Retinoid X receptor ablation in adult mouse keratinocytes generates an atopic dermatitis triggered by thymic stromal lymphopoietin

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To investigate the role of retinoid X receptors (RXRs) in epidermal homeostasis, we generated RXRαβ±/− somatic mutants in which both RXRα and RXRβ are selectively ablated in epidermal keratinocytes of adult mice. These mice develop a chronic dermatitis mimicking that observed in atopic dermatitis (AD) patients. In addition, they exhibit immunological abnormalities including elevated serum levels of IgE and IgG, associated with blood and tissue eosinophilia, indicating that keratinocyte-selective ablation of RXRs also generates a systemic syndrome similar to that found in AD patients. Furthermore, the profile of increased expression of cytokines and chemokines in skin of keratinocyte-selective RXRβ-ablated mutants was typical of a T helper 2-type inflammation, known to be crucially involved in human AD pathogenesis. Finally, we demonstrate that thymic stromal lymphopoietin, whose expression is rapidly and strongly induced in RXRβ-ablated keratinocytes, plays a key role in initiating the skin and systemic AD-like pathologies.

A topic dermatitis (AD), a chronic skin inflammatory disease with a strong genetic component that affects children (10–20%) and adults (1–3%), is characterized by pruritic and eczematoid skin lesions, associated with systemic immunological abnormalities, including peripheral eosinophilia and hyper IgE immunoglobulinemia (1). Immunological mechanisms have been involved in AD pathogenesis (2), but the possible role of epidermal keratinocytes in AD initiation and maintenance is still largely unexplored (3).

Nuclear receptors (NRs) belong to a superfamily of transcriptional regulators that include ligand-dependent and orphan receptors (4, 5). NRs play critical roles as signal transducers in vertebrate development and homeostasis, including immune functions (4, 6–8). Within the NR superfamily, the retinoid X receptor isotypes (RXR α, β, and γ) play a key role as heterodimeric partners for some 15 NRs, e.g., retinoic acid receptors, 1,25-dihydroxyvitamin D3 receptor (VDR), peroxisome proliferator-activated receptors, and liver X activated receptors (4, 5, 9). Ligand-dependent transcriptional activation by NRs requires the integrity of the core of activation function 2 (AF-2) (the AF-2 domain; see refs. 9 and 10).

In epidermis, RXRα is predominant, RXRβ level is lower, and RXRγ is undetectable (11–13). Keratinocyte-selective ablation of RXRα in adult mouse skin results in a number of abnormalities, including a progressive alopecia, keratinocyte hyperproliferation, and abnormal differentiation, and an inflammatory reaction (11, 14). In contrast, the skin of RXRβ-null mice is apparently normal (11, 14, 15).

To investigate the origin of this inflammatory reaction and to avoid any functional redundancies between keratinocytic RXRα and RXRβ, we have now generated RXRαβ−/− somatic mutants in which both RXRα and RXRβ are selectively ablated in epidermal keratinocytes of adult mice. These mice develop an AD-like chronic dermatitis, as well as a systemic syndrome, which share similarities with those found in AD patients. Thus, our data not only demonstrate that RXRs play a key role in the control of cutaneous inflammation, but also point to an initiating role of keratinocytes in AD. Moreover, we show that an early and strong enhancement of expression of the cytokine TSLP in RXR-ablated keratinocytes plays a crucial role in the generation of the AD-like skin and systemic syndrome.

Materials and Methods

Experimental Animals. RXRαβ±/− mice were obtained through tamoxifen (Tam) administration to K14-Cre-ERT2(tg/0)/RXRαL2/L2/RXRβL2/L2 adult mice, which harbor floxed RXRα and RXRβ L2 alleles and the K14-Cre-ERT2 transgene expressing the Tam-inducible Cre-ERT2 recombinase under the control of the keratin 14 (K14) promoter (11, 16, 17). Two weeks after Tam administration (week 2), recombined RXRα L− and RXRβ L− alleles were detected in skin, as well as in tongue, eyes and salivary glands (Fig. 7, which is published as supporting information on the PNAS web site). In RXRαβ±/− skin, the Cre-mediated conversion to L− alleles was restricted to the epidermis, where its efficiency was >90% (Fig. 7b). Tam-treated K14-Cre-ERT2(fg/0)/RXRαL2/L2/RXRβL2/L2 and K14-Cre-ERT2(tg/0)/RXRαL2/L2/RXRβL2/L2 adult mice did not show any skin abnormalities, and were used as controls (CT). RXRαβL2/L2 and RXRβL2/L2 mouse lines have been described (18, 19). K14-TSLP transgenic mice are described in Supporting Text, which is published as supporting information on the PNAS web site.

