



Increased IL-26 Expression Promotes T Helper Type 17- and T Helper Type 2-Associated Cytokine Production by Keratinocytes in Atopic Dermatitis

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Whereas atopic dermatitis (AD) is considered as a T helper 2 (Th2)-centered disease, IL-17–producing Th (Th17) cells are also activated in AD lesional skin. However, the relationship between Th17 responses and Th2 responses in AD is still to be elucidated. Although Th17 cells are increased in AD skin, the expression and function of IL-26, which is also produced by Th17 cells, in AD are still unknown. In this report, we demonstrated that IL-26 mRNA expression levels were elevated in AD lesional skin compared with healthy controls and that IL-26-producing cells were increased in AD lesional skin by immunohistochemistry. Furthermore, IL-26 promoted IL-8, IL-1 β , chemokine (C-C motif) ligand 20, IL-33, and β -defensin 2 production in keratinocytes through phosphorylation of signal transducer and activator of transcription 1 and signal transducer and activator of transcription 3. Selective JAK inhibitors for JAK1, JAK2, and tyrosine kinase 2 blocked IL-26–induced cytokine production in keratinocytes. We also showed that injection of IL-26 exacerbated an oxazolone-induced AD mouse model and upregulated Th2 and Th17 cytokine expression in vivo. Because previous studies indicate that the above molecules induced by IL-26 can promote Th17 and/or Th2 immune responses, IL-26 may play an important role for bridging between Th17 and Th2 responses, resulting in the development of AD.

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INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, with an increased prevalence in the past several decades (Bieber, 2008). The pathogenesis of AD has been attributed to a complex interaction among immune system abnormalities, barrier impairment, environmental factors, and genetic mutations (Bieber, 2008; Leung et al., 2004; Paller et al., 2017). Typically, AD is considered a T helper 2 (Th2)-centered disease, characterized by increased immunoglobulin E production, peripheral eosinophilia, mast cell activation, and induction of Th2 cells expressing IL-4 and IL-13 (Wierenga et al., 1991). To date, there are growing evidences that IL-17–producing Th (Th17) cell activation is also prominent in the blood and skin lesion of patients with AD (Koga et al., 2008; Noda et al., 2015; Toda et al., 2003). Th17 cells are characterized by the production of inflammatory cytokines such as IL-17A, IL-17F, IL-22, and IL-26 (Di Cesare

et al., 2008). The severity of AD is thought to be associated with the presence of Th17 cells because the highest percentage of IL-17A–producing cells is found in patients with severe AD (Koga et al., 2008). Notably, IL-17A expression is much more evident in acute than in chronic lesions, suggesting that Th17 cells are more involved in the acute phase of AD (Koga et al., 2008; Toda et al., 2003). In murine models, IL-17A expression is highly detected in the spontaneously developed AD-like skin lesions in flaky-tail mice, which carry mutations in filaggrin and matted genes (Moriyama and Kabashima, 2011). In addition, IL-17A deficiency attenuates AD-like dermatitis and production of IL-4 and immunoglobulin E in a repeated hapten application-induced AD model (Nakajima et al., 2014). IL-17A can strongly induce expression of IL-19 by keratinocytes, which in turn augments Th2 cytokine expression in T cells (Liao et al., 2004; Oka et al., 2017; Witte et al., 2014), suggesting that IL-19 possibly bridges between Th2 and Th17 axes. Actually IL-19 is overexpressed in patients with AD, and serum IL-19 levels are correlated with Eczema Area and Severity Index, serum levels of chemokine (C-C motif) ligand 17 (CCL17), and the number of eosinophils (Oka et al., 2017). These studies indicate that Th17 cells play an important role in the development of AD and that Th17 responses are also involved in the regulation of Th2 responses. However, the relationship between Th17 responses and Th2 responses in AD is still largely unknown.

IL-26 is a 171-amino acid protein that belongs to the IL-10 cytokine family including IL-10, IL-19, IL-20, IL-22, and IL-24 and is conserved in most vertebrate species but not in mice

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Abbreviations: AD, atopic dermatitis; Th2, T helper 2; Th17, IL-17–producing Th; CCL, chemokine (C-C motif) ligand; STAT, signal transducer and activator of transcription; NHEK, normal human epidermal keratinocytes; SD, standard deviation; TYK2, tyrosine kinase 2

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(Meller et al., 2015; Wilson et al., 2007). IL-26 is mainly produced by Th17 cells, natural killer cells, and macrophages (Corvaisier et al., 2012; Wolk et al., 2002). Like other Th17 cytokines, IL-26 expression levels are elevated in psoriasis skin lesions (Kunz et al., 2006; Wilson et al., 2007), synovia of patients with rheumatoid arthritis (Corvaisier et al., 2012), and colonic lesions from patients with inflammatory bowel disease (Dambacher et al., 2009), and associated with inflammatory activity. IL-26 signals through the IL-10 receptor 2-IL-20 receptor 1 heterodimeric receptor, which is expressed mainly by epithelial cells including keratinocytes (Hör et al., 2004; Sheikh et al., 2004). Previous studies show that IL-26 stimulates the release of IL-8 via phosphorylation of signal transducer and activator of transcription (STAT) 3 in the human keratinocyte cell line, HaCaT cells (Hör et al., 2004). Moreover, IL-26 induces the production of the proinflammatory cytokines IL-1 β , IL-6, and tumor necrosis factor- α by human monocytes and also upregulates the expression of CCL20 (Corvaisier et al., 2012). Thus, IL-26 is considered to be an important proinflammatory cytokine in Th17-driven diseases like psoriasis. Although Th17 cells are increased in AD skin, the expression and function of IL-26 in AD have not been studied.

In this report, we demonstrated that IL-26 mRNA expression levels were elevated in AD lesional skin compared with healthy controls, and IL-26-producing cells were increased in AD lesional skin. Furthermore, IL-26 promoted IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 mRNA expression and secreted them at protein levels in normal human epidermal keratinocytes (NHEK). IL-26 also induced phosphorylation of STAT1 and STAT3. Selective JAK inhibitors for JAK1, JAK2, and tyrosine kinase 2 (TYK2) blocked IL-26-induced cytokine production in NHEK. Thus, increased IL-26 expression can be associated with the development of AD.

RESULTS

Increased expression of IL-26 in lesional skin of AD

To investigate the involvement of IL-26 in AD, we first examined IL-26 mRNA expression levels using the lesional skin of patients with AD. Consistent with a previous study (Kunz et al., 2006), IL-26 mRNA expression levels in AD skin were significantly higher than those in healthy controls (Figure 1a). After examining correlations between IL-26 expression and Th1, Th2, or Th17 cytokine expression levels in patients with AD, we found that IL-26 mRNA expression levels positively correlated with IL-4 and CCL17 mRNA expression levels, which are important to mediate Th2-dominant responses in AD (Figure 1a; $P = 0.0278$ and 0.0142 , respectively). Additionally, IL-26 mRNA levels tended to be associated with IL-33 mRNA levels (Figure 1a; $P = 0.0591$). IL-26 mRNA expression levels also correlated with IL-22 mRNA expression levels (Figure 1a; $P = 0.0036$). We found no correlation between IL-26 mRNA expression levels and IL-13, IL-17A, or IFN- γ mRNA expression levels (Figure 1a). We next immunolabeled samples of AD lesional skin for IL-26. IL-26-positive cells in the dermis were increased in the lesional skin of AD compared with normal skin (Figure 1b). In addition, immunofluorescent staining revealed that IL-26 was expressed mainly on RAR related orphan receptor C2-positive Th17 cells (Figure 1c). We also

investigated IL-26 protein levels in the sera of patients with AD. However, serum IL-26 levels in patients with any severity of AD were comparable with those in healthy controls (Figure 1d and e). Thus, IL-26 mRNA expression levels and IL-26-producing cells were increased in AD lesional skin, and IL-26 expression was associated with Th2 and/or Th17 skewing to some extent.

