



Establishment of a Mouse Model of Atopic Dermatitis by Deleting *Ikk2* in Dermal Fibroblasts

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Atopic dermatitis is a chronic inflammatory skin disease with persistent pruritus. To clarify its molecular mechanism, it is important to establish a mouse model similar to the phenotypes of atopic dermatitis patients, particularly in exhibiting scratching behavior. *Ikk2*, a component of the I κ B kinase complex, exerts pro-inflammatory responses, whereas its deficiency in keratinocytes paradoxically causes skin inflammation. In this study, we sought to generate a mouse model exhibiting skin inflammation by which dermal fibroblasts lack *Ikk2* expression and evaluate whether cutaneous inflammatory phenotypes are similar to those of atopic dermatitis patients. To generate *Ikk2*-deficient mice (*Nestin^{cre};Ikk2^{FL/FL}*) in which *Ikk2* is deleted in dermal fibroblasts, we crossed female *Ikk2^{FL/FL}* mice to male *Nestin^{cre};Ikk2^{FL/+}* mice. These mice spontaneously developed skin inflammation limited to the face, with the appearance of *Ikk2*-deficient fibroblasts in the facial skin. These mice showed phenotypes similar to those of atopic dermatitis patients, including scratching behaviors, which are resistant to immunosuppressive or molecularly targeted drugs. These findings suggest that the *Nestin^{cre};Ikk2^{FL/FL}* mouse is an atopic dermatitis model that will be useful in clarifying atopic dermatitis pathogenesis and in developing a novel therapeutic agent for atopic dermatitis symptoms.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with persistent pruritus (Furie et al., 2017). Type 2 and T helper type 22 inflammation are dominant in its pathogenesis and IL-4 and IL-13, signature type 2 cytokines, play important roles in generating the clinical features of AD (Furie et al., 2017). Topical corticosteroids and immunosuppressive drugs are used as basic treatments. Recently, dupilumab, an antagonist of both IL-4/IL-13 signals, was approved as the first molecularly targeted drug for treating AD patients. However, a large number of AD patients are resistant to topical steroids (Izadi and Leung, 2018) or immunosuppressive drugs

(Reitamo et al., 2000). The efficacy of dupilumab was reported to be limited in its phase 3 trial (Simpson et al., 2016). This could be attributed to heterogeneity of the pathogenesis of AD (Bieber, 2012). Therefore, it is necessary to clarify the molecular mechanism behind the heterogeneity of AD and to develop novel therapeutic agents for each subtype.

Thus far, many mouse models are available in which cutaneous allergic inflammation occurs spontaneously (Jin et al., 2009). However, although AD-like inflammation develops in their skins, fewer than half of these models exhibit scratching behavior. Moreover, there is a gap in the transcriptome profiles of inflamed skins between model mice and AD patients (Ewald et al., 2017). Therefore, it is important to establish a mouse model similar to the phenotypes of AD patients, particularly in exhibiting persistent pruritus.

Nestin is known as a marker of neural stem cells. However, it turns out that *nestin* is ubiquitously expressed in various embryonal and fetal tissues in which neural crest cells (NCCs) are involved (Wiese et al., 2004). NCCs represent a multifated embryonic cell population and appear in different forms in virtually all tissues, generating a wide diversity of cell types (Dupin and Sommer, 2012). The origins of fibroblasts vary depending on the site; many fibroblasts in the face originate in NCCs, whereas fibroblasts in the trunk come from the dermomyotome (Driskell and Watt, 2015). These findings suggest that most fibroblasts in the dermis of the face are *nestin*-positive, whereas most fibroblasts in the dermis of the trunk are not. *Nestin^{cre}* is a mouse strain expressing Cre recombinase under the control of rat *nestin* promoter/enhancer. In *Nestin^{cre}*, neural cells, as well as somite- and

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Abbreviations: AD, atopic dermatitis; BM, bone marrow; BMMC, bone marrow-derived mast cell; NCC, neural crest cell; TNF, tumor necrosis factor

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NCC-derived mesenchymes, have Cre recombinase activity (Dubois et al., 2006). From these results, we can target NCC-derived facial fibroblasts using *Nestin^{cre}*.

The IkkB kinase complex has three components: two serine-threonine kinases (Ikk1 and Ikk2) and the regulatory subunit NEMO/Ikk γ . The Ikk complex elicits pro-inflammatory responses by mediating NF- κ B activation. However, the NF- κ B pathway paradoxically reflects anti-inflammatory responses in skin biology (Pasparakis, 2012). Systemic deficiency of NEMO/Ikk γ causes skin inflammation, together with hyperproliferation of keratinocytes (Makris et al., 2000). This phenotype is similar to incontinentia pigmenti, which is caused by genetic mutations in the *NEMO* gene and is characterized by multiple disorders, including skin lesions (Smahi et al., 2000). Keratinocyte-specific deficiency of Ikk2 in turn causes tumor necrosis factor (TNF)-dependent skin inflammation (Pasparakis et al., 2002). Moreover, deficiencies in other NF- κ B pathway-related molecules—Ikk1, RelB, SHARPIN, and TAK1—result in similar skin inflammation (Barton, et al., 2000; Liu et al., 2011; Omori et al., 2006; Potter et al., 2014). However, it remains elusive whether impairment of the NF- κ B pathway in keratinocytes is critical or that of fibroblasts can also generate inflammation.

To examine the role of Ikk2 in Nestin-expressing cells, we generated conditional Ikk2-deficient mice using *Nestin^{cre}* (*Nestin^{cre};Ikk2^{FL/FL}*), which surprisingly showed inflammatory phenotypes close to those of AD patients. We found that the skin inflammation would appear by deficiency of Ikk2 in the facial dermal fibroblasts. Thus, we, in this study, established a model of AD by deleting Ikk2 in dermal fibroblasts.

RESULTS

Ikk2 ablation under the expression of nestin causes facial skin inflammation

To delete Ikk2, we crossed *Nestin^{cre}* with *Ikk2^{FL/FL}*. Both floxed alleles of *Ikk2^{FL/FL}* are specifically ablated in *Nestin^{cre};Ikk2^{FL/FL}* (*Ikk2^{ΔNES}*) (Figure 1a). These mice developed eczema only in the face by postnatal day 8–10 (Figure 1b). Twelve weeks after birth, scale, erythema, erosion, and severe hemorrhaging appeared in the face (Figure 1c).

X-gal- and Ikk2-stained sections from *Ikk2^{ΔNES};Rosa26^{lacZ}* showed that recombination in the Ikk2-deficient dermal mesenchymal cells, but not in epidermal cells, mast cells, or eosinophils. X-gal intensity was more prominent in the facial skin than in the chest skin (Figure 1d and Supplementary Figure S1 online). In *Nestin^{cre};Rosa26^{RFP}*, RFP⁺ cells were limited in CD45⁺ cells in the dermis of both facial and dorsal skins. The number of RFP⁺ cells was significantly higher in facial skin than in dorsal skin (Figure 1e). Moreover, the CD45⁺/RFP⁺ cells were CD140a (a mesenchymal marker)-positive, but CD31 (an endothelial marker)-negative (Figure 1f, 1g). These results demonstrate that *Nestin^{cre}*-derived Ikk2 ablation causes the appearance of Ikk2-deficient fibroblasts in the facial skin, which subsequently leads to spontaneous appearance of skin inflammation limited to the face.