Other Materials and Methods. Genotyping, Tam treatment, epidermal preparation, histopathology, immunohistochemistry, hemato logical assays, serum cytokine and immunoglobulin determination, RNA analysis and statistic analysis are all described in Supporting Text.

Results

Selective Ablation of RXRα and β in Epidermal Keratinocytes of Adult Mice Leads to a Spontaneous Dermatitis. RXRαβ±/− mutants exhibited a progressive alopecia that became obvious by 6 weeks after Tam administration (week 6) to K14-Cre-ERT2(tg/0)/RXRαL2/L2/RXRβL2/L2 adult mice (see Materials and Methods and Fig. 7), and developed a spontaneous dermatitis that occurred predominantly on and behind the ears, on the face, in the neck region, and on the back (Fig. 1a−f). At week 2, all mutant ears displayed reddening, swelling, and scaling (Fig. 1b and e), whereas dry and scaly skin with small lesions became macroscopically visible.

Abbreviations: AD, atopic dermatitis; AF-2, activation function 2; NR, nuclear receptor; RXR, retinoid X receptor; VDR, 1,25-dihydroxyvitamin D3 receptor; Tam, tamoxifen; CT, control; DC, dendritic cell; Th, T helper; TSLP, thymic stromal lymphopoietin.

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in the trunk at week 6-week 8 (not shown). The frequent face rubbing and scratching observed in these mutants most probably reflected a skin pruritus. The severity of these abnormalities worsened with age: by week 24, all mutants exhibited swollen and red ears, and 60% had developed ulcerations and crusts in the ear, neck, and back (Fig. 1 c and f). In contrast, RXReα−/− mice developed only minor focal lesions on the dorsal skin at week 20 (11), whereas spontaneous dermatitis never occurred in RXRβ−/− mice (not shown), clearly indicating a partial RXRα/RXRβ functional redundancy.

An Inflammatory Infiltrate in Skin of RXRαβ−/− Mice. At week 2, mutant ear (Fig. 1h) and to a lesser extent dorsal skin biopsies (Fig. 1k) revealed an epidermal hyperplasia, as well as infiltrated cells and dilated blood vessels in the dermis (compare with Fig. 1 g and j). At week 12, mutant ears exhibited a highly hyperplastic epidermis, together with a heavy dermal cell infiltrate (Fig. 1l), which was less dense in mutant dorsal skin (Fig. 1l). Bacterial or fungal infections were ruled out as possible causes of skin inflammation by Gram and periodic acid-schiff reagent staining and electron microscopy (not shown).

Immunohistochemistry staining for T lymphocytes performed on ear skin at week 8 revealed in RXRαβ−/− mutants numerous CD4+ helper T cells, which were more abundant in the dermis than in the epidermis, whereas CT mice showed only few resident dermal CD4+ cells (Fig. 2 a and b and data not shown). No CD8+ T cell infiltrate was observed (not shown). CD11c, a marker for dendritic cells (DC), labeled resident epidermal Langerhans cells (LC) and few dermal DCs in CT (Fig. 2c), whereas in mutants, many DCs were revealed in the dermis, and the number of epidermal LCs was also increased (Fig. 2d). Staining with MHC class II antibody showed a strong increase in MHC II+ cell number in mutant dermis (Fig. 2 e and j). MHC II expression was also detected in mutants, but not in CT keratinocytes, suggesting an “active” state for mutant keratinocytes. In addition, numerous eosinophils were revealed by Luna’s staining (Fig. 2g and h) and electron microscopy (Fig. 2i and j) in mutant, but not in CT dermis. Mast cells were also more numerous in mutant dermis (Fig. 2 k and l), whereas neutrophils were rarely found in skin sections of both CT and mutants at week 8 (data not shown).