IL-26 promotes IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 production in NHEK

Considering clinical data on IL-26, we hypothesized that IL-26 can be associated with the development of AD, especially in the aspects of Th2 and/or Th17 skewing. We focused on keratinocytes for two reasons: (i) keratinocytes are shown to play a critical role in the pathogenesis of AD because they produce various inflammatory mediators associated with Th2 and/or Th17 polarization (Bieber, 2008) and (ii) keratinocytes expressed the receptor for IL-26 and cytokine induction by IL-26 was demonstrated in HaCaT cells (Hör et al., 2004). To assess the effect of IL-26 in keratinocytes, we next stimulated NHEK with the recombinant IL-26 protein, and measured levels of various Th2 cytokines, proinflammatory cytokines, chemokines, and antimicrobial peptides by quantitative real-time-PCR and ELISA. Consistent with previous reports on HaCaT cells (Hör et al., 2004), we confirmed that IL-26 upregulated IL-8 mRNA and protein expression in both NHEK (Figures 2a and 3a). Although previously described in monocytes (Corvaisier et al., 2012), we found that the expression levels of IL-1 β , a proinflammatory cytokine, and CCL20, a chemokine that recruits CC chemokine receptor 6-bearing cells such as Th17 and Th22 cells and dendritic cells (Fujita, 2013), were increased by stimulation with IL-26 in keratinocytes (Figure 2b and c; Figure 3b and c). Furthermore, IL-26 significantly increased IL-33 mRNA and protein expression levels in keratinocytes (Figures 2d and 3d). In contrast, IL-26 did not elevate mRNA expression of IL-25, thymic stromal lymphopoietin, CCL17, or CCL26, all of which induce Th2 immune responses similar to IL-33 in AD (Doran et al., 2017) (Figure 2e–h). Concerning antimicrobial peptides, IL-26 promoted only β -defensin 2 production, whereas other antimicrobial peptides, β -defensin 3, S100A8, and S100A9 mRNA levels were not increased (Figures 2i–l and 3e). Thus, IL-26 promoted IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 mRNA expression and secreted them at protein levels in NHEK.

IL-26 increases phosphorylation of STAT1 and STAT3 in NHEK

The JAK-STAT pathway is activated in many immune cells, and plays a key role in cytokine production (Ciechanowicz et al., 2019; Furumoto and Gadina, 2013). Stimulation with IL-26 is known to induce phosphorylation of STAT3 in keratinocytes, and phosphorylation of STAT1 is also increased by IL-26 in various types of cells, such as colon and gastric cancer cells (Hör et al., 2004; You et al., 2013). Consistent with this, western blot analysis showed that the recombinant IL-26 protein induced phosphorylation of STAT1 and STAT3 in NHEK (Figure 4). Thus, IL-26 increased phosphorylation of STAT1 and STAT3 in NHEK.

