

A Mouse Model for Atopic Dermatitis Using Topical Application of Vitamin D3 or of Its Analog MC903

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Abstract

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease with pruritus and high prevalence. Indeed, 15–30% of children and 2–10% of adults from industrialized countries are affected. Acute AD lesions are characterized by epidermal hyperplasia associated with a dominant Th2/Th17 immune response and dermal inflammatory infiltrates. Moreover, the expression of alarmins such as TSLP, IL-33, and IL-25 is upregulated in acute AD lesions. Topical application of vitamin D3 or of its low-calcemic analog MC903 induces changes in skin morphology and inflammation resembling immune perturbations observed in acute lesions of patients with AD. Mice treated with MC903 or vitamin D3 additionally display increased serum IgE levels, as observed in patients with extrinsic AD. Interestingly, these symptoms are not dependent on mouse gender or on genetic background. Thus, the easiness of this mouse model renders it very attractive to study immunologic abnormalities involved in AD development or maintenance. Furthermore, this model might be useful for preclinical studies aiming at unraveling new therapeutic strategies to treat AD. In this chapter, we describe the induction and major features of MC903 and vitamin D3-induced AD-like inflammation in mice.

Key words Atopic dermatitis, MC903, Mouse model, Thymic stromal lymphopoietin (TSLP), Skin, Type 2 T helper cells (Th2), Vitamin D3

1 Introduction

Atopic dermatitis (AD) is a chronic relapsing non-contagious inflammatory skin disease accompanied by pruritus. Its onset occurs early in life, most commonly between 6 weeks and 6 months of age. Its incidence culminates around the age of 2 years and then slowly diminishes. In 50–70% of cases, the disease progressively regresses and disappears during adolescence. Atypically, AD can develop with a late onset (<18 years old). Worldwide, the prevalence of AD is relatively high by affecting 15–30% of children and 2–10% of adults. Noticeably, AD prevalence underwent a marked increase during the past 30 years, potentially resulting from increased pollution [1]. In atopic children, regression of

eczema can coincide with the development of asthma or allergic rhinitis, whose incidence starts increasing at the age of 2 and 5 years, respectively. However, incidence of asthma peaks around an age of 5 years and then slowly decreases to reach its minimal incidence at 15 years. Incidence of allergic rhinitis remains stable throughout life [2].

The pattern of AD skin lesions is related to patients' age. In the perinatal period, erythematous and papulovesicular eruptions with oozing and crusting are often observed on the face, scalp, and flexures. Later, in childhood, AD is characterized by generalized dry skin and persistent lichenified flexural lesions on arms and legs. When AD persists or appears in adults, the disease is mostly localized on extremities (feet and hands) and flexures (elbows, knees, and neck). It can also manifest as an atopic red face, as chronic lichenified eczema on the trunk, or as subacute or psoriasiform [3]. The course of AD varies in individuals according to psychological state and environment. The physical and environmental factors involved in adults differ from those in children and this may be responsible for the different patterns of skin involvement and for the atypical morphologies [3].

Primary genetic defects affecting keratinocytes and compromising the skin barrier are believed to substantially contribute to AD development. The defective epidermal barrier permeability has long been assumed to be a consequence of the immunological abnormalities observed in AD ("inside-to-outside" paradigm). Later, skin barrier dysfunction was proposed as the "driving force" of AD pathogenesis ("outside-to-inside" paradigm). Nowadays, an "outside-to-inside, back-to-outside" paradigm predominates. In this pathogenic model, initial skin barrier impairment favors allergen/hapten/chemical ingress in the stratum corneum, which are taken up by Langerhans cells (LCs) [4]. Activated LCs then migrate to regional lymph nodes to prime naïve CD4⁺ T-lymphocytes into Type 2 T helper (Th2)/allergic cells or locally activate memory Th2 cells [5–7]. Th2 cytokines such as IL-4 and IL-13, produced not only by skin T cells but also by innate lymphoid cells type 2 (ILCs2), then downregulate genes important to skin barrier function such as filaggrin (FLG), involucrin or loricrin, further increasing skin permeability to allergens/haptens/chemicals [8–10]. This vicious cycle might be amplified by the hyper-proliferation of LCs which has previously been reported in AD skin [11]. The "outside-to-inside, back-to-outside" paradigm is strengthened by recent work showing that increased transepidermal water loss (TEWL) in newborns precedes overt AD symptoms [12].

