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IL-4 Regulates Skin Homeostasis and the Predisposition toward Allergic Skin Inflammation

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IL-4 promotes the development of Th2 cells and allergic inflammation. In atopic dermatitis lesions, IL-4 decreases the expression of multiple genes associated with innate defense, including genes in the epidermal differentiation complex (EDC) that regulate epidermal barrier function. However, it is not clear whether IL-4 also contributes to homeostatic control of EDC genes. In this report, we demonstrate that expression of EDC genes and barrier function is increased in the absence of endogenous IL-4. Mice that express a constitutively active Stat6 (Stat6VT) are prone to the development of allergic skin inflammation and have decreased expression of EDC genes. IL-4 deficiency protects Stat6VT transgenic mice from the development of allergic skin inflammation and decreased recovery time in barrier function following skin irritation, with a concomitant increase in EDC gene expression. These data suggest that IL-4 plays an important role in regulating epidermal homeostasis and innate barrier function. *The Journal of Immunology*, 2010, 184: 3186–3190.

T he development of allergic responses relies upon the coordinated production of cytokines to promote effector T cell development and the recruitment of inflammatory cells to the target organ. In the development of allergic inflammation, the current paradigm is that IL-4 promotes the development of Th2 cells, whereas IL-13 is critical in promoting tissue inflammation. This model predicts that these cytokines would only be present and have effects on the target organ during the occurrence of disease. However, it is possible that nominal amounts of cytokines in vivo have an effect on tissues even in the absence of pathology.

Allergic inflammation in the skin results in atopic dermatitis (AD), a chronic disease characterized by intense pruritis, dryness of the skin, and erythema in localized lesions (1–3). AD lesions have eosinophil and mononuclear cell infiltrates, and Th2 cytokines are observed in lesional tissue. IL-4 plays multiple roles in promoting AD. In isolated keratinocytes, IL-4 decreases the expression of genes in the epidermal differentiation complex (EDC) that contribute to barrier function and innate immune defense (4–6). Expression of transgenic IL-4 in the skin results in allergic inflammation that resembles AD (7), whereas systemic expression results in remodeling of the skin (8). Moreover, mice transgenic for the human *IL4-IL13-RAD50-IL5* loci develop pathologies similar to AD (9). Together, these studies show

that IL-4 is an important contributing factor to the development of allergic inflammation in the skin.

In this report, we show that in the absence of endogenous IL-4, mice have increased expression of genes that contribute to skin barrier integrity resulting in altered function compared with wild-type (WT) mice. Mice that express a constitutively active Stat6 (Stat6VT) develop AD-like lesions and have decreased expression of EDC genes. Constitutively active Stat6VT transgenic mice that lack endogenous IL-4 are protected from skin inflammation and recover barrier function faster following skin irritation than mice expressing active Stat6 on a WT background. Thus, IL-4 regulates barrier function homeostasis.

Materials and Methods

Generation of Stat6VT transgenic mice

The generation of Stat6VT transgenic mice was previously described (10). Transgene-positive founders (CD2:Stat6VT [78] line), where the human Stat6 gene with V547 and T548 mutated to alanine is under transcriptional control of the CD2 locus control region, were backcrossed to C57BL/6 mice (Harlan Breeders, Indianapolis, IN). IL-4–deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and mated to Stat6VT transgenic mice. All mice were maintained in specific pathogen-free conditions, and experiments were approved by the Indiana University Institutional Animal Care and Use Committee.

Selectin ligand expression

P- and E-selectin ligand expression was assessed directly ex vivo from WT and transgenic T cells on CD4+ T cells as described previously (11).

Isolation of RNA from skin and real-time PCR

For real-time PCR measurements, involved or uninvolved skin was homogenized in a tissue lyser (Qiagen, Valencia, CA), and RNA isolated with the RNeasy fibrous tissue kit (Qiagen) was used to synthesize cDNA with the First-Strand Cloned AMV kit (Invitrogen, Rockville, MD). Message levels of cytokines and barrier function genes were determined by Taqman assay (Applied Biosystems, Foster City, CA). Cycle number of duplicate samples was normalized to the expression of an endogenous control, either β_2-microglobulin or GAPDH.

