunohistological analysis, conjunctival tissues obtained as above were used. For real-time quantitative PCR (RT-qPCR), primary human conjunctival epithelial cells (PHCjECs) were cultured by a modified, previously-described method (18). Briefly, detached epithelial cells were grown in CnT-prime epithelial culture medium (CELLNTEC, Bern, Switzerland) and 1% antibiotic-antimycotic solution. Cell colonies usually became obvious within 3 to 4 days. After reaching 80% confluence in 7 to 10 days, the cultured PHCjECs were used in the subsequent procedures.

Primary human epidermis

Human skin tissues were washed, immersed overnight in 1.0 U ml⁻¹ purified dispase (Roche Diagnostic) at 4°C, and then the epidermis was detached. For immunohistochemistry, human skin tissues were prepared from normal skin samples obtained at the time of surgical resection of skin tumors. For rqPCR we used adult human epidermal keratinocytes (HEKa) (Life Technologies Corp., CA, USA).

Purification of human peripheral mononuclear cells

Procedures described elsewhere were applied (18). We obtained peripheral venous blood samples from consenting volunteers. The samples were anti-coagulated with 2Na-EDTA and mononuclear cells were isolated in lymphoprep tubes (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions.

RT-PCR

We analyzed the *in vivo* human conjunctival epithelium and the *in vivo* primary human epidermis for *IKZF1* mRNA expression as described elsewhere (19). Briefly, total RNA was isolated from tissues and human peripheral mononuclear cells using the RNeasy Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. For the RT reaction we used ReverTraAce (TOYOBO, Japan). Cultured HEKa cells and PHCjECs were harvested after PolyI:C (InvivoGen, San Diego, CA) stimulation. A StepOne plus (Applied Biosystems) instrument was used for rqPCR. The primers and probes were purchased from Applied Biosystems. The quantification data were normalized to the expression of the housekeeping gene GAPDH. PCR amplification was with DNA polymerase (Takara TaqTM; (Takara Shuzo

Co. Ltd., Shiga, Japan); the conditions were 95°C for 5 min, 40 cycles for IKZF1 and 28 cycles for GAPDH followed by 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, with final extension at 72°C for 7 min on a commercial PCR machine (GeneAmp PCR system9700; PE Applied Biosystems, Foster City, CA). The specific primers for IKZF1 were 5'-AATGAATGGCTCCCACAGGG-3' and 5'-AGCTCTTACGTTTGGCGACA -3'; for GAPDH they were 5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTCACCACCTTCTTG-3'. RNA integrity was assessed in ethidium bromide-stained 1.5% agarose gels.

Immunohistology of IKZF1 in human skin and conjunctival tissue sections

Sections were fixed for 10 min at 4°C with 4% PFA/0.1M phosphate-buffered saline (PBS), incubated overnight in a moist chamber at 4°C with goat anti-human Ikaros polyclonal antibody (ab106787; abcam, Cambridge, UK) or isotype control goat IgG (Dako Cytomation, Kyoto, Japan), and then washed in PBS without Ca and Mg [PBS(-)]. Alexa Fluor 488 donkey anti-goat IgG (H+L) (Molecular Probes Inc., Eugene, OR) was applied for 1 hr at room temperature. After washing the slides, antifade mounting medium with propidium iodide was applied (Vectashield; Vector Laboratories Inc., Burlingame, CA).

Generation of K5-Ikzf1-EGFP transgenic mice

The vector constructed to generate the transgenic mouse includes a human *keratin5* (*KRT5*) gene promoter sequence (20), 1545 bp mouse cDNA sequence encoding *Ikzf1* isoform 1 protein, 57 bp porcine teschovirus-1 cDNA sequence encoding 2A peptide, 720 bp cDNA

sequence encoding EGFP and bovine growth hormone polyA (bGH pA) sequence. Expression unit composed of Iκzf1, 2A peptide, EGFP cDNA sequences and bGH pA was chemically synthesized and cloned into plasmid vector containing *KRT5* promoter. Promoter and expression unit were excised out from plasmid vector backbone and microinjected into the pronuclei of fertilized eggs of BALB/c mice (Transgenic Inc. Fukuoka, Japan). Founder transgenic mice were selected by PCR using following primer sets, K5p-F1; ATG CCA ATG CCC CCT CAG TTG CT and BGH-R3; AGC TGG TTG TTT CCG CCT CAG to amplify entire expression unit, and K5p-F6; AAT AAC TTG CCC CAG GTG ACG and K5p-R2; AGG CTT CAGAGT CTA TAC TCC to amplify 5' region of KERATIN5 promoter sequence. These primer sets were also used for genotyping of offspring of founder mice.

For real-time PCR, the skin were harvested from mice which is within 1 week old, and immersed in 1.0 U ml⁻¹ purified dispase for overnight at 4°C, and then epidermis were detached and collected.

Microarray analysis

Microarray analysis was with Affymetrix GeneChip® mouse gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). We used approximately 35,557 probe sets with a resolution of 26 probes per gene, covering more than 28,853 genes. Throughout the process we followed Affymetrix instructions. Microarray-scanned images were obtained on a GeneChip Scanner 3000 7G (Affymetrix) using the default settings. Images were visually inspected to eliminate hybridization artifacts.

Induction of allergic contact dermatitis with 2, 4, 6-trinitro-1-chlorobenzene (TNCB)

Mice were sensitized by applying 25 µl of 1% (w/v) TNCB in acetone/olive oil (4:1) or 25 µl of vehicle alone to the shaved abdominal skin. Seven days later, 20 µl (10 µl on the dorsal- and 10 µl on the ventral side) of 1% TNCB were applied to the right ear. The same amount of acetone/olive oil (4:1) was placed on the left ear as a control. The ear thickness was measured at a predetermined site with a dial thickness gauge (Shinwa Rules, Niigata, Japan). The ear-swelling response, measured before- and 24 hr after the elicitation of contact dermatitis, was defined as the pre- and post-elicitation ear thickness.

Data analysis

Data were expressed as the mean $\pm$ SE and evaluated by the Student's *t*-test using the Excel program.

Results

1. IKZF1 expression in human keratinocytes and conjunctival epithelial cells

For the first time, we confirmed that human epidermis and conjunctival epithelium express *IKZF1* mRNA, like in human mononuclear cells. The expected length of the PCR products for *IKZF1* (554 bp) was confirmed for both tissues; their length was as in human mononuclear cells (Fig. 1A). Sequencing of the PCR products identified the molecular entity of the samples. Immunohistochemistry detected the *IKZF1* protein in the nucleus of both epidermal- and conjunctival epithelial cells (Fig. 1B).

