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Increased Th2 activity and diminished skin barrier function cooperate in allergic skin inflammation

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease induced by a complex interaction between susceptibility genes encoding skin barrier components and environmental allergen exposure that results in type 2 cytokine production. Although genetic lesions in either component can be risk factors for disease in patients, whether these pathways interact in the development of AD is not clear. To test this, we mated mice with T-cell specific expression of constitutively active Stat6 (Stat6VT) that spontaneously develop allergic skin inflammation with Flaky tail (Ft) mice that have mutations in *Flg* and *Tmem79* genes that each affect skin barrier function. Our results demonstrate that over 90% of the Stat6VT transgenic mice carrying the Ft alleles (Stat6VTxFt$^{-/-}$) develop severe atopic dermatitis lesions by 3-5 months of age, compared with only 40% of Stat6VT mice that develop disease by 6-7 months of age. Further, histopathological analysis of skin tissues from Stat6VTxFt$^{-/-}$ mice revealed extensive thickening of the dermis with increased inflammatory infiltrates as compared with Stat6VT mice. Our study suggests that skin barrier defects and altered Th2 responses independently cooperate in the pathogenesis of allergic skin inflammation, similar to effects observed in patients with AD.

Keywords

allergic skin inflammation; atopic dermatitis; genetic lesions; Stat6; Th2 response

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CONFLICT OF INTEREST

The authors state no conflicts of interest.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects 25% of children and 1–3 % of adults worldwide [1, 2]. The incidence of AD has increased by 2- to 3- fold over the past several decades, especially in industrialized countries. The pathogenesis of AD may result from complex interactions between environmental and genetic factors, barrier defects and immune dysregulation resulting in epidermal hyperplasia and increased penetration of allergens and microbial pathogens [3, 4]. Recent studies have implicated a strong association between a defect in the skin barrier and the pathogenesis of AD [5]. The defect is caused by genetic loss-of-function mutations in the *FLG* gene encoding filaggrin, a key structural protein required for epidermal barrier function. These mutations are found in a substantial proportion of AD patients [6, 7]. Flaky tail mice (Ft) have a mutation in the *Flg* gene similar to mutations seen in patient DNA [8, 9], but also have a second mutation, the matted mutation in the *Tmem79* gene [10, 11] that is closely linked to *Flg* on mouse chromosome 3. Both mutations contribute to changes in barrier function and mild spontaneous dermatitis in these mouse models. Ft or *Flg*-mutant mice have increased susceptibility to allergen sensitization, and have a propensity to develop spontaneous AD-like lesions, varying with the genetic background [8, 12, 13].

We have previously shown that Stat6VT transgenic mice develop AD-like lesions secondary to the increased Th2 activity associated with expression of a constitutively active Stat6 protein in T cells [14-17]. Approximately, 40-50% of these mice spontaneously develop AD-like lesions due to increased Th2 responses, and disease correlates with decreased expression of barrier function genes [14, 16]. Thus, as in patients [18], reduced filaggrin expression in Stat6VT mice is attributed to increased Th2 activity leading to decreased expression of barrier function genes.

In AD patients, reduced filaggrin expression could result either from mutations in the filaggrin gene (*FLG*) or from the effects of Th2 cytokines [6, 18]. However, it is not clear if altered barrier function and increased Th2 activity function independently in the development of AD-like symptoms. The aim of the present study was to ascertain how altered barrier function as a result of the Ft mutations concomitant with increased pro-allergic activity affected the development of AD-like disease.

RESULTS AND DISCUSSION

Ft mutation increases incidence and severity of allergic skin inflammation in Stat6VT mice

To determine the effect of Ft mutations in Stat6VT mice, we crossed Stat6VT transgenic and Ft mice. For simplicity, we refer to mice that are homozygous or heterozygous for the Ft mutations as Ft−/− and Ft+/−, respectively. Ft+/− skin has Flg protein expression that is intermediate between WT and Ft−/− skin [9]. WT, Stat6VT, Ft+/−, Ft−/−, Stat6VTxFt+/− and Stat6VTxFt−/− mice were monitored for the development of AD-like lesions. By 3-5 months of age, Stat6VTxFt−/− mice developed severe allergic skin inflammation, characterized by erythema, scaling and crusting of lesions on the face and body (Fig 1A-C), in contrast to Stat6VT mice where only blepharitis was apparent at this time and skin lesions did not develop until later (6-7 months) (Fig. 1B-C). Unlike Stat6VT and Stat6VTxFt−/−

