Immunization with a Pneumococcal pep27 Mutant Strain Alleviates Atopic Dermatitis through the Upregulation of Regulatory T-Cell Activity and Epithelial Barrier Function and Suppressing TSLP Expression

Ji-Hoon Kim, Saemi Ahn, Prachetash Ghosh, and Dong-Kwon Rhee

Atopic dermatitis (AD) is an inflammatory disease driven in part by type 2 helper T (Th2) cytokines and skin barrier disruption alleviating the entry of allergens. Thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine, is known to aggravate AD symptoms by activating Th2. In addition, regulatory T cells (Tregs) inhibit inflammatory cells such as Th2. However, the relationship between TSLP and Tregs in AD is unclear. A murine dermatitis model was induced by applying oxazolone to the ear skin of mice. Prophylactic and therapeutic responses were analyzed by immunizing mice intranasally with a pneumococcal pep27 mutant (Δpep27 mutant), attenuated strain by reducing the virulence of a pathogen. Intranasal immunization with a pneumococcal pep27 mutant could elicit anti-inflammatory Treg-relevant factors and epithelial barrier genes (loricrin, involucrin, filaggrin, and small proline-rich repeat proteins). Thus, pneumococcal pep27-mutant immunization suppressed epidermal collapse, IgE, TSLP, and upregulation of Th2 expression by upregulating Treg activity. In contrast, Treg inhibition aggravated AD symptoms through the upregulation of TSLP and Th2 and the repression of epithelial barrier function compared with that of the noninhibited pneumococcal Δpep27-mutant group. Taken together, immunization with pneumococcal Δpep27 mutant upregulated Treg and epithelial barrier function and inhibited TSLP and Th2 to relieve AD symptoms.


INTRODUCTION

Atopic dermatitis (AD) (alias atopic eczema) is increasing worldwide and is prevalent in about 20% of the population (Weidinger et al., 2018). AD is a chronic, inflammatory skin disease with intense pruritic and recurrent eczematous lesions. It is prevalent in children but can also occur with high frequency in adults (Leung et al., 2004).

The cause of AD remains unclear. Allergens stimulate epithelial cells to secrete thymic stromal lymphopoietin (TSLP), which is associated with AD pathogenesis by initiating T helper (Th) 2 cell hypersensitivity (Ho and Kupper, 2019; Werfel et al., 2016). Increased TSLP level is observed in the epidermis of patients with AD (Corren and Ziegler, 2019). Overexpression of IL-4, IL-5, and IL-13 plays an important role in the Th2-mediated inflammatory responses by enhancing IgE production, leading to accumulation of eosinophils in the dermis (Woodfolk, 2007; Wu and Zarrin, 2014). TSLP receptors are expressed on a wide range of hematopoietic lineage cells, including CD11c+ dendritic cells, monocytes, and Th or B cells (Levin et al., 1999; Pandey et al., 2000; Soumelis et al., 2002). Therefore, TSLP can interact with various immune cells and is expected to have different mechanisms for each disease (Soumelis et al., 2002). When exposed to allergens, TSLP secretion by keratinocytes is increased, and a Th2 inflammatory response is induced in allergic diseases (Comeau and Ziegler, 2010). The TSLP receptor is expressed specifically in skin-associated regulatory T cells (Tregs) for repression of proinflammatory conditions. Thus, TSLP activates Treg cells, and keratinocytes can directly repress the immune response (Kashiwagi et al., 2017). Moreover, TSLP produced by the skin stimulates Tregs to expand in a signal-dependent manner through the TSLP receptor (Leichner et al., 2017). However, when measuring Treg frequency in patients with AD, conflicting results can occur (Loser and Beissert, 2012). Thus, for effective AD therapy, a complete understanding of the interaction between TSLP and Treg is required.

Although the relationship between Treg number and AD disease severity is controversial (Gáspár et al., 2015; Reeler et al., 2008; Ma et al., 2014), the ineffective function of Tregs or reduced Tregs is characteristic of severe allergy disease, including AD, in animals as well as patients (Malhotra...
Immunization with \( \Delta \text{pep27} \) reduces ear inflammation in the AD model. (a) Mice were immunized I.N. with \( \Delta \text{pep27} \) mutant three times once a week and sensitized with 1% OXA to the inner and outer surfaces of both ears 7 days after the last immunization. After 5 days from OXA sensitization, the mice were challenged with 0.2% OXA a total of eight times at 2-day intervals. (b) The ear of the mouse was photographed after 24 h of the final sensitization. (c) Mouse ear thickness (\( n = 10 \)). * and # indicate significant differences between CON and OXA subjects (*) or between OXA and \( \Delta \text{pep27}/\text{OXA} \) subjects (#). (d) Representative photo of H&E-stained sections from ears for determining cell infiltration. Microscopic photographs of H&E staining at \( \times 200 \) magnification. Bars = 100 \( \mu \text{m} \). Each value is expressed as the average ± SEM. (e) TEWL was measured at the endpoint (42 days). Statistical comparison was performed using one-way ANOVA and Tukey’s test (****P < 0.0001). AD, atopic dermatitis; CFU, colony-forming unit; CON, control; h, hour; I.N., intranasally; OXA, oxazolone; TEWL, transepidermal water loss.