Analyses on mutant ear skin at week 2 showed that the appearance of CD4+ T cell and DC infiltrate preceded the increase in eosinophils and mast cells. Furthermore, although less dense than in ear skin, similar cell infiltrates were also observed in mutant dorsal skin, involving CD4+ T cells, DCs, eosinophils, and mast cells (Table 1, which is published as supporting information on the PNAS website), thus evoking an immune cell pathological pattern characteristic of human AD (1, 20).

Preferential Expression of T Helper 2 (Th2)-Type Cytokines and Chemokines in RXRαβ−/− Skin. Because cytokines and chemokines orchestrate and determine the type and outcome of the inflammatory response, their relative RNA transcript level was determined by quantitative RT-PCR in CT and mutant ear skin from week 2 to week 12 (Fig. 3a). At weeks 4 and 12, analyses of cytokines
Th2 cells, (cells, (response and to instruct dendritic cells to preferentially induce a Th2 mutant ear skin at weeks 2–12 (Fig. 3 increased at any time in the mutants, whereas that of IL-1

Li also Fig. 5 mutant at week 4-week 12, but not in CT skin (data not shown, see preferentially produced by Th2 cells (24), was also detected in

Th2-type cytokines (21–23), including IL-5, IL-13, and IL-10, as inflammatory infiltrate), revealed an increase of transcripts of produced by CD4+ helper T cells (predominant in the RXRαβp−/− inflammatory infiltrate), revealed an increase of transcripts of Th2-type cytokines (21–23), including IL-5, IL-13, and IL-10, as well as IL-4, which was present in mutant skin, but could not be detected in CT skin. IL-31, a cytokine recently reported to be preferentially produced by Th2 cells (24), was also detected in mutant at week 4-week 12, but not in CT skin (data not shown, see also Fig. 5h). Transcripts of the Th1-type cytokine IFN-γ and TNF-β (21–23), were either similarly up-regulated at weeks 4 and 12 (IFN-γ) or only weakly at week 12 (TNF-β) (Fig. 3a).

Increased transcript levels of a number of chemokines (25, 26) were also found in mutant ear skin at week 2–12 (Fig. 3a), including (i) CCL17 (TARC) and CCL22 (MDC), chemoattractants for Th2 cells, (ii) CCL8 (MCP2), involved in recruitment of eosinophils and Th2 cells, (iii) CXCL10 (IP10, a chemoattractant for Th1 cells), and (iv) CCL20 (MIP3α, a chemoattractant for immature dendritic cells and Th1 cells). In contrast, RNA levels of chemokines CCL27, CCL5 (RANTES) and CCL11 (eotaxin) were unchanged (data not shown).

The RNA level of the proinflammatory cytokine TNF-α was not increased at any time in the mutants, whereas that of IL-1α was increased at weeks 4 and 12, and that of IL-1β was only increased at week 12 (Fig. 3a). Most interestingly, the transcript level of thymic stromal lymphopoietin (TSLP), a cytokine that was recently shown to be expressed at high levels in keratinocytes of AD patients and to instruct dendritic cells to preferentially induce a Th2 response in vitro (27), was strongly up-regulated (>15-fold) in mutant ear skin at weeks 2–12 (Fig. 3a). In contrast, the RNA level of IL-7, a cytokine functionally related to TSLP (28), was unchanged (not shown).

Early Enhancement of TSLP Expression in RXRαβp−/− Epidermal Keratinocytes. In ear skin (ES) at week 2, TSLP RNA levels were much higher in mutant than in control epidermis (Fig. 3b, ES; very little TSLP RNA was found in the dermis, not shown). Dorsal skin (DS) epidermis also showed an increased expression of TSLP, but to a lesser degree (Fig. 3b). TSLP transcript levels were unchanged in tongue and salivary glands in which ablation of RXRα and RXRβ was also detected (Fig. 7), and in other tissues including thymus, spleen, lymph node, liver, and lung (Fig. 3b, and data not shown).

An antibody against TSLP did not reveal its expression in either epidermis or dermis of CT ear skin at week 2, whereas it was readily detected in basal keratinocytes of mutant ear epidermis, but not in dermis (Fig. 3c), demonstrating that the expression of TSLP protein was strongly induced in RXRαβp−/− shortly after RXRαβ ablating. In addition, TSLP serum levels were strongly increased as early as 1 week after this ablation (386 ± 138 pg/ml in mutant mice vs. <7.8 pg/ml in CT), and remained high (e.g., 449 ± 174 pg/ml at week 8) up to at least week 20 (data not shown).

