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ORIGINAL ARTICLE

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Periostin Contributes to the Pathogenesis of Atopic Dermatitis by Inducing TSLP Production from Keratinocytes

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ABSTRACT

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease where Th2-type immune responses are dominant. Keratinocytes persistently secrete proinflammatory cytokines and chemokines, amplifying Th2-type responses in AD. We have recently reported that periostin, an extracellular matrix protein induced by Th2 cytokines, plays a critical role in AD. In the present study, we have further investigated the characteristics of our allergen-induced AD model mice and the role of periostin in the pathogenesis of AD.

Methods: The ears of C57BL/6 mice, BALB/c mice, and Rag-2$^{-/-}$ $\gamma c^{-/-}$ mice (BALB/c background) were epicutaneously sensitized repeatedly with HDM. Mice were analyzed after the final sensitization. To examine the direct role of periostin, we reconstituted skin *in vitro* by coculture of keratinocytes with wild-type or periostin-deficient fibroblasts.

Results: Epicutaneous sensitization with HDM induced AD-like phenotypes and accumulation of periostin in dermis in C57BL/6 mice but not in Rag-2$^{-/-}$ $\gamma c^{-/-}$ mice. *In vitro* organotypic coculture systems revealed that periostin promoted survival and proliferation of keratinocytes and directly induced production of thymic stromal lymphopoietin (TSLP).

Conclusions: Our results suggest that periostin exacerbates the pathogenesis of AD through TSLP production from keratinocytes.

KEY WORDS

atopic dermatitis, house dust mite, periostin, Th2 cells, TSLP

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease that commonly presents during infancy and childhood.[1,2] It is estimated that worldwide, 5-20% of children are affected by AD, and its prevalence has been increasing for several decades.[3,4] Th2 immune response is believed to be involved in the pathogenesis of AD.[1,2,5] Indeed, transgenic expression of Th2 cytokine, IL-4 or IL-13, in the skin induces pruritic

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dermatitis in mice.[6,7] However, it is poorly understood how Th2 cytokines induce AD phenotypes and how chronic inflammation is sustained.

Keratinocytes actively participate in AD inflammation through production of proinflammatory cytokines and chemokines.[8] Keratinocyte-derived TSLP is of particular importance in AD.[9] TSLP is an IL-7-like cytokine produced by epithelial cells and activates dendritic cells to induce an inflammatory Th2 response. It has been reported that TSLP is expressed in keratinocytes of AD patients but not those of healthy subjects.[10] Furthermore, TSLP transgenic mice develop AD-like dermatitis, whereas TSLP receptor-deficient (TSLPR-deficient) mice fail to develop allergic skin inflammation induced by epicutaneous sensitization with allergens.[11,12] However, it has not been fully understood what endogenous trigger sustains production of TSLP from keratinocytes in AD.

Periostin is an extracellular matrix (ECM) protein belonging to the fasciclin family, which is composed of an EMI domain, four tandem fasciclin I (FAS1) domains, and the alternative splicing domain.[13,14] Periostin binds to other ECMs, collagen I, fibronectin, and tenascin-C, and enhances collagen fibrillogenesis by activating lysyl oxidase.[15-18] In addition to such structural roles as occur in ECMs, periostin acts as a matricellular protein to modulate cell function via integrins and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[14,19,20] This biphasic function of periostin contributes to various pathophysiological conditions such as cardiac development, tumorigenesis, and bronchial asthma.[14,18,20-22] Moreover, we have recently suggested that periostin sets up a vicious circle that links Th2 immune response and keratinocyte activation.[23]

In the current study, we further investigated the possible involvement of periostin in the pathogenesis of AD, using HDM-induced AD model mice and an *in vitro* organotypic coculture system of keratinocytes. Just like Th2-prone BALB/c mice sensitized with HDM, Th1-prone C57BL/6 mice sensitized with HDM exhibited AD-like phenotypes and accumulation of periostin in dermis. These phenotypes were dependent on lymphocytes. *In vitro* analyses revealed that periostin promotes survival and proliferation of keratinocytes and production of TSLP from keratinocytes. Our data confirmed the previous report that periostin accumulated in skin tissues by allergen exposure enhances Th2 immune response by activating keratinocytes.[23]

METHODS

MICE AND ANTIGENS

Eight- to twelve-week-old female BALB/c or C57BL/6 mice from SLC Japan (Hamamatsu, Japan) were used in the experiments. BALB/c background Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice were purchased from Central Institute for Experiment Animals (Kawasaki, Kanagawa, Japan). Experiments were undertaken following the guidelines for care and use of experimental animals of the Japanese Association for Laboratory Animals Science (1987). *Dermatophagoides farinae* crude extracts (house dust mite antigen, HDM; Torii, Tokyo, Japan) was used as an antigen.[24] HDM was dissolved in phosphate buffered saline (PBS) containing 50% Glycerol.