Ikk2^{ΔNES} mice exhibit AD-like skin inflammation and scratching

AD patients suffer from severe pruritus and systemic changes, such as elevation of serum IgE and periostin; as well as histologic changes, such as acanthosis, spongiosis, and

hyper- and parakeratosis (Furie et al., 2017; Murota and Katayama, 2017). We examined whether *Ikk2^{ΔNES}* mice exhibit these AD-like phenotypes. *Ikk2^{ΔNES}* exhibited extensive itch behaviors as they grew up (Figure 2a and Supplementary Figure S2 online). Serum IgE and periostin levels, systemic biomarkers reflecting type 2 inflammation, were significantly increased in *Ikk2^{ΔNES}* (Figure 2b). Moreover, histologic analyses showed increases in keratinocyte proliferation, eosinophil/mast cell number, and periostin deposition, like the features observed in AD skin (Figure 2c–2e and Supplementary Figure S1).

However, expression of cytokeratin 10, loricrin, and filaggrin proteins increased in *Ikk2^{ΔNES}* face skin. Expression of *Flg*, *Lor*, and *Ivr*, terminal differentiation genes that are important for barrier function, was increased also at mRNA level, which differs from the characteristics of both *K14-Cre;Ikk2^{FL/FL}* (Pasparakis et al., 2002) and AD patients (Furie et al., 2017) (Figure 2e and Supplementary Figure S3 online). These results suggest that *Ikk2^{ΔNES}* mice exhibit AD-like skin inflammation and scratching, but not barrier dysfunction.

Characterization of inflammation in the lesional skin of *Ikk2^{ΔNES}* mice

Types 2 and T helper type 22 inflammation are dominant in the pathogenesis of AD (Furie et al., 2017). However, some reports demonstrate the involvement of other types of inflammation (Bieber, 2012; Guttman-Yassky and Krueger, 2017), suggesting the heterogeneity of AD pathogenesis. We next characterized inflammation in the lesional skin of *Ikk2^{ΔNES}* (Figure 3a). Expression of all type 2 inflammation-related genes (*Il4*, *Il5*, *Il9*, *Il13*, *Tslp*, and *Postn*), type 17 inflammation-related genes (*Il17a*), and the IL-10/20 family of genes (*Il10*, *Il19*, *Il20*, and *Il24*) increased considerably, whereas expression of type 1 inflammation-related genes (*Ifng* and *Il12b*) and T helper type 22 type inflammation-related gene (*Il22*) did not change.

It is of note that NF- κ B-targeted genes (*Il1b*, *Il18*, *Il36a*, *Il36b*, *Il36g*, *Il6*, and *Tnf*) were also increased, although *Ikk2*-knockout mouse embryonic fibroblasts showed impaired nuclear translocation of NF- κ B p65 and p50 subunits in response to TNF or IL-1 β stimulation (Figure 3b). Interestingly, expression of itch-related genes (*Trpv1/2*, *Il31*, and *Il31ra*) was not enhanced, suggesting that the pruritus mechanism would be independent of the TRPV1/2 or IL-31.

Both the type 2 cytokines and the IL-10/20 family of cytokines exert their effects via the JAK-STAT signaling pathway, particularly STAT1 and STAT3 (Commins et al., 2008). We have shown that phosphorylation of STAT3 and STAT6, a critical transcriptional factor for the IL-4/IL-13 signals, increases in inflamed AD skin (Mitamura et al., 2018). In the skin of *Ikk2^{ΔNES}*, pSTAT3⁺, and pSTAT6⁺ cells increased in the epidermis and the dermis, respectively (Figure 3c). These results suggest that the inflamed skin of *Ikk2^{ΔNES}* dominantly exhibits type 2, type 17, and the IL-10/20 family type, as well as NF- κ B-related inflammation.

Tissue-resident cells in *Ikk2^{ΔNES}* mice are crucial for the development of skin inflammation

To exclude the possibility that bone marrow (BM)-derived leukocytes might contribute to the development of skin

