

Smad3 Regulates Dermal Cytokine and Chemokine Expression and Specific Antibody Production in Murine Responses to a Respiratory Chemical Sensitizer

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Key Words

Smad3 · Trimellitic anhydride, allergy · Skin inflammation

Abstract

Background: The cytokine transforming growth factor- β (TGF- β) has important regulatory roles in the immune system. To investigate the role of intact TGF- β signaling during the contact hypersensitivity (CHS) response to a respiratory allergen, we exposed Smad3^{-/-} mice to topical trimellitic anhydride (TMA). **Methods:** CHS was induced by topical application of TMA. The swelling of the TMA-exposed ears was analyzed, and lymph node, ear tissue and skin biopsies were collected for RNA isolation, histology and histochemical analyses. Lymph node cell proliferation was measured and blood samples were collected for analysis of TMA-specific immunoglobulin. **Results:** Topical TMA exposure resulted in increased mRNA expression of proinflammatory and suppressive cytokines (IL-1 β , TNF- α , IL-6, IFN- γ , IL-4, IL-10, IL-17, IL-23, TGF- β), chemokines (CXCL9, CXCL10, CCL24) and chemokine receptors (CCR7, CCR8, CXCR2), increased numbers of CD3⁺ T cells in ear tissue, and lymphadenopathy in the Smad3^{-/-} mice. The IL-10 result was confirmed at the protein level by immunohistochemistry. However, the ear-swelling response and infiltration of eosinophils, F4/80⁺ cells, CD11c⁺

cells and mast cells were similar in the Smad3^{-/-} mice compared to their wild-type (WT) siblings. While TMA-specific IgE was induced equally in the WT and Smad3^{-/-} mice, the concentration of TMA-specific IgG2a was significantly lower in the Smad3^{-/-} mice. **Conclusions:** The Smad3 molecule contributes significantly to the regulation of the cytokine and chemokine network during the CHS response to TMA. The lack of Smad3 resulted in a potent Th2 shift, confirmed by strongly impaired IgG2a levels.

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Introduction

Occupational contact and respiratory allergies are important health problems, commonly caused by chemicals. Typical contact allergens that cause contact hypersensitivity (CHS) induce a delayed T-helper-cell-1 (Th1)-type response, and comparatively low levels of Th2-type cytokines. Chemicals that induce respiratory sensitization are characterized by the preferential induction of a Th2-type response, as well as an increased serum con-

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centration of IgE. When inhaled, respiratory allergens elicit asthmatic responses while topical exposure elicits both an IgE-mediated immediate response and a delayed-type cutaneous hypersensitivity response [1, 2].

Transforming growth factor- β (TGF- β) regulates a wide range of biologic processes such as inflammation, development, tissue repair and tumorigenesis. It may act either as a potent anti-inflammatory or proinflammatory agent depending on the circumstances [3, 4]. The differential actions of TGF- β depend on factors such as local or systemic expression, local concentration, and the timing between activating and inhibiting signals on the cell surface [4].

The proinflammatory properties of TGF- β include strong chemoattraction of leukocytes. In mice, TGF- β is also essential for the differentiation of the highly inflammatory Th17 cell lineage [5] which has been linked to the pathogenesis of several autoimmune and inflammatory diseases, including CHS [6]. On the other hand, TGF- β also contributes to the downregulation and resolution of inflammation, through direct inhibition of T cell proliferation and cytokine production [3, 7–9]. The role of TGF- β in T-regulatory (T_{reg})-cell-mediated suppression has been controversial [10] although several studies have demonstrated its importance in T_{reg} function and differentiation [11–15]. Thus, TGF- β has multiple functions, its expression is widespread, and almost all tissue types of the body express receptors for TGF- β , which makes the analysis of its role in the immune system complicated.

TGF- β signaling is mediated by Smad proteins, which control the expression of their target genes [16]. Smad3 plays an important part in mediating TGF- β signals, and the lack of Smad3 in gene-targeted mice eventually results in immune dysregulation and susceptibility to opportunistic infection [17–19]. We have earlier shown that Smad3 deficiency reduces the magnitude of the allergic response to topical exposure to protein allergen [20], but in contrast, results in increased expression of proinflammatory cytokines during the CHS response to the contact allergen oxazolone [21]. In this study, employing a model of contact sensitivity to the respiratory allergen trimellitic acid anhydride (TMA), we observed significantly increased recruitment of skin-infiltrating T cells, significantly increased expression of proinflammatory and suppressive cytokines and chemokines at the mRNA and protein level, as well as a shift towards a Th2 response, confirmed by strongly impaired serum ovalbumin-IgG2a levels when Smad3 is lacking. The results indicate that Smad3 is an important player in the regulation of the cytokine and chemokine network during CHS response to TMA.

Materials and Methods

Mice

Smad3 knockout mice on a C57BL6 background were kindly provided by Dr. Chuxia Deng (NIH, Bethesda, Md., USA), and bred in our facilities. The resulting progeny were screened by polymerase chain reaction (PCR) to identify Smad3^{-/-} and wild-type (WT) mice. The mice were housed in pathogen-free facilities. Animals were used between 7 and 10 weeks of age. The mice were age- and sex-matched within each experiment. All animal protocols were approved by Health Services of the State Provincial Office of Southern Finland.

Induction and Elicitation of TMA Hypersensitivity

Epicutaneous treatment and sensitization of the mice (3–12 mice per group) were performed according to a previously described method [22]. In brief, the mice were lightly anesthetized with inhalation of isoflurane (Abbott Laboratories, Abbott Park, Ill., USA) and sensitized on shaved and 3 times tape-stripped skin of the back with 50 μ l TMA (500 mg/ml) in a 4:1 acetone:olive oil solution on day 0. A control group was treated similarly with vehicle. A second application of TMA was performed on day 7. On day 14, the mice were challenged with 50 μ l TMA (250 mg/ml) on the backs and 25 μ l TMA (250 mg/ml) on the dorsa of both ears. The experiments were repeated twice.

Sample Collection and Preparation

Ear thickness was measured with a micrometer (Mitutoyo, Kanagawa, Japan) before and after TMA application. The ear-swelling response was measured at 1, 2, 4, 24 and 48 h after TMA exposure. The mice were sacrificed after the last time point, and blood, ear-draining lymph nodes (LNs), ears and skin biopsies were collected for further analysis. The levels of TMA-specific IgE and IgG2a were determined from sera, and RNA was isolated from ear and skin tissue from TMA-treated areas, as well as from ear-draining LNs. Ear-draining LN size and LN cell proliferation were measured.