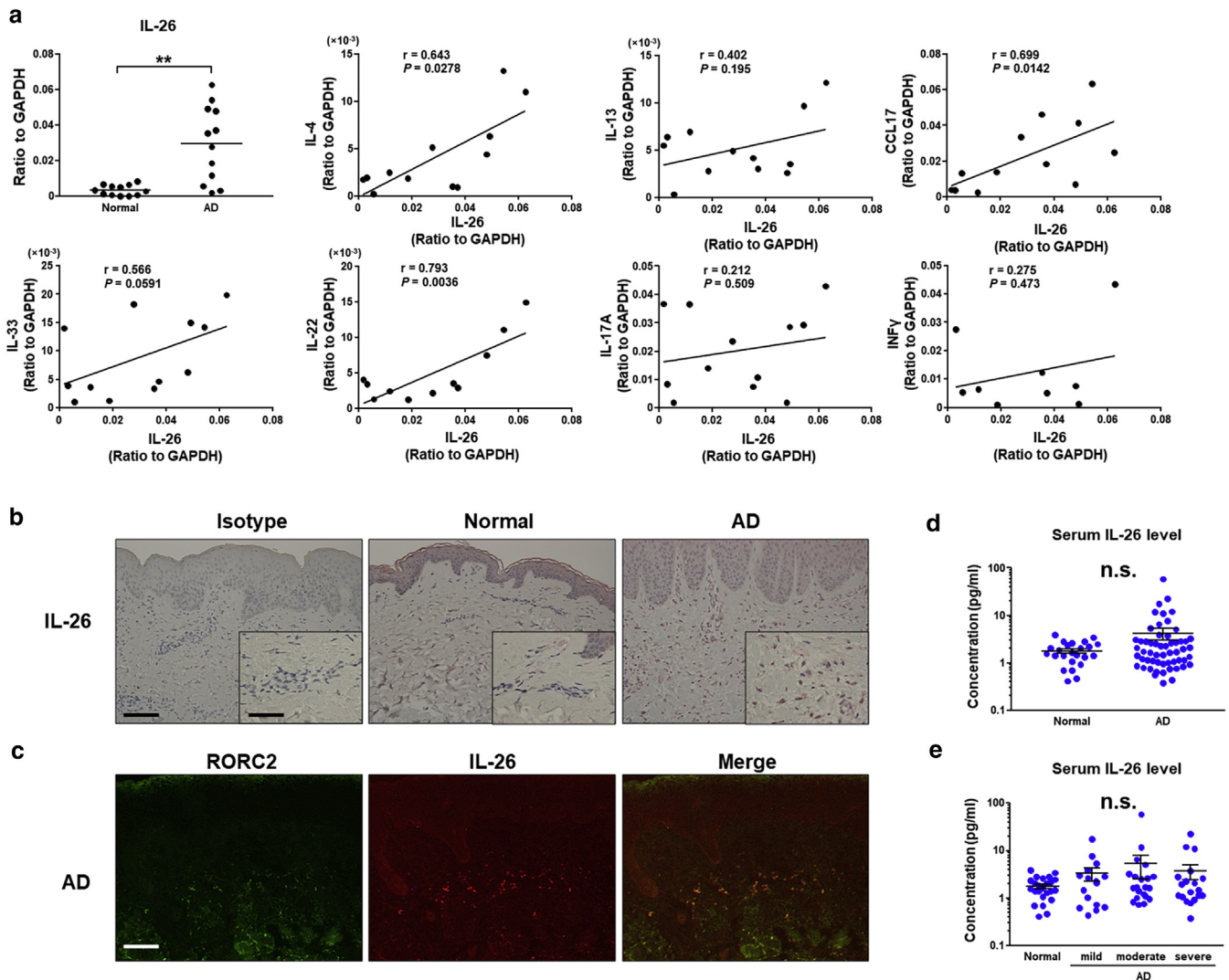


Figure 1. Increased expression of IL-26 in lesional skin of AD. (a) Quantitative real-time-PCR was performed to measure expression levels of IL-26 using mRNA extracted from AD lesional skin ($n = 12$) and normal skin ($n = 12$). Correlations between IL-26 and IL-4, IL-13, CCL17, IL-33, IL-22, IL-17A, or IFN- γ mRNA levels in AD lesional skin. Data are presented as mean $\pm$ SD ($n = 3$). $**P < 0.01$. (b) IL-26 expression in lesional skin of AD was determined by immunohistochemistry. Representative results are shown. Bar = 100 μ m. Insets show high magnification images. (c) IL-26 and RORC2 expression in lesional skin of AD was determined by fluorescent histochemical staining. Representative results are shown. Bar = 100 μ m. (d) Serum IL-26 levels in patients with AD ($n = 56$) and normal controls ($n = 24$). (e) Serum IL-26 levels in mild ($n = 16$), moderate ($n = 21$), and severe ($n = 19$) patients with AD and normal controls ($n = 24$). Data are presented as mean $\pm$ SEM. AD, atopic dermatitis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; n.s., not significant; RORC2, RAR related orphan receptor C2; SD, standard deviation, SEM, standard error of the mean.

IL-26 enhanced IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 expression via JAK1, JAK2, and TYK2 in NHEK

Recently, various studies exhibited the efficacy of JAK inhibitors for treatment of AD and psoriasis (Bissonnette et al., 2016; Ciechanowicz et al., 2019). The JAK family is composed of four members: JAK1, JAK2, JAK3, and TYK2 (Ciechanowicz et al., 2019). We assessed the effects of IL-26 in phosphorylation of JAK1, JAK2, JAK3, and TYK2 by western blot analysis. IL-26 increased phosphorylation of JAK1, JAK2, and TYK2 in NHEK (Figure 5a and b). However, phosphorylation of JAK3 was not increased by IL-26 stimulation. To investigate whether selective JAK inhibitors blocked elevated cytokine production by IL-26, we preincubated NHEK with selective JAK1, JAK2, and TYK2 inhibitors followed by stimulation of IL-26 and measured IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 expression levels by

quantitative real-time-PCR. Either inhibitor suppressed IL-26-induced production of all examined molecules in NHEK (Figure 5c). Thus, IL-26 promoted phosphorylation of JAK1, JAK2, and TYK2, and selective JAK inhibitors decreased various protein expression induced by IL-26 in NHEK.

IL-26 exacerbated an oxazolone-induced AD mouse model

The IL-26 effects on keratinocytes in vitro suggested that IL-26 can cause development of AD through inducing various inflammatory protein expressions. Therefore, we finally assessed the effects of IL-26 in vivo using an oxazolone-induced AD mouse model (Fujita et al., 2007; Jin et al., 2009; Matsumoto et al., 2004). Exogenous administration of IL-26 significantly increased the clinical score of oxazolone-induced dermatitis (Figure 6a). Histopathological findings also supported the clinical observation (Figure 6b).

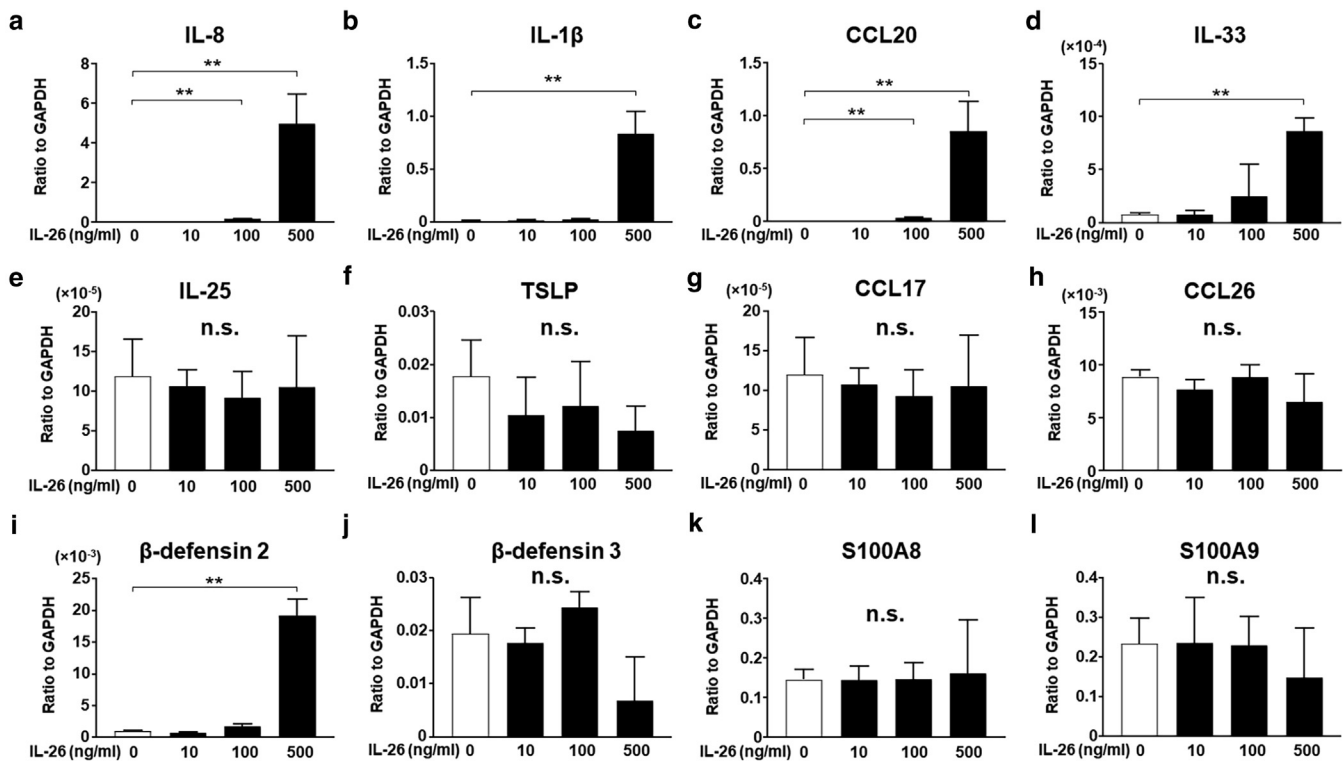


Figure 2. IL-26 promotes IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 mRNA levels in NHEK. (a–l) NHEK were cultured with recombinant IL-26 protein (0, 10, 100, or 500 ng/ml) for 24 hours. (a) IL-8, (b) IL-1 β , (c) CCL20, (d) IL-33, (e) IL-25, (f) TSLP, (g) CCL17, (h) CCL26, (i) β -defensin 2, (j) β -defensin 3, (k) S100A8, and (l) S100A9 mRNA expression was evaluated by quantitative real-time-PCR. One representative result from three independent experiments. Data are presented as mean $\pm$ SD ($n = 3$). ** $P < 0.01$. CCL, chemokine (C-C motif) ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NHEK, normal human epidermal keratinocytes; n.s., not significant; SD, standard deviation; TSLP, thymic stromal lymphopoietin.