HDM-INDUCED AD MODEL MICE

Epicutaneous sensitization of mice was performed as described previously, with minor modifications.[25] Briefly, mice were anesthetized with halothane (2-Bromo-2-chloro-1, 1, 1-trifluoroethane; Sigma-Aldrich, St Louis, MO, USA). Both surfaces of mouse ear lobes were stripped three times with cellophane tape (Kyowa, Osaka, Japan), and then 0.25 mg in 25 µl HDM solution was painted onto each surface of both ear lobes. Tape stripping and HDM painting were performed once a week for five weeks and ear thickness was measured using a dial thickness gauge (Ozaki MFG, Tokyo, Japan) immediately before each tape stripping. Mice were sacrificed 24 h after the fifth sensitization.

HISTOLOGICAL ANALYSIS

For histological examination, specimens were obtained from ears 24 h after the fifth sensitization. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 5-µm sections were stained with hematoxylin and eosin (H&E) or Masson Trichrome. The number of eosinophils infiltrated in skin was counted in each section. Immunostaining for periostin was performed with rabbit polyclonal anti-periostin antibody (Ab), as described previously.[18]

REAL–TIME PCR ANALYSIS

Draining lymph nodes and ears were obtained 24 h after the fifth sensitization. Total RNA was isolated and reversely transcribed as previously described.[26] Quantitative real-time PCR was performed using the ABI PRISM™ 7700 sequence detection system (Perkin-Elmer Japan, Yokohama, Japan). Samples were normalized to *Actb* and reported according to the $\Delta\Delta C_T$ method as RNA fold increase: $2^{\Delta\Delta CT} = 2^{\Delta CT\ sample\ -\ \Delta CT\ reference}$.

PREPARATION OF KERATINOCYTES AND FIBROBLASTS

Mouse keratinocytes were obtained and cultured by modifying the previous method.[27] Briefly, skin removed from P2 newborn BALB/c mice was cut down and incubated with DMEM containing 3.5 mg/ml dispase (Invitrogen, Carlsbad, CA, USA) at room temperature for 2 h. Epidermis separated from dermis was trypsinized at 37℃ for 2 min, followed by filtra-

tion. Wild-type or *Postn*$^{-/-}$ mouse embryonic fibroblasts were obtained from E14.5 embryos.

RECOMBINANT PROTEINS

The recombinant protein of periostin was prepared as previously described.[18]

ORGANOTYPIC COCULTURE

A reconstruction model of skin was performed as previously described.[23,28] Two ml of type I collagen gel solution (Nitta Gelatin, Osaka, Japan) containing 2×10^6 of wild-type or *Postn*$^{-/-}$ fibroblasts with or without recombinant mouse periostin (R&D Systems, Minneapolis, MN, USA) was poured into a Millicell-CM 30 mm in diameter (Millipore, Bedford, MA, USA) and then 2×10^6 keratinocytes were seeded on the collagen gel. The culture assembly was fixed at day 4 or 7 with 10% buffered formalin and embedded in paraffin. Multiple 5-μm sections were stained with H&E or immunostained with anti-cytokeratin 10 (CK10) Ab, anti-cytokeratin 14 (CK14) Ab, anti-proliferating cell nuclear antigen (PCNA) Ab, or anti-phospho-Akt Ab (Cell Signaling, Beverly, MA, USA). Apoptotic cells were detected by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

MONOLAYERED CULTURE OF KERATINOCYTES

Primary keratinocytes were cultured on plates coated with recombinant periostin or BSA. Total RNA was obtained one day after stimulation, and *Tslp* mRNA levels were measured with real-time PCR. Supernatants were harvested three days after stimulation, and TSLP protein levels were measured by ELISA (Mouse TSLP ELISA MAX™ Delux; BioLegend, San Diego, CA, USA).

STATISTICS

All the results were analyzed by two-tailed Student's test.

