Smad3 Signal Transducer Regulates Skin Inflammation and Specific IgE Response in Murine Model of Atopic Dermatitis

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Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by itchy, dry, and inflamed skin. Transforming growth factor (TGF)-β is an important fibrogenic and immunomodulatory factor that regulates cellular processes in the injured and inflamed skin. This study examines the role of the TGF-β–Smad signaling pathway using Smad3-deficient mice in a murine model of AD. Dermatitis was induced in mice by epicutaneous application of ovalbumin (OVA) applied in a patch to tape-stripped skin. OVA-specific IgE and IgG_{2a} antibody levels were measured by ELISA. Skin biopsies from sensitized skin areas were used for RNA isolation, histology, and immunohistochemical examination. The thickness of dermis was significantly reduced in OVA-sensitized skin of Smad3$^{-/-}$ mice. The defect in the dermal thickness was accompanied by a decrease in the expression of mRNA for proinflammatory cytokines IL-6 and IL-1β in the OVA-sensitized skin. In contrast, the number of mast cells was significantly increased in OVA-sensitized skin of Smad3$^{-/-}$ mice, which also exhibited elevated levels of OVA-specific IgE. These results demonstrate that the Smad3-pathway regulates allergen-induced skin inflammation and systemic IgE antibody production in a murine model AD. The Smad3 signaling pathway might be a potential target in the therapy of allergic skin diseases.

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INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by dry inflamed skin, which is always itchy (Hanifin and Rajka, 1980). AD usually starts in early childhood and its prevalence has been increasing in recent years in parallel with other atopic conditions, such as asthma, rhinitis, and allergies. Both genetic and environmental factors play an important role in developing AD (Spergel and Paller, 2003; Foroughi *et al.*, 2005; Linneberg *et al.*, 2006). Approximately, 80% of patients with AD have elevated levels of serum IgE and specific IgE antibodies to environmental allergens suggesting an important role of allergens in AD. In the acute phase of AD, skin lesions show a marked infiltration with activated CD4+ T cells within the dermis, and Th2-type cytokines characterize the inflammatory response. In the chronic phase of the disease, however, Th1-type cytokines are highly expressed and predominate over Th2 cytokines. The molecular and cellular mechanisms underlying AD are unfortunately not well understood, which hampers the development of specific treatments for the disease.

It has been reported recently that children with AD exhibit a significantly higher proportion of a low transforming growth factor (TGF)-β1 cytokine-producing allele compared with healthy controls (Arkwright *et al.*, 2001). TGF-β is an important fibrogenic and immunomodulatory factor that regulates cellular processes in injured and inflamed skin. TGF-β family proteins have multiple functions, which in some cases can be contradictory, either stimulating or inhibiting immune cell functions (Smeltz *et al.*, 2005; Ito *et al.*, 2006; Meadows *et al.*, 2006). TGF-β regulates numerous cellular responses, such as differentiation, proliferation, migration, and apoptosis. TGF-β is produced by most cell types, such as immune and structural cells in different tissues (Chu *et al.*, 2000; Harrison *et al.*, 2006; Meadows *et al.*, 2006). TGF-β proteins are able to elicit different effects at different concentrations (Chen and Khalil, 2006; Funaba *et al.*, 2006). Thus, TGF-β is secreted by many sources and

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Abbreviations: AD, atopic dermatitis; EC, epicutaneous; OVA, ovalbumin; TGF-β, transforming growth factor -β; WT, wild-type

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different mediators such as pro- and antiinflammatory compounds can modulate its synthesis.

Mature TGF-β binds TGF-β receptor types I and II to initiate signaling. TGF-β signal transducers called Smads are intracellular proteins, transcription factors of $\sim$500 amino acids in length, with two globular domains, an N-terminal mad homology 1 domain and a C-terminal mad homology 2 domain (Shi and Massagué, 2003; Massagué *et al.*, 2005). There are three families of Smads: receptor-activated (R-) Smads (Smads 1, 2, 3, 5, and 8), common-partner (Co-) Smads (Smad 4), and the inhibitory (I-) Smads (Smads 6 and 7). Smads 2 and 3 are activated by TGF-β and activin receptors, whereas Smads 1, 5, and 8 are activated by bone morphogenetic protein (Piek *et al.*, 1999). Smads deliver TGF-β signals from the cytoplasm to the nucleus controlling the expression of target genes in a cell type-specific manner. Increased activation of the TGF-β pathway has been observed in the skin and lung, especially during tissue injury and inflammation (Munger *et al.*, 1999).