Primary skin barrier disturbances observed in AD are, in part, driven by loss-of-function mutations in the gene encoding the epidermal protein filaggrin (FLG) [13]. However, other genes important for skin barrier function are potentially involved. Meta-analysis of genome-wide association studies identified other risk loci linked

to AD and involved in epidermal proliferation and differentiation [14, 15]. However, to date, only FLG mutations were found to predict dose-dependent alterations in the permeability of epidermal barrier [16–18]. In patients with AD, the epidermal barrier defect is also provoked by abnormal lipid composition and organization of the stratum corneum and by an altered epidermal lipid metabolism [9, 19–22]. These cutaneous lipid abnormalities can result from upstream genetic alterations in lipid-related genes but also in genes related to keratinocyte differentiation [16].

During flares, two skin states, i.e., nonlesional vs. lesional coexist in AD patients. Nonlesional skin, despite a normal appearance, shows epidermal hyperplasia, increased TEWL, and is associated with deregulated keratinocyte differentiation, a less acidic skin surface pH and subclinical signs of inflammation [23]. Increased numbers of Ki67⁺ keratinocytes and upregulation of keratin 16 expression demonstrate a stimulation of keratinocyte proliferation in nonlesional skin. This keratinocyte hyper-proliferation is not counteracted by increased apoptosis [24]. Moreover, CD3⁺ and CD11c⁺ cells are more abundant in nonlesional AD than in healthy skin [23]. Furthermore, the transcription of genes involved in both epidermal homeostasis and immune response is largely altered in nonlesional AD when compared to healthy skin. The expression of genes encoding for major terminal differentiation proteins, including loricrin, FLG, involucrine, corneodesmosine, late cornified envelope and small proline rich proteins is downregulated [23]. Interestingly, protein levels of Claudin 4, a protein essential for tight junctions, are enhanced in nonlesional AD when compared to healthy and lesional skin [25]. The expression of the T-cell trafficking chemokines, CCL17, CCL5, CCL11, CCL18 and the expression of IL-13 considered as Th2 mediators are markedly increased whereas expression of genes involved in Th1/IFN γ signaling is moderately triggered in nonlesional AD [23]. Furthermore, expression of IL-23/p19 and p40 is increased, suggesting a partial upregulation of the Th17 immune response [23]. A Th22 immune response is not induced in nonlesional skin with expression of IL-22 similar in nonlesional AD and healthy skin [26, 27]. Thus, nonlesional skin mainly displays a Th2/Th17 immune response. Expression of antimicrobial peptides such as hBD-2 and LL-37 [28, 29] and neutrophil numbers [30] are unaltered in nonlesional skin when compared to healthy skin and it remains unknown whether nonlesional AD skin is infiltrated by increased numbers of ILCs2. Thus, the contribution of innate immunity to nonlesional AD might rather be minor.

Lesional AD skin exhibits major epidermal hyperplasia, spongiosis, lichenification, overt inflammation, possibly bacterial as well as viral superinfection. Moreover, lesional AD is characterized by severe skin barrier impairment associated with dramatic increase of TEWL and strong downregulation of keratinocyte differentiation.

Skin lesions of AD patients are infiltrated with various immune cells, such as eosinophils, basophiles, mast cells, dendritic cells (DCs), CD8⁺ T cells, CD4⁺ T cells, and ILCs2 [31]. Recent work has shown that neutrophils and neutrophil-related molecule such as DEFB4 and CXCL1 are increased in skin lesions of AD patients when compared to nonlesional skin, thus changing the classical view on the contribution of neutrophils to AD [30]. Moreover, higher levels of neutrophils in lesional AD are associated with *Staphylococcus aureus* infection and *Staphylococcal* coagulase-induced supraepidermal crust formation [30]. Others reported lower numbers of polymorphonuclear leukocytes, NK cells and plasmacytoid DCs (pDCs) in AD lesions, potentially explaining microbial superinfection [32]. Thus, both abnormal adaptive and innate immunity contribute to lesional AD [32].