Histology and Immunohistochemistry

For histological analyses, skin tissue samples from TMA-treated skin areas (ear tissue and dorsal skin) were fixed in 10% formalin, embedded in paraffin wax, routinely processed, cut into 5- μ m sections, stained with HE and examined for eosinophils and neutrophils under light microscopy. Inflammatory cells were counted in 15 high-power fields at $\times$ 1,000 magnification and expressed as cells per high-power field. Mast cells were counted at $\times$ 400 magnification. Frozen, allergen-exposed ear pinnae were stored in -80°C and embedded in OCT compound until sectioned, fixed in cold acetone, and stained with peroxidase with rat antimouse CD3 antibody (Ab, clone 17A2), rat antimouse CD4 Ab (clone RM4–5), rat antimouse CD8 Ab (clone 53–6.7), rat antimouse F4/80 (clone BM8), rat antimouse Foxp3 (clone FJK-16s), rat antimouse IL-10 (clone JES5–2A5), and hamster antimouse CD11c (clone HL3). All antibodies were purchased from BD Pharmingen, except rat antimouse F4/80 which was from Acris Antibodies, rat antimouse Foxp3 from eBioscience, and IL-10 from Abcam. Biotin-conjugated secondary Ab antirat IgG (H+L) was purchased from Vector Laboratories.

Proliferation Test

Draining LN cell proliferation was measured with no stimulating antigen immediately after isolation. The cells were seeded at 10^5 cells per well in 200 μ l complete RPMI-1640 medium with Glutamax (Invitrogen Life Technologies), incubated in 37°C/5% CO₂ for 36 h, and pulsed with 1 μ Ci [³H] thymidine per well (Amersham Biosciences Europe, Freiburg, Germany) during the last 18 h. Incorporated radioactivity was determined using a liquid scintillation counter (Trilux 1450 Microbeta, Wallac, Turku, Finland). Results are expressed as mean counts per minute of triplicate wells and represent means $\pm$ SEM.

RNA Isolation and cDNA Synthesis

Skin and ear biopsies that were obtained after sacrifice of the mice at 48 h after the final allergen exposure were snap frozen on dry ice and kept at -80°C pending homogenization with an Ultra-Turrax T8 (IKA Labortechnik) in TRIzol (Invitrogen Life Technologies). RNA extraction was performed according to TRIzol instructions, followed by DNaseI (RNase-free; Invitrogen Life Technologies) treatment to remove contaminating genomic DNA, and further extraction with phenol-chloroform-isoamylalcohol (25:24:1). One microgram of extracted RNA was reverse-transcribed into cDNA using MultiScribe reverse transcriptase and random hexamers (Applied Biosystems).

Real-Time Quantitative PCR

Real-time quantitative PCR was performed with AbiPrism 7700 Sequence Detector System (Applied Biosystems, Foster City, Calif., USA) as described previously [23]. PCR primers and target-specific probes were purchased as predeveloped reagents [CXCL5, CXCL9, CXCL10, CCL24, CCR7, CCR8, CXCR2, IL-1 β , IL-6, TNF- α , IL-4, IL-10, FoxP3, IL-17, IL-23 (Applied Biosystems)], or were designed as described previously (TGF- β , IFN- γ , CXCR3, CCR3, CCR5) [24]. Endogenous 18S rRNA was used as the house-keeping gene and target gene expression was expressed as relative quantities.

TMA-Specific IgE and IgG2a ELISA

TMA-specific IgE and IgG2a serum levels were measured by the ELISA method. First, a TMA-mouse serum albumin (MSA)-conjugate was prepared by adding 10 μ l TMA in acetone (117 mg/ml) to 10 ml MSA in 0.05 M NaHCO₃ (500 μ g/ml, pH 9.0). The mixture was allowed to react on ice for 60 min. The TMA-MSA conjugates were purified by high-performance liquid chromatography and adhered to microtiter plates at 10 μ g/ml in 0.05 M NaHCO₃ (pH 9.6) at 4°C overnight. Then the microtiter plates were blocked with 3% BSA in PBS for 2 h at 20°C, and 100 μ l per well of the sera (diluted in 1% BSA-PBS) were added and incubated at 4°C overnight. TMA-specific IgE and IgG2a were visualized by the HRP-peroxidase enzymatic method, using biotinylated rat anti-mouse IgE or IgG2a monoclonal antibody (2 μ g/ml) as secondary antibody. Absorbance was read at 405 nm with an ELISA reader (Multiskan MS, Labsystems, Vantaa, Finland).

Data Analysis

Data were analyzed with the GraphPadPrism software and expressed as means $\pm$ SEM. Statistical significance was accepted when $p < 0.05$ using the Mann-Whitney U test.

Results

Topical Exposure to TMA Induces an Immediate and Delayed-Type Ear Swelling Response in Smad3 $^{-/-}$ Mice

Topical application of TMA resulted in significant swelling of the ears in the WT mice, but not in the Smad3 $^{-/-}$ mice at 1 h after exposure (online suppl. fig. 1, www.karger.com/doi/10.1159/000236006). Median ear swelling at 1 h after exposure was 22% in the WT mice, and 11% in the Smad3 $^{-/-}$ mice. During the late-phase response to TMA exposure, the swelling of the ears was significantly increased both in the WT and the Smad3 $^{-/-}$ mice compared to the controls. Median ear swelling was 37% in the Smad3-deficient mice and 25% in the WT mice at 24 h (fig. 1a), and 37 and 22%, respectively, at 48 h after exposure (data shown in millimeters $\times 10^{-2}$ in online suppl. fig. 1). Due to fairly large variations in the Smad3 $^{-/-}$ mice, these differences did not, however, reach statistical significance.