Systemic Abnormalities in RXRαβp−/− Mice. At week 12, IgE and IgG levels were on average 5- and 4-fold higher in RXRαβp−/− mice than in CT, respectively, whereas IgM and IgA levels were unchanged (Fig. 4a and data not shown). IgG subtype IgG1, but not IgG2a, 2b, and 3, contributed to IgG increase (data not shown). IgE increased earlier than IgG, as a 3-fold increase of IgE was already of IL-7, a cytokine functionally related to TSLP (28), was unchanged (not shown).
seen at week 4 in mutants, when no IgG elevation could be detected (data not shown). Further elevation of IgE and IgG1 levels was observed in RXRαβp−/− mice at week 20 (>20- and 10-fold higher than in CT; respectively, data not shown), suggesting a Th2-like systemic immune reaction (29).

Total white blood cell (WBC) counts at week 12 showed an average of 25,400 and 9,700 cells per µl in RXRαβp−/− mutants and CT, respectively. Eosinophil counts strikingly increased from 1.1% to 14.8% of total WBC, reaching an average of 3,760 cells per µl. Peripheral lymphocytes (19,600 cells per µl) and neutrophils (2,360 cells per µl) were also increased relative to CT (8,150 and 1,430 cells per µl, respectively).

At week 12 the serum concentration of IL-5, a critical factor for eosinophil expansion (30, 31), was much higher in RXRαβp−/− mutants (124 ± 19 pg/ml) than in CT (<16 pg/ml). IL-2, IL-4, IFN-γ, and TNF-α levels were too low to be detected in both CT and mutant sera (data not shown).

RXRαβp−/− mice exhibited hyperplasia of regional lymph nodes (LN), which invariably included cervical LN and spleen (Fig. 4d and data not shown). Mutant cervical LN were 2- and 10-fold enlarged at weeks 2 and 24, respectively. Mutant mice also developed a progressive splenomegaly after weeks 6–8 (Fig. 4b), and the liver of 50% of mutant mice was up to 2-fold enlarged at week 20 (data not shown).

Histological examination at week 12 revealed numerous eosinophils in mutant cervical LN and spleen (Fig. 4c), in which an increased number of plasma cells was also seen in hematoyxin and eosin-stained sections (data not shown). A dense perivenous infiltrate rich in eosinophils, lymphocytes, and neutrophils was present in mutant liver (Fig. 4c). Eosinophil infiltrates were also observed to a lesser extent in mutant lung and heart at a later stage (week 20), whereas thymus examination at week 12 did not reveal any overt abnormality (data not shown).

Mice Lacking RXRα and RXRβ Activation Function 2 (AF-2) in Keratinocytes Develop an Alopoeia But Not the Th2-Like Skin Inflammation and Systemic Abnormalities. To investigate whether the ligand-dependent AF-2s of RXRα and RXRβ are required for the skin inflammation observed in RXRαβp−/− mutants, we crossed RXRαepaf2o and RXRβepaf2o mice (heterozygous for the α2o allele, refs. 18 and 19) with K14-Cre-ERT2(tg/0) mice. Further elevation of IgE and IgG1 levels was seen at week 4 in mutants, when no IgG elevation could be detected (data not shown). Mutant cervical LN were 2- and 10-fold enlarged at weeks 2 and 24, respectively. Mutant mice also developed a progressive splenomegaly after weeks 6–8 (Fig. 4b), and the liver of 50% of mutant mice was up to 2-fold enlarged at week 20 (data not shown).

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Fig. 5. RXRαβp−/− mice do not develop a skin inflammation. (a–c) Appearance of control (CT) (a), RXRαβp−/− mutant (b), and RXRαβp−/− mutant (c) at week 5. Close views of the ears are shown in Insets. Black arrows in b and c point to regions with hair loss, and white arrowhead in b inset points to the red and swollen ear. (d–f) Hematoxylin/eosin staining of ear sections of CT (d), RXRαβp−/− mutant (e), and RXRαβp−/− mutant (f) at week 5. White arrows point to dermal–epidermal junction, hf, hair follicle; u, utricle. (Scale bar, 50 µm.) (g) Comparison of immune cell infiltrate (g) and RNA levels of cytokines and chemokines (h) in ear skin of RXRαβp−/− and RXRαβp−/− mutant mice at week 5. The numbers in g represent the average counting of the corresponding cells from three microscopic fields (objective ×20) of ear sections. *P < 0.05.