In this study, we examined the role of TGF-β–Smad signaling in AD using Smad3-deficient mice and a murine model of AD. We demonstrate here that Smad3-deficiency modulates allergen-induced skin inflammation and specific IgE responses after epicutaneous (EC) ovalbumin (OVA) sensitization.

RESULTS

Dermal thickness is reduced in OVA-sensitized skin sites of Smad3$^{-/-}$ mice

The thickness of dermis and epidermis was significantly increased in OVA-sensitized skin sites of wild-type (WT) mice compared with saline-treated controls (Figure 1). Similarly, the thickness of the epidermis was enhanced in the OVA-sensitized skin of Smad3$^{-/-}$ mice compared with their saline-treated controls although the difference was not statistically significant (Figure 1g). No significant difference in the epidermal thickness was found in Smad3$^{-/-}$ mice compared with WT mice after OVA sensitization (Figure 1g). In contrast, however, dermal thickening was significantly reduced after EC OVA sensitization in Smad3$^{-/-}$ mice compared with their WT littermates (Figure 1h). A representative picture of the skin morphology after EC sensitization with OVA and saline is provided in Figure 1a–e.

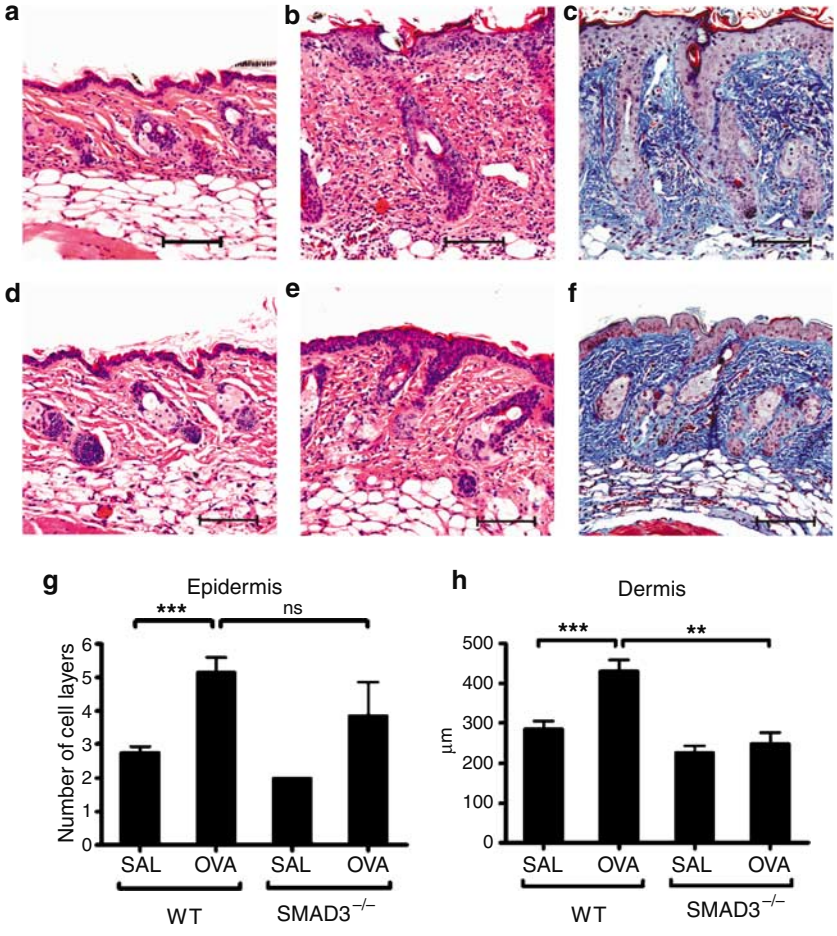

Figure 1. Histological changes in the (g) epidermis and (h) dermis of mice skin after EC exposure to OVA or saline. The results are shown as mean $\pm$SEM. $^{**}P<0.01$, $^{***}P<0.001$, ns: not statistically significant. (**a** and **d**) Hematoxylin and eosin-stained skin sections are from saline groups and (**b** and **e**) OVA groups; Masson-trichrome-stained skin sections are from OVA-treated WT and Smad3$^{-/-}$mice; (**d**–**f**) Smad3$^{-/-}$ mice and (**a**–**c**) WT mice; bar = 100 μm; n = 8–14 mice per group.