Immunohistochemical staining revealed the upregulated phosphorylation of STAT1 and STAT3 in keratinocytes in IL-26-treated mice (Figure 6b). The IL-26-treated skin contained significantly higher mRNA levels of IL-4, IL-13, IL-33, IL-17A, and CCL20 than phosphate buffered saline-treated skin, indicating an augmented Th2 and Th17 response (Figure 6c). Thus, treatment with IL-26 exacerbated oxazolone-induced dermatitis by enhancing Th2 and Th17 response.

DISCUSSION

In this study, we first showed that IL-26 mRNA levels were significantly elevated in the lesional skin of AD compared with that of healthy controls. By immunohistochemistry, IL-26 was expressed on RAR related orphan receptor C2-positive Th17 cells in the dermis of AD lesional skin, consistent with the previous report describing that Th17 cells exist in the lesional skin of AD (Koga et al., 2008; Noda et al., 2015). However, serum IL-26 levels were comparable between patients with AD and healthy individuals. Similarly, some previous reports show that serum IL-17A levels are not elevated in patients with AD compared with healthy controls (Tan et al., 2017; Ungar et al., 2017), although IL-17A plays an important role in the development of AD. Meanwhile, serum levels of other Th17 cytokines, such as IL-17F and IL-22, are elevated in patients with AD (Hayashida et al., 2011; Park et al., 2015). Our results and previous reports suggest that not

all but some Th17 cytokines including IL-26 may be produced and act as proinflammatory cytokines mainly in lesional skin.

In this study, all clinical samples were obtained from Japanese subjects. Considering that Th17 skewing is more pronounced in Asian patients with AD than Western patients with AD (Noda et al., 2015), there is a possibility that IL-26 elevation in AD skin may not be observed in other races. Although Kunz et al. (2006) found the elevation of IL-26 mRNA expression in AD skin in German patients, further studies will be needed to clearly determine whether increased IL-26 expression is also seen in patients with AD from other populations.

We next found that IL-26 expression levels significantly correlated with IL-4 and CCL17 expression levels and tended to correlate with IL-33 mRNA levels in lesional skin. IL-4 is a key cytokine in the development of Th2 inflammation. CCL17 is a chemokine that binds to CC chemokine receptor 4 mainly expressed in Th2 cells and is considered to have a causative role in the accumulation of Th2 cells to lesional skin of AD (Kakinuma et al., 2001). IL-33 is also involved in Th2 immunity through activating dendritic cells and group 2 innate lymphoid cells (Mjösberg et al., 2011; Rank et al., 2009), and the expression level is increased in patients with AD (Savinko et al., 2012). Taken together, our results led to the hypothesis that IL-26 can be associated with the augmentation of Th2 inflammation in AD. Consistently, we exhibited that IL-33 expression levels in NHEK were elevated

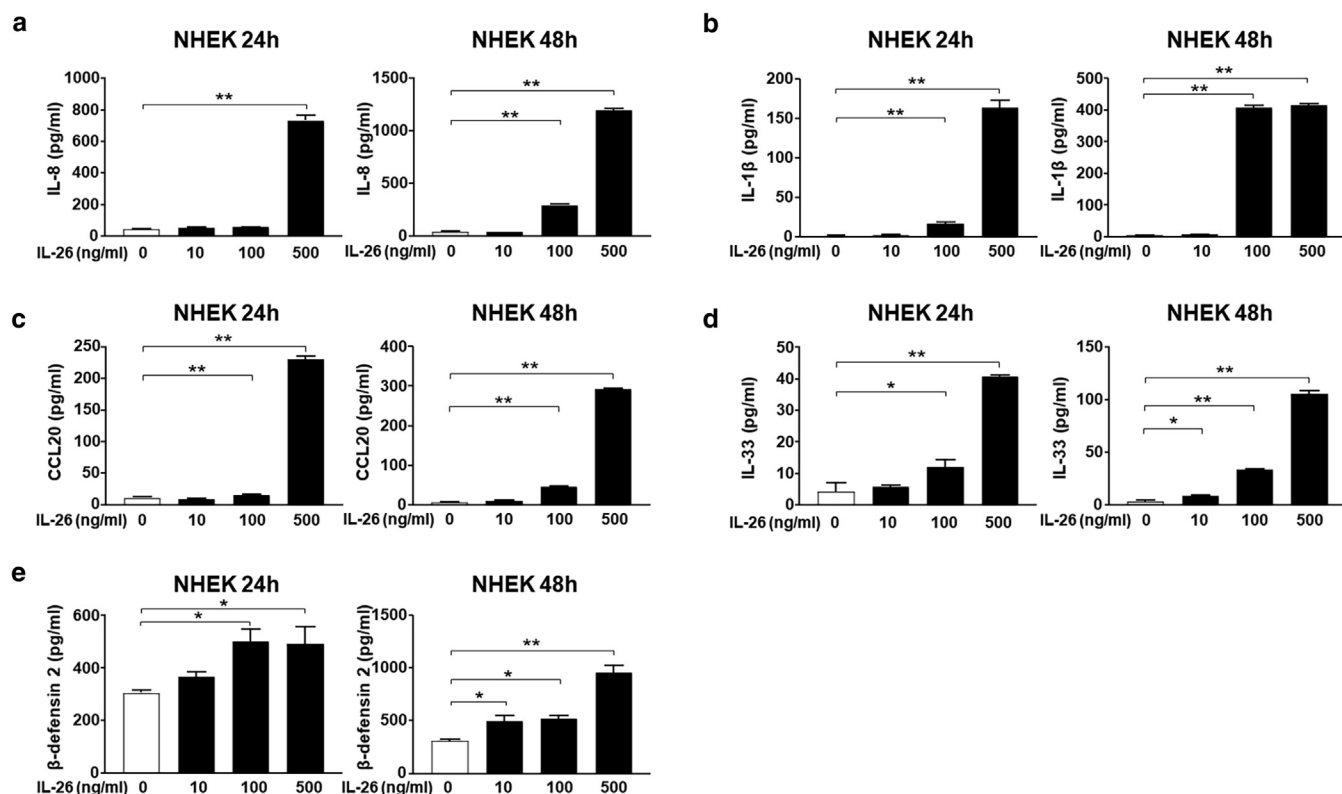
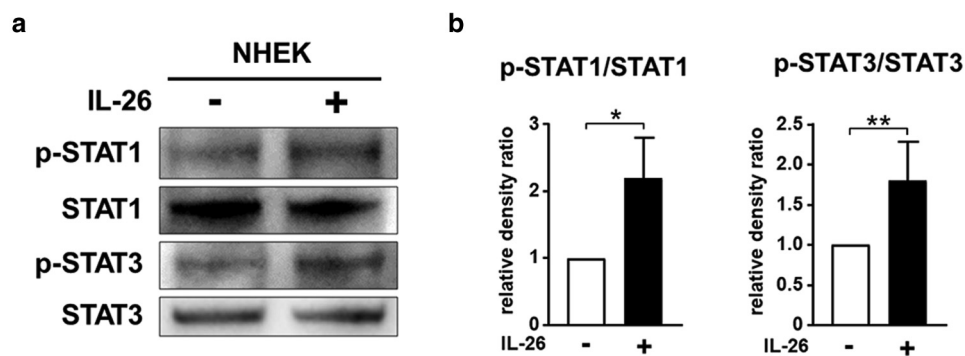