2. Upregulation of *IKZF1* expression by polyI:C stimulation

Elsewhere we reported that TLR3 was strongly expressed on the epithelium by, for example, ocular surface epithelial cells (14) and keratinocytes (16) and that TLR3 SNPs were significantly associated with SJS/TEN with SOC in a Japanese study population (21, 22). Therefore, we examined whether polyI:C would upregulate the expression of *IKZF1*. We found that its expression was maximally upregulated 24 hr after polyI:C stimulation in primary HEKa cells (Supplementary Fig. 1). The significant upregulation of *IKZF1* expression was confirmed both in HEKa cells (Fig. 2a) and in PHCjECs (Fig. 2b).

3. Dermatitis and ocular surface inflammation in K5-*Ikzf1*-EGFP transgenic mice

To investigate the function of *IKZF1* in the epidermis and conjunctival epithelium, we produced K5-*Ikzf1*-EGFP transgenic (*Ikzf1* Tg) mice. They developed dermatitis with hair loss (Fig. 3A); some developed blepharoconjunctivitis (Fig. 3B).

Histological analysis also showed dermatitis (Fig. 4) in the transgenic mice; we observed mild thickening of the epidermis and moderate infiltration of the dermis by inflammatory cells. Moreover, histologic study showed that the tissue inflammation in the blepharoconjunctiva (Fig.5a), tongue (Fig.5b), and paronychia (Fig.5c), was accompanied by mild inflammatory cell infiltration in some *Ikzf1* Tg mice.

4. GeneChip analysis of the epidermis of *Ikzf1* Tg mice

Next we investigated the difference in gene expression in the epidermis of Tg and WT mice. Microarray analysis revealed that the number of 31 genes was more than doubled in the epidermis of *Ikzf1* Tg- than WT mice ($p < 0.05$) (Supplementary Table 1). We performed

qPCR assay on 18 genes whose difference between Tg and WT mice was $p < 0.01$. The quantification showed that *Lcn2*, *Adh7*, *Epgn*, *Ifi202b*, *Cdo1*, *Gpr37*, *Duoxa1*, *Tnfrsf4*, and *Enpp5* genes were significantly upregulated in the epidermis of *Ikzf1* Tg mice (Fig. 6). At least four of these genes, i.e. *Lcn2*, *Epgn*, *Ifi202b*, *Cdo1*, and *Tnfrsf4*, might play roles in the onset or induction of tissue inflammation or cell migration, including the repression of tumor incidence.

5. Allergic contact dermatitis in *Ikzf1* Tg mice

Since TLR3 elicited a contact hypersensitivity response such as allergic contact dermatitis (16), we hypothesized that IKZF1 intervenes in the pathway from TLR3 triggering to the contact hypersensitivity response. Therefore, we challenged *Ikzf1* Tg mice with allergic contact dermatitis on the ear with TNCB and counted the infiltrated inflammatory cells. We found that while infiltrated cells in the ear skin of *Ikzf1* Tg mice treated with vehicle only were not significantly increased compared with wild type mice, there was a significant increase in their number in TNCB-challenged *Ikzf1* Tg mice compared with TNCB-challenged wild type mice. Histological study of the ear skin 24 hr post-TNCB challenge revealed extensive leukocyte infiltration in the dermis of *Ikzf1* Tg mice (Figs. 7a-c).

Discussion

Our study revealed that human epidermis and conjunctival epithelium express IKZF1 mRNA and protein, and that IKZF1 expression was upregulated by TLR3-triggering in epidermal keratinocytes and conjunctival epithelial cells. This is the first finding describing the expression of functional Ikaros in mucosal tissues. Our findings may open the new way to the better understanding of mucosal inflammation.

Functions of IKZF1 isoforms involved in hematopoiesis and hematopoietic malignancies have been studied extensively. Research on the pathological role of these isoforms has focused on SLE with complex genetic inheritance. A GWAS identified *IKZF1* as a new SLE susceptibility gene (7). Foxp3⁺ Helios⁺Treg cells with migratory potential into inflamed tissues are increased in active SLE to compensate for autoreactive effector responses (23). We also identified an association between *IKZF1* SNPs and CM-SJS/TEN with SOC not only in Japanese, but also in Korean and Indian populations (8).

Stevens-Johnson syndrome (SJS) involves an acute inflammatory vesiculobullous reaction of the skin and mucosa present on the ocular surface, in the oral cavity, and in genitals. In patients with extensive skin detachment and a poor prognosis, the condition is known as toxic epidermal necrolysis (TEN). About 40% of SJS/TEN patients diagnosed by dermatologists develop SOC (24). Cold medicines including multi-ingredient cold medications and nonsteroidal anti-inflammatory drugs (NSAIDs) were implicated in SJS/TEN with SOC and about 80% of our patients developed SJS/TEN with SOC after taking cold medicines a few days before disease onset (CM-SJS/TEN with SOC) (8, 25).

K5-Ikzf1-EGFP Tg mice developed mucocutaneous inflammation, e.g. dermatitis with hair loss, tissue inflammation in the blepharoconjunctiva, tongue, and paronychia, that are seen in the acute stage of SJS/TEN patients (9). We reported that TLR3, whose SNPs were significantly associated with CM-SJS/TEN with SOC in the Japanese (21, 22), were strongly expressed in epithelial cells on the ocular surface (14, 22), in keratinocytes (16), and in these tissues the inflammation is positively regulated by TLR3(16, 17). We suggest that those earlier and our current findings support the hypothesis that Ikaros upregulated by TLR3 triggering might participate in the mucocutaneous inflammation of CM-SJS/TEN with SOC.

In this context, it is of note that the upregulation of *Lcn2*, *Epgn*, *Cdo1*, and *Tnfrsf4* genes was more pronounced in the epidermis of *Ikzf1* Tg- than WT mice (Fig. 7). LCN2 was released from a colonic epithelial cell line by the TLR-3 ligand polyI:C (26). Local mucosal epithelial cells mediate ocular allergic inflammation via the OX40L-OX40 (*Tnfrsf4*) signaling pathway (27). Cysteine dioxygenase (*CDO1*) is involved in the biodegradation of toxic cysteine (28) and epigen (*EPGN*) is the most recently discovered EGFR ligand (29). Dual oxidase maturation factor 1 (*DUOXA1*) has been associated with the maturation of the reactive oxygen species (ROS) producing enzyme, and it was reported that *DUOXA1* overexpression increases reactive oxygen species production (30). It was also reported that interferon-inducible gene 202b controls CD8 β T cell-mediated suppression in anti-DNA Ig peptide-treated lupus mice (31). These gene products (albeit not all) may participate in the onset or induction of mucosal tissue inflammation.

