Topical Application of Dieckol Ameliorates Atopic Dermatitis in NC/Nga Mice by Suppressing Thymic Stromal Lymphopoietin Production

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TO THE EDITOR

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease that is characterized by extreme pruritus. AD is considered the first stage of atopic march (Leung et al., 2004). From an immunological aspect, AD is closely linked to the disruption of Th1/Th2 cytokine homeostasis that skews to Th2 immunity (Bieber, 2008). Immunological approaches to the treatment of AD have mainly focused on stimulating Th1 immunity to recover the Th1/Th2 balance (Leung et al., 2004). Thymic stromal lymphopoietin (TSLP) was recently shown to play a critical role in the progress of AD by inducing Th2 immune responses (Soumelis et al., 2002; Zhang et al., 2009). Increased TSLP production in keratinocytes induces the expression of the OX40 ligand (OX40L) in dendritic cells (DCs), which in turn stimulates the differentiation of naïve $CD4^+$ T cells into Th2 cells to produce Th2 cytokines such as IL-4, IL-5, and IL-13 (Leyva-Castillo, Hener, Michea, et al., 2013). Thus, the TSLP-OX40L axis is considered integral to the induction of a Th2 cell-mediated allergic cascade in AD (Murakami-Satsutani et al., 2014). Indeed, high levels of TSLP expression have been observed in epidermal keratinocytes of AD skin lesions (Leyva-Castillo, Hener, Jiang, et al. 2013a). Therefore, suppressing TSLP may represent a novel therapeutic approach for treating AD by restoring Th1/Th2 balance. Here, we investigated whether dieckol, a phlorotannin from *Ecklonia cava* (Figure 1a), can suppress TSLP production to reduce Th2 immunity and effectively alleviate AD-like symptoms in an NC/Nga mouse model in vivo.

First, we determined whether dieckol affects the production of TSLP in a mouse keratinocyte cell line, KCMH-1, that constitutively produces high amounts of TSLP (Segawa et al., 2014). Dieckol reduced TSLP mRNA and protein levels in KCMH-1 cells (Figure 1b and c). In addition, TSLP expression induced by MC903 in mouse ear skin was reduced by topical application of dieckol (see Supplementary Figure S1 online). NF-κB is an important transcription factor required for TSLP expression. Dieckol significantly reduced NF-κB-dependent luciferase expression in KCMH-1 cells that have high basal activation of NF-κB (Figure 1d). Dieckol blocked IL-1β-induced phosphorylation and degradation of IκBα and NF-κB luciferase activity in mouse keratinocytes (Pam212) (Figure 1e and f, see Supplementary Figure S2a online). Similarly, Joe et al. (2006) showed that dieckol inhibited phorbol myristate acetate-induced NF-κB activation in HeLa cells, leading to the decrease of matrix metalloproteinase-1 in human dermal fibroblasts (Joe et al., 2006). The results confirm that dieckol suppresses TSLP production by inhibiting NF-κB activation in keratinocytes. In contrast, IL-1β-induced early growth response protein 1 (EGR-1) expression and phosphorylation of p38, extracellular signal–related kinase, and c-Jun N-terminal kinase were not inhibited by dieckol in Pam212 cells (see Supplementary Figure S2b). To delineate how dieckol interacts with the NF-κB signaling cascade, ligand-independent NF-κB activation was induced by exogenously overexpressing the signaling component in 293T cells. Dieckol suppressed NF-κB activation induced by inhibitor of NF-κB kinase subunit beta (IKKβ) but not p65 (see Supplementary Figure S3 online), suggesting that dieckol may target IKKβ itself or the complex involving IKKβ but not p65 and its downstream components. We further examined whether the suppression of TSLP production would lead to a decrease in OX40L expression in mouse primary bone marrow-derived DCs (BMDCs). Incubation of BMDCs with conditioned medium from KCMH-1 cells resulted in the induction of OX40L mRNA expression in BMDCs. This induction of OX40L mRNA expression was abolished by dieckol treatment of KCMH-1 cells (Figure 1g), suggesting that inhibition of TSLP production by dieckol in keratinocytes can reduce OX40L expression in DCs.