RESULTS

EPICUTANEOUS SENSITIZATION OF HOUSE DUST MITE ANTIGEN (HDM) INDUCES AD-LIKE PHENOTYPES IN C57BL/6 MICE

House dust mite allergen (HDM) is the most common aeroallergen resulting in allergic sensitization in children with AD.[1] It has been reported that epicutaneous sensitization with HDM induces AD-like phenotypes in Nc/Nga mice and BALB/c mice.[25,29] Indeed, we recently demonstrated that epicutaneous sensitization by repeated painting with HDM induced ear swelling in BALB/c mice[23] (Fig. 1A). We first examined whether HDM induces AD-like phenotypes in Th1-prone C57BL/6 mice as well. Epicutaneous sensitization with HDM significantly increased ear thickness in C57BL/6 mice as well as in BALB/c mice (Fig. 1B). Histological examination of ear skin tissues from HDM-sensitized C57BL/6 mice showed phenotypes similar to HDM-sensitized BALB/c, such as thickened epidermis and dermis, fibrosis, and infiltration of mononuclear cells and eosinophils in the dermis (Fig. 1C, D), although their extent in C57BL/6 mice was slightly less than that in BALB/c mice[23] (data not shown). Furthermore, periostin was significantly accumulated in the dermis of HDM-sensitized C57BL/6 mice (Fig. 1C). The accumulation of periostin in the dermis of HDM-sensitized skin was due to increased mRNA levels (Fig. 1E). This induction was observed from 24 h after second sensitization of HDM. Next we examined the type of immune response in HDM-sensitized mice. Th2 immune response is believed to play a key pathogenic role in AD, which is supported by elevated IgE levels in the majority of AD patients.[1] As in BALB/c mice, increased expression of mRNA for *Il4* and *Il13* or decreased expression of mRNA for *Ifng* was observed in the draining lymph nodes of HDM-sensitized C57BL/6 mice, which indicates a systemic Th2 immune response (Fig. 2A). Similarly, the expression of mRNA for *Il13* was increased in the skin lesions of HDM-sensitized mice (Fig. 2B). Moreover, the expression of mRNA for *Tslp*, which is important for allergic inflammation, was increased in HDM-sensitized mice. These data demonstrate that epicutaneous sensitization of HDM causes allergic dermatitis and the expression of periostin and TSLP, irrespective of backgrounds of the strain in mice, and suggests that periostin is involved in the pathogenesis of allergic dermatitis in AD.

LYMPHOCYTES ARE REQUIRED FOR INFILTRATION OF MONONUCLEAR CELLS AND EOSINOPHILS AND FOR PERIOSTIN EXPRESSION IN HDM-SENSITIZED SKIN

It is known that a marked infiltration of mononuclear cells, especially $CD4^+$ activated memory T cells, is observed in human AD skin lesions.[1,2] To investigate whether HDM-induced AD phenotypes in model mice are dependent on lymphocytes, we sensitized Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice, which lack lymphocytes, with HDM. As shown in Figure 3A, epicutaneous sensitization of HDM did not induce ear swelling in Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice. These mice failed to display dermal infiltration by eosinophils or mononuclear cells (Fig. 3B). The necessity of lymphocytes for allergen-induced dermatitis in mice is consistent with a previous study using OVA as an allergen.[30] Furthermore, the expression of periostin and TSLP was not induced by sensitization of HDM in the skin lesions of Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice (Fig. 3B, C). These results suggest that in our AD model mice, lymphocytes are essential for the development of skin inflammation, thickening of dermis and epidermis, and concomitant expression of periostin and TSLP.