Collagen fiber staining revealed a clear-cut increase in the collagen fiber deposition in OVA-sensitized skin of WT mice compared with saline controls. In contrast, no difference in the collagen deposition was observed in the OVA-sensitized skin of Smad3$^{-/-}$ mice compared with their saline-treated controls. Moreover, collagen deposition was significantly reduced in the skin of Smad3-deficient mice compared with WT mice after EC OVA sensitization. A representative picture of the collagen fiber deposition after EC sensitization with OVA in WT and Smad3$^{-/-}$ mice is provided in Figure 1c and f.

Smad3$^{-/-}$ mice exhibit increased numbers of mast cells in the OVA-sensitized skin

The number of mast cells was significantly increased in the OVA-sensitized skin of Smad3$^{-/-}$ mice compared with the saline group (Figure 2). Interestingly, mast cell numbers remained at a low level in OVA-sensitized WT mice and the difference in mast cell numbers between OVA-sensitized skin of WT mice and Smad3$^{-/-}$ mice was statistically significant (Figure 2).

The number of skin-infiltrating eosinophils was significantly increased in OVA-sensitized WT mice compared with

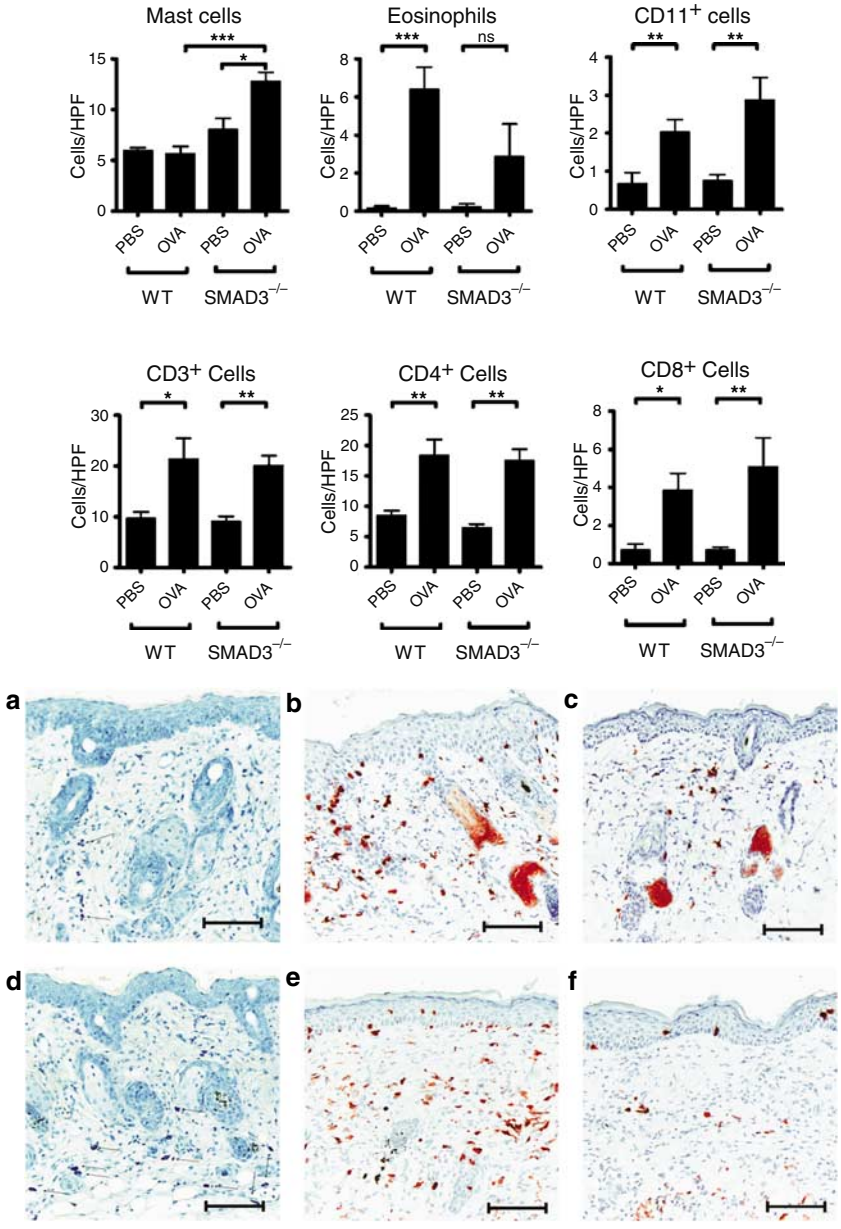

Figure 2. Mast cells were significantly increased in OVA-sensitized Smad3$^{-/-}$ mice, but remained at a low level in OVA-sensitized WT mice. The number of eosinophils, CD11c-, CD3-, CD4-, and CD8-positive cells was significantly increased in mice skin after repeated EC exposures to OVA. The cells were counted under a light microscope and the results are expressed as cells per high-power field (original magnification ×40, eosinophils original magnification ×100). (**a** and **d**) Mast cells, (**b** and **e**) CD4+, (**c** and **f**) CD8+. (**d**–**f**) OVA-treated Smad3$^{-/-}$ mice and (**a**–**c**) OVA-treated WT mice; bar = 100 μm. The data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001, ns: not statistically significant; n = 6–14 mice per group.

saline-sensitized controls (Figure 2). An increase in the numbers of eosinophils after OVA sensitization was also seen in Smad3$^{-/-}$ mice compared with their saline-treated controls, but the difference was not statistically significant. Immunohistochemical staining demonstrated that the numbers of CD11+ cells were equally elevated in the skin of Smad3$^{-/-}$ mice and their WT littermates after OVA sensitization (Figure 2). Moreover, increased numbers of CD3+, CD4+, and CD8+ cells were observed in the skin of Smad3$^{-/-}$ mice and WT mice after EC OVA sensitization. However, there were no significant differences in lymphocyte numbers between the Smad3$^{-/-}$ mice and WT mice (Figure 2). We also analyzed expression of forkhead box P3 mRNA, a marker for regulatory T cells, in the sensitized skin sites of WT and Smad3-deficient mice. No significant differences between any of the study groups were detected.

Expression of IL-6 and IL-1β mRNA is drastically reduced in the OVA-sensitized skin of Smad3$^{-/-}$ mice

mRNA expression of proinflammatory cytokines TNF-α, IL-6, and IL-1β was significantly increased in OVA-sensitized skin sites of WT mice compared with saline-treated controls (Figure 3). Interestingly, expression of IL-6 and IL-1β mRNA was dramatically decreased in OVA-sensitized skin of Smad3$^{-/-}$ mice compared with their WT littermates (Figure 3). There were no significant difference in the expression of IL-6, IL-1β, and TNF-α mRNA between OVA- and saline-sensitized skin in Smad3$^{-/-}$ mice (Figure 3).

The expression levels of a major Th2 cytokine IL-4 and Th1 cytokine IFN-γ were significantly higher in the skin of OVA-sensitized mice compared with saline-treated controls, but no significant difference between Smad3$^{-/-}$ and WT mice was observed (Figure 3).

OVA-specific IgE levels are elevated in OVA-sensitized Smad3$^{-/-}$ mice

OVA-specific IgE levels were increased both in Smad3$^{-/-}$ and WT mice sensitized with OVA (Figure 4). However, OVA-specific IgE levels were significantly elevated in Smad3$^{-/-}$ mice compared with their WT littermates (Figure 4). In addition, the levels of OVA-specific IgG2a were induced after OVA sensitization in both mice groups, but there were no significant differences in antibody levels between WT and Smad3$^{-/-}$ mice (Figure 4).

DISCUSSION

The role of TGF-β–Smad3 signaling in the immunoregulation and pathophysiology of allergic skin disorders is unknown. However, mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response (Ashcroft *et al.*, 1999). Although the mechanisms of fibrosis and wound healing may differ from the mechanisms involved in inflammation, we anticipated, based on the wound repair