The \( \Delta \text{pep27} \) mutant could mediate therapeutic and prophylactic effects on AD. Results showed that \( \Delta \text{pep27} \) mutant could repress allergic mediators and remediate AD by upregulating Treg and the epithelial barrier response and downregulating TSLP.

**RESULTS**

**Immunization with \( \Delta \text{pep27} \) mutant ameliorates oxazolone-induced AD symptoms**

To assess the effect of \( \Delta \text{pep27} \)-mutant immunization on oxazolone (OXA)-induced AD, mice were immunized with \( \Delta \text{pep27} \) mutant, followed by AD induction through sensitization and challenge with OXA 7 days after immunization (Figure 1a). The OXA group showed severe atopic symptoms such as itching, erythema, edema, and dryness. However, immunization with \( \Delta \text{pep27} \) mutant reduced the atopy-like symptoms (Figure 1b). In addition, OXA increased ear thickness significantly and increased epidermal epithelium to 5–9 layers compared with the normal control, whereas \( \Delta \text{pep27} \)-mutant immunization reduced ear thickness compared with the OXA group (Figure 1c). To further confirm the alleviation of AD, H&E staining was performed. Treatment with OXA resulted in the typical characteristics of AD comprising hyperkeratosis and parakeratosis as well as pustules. In contrast, the \( \Delta \text{pep27} \) group showed reduced epidermal thickness and absence of pustules (Figure 1d). Determination of transepidermal water loss, a clinically practical disease parameter (Holm et al., 2006), showed decreased transepidermal water loss in the \( \Delta \text{pep27} \)-mutant immunization group compared with that in the OXA-treated group (Figure 1e). Thus, OXA-induced AD can be attenuated by \( \Delta \text{pep27} \)-mutant immunization.

Treg is upregulated, and Th2 is suppressed by \( \Delta \text{pep27} \)-mutant immunization

To test the hypothesis that Treg upregulated by \( \Delta \text{pep27} \) mutant could alleviate the Th2 response, the levels of Th2 and Treg transcripts in mice ears were determined by RT-qPCR. Transcripts of the Th2-related markers Il4, Il5, and Gata3 were significantly increased in the OXA-treated group compared with that in the control group. In contrast, immunization with \( \Delta \text{pep27} \) mutant downregulated all Th2-related markers. In addition, the transcripts of Treg-related genes Il10 and Tgf\( \beta \) and transcription factor Foxp3 were significantly increased in the \( \Delta \text{pep27}/\text{OXA} \)-treated group compared with that in the OXA-treated group (Figure 2a).

To corroborate these results, serum cytokine levels were investigated by ELISA. OXA treatment increased IL-4 and IL-5 levels compared with control treatment, but immunization
with Δpep27 mutant repressed them and significantly increased IL-10 levels compared with that of other groups (Figure 2b).

In patients with AD, conflicting results exist regarding the relationship between Tregs and AD symptoms (Brandt et al., 2009; Ou et al., 2004; Reefer et al., 2008). In the AD-like mouse model used in this study, Treg markers were slightly increased in the OXA-treated group compared with that in the control group (Figure 2a). However, the group immunized with Δpep27 mutant had significantly increased Treg-relevant transcripts levels compared with the OXA-treated group (Figure 2a).

Consistently, increased IgE level in the OXA-treated group was significantly repressed by Δpep27-mutant immunization (Figure 2c). These results show that immunization with Δpep27 mutant downregulated Th2 response and upregulated Treg function to relieve AD-like symptoms.