Western analysis of EDC proteins

Shaved dorsal skin from WT, $Il4^{-/-}$, or Stat6VT mice was stretched and frozen in liquid nitrogen. Epidermis was mechanically separated from the dermis by scratching. Epidermis was then homogenized in ice-cold lysis

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Abbreviations used in this paper: AD, atopic dermatitis; DC, dendritic cell; EDC, epidermal differentiation complex; MHC-II, MHC class II; qPCR, quantitative PCR; TEWL, transepidermal water loss; WT, wild-type.

buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 10% glycerol, 150 mM NaCl, 50 mM Tris (pH 7.4), and protein inhibitors (1 mM phenylmethanesulphonylfluoride, aprotinin at 1 mg/ml, and leupeptin at 1 mg/ml). Western blot analysis was performed as previously described (12) using specific Abs against filaggrin (Abcam, Cambridge, MA) and involucrin (Covance, Emeryville, CA), or control Ab GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). For densitometric analysis, the scanned images of immunoblots were quantified with Image J software.

Ab treatments

The dorsal side of ears from Stat6VT transgenic mice was injected with isotype control Ab (rat IgG1 isotype control; eBioscience, San Diego, CA) in the left ear and either anti–IL-4 (rat anti-mouse IL-4, clone 11B11) or anti–IL-13 Ab (rat anti-mouse IL-13 Ab; eBioscience) in the right ear on days 0 and 1, respectively. On day 2, the ear skin corresponding to the injection site was excised 18 h later, and RNA was isolated from the skin for real-time PCR measurements.

Transepidermal water loss measurements

Female WT C57BL/6, Stat6VT, and $Il4^{-/-}$ Stat6VT mice (4–6 mo old) were used for transepidermal water loss (TEWL) measurements. Briefly, the mice were anesthetized with ketamine/xylazine (100/10 μg/kg body weight) before the backs of the mice were shaved. Twenty-four hours later, baseline TEWL measurements were taken with an evaporimeter, and 0.1 ml 0.1% retinoic acid (Sigma-Aldrich, St. Louis, MO) was applied topically to irritate skin. The following day, TEWL was measured. The process of application of retinoic acid and TEWL measurements was repeated for 5 d until the levels for TEWL reached 100–150 g/m²/h. The recovery of TEWL was determined at the time points indicated.

Epicutaneous sensitization and skin dendritic cell migration to draining lymph nodes

WT and $Il4^{-/-}$ mice were epicutaneously sensitized with OVA-Alexa Fluor 647 (Invitrogen, Carlsbad, CA). Briefly, the back skin of anesthetized mice was shaven and tape stripped three times before painting with 500 μg OVA-Alexa Fluor 647. Twenty-four hours later, mice were sacrificed before draining lymph nodes were harvested. Cells were first incubated with anti-CD16/CD32 mAb (2.4G2; BD Biosciences, San Jose, CA) and stained with FITC anti-mouse MHC class II (MHC-II) and PE anti-mouse CD11c (BD Biosciences). The proportion of OVA-Alexa Fluor 647$^+$ cells was quantified by gating on 7-aminoactinomycin–negative MHC-II$^+$CD11c$^+$ cells on a FACSCalibur (BD Biosciences).

Results

Altered barrier function in the absence of endogenous IL-4

Although IL-4 regulates the expression of genes involved in skin barrier integrity during AD, it is not clear if nominal levels of IL-4 contribute to homeostatic barrier integrity. To test this, we isolated RNA from the ear tissue of WT and $Il4^{-/-}$ C57BL/6 mice and tested for the expression of genes associated with skin barrier integrity. Although the expression of keratin 14 (*Krt14*) was not different between WT and $Il4^{-/-}$ tissue, we observed 2- to 3-fold increases in loricrin (*Lor*), *Spink5*, kallikrein 7 (*Klk7*), and transglutaminase 3 (*Tsg3*) (Fig. 1*A* and data not shown). We observed 5- to 15-fold increases in the expression of filaggrin (*Flg*) and involucrin (*Ivl*). To confirm that changes in mRNA levels resulted in altered protein expression, we examined protein levels of *Flg* and *Ivl* in epidermal protein extracts from WT and $Il4^{-/-}$ mice. Expression assessed by immunoblot was averaged from three to five mice and showed a 40% increase in the expression of both proteins (Fig. 1*B*).