There is evidence that the regulatory networks up- and downstream of Ikaros are critical for normal lymphopoiesis (32). These networks may also play a pivotal role in the skewing of activated T-cell subsets that are involved in Th1-, Th2-, and Th17-type inflammation. In *Ikzf1* Tg mice, both Th1 and Th2 cells were significantly upregulated in splenic CD4 $^{+}$ and CD8 $^{+}$ T-cells (Supplementary Fig. 2). On the other hand, Th17 cells were upregulated only in CD4 $^{+}$ T- but not in CD8 $^{+}$ T-cells. The smaller P value in Th1 cells than in Th2 cells might indicate that Ikaros is more relevant in the activation of Th1 than Th2 cells. To finalize the precise contribution of Th subsets to the mucocutaneous inflammation in *Ikzf1* Tg mice, far more detailed study on local and systemic polarization will be needed.

Our preliminary experiments show that *Ikzf1* gene expression in the *K5-Ikzf1-EGFP* Tg was partially downregulated in *K5-Ikzf1-EGFP* Tg, and that among upregulated genes in *K5-Ikzf1-EGFP* Tg compared with wild type, *Ifi202b* and *Gpr37* were significantly downregulated in *tlr3KO K5-Ikzf1-EGFP* Tg mice, on the other hand, *Agh7* were significantly

upregulated in *tlr3KO K5-Ikzf1-EGFP* Tg mice (Supplementary Fig.3). These findings might suggest that *Ikzf1* inducible genes could be partially controlled by TLR3.

The silencing of IKZF1 mRNA with siRNA in HEKa cells led to the reduced gene expression of MCP-1 and IL-6 mRNA but not of IL-8 (Supplementary Fig.4). This suggests that the elevated expression of Ikaros in epithelial cells might lead to the upregulation of the pro-inflammatory cytokines MCP-1 and IL-6 that have been found to be upregulated in the tears of human SJS/TEN patients in the acute stage (33).

Elsewhere we presented our hypothesis that the onset of SJS/TEN with SOC is strongly associated with anomalies in the innate immune system (9). IKZF1, as a susceptibility gene for CM-SJS/TEN with SOC, is upregulated by TLR3 triggering. Considering that SJS/TEN may also be induced by infectious pathogens or danger-associated molecular patterns (DAMPs), the findings we present here do not exclude the possibility that other innate immune receptors on mucosal tissues participate in the upregulation of Ikaros. Functional gene-gene interactions such as TLR3-EP3 (22) and TLR3-IKZF1 in CM-SJS/TEN with SOC as reported here, suggest that an imbalance in the network comprised of susceptibility genes contributes to the pathogenesis of SJS/TEN with SOC.

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Figure Legends

Figure 1.

IKZF1 expression in human keratinocytes and conjunctival epithelial cells

- A. Human epidermal- and conjunctival epithelial cells, like human mononuclear cells, express *IKZF1* mRNA.
- B. Immunohistochemistry detected IKZF1 protein in the nucleus of both epidermal-and conjunctival epithelial cells.

Figure 2

The upregulated IKZF1 mRNA expression

- a. The expression of *IKZF1* was significantly upregulated in HEKa cells 24 hr after polyI:C stimulation. Each group n=4. Representative data of 6 independent experiments are shown. *p < 0.01
- b. The expression of *IKZF1* was significantly upregulated in primary human conjunctival epithelial cells 24 hr after polyI:C stimulation. Each group n=6. Representative data of 6 independent experiments are shown. ***p < 0.0000001

Figure 3

Phenotype of K5-Ikzf1-EGFP transgenic (Ikzf1 Tg) mice

- a. Dermatitis with hair loss
- b. Blepharoconjunctivitis

Figure 4

Histological analysis of murine skin

Note mild thickening of the epidermis and moderate infiltration by inflammatory cells in the dermis of the abdominal- (a) and dorsal skin (b). Bar: 100 μ m

Figure 5

*Tissue inflammation detected by histological analysis in *Ikzf1* Tg mice*

- a. Blepharoconjunctiva: Mild inflammatory cell infiltration
- b. Tongue: Mild inflammatory cell infiltration
- c. Paronychia: Mild inflammatory cell infiltration

Bar: 100 μ m

Figure 6

*Elevated allergic contact dermatitis in *Ikzf1* Tg mice*

- a. There was no significant difference in the number of cells infiltrating the ear skin of vehicle-only-treated control WT- and *Ikzf1* Tg mice.
- b. In *Ikzf1* Tg mice challenged with TNCB, but not in WT mice, there is a significant increase in the number of infiltrating cells.

*p < 0.01, **p < 0.005

- c. Histology of the ear skin 24 hours post-TNCB challenge revealed extensive leukocyte infiltration in the dermis of *Ikzf1* Tg mice.

Figure 7

GeneChip analysis of the epidermis of Ikzf1 Tg mice

Gene expression analysis of the epidermis of Tg and WT mice not older than 2 weeks showed that the upregulation of *Lcn2*, *Adh7*, *Epgn*, *Cdo1*, *Gpr37*, *Tnfrsf4*, and *Enpp5* genes was more marked in the epidermis of *Ikzf1* Tg- than WT mice. Representative data of 4 independent experiments are shown.