mice, Ft+/− or Ft−/− mice exhibited variable development of blepharitis and dry scaly skin, but no other manifestations of AD-like inflammation (Fig 1B). In previous reports, spontaneous skin inflammation was observed in older Ft−/− mice on a B6 genetic background [12]. Stat6VT transgenic mice with one or two Ft alleles also had an increased frequency of severe AD-like disease (Fig. 1B). We further observed a significantly higher percentage of Stat6VT and Stat6VT x Ft−/− mice requiring euthanasia due to severe disease, compared with WT or Ft−/− mice (Fig 1D). Although there was a trend for increased morbidity in the Stat6VT x Ft−/− mice compared with the Stat6VT mice, the difference was not significant. Our results demonstrate that the Ft mutation results in an earlier onset, higher incidence and increased severity of development of AD-like lesions in Stat6VT mice.

Exacerbated skin pathology and decreased barrier function in Stat6VT x Ft−/− mice

To further characterize the histopathology associated with increased disease in Stat6VT x Ft −/− mice, we performed histological analysis of ear skin tissue from WT, Ft−/−, Stat6VT and Stat6VT x Ft−/− mice aged 3-5 months. We observed a thickening of the epidermis and dermis with infiltration of inflammatory cells in Stat6VT mice as previously described [14], that was further exacerbated in Stat6VT x Ft−/− mice with more severe inflammatory infiltrates than Stat6VT mice (Fig 2A). Infiltrates in Stat6VT skin include increases in T cells, eosinophils, mast cells, but no increases in type 2 innate lymphoid cells ([14, 19] and data not shown). Changes in the pathology of skin from Ft−/− mice compared with WT mice were minimal (Fig. 2A).

Although the Ft mutant decreases *Flg* expression below that observed in Stat6VT transgenic mice, it is not clear how much of an effect there is on the expression of other EDC genes. Quantitative PCR indicated a further reduction in the expression of *Ivl* and *Hrnr* in Stat6VTxFt−/− mice as compared with Stat6VT mice (Fig, 2B). The expression of *Flg* in Stat6VTxFt−/− mice was similar to that of Ft−/− mice, but the expression of *Hrnr* and *Ivl* were further decreased in Stat6VTxFt−/− mice as compared with Ft−/− mice. To verify the results with protein expression, we performed Flg immunoblots with skin from WT, Ft−/−, and Stat6VT transgenic mice. We observed the fully processed Flg protein at 32 kDa in the WT but not the Ft−/− samples, and not in the Stat6VT transgenic samples (Fig. 2C), consistent with previous results [8, 9, 14]. We did observe decreased expression of higher molecular weight unprocessed Flg proteins, also consistent with previous results [9]. The accumulated density for all bands in each lane was measured and averaged across multiple samples (Fig. 2D).

To determine if the decreased expression of epidermal barrier genes in mice had functional consequences, we tested the ability of the protein antigen to cross the skin and be taken up by dendritic cells as described earlier [14, 17]. Twenty-four hours after applying Alexa647-labeled OVA to the shaved backs of WT, Stat6VT, Ft−/− and Stat6VTxFt−/− mice, dendritic cells (CD11c+ MHC II-hi cells that would include both Langerhans cells and dermal dendritic cells) in the draining lymph nodes were examined for the uptake of labeled OVA. Although we observed an increase in the percentage of Alexa647^{+} CD11c^{+} cells in Stat6VT mice compared with WT mice, the percentages of Alexa647^{+} CD11c^{+} cells in Stat6VTxFt−/− mice were further increased compared with WT mice, Stat6VT or Ft−/− mice (Fig 2E).

These results indicate that in Stat6VT x Ft−/− transgenic mice, there is increased protein translocation across the skin barrier.