To determine if increased expression of these genes had functional consequences, and knowing that dendritic cell (DC) function is normal in the absence of IL-4 (13), we tested the ability of protein Ag to cross the skin and be taken up by DCs. The shaved backs of WT and $Il4^{-/-}$ mice were painted with Alexa 647-labeled OVA, and 24 h later, Alexa 647 fluorescence was assessed in CD11c$^+$ cells present in the draining lymph nodes. Significantly fewer Alexa 647$^+$CD11c$^+$ cells were observed in $Il4^{-/-}$ mice than in WT mice (Fig. 1*C*). These results suggest that in the absence of endogenous IL-4, there is increased barrier function.
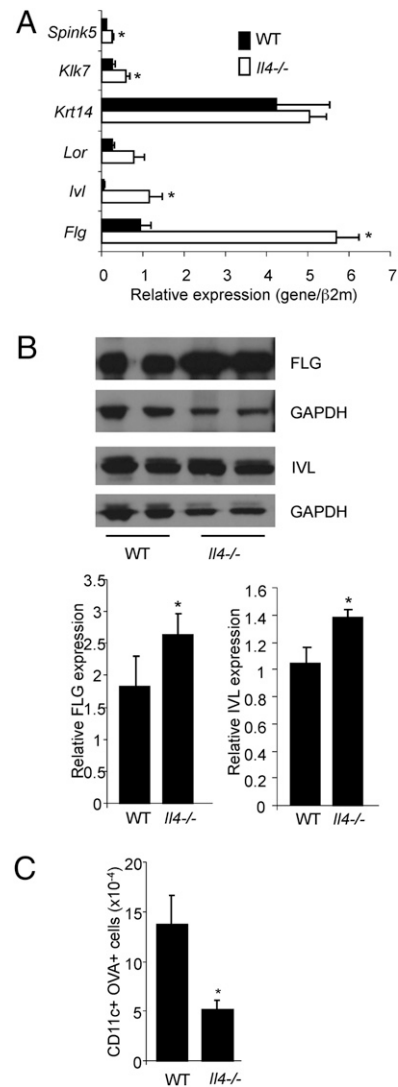

FIGURE 1. IL-4 deficiency increases skin barrier function. *A*, RNA was isolated from WT and $Il4^{-/-}$ ear tissue before analysis of gene expression by qPCR. Results are the average ± SEM from samples of four to six mice. Results are presented as expression of each gene relative to the control (β$_2$-microglobulin) using the $2^{-\Delta Ct}$ method. *B*, Immunoblots of protein extracts from epidermis of WT and $Il4^{-/-}$ mice for FLG and IVL with GAPDH as a loading control. Bar graphs represent the average densitometry of three to five samples normalized to expression of GAPDH. *C*, The backs of WT and $Il4^{-/-}$ mice were shaved and applied with OVA-Alexa 647. Twenty-four hours later, draining lymph nodes were isolated, and DCs (CD11c$^+$MHC-II^{hi}) were examined for Alexa 647 fluorescence. Numbers are the average ± SEM of four mice, calculated as percentage of Alexa 647$^+$ DC × cell number, and are representative of three experiments. *Significantly different from WT, $p < 0.05$.

AD in mice expressing Stat6VT in T cells

We next wanted to test if increased IL-4 would promote allergic skin inflammation. We have previously described mice that express Stat6VT in T cells (10). These mice are prone to allergic inflammation, including blepharitis and recruitment of eosinophils and lymphocytes to the lung (14). These mice are also prone to the development of skin inflammation that appears similar to AD. Stat6VT transgenic mice display increased scratching behavior, resulting in dermatitic plaques with alopecia on the face, back, or other areas of the body (Fig. 2*A*). Lesions can become chronically irritated and infected, resulting in more severe inflammation (Fig. 2*A*). Examination of ear tissue by histology, even in mice that do