Lesional AD skin develops in two phases, i.e., the acute phase which lasts only a few hours followed by the chronic phase. Acute lesions are characterized by a Th2 dominant immune response and epidermal hyperplasia, very similar to nonlesional AD. However, acute lesions additionally exhibit increased expression of alarmins such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) which are produced by damaged epidermal cells, mostly keratinocytes [33]. These alarmins are believed to promote Th2 immune response, thus perpetuating immune abnormalities [33]. Because the acute phase of lesional AD only lasts a few hours, the literature mostly reports data on the chronic phase of lesional AD. Immune abnormalities observed in chronic AD lesions are more complex than in acute lesions because Th1, Th17, Th22, and Th2 immune responses coexist [26, 27, 34]. Acute and chronic AD lesions can be distinguished by differential expression pattern of various molecules (Table 1) [35, 36]. Most of AD skin lesions are colonized with microbes [37]. Microbial superinfection triggers receptors such as the protease-activated receptors (PARs), the Toll-like receptors (TLRs), the C-type lectins (CLRs), and inflammasomes which, in turn, induce NF- κ B activation, reactive oxygen species (ROS) production, and the release of innate pro-Th2 cell cytokines including alarmins and granulocyte-macrophage colony-stimulating factor (GM-CSF) by epidermal cells [33, 37]. Subsequently, ILCs2 are recruited to AD skin lesions by TSLP, IL-25, IL-33 but also by prostaglandin D2 [31–33, 38, 39]. Thus, in chronic AD lesions, microbial superinfection sustains inflammation by triggering innate immunity via keratinocyte activation.

Various mouse models have been developed in order to better understand the pathogenesis of AD or to evaluate efficacy of potential therapies. However, there are no perfect mouse models for AD because genetically- or chemically-induced AD in mice only recapitulates part of the features observed in AD patients but never all [7, 40]. The choice of a mouse model to study AD should be tailored to the question asked. The mouse model using vitamin D3

Table 1
Cytokine expression profile in acute versus chronic AD lesions

	Acute lesions	Chronic lesions
IL-4	↑	~↑
IL-5	=	↑
IL-10	↑	↑
IL-13	~↑	↑
IL-31	↑	↑
CCL18	~↑	↑
CCL5	~↑	↑
CCL13	=	↑
CCL11	↑	↑
TSLP	↑	↑
TSLPR	~↑	↑
OX40L	=	=
IL-9	~↑	~↑
IL-22	↑	↑
S100A7	↑	↑
S100A8	↑	↑
S100A9	↑	↑
S100A12	↑	↑
IL-32	↑	↑
IFN- γ	=	↑
IL-8	~↑	~↑
IL-1 β	~↑	~↑
IFN- α	=	~↑
CXCL9	=	↑
CXCL10	=	↑
CXCL11	=	↑
IL-17A	~↑	~↑
IL-23p19	~↑	=
IL-23p40	~↑	~↑
CCL20	↑	↑
Foxp3	=	↑

Expression compared to nonlesional AD [35, 36], = unchanged expression, ~↑ non significantly increased, ↑ significantly increased

or its low-calcemic analog, MC903 has been developed by Li et al. in 2006 [41]. In this model, vitamin D3 or MC903 induces over-expression of TSLP by keratinocytes leading to the development of AD-like inflammation [41, 42] and of asthma [43, 44]. Mice treated with MC903 or vitamin D3 exhibit epidermal hyperplasia associated with increased dermal inflammatory infiltrates consisting of eosinophils and CD3⁺, CD4⁺, CD11c⁺, GR-1⁺, and mast cells [41]. Skin treated with MC903 shows increased levels of TSLP, IL-4, IL-5, IL-13, IL-31, IL-10, IL-8, IFN- γ , and TNF- β [5, 41]. Thus, in this model, a mixed Th2/Th1 immune response is triggered by MC903/vitamin D3 treatment. However, it remains unknown whether MC903 or vitamin D3 enhances a Th17 or a Th22 immune response in mouse skin. Moreover, vitamin D3- or MC903-treated mice display increased serum IgE [5, 41], as observed in extrinsic AD. The induction of AD-like symptoms by vitamin D3 or MC903 requires both adaptive and innate immunity. Indeed, this model has been used to demonstrate the pathogenic role of LCs in the development of AD [5] and their hyper-proliferation in AD skin [11]. Furthermore, topical applications of MC903 and TSLPR deficient mice have been employed to demonstrate that TSLP signaling in CD4⁺ T cells is required for memory formation of Th2 cells [45]. Recently, this model has been utilized to show a TSLP-dependent, IL-33-independent recruitment of ILCs2 to AD skin [39] and that AD-like inflammation is improved by microRNA-146a through suppression of innate immune responses in keratinocytes [46]. Finally, RAG1^{-/-} mice treated with MC903 display epidermal hyperplasia, increased skin expression of TSLP, IL-4, IL-6, IL-10, and IFN- γ , and increased skin infiltration with ILCs2, eosinophils, mast cells, and CD11c⁺ cells [39, 41].