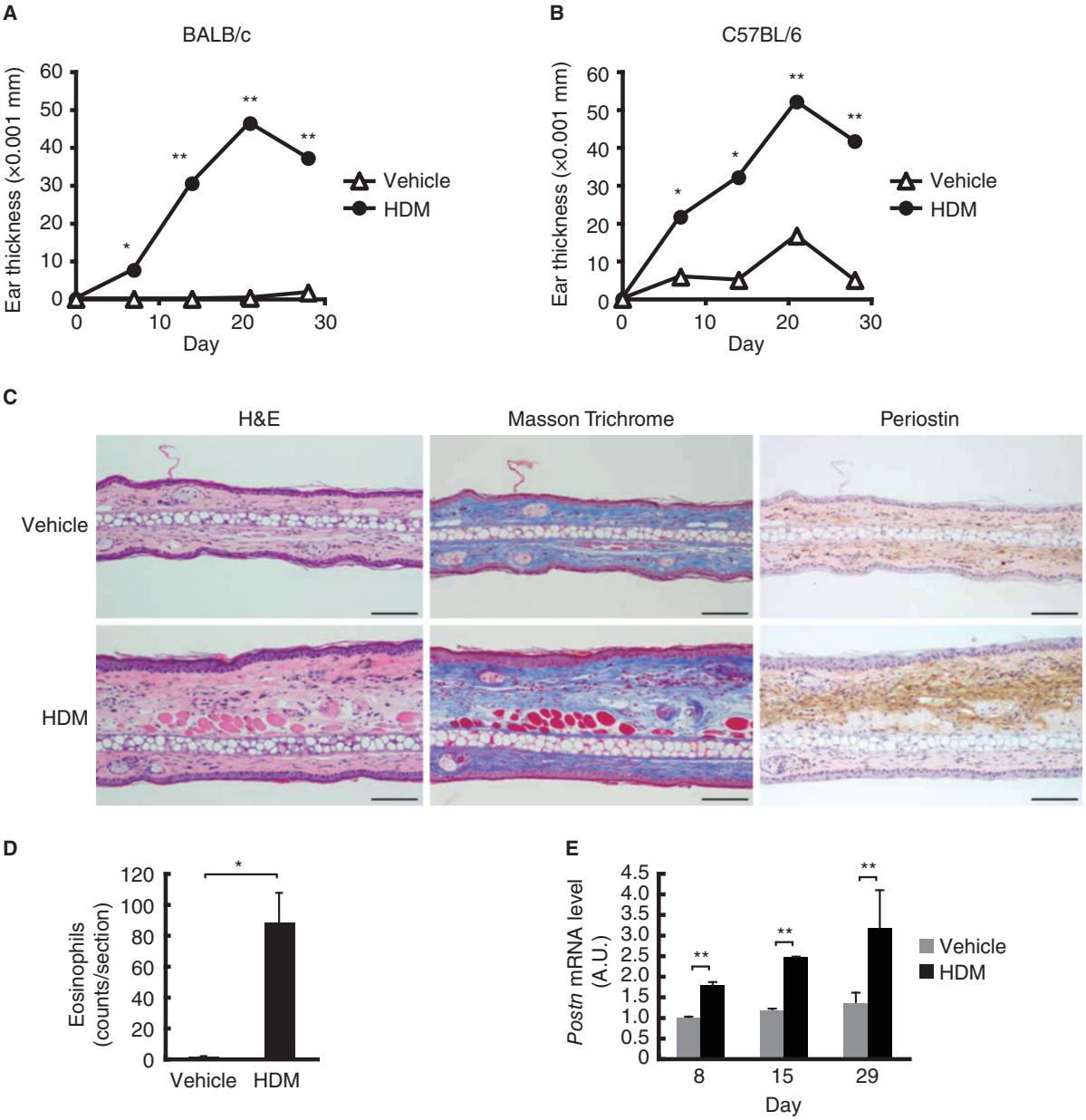

Fig. 1 Epicutaneous sensitization of house dust mite antigen (HDM) induces AD-like phenotypes in C57BL/6 mice. (**A**) Change in ear thickness caused by repeated painting with vehicle (open triangle, $n = 10$) or HDM (closed circle, $n = 18$) in BALB/c mice. (**B**) Change in ear thickness caused by repeated painting with vehicle (open triangle, $n = 6$) or HDM (closed circle, $n = 12$) in C57BL/6 mice. (**C**) Representative photomicrographs of H&E, Masson trichrome, and immunostaining for periostin of ear skin biopsies taken from C57BL/6 mice treated with vehicle or HDM 24 h after the fifth sensitization. Scale bar: 50 μm. (**D**) The number of eosinophils infiltrated in skin tissues of C57BL/6 mice was counted in each section. (**E**) Quantitative analysis of mRNA levels of *Postn* in the skin lesions from C57BL/6 mice at the indicated time after first sensitization (Vehicle, $n = 4$; HDM, $n = 6$). Values are means + s.d. of all experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

PERIOSTIN ENHANCES PROLIFERATION AND DIFFERENTIATION OF KERATINOCYTES

Aberrant differentiation (hyperkeratosis/parakeratosis) and hyperproliferation of keratinocytes (acanthosis) are typical features of AD.[1,2] Keratinocytes are important for inflammatory response in AD as enhancer cells, secreting various cytokines and chemokines,[8,31] and hyperproliferation of keratinocytes is assumed to provide the basis for excessive production of proinflammatory cytokines and chemokines. To