*p < 0.05, **p < 0.005, ***p < 0.0005

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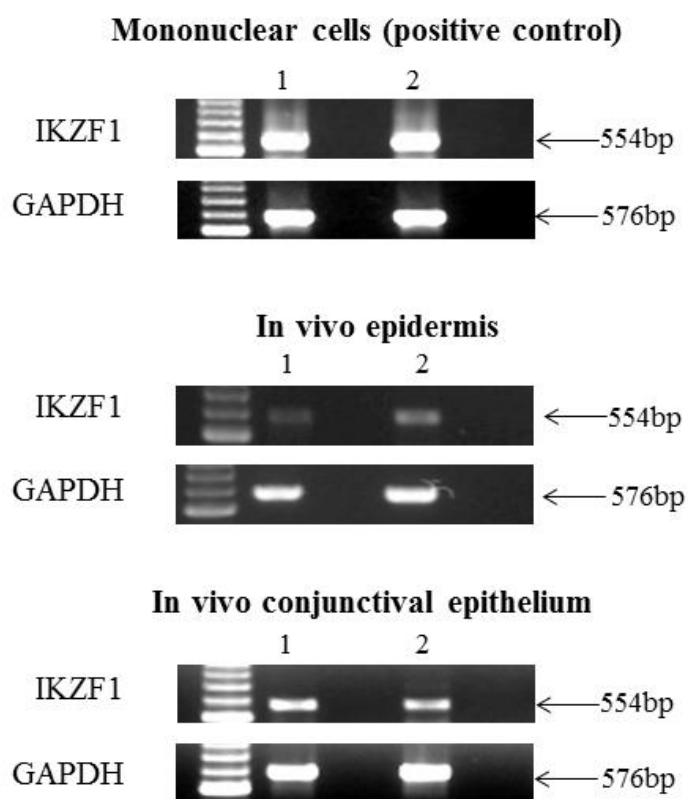
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Figure 1

A.



B.

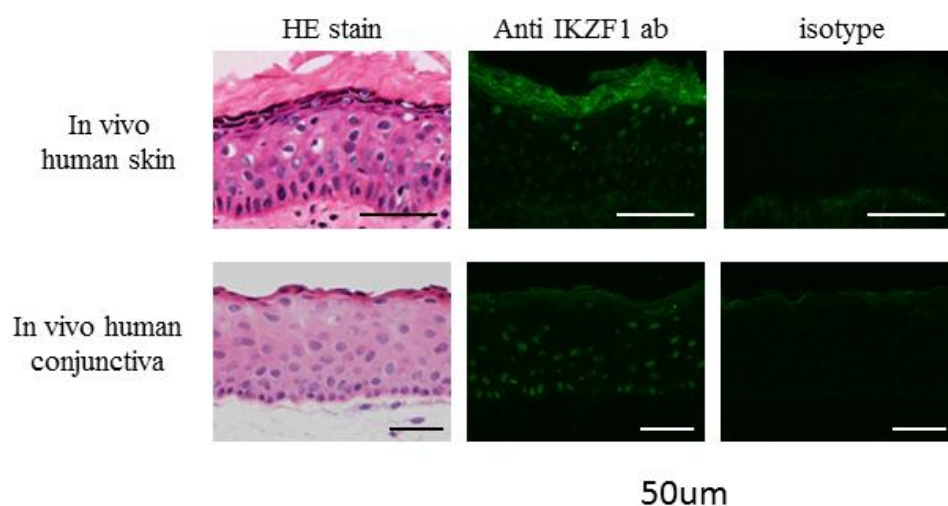


Figure 2

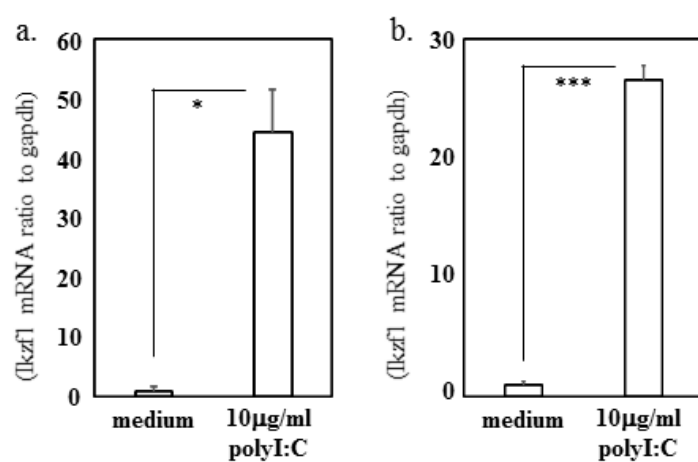
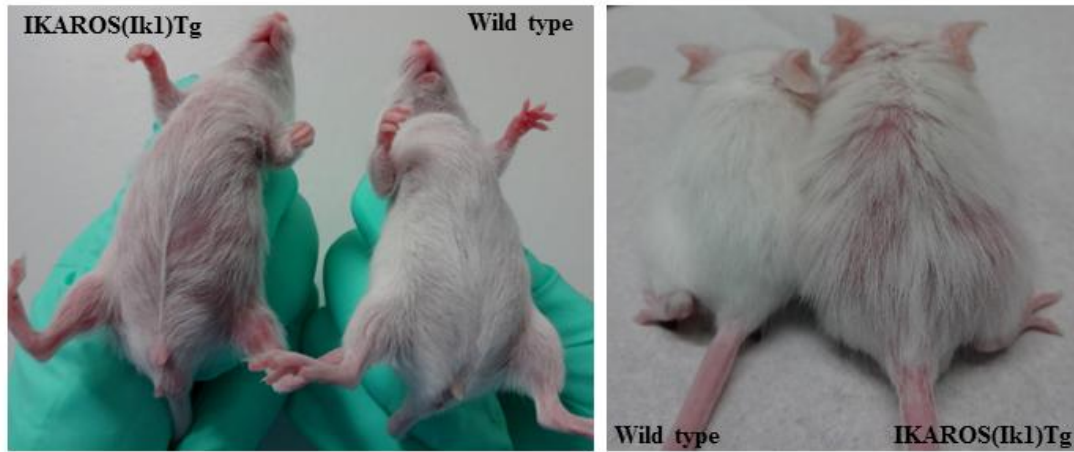


Figure 3

a.



b.