Therapeutic effect of Δpep27-mutant immunization on AD
To evaluate the therapeutic potential of Δpep27 mutant, after OXA sensitization, Δpep27-mutant immunization and OXA challenge were performed simultaneously (Supplementary Figure S1a). The OXA treatment elicited severe dermatitis compared with control treatment; however, Δpep27-mutant immunization reduced the degree of redness. Ear thickness was more than double in the OXA-treated group compared with that in the control group but was reduced significantly in the OXA/Δpep27treated group compared with that in the OXA-treated group (Supplementary Figure S1b). Histological analysis of the mice ears revealed that the extent of epidermal lesions was augmented significantly in the OXA-treated group compared with that in the control. In contrast, they were decreased by Δpep27-mutant immunization (Supplementary Figure S1c). Moreover, the OXA/Δpep27-treated group showed a significantly reduced disease index in the epidermis and dermis compared with that in the OXA-treated group (Supplementary Figure S1d). These results show the therapeutic effect of Δpep27-mutant immunization. To further confirm the therapeutic effect of Δpep27 mutant, RT-qPCR was conducted to access the role of AD-related proinflammatory genes such as TSLP in the initiation and maintenance of AD (Mizutani et al., 2015). Immunization with Δpep27 mutant downregulated TSLP, IL1β, and TNFa with compared with that in the OXA-treated group. In addition, Δpep27-mutant immunization significantly increased IL10 level, although there was no significant change in the level of IL17 compared with the levels in the other groups (Supplementary Figure S2a). To corroborate these findings, serum IgE and Th2 cytokine levels were assessed. Consistently, immunization with Δpep27 mutant significantly reduced serum IgE, IL-4, and IL-5 levels but not IL17 in the OXA-treated group (Supplementary Figure S2b and c).
**Immunization with Δpep27 mutant alleviates AD through Th2/Treg regulation**

To determine the underlying mechanism of Δpep27-mutant immunization in a prophylactic and/or therapeutic model, the transcript profiles of specific transcription factors associated with Th1 (T-bet), Th2 (Gata-3), and Th17 (Rorγt) cells and Tregs (Foxp3) were assessed by RT-qPCR. In both AD models, exposure to OXA substantially induced Gata-3 transcripts, whereas Δpep27 mutant repressed it. Moreover, Δpep27 mutant significantly induced Foxp3 transcripts (Figure 2a and Supplementary Figure S3a). However, no differences in the transcript levels of T-bet and Rorγt were noted in the therapeutic model (Supplementary Figure S3a). Th1- and Th17-related markers were increased in the OXA-treated group compared with those in the control group but showed similar levels in the Δpep27/OXA-treated group (Supplementary Figure S3b). Therefore, Δpep27 mutant seems to prevent and/or mitigate AD through Th2 inhibition and Treg upregulation but neither through Th1 nor Th17 mediation.

**Figure 3. Immunization with Δpep27 alleviates AD symptoms through the downregulation of TSLP and upregulation of Tregs.**

(a) The AD model. For TSLP neutralization, anti-TSLP antibody (20 μg) was given intraperitoneally. For Treg inactivation, PC61 (30 μg) was administered three times intraperitoneally. (b) Photographs of mouse ears 24 hours after the last OXA challenge. (c) Mouse ear thickness. The right panel is the ear thickness at 15 days (n = 10). (d) Histological analysis of epidermal and dermal sections at ×200 (top) and ×400 magnification (bottom) (n = 3). Bars = 50 μm. (e) Graph of the number of eosinophils found in the dermal layer and measurement of epidermal thickness (n = 9). Statistical comparison was performed using one-way ANOVA and Tukey’s test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001). AD, atopic dermatitis; CFU, colony-forming unit; CON, control; I.N., intranasally; ns, not significant; OXA, oxazolone; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.
AD is attenuated by Δpep27-mutant immunization through TSLP downregulation and Treg upregulation

TSLP directly and selectively impairs IL-10 production of Treg and subsequently inhibits their suppressive activity (Nguyen et al., 2010). Thus, to determine whether Δpep27 mutant can upregulate Treg and repress TSLP, transcripts were measured by RT-qPCR. OXA elicited TSLP upregulation, whereas Δpep27 mutant reversed this induction, possibly through Treg upregulation (Supplementary Figures S2 and S3a). However, no relation between Treg and TSLP was shown. Thus, to elucidate the mechanism, antibody neutralization studies using either anti-TSLP (a-TSLP) or anti-CD25 (PC61: a-CD25) were performed using IgG as a negative control (Figures 3a and 4a and Supplementary Figures S4 and S5). Treatment with Δpep27/OXA/a-CD25 produced more severe AD symptoms, such as erythema, keratinization, and even hair loss than in the OXA-treated group. On the other hand, Δpep27/OXA/a-TSLP treatment significantly relieved AD symptoms compared with the Δpep27/OXA treatment (Figure 3b). Ear thickness continued to increase in all groups except in the control group. However, ear thickness in the Δpep27/OXA and Δpep27/OXA/a-TSLP groups tended to decrease gradually, eventually showing a significant decrease in skin thickness in the Δpep27/OXA/a-TSLP group compared with that in the Δpep27/OXA group. The Δpep27/OXA/a-CD25 group showed the fastest increase in ear thickness from the onset of AD and the highest thickness from the start to the end of the OXA challenge (Figure 3c).

