

Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma

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Contributed by Pierre Chambon, December 12, 2008 (sent for review November 27, 2008)

Atopic dermatitis (AD) is often the initial step in the “atopic march,” given that more than half of AD patients with moderate to severe AD develop asthma later in life. Both AD and asthma share a similar “atopy” phenotype that includes T helper type 2 inflammation with eosinophilia and hyper-IgE immunoglobulinemia, but the molecular mechanisms underlying the “atopic march” remain elusive. In the present study, we show that induced expression of thymic stromal lymphopoietin (TSLP) in mouse epidermal keratinocytes upon topical application of MC903 (a low calcemic analogue of vitamin D3) not only triggers AD as we previously reported but also aggravates experimental allergic asthma induced by ovalbumin sensitization and challenge. Our study, which provides a mouse model to study human “atopic march,” indicates that keratinocyte-produced TSLP may represent an important factor in the link of atopic dermatitis to asthma.

atopic dermatitis | atopic march | mouse model | TSLP | vitamin D3

Atopic dermatitis (AD) is a common skin disease that is often associated with other atopic disorders, such as asthma and allergic rhinitis. AD, characterized by pruritic and eczematoid skin lesions, affects children (10%–20%) and adults (1%–3%) (1, 2). Asthma is a chronic airway inflammatory disease, exhibiting lung allergic inflammation, mucus hypersecretion, and airway hyperactivity (AHR) (3, 4). Epidemiologic analysis shows that AD is often the initial step in the so-called “atopic march” (1, 5–7), given that more than 50% of AD patients with moderate to severe AD develop asthma later in life, and the severity of AD influences the course of respiratory allergy. Both AD and asthma share an “atopy” phenotype that includes a T helper type 2 (Th2) inflammation with eosinophilia and hyper-IgE immunoglobulinemia (5, 6), but the molecular mechanisms underlying the “atopic march” remain unclear.

Recently, thymic stromal lymphopoietin (TSLP) emerged as a likely master regulator of AD inflammation (8–10). Overproduction of TSLP was found in keratinocytes of human AD skin lesions (11). We reported that selective ablation of retinoid X receptors (RXRs) in epidermal keratinocytes in adult mice induces TSLP expression in epidermis and triggers an AD-like syndrome (12). Our study (12) and a study by Yoo *et al.* (13) demonstrated that mice overexpressing TSLP in keratinocytes developed a similar AD-like dermatitis. More recently, we showed that topical application of MC903 (calcipotriol; a low-calcemic analogue of vitamin D3) (14) triggers an AD-like syndrome through the induction of keratinocytic TSLP expression (15, 16). On the basis of these results, which indicate that TSLP is both sufficient and necessary in triggering an AD inflammation, and the fact that asthma is often associated with AD, we speculated that the occurrence of asthma may be increased in mice exhibiting TSLP-induced AD.

To investigate this possibility, mice topically treated with MC903 (16) were subjected to an ovalbumin (OVA)-induced asthma protocol, in which OVA sensitization and intranasal challenge

induce characteristic features of allergic asthma (17). We show here that MC903 topical treatment triggers an aggravation of OVA allergic asthma phenotype, which is mediated by MC903-induced TSLP in epidermal keratinocytes. Interestingly, the induced expression of epidermal TSLP during the OVA sensitization period was sufficient to drive an aggravation of the asthma that was generated upon OVA challenge at a time when the skin AD had resolved and no skin and blood TSLP could be detected. Our results indicate that epidermis-produced TSLP could be instrumental in the onset of the “atopic march.”

Results

Mice Bearing MC903-Induced AD Develop Aggravated Experimental Asthma. Wild-type BALB/c mice, topically treated with MC903 to induce AD-like dermatitis, were concomitantly subjected to an OVA experimental asthma protocol (Fig. 1A). Upon MC903 application, both OVA- and alum (aluminum hydroxide, as control)-treated mice similarly exhibited an induction of TSLP expression in keratinocytes (16) together with a subsequent increase of serum TSLP (Fig. 1B) and developed an AD-like skin inflammation (16) (Fig. 1C and data not shown). In contrast, ethanol (EtOH) application (as a vehicle control for MC903) induced neither TSLP expression nor an AD phenotype (16) (Fig. 1B and C and data not shown).

To assess lung allergic inflammation, bronchoalveolar lavage (BAL) fluid was analyzed at Day (D)22. An influx of inflammatory cells was seen in BAL of OVA+EtOH but not of alum+EtOH mice (Fig. 1D). Interestingly, upon MC903 application, the total BAL cell number, particularly that of eosinophils, was further increased in OVA mice but not in alum mice (Fig. 1D; compare OVA+MC903 with OVA+EtOH). Because a low amount of OVA (5 μ g) was used for allergen challenge, OVA+EtOH lungs showed only mild peribronchiolar and perivascular inflammatory cell infiltration (Fig. 1E), which was markedly increased in OVA+MC903 lungs (Fig. 1E and data not shown), consisting mostly of eosinophils [as shown in Fig. 1E (*Insets*) and confirmed by Congo red staining (Fig. 1F)] and CD3⁺ T lymphocytes, mainly CD4⁺ helper T cells (Fig. 1G). Periodic acid-Schiff (PAS) staining revealed enhanced hyperplasia of mucus-secreting goblet cells (PAS⁺) in medium- and large-sized bronchioles of OVA+MC903 lungs when compared with OVA+EtOH lungs (Fig. 1H), whereas very few PAS⁺ cells

Author contributions: Z.Z., M.L., and P.C. designed research; Z.Z. and P.H. performed research; Z.Z., N.F., M.L., and P.C. analyzed data; S.K. and D.M. contributed new reagents/analytic tools; and M.L. and P.C. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812668106/DCSupplemental.