Figure 3. IL-26 promotes IL-8, IL-1β, CCL20, IL-33, and β-defensin 2 levels in NHEK. (a–e) NHEK were cultured with recombinant IL-26 protein (0, 10, 100, or 500 ng/ml) for 24 or 48 hours. (a) Serum IL-8, (b) IL-1β, (c) CCL20, (d) IL-33, and (e) β-defensin 2 expression was evaluated by ELISA. One representative result from three independent experiments. Data are presented as mean ± SD (n = 3). **P* < 0.05, ***P* < 0.01. CCL, chemokine (C-C motif) ligand; h, hour; NHEK, normal human epidermal keratinocyte; SD, standard deviation.

by stimulation with IL-26. We also revealed that IL-26 induced expression of IL-1β, which has the capacity to induce thymic stromal lymphopoietin, a master cytokine of Th2 immune responses, in human epidermis (Bernard et al., 2017). Additionally, IL-26 enhanced an oxazolone-induced AD mouse model and expression of Th2 cytokines in vivo. Taken together, IL-26 can promote Th2 immune polarization in AD lesional skin. Meanwhile, we showed that IL-26

expression levels were correlated with IL-22 expression in AD skin and that IL-26 induced CCL20 expression in NHEK and CCL20 and IL-17A expression in the oxazolone-induced AD mouse model. Because keratinocyte-derived CCL20 recruits Th17 and Th22 cells in psoriasis skin (Harper et al., 2009), IL-26 may also contribute to enhanced Th17 immune responses in addition to the augmentation of Th2 immune responses in lesional skin of AD.

Figure 4. IL-26 increases phosphorylation of STAT1 and STAT3 in NHEK. (a) Western blotting analysis was conducted on the lysates of NHEK treated with recombinant IL-26 protein (0 or 500 ng/ml) for 30 minutes. Phosphorylation of STAT1 and STAT3 was measured. The representative blot from three independent experiments. (b) Band intensities were calculated by Image J software. Relative optical density of phospho-STAT1 (p-STAT1)/STAT1 and p-STAT3/STAT3 was calculated and normalized to the value of control cells. Data are presented as mean ± SD (n = 3). **P* < 0.05, ***P* < 0.01. NHEK, normal human epidermal keratinocytes; SD, standard deviation; STAT, signal transducer and activator of transcription.



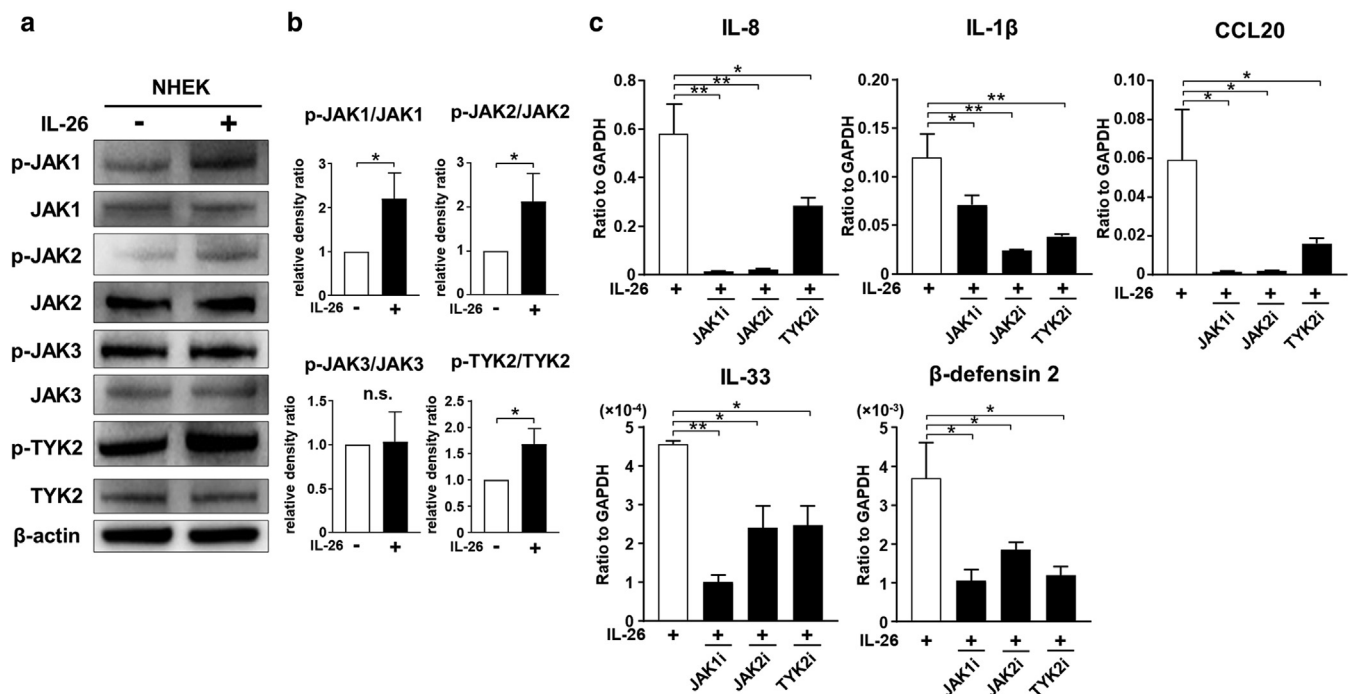


Figure 5. IL-26 enhanced IL-8, IL-1β, CCL20, IL-33, and β-defensin 2 expression via JAK1, JAK2, and TYK2 in NHEK. (a) Western blotting analysis was conducted on the lysates of NHEK treated with recombinant IL-26 protein (0 or 500 ng/ml) for 30 minutes. Phosphorylation of JAK1, JAK2, JAK3, and TYK2 was measured. The representative blot from three independent experiments. (b) Band intensities were calculated by Image J software. Relative optical density of phospho-JAK1 (p-JAK1)/JAK1, p-JAK2/JAK2, p-JAK3/JAK3, and p-TYK2/TYK2 was calculated and normalized to the value of control cells. (c) NHEK were precultured with upadacitinib (a selective JAK1 inhibitor, 50 nM), JAK2 inhibitor V (a selective JAK2 inhibitor, 50 μM), Tyrphostin A1 (a selective TYK2 inhibitor, 50 μM) for 1 hour and were cultured with recombinant IL-26 protein (500 ng/ml) for 12 hours. IL-8, IL-1β, CCL20, IL-33, and β-defensin 2 mRNA expression was evaluated by quantitative real-time-PCR. One representative result from three independent experiments. Data are presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01. CCL, chemokine (C-C motif) ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NHEK, normal human epidermal keratinocytes; n.s., not significant; SD, standard deviation; TYK, tyrosine kinase.