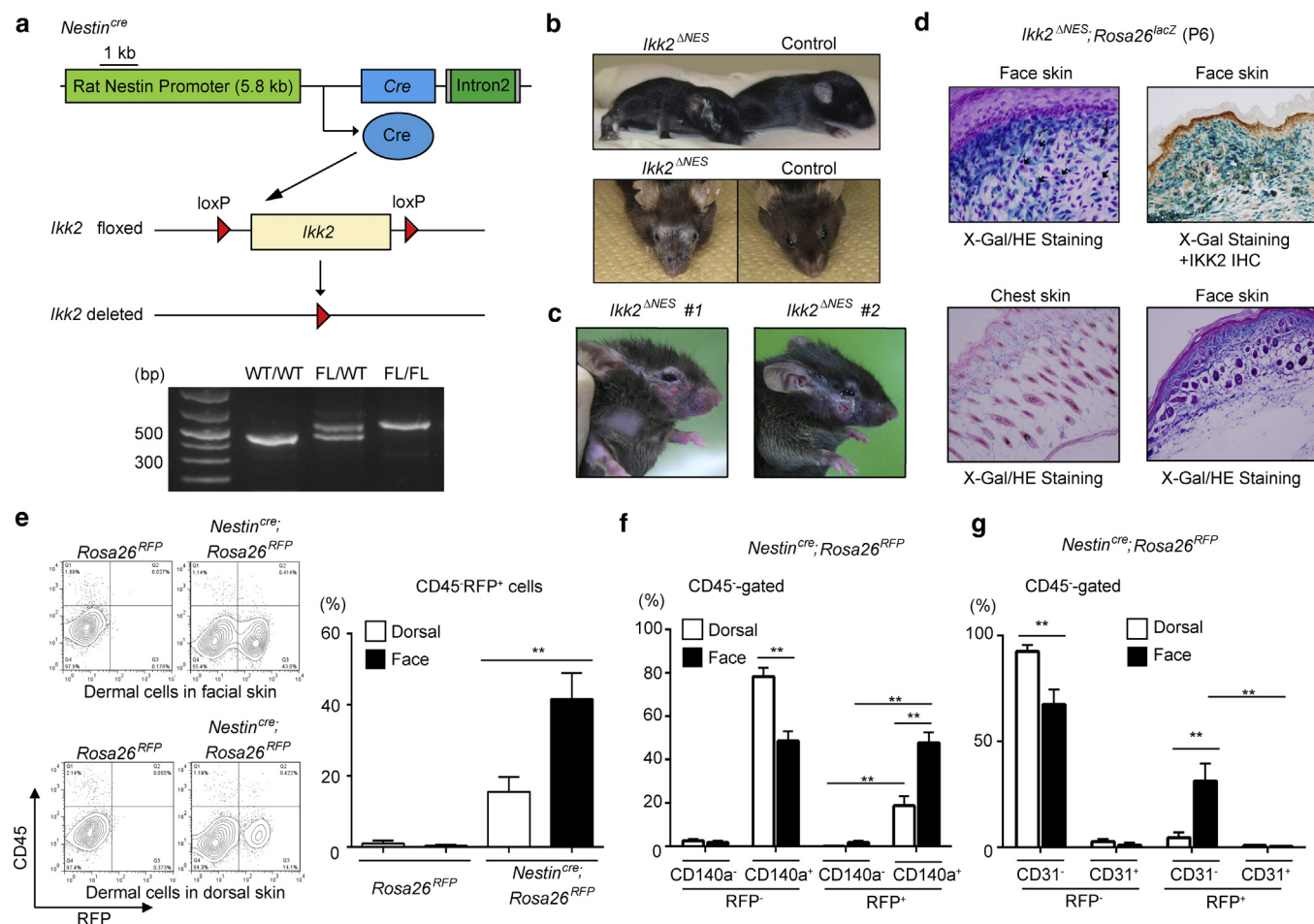


Figure 1. Spontaneous skin inflammation in the faces of *Ikk2^{ΔNES}* mice. (a) Specific deletion of the loxP-flanked *Ikk2* gene by *Nestin^{Cre}*. (b, c) The appearances of *Ikk2^{ΔNES}* and control mice. Postnatal day 10 (b, upper), 24-week-old (b, lower), 12-week-old (c). (d) X-gal (blue) and *Ikk2* (brown) staining of *Ikk2^{ΔNES}; Rosa26^{lacZ}* mice (postnatal day 6). Face skin images with high magnification (X-gal; upper left, X-gal plus *Ikk2* stain; upper right) and chest (lower left) with low magnification are depicted. Black arrows indicate mast cells. (e–g) Lineage tracing of Cre-expressing cells. (e) Increased proportions of CD140a⁺ cells (f) and CD31⁺ cells (g) in CD45⁺RFP⁺ facial cells. Data shown are mean ± standard deviation (n = 3) from three different experiments. Statistical analysis was performed using a two-sided, Student *t* test; ***P* < 0.01. HE, hematoxylin and eosin.

inflammation in *Ikk2^{ΔNES}*, we performed functional analyses of BM-derived mast cells (BMMCs) and a BM transplantation experiment. *Ikk2^{ΔNES}* and control BMMCs similarly expressed c-kit and FcεRIα on the cell surface (Supplementary Figure S4a online). Histidine decarboxylase and mast cell protease 6 expression were also comparable (Supplementary Figure S4b). Additionally, there was no difference in IgE-dependent degranulation response between control and *Ikk2^{ΔNES}* BMMCs, even when the response was further enhanced by adhering to periostin (Supplementary Figure S4c, S4d). As shown in Figure 4a, CD45⁺ peripheral blood leukocytes in B6-Ly5.1 mice (recipient) were mostly replaced with *Ikk2^{ΔNES}* BM-derived cells 8 weeks after BM transplantation. In this context, B6-Ly5.1 mice did not develop skin inflammation, suggesting that *Ikk2^{ΔNES}* BM-derived leukocytes are not pathological in the presence of *Ikk2*-present recipient cells. Conversely, transplantation of leukocytes derived from B6-Ly5.1 mice failed to block inflammation in *Ikk2^{ΔNES}* mice (Figure 4b). Of interest, eosinophils derived from B6-Ly5.1 BM rapidly increased in *Ikk2^{ΔNES}* mice, suggesting that growth factors or other environments to enhance expansion of eosinophils are present in

Ikk2^{ΔNES} mice. These results suggest that BMMC functions are not dysregulated and that cells residing in the skin are essential for the development of skin inflammation of *Ikk2^{ΔNES}* mice, which is conceivable, given the previous finding that NCC-derived facial fibroblasts are critical for the onset of inflammation.

Gene expression profile of *Ikk2^{ΔNES}* mice is close to that of AD patients

Data on the transcriptome analyses of several mouse models and AD patients reveals some gaps (Ewald et al., 2017). Thus, we first comprehensively analyzed the transcriptome profile of the inflamed skin of *Ikk2^{ΔNES}* using cap analysis of gene expression method (Figure 5a). Gene set enrichment analysis showed the five most significantly upregulated gene sets (Figure 5b): extracellular space (*Klk6* and *Muc5ac*), the defense response (*Defb4a* and *Defb103a*), the immune response (*IL2ra* and *Icos*), the immune system process (*Csf3r*, *Cxcr2*, and *Mmp9*), and the response to external stimulus (*Aqp3* and *Pax6*) (Supplementary Figure S5 online). On the other hand, the five most significantly downregulated gene sets were intrinsic components of plasma membrane (*Cd226*, *Fgfr4*, and *Gpr64*),