Thus, the MC903- or vitamin D3-based AD mouse model nicely recapitulates important hallmarks of lesional AD. Therefore, it can be used to address still unanswered questions related to the complex immune interplay that characterizes lesional AD. Furthermore, this model might help in deciphering abnormal adaptive and innate immune responses in AD. The epidermal barrier function has not yet been investigated in this model, which could be useful to detail the effects of cutaneous immunity on stratum corneum and tight junction barriers. Moreover, this mouse model could be used to evaluate the relation between AD and asthma.

To conclude, the big advantage of this model is its easiness. Indeed, it suffices to topically apply vitamin D3 or MC903 to mouse skin for several days to obtain AD-like inflammation recapitulating major AD features [41]. This model does not require tape stripping which can be tricky, application of occlusive patches onto mouse back that are sometimes not easy to manage, topical application of hazardous agents such as oxazolone or 2,4-dinitrofluorobenzene (DNFB), or complex genetic manipulations that take months or

years to be established. In this chapter, we will provide all technical details for topically applying vitamin D3 or MC903 to mouse skin in order to obtain AD-like inflammation.

2 Materials

1. 8–10-week-old, sex- and weight-matched mice (*see Note 1*).
2. Ethanol 99% grade pure.
3. A high precision balance.
4. Safe-lock 1.5 ml black Eppendorf tubes (light safe).
5. Eventually a shaving devise and a depilatory cream.
6. MC903 (also called calcipotriol, >98%, Tocris). The working concentration for MC903 is 45 μ M. It can be prepared with an initial concentration of 450 μ M as follows: Dissolve 1 mg MC903 (MM=412.6 g/mol) in 5.386 ml ethanol. Further dilute this stock solution ten times in ethanol to reach the working concentration of 45 μ M. Prepare 1 ml aliquots in small sterile black Eppendorf tubes (safe-lock, light safe) and store at -20°C . Aliquots should not repeatedly be thawed and frozen because of subsequent ethanol evaporation, which will concentrate the chemical (*see Notes 2–4*). Argon can be applied to the Eppendorf tubes to avoid oxidation of MC903 for long-term storage (*see Note 5*).
7. Vitamin D3 (also called $1\alpha,25$ -dihydroxyvitamin D3 or cholecalciferol, >98% HPLC, Sigma-Aldrich). Dissolve vitamin D3 in ethanol to a working concentration of 50 μ M. It can be prepared as an initial solution of 500 μ M as follows: Dissolve 1 mg vitamin D3 (MM=384.339 g/mol) in 5.204 ml ethanol. Dilute this solution ten times with ethanol to reach the working concentration of 50 μ M. Use the same storage conditions as described for MC903 (*see Notes 2–5*).

3 Methods

Compounds can be topically applied to mouse ears under short anesthesia with ketamine or isoflurane. Because MC903 is a low-calcemic vitamin D3 analog, it can be applied to mouse skin at higher concentrations and for a longer time than vitamin D3. Therefore, MC903 can be applied not only to mouse ears but also to mouse back skin after hair removal (please see below). Vitamin D3 should only be applied to mouse ears because of its adverse effects on systemic calcium metabolism.