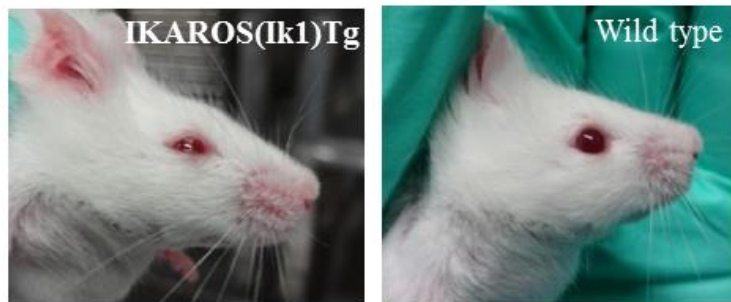


Figure 4

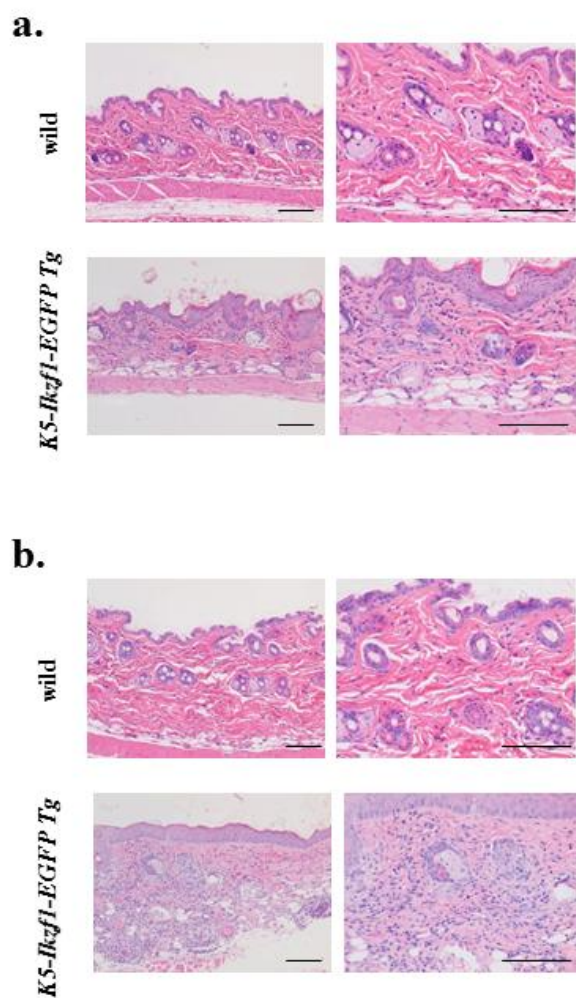


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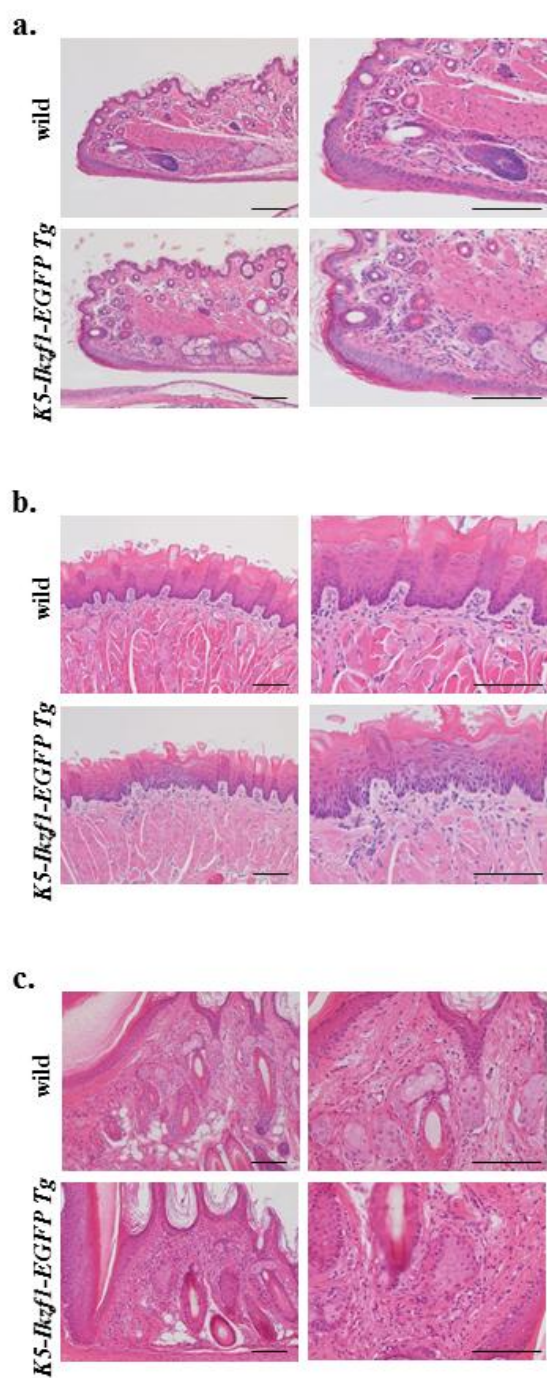


Figure 6

A.