Furthermore, we revealed that expression levels of β-defensin 2, one of the antimicrobial peptides, in NHEK were triggered by IL-26 stimulation. Similarly, other Th17 cytokines including IL-17 and IL-22 have the capacity to augment the production of β-defensin 2 in keratinocytes (Liang et al., 2006). Although patients with AD are susceptible to superficial bacterial, fungal, and viral infection, our results indicated that IL-26 also contributes to protect against skin infections in AD, similar to other Th17 cytokines. However, increasing evidence suggests that β-defensin 2 may also contribute to AD pathogenesis through upregulating both Th17 and Th2 immune responses. For example, β-defensin 2 causes the release of IL-22, IL-4, IL-13, and/or IL-31 from T cells and mast cells (Kanda and Watanabe, 2012; Niyonsaba et al., 2010). Additionally, β-defensin 2 can attract CC chemokine receptor 6-positive T cells and mast cells (Niyonsaba et al., 2002; Pérez-Cañadillas et al., 2001). Thus, increased β-defensin 2 expression by IL-26 may also augment the Th17 and Th2 axis in AD.

The JAK-STAT pathway plays a key role in the development and differentiation of immune cells. Selective JAK inhibitors suppress phosphorylation and activation of JAKs, resulting in the blockage of the inflammatory cytokine cascade (Furumoto and Gadina, 2013). As such, JAK inhibitors are already being used in clinical practice for rheumatoid arthritis, myelofibrosis, and polycythemia vera (Ciechanowicz et al., 2019; Schwartz et al., 2017). Within

dermatology, JAK inhibitors have been studied most rigorously in psoriasis, and multiple lines of evidence support that systemic and topical JAK inhibitors can improve the lesional skin of psoriasis (Ciechanowicz et al., 2019). In the treatment of AD, clinical trials of JAK inhibitors, such as tofacitinib (JAK1/3 inhibitor) and upadacitinib (JAK1 inhibitor), have been designed and implemented (Bissonnette et al., 2016; Ciechanowicz et al., 2019; Paller et al., 2017). In this study, we found that stimulation with IL-26 induced phosphorylation of JAK1, JAK2, and TYK2 in NHEK and that selective JAK inhibitors for them suppressed the elevation of inflammatory protein expression levels induced by IL-26. Phosphorylation of STAT1 and STAT3 in keratinocytes was augmented by IL-26 administration in an oxazolone-induced AD model. Thus, selective JAK inhibitors can downregulate IL-26-mediated Th17 and Th2 inflammation in AD skin. Additionally, because IL-26 mRNA levels are increased in the lesional skin of psoriasis (Wilson et al., 2007) and IL-26 exacerbates imiquimod-induced psoriasis-like inflammation because of enhanced angiogenesis and immune cells infiltration in a human IL-26 transgenic mice model (Itoh et al., 2019), IL-26 may be a promising therapeutic target even in psoriasis. Our finding also supports the efficacy of selective JAK inhibitors in psoriasis.

In summary, IL-26 expression levels were upregulated in the lesional skin of AD and correlated with IL-4 and CCL17 expression levels. IL-26 promoted IL-8, IL-1β, CCL20, IL-33,

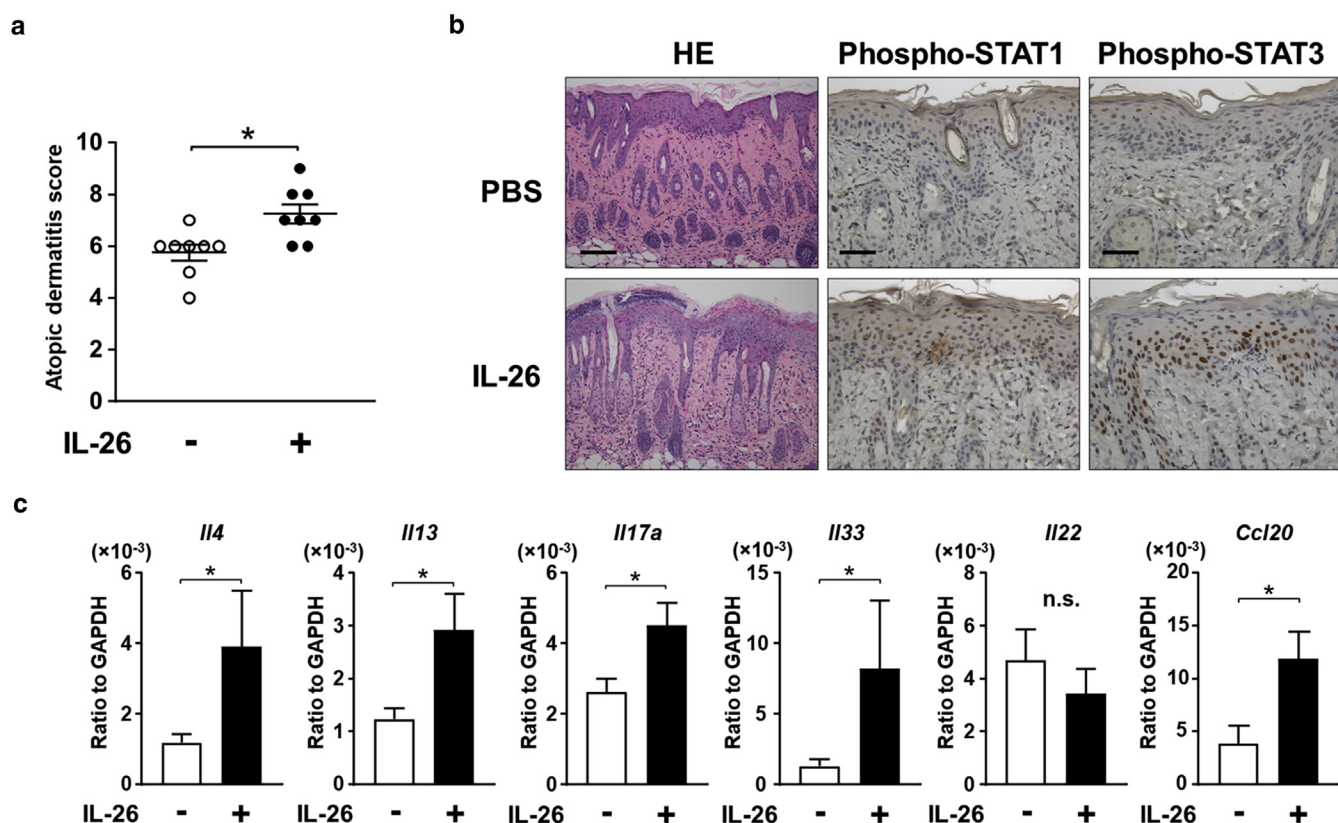


Figure 6. IL-26 exacerbated oxazolone-induced AD mouse model. After mice were sensitized with oxazolone for 7 days, oxazolone was further topically applied to the shaved dorsal skin with or without injection of recombinant IL-26 for 10 days. Skin tissue were harvested on day 11. (a) The severity of dermatitis was determined by a scoring index of AD. (b) Tissue sections from the back skin were stained with HE (Bar = 100 μ m), phospho-STAT1, and phospho-STAT3 (Bar = 200 μ m). Representative results are shown. (c) IL-4, IL-13, IL-17A, IL-33, IL-22, and CCL20 mRNA expression in the back skin was evaluated by quantitative real-time-PCR. Data are presented as mean $\pm$ SEM (n = 8). *P < 0.05. AD, atopic dermatitis; CCL, chemokine (C-C motif) ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; n.s., not significant; PBS, phosphate buffered saline; SEM, standard error of the mean; STAT, signal transducer and activator of transcription.