It is interesting that protein translocation was not increased in Ft−/− mice as previous reports showed increased cutaneous sensitization in Ft−/− and *Flg*−/− mice [8, 12]. Notably, these earlier studies utilized repeated allergen applications where a Th2 response developed, whereas the experiment described here (Fig. 2E) is based on a short-term 24-hour assay where endogenous cytokine responses would not likely develop. These differences suggest a requirement for an ongoing Th2 response to facilitate increased protein translocation. This interpretation is consistent with acute protein translocation being increased in Stat6VT transgenic mice, where a Th2 cytokine milieu is present, and even more pronounced when combined with Ft mutations. As Ft−/− mice do develop Th2 responses following sensitization, it would be interesting to assess the effect of Stat6-deficiency on AD like lesions in the Ft−/− mice. Our results suggest that the two genetic lesions causing decreased barrier function and increased Th2 activity work independently and cooperatively in the development of AD-like disease. Although the increased Th2 activity in the Stat6VT mice decreases EDC and other skin barrier function gene expression, we speculate that the mutations in the Ft mice result in earlier impairment of barrier function in Stat6VT transgenic mice that contribute to the earlier initiation of disease.

CONCLUDING REMARKS

In the present report, we demonstrate that the mutations in the Ft mice that result in decreased barrier function exacerbate the development of spontaneous allergic skin disease in Stat6VT mice. This is consistent with the increased propensity of Ft mice, and mice with specific mutations in *Flg* and *Tmem79*, to develop AD-like lesions following sensitization [8, 10, 11]. Our results are also consistent with human studies where *FLG* mutations that are neither required nor sufficient for disease, are strong predisposing factors for AD, highlighting the idea that changes in barrier function alone are not sufficient to generate a severe and high frequency early-onset disease [6, 7]. As therapies develop to block Th2 responses in AD, it will be interesting to compare the effects of treatment on patients with or without genetic mutations in barrier function genes [20].

MATERIALS AND METHODS

Generation of Stat6VT and Stat6VT x Ft−/− mice

The generation of Stat6VT transgenic mice on a C57BL/6 background was previously described [14, 15]. 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). To obtain Stat6VT mice with barrier function mutations, Stat6VT mice were mated to Flaky tail mice that were obtained from Dr. John Sundberg at the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free conditions, and experiments were approved by the Indiana University Institutional Animal Care and Use Committee.

Quantification of incidence and mortality in Stat6VT, Stat6VT x and Stat6VT x Ft−/− mice

Mice were monitored for the onset and development of AD lesions between 3-13 months. The percentage of mice that develop no disease, blepharitis, dry flaky skin or severe AD lesions was determined. Kaplan-Meier morbidity estimates were used to evaluate the mice that required euthanasia or those that died due to severe lesions and analysis was performed with GraphPad Prism 6. For other analyses, data were expressed as means of 3 independent experiments and analyzed with the Student *t*-test or Chi-square test. A p value of less than 0.05 was considered statistically significant.

Histological examination of skin sections

Skin tissues were fixed in neutral buffered Formalin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H & E) to evaluate the infiltration of inflammatory cells, by light microscopy. Filaggrin protein expression was analyzed as previously described [19].

Isolation of RNA from skin and real-time PCR

For real-time PCR measurements, the 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 barrier function genes were determined by Taqman assay (Applied Biosystems, Foster City, CA). Cycle number of the samples was normalized to the expression of β_2- microglobulin.

Epicutaneous allergen exposure and skin dendritic cell migration to draining lymph nodes

Mice were epicutaneously exposed to 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 dissolved in PBS. Twenty-four hours later, mice were sacrificed and 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 Flour 647+ cells was quantified as described previously by gating on CD11c+ MHC II-hi cells [14].