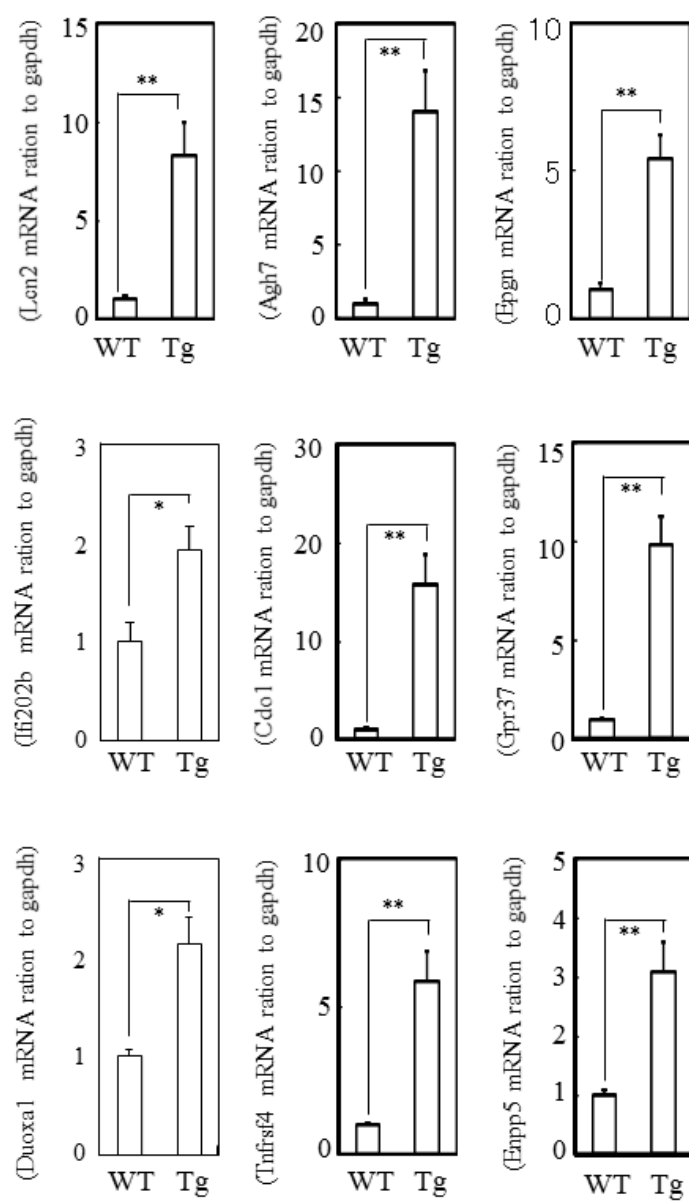
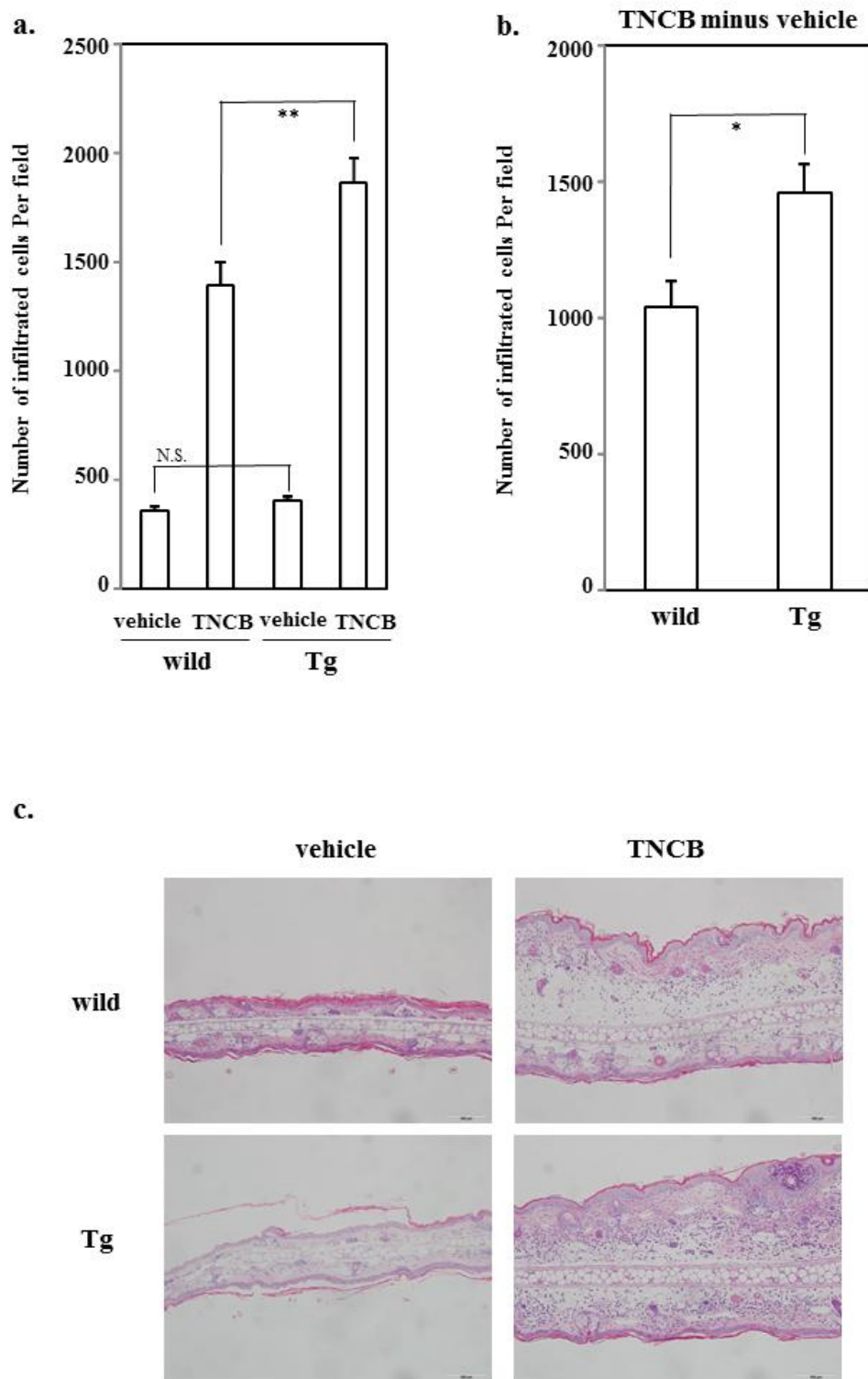
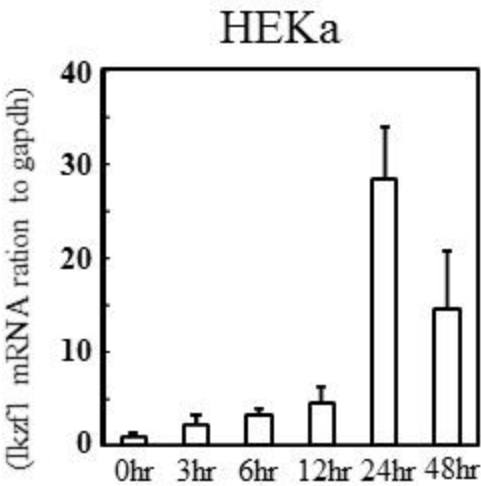