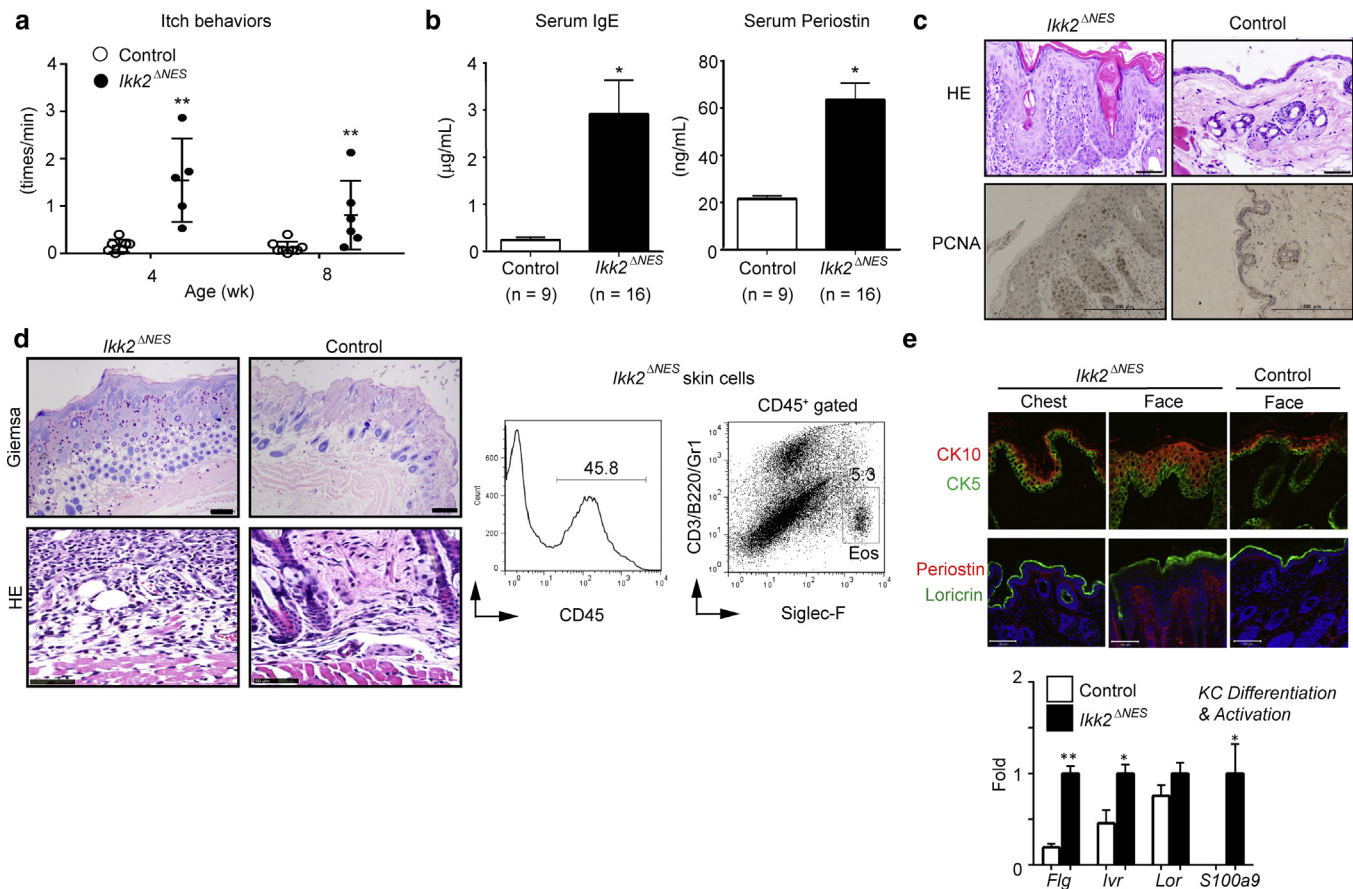


Figure 2. *Ikk2*^{ΔNES} mice exhibit atopic dermatitis-like phenotypes. (a) Frequencies of itch behaviors of *Ikk2*^{ΔNES} (n = 5–6) and control mice (n = 7–8). (b) Serum IgE and periostin levels. The data shown are mean ± standard deviation from three different experiments. Statistical analysis was performed using a two-sided, Mann-Whitney *t* test; **P* < 0.05, ***P* < 0.01 versus control mice. (c, d) Histologic analysis of the facial skins. (c) Hematoxylin and eosin (upper) and proliferating cell nuclear antigen (lower) staining. (d) Giemsa (upper) and hematoxylin and eosin (lower). Facial skin eosinophils (CD45⁺Siglec-F⁺CD3⁺B220⁺Gr1⁺) (d, right panel). (e) Expression of cytokeratin (CK) 5, CK10 (upper), periostin, and loricrin (lower) by immunofluorescence confocal microscopy. Expression of genes related to keratinocyte differentiation/activation. Scale bars = 50 μm (c, upper and d, lower), 100 μm (e), and 200 μm (c, lower and e, upper). HE, hematoxylin and eosin; KC, keratinocyte.

the system process (*Col4a3* and *Col1a2*), the muscle system process (*Myl1* and *Actn3*), cell-to-cell signaling (*Fgf18* and *Nova1*), and negative regulation of cell communication (*Tra-f3ip1* and *Dab1*) (Supplementary Figure S5).

A recent meta-analysis has identified the top 30 upregulated and downregulated genes in human AD skins (Ewald et al., 2017). We displayed the heat maps of genes focused on cellular signaling pathways (Figure 5c). In *Ikk2*^{ΔNES}, canonical NF-κB signals (*Ikbkb/Ikk2*, *Ikbkg/Ikkγ*, and *Ikbip*) were downregulated, whereas non-canonical NF-κB signals (*Chuk/Ikk1*, *Bcl3*, and *Nfkb2*) were upregulated. JAK-STAT pathway (*Jak1*, *Stat1*, *Stat3*, and *Stat6*) and chemokines/ILs (*Ccl4*, *Ccl11*, *Ccl8*, *Ccl20*, *Cxcl1*, *Cxcl2*, *Il1a*, *Il33*, and *Il1rl1*) were upregulated in *Ikk2*^{ΔNES}.

Figure 5d illustrates the 60 genes altered in human AD skins and their expression in *Ikk2*^{ΔNES} and previously reported mouse models. Among these models, the profile of *Ikk2*^{ΔNES} was most consistent with that of AD patients in both the upregulated (16 of 30) and the downregulated (19 of 30) genes. These results suggest that among the investigated mouse models, the gene expression profile in the lesional skin of *Ikk2*^{ΔNES} is the closest to that of AD.

Effects of pharmacological treatments on skin inflammation in *Ikk2*^{ΔNES} mice

Tacrolimus ointment is a widely used immunosuppressive drug in treating AD patients (Furue et al., 2017). Tofacitinib, a potent pan-JAK inhibitor, has been reported to improve skin inflammation in AD (Bissonnette et al., 2016). Epidermal Stat3 activation is linked to the pathogenesis of AD by causing barrier dysfunction (Amano et al., 2015) and epidermal hyperplasia (Tarutani et al., 2013). Therefore, we assessed the effects of tacrolimus, tofacitinib, and STAT3 inhibitor (Stattic) on skin inflammation in *Ikk2*^{ΔNES} (Figure 6a). Topical application of these drugs partially, but not completely, decreased infiltration of leukocytes, including eosinophils, into the dermis (Figure 6b, 6c). Likewise, all three of these drugs partially, but not completely, suppressed epidermal swelling (Figure 6d). They had no effects on dermis swelling (Figure 6e). These results suggest that skin inflammation in *Ikk2*^{ΔNES} is resistant to treatment with representative AD drugs, an immunosuppressive drug, and drugs targeting Jaks or STAT3.