Transgenic Mice Overexpressing TSLP in Epidermal Keratinocytes Exhibit Skin and Systemic Abnormalities Similar to Those of RXRαβp−/− Mice. The close association of the early increase in TSLP expression, which was highly increased in RXRαβp−/− keratinocytes, was not enhanced in RXRαβp−/− skin (Fig. 5h). In keeping with these transcript data, the TSLP serum level was also unchanged in RXRαβp−/− mice when compared to controls (data not shown). Moreover, serum levels of IL-5 and IgE were not elevated in RXRαβp−/− mice, and there was no eosinophilia in peripheral blood and in various organs (data not shown), indicating that RXRαβp−/− mice did not develop the systemic AD-like syndrome seen in RXRαβp−/− mice.
later stages (e.g., 7 weeks old) (data not shown). Cytokine and chemokine profiles, similar to those observed in RXRαβp−/− skin, were found in ear skin of 3-week-old K14-TSLP mice, with increased levels of IL-4, IL-5, IL-10, and IL-31 transcripts, as well as IFN-γ and chemokines CCL17, CCL22, CXCL10, CCL8, and CCL20 transcripts, whereas there was little change in TNF-α, IL-1α, and IL-1β transcripts (Fig. 6).

Moreover, K14-TSLP mice developed the systemic syndrome previously seen in RXRαβp−/− mice, i.e., elevated serum levels of IgE, IgG, and IL-5, and increased number of lymphocytes and eosinophils in the blood of 5-week-old mice (Fig. 8, which is published as supporting information on the PNAS web site). K14-TSLP mice also exhibited hyperplasia of lymph nodes, spleen, and liver, with numerous eosinophils present in these tissues (Fig. 8).

**Discussion**

We have shown here that RXRαβp−/− mice develop a skin and systemic syndrome similar to that of human AD. Several lines of evidence strongly support the conclusion that the chronic dermatitis developed by RXRαβp−/− mice is very close to that of AD patients. First, these mice display the major clinical features of patients suffering from AD (32–34), including eczematosus-like lesions, xerosis, and pruritus. Second, in these mice, the skin inflammatory cell infiltrate is mostly composed of CD4+ T and dendritic cells, associated with eosinophils and mast cells, which together are characteristic of skin lesions of AD patients (1, 20).

Third, the profile of increased expression of cytokines and chemokines found in mutant inflammatory skin is typical of a Th2-type inflammation, known to be crucially involved in human AD pathogenesis (1, 24): IL-4, IL-5, IL-13, IL-10, and IL-31 are known to be Th2-type cytokines, whereas CCL17, CCL22 and CCL8 are known to preferentially chemottract Th2 cells. Importantly, the expression of TSLP, a recently identified cytokine that was shown to be highly expressed in keratinocytes of AD patients (27), is dramatically increased in keratinocytes of mutant mice. As in chronic human AD skin lesions (35), a delayed minor component of Th1-type inflammation is also found, as indicated by an increase in Th1-type cytokines (IFN-γ and TNF-β) and chemokines (CCL10 and CCL20). Finally, most AD patients exhibit a systemic Th2-like immune syndrome similar to that found in RXRαβp−/− mice, including increased serum levels of IgE and blood eosinophilia, possibly because of IL-4 and IL-13 (switching B lymphocytes into IgE production; ref. 36) and IL-5 (promoting the growth and activation of eosinophils; ref. 37) increases.

Interestingly, our data indicate that RXRs are differentially required in keratinocytes for maintenance of the hair cycle and prevention of the occurrence of an AD-like skin and systemic syndrome. We previously reported that keratinocyte-selective ablation of RXRα is sufficient on its own to interrupt the hair cycle and to generate an alopecia, whose severity was increased by further RXRβ ablation (11, 14). Because this alopecia was similar to that observed in VDR-null (11) and VDRp−/− (our unpublished results) mutants, RXR/VDR heterodimers are most probably instrumental to hair cycle maintenance (11). Here, we show clearly that, in addition, RXR AF-2 must be functional in this heterodimer, because the keratinocyte-selective inactivation of AF-2 in RXRα and RXRβ (RXRαβpaf2−/− mutant) results in a similar alopecia.