Histological observation revealed that thicker skin induced by OXA was further exacerbated by a-CD25 treatment, although nonsignificantly (Figure 3c). However, both Δpep27/OXA/a-TSLP and Δpep27/OXA markedly decreased skin thickness compared with the OXA. Skin thickness tended to decrease in the order of Δpep27/OXA/a-CD25, OXA, Δpep27/OXA, Δpep27/OXA/a-TSLP, and the control group (Supplementary Figure S4a and b), suggesting that a-TSLP relieves AD symptoms, but a-CD25 aggravates them.

Consistently, the number of eosinophils in the skin dermis was significantly increased by OXA and was further increased by a-CD25 treatment (Figure 3e). The Δpep27/OXA/a-TSLP group showed a substantial decrease in eosinophil number compared with the Δpep27/OXA group, suggesting that TSLP is an aggravating factor in the OXA-induced AD model. The epidermal thickness results were similar to the eosinophil results. The epidermis was thickest in the Δpep27/OXA/a-CD25 group but decreased significantly in the Δpep27/OXA/
\(\Delta\text{pep27}\) group compared with that in the \(\Delta\text{pep27}/\text{OXA}\) group (Figure 3d and e). In addition, \(Bax\), a proapoptotic gene, was decreased, whereas \(Bcl2\), an antiapoptotic gene, was increased in the \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) group compared with those in the \(\Delta\text{pep27}/\text{OXA}\) group (Supplementary Figure S4d), indicating that \(\Delta\text{pep27}\) mutant represses OXA-induced inflammation and apoptosis.

With infiltration of inflammatory cells into the dermis, neutrophils were the main infiltrated immune cells, and both OXA and \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) showed severe grades of infiltration (Supplementary Figure S4c). Of the groups, infiltration of inflammatory cells in the \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-TSLP}\) group was the lowest (Supplementary Figure S4c), showing that TSLP inhibition and Treg induction are the major factors for alleviating AD symptoms.

Immunization with \(\Delta\text{pep27}\) mutant alone can repress TSLP and increase Treg relevant factors

To further evaluate the background effect, RT-qPCR and ELISA analyses were performed after Treg- or TSLP-neutralizing antibody injection into the OXA-alone or \(\Delta\text{pep27}\)-mutant–alone (without OXA) group. In the normal groups, antibody treatments did not elicit any significant changes in any of the markers tested (Supplementary Figure S5a–d). The TSLP transcript was significantly upregulated after OXA/\(\alpha\text{-CD25}\) treatment compared with that in the OXA/IgG-treated group (Supplementary Figure S5a), suggesting that Treg depletion can induce TSLP. Although OXA/\(\alpha\)/TSLP treatment significantly decreased the number of eosinophils compared with that in the OXA/IgG-treated group (Supplementary Figure S5b and d), it did not produce a significant modulation of Treg-relevant factors (Foxp3 and IL-10) compared with that in the OXA/IgG-treated group. In contrast, \(\Delta\text{pep27}\) mutant alone downregulated TSLP in the absence of OXA insult, whereas IL-10 level was significantly upregulated at both mRNA and protein levels (Supplementary Figure S5a, d, and e), suggesting induction of the anti-inflammatory milieu. Consistently, \(\Delta\text{pep27}\) mutant alone did not increase eosinophil or apoptosis (Supplementary Figure S5b–e), indicating no pathological change by \(\Delta\text{pep27}\) mutant. Thus, in allergic AD, immunization with \(\Delta\text{pep27}\) mutant seems to upregulate Treg to suppress TSLP and Th2 transcripts and repress inflammation and apoptosis.