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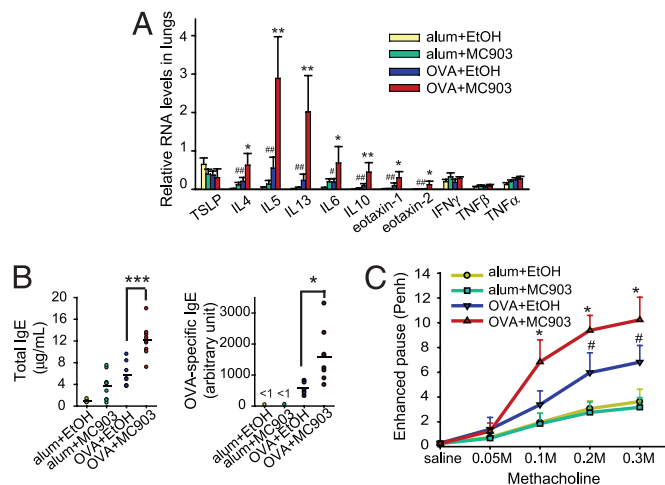


Fig. 2. Ear topical MC903 treatment aggravates Th2 inflammation and AHR in OVA-induced asthma. (A) Quantitative RT-PCR analyses of cytokines and chemokines in lungs at D22. $^{*}P < 0.05$, $^{**}P < 0.01$, OVA+EtOH vs. alum+EtOH; $^{*}P < 0.05$, $^{**}P < 0.01$, OVA+MC903 vs. OVA+EtOH ($n = 6$ mice per group). (B) Serum total IgE and OVA-specific IgE at D22. $^{*}P < 0.05$, $^{***}P < 0.001$ ($n \geq 6$ mice per group). (C) AHR evaluated by plethysmography after exposure to increasing dose of methacholine. $^{*}P < 0.05$, OVA+EtOH vs. alum+EtOH; $^{*}P < 0.05$, OVA+MC903 vs. OVA+EtOH ($n = 6$ mice per group). Error bars indicate SD.

ocytes, and the subsequent elevated TSLP protein in blood, did not lead, on their own, to any spontaneous lung inflammation or enhanced AHR in nonsensitized and nonchallenged mice (Figs. 1 and 2; compare alum+MC903 and alum+EtOH). Also note that, in the absence of OVA sensitization, mice topically treated with EtOH or MC903 on the ear did not develop any asthmatic phenotype upon OVA intranasal challenge (data not shown).

MC903-Induced Aggravation of OVA Asthma Is Mediated by TSLP Produced in Keratinocytes. Vitamin D3 has pleiotropic actions (21) and can act directly on naive CD4 $^{+}$ T cells to enhance the development of Th2 cells (22) and on antigen-presenting cells (e.g.,

macrophages and dendritic cells) (21). To investigate whether MC903-triggered aggravation of OVA-induced allergic asthma is mediated by TSLP produced in keratinocytes (16), TSLP $^{ep-/-}$ mutant mice (TSLP-MT), in which TSLP is selectively ablated in keratinocytes at adult stage (15, 23), were subjected to the same experimental protocol (Fig. 1A). Upon MC903 treatment, (i) TSLP-MT mice did not develop AD-like dermatitis, (ii) there was no induction of TSLP expression (15) (Fig. 3A), and (iii) these mice did not exhibit aggravated asthma upon OVA treatment. MC903 induced an increase of BAL total cell and eosinophil numbers in OVA-treated wild-type control (CT) mice but not in OVA-treated TSLP-MT mice (Fig. 3B), and TSLP-MT mouse lungs failed to exhibit an aggravation of inflammatory cell infiltration (Fig. 3C) and goblet cell hyperplasia (Fig. 3D). Moreover, MC903-induced enhancement of lung Th2 cytokine and chemokine expression in OVA-treated CT mice (Fig. 3E), as well as that of total IgE and OVA-specific IgE (Fig. 3F), were all abrogated in OVA-treated TSLP-MT mice. We could not assess AHR in CT and TSLP-MT mice, because irrespective of their treatment they did not exhibit AHR in response to methacholine (data not shown), which was most probably because of the mixed genetic background ($\approx 85\%$ C57BL/6 + 15% 129/sv) (24). Indeed, upon OVA treatment, C57BL/6 mice did not exhibit AHR to methacholine challenge (data not shown), even though other asthma-like features were aggravated upon MC903 ear topical application, including increased BAL counts, inflammatory infiltration in the lung, elevated expression of Th2 cytokines and eosinophil-attractant chemokines [Fig. S1 A–C], and increased production of total IgE and OVA-specific IgE (Fig. S1 D–E), although their levels were lower as compared with BALB/c mice (Fig. 2B).

Thus, TSLP produced by keratinocytes is necessary for the aggravation of OVA-induced experimental asthma upon skin MC903 treatment. Another line of evidence further supports this conclusion. We previously demonstrated that vitamin D receptor (VDR) present in keratinocytes mediates MC903-induced TSLP expression (16): VDR $^{ep-/-}$ (VDR-MT) mice in which VDR is selectively ablated in epidermal keratinocytes exhibit neither TSLP expression nor an AD-like syndrome upon MC903 treatment (Fig. S2 A and B). Accordingly, and similarly to TSLP-MT mice,