Furthermore, our finding of a Th1-like inflammatory reaction in RXRαβpaf2−/− skin with increased expression of IFN-γ and IFN-γ-induced chemokines (CCL10 and CCL20) (38, 39), suggests that the occurrence of this alopecia could be related to an increase in IFN-γ signaling (40). In this respect, we note that there is a similar Th1-like inflammatory reaction in VDRp−/− skin (our unpublished data).

On the other hand, AF-2 is clearly dispensable in the process by which RXRα and β prevent the appearance of a Th2-like inflammatory reaction with increased expression of TSLP and Th2-type cytokines and chemokines, which strongly suggests that these cytokines and chemokines, as well as TSLP, are involved in the generation of the Th2-type AD-like skin and systemic syndrome. The identity of the heterodimeric partner(s) of RXRα and RXRβ is currently unknown, as germ-line or keratinocyte-selective ablation of retinoic acid receptors α, β, or γ, VDR, peroxisome proliferator-activated receptors α, β, or γ, liver X receptors α or β, and thyroid hormone receptors α or β did not result in the AD-like syndromes exhibited by RXRαβp−/− mutants or increased expression of Th2-type cytokines and TSLP in their skin (data not shown).
Other RXR NR partners might be involved, and/or these NRs could be functionally redundant.

Several lines of evidence strongly support the conclusion that TSLP plays a key role in the chain of events that generate the Th2-type skin inflammation and atopic syndrome. First, among the various cytokines aberrantly expressed in RXRαββΔ20 mutants, TSLP is the earliest to be strongly overproduced in basal keratinocytes (Fig. 3), and moreover, its up-regulation is tightly correlated with the occurrence of the atopic skin and systemic syndrome (TSLP is overproduced in RXRαββΔ20/−/− mice, but not in RXRαββΔ20 mice). Furthermore, the same functional redundancy exists between RXRα and RXRβ for the presence of increased levels of TSLP transcripts in keratinocytes and TSLP proteins in serum (Fig. 9, which is published as supporting information on the PNAS web site) and for the occurrence of the AD-like skin and systemic syndrome. Finally, and most importantly, expressing TSLP in epidermal keratinocytes of transgenic mice generates AD-like systemic syndrome. Finally, and most importantly, expressing TSLP in epidermal keratinocytes of transgenic mice generates AD-like systemic syndrome. Moreover, these transgenic mice have shown that human TSLP activates dendritic cells, which can induce differentiation of allogenic proallergic Th2 cells (41) as well as homeostatic proliferation of autologous CD4+ T cells (42). Whether similar mechanisms mediate the effect of TSLP overproduced in murine and human keratinocytes to trigger the AD skin and systemic immune reactions remains to be investigated.

It remains also to be seen how the overproduction of TSLP is triggered in human skin of AD patients and to what extent a dysregulation of NR pathways involving TSLP could be implicated in the pathogenesis of AD. That TSLP expression in RXRαββΔ20/−/− mice, but not in RXRαββΔ20 mice, and moreover, its up-regulation is tightly correlated with the occurrence of the atopic skin and systemic syndrome (TSLP is overproduced in RXRαββΔ20/−/− mice, but not in RXRαββΔ20 mice). Furthermore, the same functional redundancy exists between RXRα and RXRβ for the presence of increased levels of TSLP transcripts in keratinocytes and TSLP proteins in serum (Fig. 9, which is published as supporting information on the PNAS web site) and for the occurrence of the AD-like skin and systemic syndrome. Finally, and most importantly, expressing TSLP in epidermal keratinocytes of transgenic mice generates AD-like systemic syndrome. Finally, and most importantly, expressing TSLP in epidermal keratinocytes of transgenic mice generates AD-like systemic syndrome. Moreover, these transgenic mice have shown that human TSLP activates dendritic cells, which can induce differentiation of allogenic proallergic Th2 cells (41) as well as homeostatic proliferation of autologous CD4+ T cells (42). Whether similar mechanisms mediate the effect of TSLP overproduced in murine and human keratinocytes to trigger the AD skin and systemic immune reactions remains to be investigated.

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