not have obvious lesions, shows considerable thickening of the dermis and epidermis with cellular infiltration of eosinophils and lymphocytes (Fig. 2B). In 3- to 6-mo-old mice that do not have AD lesions, there is a significant increase in *Il4* mRNA in ear tissue isolated from Stat6VT transgenic mice compared with WT mice (Fig. 2C). In contrast, expression of other Th2 cytokines, including *Il13*, Th17 cytokines *Il17* and *Il17f*, and the Th1 cytokine *Ifng*, were not significantly increased in nonlesional skin (data not shown). However, in mice with AD lesions, *Il4* and *Il13* mRNA were increased in both lesional and nonlesional tissue (Fig. 2D). Peripheral T cells from Stat6VT transgenic mice had increased percentages of selectin ligand-positive CD4$^+$ T cells, suggesting an increased propensity for migration to the skin (Fig. 2E).

Because IL-4 deficiency increased the expression of *Flg*, we tested whether expression of this gene was altered in skin from Stat6VT transgenic mice. At early time points (2–3 mo), *Flg* expression was not different between wild-type and Stat6VT skin (Fig. 2F). Expression of *Flg* increased over time in WT mice, but not in Stat6VT transgenic mice, resulting in significant *Flg* expression differences between transgenic and nontransgenic mice (Fig. 2F). We observed a similar decrease in filaggrin protein

expression (Fig. 2G). The time frame in which we observed a difference in *Flg* expression (4–6 mo) was also the time frame during which symptoms of skin inflammation became apparent.

IL-4 deficiency protects from the onset of allergic skin inflammation in Stat6VT transgenic mice

We next wanted to determine if blocking cytokines that promote allergic inflammation had an effect on EDC gene expression. To test this, we performed intradermal injections of control Ab, anti–IL-4, or anti–IL-13 into the dorsal side of ears from Stat6VT transgenic mice (age 4–5 mo). Mice received two daily injections, and skin was harvested for analysis of mRNA using quantitative PCR (qPCR). We observed that either anti–IL-4 or anti–IL-13 treatment led to increased *Ivl* expression (Fig. 3), whereas only anti–IL-4 significantly increased *Flg* expression (Fig. 3).

Ideally, mating Stat6VT transgenic mice to *Il4*$^{-/-}$ and *Il13*$^{-/-}$ mice would define the relative role of each cytokine in allergic skin inflammation. However, *Il13*$^{-/-}$ mice also have deficiencies in IL-4 production (15) that would complicate the interpretation of that analysis. Thus, to determine if IL-4 deficiency would protect from the development of allergic skin inflammation, we generated *Il4*$^{-/-}$
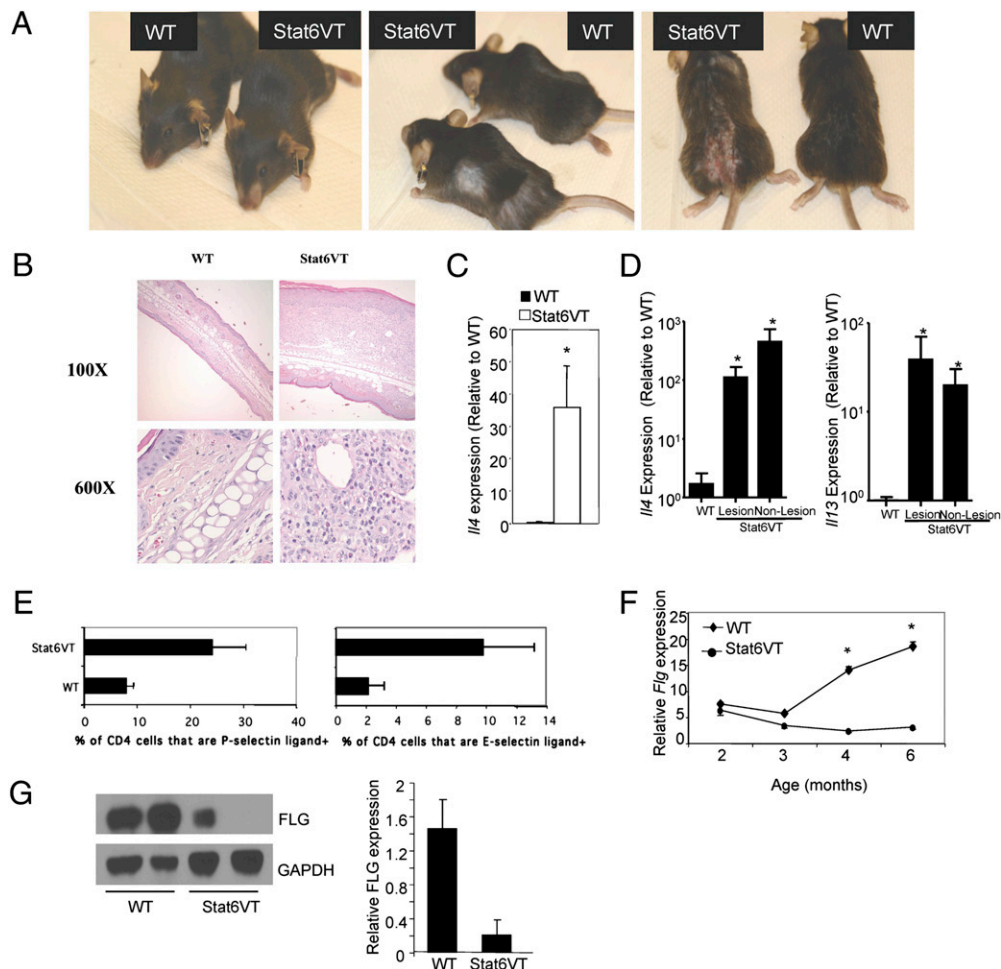