3.1 Induction of AD-Like Skin Inflammation

3.1.1 Topical Application of MC903

1. When MC903 is applied to mouse back skin, hair should be removed 3 days before starting the topical applications (*see Note 6*).
2. To induce AD-like inflammation with MC903, compound should topically be applied once daily as follows for (1) mouse ears (12.5 μ l on dorsal and ventral ear side for a total volume of 25 μ l per ear) or (2) back skin (25 μ l for a skin area the size of a quarter). Altogether, 1.125 nmol MC903 will be applied per ear or per back skin area every day (*see Note 1*).
3. The sequence of treatment will be the following: 5 days treatment, 2 days interruption, 5 days treatment, 2 days interruption, 1 or 2 days treatment (for a 15–16 day-treatment period). Control animals are treated in parallel with ethanol only (*Fig. 1*).

3.1.2 Topical Application of Vitamin D3

1. When vitamin D3 is used, only one ear should be treated because of adverse effects on systemic calcium metabolism (*see Notes 1 and 2*).
2. To induce AD-like inflammation with vitamin D3, the compound should topically be applied to one mouse ear (10 μ l on dorsal and ventral ear side for a total volume of 20 μ l per ear) once daily. Altogether, 0.4 nmol vitamin D3 will be applied per ear and per day according to the following treatment sequence: 4 days treatment, 3 days interruption, 4 days treatment (for a 11 day-treatment period) (*see Notes 1 and 2*). Control animals are treated in parallel with ethanol only (*see diagram below*).

3.2 Evaluation of Atopic Inflammation

The time course of inflammation can be monitored by measuring ear thickness with a caliper under light anesthesia (*Fig. 2a*). Upon treatment, inflammation develops as skin hyper-vascularization and subsequent skin redness becomes visible (*Fig. 2b*). Auricular lymph nodes are also enlarged after treatment (*Fig. 2c*) (*see Note 7*).

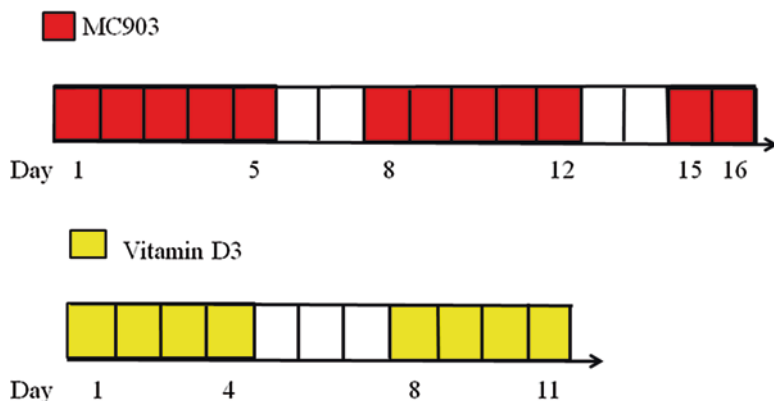


Fig. 1 Diagrams showing the daily treatment scheme for MC903 (in red) and for vitamin D3 (in yellow)