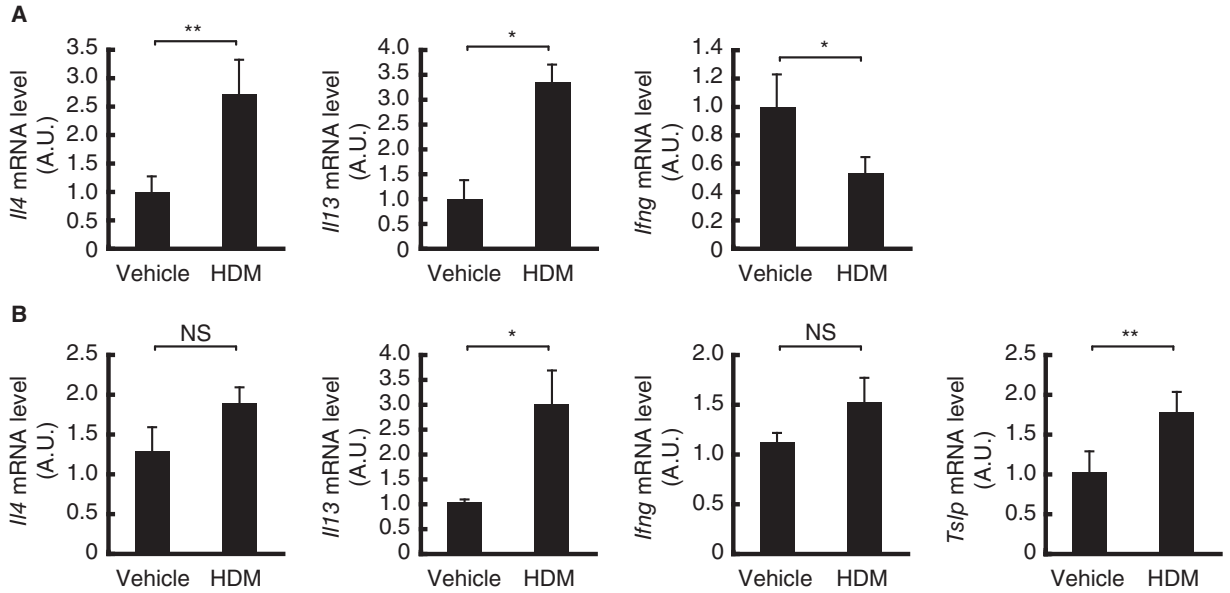

Fig. 2 Epicutaneous sensitization of HDM induces Th2 skewed cytokine profiles. Quantitative analysis of mRNA levels of *Il4*, *Il13*, *Ifng*, or *Tslp* in the draining lymph nodes (**A**) or the skin lesions (**B**) from C57BL/6 mice 24 h after the fifth sensitization (vehicle, $n = 4$; HDM, $n = 6$). Values are means + s.d. of all experiments. $^*P < 0.05$, $^{**}P < 0.01$.

examine the direct role of periostin in proliferation of keratinocytes, we reconstituted skin *in vitro* by coculturing keratinocytes with fibroblasts.[28] Primary keratinocytes were cocultured on collagen gel containing wild-type fibroblasts or *Postn*$^{-/-}$ fibroblasts because the main source of periostin is fibroblasts[23] (Fig. 4A). The concentratration of periostin in the supernatant in this condition was not so different from that in the serum from AD patients[23] (data not shown). Keratinocytes cocultured with wild-type fibroblasts proliferated and stratified, which result in the formation of an epidermal layer consisting of basal, pickle cell, granular, and cornified layers by day 4 (Fig. 4B). Keratinocytes further differentiated and formed more thickened cornified layers by day 7. The expression pattern of CK14, a marker of basal layer cells, and CK10, a marker of suprabasal layer cells, was similar to that of keratinocytes *in vivo* (Fig. 4C). In contrast, keratinocytes cocultured with *Postn*$^{-/-}$ fibroblasts proliferated less and formed a thinner epidermis compared with wild-type fibroblasts. Addition of recombinat periostin in coculture with *Postn*$^{-/-}$ fibroblasts completely restored them to a level comparable to that of wild-type fibroblasts. To further analyze the effect of periostin on keratinocytes, we quantified survival and proliferation of keratinocytes by immunostaining for PCNA and TUNEL assay, which detect proliferated and apoptotic cells, respectively. Proliferating keratinocytes significantly decreased in the periostin-deficient condition (56%) as compared with the periostin-present conditions (WT-rPeriostin, 87%; WT+rPeriostin, 90%; KO+rPeriostin, 89%), although proliferation of fibroblasts did not change in any conditions (Fig. 4D, E). In contrast, apoptotic cells significantly increased in the periostin-deficient condition (Fig. 4F). It is known that periostin activates the Akt pathway via α_v integrins and that the Akt signals promote survival of differentiating keratinocytes.[32] Indeed, phosphorylated Akt decreased in the periostin-deficient condition (18%) as compared with the periostin-present conditions (WT-rPeriostin, 38%; WT+rPeriostin, 40%; KO+rPeriostin, 46%, Fig. 4 G). These results suggest that periostin induces thickening of epidermis by suppressing apoptosis and enhancing proliferation of keratinocytes through Akt signaling.

PERIOSTIN INDUCES PRODUCTION OF TSLP FROM KERATINOCYTES

TSLP has been implicated as a critical mediator secreted from keratinocytes in AD, and overexpression of TSLP in keratinocytes results in the appearance of AD-like dermatitis in mice.[12,33] To further analyze the role of periostin in keratinocayte activation, we stimulated monolayer-cultured keratinocytes with plate-bound recombinant periostin. As shown in Figure 5A, recombinant periostin induced expression of *Tslp* at the transcriptional level. Furthermore, periostin induced production of TSLP in TLR4-deficient keratinocytes, which indicates that TSLP induced by recombinant periostin is not due to contamination of LPS in recombinant protein solution (Fig. 5B). These results suggest that periostin enhances allergic dermatitis by induction of TSLP from keratinocytes.