FIGURE 2. Mice expressing Stat6VT in T cells develop allergic skin inflammation. *A*, Photographs of Stat6VT transgenic mice and littermate controls showing areas affected by skin inflammation. *B*, Histological analysis of ear tissue from WT and Stat6VT transgenic mice. Samples were fixed and stained with H&E, original magnification ×100 (*top*) and ×600 (*bottom*). *C*, RNA was isolated from skin of WT and Stat6VT transgenic mice that did not have lesions before analysis of cytokine mRNA using qPCR. *D*, RNA was isolated from skin of WT and lesional or nonlesional skin of Stat6VT transgenic mice that had active lesions before analysis of cytokine mRNA using qPCR. Results are the average ± SEM of four to five mice. *E*, Selectin ligand expression was examined on CD4$^+$ splenic T cells using flow cytometry. Results are the average ± SD of two to four mice and are representative of two experiments. *F*, RNA was isolated from skin of WT and Stat6VT transgenic mice that did not have lesions before analysis of *Flg* mRNA using qPCR. *G*, Immunoblots of protein extracts from epidermis of WT and Stat6VT transgenic mice for FLG with GAPDH as a loading control. Bar graphs represent the average densitometry of three to five samples normalized to expression of GAPDH. Results in *C*, *D*, and *F* are the average ± SEM of five to nine mice. *Significantly different from WT, $p < 0.05$.
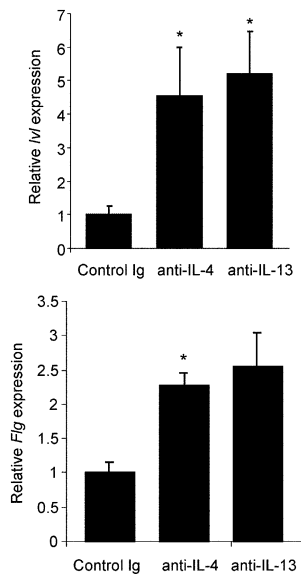
FIGURE 3. Abs to Th2 cytokines increase EDC gene expression in Stat6VT transgenic mice. Abs (control, anti–IL-4, anti–IL-13; all IgG1) were injected intradermally into the dorsal side of the ears of Stat6VT transgenic mice daily for 2 d. Eighteen hours after the last injection, RNA was isolated from ear tissue and used for qPCR of *Ivl* and *Flg* expression. Results are the average ± SEM of three to five mice. *Significantly different from control Ig treated mice, $p < 0.05$.