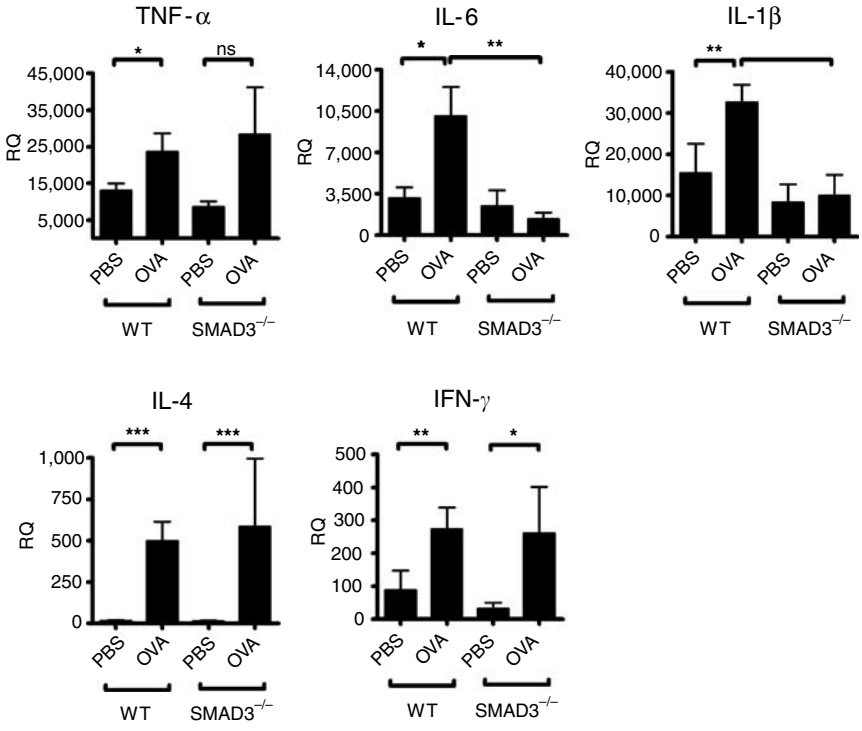

Figure 3. mRNA expression of cytokines in mice skin after EC exposure to OVA or saline. Real-time quantitative RT-PCR was used to analyze the mRNA expression levels. IL-6 and IL-1β mRNA levels were significantly increased in OVA-sensitized WT mice compared with OVA-sensitized Smad3$^{-/-}$ mice. The levels of IL-4, IFN-γ, and TNF-α mRNA were significantly higher in OVA-sensitized mice compared with saline group, but no significant difference between Smad3$^{-/-}$ and WT mice was detected. Bars and errors represent mean$\pm$SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$. NS: Not statistically significant; $n=6$–16 mice per group.

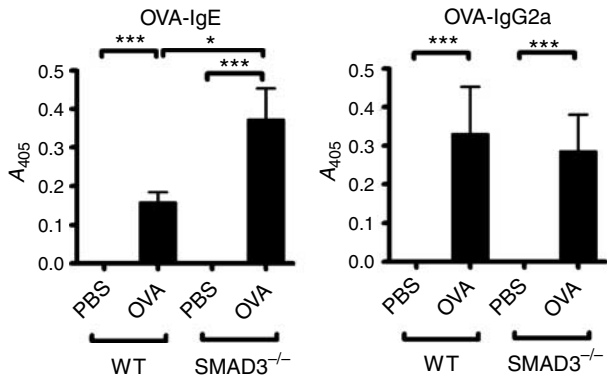

Figure 4. Mice serum levels of OVA-specific IgE and IgG2a after EC exposure to OVA or saline. OVA-specific IgE and IgG_{2a} levels were significantly increased in Smad3$^{-/-}$ and WT mice sensitized with OVA. There were no significant differences in antibody levels between OVA-sensitized WT and Smad3$^{-/-}$ mice. The data are shown as mean ± SEM, ***$P<0.001$; $n=7$–13 mice per group.

studies, that the defect in TGF-β–Smad3 signaling causes milder skin injury and inflammatory response in the experimental model of AD. Indeed, EC exposure to OVA resulted in significantly reduced dermal thickening and drastically decreased expression of IL-6 and IL-1β mRNA in the skin of Smad3$^{-/-}$ mice. Interestingly, however, the numbers of dermal mast cells and OVA-specific IgE levels were significantly increased in Smad3$^{-/-}$ mice after OVA-sensitization.