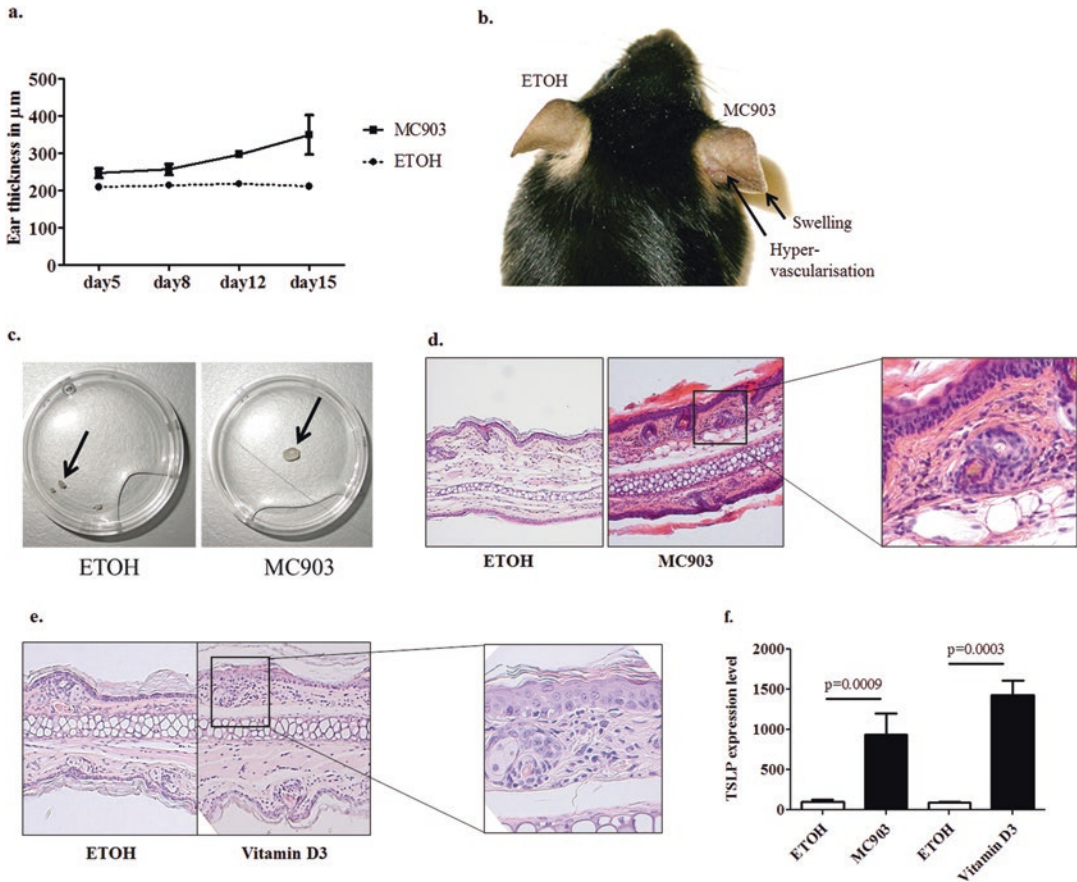


Fig. 2 MC903 induces atopic dermatitis-like inflammation in mice. C57BL/6 mice were topically treated with MC903 on right ear or with ethanol on left ear for 15 days as follow: 5 days topical treatment, 2 days interruption, 5 days treatment, 2 days interruption, 1 day topical treatment. (a) Time course of ear thickness. Days on x-axis refer to days after start of the treatment. (b) Pictures of a representative mouse on day 15 showing ear thickening and hyper-vascularization (arrows) for the ear side treated with MC903. (c) Pictures of representative auricular lymph nodes from a mouse treated for 15 days with MC903 or ethanol (ETOH). Pictures of representative H&E staining from mouse ears after topical treatment with ethanol or MC903 (d) or vitamin D3 according to the 11-day protocol (e). Magnification $\times 100$ (left panel) or $\times 400$ (right panel). (f) Histogram showing expression of TSLP, measured by quantitative PCR, in mouse ears treated with ethanol, or MC903 or vitamin D3. Results are expressed as % of controls and shown as Mean $\pm$ SEM. Statistical significance was determined by a Student-*t*-test

Histological analysis of mouse skin shows epidermal hyperplasia and dermal inflammatory infiltrates mostly consisting of lymphocytes, macrophages, eosinophils and neutrophils (Fig. 2d) (see Note 8). The vitamin D3-related symptoms are attenuated due to shorter treatment time and lower dose of the compound used (Fig. 2e). However, this protocol is sufficient to induce a robust TSLP overexpression in mouse skin (Fig. 2f) (see Note 9).