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Abbreviations

AD	atopic dermatitis
EDC	epidermal differentiation complex
Flg	filaggrin

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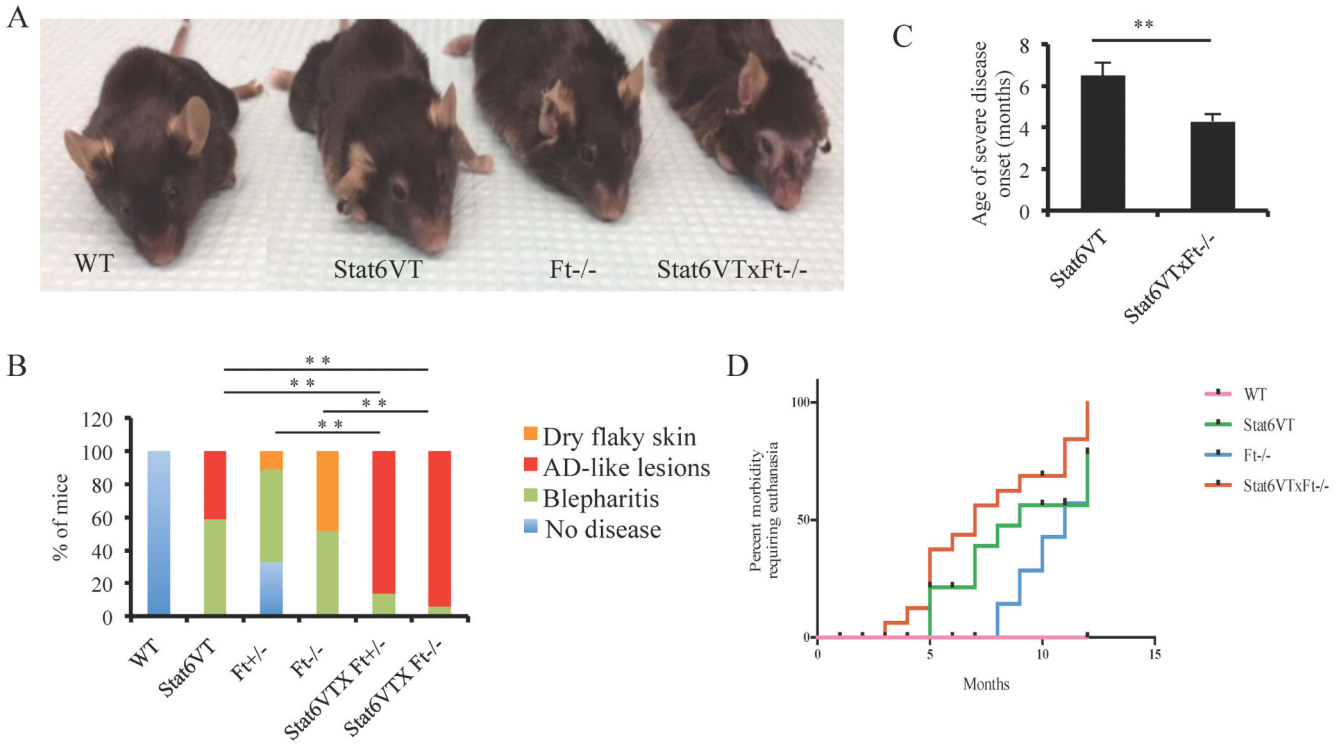

Figure 1.

Stat6VT mice with Flaky tail mutations develop severe allergic skin inflammation. (A) Photographs of WT, Stat6VT, Flaky tail (Ft−/−) and Stat6VTxFt−/− mice. B, Percent of mice that develop no disease, blepharitis, dry flaky skin or AD lesions is shown. Data were analyzed by Chi-square test, ** $p < 0.0001$, n=7-18. C, Age of onset of severe disease in Stat6VT and Stat6VTxFt−/− mice. Data are mean ± SEM (n=7-17 mice/group) and are pooled from 2-3 independent experiments. ** $p < 0.01$; Student's *t*-test. D, Morbidity curves of the indicated genotypes by Kaplan-Meier analysis is shown. Data shown are pooled from 2-3 independent experiments; n=8-17 per group, and data were analyzed by Chi-square test.