The increased deposits of collagen may cause skin thickening, which is a characteristic feature of human AD. Extracellular matrix and the regulation of the synthesis of matrix molecules are key elements in the pathology of many skin diseases. The balance in the synthesis and breakdown of connective tissue is tightly controlled by the release of mediators, including TGF-β, from inflammatory cells or connective tissue cells, which can influence collagen and matrix metalloproteinase production in both paracrine and autocrine fashions (Eckes *et al.*, 2000). Disorders cause wound-healing defects or the development of fibrosis. Fibrosis is a normal consequence of chronic inflammation and tissue injury with excessive cell accumulation, skin thickening, and deposition of extracellular matrix proteins. There are a number of studies showing the involvement of Smad3–TGF-β signaling in the collagen transcription and in the mechanisms of fibrosis (Chen *et al.*, 1999; Verrecchia *et al.*, 2000). Smad3 transmits TGF-β signals from the receptor to the procollagen gene in human fibroblasts (Chen *et al.*, 1999). It is, thus, possible that Smad3 regulates collagen production in our model of AD. Decreased secretion of collagen in Smad3$^{-/-}$ mice may result in reduced dermal thickness after EC OVA sensitization.

The proinflammatory cytokines TNF, IL-1, and IL-6 are known to participate in the pathogenesis of AD by orchestrating the interactions between resident and infiltrating cells. Interestingly, the expression of skin IL-6 and IL-1β mRNA was drastically decreased in the OVA-sensitized skin of Smad3$^{-/-}$ mice compared with WT mice. Several studies have demonstrated the interaction between IL-6 and TGF-β in disease states. An intestinal epithelial cell line study demonstrates a cross-talk between TGF-β and IL-6, and it is suggested that TGF-β may play a role in the negative regulation of IL-6 signaling in intestinal epithelial cells (Walia *et al.*, 2003). Moreover, a recent study showed that IL-6 increased TGF-β1-dependent Smad3 signaling in proximal tubular cells. Zhang *et al.* (2005) suggested that synergistic effect between IL-6 and TGF-β1 might lead to an enhanced profibrotic response in the kidney. Furthermore, it has been recently reported that IL-1β-induced inflammation toward fibrosis is Smad3 dependent, because it developed only in WT control mice and not in Smad3-null mice (Bonniaud *et al.*, 2005). Taken together, present results support the view that Smad3 regulates IL-6 and IL-1β expression in allergen-induced skin inflammation. Reduced production of these proinflammatory cytokines in the skin may in turn cause diminished skin fibrosis leading to reduced dermal thickening.

Mast cells play an important role in allergic responses owing to the secretion of a variety of inflammatory mediators after appropriate activation. Depending on the circumstances, the cell could phenotypically express a wide spectrum of variation in the types, kinetics, and/or magnitude of its secretory functions (Galli *et al.*, 2005). We found increased numbers of mast cells in the skin of OVA-sensitized Smad3$^{-/-}$ mice suggesting that the TGF-β–Smad3 pathway regulates mast cell fate in the allergen-sensitized skin. Mast cells, eosinophils, and other cells recruited to the site of inflammation are potential sources of TGF-β. The role of TGF-β and factors affecting mast cell migration are largely unknown. Smads appear to be involved in TGF-β1-mediated mast cell migration and also TGF-β1-mediated mast cell-growth inhibition (Olsson *et al.*, 2001). Olsson *et al.* (2001) found that TGF-β1 induced migration of the human mast cell line at 40 fM, whereas growth inhibition of mast cells was detected at 400 pM. They also demonstrated that signals induced by TGF-β1 that lead to cell migration diverge from the signals for growth inhibition in mast cells.

Severity of AD correlates with elevated levels of IgE (Flohr *et al.*, 2004; Boguniewicz *et al.*, 2006). It is of interest that in this study, OVA-specific IgE levels, but not OVA-specific IgG2a levels, were scientifically upregulated in Smad3$^{-/-}$ mice compared with their WT controls after EC OVA sensitization. These results suggest that Smad3 signaling has an inhibitory effect on the production of allergen-specific IgE in our model. TGF-β is known to suppress IgE synthesis. Thus, impaired TGF–βSmad3 signaling may result in upregulation of OVA-specific IgE antibodies. In line with this, a significantly higher proportion of children with AD who frequently exhibit elevated levels of IgE antibodies demonstrate a TGF-β1 gene allele, which is associated with lower production of TGF-β1 cytokine (Arkwright *et al.*, 2001).

In conclusion, the present data indicate that Smad3, an important mediator of TGF-β signaling, regulates chronic skin inflammation and systemic IgE antibody production in a murine model AD. Smad3 deficiency affects the thickness of dermis, immunological status of the skin through proinflammatory cytokines, and mast cell infiltration as well

as specific IgE levels after EC allergen sensitization. The Smad3 signaling pathway might be a potential target in the therapy of allergic skin diseases.