4 Notes

1. This model works in mice regardless of sex and genetic background (Balb/c versus C57BL/6).
2. Mice treated with vitamin D3 will lose weight over time during the experiment due to interactions between vitamin D3 and calcium metabolism. Therefore, MC903 should be preferred to vitamin D3. In case, MC903 is not available, a careful follow up of the mice should be done; only mice weighting more than 25 g should be used for the experiment.
3. Compounds should be stored at -20°C protected from light and in aliquots. If possible, remove oxygen in tubes with argon prior storage (*see* **Note 5**). Tubes should have a safe lock and be additionally sealed with Parafilm to avoid ethanol evaporation.
4. Aliquots of working solution should be prepared to be only used once. Otherwise, air can induce compound oxidation and ethanol evaporation.
5. Argon is sold as a bottle of gas. The bottle output is usually connected to flexible plastic tubing at the end of which a glass Pasteur pipette or a pipette tip is inserted. After opening of the bottle to release the gas, apply the Pasteur pipette in the Eppendorf tubes for 30 s at least. It takes a bit of time to eliminate all oxygen. The duration of argon diffusion depends on the volume of liquid in the tube.
6. It is important to remove hair 3 days prior to topical treatment. These 3 days are required for skin recovery after hair removal which inflicts skin traumas. Mice are anesthetized and shaved on a large back skin area with a shaving device. Then, a thick layer of depilatory cream is applied onto skin for 4–7 min and washed away with water only. Cream should be entirely removed and mice carefully dried using handkerchiefs. To help mice to recover from anesthesia and hair removal, they can be placed on a warming table at 35°C . If no warming table is available, mice can be placed in between two latex gloves filled with warm water. This recovery procedure should not exceed 20 min.
7. Auricular lymph nodes can be further used for flow cytometry analyses or for cell cultures as follows: place auricular lymph node(s) in a 6 well-plate containing 2 ml Hank's buffer without calcium and magnesium and 2% FCS. Then, dislocate lymph nodes with curved pincers and pipet the bulk into a 50 ml Falcon tube. Wash the well that has contained lymph nodes with 2 ml Hank's buffer and 2% FCS and add to Falcon tube. Auricular lymph nodes are then digested with collagenase D (120 μl of a 40 mg/ml solution) for 25 min at 37°C under shaking. Stop the digestion by adding EDTA (80 μl of a 0.5 M solution) for 5 min at 37°C under shaking. Single cell

suspension is obtained by filtering cells through a 40 μm cell strainer, washed with cold PBS and centrifuged. Finally, dissolve the pellet in cold PBS or R10 medium, count cells in a hemocytometer.

8. To perform H&E staining on mouse ears, disinfect tissue with 70 % ethanol, remove and fix it overnight at 4 °C in 1 ml of a 4 % formalin solution. Then, embed $\frac{1}{2}$ ear in paraffin and cut in 3 μm sections with a microtome. Hematoxylin and eosin staining is then done as follows: after deparaffinization with successive xylene and ethanol baths, sections are first stained with haematoxylin (3 $\times$ 5 min), washed with 1 % acid alcohol for 2 min and then further stained with eosin for 2 min. After extensive washings, sections are mounted and examined under a light microscope.
9. To measure expression of TSLP in mouse ears, the following protocol can be utilized: RNase-free tubes and solutions should be used throughout all RNA preparation and RNA Zap[®] (Ambion) should be used to clean up instruments. First disinfect ears with 70 % ethanol, remove and immediately shock freeze it in liquid nitrogen. Ears can be stored at -80 °C for a few days or used on the same day to prepare RNA. Skin contains RNases; therefore it is always better to prepare RNA from skin as soon as possible after removal because the storage can induce RNA degradation. RNA is then prepared as follows (adapted from TRIzol data sheet): add 1 ml TRIzol to $\frac{1}{2}$ mouse ear and homogenize with a homogenizer. Incubate samples for at least 5 min at room temperature. Then, add 200 μl chloroform and mix tubes by inverting for 15 s. Incubate samples at room temperature for at least 2 min and centrifuge at 12,000 $\times g$ for 15 min at 4 °C. Pipet the aqueous phase (the upper color-less phase) and transfer into a clean tube. Add 500 μl isopropanol and mix by inverting 5–10 times. Let RNA precipitation at -20 °C overnight. Then, centrifuge samples at 12,000 $\times g$ for 10 min at 4 °C. A white pellet should be visible at the bottom of the tube. To ensure visualization of a pellet, GlycoBlue[™] (Thermofisher) (1:1000) can be added to the mixture prior overnight precipitation. After centrifugation, remove supernatant, add 1 ml 75 % ethanol and centrifuge samples at 7500 $\times g$ for 10 min at 4 °C. Carefully remove ethanol and let the tube drying by inverting on a clean tissue for 5–10 min at room temperature. Clean swabs can alternatively serve at drying residual ethanol on tube walls. When the pellet is dried up, add 50 μl TE buffer and vortex well. Samples can be further incubated at 55–60 °C for 10 min to inactivate proteins (Table 2). Then, genomic DNA can be removed from samples by DNase treatment according to manufacturer's instructions (Ambion, Austin, TX). The removal of genomic