TSLP and Th2 are repressed by \(\Delta\text{pep27}\)-dependent Treg upregulation

To assess whether \(\Delta\text{pep27}\)-dependent TSLP downregulation is mediated by Treg, relevant transcripts were determined after antibody neutralization. The \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) treatment significantly increased Tslp transcripts compared with \(\Delta\text{pep27}/\text{OXA}\) treatment (Figure 4a), indicating that Treg is a negative regulator of TSLP. Consistently, the \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) group showed the highest levels of IgE and Th2 transcripts (\(\text{Gata}-3\) and \(\text{Il}4\)) due to repressed Treg-relevant genes (\(\text{Foxp}3\), \(\text{Tgfb}\), and \(\text{Il}10\)) (Figure 4b–d), indicating the repression of TSLP and Th2 by \(\Delta\text{pep27}\)-dependent Treg. Although \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-TSLP}\) showed a significantly reduced \(\text{Gata}-3\) transcript level compared with the \(\Delta\text{pep27}/\text{OXA}\), it revealed the lowest IgE (Figure 4b), indicating TSLP as an aggravating agent for AD. In addition, the \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-TSLP}\) group showed decreased \(\text{Gata}-3\) and \(\text{Il}5\) transcripts compared with the \(\Delta\text{pep27}/\text{OXA}\) group, indicating a role of TSLP in Th2 expression. In contrast, the \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-TSLP}\) group induced the highest \(\text{Foxp}3\) and \(\text{Il}10\) transcripts, even higher than in the \(\Delta\text{pep27}/\text{OXA}\) group (Figure 4c and d), suggesting that TSLP is a negative regulator of Treg.

Skin barrier–related proteins are upregulated by \(\Delta\text{pep27}\) mutant

Patients with AD show characteristically reduced levels of skin barrier molecules, such as loricrin (\(\text{Lor}\)), involucrin (\(\text{Ivl}\)), and filaggrin (\(\text{Flg}\) (Furue, 2020), which are inhibited by Th2-related cytokines (IL-4 and IL-13) (Kim et al., 2008). A family of SPRRs and involucrin gene \(\text{Ivl}\) are soluble and involved in the formation of the initial scaffold during skin layer formation. When the scaffold is complete, insoluble proteins such as involucrin are cross-linked with this structure to form a skin barrier (Eckert et al., 2005; Rinnerthaler et al., 2015). Confocal microscopy revealed that \(\Delta\text{pep27}\)-mutant immunization (\(\Delta\text{pep27}/\text{OXA}\)) resulted in the highest loricrin gene \(\text{Lor}\), involucrin gene \(\text{Ivl}\), and \(\text{Flg}\) levels. However, \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) treatment reduced the expression of loricrin gene \(\text{Lor}\) to the lowest level (Figure 5a and b). Thus, \(\Delta\text{pep27}\) mutant upregulates loricrin gene \(\text{Lor}\), involucrin gene \(\text{Ivl}\), and \(\text{Flg}\) expression, whereas Treg inactivation repressed this expression significantly (Figure 5c). Similar to other molecules, \(\Delta\text{pep27}/\text{OXA}\) increased the expression of all SPRRs, whereas \(\Delta\text{pep27}/\text{OXA}/\alpha\text{-CD25}\) treatment decreased \(\text{Sprr}1\) and \(\text{Sprr}2\) transcripts compared with the \(\Delta\text{pep27}/\text{OXA}\) treatment (Figure 5c). In contrast, in non-OXA conditions, immunization by \(\Delta\text{pep27}\) mutant followed by PBS or \(\alpha\text{-CD25}\) treatment did not elicit any modulation in skin barrier–related marker expression (Supplementary Figure S6a and b). Taken together, these results indicate that \(\Delta\text{pep27}\)-mutant immunization relieves AD symptoms by upregulating the expression of epithelial barrier molecules through Treg upregulation.

DISCUSSION

Children with AD have a delayed IgG response to pneumococcal polysaccharide vaccine (Arkwright et al., 2000). Exposure to pneumococci at an early stage of life can protect against allergic diseases during the adult stage because antibodies are generated against the conserved epitopes in allergens (Patel and Kearney, 2015). However, the underlying mechanism of pneumococcus in AD remains incomplete. In this study, we showed how \(\Delta\text{pep27}\) mutant can prevent or attenuate AD-like symptoms in an animal model. In addition, immunization with \(\Delta\text{pep27}\) mutant inhibited the Th2 response by promoting Tregs and the epithelial barrier and suppressing TSLP expression, mitigating AD symptoms.