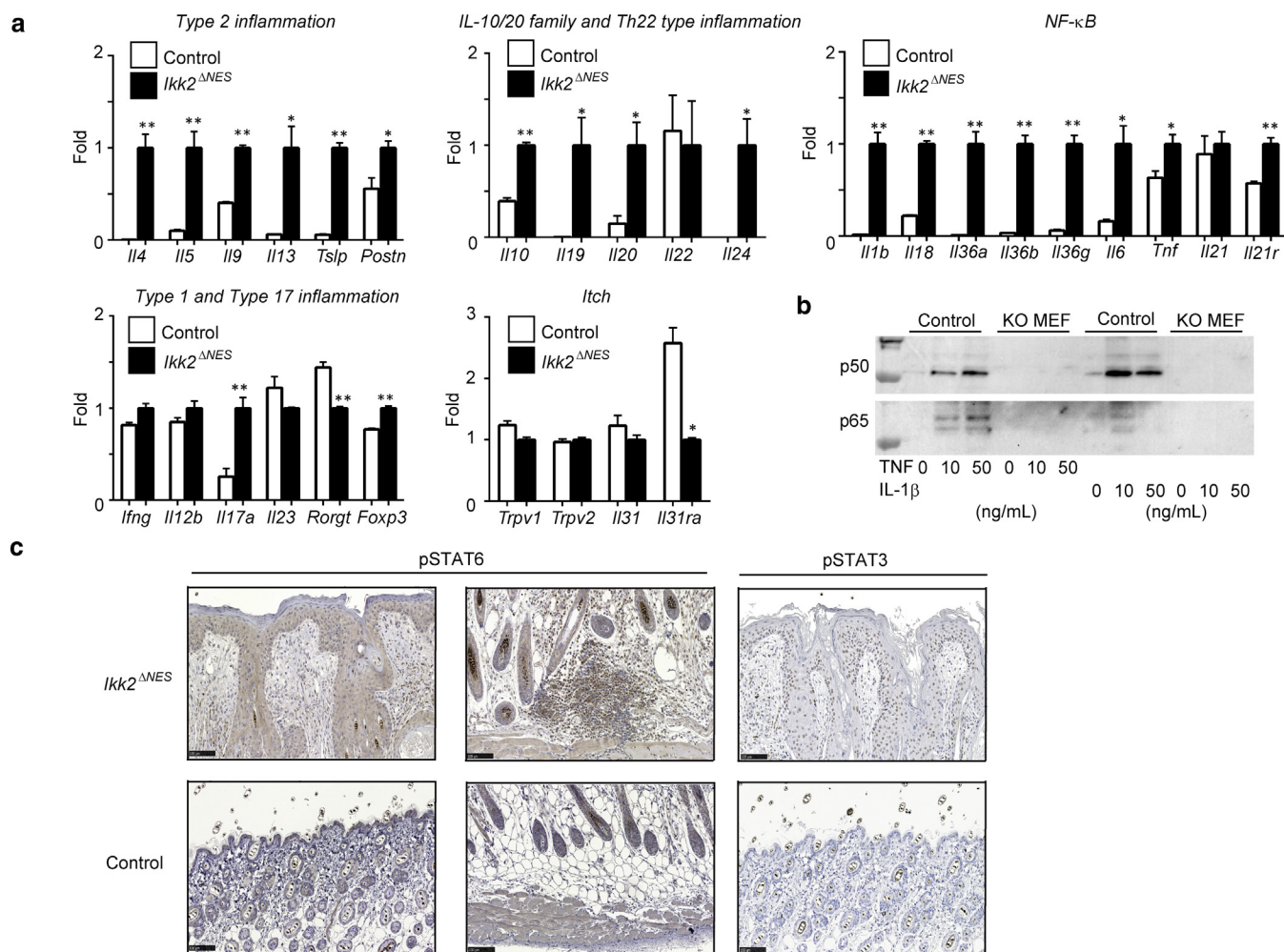


Figure 3. Increased expression of type 2 and NF-κB inflammation-related cytokines in the facial skin of *Ikk2*^{ΔNES} mice. (a) Expression of genes related to type 2 inflammation, IL10/20 family, NF-κB, type 1 inflammation, type 17 inflammation, T helper type 22 type inflammation, and itch. Data shown are mean ± standard deviation (n = 4). Statistical analysis was performed using a two-sided, Student *t* test; **P* < 0.05, ***P* < 0.01 versus control mice. (b) Western blot analysis of the nuclear localization of NF-κB p50 and p65 subunits in control and *Ikk2*-deficient mouse embryonic fibroblasts. (c) pSTAT3/6⁺ cells in facial skins of *Ikk2*^{ΔNES} and control mice. Epidermal pSTAT6⁺ (left panels), pSTAT3⁺ (right panels), and dermal pSTAT6⁺ (center panels) cells are depicted. Scale bar = 100 μm. KO MEF, knockout mouse embryonic fibroblast; Th22, T helper type 22; TNF, tumor necrosis factor.

DISCUSSION

In this study, we generated a mouse model of AD (*Ikk2*^{ΔNES}) by deleting *Ikk2* in dermal fibroblasts of the face using *Nestin*^{cre}. We cannot exclude the possibility that some cells other than dermal fibroblasts contribute to the onset of skin inflammation because nestin is expressed in various embryonal and fetal tissues (Wiese et al., 2004). However, we strongly believe that skin inflammation is attributable to dermal fibroblasts, based on the following findings: (i) skin inflammation occurred in the face, where dermal fibroblasts dominantly express nestin (Figure 1) and (ii) *Nestin*^{cre} has Cre recombinase activity in the NCC-derived mesenchyme (Dubois et al., 2006), whereas *NesCre*, the alternative *Nestin*-Cre mouse strain, lacks it and did not lead to skin inflammation (Zhang et al., 2008). Therefore, skin inflammation in *Nestin*^{cre} would be ascribed to their activity to target *Ikk2* gene in NCC-derived facial fibroblasts. *Ikk2* ablation in the central nervous system is not enough for the onset of facial skin inflammation because both *Nestin*^{cre} and *NesCre* are sufficient for recombination in central nervous system

(Dubois et al., 2006; Tronche et al., 1999). Based on these findings, we conclude that the primary lesions in facial skin are caused by dermal fibroblasts. Keratinocyte-specific deficiency of *Ikk1*, *Ikk2*, and *TAK1*, or systemic deficiency of *NEMO/Ikkγ*, *RelB*, and *SHARPIN*, shows that mice exhibiting skin inflammation throughout their bodies live for only a short time (Barton et al., 2000; Liu et al., 2011; Makris et al., 2000; Omori et al., 2006; Paspaparakis et al., 2002; Potter et al., 2014). In contrast, *Ikk2*^{ΔNES} mice with skin inflammation limited to the facial skin live as long as wild-type mice (data not shown). Thus, the recombination system targeting nestin has the advantage of deleting certain molecules limited to dermal fibroblasts of the facial skin.

Although the pathological roles of fibroblasts in the pathogenesis of AD were underestimated before compared to immune cells and epithelial cells, it is now believed that fibroblasts play pivotal roles in allergic skin inflammation by communicating with epidermal cells and immune cells via a variety of soluble mediators, such as eotaxin/Ccl11, stem cell factor, and periostin (Izuhara et al., 2017; Kanbe et al., 2001;