Table 2
Summary for RNA and cDNA preparation from mouse ears

RNA preparation 50 µl		cDNA preparation 100 µl	
<i>Step 1:</i> Homogenization	½ ear + 1 ml TRIzol	<i>Step 1:</i> Denaturation step	1 µg RNA + 4 µl Hexamer (<i>see</i> Note 11) + 10 µl 10 mM dNTP + (qsp 54 µl RNase-free water) Incubate for 5 min at 65 °C
<i>Step 2:</i> Dissociation of nucleoprotein complex	Incubation for 5 min at room temperature	<i>Step 2:</i> To bring all reagents at room temperature	20 µl 5× buffer + 10 µl 0.5 M DTT Incubate for 10 min at 25 °C
<i>Step 3:</i> Isolation of nucleic acids	+200 µl chloroform Mix Centrifuge at 12,000× <i>g</i> for 15 min at 4 °C Transfer color-less phase into a fresh tube	<i>Step 3:</i> cDNA synthesis	2 µl Superscript II (200 U/ml) Incubate for 50 min at 42 °C
<i>Step 4:</i> Precipitation of nucleic acids	+500 µl isopropanol Mix Precipitate overnight at −20 °C	<i>Step 4:</i> Enzyme inactivation	Incubate for 5 min at 96 °C
<i>Step 5:</i> Isolation of nucleic acids	Centrifuge at 12,000× <i>g</i> for 10 min at 4 °C	<i>Step 5:</i> Storage	cDNA can be stored at 4 °C for short term and at −20/−80 °C for longer storage
<i>Step 6:</i> Wash	+1 ml 75% ethanol Centrifuge at 7500× <i>g</i> for 10 min at 4 °C		
<i>Step 7:</i> RNA solution	Dry pellet + TE buffer		
<i>Step 8:</i> Inactivation of proteins	10 min at 55–60 °C		
<i>Step 9:</i> RNA concentration	Dilute 1:100 RNA in RNase-free distilled water and measure absorbance at 260 vs. 280 nm		

DNA allows a more sensitive detection of changes in gene expression. Ultimately, prepare random primed cDNA from total RNA (Superscript II RNase H-reverse transcriptase; Life Technologies) (Table 2). Real-time PCR (Real-time PCR detection system CFX96; Biorad) is performed using the Brilliant III Ultra-Fast Quantitative PCR Kit from Agilent

technologies according to manufacturer's instructions. Taqman primer and probe mix are used with this kit and increase PCR specificity. Sequences for probes and primers specific for mouse TATA binding protein (*see Note 10*) and for mouse TSLP mRNA molecules are synthesized by Microsynth (Balgach, Switzerland): TSLP forward primer 5'CCT TCA CTC CCC GAC AAA AC3', TSLP reverse primer 5'GCC TGG GCA GTG GTC ATT3' and TSLP Taqman probe FAM-5'TTG CCC GGA GAA CAA GAG AAG CCC T3'-TAMRA; TATA binding protein forward primer 5'ACTTCGTGCAAGA AATGCTGAA3', TATA binding protein reverse primer 5'TGTCCGTGGCTCTCTTATTCTCA3' and TATA binding protein Taqman probe FAM-5'TCCCAAGCGATTTGC TGCAGTCATC3'-TAMRA. Alternatively, pre-made primer and probe mix are commercially available (Applied Biosystems). RT-PCR is performed with a first cycle at 95 °C for 3 min and then 39 cycles as follows: 5 s at 95 °C, 10 s at 60 °C with the current kit.

10. TATA binding protein is a good housekeeping gene for skin. Alternatively, cyclophilin can be used. These two housekeeping genes are best for the skin. Other housekeeping genes such as 36B4 and beta-2-microglobulin work also satisfyingly.
11. Hexamers are synthesized by Microsynth (Balgach, Switzerland) and prepared as a 100 µM stock solution in RNase-free distilled water. Dissolve hexamers in distilled water, vortex, incubate for 5 min at 55 °C, vortex, further incubate for 3 min at 55 °C, cool down on ice and store at −20 °C.

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