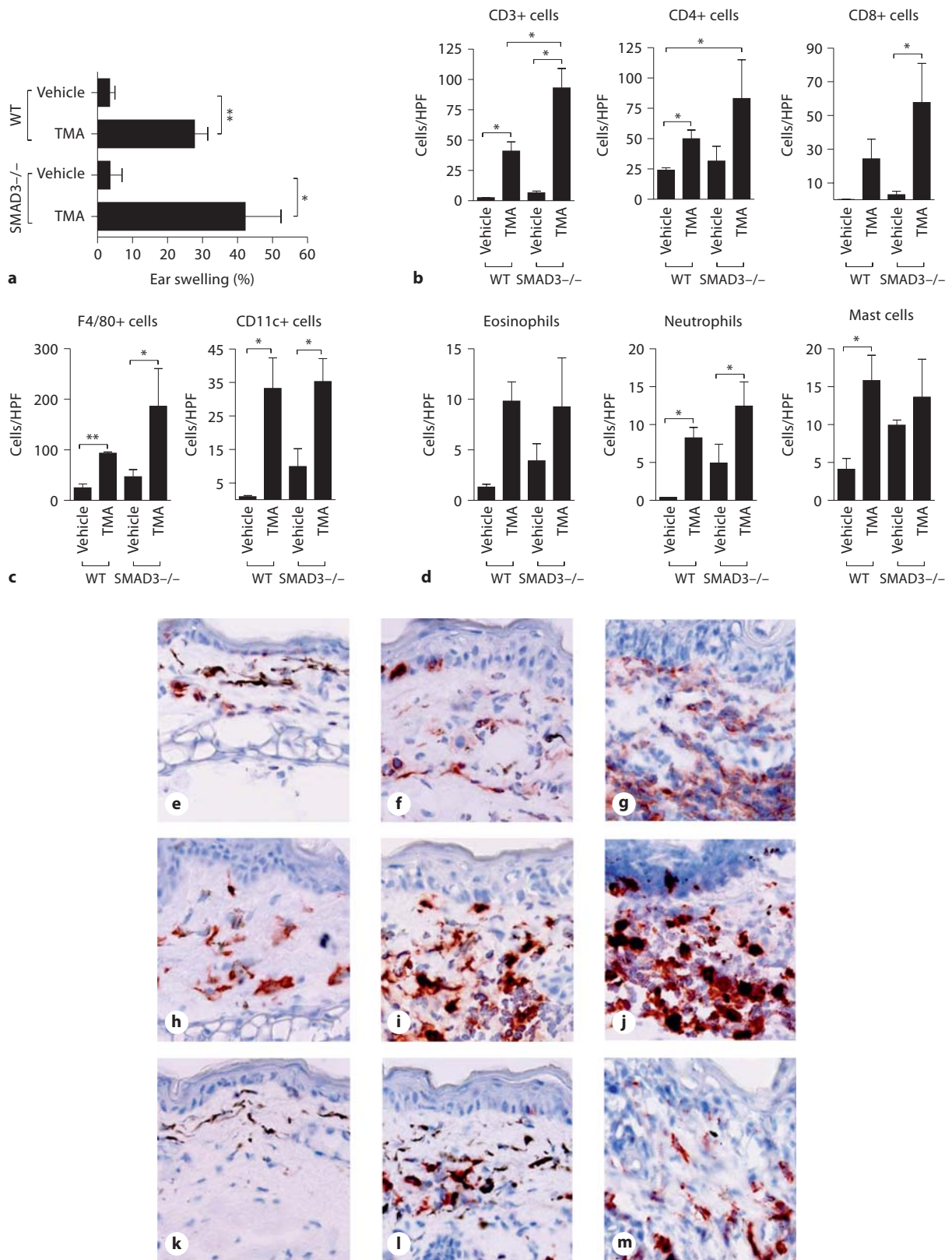
CD3 $^{+}$ T Cell Infiltration Is Significantly Increased in the Smad3 $^{-/-}$ Mice Ear Tissue after Topical TMA Exposure

Skin-infiltrating T cells are central mediators of CHS. The number of CD3 $^{+}$ T cells was significantly higher by histochemical analysis in TMA-treated ear tissue in the Smad3 $^{-/-}$ mice compared to the WT mice (fig. 1b, e–g). Both CD4 $^{+}$ and CD8 $^{+}$ cells showed a tendency to increase in the Smad3 $^{-/-}$ mice compared to the WT mice although not statistically significantly so (fig. 1b, h–m).

Macrophages and Langerhans cells (LCs) can be identified by their expression of the F4/80 antigen in mouse skin. F4/80 $^{+}$ cells increased slightly in ear tissue in the Smad3 $^{-/-}$ mice (fig. 1c), but not statistically significantly so. Furthermore, CD11c is a general marker of mouse dendritic cells (DCs), including LCs. The number of CD11c $^{+}$ cells in ear tissue was similar in the WT and the Smad3 $^{-/-}$ mice (fig. 1c), suggesting that the slight increase in F4/80 $^{+}$ cells is not due to increased numbers of LCs in the Smad3 $^{-/-}$ mice. Finally, the numbers of neutrophils, eosinophils and mast cells were similar in TMA-exposed skin sites of the Smad3 $^{-/-}$ and the WT mice (fig. 1d).

Proinflammatory Cytokine Expression at the mRNA Level Is Significantly Increased in TMA-Exposed Ear Tissue in the Smad3 $^{-/-}$ Mice

Ear tissue and biopsies from skin sites that were exposed to TMA were collected and analyzed for RNA content by real-time PCR. The expression of proinflamma-



tory cytokines IL-1 β , TNF- α and IL-6 mRNA (fig. 2a), Th1-type cytokine IFN- γ mRNA (fig. 2b), Th2-type cytokine IL-4 mRNA (fig. 2c) and Th17-type cytokine IL-17 mRNA (fig. 2d) was significantly increased in TMA-exposed ear tissue in Smad3 $^{-/-}$ mice. IL-23, which is required for the maintenance of Th17 cells, was also significantly increased in ear tissue of Smad3 $^{-/-}$ mice (fig. 2d). The expression profile of the above cytokines was similar in skin sites that were exposed to TMA 3 times altogether (online suppl. fig. 2a–c), and TGF- β mRNA was significantly upregulated in the skin of Smad3 $^{-/-}$ mice after TMA exposure (fig. 2e).

T_{reg}-Specific Foxp3 and Suppressive Cytokine IL-10 Expression Is at a Significantly Higher Level in Smad3 $^{-/-}$ Mice

The expression of T_{reg}-specific transcription factor Foxp3 mRNA was at a significantly high level in ear tissue in Smad3 $^{-/-}$ mice compared to WT mice, particularly in the control group. In fact, one topical application of TMA resulted in decreased Foxp3 mRNA expression in ear tissue in the Smad3 $^{-/-}$ mice compared to the control group (fig. 2f), and repeated TMA exposure dampened the expression of Foxp3 mRNA additionally (online suppl. fig. 2d). In contrast, in the WT mice, one topical application of TMA did not result in any changes in the expression of Foxp3 in ear tissue (fig. 2f). However, repeated exposure to TMA induced significantly decreased levels of Foxp3 mRNA, also in the WT mice (online suppl. fig. 2d). In histochemical analyses of ear tissue sections, the number of Foxp3-staining cells in ear tissue in the Smad3 $^{-/-}$ mice was slightly, but not statistically significantly increased (fig. 2g).