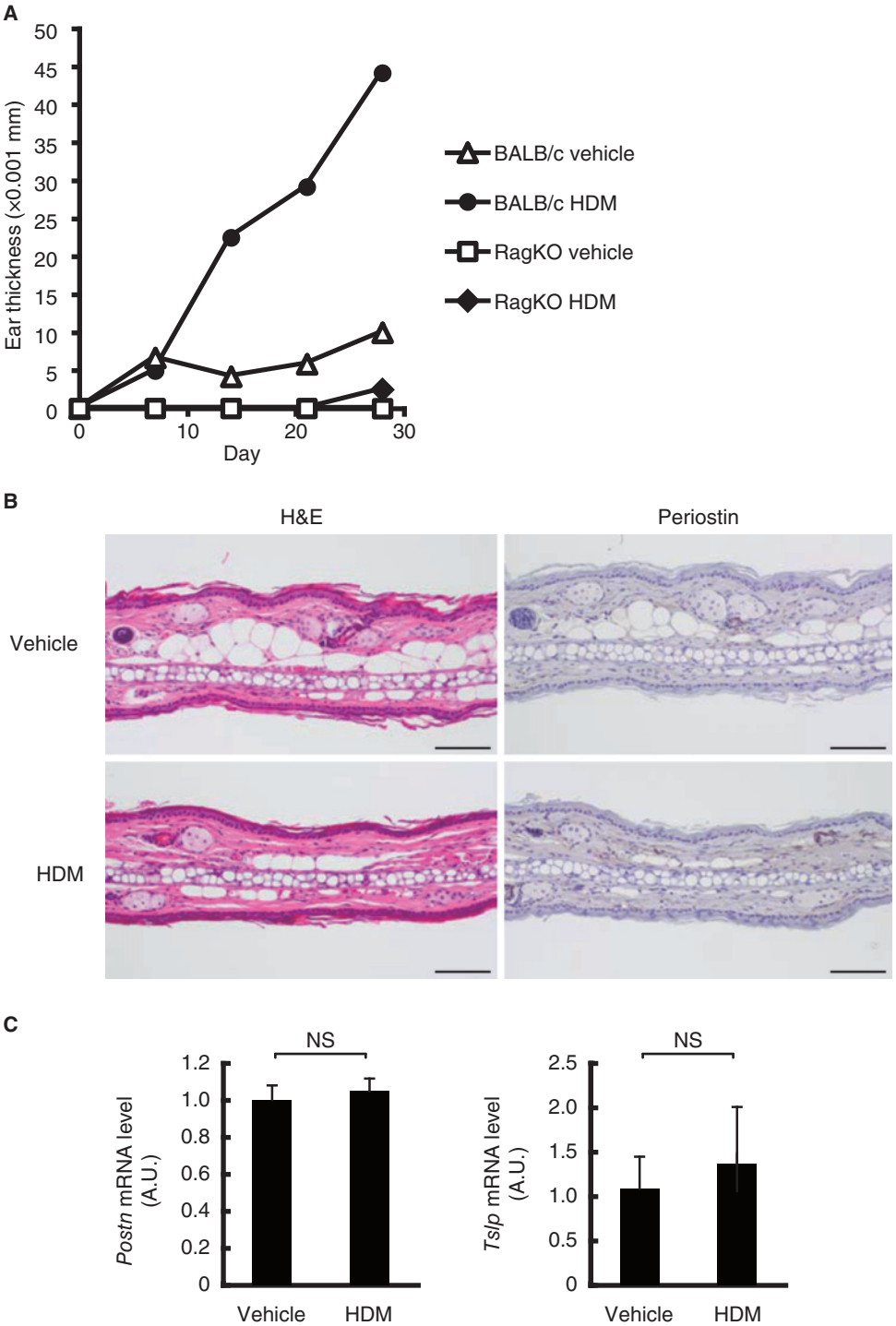

Fig. 3 HDM-induced allergic dermatitis requires lymphocytes. (**A**) Change in ear thickness caused by repeated painting with vehicle or HDM in Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice or BALB/c mice. The open squares, closed diamonds, open triangles, and closed circles show vehicle- (n = 3) or HDM-sensitized Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice (n = 6) or vehicle- (n = 1) or HDM-sensitized BALB/c mice (n = 1), respectively. (**B**) Representative photomicrographs of H&E and immunostaining for periostin of ear skin biopsies taken from Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice treated with vehicle or HDM 24 h after the fifth sensitization. Scale bar: 50 μm. (**C**) Quantitative analysis of mRNA levels of *Postn* or *Tslp* in the skin lesions from Rag-2$^{-/-}$ $\gamma_c^{-/-}$ mice 24 h after the fifth sensitization (Vehicle, n = 3; HDM, n = 6). Values are means + s.d. of all experiments.