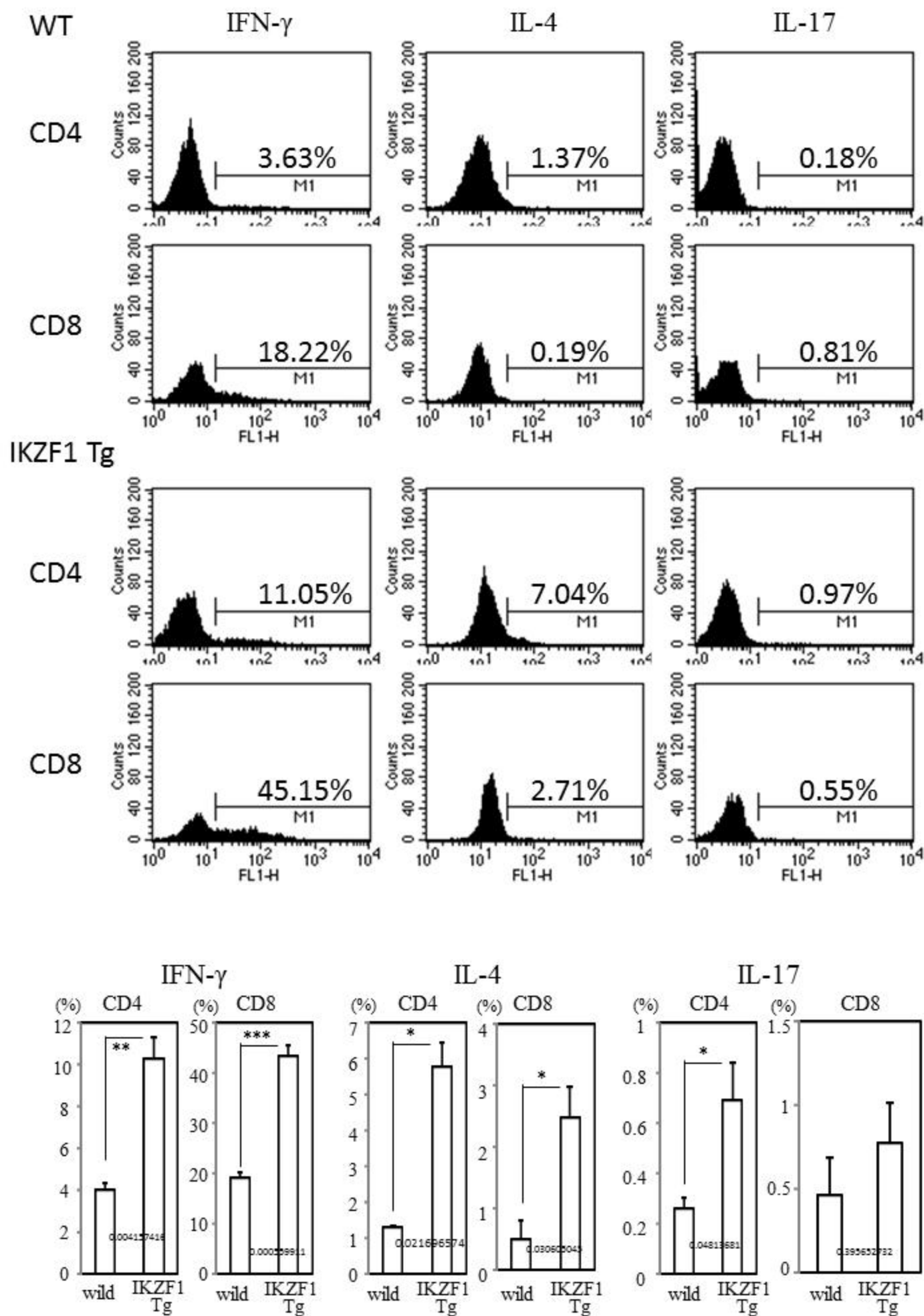
Figure 7



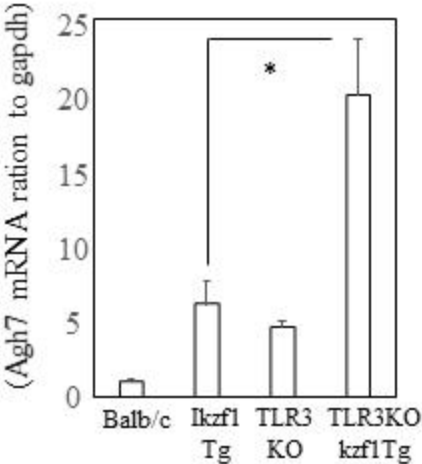
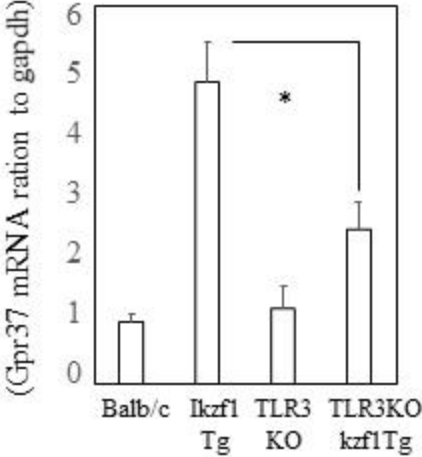
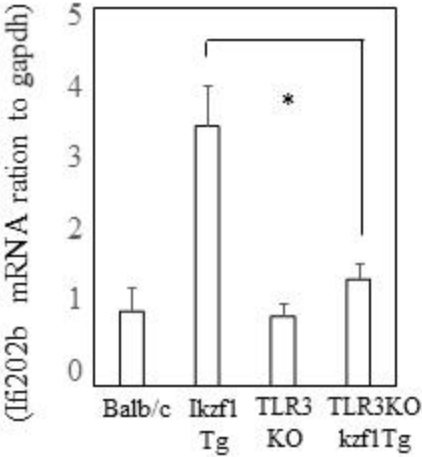
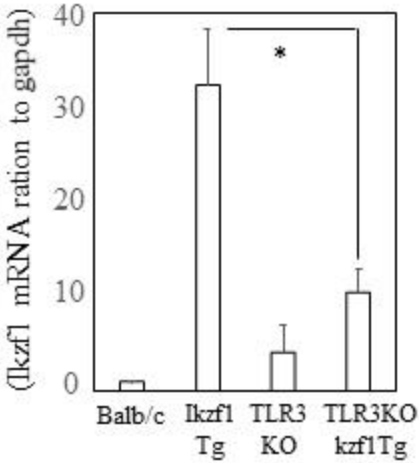
Supplementary Figure 1