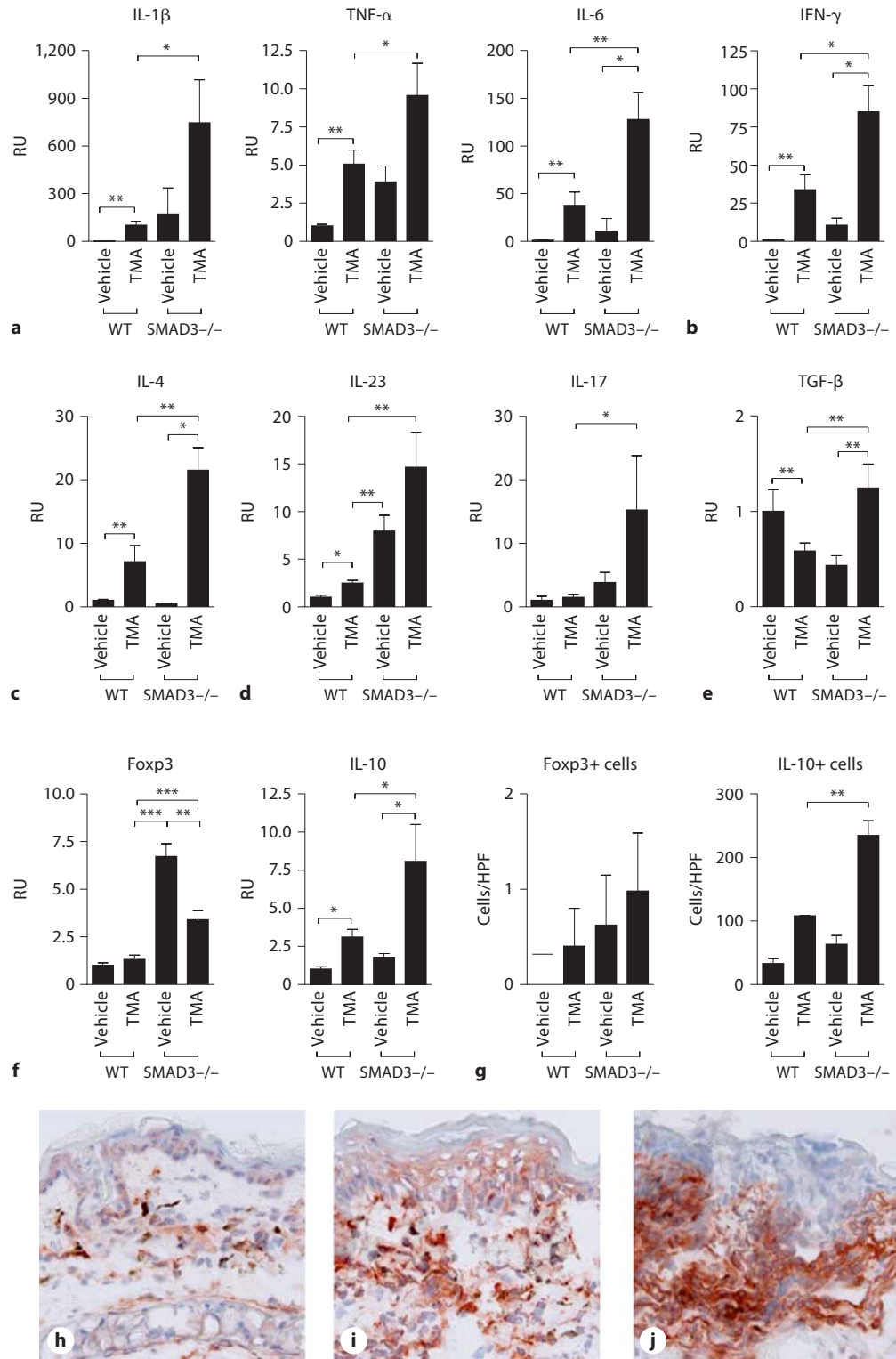
Fig. 1. Ear-swelling response and histological changes in ear tissue and skin after topical exposure to TMA in WT and Smad3 $^{-/-}$ mice. Topical application of TMA to the ears induced significant ear swelling in WT and Smad3 $^{-/-}$ mice at 24 h after exposure to TMA (**a**), and the number of CD3+ T cells increased significantly in TMA-exposed ear tissue in Smad3 $^{-/-}$ mice (**b**). The number of CD4+, CD8+ (**b**), F4/80+ and CD11c+ (**c**) cells was also determined by immunohistochemical analysis in frozen ear sections. Neutrophils, eosinophils and mast cells were enumerated in HE-stained skin sections (**d**). Bars represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$. **e–g** CD3 staining of vehicle-treated WT (**e**), TMA-exposed WT (**f**) and TMA-exposed Smad3 $^{-/-}$ ear tissue (**g**). **h–j** CD4 staining of vehicle-treated WT (**h**), TMA-exposed WT (**i**) and TMA-exposed Smad3 $^{-/-}$ ear tissue (**j**). **k–m** CD8 staining of vehicle-treated WT (**k**), TMA-exposed WT (**l**) and TMA-exposed Smad3 $^{-/-}$ ear tissue (**m**). **e–m** Magnification: $\times 500$.

The expression of suppressive cytokine IL-10 mRNA was significantly increased in TMA-treated ear tissue in Smad3 $^{-/-}$ mice compared to WT mice (fig. 2f). Immunohistochemical analysis of frozen sections of the ears confirmed the results at the mRNA level, showing significantly increased numbers of IL-10+ cells in the Smad3 $^{-/-}$ mice (fig. 2g, h–j).