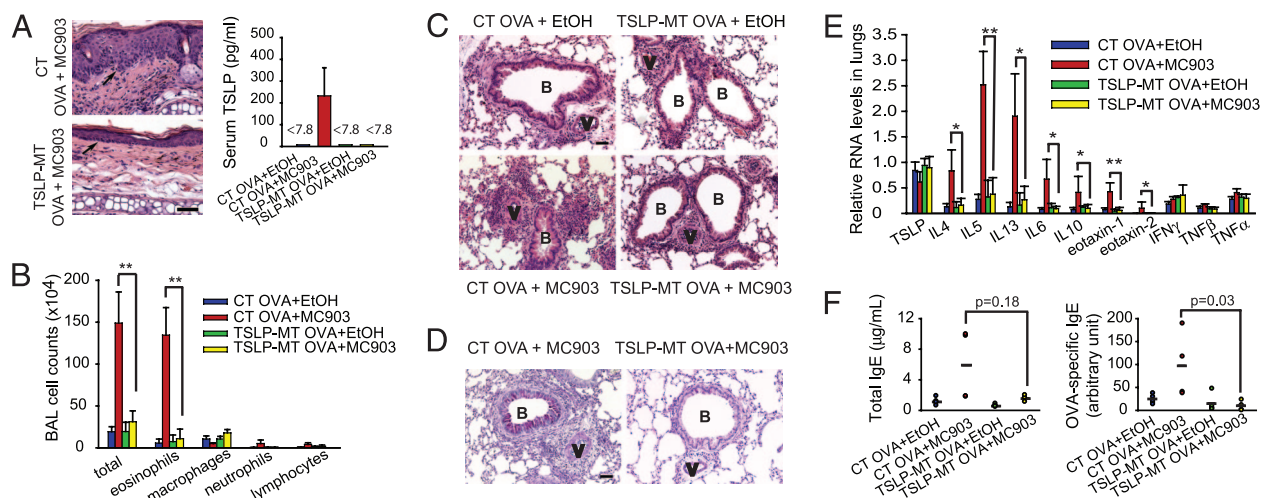


Fig. 3. Selective ablation of TSLP in epidermal keratinocytes suppresses the aggravation of OVA-induced asthma upon ear MC903 topical treatment. TSLP-MT [Tam-injected K14-Cre-ER $^{T2(g0)}$ /TSLP $^{L2/L2}$ mice; mixed genetic background of $\approx 85\%$ C57BL/6 and 15% 129/sv] and wild-type CT [Tam-injected K14-Cre-ER $^{T2(g0)}$ /TSLP $^{L2/L2}$ littermate mice] were subjected to the experimental protocol described in Fig. 1A. (A) OVA-treated TSLP-MT mice do not develop an ear AD-like dermatitis, and their serum TSLP at D22 is not increased upon MC903 application ($n = 6$ mice in each group). Ear sections were stained by H&E. Arrows point to dermal/epidermal junction. (Scale bar, 50 μ m.) (B) Cell counts in BAL fluid. $^{**}P < 0.01$ ($n \geq 4$ mice per group). (C) H&E and (D) PAS stainings of lung paraffin sections. (Scale bars, 50 μ m.) B, bronchiole; V, blood vessel. (E) Relative RNA levels of cytokines and chemokines in lungs at D22. $^{*}P < 0.05$, $^{**}P < 0.01$ ($n \geq 4$ mice per group). (F) Serum levels of total IgE and OVA-specific IgE at D22 ($n \geq 4$ mice per group). Error bars indicate SD.

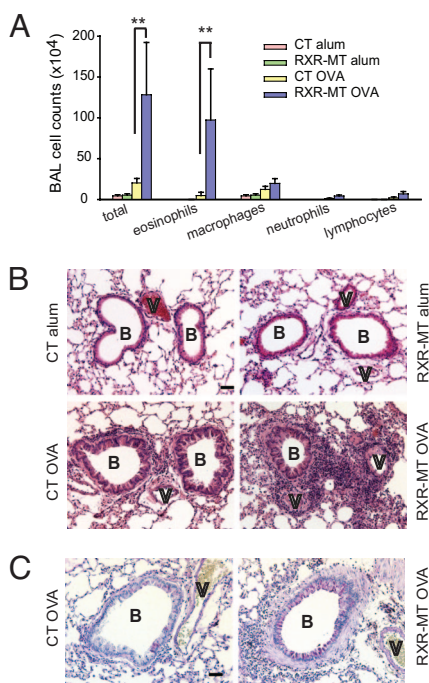


Fig. 4. Aggravated airway inflammation in OVA-sensitized and -challenged RXR α ^{esp-/-} mice. RXR-MT [Tam-injected K14-Cre^{T2(tg0)}/RXR α ^{L2/L2}/RXR β ^{L2/L2} mice; mixed genetic background of $\approx$ 50% C57BL/6 and 50% 129/sv] and wild-type CT [Tam-injected K14-Cre^{T2(0/0)}/RXR α ^{L2/L2}/RXR β ^{L2/L2} [littermate mice] were treated with OVA or alum as described in Fig. 1A, but without topical application of MC903 or EtOH. (A) Cell counts in BAL fluid. ***P* < 0.01 (*n* = 5 mice per group). Error bars indicate SD. (B) H&E staining and (C) PAS staining of lung paraffin sections. B, bronchiole; V, blood vessel. (Scale bars, 50 μ m.)

VDR-MT mice did not exhibit an aggravation of the asthmatic phenotype upon MC903 treatment (Fig. S2 C–G).

Mice with Selective Ablation of RXRs α and β in Keratinocytes Develop Aggravated Airway Inflammation upon OVA Treatment. We previously reported that selective ablation of both RXRs α and β in adult

mouse keratinocytes (RXR α sup^{-/-}, hereafter called RXR-MT) induced the expression of TSLP in keratinocytes and generated a spontaneous AD-like syndrome (12). Upon OVA sensitization and challenge, RXR-MT mice developed a more severe lung inflammation, showing an increase of BAL cell counts, particularly in eosinophils (Fig. 4), inflammatory infiltration in the lung (Fig. 4B), hyperplasia of mucus-secreting goblet cells (Fig. 4C), expression of Th2 cytokines and eosinophil-attractant chemokines in lungs, and production of total IgE and OVA-specific IgE (Fig. S3A and B). In contrast, alum-treated RXR-MT mice did not exhibit obvious lung inflammation (Fig. 4A and B and data not shown). These results further support that increased production of TSLP in keratinocytes, which is instrumental in triggering an AD, also aggravates an OVA-induced allergic asthma.