Stat6VT transgenic mice. We have previously shown that in the absence of endogenous IL-4, Stat6VT-expressing T cells are still capable of developing into Th2-like cells expressing IL-5 and IL-13 (14). Although AD-like symptoms appear in ∼40% of Stat6VT transgenic mice, there was no observed skin inflammation in WT or $Il4^{-/-}$ Stat6VT transgenic mice. Histology indicated that the dermis of $Il4^{-/-}$ Stat6VT transgenic mice was thickened compared with WT skin, although still less than that observed in Stat6VT transgenic mice on a WT background, and lacked eosinophilic infiltration (Fig. 4A). Skin from Stat6VT transgenic mice had decreased expression of several EDC genes, including *Lor*, *Ivl*, and, most prominently, *Flg* (Fig. 4B). However, IL-4 deficiency was able to increase the expression level of these genes in Stat6VT transgenic mice above WT levels, similar to the pattern observed in nontransgenic $Il4^{-/-}$ skin (Figs. 1A, 4B), suggesting that eliminating IL-4 increased the barrier function of skin in Stat6VT transgenic mice and attenuated the propensity for the development of allergic inflammation.

To directly test if IL-4 deficiency affects skin homeostasis, we irritated shaved skin by applying topical retinoic acid for 1 wk and examined recovery of TEWL over the following week. Application of the retinoic acid resulted in a 10-fold increase in TEWL from baseline levels that recovered to near baseline levels after 8 d without treatment (Fig. 4C). Stat6VT transgenic mice had a slower recovery, still having a 4-fold elevated TEWL after 8 d. In contrast, $Il4^{-/-}$ Stat6VT transgenic mice had recovery in TEWL similar to WT mice (Fig. 4C).

Discussion

Allergic inflammation results from a complex interaction among environment, immune system, and target organ that has not been well defined. It is still unclear whether a primary defect lies in a target organ that provides insufficient barrier function or in an inappropriate Th2 immune response that develops to innocuous Ag. Although polymorphisms in genes required for skin barrier function are associated with AD (16–18), it is unclear if these result in altered gene expression or perhaps altered sensitivity to cytokines produced during an allergic response. In this report, we used
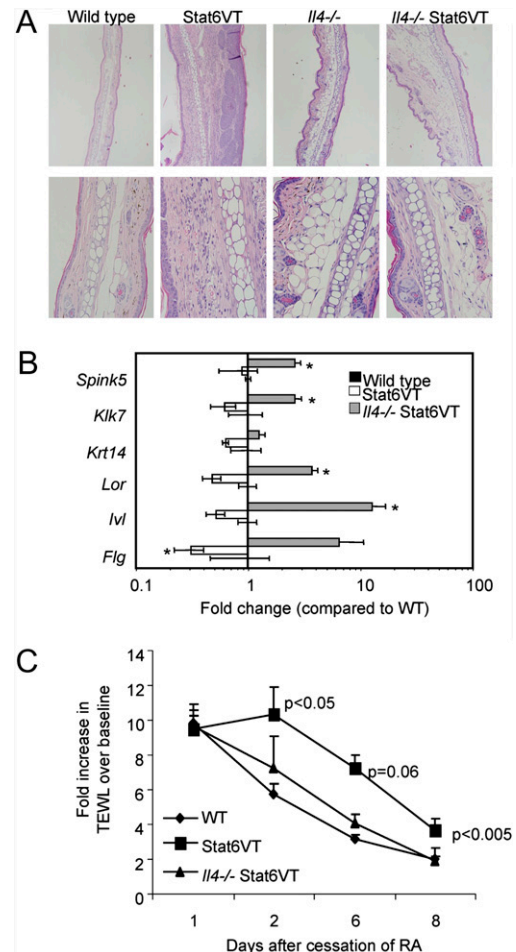

FIGURE 4. IL-4 deficiency protects from the development of allergic skin inflammation and promotes recovery from injury. A, Histology of ear tissue from mice of the indicated genotypes. Samples were fixed and stained with H&E, original magnification ×100 (*top*) and bottom ×400 (*bottom*). B, RNA was isolated from ear tissue of WT, Stat6VT transgenic, and $Il4^{-/-}$ Stat6VT transgenic mice before analysis of gene expression by qPCR. Results are the average ± SEM from samples of four to six mice. WT values are equal to 1 and are not above the axis. C, Fold increase in pretreatment TEWL following retinoic acid application was examined at the indicated time points. Results are the average ± SEM of four to six mice. *Significantly different from WT, $p < 0.05$.

a model of AD arising from T cell-specific expression of an active Stat6 to test whether predisposition to a Th2 response would result in specific changes in target organ gene expression. We demonstrated increased EDC gene expression and barrier function in the absence of endogenous IL-4, contrasting with decreased EDC gene expression and allergic skin inflammation when the immune system is predisposed toward a Th2 response. Importantly, IL-4 deficiency protects from the development of allergic skin inflammation and hastens the recovery of barrier function following skin irritation.