The Chemokines CXCL9, CXCL10 and CCL24 Are Significantly Increased at the mRNA Level in TMA-Exposed Ear Tissue in the Smad3 $^{-/-}$ Mice

Chemokines orchestrate the recruitment of T cells and other leukocytes, and are thus key players in the disease process [25]. In the Smad3 $^{-/-}$ mice, the production of Th1-type chemokines C-X-C chemokine ligand 9 (CXCL9) and CXCL10 was significantly increased at the mRNA level in TMA-exposed ear tissue (fig. 3a). Also the Th2-type C-C chemokine ligand 24 (CCL24), which preferentially attracts eosinophils and basophils [26], was significantly increased in the Smad3 $^{-/-}$ mice (fig. 3b). Finally, CXCL5, which selectively attracts neutrophils [27], was slightly, but not statistically significantly increased in ear tissue in the Smad3 $^{-/-}$ mice (fig. 3c). However, 3 sequential applications of TMA onto the skin resulted in significantly increased CXCL5 in the Smad3 $^{-/-}$ mice (online suppl. fig. 2e).

The selective migration of T cells and other immune cells can be explained by the combined expression of selectins, integrins and chemokine receptors on their surface. C-C chemokine receptor 7 (CCR7) directs naive T cells and DCs to secondary LNs, while skin-infiltrating T cells preferentially express cutaneous lymphocyte antigen (CLA), CCR4 and CCR10 [28]. The expression of CCR7 was significantly increased in TMA-exposed ear tissue in the Smad3 $^{-/-}$ mice (fig. 3d), while CCR10 expression was similar in the Smad3 $^{-/-}$ and the WT mice (data not shown). The expression of CCR8, which is possibly also required for entry into the dermis [29], and preferentially expressed in Th2 cells [30], was significantly increased in the Smad3 $^{-/-}$ mice (fig. 3e). Also CCR3, which is highly expressed in Th2 cells, was slightly, although not statistically significantly, increased in Smad3 $^{-/-}$ ear and skin tissue (fig. 3e, online suppl. fig. 2f). By contrast, the expression of C-X-C chemokine receptor 3 (CXCR3) and CCR5, which are highly expressed by Th1 cells [31], was not significantly different in the skin in the Smad3 $^{-/-}$ mice compared to the WT (online suppl. fig. 2f). Finally, CXCR2, which is highly expressed by neutrophils [32], was significantly increased at the mRNA level in ear tissue of the Smad3 $^{-/-}$ mice (fig. 3f).



Smad3^{-/-} Mice Develop Significant Lymphadenopathy after Topical Exposure to TMA and the Expression of Cytokines IL-1 β , IL-4, IL-17 and IL-23 mRNA Is Significantly Increased in the Draining LNs

Presentation of hapten takes place both in LNs and at the site of allergen exposure to memory T cells during secondary exposure to hapten [33]. In LNs, antigen-experienced T cells can be stimulated by antigen to divide,

produce effector cytokines and migrate to peripheral tissues. By contrast, activated T cells that have migrated to nonlymphoid tissues, produce substantial effector cytokines upon antigen challenge, but are unable to divide or migrate back to the LNs [34]. At 48 h after topical TMA exposure, the ear-draining LNs in the *Smad3^{-/-}* mice were significantly larger than in the WT mice (fig. 4a). The rate of LN cell proliferation was significantly higher in the *Smad3^{-/-}* mice (fig. 4a), and the expression of