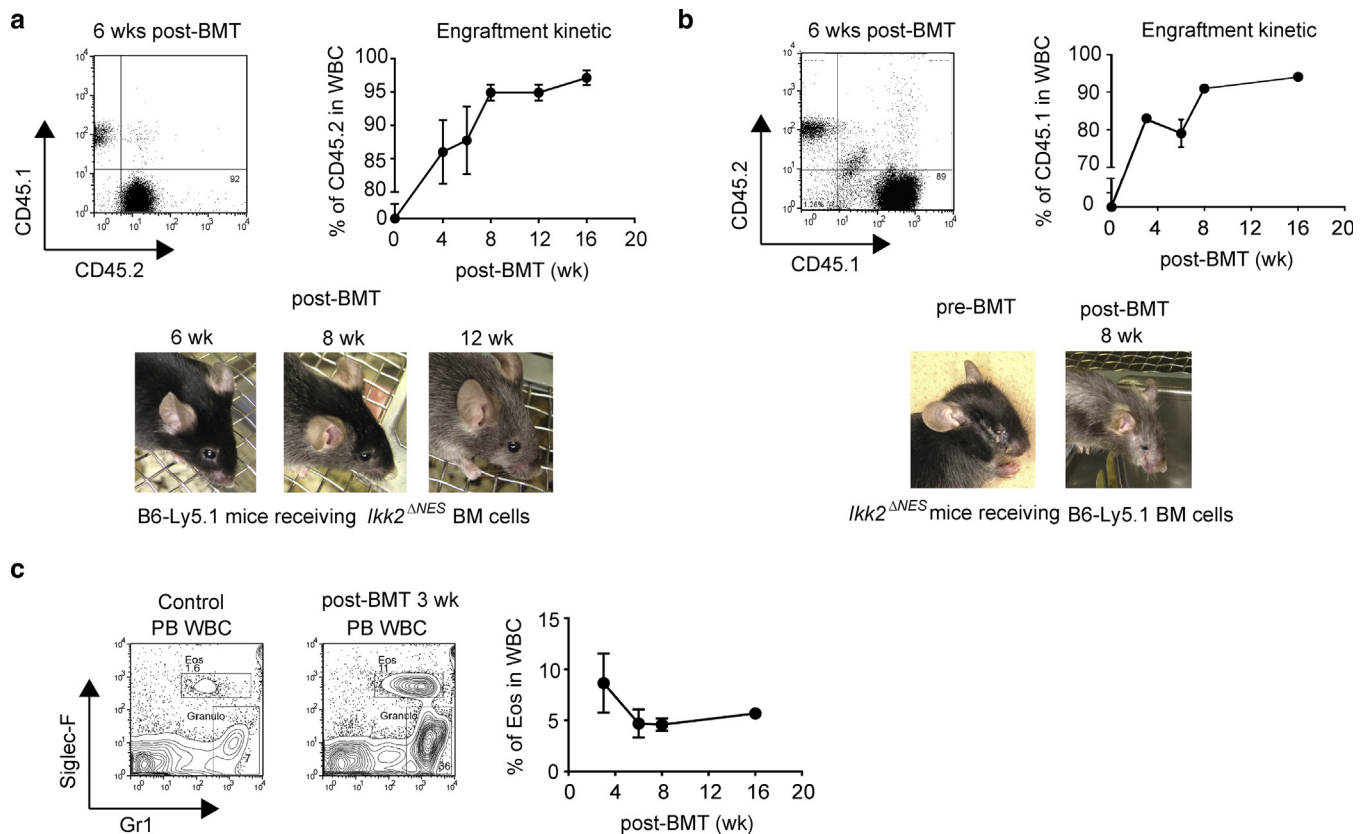


Figure 4. Tissue-resident cells are required for the onset of skin inflammation in *Ikk2*^{ΔNES} mice. (a) Transplantation of *Ikk2*^{ΔNES} bone marrow cells (CD45.2⁺) into the recipient B6-Ly5.1 mice. (b, c) Transplantation of B6-Ly5.1 bone marrow cells (CD45.1⁺) into the recipient *Ikk2*^{ΔNES} mice. Flow cytometric analysis of donor cells in white blood cells in recipient mice 6 weeks after bone marrow transplantation (a and b, upper left panels). Engraftment kinetic of donor cells (a and b, upper right panels) and appearance (a and b, bottom panels) in recipient mice are depicted. Engraftment kinetic of donor eosinophils (CD45.1⁺Siglec-F⁺Gr1^{mid}) in white blood cells in *Ikk2*^{ΔNES} mice after bone marrow transplantation (c). Data shown are mean ± standard deviation (n = 2–3). BMT, bone marrow transplantation; WBC, white blood cell.

Yawalkar et al., 1999). We previously demonstrated that periostin derived from IL-4/IL-13–stimulated fibroblasts, a matricellular protein acting on keratinocytes, was followed by induction of pro-inflammatory cytokines, such as TSLP, generating a vicious cycle in the pathogenesis of AD (Izuhara et al., 2017). *Ikk2*^{ΔNES} overexpressed periostin in the facial dermis, suggesting its pathological role in skin inflammation (Figure 2e). Thus, *Ikk2*^{ΔNES} have highlighted the significance of dermal fibroblasts in the pathogenesis of skin allergic inflammation.

Although many rodent models that spontaneously develop dermatitis are currently available for investigating AD pathogenesis (Jin et al., 2009), there are disparities in the scratching behaviors and transcriptome profiles of inflamed skin between these model mice and AD patients (Ewald et al., 2017). Our mouse model is superior to the established mouse models in these respects: *Ikk2*^{ΔNES} mice exhibit severe pruritus, as do AD patients, and the gene expression profile in their lesional face skin is similar to that of AD patients (Figures 2a and 6), although previous reports examined different portions of skin, such as back or ear.

At this moment, it remains unknown how the genetic variation in these mice corresponds to the genetic factors of AD patients. In several IKK2-deficient individuals exhibiting severe immunodeficiency, no apparent skin inflammation

was reported (Zhang et al., 2017). In contrast, genetic mutations in the *NEMO* gene cause incontinentia pigmenti, characterized by multiple disorders, including skin lesions (Smahi et al., 2000). Moreover, the genome-wide association study identified *CARD11*, the TCR/BCR signaling molecule upstream NF-κB pathway, as a susceptibility locus for AD (Lyons and Milner, 2018). Hypomorphic mutations in *CARD11* and *MALT1*, a component of the CARD11-BCL10-MALT1 signalosome complex, have been identified in severe AD patients (Lyons and Milner, 2018). These findings suggest that our model mice may provide a clue to identifying genetic variants in NF-κB pathway–related molecules contributing to susceptibility to AD.

The current first-line AD treatments, topical steroids and calcineurin inhibitors such as tacrolimus, are targeting mainly immune cells, with limited efficacy (Furie et al., 2017). Dupilumab, an IL-4Rα antagonist, also showed limited efficacy (Simpson et al., 2016).

Both findings are ascribed to the heterogeneity of the pathogenesis of AD, in which non-immune cells and/or many mediators are involved. In this study, we investigated the pharmacological effects of tacrolimus, an immunosuppressive agent usually used for AD patients, as well as tofacitinib and STAT3 inhibitor (Stattic), both now under development as anti-AD drugs, on skin inflammation in *Ikk2*^{ΔNES}, but only