Next, we investigated whether dieckol can suppress TSLP production and AD-like symptoms *in vivo* using an NC/Nga mouse AD model. Animal care and the experimental protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Catholic University of Korea (permission no. 2013-020-02). AD-like symptoms were induced in male NC/Nga mice by repeatedly applying a topical allergen—extract of *Dermatophagoides farina*, a major species of house dust mites (HDM)—and 2,4-dinitrochlorobenzene (DNCB) on the shaved dorsum. Dieckol (1%) was applied topically on NC/Nga mice daily for 4 weeks. Repeated application of HDM/DNCB to NC/Nga mice induced skin dryness, severe erythema,

Abbreviations: AD, atopic dermatitis; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene; HDM, house dust mite; IKKβ, inhibitor of NF-κB kinase subunit beta; OX40L, OX40 ligand; TSLP, thymic stromal lymphopoietin

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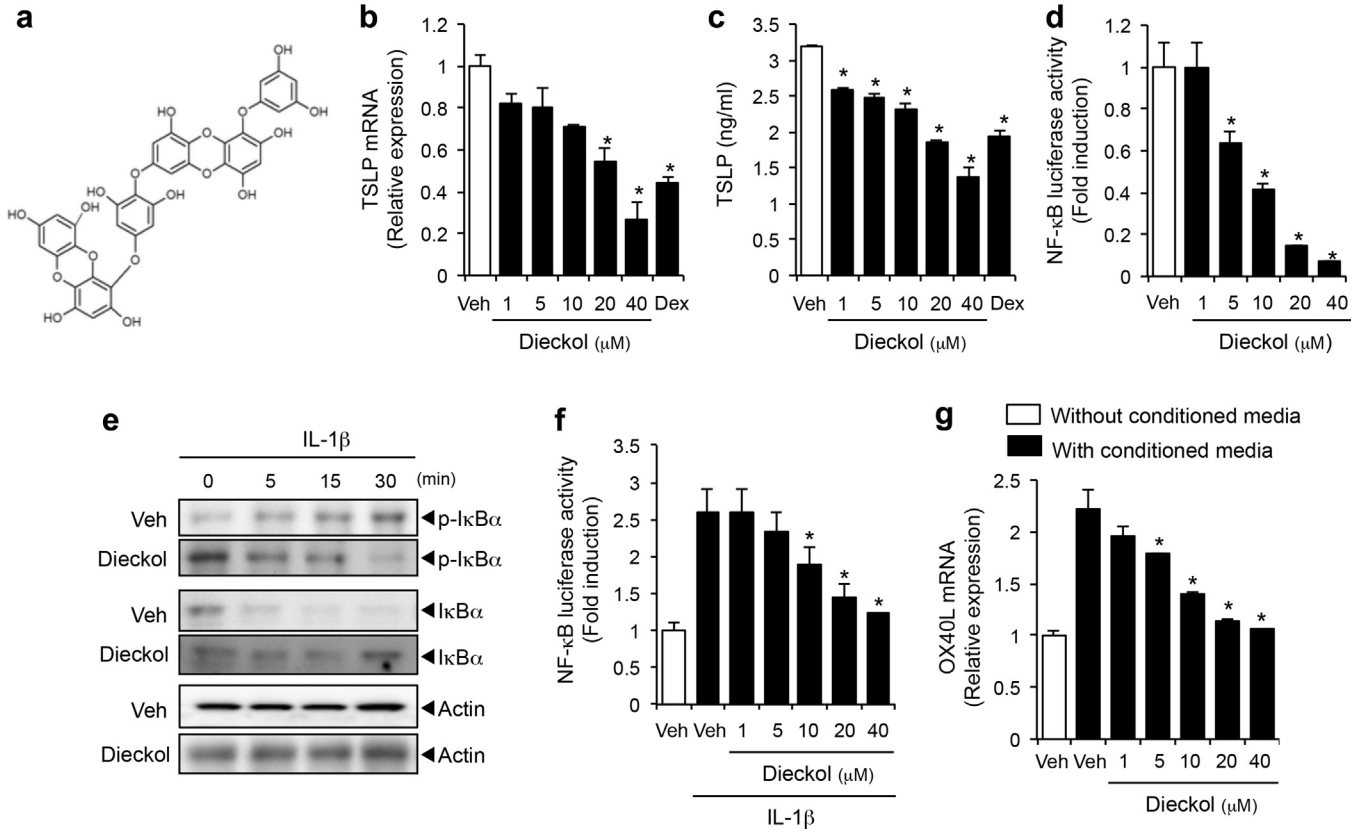