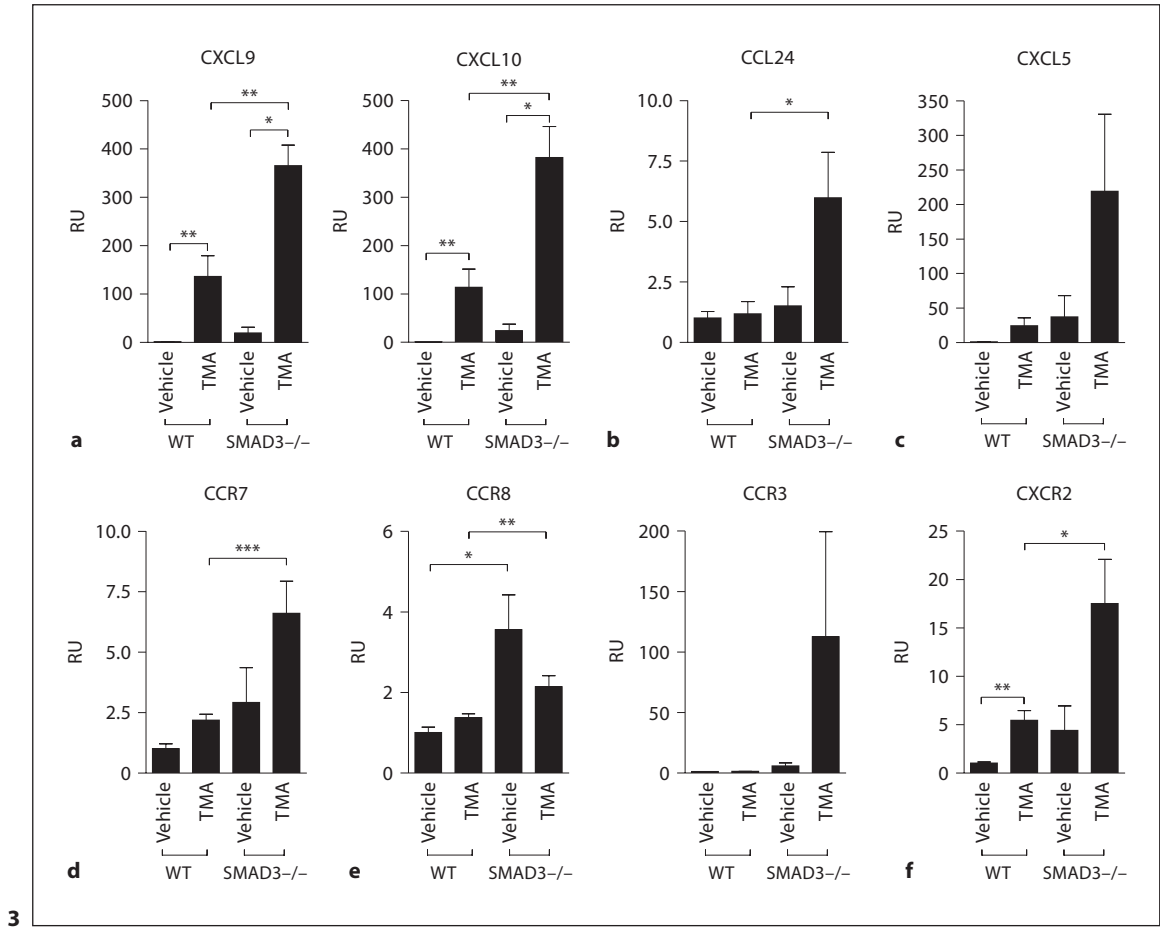


Fig. 2. Expression of proinflammatory and suppressive cytokines, and Foxp3 in ear tissue in *Smad3^{-/-}* and WT mice after topical exposure to TMA. mRNA expression of IL-1 β , TNF- α , IL-6 (a), IFN- γ (b), IL-4 (c), IL-23, IL-17 (d), Foxp3 and IL-10 (f) in TMA-exposed ear tissue, and TGF- β in skin (e) in *Smad3^{-/-}* and WT mice. Immunohistochemical analysis of Foxp3⁺ and IL-10⁺ cells (g) showed significantly increased numbers of IL-10⁺ cells in *Smad3^{-/-}* mice. h–j IL-10 staining of WT vehicle (h), TMA-exposed WT (i) and TMA-exposed *Smad3^{-/-}* (j) ear sections. RU = Relative units. Bars and errors represent mean $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. h–j Magnification: $\times$ 500.

Fig. 3. mRNA expression of chemokines and chemokine receptors in ear tissue in the *Smad3^{-/-}* and WT mice after topical exposure to TMA. CXCL9, CXCL10 (a) and CCL24 (b) increased significantly in TMA-exposed ear tissue in *Smad3^{-/-}* mice. CXCL5 mRNA expression increased slightly, but nonsignificantly in ear tissue in *Smad3^{-/-}* mice (c). The mRNA expression of chemokine receptors CCR7 (d), CCR8 (e) and CXCR2 (f) increased significantly in *Smad3^{-/-}* mice. RU = Relative units. Bars represent mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

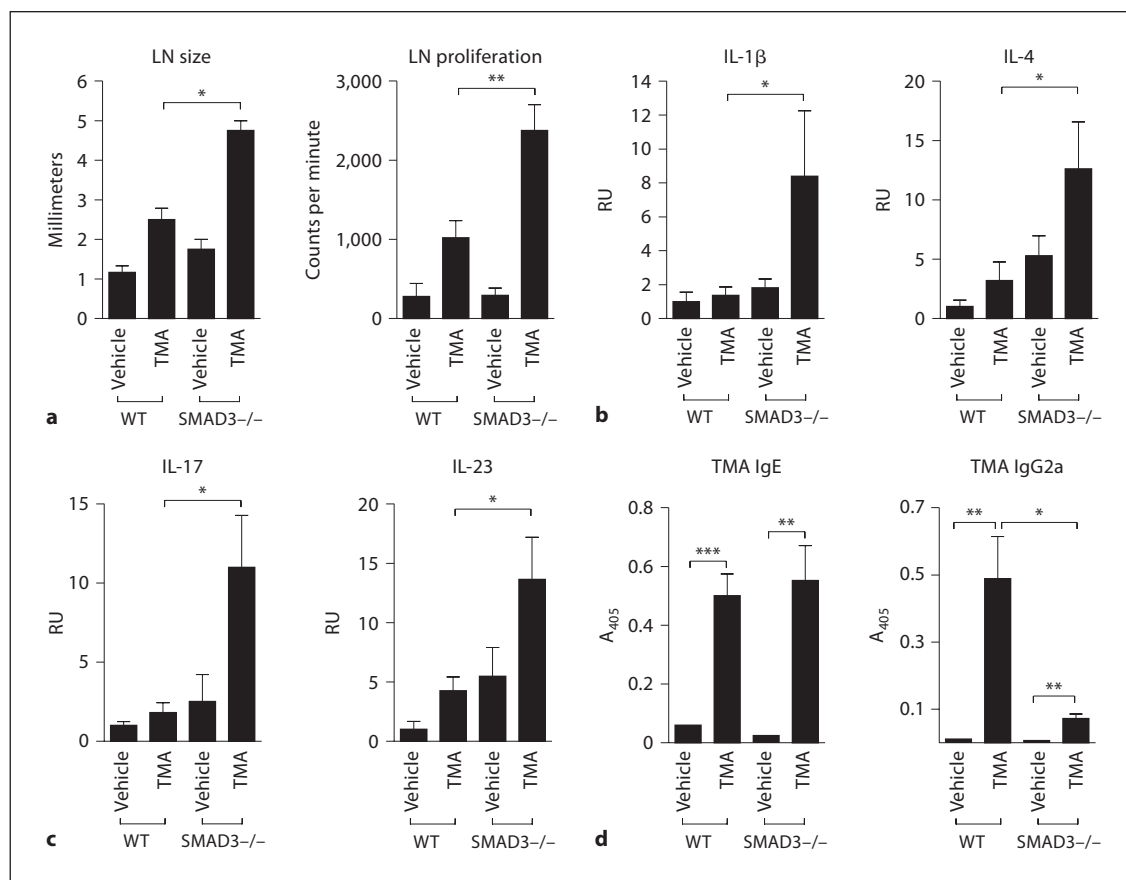


Fig. 4. Ear-draining LN size, rate of LN cell proliferation, expression of cytokine mRNA in LNs, and serum levels of TMA-specific IgE and IgG2a in Smad3^{-/-} and WT mice. LN size and the rate of LN cell proliferation were significantly increased in Smad3^{-/-} mice (a, b). The expression of IL-1β, IL-4 (b), IL-17 and

IL-23 (c) mRNA was significantly higher in Smad3^{-/-} mice. TMA-specific IgE was significantly increased after topical TMA exposure (d). The production of TMA-specific IgG2a was significantly impaired in Smad3^{-/-} mice (d). RU = Relative units. Bars represent mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

cytokines IL-1β, IL-4, IL-17 and IL-23 (but not IFN-γ, data not shown) was significantly elevated in LNs of Smad3^{-/-} mice (fig. 4 b, c).

TMA-Specific IgG2a Production Is Impaired in the Smad3^{-/-} Mice

TMA is known to cause a significant increase in the serum concentration of IgE, while IgG2a production remains at a lower level [35]. TMA-specific IgE production was similar in Smad3^{-/-} and WT mice after topical exposure to TMA (fig. 4d). Topical TMA exposure also induced significantly increased production of TMA-specific IgG2a in both groups of mice. However, in Smad3^{-/-} mice, the level of TMA-specific IgG2a was significantly lower than in WT mice (fig. 4d).