Increased Production of Epidermal TSLP During the OVA Sensitization Phase Aggravates OVA-Induced Allergic Asthma.

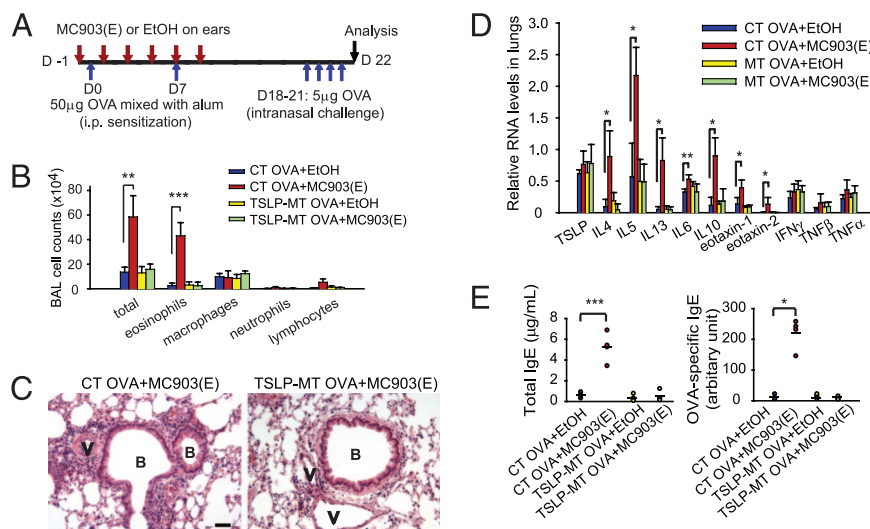


Fig. 5. Epidermal production of TSLP during sensitization phase of OVA treatment aggravates airway allergic inflammation. (A) Experimental protocol. OVA sensitization and challenge in wild-type CT or TSLP-MT mice were performed as described in Fig. 1A. MC903 was topically applied on both ears (1.125 nmol per ear) every other day at an early phase (during sensitization) from D - 1 to D9 [MC903(E)]. (B) Cell counts in BAL fluid. * $P < 0.01$, *** $P < 0.001$ ($n = 5$ mice per group). (C) H&E staining of lung paraffin sections. B, bronchiole; V, blood vessel. (Scale bar, 50 μm .) (D) Quantitative RT-PCR analyses of cytokines and chemokines in lungs at D22. * $P < 0.05$, ** $P < 0.01$ ($n \geq 4$ mice per group). (E) Serum levels of total and OVA-specific IgE at D22. * $P < 0.05$, *** $P < 0.001$ ($n \geq 4$ mice per group). Error bars indicate SD.

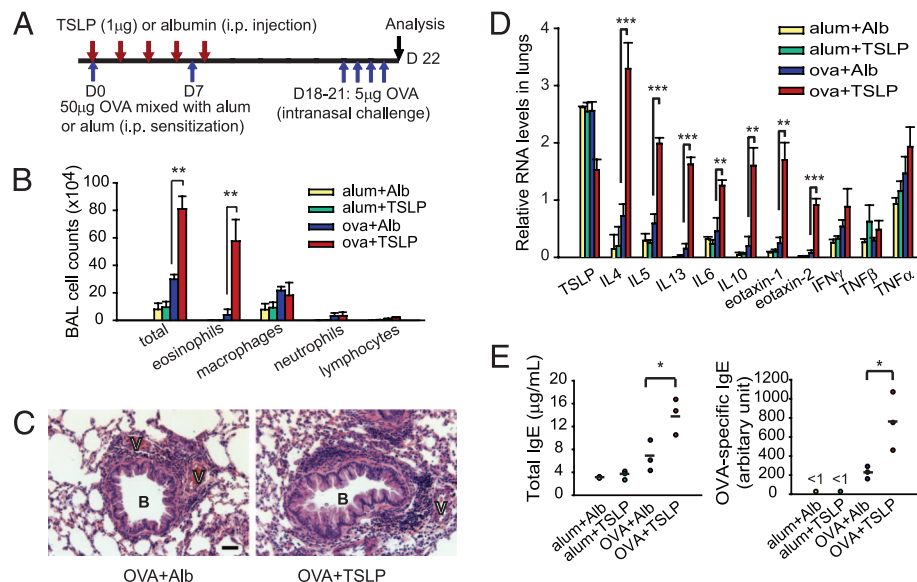


Fig. 6. Exogenous TSLP during sensitization phase of OVA treatment aggravates airway allergic inflammation. (A) Experimental protocol. OVA sensitization and challenge in wild-type BALB/c mice were performed as described in Fig. 1A. Recombinant mouse TSLP [1μ g mixed with 10μ g albumin (Alb)] or Alb (10μ g, as control) was i.p. injected every other day during the sensitization phase from D0 to D8. (B) Cell counts in BAL fluid. (C) H&E staining of lung paraffin sections. B, bronchiole; V, blood vessel. (Scale bar, 50μ m.) (D) Quantitative RT-PCR analyses of cytokines and chemokines in lungs at D22. (E) Serum levels of total and OVA-specific IgE at D22. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Error bars indicate SD. Results are representative of 2 independent experiments.

ment, compared with OVA treatment alone (Fig. S4). Because OVA+MC903(E)-treated TSLP-MT mice failed to exhibit an aggravated OVA-induced lung inflammation [Fig. 5B–E; compare TSLP-MT OVA+MC903(E) with CT OVA+MC903(E)], we concluded that increased epidermal TSLP present during the allergen sensitization phase can trigger an aggravation of the asthmatic phenotype that is generated upon allergen challenge in the airway.