The expression of Treg marker was slightly increased in the OXA-treated group compared with that in the control group (Figures 2 and 4). Presumably, this is similar to a situation where increased Treg was observed to compensate for the reduced anti-inflammatory response in the context of a strong Th2 inflammatory response (Roesner et al., 2015). However, \(\Delta\text{pep27}\)-mutant immunization alleviates AD-like symptoms by inducing Treg expression compared with OXA treatment. Th17 cytokines are overexpressed in patients with AD (Dehingra and Guttman-Yassky, 2014; Gittler et al., 2012). However, when the level of \(\text{Ror}t\) transcripts was measured, there was no significant difference in the therapeutic and
prophylactic AD models (Supplementary Figure S3a and b). Therefore, Th17 does not appear to be involved in the relief of symptoms after immunization with \( \text{D}^{\text{pep27}} \) mutant in the OXA-induced AD model. In both human and animal asthma studies, Treg induction has been suggested as protective immunotherapy (Ray et al., 2010). Similarly, \( \text{D}^{\text{pep27}} \)-mutant immunization preferentially alleviates AD symptoms by induction of Treg rather than by repression of Th17.

Skin exterior disruption endorses inflammation through uncontrolled regulation of immunomodulatory proteins and secretion of IL-1\( \beta \), TSLP, and TNF-\( \alpha \) (Ito et al., 2005; Oyoshi et al., 2010; Yoon et al., 2016). In particular, TSLP is produced in epithelial cells and induces type 2 inflammation by activating TSLPR-expressing dendritic cells, basophils, and CD4 T cells (Al-Shami et al., 2004; Ito et al., 2005; Masuoka et al., 2012; Noti et al., 2013). The relationship between TSLP and Tregs is controversial. For example, TSLP promotes pulmonary Treg development (Leichner et al., 2017; Spadoni et al., 2012) and impairs IL-10 (Nguyen et al., 2010). Interestingly, TSLP neutralization elicited the highest Treg induction, which was higher than that found in the \( \text{D}^{\text{pep27}} \)-treated group (Figure 4c), showing that TSLP inhibited Tregs and exacerbated the symptoms of AD in the OXA-induced AD model. Consistent with our results, children with asthma have shown increased TSLP levels with decreased Treg population (Chauhan et al., 2015). In contrast, TSLP antibody treatment in patients with asthma did not alter the circulating Treg frequency (Baatjes et al., 2015). However, the relationship between TSLP and Treg needs to be further clarified in patients with AD.

In AD, impairment of the skin barrier function and the subsequently increased infiltration of allergens into the skin lead to the allergic inflammatory responses that are the main features of Th2 inflammation (Traidl-Hoffmann et al., 2005; Werfel et al., 2016). Patients with AD have a reduced Th1/Th2 ratio, which implies a Th1 and Th2 imbalance (Czarnowicki et al., 2020). For example, in a model using the atopy-patch test technique in patients with AD, Th1 cells were significantly increased after 24–48 hours (Leung and Bieber, 2003). In addition, when AD was induced in IFN\( \gamma \)−/− mice, dermal thickness decreased (Spergel et al., 1999). Therefore, the Th1 response is also involved in AD. In most experimental AD systems, haptens such as OXA induce a Th1-dominant response (Gittler et al., 2013). In fact, in the OXA-treated group of the preventive AD model, the Th1 cell markers were increased compared with those in the control group, but there was no difference in the \( \text{D}^{\text{pep27}} \)-treated group (Supplementary Figure S3b). Therefore, \( \text{D}^{\text{pep27}} \) mutant does not contribute to symptom relief by modulating the Th1 response.

In summary, \( \text{D}^{\text{pep27}} \) mutant is a prospective mucosal vaccine that has a substantial anti-inflammatory effect in AD by subduing inflammatory Th2 cytokines and TSLP and inducing anti-inflammatory Tregs and an epithelial barrier.
MATERIALS AND METHODS

Materials, bacterial strain, and mice
All the chemicals used for bacterial culture were bought from Difco BD (Franklin Lakes, NJ). The pep27 mutant (THpep27) (Choi et al. 2013) derived from S. pneumoniae strain D39 (type 2) (GenBank: CP000410.2) was used. Bacteria were cultured in THY medium cultured at 37 °C without aeration. Female BALB/c mice (Orient Bio, Seongnam, Republic of Korea) aged 5 weeks were maintained for 1 week to adapt to the animal room environment. The use of mice was approved in accordance with the guidelines of the Animal Ethics Committee of Sungkyunkwan University (Suwon, Republic of Korea) and the guidelines of the Korean Animal Protection Act. Euthanasia was performed by the carbon dioxide inhalation method.

Δpep27-mutant immunization
Mice were intranasally immunized with 1 × 10^8 colony-forming units of Δpep27-mutant vaccine per mouse every week. All vaccinations were performed using 100 μl of a ketamine–xylazine mixture (10 ml of ketamine, 2.5 ml of xylazine, 12.5 ml of PBS, 100 mg/kg) as an anesthetic.