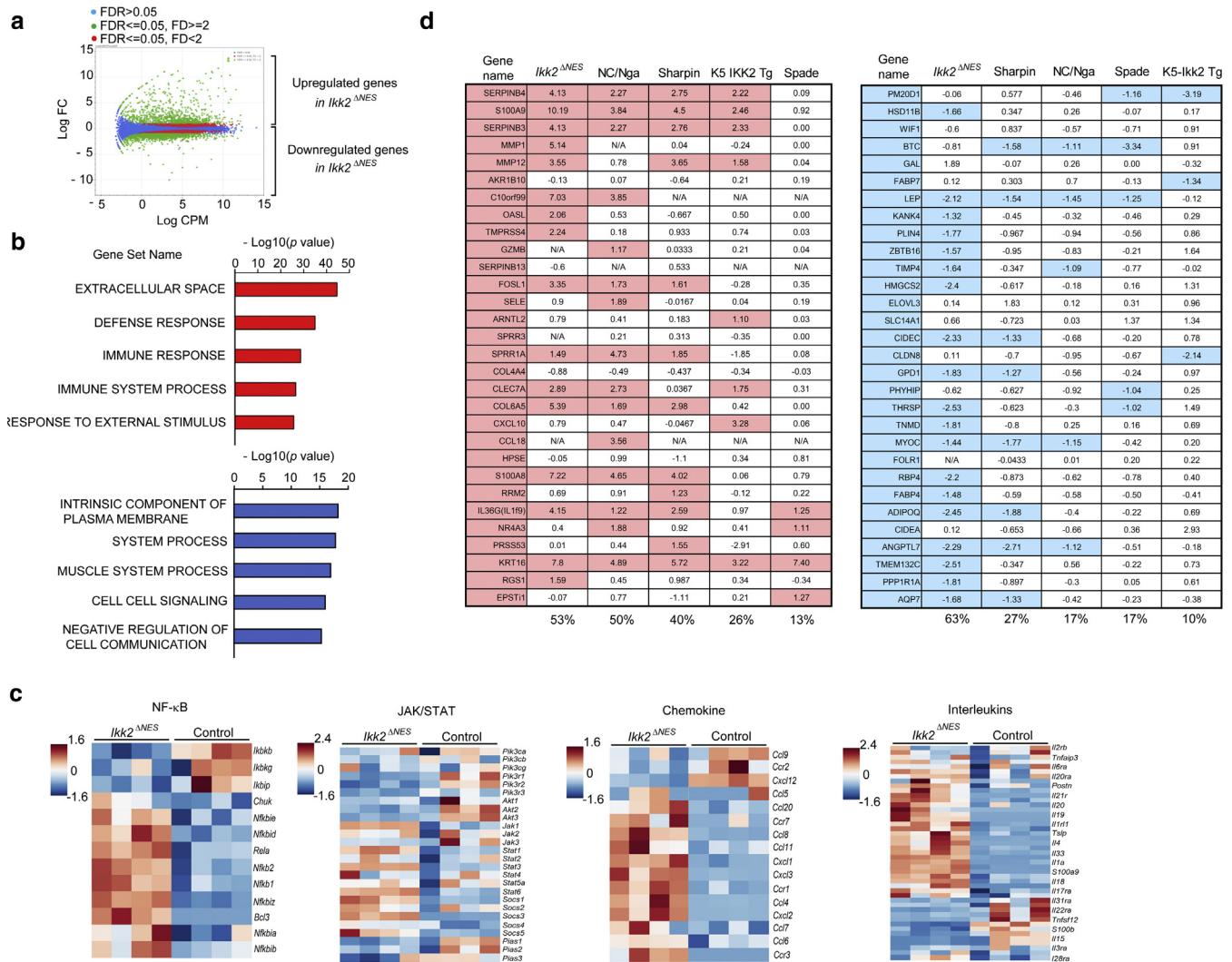


Figure 5. Gene expression profiles in lesional facial skin of *Ikk2*^{ΔNES} mice. Expression analysis of data from cap analysis of gene expression. (a) MA plot of differential expression analysis between lesional and non-lesional facial skins. The MA plot depicts log₂ fold-change versus log counts per million. (b) Gene set enrichment analysis was performed to identify gene sets enriched for significantly dysregulated genes in the *Ikk2*^{ΔNES} facial skin samples. The top five upregulated (upper) and downregulated (lower) gene sets in lesional skin. (c) Heat maps of NF-κB pathway, JAK-STAT pathway, chemokine, and IL genes expressed differentially in *Ikk2*^{ΔNES} facial skin. (d) Heat map of the top 30 upregulated (red) and downregulated (blue) genes in human atopic dermatitis and their orthologs in lesional skin of murine models (mite-exposed NC/Nga, *Sharpin*^{cpdm/cpdm}, *K5-Ikk2*, and *Jak1*^{spade/spade}). Log fold-change values are shown. CPM, counts per million; FC, fold-change; FDR, false discovery rate; N/A, not applicable.

partial effectiveness was found (Figure 6). These results suggest that leukocyte-independent pathway and/or the Jak-STAT-independent pathway is involved in the pathogenesis of inflammation and that *Ikk2*^{ΔNES} would be useful in developing novel therapeutic drugs for AD that would target such pathways. IL-31 induces itch by activating sensory neurons expressing IL-31Rα, TRPV1, and TRPA1 and is upregulated in pruritic AD (Kittaka and Tominaga, 2017). Nemolizumab, an anti-IL-31Rα antibody, inhibits pruritus in AD by blunting the IL-31/IL-31Rα-pathway (Furue et al., 2017). However, expression of *Il31*, *Il31ra*, and *Trpv1/2* was not increased in lesional skins of *Ikk2*^{ΔNES} (Figure 3a), suggesting that chronic pruritus of *Ikk2*^{ΔNES} occurs independent of the TRPV1/2 or IL-31. *Ikk2*^{ΔNES} mice would be useful to search for a novel therapeutic drug for itching in AD.

One limitation of this study is that the mice lack barrier dysfunction, which is a typical characteristic of AD

(Rerknimitr et al., 2017). Congenitally or acquired barrier dysfunction is important for the pathogenesis of AD by prompting invasion of allergens (Furue et al., 2017). However, *Ikk2*^{ΔNES} showed dermatitis by postnatal day 10 without apparent scratching behaviors. In addition, upregulation rather than downregulation of skin barrier-related molecules—*Flg*, *Lor*, and *Ivr*—throughout the inflammation (Figure 2e) suggested that allergic inflammation is responsible for the onset and exacerbation of facial dermatitis in these mice, while barrier dysfunction is unlikely to be associated.

Another limitation of this study is that the underlying mechanism of how deficiency of *Ikk2* in dermal fibroblasts causes AD-like inflammation is not elucidated enough. It has been shown that deficiency of the NF-κB pathway-related molecules—IKK1/2, NEMO, RelB, SHARPIN, and TAK1—in keratinocytes paradoxically causes skin inflammation. In this study, *Ikk2* deficiency in dermal fibroblasts adjacent to