We next asked whether MC903-induced AD before the OVA sensitization phase would also affect OVA-induced asthma. Wild-type BALB/c mice were topically treated with MC903 every other day for 12 days to allow the development of an ear AD, and serum TSLP level reached 1030 ± 370 pg/mL at D13. However, 16 days after the last MC903 treatment (D28), serum TSLP could not be detected any longer, and the dermatitis had almost disappeared (data not shown). These mice were then subjected to OVA i.p. sensitization (at D28 and D35) and intranasal challenge (at D46–D49). We found that the OVA-induced asthma did not worsen (data not shown). Thus, even though there had been a history of AD with induced epidermal TSLP, these mice did not exhibit an aggravation of lung allergic inflammation to a specific allergen (i.e., OVA), when allergen sensitization took place after AD had resolved and TSLP was not produced any longer.

Discussion

We have shown here that increased expression of TSLP in skin keratinocytes, either upon MC903 topical treatment or by ablation of RXR α and RXR β in adult mouse keratinocytes, not only locally triggers an AD but also leads to an aggravation of a concomitant OVA-induced asthma-like lung inflammation. Clearly, keratinocytic TSLP may act as an important player in the link of AD to asthma. It is noteworthy that, unlike transgenic mice overexpressing TSLP under the lung-specific SPC promoter, which develop a spontaneous lung inflammation (19), the increased epidermal TSLP expression and subsequent increase in blood circulating TSLP in our present mouse model do not lead to any spontaneous lung inflammation in the absence of OVA sensitization and challenge (Figs. 1 and 2; compare alum+MC903 with alum+EtOH). However, our data do not exclude that lung locally produced TSLP could be important for the development or/and maintenance of

asthma, given that an increase of TSLP RNA was found in the bronchial epithelium and submucosa of asthmatic patients (25). In this respect, we note that TSLPR $^{-/-}$ (TSLP receptor null) mice (18, 19) and TSLP $^{-/-}$ null mice (our unpublished data) fail to develop experimental asthma in response to OVA treatment, also suggesting that TSLP signaling could play an important role in allergic lung inflammation. However, it remains to be determined in which cells and at which stage TSLP and TSLPR are involved. In any event, the present results clearly show that (i) in this OVA mouse model, epidermal TSLP is dispensable for the generation of an allergic asthma, because the OVA-induced airway inflammation is similarly developed in TSLP $^{ep-/-}$ (selective ablation of TSLP in epidermal keratinocytes) and wild-type CT mice [see Fig. 3 (compare CT OVA+EtOH and TSLP-MT OVA+EtOH), and data not shown]; (ii) although, on its own, increased expression of TSLP in keratinocytes is not sufficient in driving an asthma-like lung inflammation, it can lead to aggravation of an asthma phenotype induced by allergen sensitization and challenge.

We also found that the selective production of epidermal TSLP during the allergen sensitization phase is able to aggravate an airway allergic inflammation, even though AD has resolved and the TSLP level has returned to normal at the time of allergen challenging (Fig. 5). Importantly, it seems that the production of epidermal TSLP enhanced the systemic immune response to the sensitizing allergen [as indicated by elevated OVA-specific IgE levels (Fig. 5E)]. Moreover, in another BALB/c mouse experiment, in which the interval between OVA sensitization and challenge was increased from 10 days (as shown in Fig. 5A) to 50 days, we still observed an aggravation of airway inflammation and increased AHR upon overproduction of epidermal TSLP during the OVA sensitization period (data not shown), thus suggesting that TSLP can induce enhanced memory to the sensitizing allergen.

Further studies are required to uncover the cellular and molecular mechanisms underlying the effects of keratinocyte-produced TSLP on allergic asthma pathogenesis. Because increased expression of TSLP in epidermal keratinocytes is accompanied by circulating TSLP in the blood, the aggravating effect of TSLP on asthmatic lung inflammation could be mediated (i) through TSLP–TSLPR activation of immune cells that may be involved in allergic

asthma pathogenesis (26, 27), including dendritic cells (10), T cells (28–30), natural killer T cells (27, 31), eosinophils (32), B cells (3, 33, 34), and mast cells (35), located either in lungs or in other tissues; or (ii) through other TSLP-induced factor(s) produced locally in the skin and/or systemically in AD mice. To examine the possible involvement of systemic TSLP, BALB/c mice were i.p. injected with recombinant TSLP protein every other day from D0 to D7 during OVA sensitization (Fig. 6A), which led to a slight increase of serum TSLP level [10–20 pg/mL at D1 and D5, and undetected (<7.8 pg/mL) after D10], although no skin AD inflammation could be observed. After OVA intranasal challenge (D18 to D21), these mice developed enhanced airway inflammation, exhibiting an increase of BAL cell counts, lung inflammatory infiltrate, and total and OVA-specific IgE and lung Th2 cytokine expression (Fig. 6B–E), thus indicating that systemic TSLP, on its own, is sufficient to trigger the aggravation of OVA-induced asthma.

It is worth mentioning that in the present model, TSLP was induced in mouse ear skin, whereas the OVA sensitization of the experimental asthma was via i.p. injection. Because, in real life, skin is recognized as one of the initiation sites for allergen sensitizing (2), and there is evidence for a role of epicutaneous sensitization in the atopic march (36, 37), locally increased production of TSLP in AD skin (11) could in fact enhance epicutaneous sensitization to allergens, thereby aggravating the risk to develop asthma and/or its severity, when later in life the same allergens are encountered in airways. This possibility is currently under investigation.

In conclusion, our present study not only provides a mouse model for studying possible mechanisms involved in the “atopic march” but also suggests that overproduction of TSLP in the skin of patients with AD could be a risk factor for the development of allergic airway inflammation. It needs to be studied clinically whether the level of TSLP in lesioned skin and/or in the serum of AD patients is predictive of subsequent occurrence and severity of asthma in these patients, and whether targeting skin TSLP expression and/or

activity in AD patients could potentially help to reduce the occurrence and/or severity of allergic airway disease.