and β -defensin 2 production by keratinocytes via the phosphorylation of STAT1 and STAT3. Selective JAK inhibitors for JAK1, JAK2, and TYK2 suppressed these effects. Moreover, IL-26 exacerbated an oxazolone-induced AD mouse model and upregulated Th2 and Th17 cytokine expression in vivo. Thus, IL-26 may play an important role for bridging between Th17 and Th2 responses in the lesional skin of AD, and the blockade of JAK-STAT pathways might be a promising treatment option.

MATERIALS AND METHODS

Patients and samples

mRNA was obtained from biopsy materials of lesional skin of AD (n = 12; mean $\pm$ standard deviation [SD] age: 37.5 $\pm$ 15.4 years; five males and seven females) and normal skin adjacent to benign skin tumors (n = 12; mean $\pm$ SD age: 50.6 $\pm$ 15.1 years; six males and six females) using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Skin samples for immunohistochemistry included lesional skin of AD (n = 17; mean $\pm$ SD age: 37.6 $\pm$ 13.0 years) and normal skin adjacent to benign skin tumors (n = 7; mean $\pm$ SD age: 47.9 $\pm$ 10.7 years). Skin samples for fluorescent histochemical staining included lesional skin of AD (n = 7; mean $\pm$ SD age: 37.7 $\pm$ 10.8 years; five males and two females). Serum samples were obtained from 56 patients with AD (16 mild cases, 21 moderate cases, and 19 severe cases; mean $\pm$ SD age: 36.6 $\pm$ 12.5 years; 34 males

and 22 females) and 24 healthy control subjects (mean $\pm$ SD age: 38.4 $\pm$ 16.1 years; 11 males and 13 females). The healthy controls had no history of allergy and AD. All patients with AD were Japanese and enrolled according to the criteria of Hanifin and Rajka (1980). The disease severity of AD was determined by Physician Global Assessment. All samples were collected during daily clinical practice in the University of Tokyo Hospital. The medical ethics committee of the University of Tokyo approved all described studies, and the study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained to use blood and skin samples from patients and healthy controls.

Analysis of IL-26 expression in lesional skin and sera of AD

Quantitative real-time-PCR and immunohistochemistry for IL-26 were performed using the lesional skin of AD. Fluorescent immunostaining for IL-26 and RAR related orphan receptor C2 was performed using a frozen section of AD lesional skin. A specific ELISA for IL-26 was performed using the sera of patients with AD. Detailed methods are given in the [Supplementary Materials and Methods](#).

Analysis of the effects of IL-26 on NHEK

NHEK were purchased from Kurabo Industries (Osaka, Japan) and cultured in Humedia-KB2 (Kurabo Industries) supplemented with Human Keratinocyte Growth Supplement sets (Kurabo Industries). When 80% confluence was achieved, the cells were trypsinized, washed, and resuspended in the medium at 1×10^5 cells/ml, and

2 ml was added to each well of the 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). When the cells reached confluence, the medium was completely removed, and 2 ml serum-free medium was added to each well. Simultaneously, the recombinant human IL-26 monomer with very low endotoxin content (MyBioSource, San Diego, CA) was added, and the cells were incubated at 37 °C and 5% carbon dioxide. In some experiments, upadacitinib (a selective JAK1 inhibitor; BioVision, Exton, PA), JAK2 inhibitor V (a selective JAK2 inhibitor; Cayman, Ann Arbor, MI), and Tyrphostin A1 (a selective TYK2 inhibitor; Tokyo Kasei, Tokyo, Japan) were used. After indicated time points, cell culture supernatants, total mRNA, and proteins were collected for quantitative real-time-PCR, ELISA, or western blotting. Detailed methods are given in the [Supplementary Materials and Methods](#).

Oxazolone-induced AD mouse model

Ten-week-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). An oxazolone-induced AD mouse model was induced as previously described ([Fujita et al., 2007](#)). Recombinant IL-26 protein (2 µg/ml; MyBioSource) in 100 µl of phosphate buffered saline was injected subcutaneously in IL-26-treated group, whereas 100 µl of phosphate buffered saline was injected in the control group on each day. On day 11, the score of AD-like phenotype was measured according to the criteria described previously ([Jeong et al., 2019](#)). Skin samples were harvested for further analysis. All studies and procedures were approved by the Committee on Animal Experimentation of the University of Tokyo Graduate School of Medicine. Detailed methods are given in the [Supplementary Materials and Methods](#).

Statistical analysis

GraphPad Prism software, version 7.01 (GraphPad Software, San Diego, CA) program was used for statistical analyses. All experiments were repeated at least three times and the mean ± SD was determined. Statistical analysis between 2 groups was performed using the Student *t* test. For comparing two group values that did not follow Gaussian distribution, a two-tailed Mann-Whitney *U* test was used. Correlation coefficients were determined by using the Spearman rank correlation test. *P*-values < 0.05 were considered statistically significant.

Data availability statement

This study does not include large data sets, such as gene expression arrays, single nucleotide polymorphism arrays, proteomic data sets, high throughput sequencing, or genome-wide association study data.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: TM; Data Curation: HK; Formal Analysis: HK; Funding Acquisition: TM; Investigation: HK; Methodology: HK; Project Administration: TM; Resources: HK, TM, YH, TA, SWO, TO, NST, HS, MS; Supervision: TM, SS; Validation: HK; Visualization: HK; Writing - Original Draft Preparation: HK, TM; Writing - Review and Editing: TM, YH, TA, SWO, TO, NST, HS, MS, SS.

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.2019.07.713>.

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SUPPLEMENTARY MATERIALS AND METHODS

Quantitative real-time–PCR assay

Total mRNA was obtained from human and mouse skin samples with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) and from cells with TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The mRNA levels were analyzed using quantitative real-time–PCR with THUNDERBIRD SYBR qPCR Mix (TOYOBO). The mRNA levels were normalized to those of the *GAPDH* gene. The relative change in the levels of genes of interest was determined by the $2^{-\Delta\Delta CT}$ method. Primers are listed in [Supplementary Table S1](#).