Supplementary Figure 2

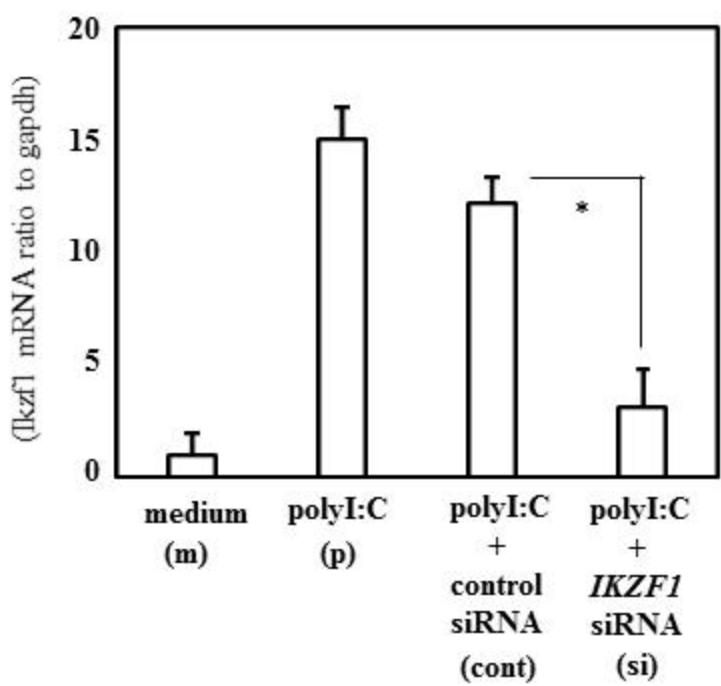


Supplementary Figure 3



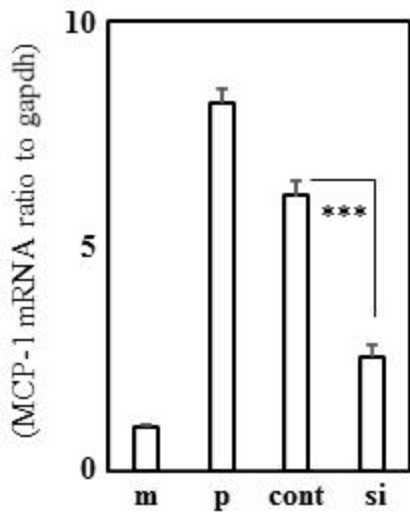
Supplementary Figure 4

A.

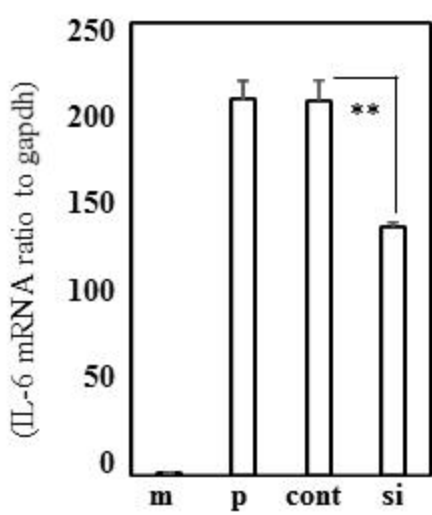


B.

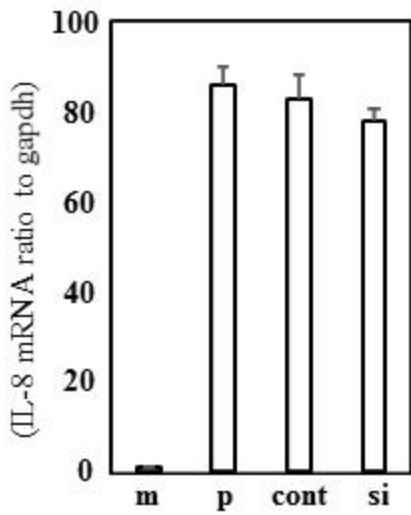
MCP-1



IL-6



IL-8



Elevated Expression of Ikaros Family Zinc Finger 1 (*IKZF1*) Exacerbates Mucocutaneous Inflammation

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Supplementary Methods

Flow cytometry and intracellular cytokine analysis

Phycoerythrin (PE)-conjugated anti-CD4- (eBioscience, San Diego, CA), anti-CD8a (eBioscience)-, fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ - (eBioscience), anti-IL-4- (eBioscience), and anti-IL-17A antibodies (Biolegend, London, UK) were used. Isotype-matched antibodies were the control reagents. Single-cell suspensions of mouse spleens were prepared. For intracellular cytokine staining, cells were incubated for 4 hr with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich). Surface staining was with anti-CD4 antibody (30 min, 4°C). After permeabilization with IntraStain according to the manufacturer's instructions (Yamasa Corp. Tokyo, Japan), the cells were stained for IFN- γ , IL-4, and IL-17 and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

TLR3KO $Ikzf1$ Tg mice

TLR3KOIkzf1Tg mice were generated by interbreeding of TLR3KO and Ikzf1Tg mice at Kyoto Prefectural University of Medicine. For real-time PCR, the skin were harvested from mice which is within 1 week old, and immersed in 1.0 U ml⁻¹ purified dispase for overnight at 4°C, and then epidermis were detached and collected. The mice were maintained on a 12-hour/12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan. All studies were

performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Silencing of IKZF1 expression

To suppress expression of the *IKZF1* gene we used siRNA methods. For siRNA, esiRNA from human IKZF1 (MISSION(R), Sigma-Aldrich, MO) was transfected into HEKa cells using the Lipofectamin RNA9MAX reagent (Life Technology), 24 hr after transfection with siRNA we added polyI:C (10 ug/ml). HEKa cells were harvested for real-time quantitative PCR assay 24 hr post-stimulation.

Supplementary Figure Legends

Supplementary Figure 1

Time-course of *IKZF1* mRNA expression in HEKa cells stimulated with polyI:C. Each group n=4. Representative data from 3 independent experiments are shown.

Supplementary Figure 2

Peripheral T-cell balance in *Ikzf1* Tg mice

- a. In *Ikzf1* Tg mice, both Th1 and Th2 cells were significantly upregulated in both splenic CD4⁺ and CD8⁺T-cells. Representative data of 3 independent experiments are shown.
- b. The p values were calculated using the % data from 3 independent experiments.

Supplementary Figure 3

- A. *Ikzf1* mRNA expression in the TLR3KO*ikzf1*Tg mice
- B. Downregulated genes in the TLR3KO*ikzf1*Tg mice
- C. Upregulated genes in the TLR3KO*ikzf1*Tg mice

Supplementary Figure 4

- A. Confirmation that IKZF1 mRNA was significantly downregulated with IKZF1 siRNA but not with control siRNA
- B. Downregulation of MCP-1 and IL-6 mRNA but not of IL-8 by IKZF1 siRNA in HEKa cells.

in medium, n=4, polyI:C, n=4, polyI:C plus control siRNA, n=13, polyI:C plus IKZF1

siRNA; n=13. Representative data of 3 independent experiments are shown.

*p < 0.0005, **p < 0.00005, ***p<0.000005