MATERIALS AND METHODS

Mice

Smad3 knockout mice have been generated by Deng and co-workers (Yang *et al.*, 1999) and were bred in our facilities. The resulting progeny were screened by PCR to identify Smad3$^{-/-}$ and WT mice. Animals between 7 and 10 weeks of age were used and were age- and sex-matched within each experiment. The mice were housed in pathogen-free facilities. All animal protocols were approved by the Finnish Institute of Occupational Health Committee on Animal Welfare.

Sensitization

Epicutaneous treatment and sensitization of mice was performed as described previously (Spergel *et al.*, 1998). Briefly, mice were anesthetized with inhalation of Isofluran (Abbott Laboratories, Abbott Park, IL). Mouse back skin was shaved with an electric razor and the shaved area was tape-stripped four times by transparent adhesive tape (Tegaderm, 3M Health Care, St. Paul, MN) to remove hair and to introduce a standardized skin injury. A sterile gauze patch ($1 \times 1\,cm^2$) moisturized with 100 μl of 0.1% OVA in saline (OVA group) or 100 μl of 0.9% saline (saline group) was put on the back skin of mice and attached with Tegaderm adhesive tape.

The patches were kept there for 1 week and then removed. Two weeks later, an identical patch was applied to the same skin site. The whole experiment contained a total of three 1-week exposures with a 2-week interval between each exposure. The mice were killed after the last sensitization week. Blood and skin biopsies were collected for further analysis. The experiment was performed three times.

Sample collection and skin preparations

The mice were killed after the last sensitization week. Blood was collected and used for antibody analysis. Skin biopsies from treated skin areas were used for RNA isolation, histology, and immunohistochemical examination. For histology, the skin was fixed in 10% formalin, embedded in paraffin wax, routinely processed, sectioned 5 μm thick, and stained with hematoxylin and eosin, periodic acid schiff stain and Masson-trichrome stain, and examined under light microscopy. For immunohistochemical staining, the skin was embedded into OCT compound (Sakura, Finetek Europe B.V., The Netherlands) and quick frozen. The skin samples were cut into 5-μm sections and stained immunohistochemically with mAbs against CD11c, CD3, CD4, and CD8 (BD, Pharmingen, San Jose, CA) using the ChemMate (DakoCytomation, Glostrup, Denmark) staining kit.

ELISA

OVA-specific IgE, IgG_1, and IgG_{2a} serum levels were measured by ELISA method. Ninety-six-well microtiter plates (Nunc, Rochester, NY) were coated with 100 μg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6) at 4°C overnight. The plates were washed with phosphate-buffered saline–Tween-20 (0.05%) and blocked with 3% BSA in phosphate-buffered saline for 2 hours at 20°C and washed again. One hundred microliters of diluted sera (1/10, 1/20, 1/40, 1/80) in 1% BSA–phosphate-buffered saline was incubated at 4°C overnight. After washing, 2 μg of biotin-conjugated rat anti-mouse IgE mAb in 1 ml of 1% BSA–phosphate-buffered saline was incubated for 2 hours at 20°C and washed again. Streptavidin–horseradish peroxidase (BD, Pharmingen) 1/4000 in 1% BSA was added and incubated for 30 minutes at 20°C and washed. Peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and absorbance read at 405 nm with an ELISA reader (Multiskan MS, Labsystems, Vantaa, Finland). OVA-specific IgG_{2a} was measured by using the same method described. The plates were coated with 2 μg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6). Serial dilutions of sera for IgG_{2a} (1/60, 1/180, 1/540, 1/1620) were used. Bound IgG_{2a} was detected with biotin-conjugated rat anti-mouse IgG_{2a} mAb (BD, Pharmingen).

RT-PCR

Total RNA from skin was extracted and transcribed into cDNA. Real-time quantitative PCR was performed with an AbiPrism 7,700 Sequence Detector System (Applied Biosystems, Foster City, CA). PCR primers and probes were TNF-α, IL-6, IL-1β, IL-4, and IFN-γ (Applied Biosystems), and endogeneous 18S rRNA was used as the housekeeping gene. The target gene expression was expressed as relative quantitatives.

Data analysis

Data were analyzed with the GraphPadPrism software. Data are expressed as mean +/−SEM. Statistical significance was accepted when $P<0.05$ using the Mann–Whitney U test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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