ELISA

Serum IL-26 levels and supernatant levels of IL-8, IL-1 β , CCL20, IL-33, and β -defensin 2 were quantified using the human IL-26 ELISA Kit (Cloud Clone, Houston, TX), human IL-8 ELISA Kit (R&D Systems, Minneapolis, MN), human IL-1 β ELISA Kit (R&D Systems), human CCL20 ELISA Kit (R&D Systems), human IL-33 ELISA Kit (R&D Systems), and human β -defensin 2 ELISA Kit (Phoenix Pharmaceuticals, Belmont, CA), according to the manufacturer's instructions. These assays employ the quantitative sandwich enzyme immunoassay technique.

Immunohistochemistry

We performed immunohistochemical staining for IL-26 using lesional skin of AD ($n = 17$) and normal skin ($n = 7$). These sections were stained with either rabbit anti-human IL-26 antibody (clone 197505; R&D Systems) or isotype-matched control antibodies followed by an avidin-biotin-alkaline phosphatase staining (Vector Lab, Burlingame, CA). Each section was examined independently by three investigators in a blinded manner.

Fluorescent histochemical staining

Fluorescent immunostaining for IL-26 and RAR related orphan receptor C2 was performed using lesional skin of AD ($n = 7$). For double staining, 6- μ m-thick frozen tissue sections were first fixed with cold acetone for 20 minutes. These sections were incubated with Alexa Fluor 488-conjugated anti-human RAR related orphan receptor C2 antibody (BD Pharmingen, San Diego, CA) and PE-conjugated anti-human IL-26 antibody (Invitrogen) at 4 °C for overnight. The nuclear staining was performed using mounting medium with DAPI (Vector Lab). Each section was examined independently by three investigators in a blinded manner.

Western blotting

After collecting proteins, SDS-PAGE (Invitrogen) was performed as described previously ([Sugaya et al., 2006](#)). After transfer to nitrocellulose membrane (Invitrogen), the membrane was blotted with antibodies against STAT1, phosphorylated STAT1, STAT3, phosphorylated STAT3, JAK1, phosphorylated JAK1, JAK2, phosphorylated JAK2, JAK3, phosphorylated JAK3, TYK2, and phosphorylated TYK2

(Cell Signaling Technology, Beverly, MA) overnight at 4 °C and with the appropriate secondary antibodies for 1 hour at room temperature. Visualization was performed by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The density of each band was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Animal model of oxazolone-induced dermatitis

Ten-week-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were healthy and fertile and did not display evidence of infection or disease. All mice were housed in a pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of the University of Tokyo Graduate School of Medicine. For sensitization and elicitation, mice were sensitized with 25 μ l of a 1% oxazolone solution (4-ethoxymethylene-2-phenyloxazolone; Sigma-Aldrich, St. Louis, MO) in acetone/sesame seed oil (4:1) applied to the shaved belly as previously described ([Fujita et al., 2007](#)). Starting 7 days after sensitization, 50 μ l of 1% oxazolone was repeatedly applied to the shaved back skin at 2-day intervals until day 10. Simultaneously, recombinant IL-26 protein (2 μ g/ml; MyBioSource) in 100 μ l of phosphate buffered saline was injected subcutaneously in IL-26-treated group, whereas 100 μ l of phosphate buffered saline was injected in control group on each day. On day 11, the score of AD-like phenotype was measured according to the criteria described previously ([Jeong et al., 2019](#)). Briefly, scores of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) were given for each of the four symptoms: (i) erythema/hemorrhage, (ii) edema, (iii) excoriation/erosion, and (iv) scaling/dryness. Maximum score is 12. Then skin samples were obtained for RNA extraction and immunohistochemical analysis. For immunohistochemical analysis, the skin samples were fixed paraformaldehyde, and then paraffin embedded. Sections (6- μ m thick) were stained using anti-phospho-STAT1 and anti-phospho-STAT3 antibodies (Cell Signaling Technology) followed by ABC staining (Vector Lab). Tissue were also processed for standard hematoxylin and eosin staining. The skin score and each section were examined independently by three investigators in a blinded manner.

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Supplementary Table S1. List of Used Primers

Organism	Target	Forward / Reverse (5'-3')
Human	IL-26	GGTTGCTGTAGTCACTCTGTCTC / GCGGTCTTCTGGAATCGTTGC
	IL-4	CACAGGCACAAGCAGCTGAT / CTCTGGTTGGCTTCCTTCACA
	IL-33	GGAAGAACACAGCAAGCAAAGCCT / TAAGGCCAGAGCGGAGCTTCATAA
	IL-13	CGAGAAGACCCAGAGGATGCT / GGGCCACCTCGATTTTGG
	IL-22	TGAATAACTAACCCCTTTCCTG / TGGCTTCCCATTCTCCTTTTG
	IL-17A	ACTACAACCGATCCACCTCAC / ACTTTGCCTCCAGATCACAG
	IFN- γ	TCCTGTCACTGTCTCACTTAATCCTT / TTAGGTTGGCTGCCTAGTTGG
	IL-8	TAGCAAAATTGAGGCCAAGG / AAACCAAGGCACAGTGGAAAC
	IL-1 β	CTGTACCTGTCTGCGTGTT / GTGCTGATGTACCAGTTGGG
	β -defensin 2	GATGCCTCTCCAG TGTTTTT / GGATGACATATGGCTCCACTCTT
	β -defensin 3	GTGAAGCCTAGCAGCTATGAGGAT / TGATTCCTCCATGACCTGGAA
	S100A8	ATGCCGTCTACAGGGATGAC / ACGCCCATCTTTATCACCAG
	S100A9	CAGCTGGAACGCAACATAGA / TCAGCTGCTTGCTGCATTT
	IL-25	CCAGGTGGTTGCATTCTTGG / TGGCTGTAGGTGTGGGTTC
	CCL17	GCAAAGCCTTGAGAGGTCTTTGA / CGGTGGAGGTCCCAGGTAGT
	TSLP	CCCAGGCTATTCGGAAACTCA / ACGCCACAATCCTTGTAATTGTG
	CCL26	TCTGTACCCATCCAAGGAAAA / GGGTCCATGTAGCCTTCAGA
	GAPDH	ACCCACTCCTCCACCTTTGA / CATACCAGGAAATGAGCTTGACAA
Mouse	IL-4	ACGGAGATGGATGTGCCAAACGTC / CGAGTAATCCATTGTCATGATGC
	IL-13	GGATATTGCATGGCCTCTGTAAC / AACAGTTGCTTTGTGTAGCTGA
	IL-17A	CTCCAGAAGGCCCTCAGACTAC / AGCTTTCCCTCCGCATTGACACAG
	IL-22	AGCTTGAGGTGTCCAACCTC / GGTAGCACTGATCTTAGCACTG
	CCL20	TGCTCTTCCTTGCTTTGGCATGGGTA / TCTGTGCAGTGATGTGCAGGTGAAGC
	IL-33	CAATCAGGCGACGGTGTGGATGG / TCCGGAGGCGAGACGTCACC
	GAPDH	CGTGTTCCTACCCCAATGT / TGTCATCATACTGGCAGGTTTCT

Abbreviations: CCL, chemokine (C-C motif) ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSP, thymic stromal lymphopoietin.