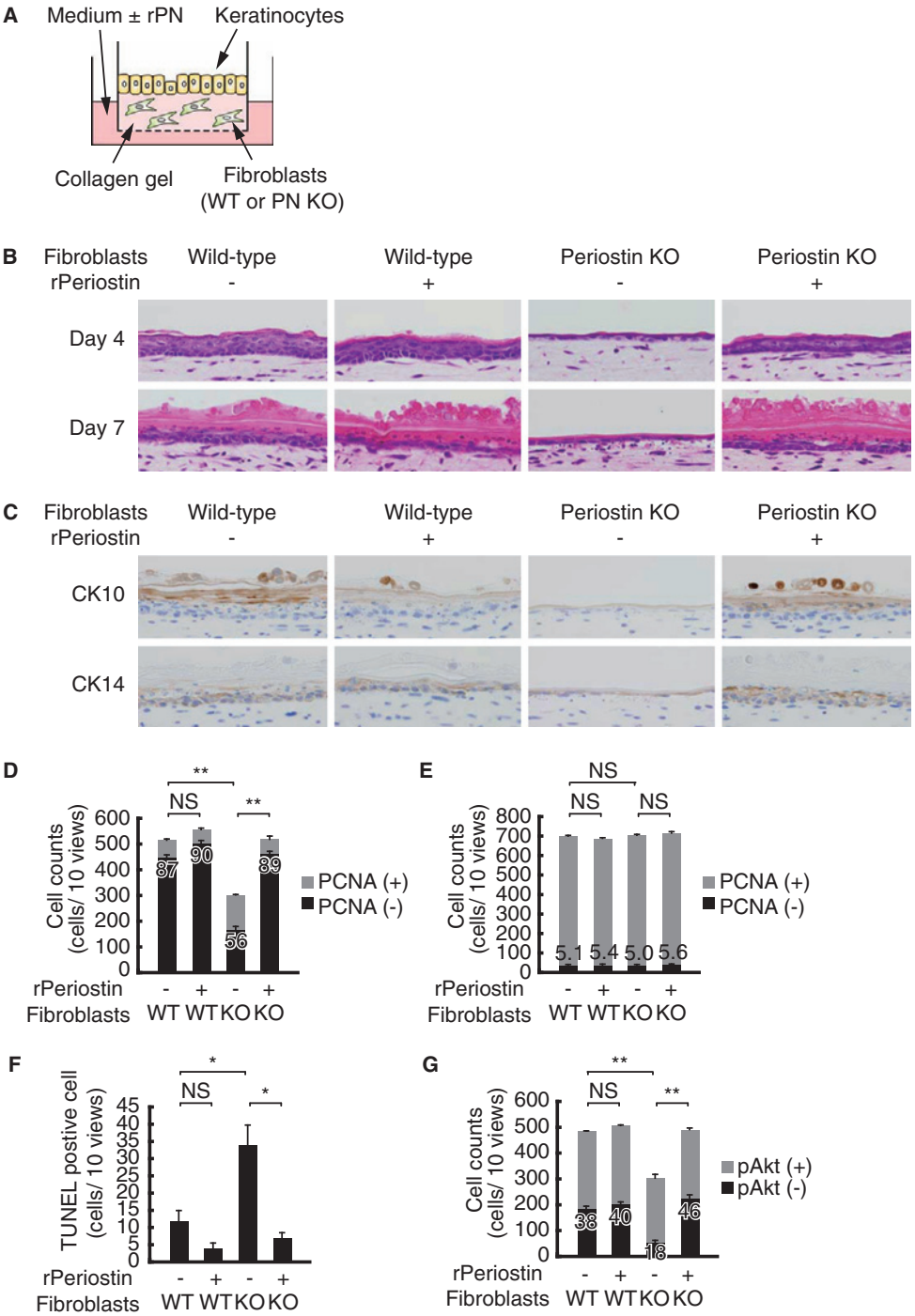

Fig. 4 Periostin enhances survival and proliferation of keratinocytes. (**A**) The system used for this analysis is illustrated. Representative photomicrographs of H&E (**B**) or immunostaining for CK10 or CK14 (**C**) in 4 days or 7 days co-culture. Keratinocytes were co-cultured with wild-type or *Postn*$^{-/-}$ fibroblasts with or without recombinant periostin. Graphs show PCNA positive and negative keratinocytes (**D**), PCNA positive and negative fibroblasts (**E**), TUNEL positive and negative keratinocytes (**F**), and phospho-Akt positive or negative keratinocytes (**G**). The numbers in each graph are percentage of positive cells in total cells. Values are means + s.d. of all experiments. *$P < 0.05$, **$P < 0.01$.