OXA-induced AD model
To assess the preventive efficacy of Δpep27 in AD, Δpep27-mutant immunization was performed once a week before the challenge. For assessment of therapeutic efficiencies, Δpep27-mutant immunization was performed once a week during the challenge. Immunization was performed three times, and each mouse was treated with 1 × 10^8 colony-forming units of Δpep27 mutant or PBS. For sensitization, 1% (w/v) OXA (Sigma-Aldrich, St. Louis, MO) was mixed in an acetone solution (4:1), and 20 μl of the solution was applied to the inside and outside of the ears of the mice. After 5 days, 20 μl of the solution was applied to the ears with 0.2% (w/v) OXA in acetone and olive oil solution for the challenge. OXA solution (0.2% w/v) was applied to the ears eight times at 2-day intervals. Twenty-four hours after the last challenge, the ears and serum were collected.

The other methods are shown in the Supplementary Materials and Methods.

Data availability statement
No generation or analysis of large datasets during this study.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
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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.2022.07.021

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SUPPLEMENTARY MATERIALS AND METHODS

Transepidermal water loss
Transepidermal water loss was measured using Aquaflux (AF200, Biyo Systems, London, United Kingdom). The day after the last oxazolone challenge, transepidermal water loss was measured in both ears of the mouse at room temperature. Two readings from each ear were taken and averaged for each mouse.

Thymic stromal lymphopoietin neutralization and regulatory T cell inactivation in vivo
For the thymic stromal lymphopoietin neutralization experiment, the mice received 20 μg of anti-thymic stromal lymphopoietin mAb (MAB555, R&D Systems, Minneapolis, MN) or the isotype control IgG2A through intraperitoneal injection (MAB006, R&D Systems). For regulatory T cell inactivation, mice were injected intraperitoneally with 30 μg of purified PC61 mAb (affinity purified, BioLegend, San Diego, CA) or control isotype 3 days after Δpep27 immunization.

ELISA
Levels of IL-4, IL-5, IL-10, IL-13, and IgE were measured in mouse serum using commercial kits (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions.

Multiplex cytokine assay
Levels of IL-4, IL-5, IL-10, IL-13, and IL-17 in mouse serum were simultaneously measured using the Luminex Multiplex Cytokine Assay (Merck Chemicals and Life Science AB, Billerica, MA).

Histological analysis
Mice ears were collected 24 hours after the last oxazolone treatment. The tissues were fixed in a 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) solution and then placed in a paraffin block. The paraffin-embedded tissue sections were cut into 10 μm slices and heat immobilized. This was followed by deparaffinization by dipping in xylene (Sigma-Aldrich) solution, rehydration by an ordered sequence of ethanol, and then incubated with 3% hydrogen peroxide, made permeable with 0.5% Triton X-100, and then incubated in a TUNEL reaction mixture (Roche, Munich, Germany). The sections were washed and visualized using a converter with 0.03% 3,30-diaminobenzidine. TUNEL-stained tissue sections counterstained with eosin were then viewed using an optical microscope (BX53, Olympus, Tokyo, Japan). This work was performed by KNOTUS (Incheon, South Korea).

Immunohistochemistry and immunofluorescence
Paraffin-embedded ear tissue sections were fixed for 15 minutes at room temperature in a 4% paraformaldehyde solution, followed by permeabilization in a cold ethanol–acetic acid mixture for 5 minutes. Antigen retrieval was conducted in citrate buffer (10 mM, pH 6.0). After that, the sections were incubated with caspase-3 antibody (Abcam, Cambridge, United Kingdom) or loricrin (Novus Biological, Littleton, CO) overnight at 4 °C. Visualizations were done using 3,30-diaminobenzidine as a chromogen. In the case of immunofluorescence, slides were incubated with Alexa flour 488–conjugated secondary antibody (Abcam) plus DAPI for DNA staining.

Skin thickness measurement
Ear skin was photographed at ×200 magnification with a digital camera (DS-Ri2, Nikon, Tokyo, Japan). Epidermal thickness and the full thickness of skin were measured using a NIS-Elements-BR, version 5.21 (Nikon) image analysis program. The full thickness of the ear skin was calculated as the thickness from the epidermis to the perichondrium. Epidermal thickness and total skin thickness were measured at three locations per stained slide.

Measurement of eosinophil count and inflammatory cell infiltration
The number of eosinophils that appeared in the skin dermis layer was determined under a microscope at ×400 magnification (high-power field) with a digital camera. Measurements were conducted at three sites per stained slide. The degree of inflammatory cell infiltrates appearing in the dermal layer of the skin was classified into four grades: 0 = absence, 1 = mild, 2 = moderate, and 3 = severe.