Figure 1. Dieckol suppresses TSLP production through the inhibition of NF-κB activation in keratinocytes. **(a)** Chemical structure of dieckol (4-[4-[6-(3,5-dihydroxyphenoxy)-4,7,9-trihydroxydibenzo-p-dioxin-2-yl]oxy-3,5-dihydroxyphenoxy]dibenzo-p-dioxin-1,3,6,8-tetrol; molar mass = 742.549 g/mol). **(b, c)** KCMH-1 cells were treated with dieckol or dexamethasone (10 μmol/L) for **(b)** 6 hours or **(c)** 24 hours. TSLP mRNA or protein levels were determined by **(b)** RT-real-time quantitative PCR (n = 3) or **(c)** ELISA (n = 6). mRNA levels of TSLP were normalized with GAPDH mRNA levels. **(d)** KCMH-1 cells were transfected with pNF-κB-luciferase plasmid with β-galactosidase expression plasmid. Dieckol was treated for 30 minutes. Luciferase activity was normalized by β-galactosidase activity (n = 6). **(e)** Pam212 cells were pretreated with dieckol (40 μmol/L) for 1 hour and stimulated with IL-1β (10 ng/ml) and subjected to immunoblotting. **(f)** Pam212 cells were transfected with pNF-κB-luciferase reporter plasmid with β-galactosidase expression plasmid. After cells were pretreated with dieckol for 2 hours, cells were treated with IL-1β (10 ng/ml) for 18 hours and dieckol for 30 minutes (n = 3). **(g)** After KCMH-1 cells were incubated with dieckol for 24 hours, the conditioned media was collected and added to mouse primary bone marrow-derived dendritic cells. OX40L mRNA levels were determined by RT-real-time quantitative PCR and normalized with GAPDH mRNA levels (n = 6). Data are shown as the mean ± SEM. $^*P < 0.05$ vs. (b, c, d) vehicle alone, **(f)** IL-1β alone, or **(g)** vehicle with conditioned media by 1-way analysis of variance followed by Dunnett's post hoc test. Dex, dexamethasone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκBα, inhibitor of kappa B alpha; μM, μmol/L; OX40L, OX40 ligand; p-IκBα, phosphor-IκBα; RT, reverse transcription; SEM, standard error of the mean; TSLP, thymic stromal lymphopoietin; Veh, vehicle.

hemorrhage, scarring, dryness, edema, excoriation, and erosion (Figure 2a). Meanwhile, topical application of dieckol greatly improved these AD-like skin symptoms induced by HDM/DNCB as determined by dermatitis score evaluation (Yamamoto et al., 2007) (Figure 2a and b). Histological assessment of skin tissues by hematoxylin and eosin staining confirmed that AD-like skin lesions characterized by epidermal hyperplasia, edema, and accumulation of inflammatory cells in the dermis/epidermis were significantly alleviated by dieckol (Figure 2c, left). Toluidine blue staining of skin tissues showed that topical application of dieckol significantly reduced the infiltration of dermal mast cells (Figure 2c, right; see Supplementary Figure S4 online). The elevation of serum IgE levels in HDM/DNCB-stimulated mice was also attenuated by topical application of dieckol (Figure 2d). Most importantly, topical application of dieckol nearly abolished HDM/DNCB-induced production of TSLP in AD-like skin lesions in NC/Nga mice (Figure 2e, see Supplementary Figure S5a online), in agreement with the effects observed in KCMH-1 cells. In addition, dieckol blocked the production of IL-33 (Figure 2f, see Supplementary Figure S5b), which is secreted by the cells of barrier tissues and activates Th2 lymphocytes, mast cells, and eosinophils (Savinko et al., 2012). IL-33 functions as a positive regulator of the TSLP-OX40L axis, which initiates and maintains Th2 cell—mediated inflammatory responses (Imai et al., 2013; Murakami-Satsutani et al., 2014).

The differentiation of naïve T cells into Th1 and Th2 cells is regulated by key transcription factors such as T-bet and GATA-3 for Th1 and Th2 cells, respectively. Topical application of dieckol blocked the enhanced expression of GATA-3 in AD-like skin lesions (Figure 2g), whereas the mRNA levels of T-bet were increased (Figure 2h). Topical application of dieckol reduced the levels of Th2 cytokines such as IL-4 and -5 in AD-like skin lesions (Figure 2i and j, see Supplementary Figure S5c and d). The level of IL-13 was also slightly decreased by dieckol