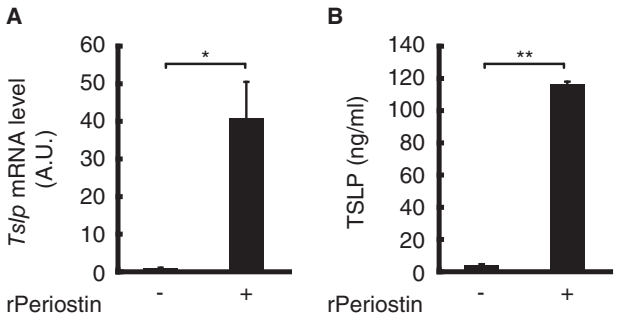

Fig. 5 Periostin induces expression of TSLP in keratinocytes. Graphs show *Tslp* mRNA level of keratinocytes stimulated with periostin for 24 h (**A**) and TSLP protein level in the supernatants of culture of keratinocytes stimulated with periostin for 72 h (**B**). Values are means + s.d. of all experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

As mentioned earlier, in children with AD, house dust mite antigen has been reported to be the most common aeroallergen resulting in allergic sensitization.[1] Epicutaneous sensitization of HDM induces AD-like phenotypes, both acute and chronic features of AD skin lesions, in Nc/Nga mice and Th2-prone BALB/c mice.[25,29] This model is Th2-dominant and STAT6-dependent.[23] In this study, we showed that HDM induces AD-like phenotype in Th1-prone C57 BL6 mice as well as BALB/c mice (Fig. 1, Fig. 2). Furthermore, we showed that HDM-induced AD-like phenotypes in model mice are dependent on lymphocytes (Fig. 3). HDM-induced allergic dermatitis exhibits both acute (redness, T cell-dominant infiltration, and enhanced expression of Th2 cytokines and chemokines) and chronic (swelling, acanthosis, and dermal fibrosis) features in AD (Fig. 1, Fig. 2 and data not shown). However, we could not detect the significant production of IFN-γ in the skin lesions, which is a feature of chronic AD skin lesions, in either HDM-sensitized C57BL/6 mice or BALB/c mice[23] (data not shown). This is consistent with the fact that most AD model mice show Th2-dominant and no production of IFN-γ.[34]

Furthermore, using an *in vitro* organotypic coculture system, we showed that periostin promotes survival and proliferation of keratinocytes and directly induce production of TSLP from keratinocytes (Fig. 4, Fig. 5). There is growing evidence supporting that the idea that keratinocytes act as enhancer cells for the inflammatory response in AD by secreting various cytokines and chemokines.[31,35] Especially, TSLP is an important keratinocyte-derived cytokine.[8] TSLP instructs dendritic cells to create a Th2-permissive microenvironment by inducing expression of OX40L, which triggers the differentiation of inflammatory Th 2 cells.[9,36] TSLP was shown to be highly expressed by keratinocytes in AD lesions.[10] In mice, the transgenic expression of TSLP in keratinocytes results in allergic dermatitis, whereas in TSLPR-deficient mice, allergic skin inflammation by epicutaneous sensitization of allergen is severely impaired.[11,12] It is conceivable that in addition to the direct induction of TSLP expression, periostin upregulates proliferation of TSLP-producing keratinocytes, contributing to the enhancement of Th2 inflammation. However, we cannot exclude the possibility that periostin acts on inflammatory cells other than keratinocytes, which causes acceleration of Th2 inflammation *in vivo*. It has been reported that periostin-null mice applied to the asthma model showed reduced eosinophilic inflammation and that periostin enhanced eosinophil adhesion to fibronectin,[22] which may support this possibility.

We have reported that periostin activates the PI3K/Akt pathway and NF-κB via α_v integrins.[23] In this study, we also showed that Akt is phosphorylated in keratinocytes in periostin-present conditions in accordance with survival, proliferation, and differentiation of keratinocytes. Considering the previous references that the PI3K/Akt pathway plays an important role in not only proliferation of keratinocytes but also their terminal differentiation,[32,37,38] the periostin/α_v integrin/PI3K/Akt pathway is likely to be involved in hyperplasia and dysregulated differentiation of epidermis in AD. Interestingly, it has been reported that βig-h3, which belongs to the same family as periostin, induces keratinocyte differentiation via the integrin-PI3K/Akt pathway.[39]

Taking together all of these results, periostin accelerates Th2 type inflammation by enhancing the production of TSLP in the following manner: 1) Allergens trigger first inflammatory responses including infiltration of lymphocytes. 2) Lymphocytes induce the expression of periostin in dermis. 3) Periostin accumulated in dermis acts on keratinocyte to induce proliferation and to produce TSLP. Several trials targeting IL-4/IL-13 signals or TSLP have been successful in AD model mice.[11,40,41] In addition to these targets, periostin could be a good target to develop therapeutic agents for AD.

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