Methods

Mice. Wild-type BALB/c and C57BL/6 male mice (8–10 weeks old) were purchased from Charles River Laboratories. Six-week-old male K14-Cre-ER^{T2}(tg0)/TSLP^{L2/L2} mice (in a mixed genetic background of ≈85% C57BL/6 and 15% 129/sv) (15) and K14-Cre-ER^{T2}(tg0)/RXR^αL2/L2/RXR^βL2/L2 mice (12) (in a mixed genetic background of ≈50% C57BL/6 and 50% 129/sv) were i.p. injected with tamoxifen (Tam, 0.1 mg for 5 days) (23), thus yielding TSLP^{ep}−/− (TSLP-MT) and RXR^{αβ}ep−/− (RXR-MT) mice, respectively. Tam-injected age- and sex-matched littermates (K14-Cre-ER^{T2}(0/0)/TSLP^{L2/L2} or K14-Cre-ER^{T2}(0/0)/RXR^αL2/L2/RXR^βL2/L2 mice) were used as wild-type CT mice. All mice were subjected to experiments 4 weeks after the first Tam injection. Ten-week-old male K14-Cre^(tg0)/VDR^{L2/L2} (in a mixed genetic background of ≈60% C57BL/6 and 40% 129/sv) were used as VDR^{ep}−/− (VDR-MT) mice (16) and their age- and sex-matched K14-Cre^(0/0)/VDR^{L2/L2} littermates as CT mice. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

Other Methods. MC903 topical application, OVA sensitization and challenge, TSLP administration, assessment of airway reactivity, analysis of BAL cells, histology, immunohistochemistry, serum IgE and TSLP determination, RNA analysis, and statistical analysis are described in the *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank the staff of the mouse knockout, histopathology, and animal facilities of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) and Institut Clinique de la Souris (ICS) for their kind help; A. Guimond (ICS) and M. Neves (Faculté de Pharmacie, Université Louis Pasteur-Strasbourg) for initial plethysmography analyses; H. Jiang (IGBMC) for recent experiments; Drs. N. Rochel-Guiberteau (IGBMC) and D. Moras (IGBMC) for vitamin D3 analogue MC903 from LEO Pharmaceutical Products; and Dr. F. Geissmann (London) for a critical reading of the manuscript. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Ministère de l'Enseignement supérieur et de la Recherche, and l'Agence Nationale de la Recherche (No. ANR-07-PHYSIO-002-01). Z.Z. is supported by a predoctoral fellowship from the Association pour la Recherche à l'IGBMC.

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Supporting Information

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SI Materials and Methods

MC903 Topical Application. MC903 (calcipotriol) (1) was dissolved in EtOH and topically applied on mouse ears (1.125 nmol in 25 μ L per ear). As vehicle control, the same volume of EtOH was applied on mouse ears.

OVA Sensitization and Challenge. Mice belonging to OVA groups were sensitized by i.p. injection of 50 μ g OVA (Sigma) emulsified with 2 mg alum (Aldrich) in 100 μ L saline on D0 and D7, and were then challenged by intranasal instillation of 5 μ g OVA dissolved in 25 μ L saline from D18 to D21. As unsensitized and unchallenged controls (alum groups), mice were injected with alum at D0 and D7 and then treated intranasally with saline from D18 to D21. At D22, airway reactivity was examined before mice were killed for other analyses.

TSLP Administration. Mice were i.p. injected with recombinant mouse TSLP (R&D Systems) [1 μ g mixed with 10 μ g Alb (Sigma)] or Alb alone (10 μ g, as control) every other day during the OVA sensitization phase from D0 to D8.

Assessment of Airway Reactivity. One day after the last intranasal challenge, airway reactivity was measured by noninvasive whole-body plethysmography (Emka Technologies) in response to inhaled methacholine (Sigma), using enhanced pause (Penh) as an index of airway responsiveness (2).

Analysis of BAL Cells. Mouse lungs were lavaged with saline containing 1 mg/mL EDTA. The lavage fluid was centrifuged, and BAL cells were counted with a hemocytometer (Neubauer's chamber; VWR). Differential cell counts were determined using cytopspin preparations stained with Microscopy Hemacolor (Merck).

Histology. After killing, mouse left lungs were inflated with 4% paraformaldehyde, fixed overnight at 4 $^{\circ}$ C, and embedded in paraffin. Sections (5 μ m) were stained with H&E, PAS, or Congo red.

Immunohistochemistry. After fixation in 4% paraformaldehyde, 10- μ m cryosections were blocked in 5% normal goat serum

(Vector Laboratories) and incubated with rat monoclonal anti-CD3 (clone KT3) or rat monoclonal anti-CD4 (clone GK1.5) antibody (BD Biosciences PharMingen). After washing, sections were incubated with CY3-conjugated goat-antirat IgG antibody (Jackson ImmunoResearch) and mounted with Vectashield medium (Vector Laboratories) containing DAPI (Invitrogen).

Serum IgE Determination. Microtiter plates (R&D Systems) were first coated with a monoclonal rat antimouse IgE (clone R35-72; BD Biosciences PharMingen) (for total IgE measurement) or with OVA (for OVA-specific IgE measurement). The coated plates were blocked with PBS-BSA and incubated with serum samples and with a biotinylated rat antimouse IgE (clone R35-118; BD Biosciences PharMingen). ExtrAvidin peroxidase (Sigma) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences PharMingen) were used for detection. Serum total IgE concentration was determined from a standard curve of mouse IgE (clone C38-2; BD Biosciences PharMingen). Serum levels of OVA-specific IgE were related to pooled serum from OVA-sensitized and -challenged BALB/c mice (internal standard) and expressed as arbitrary units.

Serum TSLP Determination. Serum TSLP levels were determined with Quantikine Mouse TSLP Immunoassay (R&D Systems).