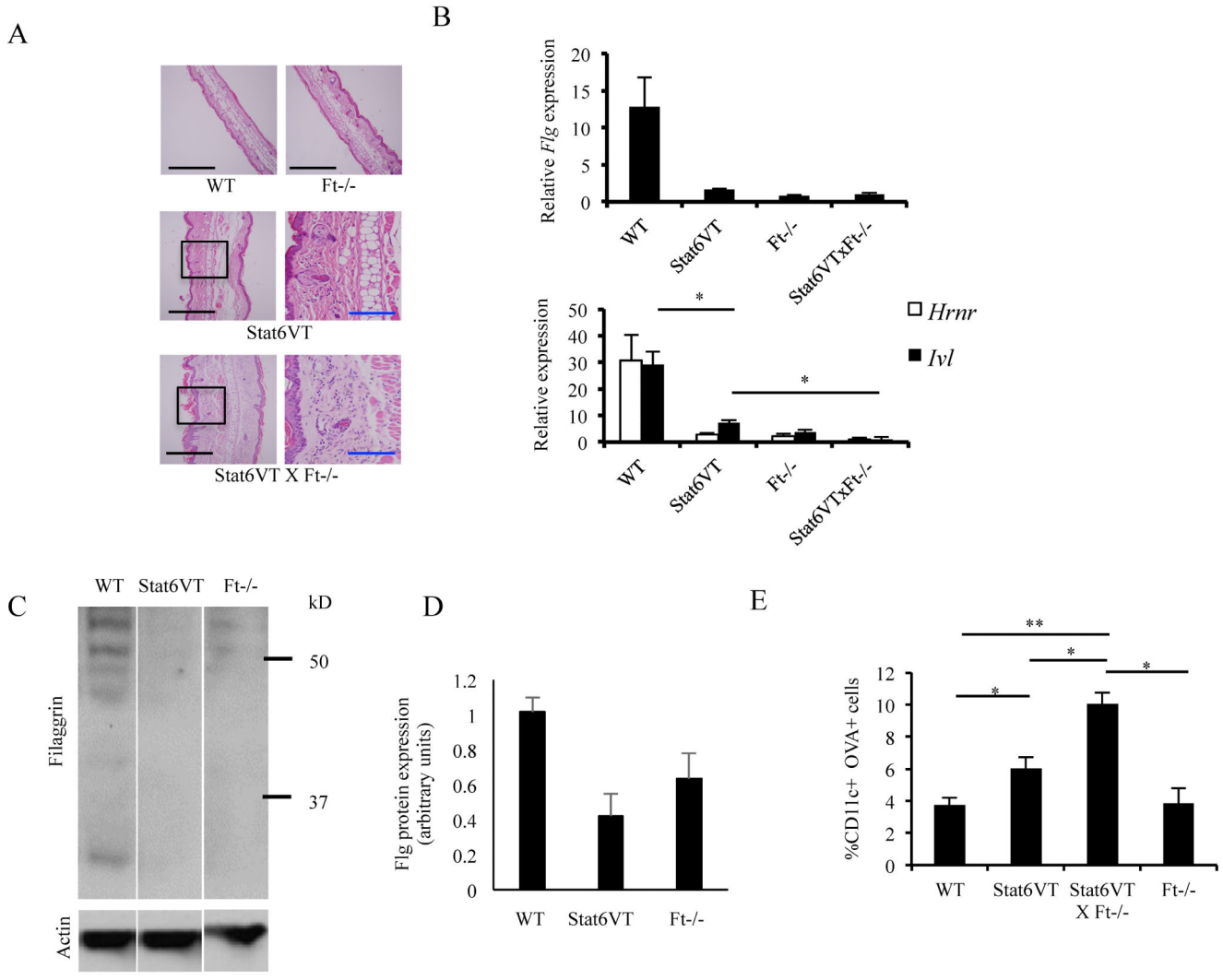

Figure 2.

Stat6VTxFt−/− mice have severe skin immunopathology and decreased barrier function. A, Histological analysis of ear tissue from the indicated mice (n= 5-10 mice/group). Samples were fixed and stained with H & E, and images were taken at x100 (WT, Ft−/−and left panels for Stat6VT and Stat6VT x Ft−/−) or x600 (right panels for Stat6VT and Stat6VT x Ft−/−). Black bars = 1 mm. Blue bars = 0.1 mm. Boxes indicate regions enlarged in photomicrograph to the right. B, Expression of EDC genes. RNA was isolated from the ear skin of mice and expression of *Flg, Hrnr*, and *Ivl* determined by quantitative PCR, Data are mean ± SEM of 2-4 mice and are representative of three independent experiments. * $p <$ 0.05; Student's *t*-test,. C, Immunoblot of filaggrin expression in skin samples from the indicated genotypes. Representative of three samples from separate mice on one immunoblot. D, Averages of densitometry for replicate samples of filaggrin expression relative to actin expression. E, Ft mutations in Stat6VT transgenic mice further decrease the barrier function. Mice from the indicated genotypes were treated with OVA-Alexa 647 and uptake by DCs was measured as described in methods. (D and E) Bar graphs represent the

mean ± SEM (n= 3-5 mice /group) and are representative of two independent experiments. *$p < 0.05$, ** $p < 0.01$; Student's I-test.