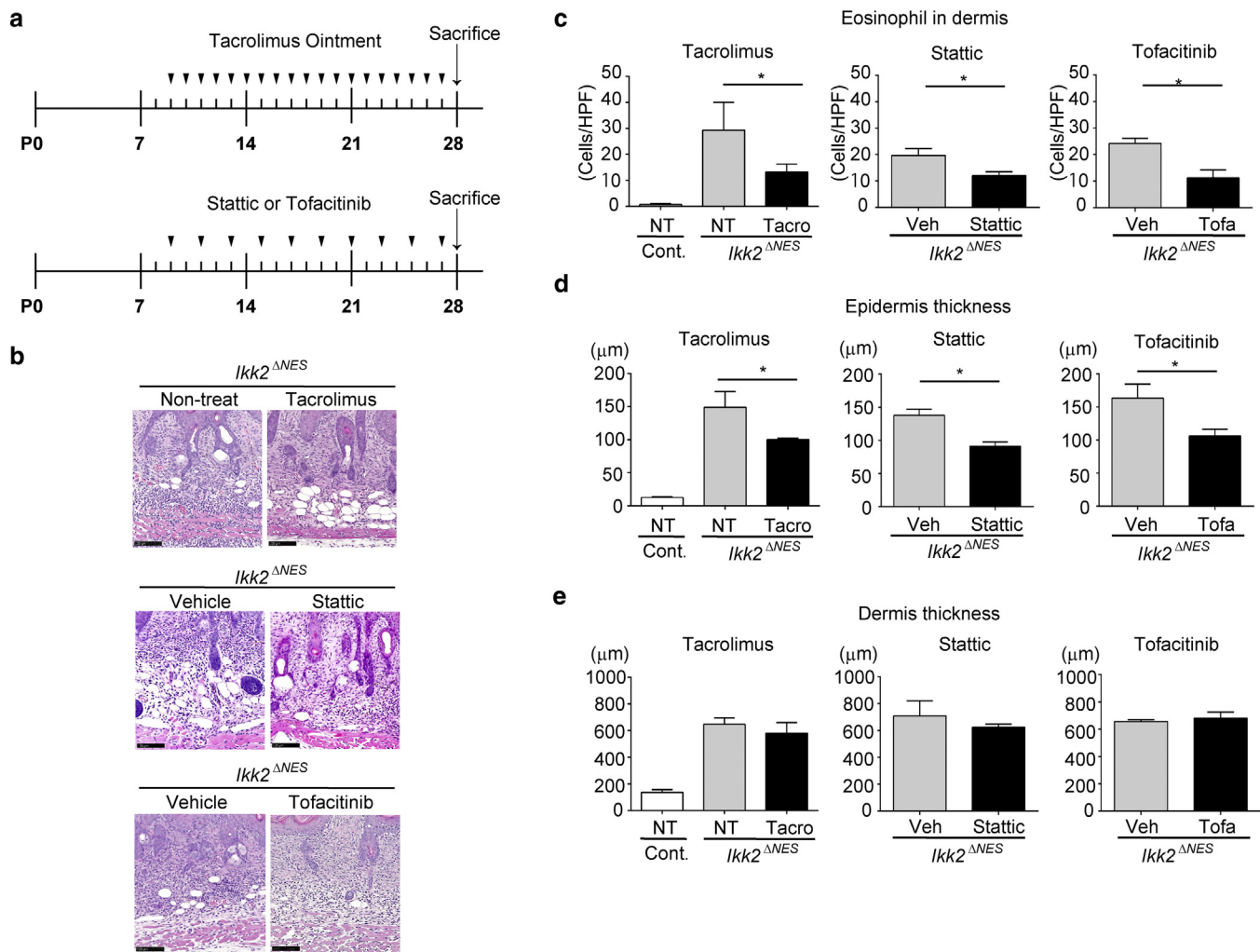


Figure 6. Effects of pharmacological treatment on *Ikk2*^{ΔNES} mice. (a) The experimental protocols for the topical application of drugs. (b) Hematoxylin and eosin stains of tacrolimus-treated and untreated skins (upper), Static- and vehicle-treated skins (middle), tofacitinib- and vehicle-treated skins (bottom). After drug treatment, eosinophil number in the dermis in 10 high-power fields ($\times 400$) (c), as well as the thicknesses of the epidermis (d) and dermis (e) were measured. Data shown are the mean $\pm$ standard error from three different experiments ($n = 5$, non-treated control mice, non-treated *Ikk2*^{ΔNES} mice, and tofacitinib-treated *Ikk2*^{ΔNES} mice; $n = 3$, Static-treated *Ikk2*^{ΔNES} mice and vehicle-treated *Ikk2*^{ΔNES} mice). Statistical analysis was performed using a two-sided, Mann-Whitney *t* test for tofacitinib or Student *t* test for Static and tacrolimus; $P < 0.05$. HPF, high-power field; NT, non-treat; Tacro, tacrolimus; Tofa, tofacitinib; Veh, vehicle.

keratinocytes can cause similar phenotypes. TNF–IL-24 pathway contributes to the onset of skin inflammation in *K14-Cre;Ikk2*^{FL/FL} mice (Kumari et al., 2013). We have recently found that IL-24 is a mediator of AD downstream of the IL-13/periostin pathway and is highly expressed in keratinocytes of both AD patients and AD model mice (Mitamura et al., 2018). *Ikk2*^{ΔNES} highly expressed *Il24* and *Tnf* together with *Il4*, *Il13*, and *Postn* (Figure 3a). These results suggest that induction of IL-24 by TNF or the IL-13/periostin pathway may be an underlying mechanism of skin inflammation in *Ikk2*^{ΔNES}. Further studies aiming at clarifying it are needed in the future.

METHODS

Study approval

All procedures for animal experiments were approved by the Saga University Animal Care and Use Committee and by the Committee for Animal Experiments at the University of Toyama.

Generation and care of mice

Rosa26^{RFP} was prepared by Hans Joerg Fehling, as described previously (Luche et al., 2007). *Nestin*^{cre} (Dubois et al., 2006) and *Ikk2*^{FL/FL} (Pasparakis et al., 2002) were provided by Gail R. Martin and Manolis Pasparakis, respectively. *Rosa26*^{lacZ} was purchased from Jackson Labs (Bar Harbor, ME). All mice were used on C57BL/6J background. Genotyping PCR conditions are described in Supplementary Methods online.

Antibodies

The primary and secondary antibodies used in this study are described in Supplementary Table S1 online.

Histology

We performed Giemsa, toluidine blue, and hematoxylin and eosin staining to detect eosinophils and mast cells. The thickness of dermis and epidermis, and the number of eosinophils were measured. Other analyses were performed as described in Supplementary Methods.

Culture and functional analysis of BMDCs

BMDCs were prepared as described in [Supplementary Methods](#).

Western blot

We analyzed NF- κ B p50 or p65 level in nuclear extracts from control and Ikk2-knockout mouse embryonic fibroblasts. See [Supplementary Methods](#).

Flow cytometry

Flow cytometric analysis for skin cells and peripheral blood cell was performed. See [Supplementary Methods](#).

Monitoring of itch behaviors

Mice were monitored for 15 minutes and itch behaviors were evaluated. See [Supplementary Methods](#).

Cap analysis of gene expression

We performed cap analysis of gene expression using lesional and non-lesional facial skins, prepared from 4-week-old *Ikk2^{ΔNES}* and control mice, respectively. Data are available at National Center for Biotechnology Information Gene Expression Omnibus: GSE109936. See [Supplementary Methods](#).

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR was performed as described in [Supplemental Materials](#) online. The SYBR primer sequences and TaqMan probe product numbers (Thermo Fisher Scientific, Waltham, MA) are described in [Supplementary Table S2](#) online.

Pharmacological treatment

The 0.01% Static and 0.5% tofacitinib were prepared as described in [Supplementary Methods](#). The 0.1% tacrolimus ointment was obtained from NIPRO (Osaka, Japan). *Ikk2^{ΔNES}* mice were epicutaneously treated with or without drugs every day (tacrolimus ointment) or every other day (Static and tofacitinib; 20 μ l/mouse) from postnatal days 9–27.

Statistical analysis

Data shown are mean $\pm$ standard deviation or standard error. Statistical analyses were performed using the two-sided, Mann-Whitney *t* test or Student *t* test. *P* values <0.05 were considered to indicate statistically significant differences.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2018.10.047>.

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