RNA Analysis. RNA was reverse-transcribed using random oligonucleotide hexamers and amplified by PCR with a Roche light cycler (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Hypoxanthine phosphoribosyltransferase was used as an internal control. Specific primers used for quantitative RT-PCR are available upon request.

Statistical Analysis. Data are represented as mean $\pm$ SD and were analyzed using SigmaStat (Systat Software) by the Student *t* test or the Mann-Whitney rank sum nonparametric test, depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene median test for equal variance. *P* < 0.05 was considered statistically significant.

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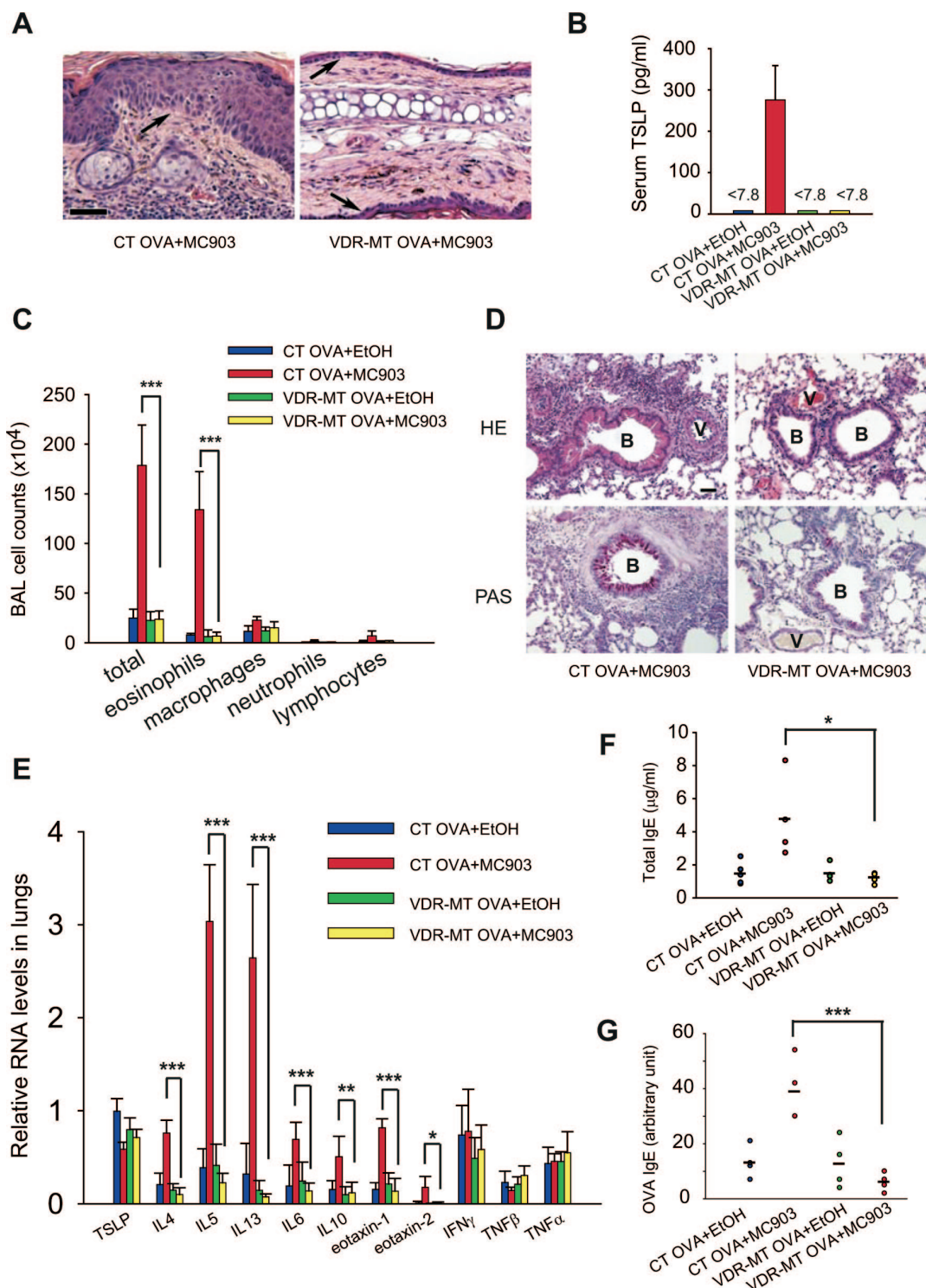


Fig. S2. VDR^{EP-/-} mutant mice do not exhibit aggravation of the OVA-induced asthmatic phenotype upon ear MC903 topical treatment. VDR-MT [K14-Cre^{tg}/0/VDR^{L2/L2} mice; mixed genetic background of ≈60% C57BL/6 and 40% 129/sv] and wild-type CT [VDR^{L2/L2} littermate mice] were subjected to the experimental protocol described in Fig. 1A. (A) H&E staining of ear paraffin sections at D22, showing that OVA/CT application did not trigger the AD-like skin inflammation in OVA-treated VDR-MT mice. Arrows point to dermal/epidermal junction. (Scale bar, 50 μm.) (B) Serum TSLP levels at D22 (*n* = 4 mice per group). (C) Cell counts in the BAL fluid. ****P* < 0.001 (*n* = 4 mice per group). (D) H&E (*Top*) and PAS (*Bottom*) staining of lung paraffin sections. B, bronchiole; V, blood vessel. (Scale bar, 50 μm.) (E) Quantitative RT-PCR analyses of cytokines and chemokines in lungs. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (*n* = 4 mice per group). (F) Serum total IgE and (G) OVA-specific IgE at D22. **P* < 0.05, ****P* < 0.001 (*n* = 4 mice per group). Error bars indicate SD.