RT-qPCR
The RT-qPCR was performed as described in a previous study (Kim et al., 2019) using the primers (Supplementary Table S1).

Statistical analysis
Each experiment was performed at least three times. Statistics were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and presented as mean ± SEM. One-way analysis of variance was used in data analysis.

SUPPLEMENTARY REFERENCE
Supplementary Figure S1. Treatment with Δpep27 attenuates AD symptoms in a therapeutic model. (a) Outline of the therapeutic effects of Δpep27 on the OXA-induced therapeutic AD model. Mice were immunized with Δpep27 at 6, 13, and 20 days during 10 times challenges at 2-day intervals from day 6 to day 24 after sensitization with 1% OXA. (b) The ear of the mouse was photographed 48 h after the last sensitization. Therapeutic mice ear thickness was measured on days 1, 6, 13, 19, and 25 (n = 4). (c, d) Histological analysis of epidermal and dermal indices and infiltration of the ear cells were investigated by H&E staining (n = 3). Microscopic photographs of H&E staining for inflammation were captured at ×200 magnification. Bars = 100 μm. The thickness of the epidermis and dermis was measured: normal = 1, two times greater than normal = 2, three times greater than normal = 3. Data are expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001). AD, atopic dermatitis; CFU, colony-forming unit; CON, control; h, hour; I.N., intranasally; OXA, oxazolone.
Supplementary Figure S2. Immunization with Δpep27 represses Th2 cytokines and TSLP and induces Treg-relevant cytokines in a therapeutic AD model. The mice were immunized three times with Δpep27 during OXA-induced AD skin lesion. (a) The mRNA expression levels in mouse ear tissues were analyzed by RT-qPCR (n = 3). (b) The total serum levels of IL-4, IL-5, and IL-17A were measured by ELISA (n = 3). (c) Serum IgE level was measured by ELISA (n = 3). Each value is expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey’s test (*P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001). AD, atopic dermatitis; CON, control; conc., concentration; ns, not significant; OXA, oxazolone; Th2, T helper 2; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.

Supplementary Figure S3. Immunization with Δpep27 restores Th2/Treg balance in the preventive and therapeutic models. (a) mRNA expression levels of Th cell–specific makers (Gata-3, T-bet, Foxp3, and RORgt) were analyzed by RT-qPCR in the therapeutic model (n = 3). (b) Th1-related (T-bet and Tnfa) and Th17-related (Rorγt and Il17) markers were analyzed by RT-qPCR in the preventive model (n = 5). Data are expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey’s test (*P ≤ 0.05 and **P ≤ 0.01). CON, control; ns, not significant; Th, T helper; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.
Supplementary Figure S4. TSLP aggravates skin inflammation and inflammatory cell infiltration, whereas Treg relieves them. (a) Images of H&E staining show the full thickness of the ear skin. Bars = 50 μm. (b) The value of the thickness (n = 9). (c) A graph of the degree of inflammatory cell infiltration (n = 3). (d) Proapoptotic (Bax) and antiapoptotic (Bcl2) genes were analyzed by RT-qPCR (n = 5). Data are expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey’s test (**P < 0.01, ***P < 0.001, and ****P < 0.0001). CON, control; ns, not significant; OXA, oxazolone; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.

Supplementary Figure S5. Immunization with Δpep27 alone can repress TSLP and increase Treg-relevant factors. (a, d) mRNA expression levels of Tslp, Treg-, and Th2 cell-specific markers (Foxp3, Il10, Gata-3, and Il4) were analyzed by RT-qPCR in antibody-treated groups (n = 5). (b) Graph of the number of eosinophils found in the dermal layer (n = 5). (c) The percentage of TUNEL-positive cells in four random fields from each mouse ear section (n = 4). (e) The IL-10 and IL-4 cytokine levels in serum were measured by ELISA (n = 5). Data are expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey’s test (*P < 0.05, **P < 0.01, and ****P < 0.0001). conc., concentration; OXA, oxazolone; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.
Immunization with Δpep27 did not affect the normal skin barrier. (a) Immunofluorescence images of ear tissue stained with Lor. DNA was counterstained with DAPI. Bars = 100 μm. (b) Skin barrier-related genes were analyzed by RT-qPCR (n = 4 or 5). Data are expressed as mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Tukey’s test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001). CON, control; Lor, loricrin; ns, not significant; OXA, oxaazolone.
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Abbreviations: F, forward; Iv1, involucrin; Lor, loricrin; R, reverse.