Discussion

The importance of TGF-β as an immunoregulatory agent is evident from the severe immunopathology seen in TGF-β knockout mice [3]. TGF-β has been assigned both pro- and anti-inflammatory roles, such as providing a chemotactic gradient for leukocytes [3], and contributing essentially to the differentiation of Th17 cells in mice [5] on the one hand, and downregulating proinflammatory cytokine production [3, 36] and inhibiting the differentiation of both Th1 and Th2 cells [7] on the other. Many studies have suggested that TGF-β is involved in the immunoregulation of allergies and induction of tolerance to allergens [37]. Smad3 plays an important part in mediating TGF-β signals [17–19], and targeted disruption

tion of Smad3 results in altered leukocyte function, including abnormal regulation of cytokine production and immune cell proliferation [17, 19].

A noticeable phenotype in Smad3^{-/-} animals is their decreased size and growth rate of young mice [19]. We noted that the ears in Smad3^{-/-} mice were significantly thinner than in their WT siblings at the starting point, and at 1 and 2 h after TMA exposure (online suppl. fig. 1). During the late-phase response, at 24 and 48 h after exposure, the initially thinner ears in Smad3^{-/-} mice, were, however, on average, as thick as in their WT siblings (online suppl. fig. 1), indicating a slightly more potent net swelling of the ears. Thus, the dynamics of the ear-swelling response in Smad3^{-/-} mice appeared different from that in WT mice, by not swelling as distinctively at 1 h and by swelling slightly more at 24 and 48 h after exposure. However, the small differences in ear-swelling responses between Smad3^{-/-} mice and their WT siblings did not reach statistical significance although many other inflammatory parameters increased significantly. Smad3 is known to play an essential role during tissue injury, as demonstrated by earlier work [18, 38], and is involved in the migratory response of epidermal cells during re-epithelialization of wounds [39]. Perhaps, for the same reason, the Smad3^{-/-} mice were unable to mount a significantly potent swelling of the ears that would have corresponded to the magnitude of the expression of inflammatory mediators in ear tissue, when compared to WT mice.

During the sensitization process, hapten-processing LCs migrate to LNs where they present hapten on their surface to naive T cells which are activated, begin to proliferate and differentiate into T effector cells. During secondary exposure to hapten, memory T cells are activated by local antigen-presenting cells (APCs) in the skin, as well as in the regional LNs [33]. T cell fate, i.e. the differentiation into Th1, Th2, Th17 or T_{reg} effector subsets, is determined by the quantity and quality of stimulation, including concentration of antigen, costimulatory molecules, and cytokines, as well as the frequency of responding T cells and density of antigen-presenting DCs [40]. In the Smad3^{-/-} mice, we observed significantly larger LNs after topical TMA exposure, and higher rates of LN cell proliferation. We did not analyze the cell content in the LNs, but may speculate, based on earlier work, in which Smad3^{-/-} T cells were completely unresponsive to anti-proliferative effects of TGF- β [19], that primarily T cells might have contributed to the lymphadenopathy that we observed in Smad3^{-/-} mice. Smad3^{-/-} T cell also display defects in TGF- β -mediated inhibition of cytokine production [19], and subtle changes in cytokine expression

can result in modulation of the immune response [41]. Indeed, in the LNs of Smad3^{-/-} mice, the expression of a number of cytokines was modulated, compared to WT mice. Thus, the increased numbers of responding lymphocytes, as well as altered cytokine production in the Smad3^{-/-} mice, might have influenced the outcome of the immune response, resulting in a significant Th2 shift, confirmed by unaltered IgE production but greatly impaired serum IgG2a levels. At 48 h after TMA exposure, the expression of a large repertoire of inflammatory mediators was significantly altered in all Smad3^{-/-} mouse tissues that were analyzed.

The rapid recruitment of skin-infiltrating CD8⁺ T cells initiates the CHS response, while CD4⁺ T cell infiltration occurs at a later stage [42]. T-cell-derived cytokines such as IFN- γ mediate the late classical aspects of CHS at the site of allergen exposure. The expression of IFN- γ , IL-4 and IL-17 mRNA and the number of CD3⁺ T cells were significantly increased in ear tissue in the Smad3^{-/-} mice. According to the literature, CD8⁺ T cells predominantly produce IFN- γ while CD4⁺ T cells produce IFN- γ , IL-4 [43] or IL-17 [44]. Both CD4⁺ and CD8⁺ cell numbers were slightly increased in the Smad3^{-/-} mice, indicating that both T cell subsets might have contributed to the modulated immune response.

T-cell-derived IFN- γ and TNF- α activate keratinocytes and other cells to produce a vast array of growth factors, chemokines and cytokines such as IL-1 β and TNF- α [45, 46], and IL-17 can reinforce the effects of IFN- γ on keratinocytes [47]. In the Smad3^{-/-} mice, we observed significantly increased expression of IL-1 β , TNF- α and IL-6, which among many other important biological functions, is crucial for the differentiation of the Th17 lineage [44, 48]. Th17 cells play an active role in skin immune responses [6], and in particular, a CD8⁺ subpopulation of T cells that produce IL-17 is important for the elicitation of CHS responses [6]. Th17 differentiation in mice also requires TGF- β , and IL-23 is important for maintaining the Th17 lineage [49]. IL-23 is primarily secreted by activated APCs such as DCs (including LCs), monocytes and macrophages [50], which are F4/80⁺ in mouse skin tissue. In the Smad3^{-/-} mice, TGF- β and IL-23 were significantly increased at the mRNA level, and the number of F4/80⁺ cells was slightly increased in ear tissue. As the number of CD11c⁺ cells was similar in Smad3^{-/-} and WT mice, the source of excessive IL-23mRNA expression in Smad3^{-/-} mice might be monocytes or macrophages. Altogether, the local cytokine milieu in skin, ear and LN tissue provided optimal conditions for Th17 differentiation in Smad3^{-/-} mice.