The ability of IL-4 to regulate basal expression of EDC genes could result from several mechanisms. Effects could be direct, resulting from homeostatic levels of IL-4 present in the circulation or the tissue that act directly on the keratinocytes (5). It has not been demonstrated whether IL-4–induced changes in keratinocyte gene expression are a result of STAT6 binding to EDC gene promoters or interference with other transcription factors that positively regulate mRNA levels. Effects could also be indirect, and in the absence of IL-4, the production of Th1 or Th17 cytokines that affect expression of EDC genes could be altered (5, 19). However, we did not observe

increases in mRNA levels of *Ifng* or *Il22* in skin from *Il4*$^{-/-}$ mice (data not shown). That expression of *Ivl* was increased most by the absence of IL-4, whereas *Flg* was decreased the most in the presence of a high Th2 environment (Stat6VT transgenic mice), suggests that EDC gene regulation is likely more complex than simply the presence or absence of IL-4.

The model of AD in Stat6VT transgenic mice is unique in that disease results from a perturbation of T cell function, not of ectopic gene expression within the skin, and is dependent on IL-4 for development. This is important, as allergic skin inflammation in other mouse models of AD may appear as Th1-mediated inflammation in mice that are genetically deficient in the ability to develop Th2 responses (3). Thus, the dependence on IL-4 for disease development in this model is significant. The requirement for a specific effector cytokine suggests that although genetic predisposition to changes in barrier function, including mutations in *FLG*, is an important risk factor in AD development (17, 18), a strong skewing of immune function can have similar effects. The increased Th2 cytokine production in Stat6VT transgenic mice decreases expression of multiple genes in the EDC complex, resulting in decreased barrier function, which at least partially contributes to the development of allergic skin inflammation. Our results also provide a mechanism for the AD-like disease that develops in IL-4 transgenic models (9, 20).

Although IL-4 is clearly required for the development of allergic skin inflammation in Stat6VT transgenic mice, the relative roles of IL-4 and IL-13 are not fully defined. We demonstrate that acute treatment of skin from Stat6VT transgenic mice with either anti–IL-4 or anti–IL-13 increased the level of *Ivl* and *Flg* mRNA. Other reports have shown that treatment of keratinocytes with IL-4 and IL-13 can decrease EDC gene expression (4, 6). But although transgenic mice expressing IL-4, or IL-4 and IL-13, develop allergic skin inflammation (7, 9), the ability of IL-13 alone to promote allergic skin inflammation has not been determined. The decreases in IL-4 production caused by targeting the *Il13* locus prevent a precise determination of the role of IL-13 in this model (15). Importantly, the ability of IL-4 deficiency to prevent allergic skin inflammation in Stat6VT transgenic mice, and that IL-13–secreting cells develop in *Il4*$^{-/-}$ Stat6VT transgenic mice (14), suggests that although both cytokines can induce regulation of EDC genes, they are not functionally redundant in the development of allergic skin inflammation.

The development of AD in patients relies on multiple factors. As mentioned above, mutations in *FLG* are a significant risk factor for AD (17, 18), and barrier function, as assessed by increased TEWL, is decreased (21–24). Similarly, a family history of allergic disease, which likely reflects an increased propensity for development of allergic responses, is a significant risk factor (25, 26). Importantly, our studies show that the effect of IL-4 on keratinocyte gene expression is not restricted to active lesions (4–6), but that it also contributes to homeostatic expression of genes involved in skin barrier function. Thus, increases in systemic or local IL-4 through the ability to regulate barrier function may be a predisposing factor for the development of AD. Modulating the levels of IL-4 in patients might be an effective method for not only treating lesions, but also limiting future exacerbations.

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Disclosures

The authors have no financial conflicts of interest.

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