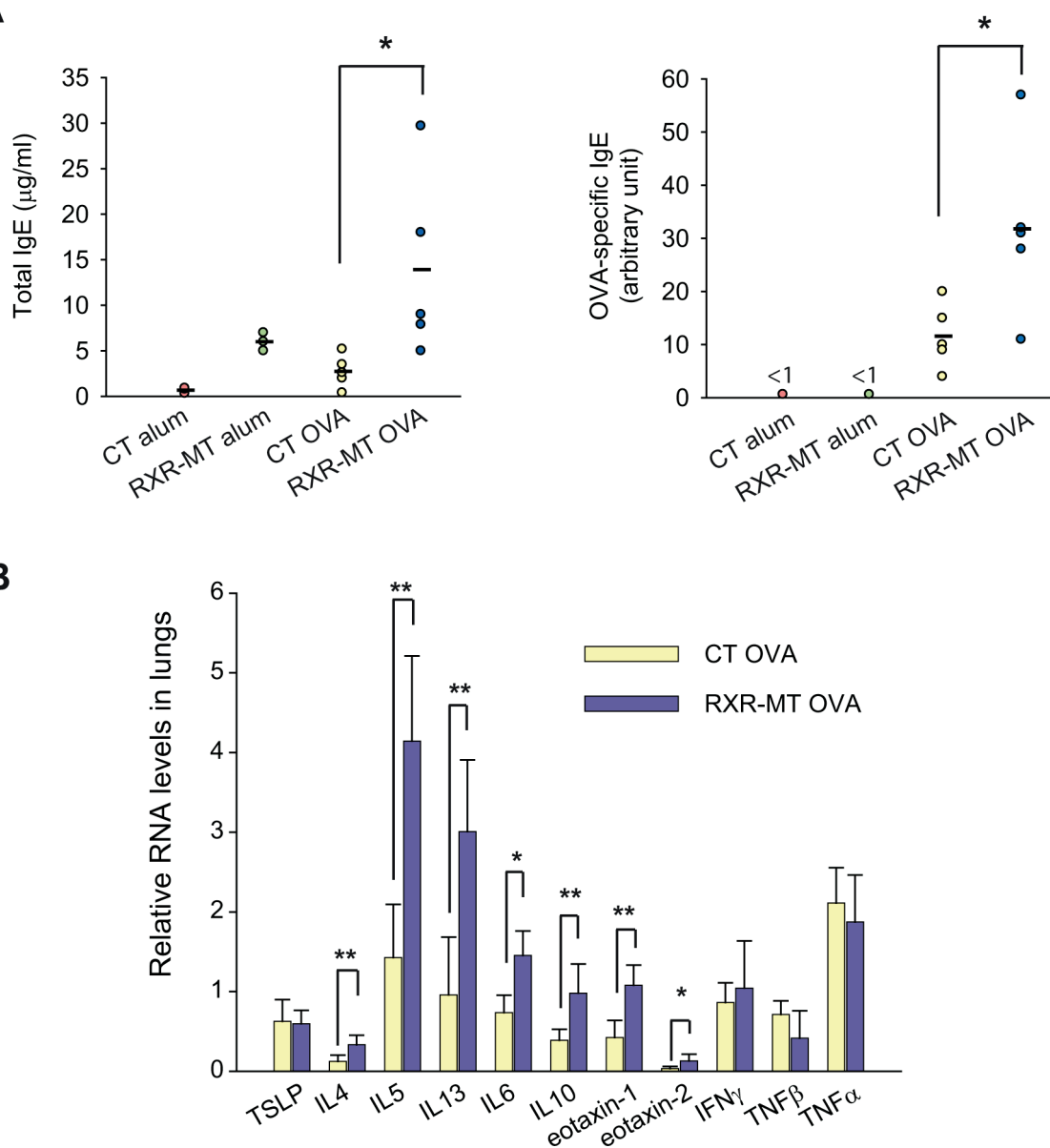


Fig. S3. OVA-sensitized and -challenged RXR $\alpha^{\text{ep-/-}}$ mutant mice develop a more severe Th2 allergic inflammation. RXR-MT [Tam-injected K14-CreER^{T2(gf/0)}/RXR $\alpha^{\text{L2/L2}}$ /RXR $\beta^{\text{L2/L2}}$ mice; mixed genetic background of $\approx 50\%$ C57BL/6 and 50% 129/sv] and wild-type CT [Tam-injected RXR $\alpha^{\text{L2/L2}}$ /RXR $\beta^{\text{L2/L2}}$ littermate mice] were treated with OVA or alum as described in Fig. 1A, but without application of MC903 or EtOH. (A) Serum total and OVA-specific IgE at D22. * $P < 0.05$ ($n = 5$ mice per group). (B) Quantitative RT-PCR analyses of cytokines and chemokines in lungs at D22. * $P < 0.05$, ** $P < 0.01$ ($n = 5$ mice per group). Error bars indicate SD.

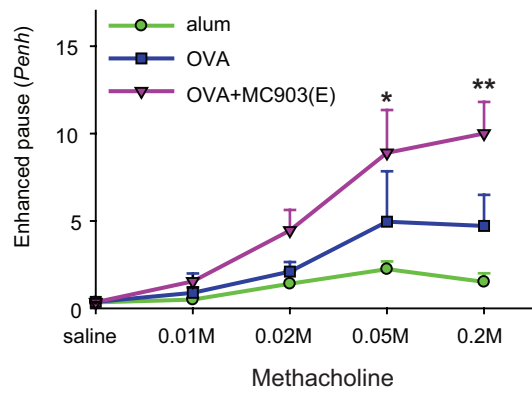


Fig. S4. AHR is enhanced in OVA-sensitized and -challenged BALB/c mice upon MC903 skin topical treatment at an early (E) phase (during sensitization) of the OVA experimental asthma protocol (as described in Fig. 5A). AHR is evaluated by plethysmography after exposure to increasing doses of methacholine. * $P < 0.05$, ** $P < 0.01$, OVA+MC903(E) vs. OVA ($n \geq 6$ mice per group). Error bars indicate SD.