IL-4 can act synergistically with IFN- γ to enhance the release of Th1 attracting chemokines, such as CXCL9 and CXCL10, which promote the recruitment and retention of Th1 cells in the skin [51]. While CXCL9 and CXCL10 were significantly increased at the mRNA level in TMA-exposed ear tissue in Smad3 $^{-/-}$ mice, the expression of their corresponding receptor CXCR3 was not. Moreover, the expression of CCR5, which is highly expressed by Th1 cells, was not increased in Smad3 $^{-/-}$ mice although IFN- γ mRNA expression in ear and skin tissue was significantly increased compared to WT mice. Instead CCR8, which is preferentially expressed by Th2 cells, was significantly increased at the mRNA level in Smad3 $^{-/-}$ mice, indicating that the skin-infiltrated CD3 $^{+}$ T cells in Smad3 $^{-/-}$ mice, might predominantly be of the Th2 phenotype. Furthermore, the expression of CCR7 was significantly high in Smad3 $^{-/-}$ mice. This chemokine receptor is expressed on DCs, B cells and T cells, and involved in homing of T cells and antigen-presenting DCs to the LNs [52, 53]. The expression of CCR7 is mainly associated with naive T cells, but has been observed to be highly expressed by activated CLA $^{+}$ skin-homing T cells in individuals with cutaneous atopic disease [54]. TGF- β [55], and IL-4 [54], which were both expressed at a high level in Smad3 $^{-/-}$ mice, promote the expression of CCR7 on T cells. Thus, the significantly increased CCR7 mRNA expression might also reflect the increased number of T cells in ear tissue in Smad3 $^{-/-}$ mice.

The 'Th2-type' chemokine CCL24 binds to CCR3, which is most abundantly expressed by eosinophils, and to a lesser extent by basophils and Th2 cells [26, 52]. CCL24 mRNA was significantly increased in TMA-exposed ear and skin tissue in the Smad3 $^{-/-}$ mice, while the expression of CCR3 at the mRNA level was slightly, but not statistically significantly increased. However, the number of recruited eosinophils was similar in the skin of Smad3 $^{-/-}$ and WT mice by histological examination. Thus, the slightly elevated expression of CCR3 mRNA in ear and skin tissue might instead reflect the increased number of Th2-type CD3 $^{+}$ T cells in Smad3 $^{-/-}$ mice.

The expression of neutrophil-attracting chemokine CXCL5 was slightly although not statistically significantly increased in Smad3 ear tissue. In the skin, however, which received 3 applications of TMA, the expression of CXCL5 was significantly increased in Smad3 $^{-/-}$ mice. Also the expression of CXCR2, which is attracted by CXCL5 and highly expressed by neutrophils, was significantly increased in ear tissue in the Smad3 $^{-/-}$ mice. However, in skin samples, the number of neutrophils was only slightly, but not statistically significantly increased in

the Smad3 $^{-/-}$ mice, and in ear tissue, the number of neutrophils was not determined. Taken together, the chemokine receptor mRNA expression profile in Smad3 $^{-/-}$ mice (i.e. high expression of CCR8 and CCR3 mRNA, and similar expression of CCR5 and CXCR3 mRNA compared to the WT mice) suggests that the Th2 phenotype might be well represented among the recruited T cells in Smad3 $^{-/-}$ mice. In addition, the number of skin-infiltrated eosinophils and neutrophils did not correspond well to the expression level of eosinophil- and neutrophil-attracting chemokines CCL24 and CXCL5, respectively, suggesting impaired migration of the Smad3 $^{-/-}$ inflammatory cells, which is in accordance with earlier studies that have demonstrated that Smad3 is involved in the migratory response of various cell types [18, 39, 56, 57].

T_{reg} suppress immune activation and are characterized by the expression of transcription factor Foxp3 [58, 59]. TGF- β is a potent inducer of Foxp3 expression both in vitro and in vivo [11, 14], but its importance in T_{reg} suppression remains controversial [10, 60]. Several studies have shown that TGF- β production by T_{reg} plays a minor, if any, role in the in vitro suppressive function of T_{reg} [10, 60]. The major function of T_{reg}-produced TGF- β is probably related to its capacity to induce the differentiation of T_{regs} [13, 61]. It has been well documented that CD4 $^{+}$ T cells from Smad3 $^{-/-}$ mice are completely resistant to the antiproliferative effects of exogenous TGF- β [10, 17, 19], and this may also include CD4 $^{+}$ T_{regs}. Of note, the expression of Foxp3 mRNA was at a significantly higher level in the Smad3 $^{-/-}$ control group, and the exposure to TMA reduced the level of Foxp3 mRNA expression in Smad3 $^{-/-}$ mice. However, compared to WT mice, the expression of Foxp3 mRNA was significantly increased in both the control group and the TMA-exposed groups of Smad3 $^{-/-}$ mice, suggesting the frequency of T_{regs} might be unusually high in Smad3 $^{-/-}$ mice (fig. 2f). However, immunohistochemical analysis of frozen ear sections showed only slightly, but not statistically significantly, increased numbers of Foxp3 $^{+}$ cells in Smad3 $^{-/-}$ mice (fig. 2g).

IL-10 has broad anti-inflammatory properties, with APCs and lymphocytes as main targets [62]. Although IL-10 was originally isolated from Th2 cells [63], it is known to be produced by many cell types, and T_{regs} constitute an important segment in IL-10-dependent suppression of inflammation [64]. In TMA-exposed ear tissue of Smad3 $^{-/-}$ mice, the expression of IL-10 mRNA was significantly increased compared to WT mice. The increased expression of IL-10 in Smad3 $^{-/-}$ mice was con-

firmed on the protein level by immunohistochemical staining of IL-10+ cells (fig. 2g, h–j). The IL-10+ cells infiltrate localized mainly to the subepidermal parts of the ear tissue in Smad3^{–/–} mice, and might consist of T cells and APCs, in addition to, perhaps residential cells.

Taken together, the lack of Smad3 resulted in significantly increased expression of proinflammatory, Th1, Th2 and Th17 cytokines, as well as Foxp3, IL-10 and TGF- β mRNA in ear, skin and LN tissue after topical exposure to TMA. The increased production of IL-10 in Smad3^{–/–} mice was confirmed at the protein level by immunohistochemical staining. In addition, T-cell-attracting chemokines CXCL9 and CXCL10, as well as CCL24 and CXCL5 were significantly increased in Smad3^{–/–} mice. The number of skin-infiltrated CD3+ T cells and mRNA expression of chemokine receptors that are highly expressed by Th2 cells were significantly increased in Smad3^{–/–} mice, indicating a predominating Th2 phenotype of the accumulated T cells. Finally, the level of TMA-specific IgE in the serum was similar in Smad3^{–/–} and

WT mice, while the production of TMA-specific IgG2a was greatly impaired in Smad3^{–/–} mice, indicating a strong Th2 shift. Our results add to the accumulating evidence that Smad3 is essential for proper TGF- β signaling during the immune response to contact sensitizers. Unraveling the mechanisms of Smad3 may open new possibilities for treating and preventing allergic responses, which may lead to severe illness and loss of work ability.

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