Spontaneous atopic dermatitis is mediated by innate immunity, with the secondary lung inflammation of the atopic march requiring adaptive immunity

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Background: Atopic dermatitis (AD) is an inflammatory skin condition that can occur in early life, predisposing to asthma development in a phenomenon known as the atopic march. Although genetic and environmental factors are known to contribute to AD and asthma, the mechanisms underlying the atopic march remain poorly understood. Filaggrin loss-of-function mutations are a major genetic predisposer for the development of AD and progression to AD-associated asthma.
Objective: We sought to experimentally address whether filaggrin mutations in mice lead to the development of spontaneous eczematous inflammation and address the aberrant immunologic milieu arising in a mouse model of filaggrin deficiency.
Methods: Filaggrin mutant mice were generated on the proallergic BALB/c background, creating a novel model for the assessment of spontaneous AD-like inflammation. Independently recruited AD case collections were analyzed to define associations between filaggrin mutations and immunologic phenotypes.
Results: Filaggrin-deficient mice on a BALB/c background had profound spontaneous AD-like inflammation with progression to compromised pulmonary function with age, reflecting the atopic march in patients with AD. Strikingly, skin inflammation occurs independently of adaptive immunity and is associated with cutaneous expansion of IL-5–producing type 2 innate lymphoid cells. Furthermore, subjects with filaggrin mutations have an increased frequency of type 2 innate lymphoid cells in the skin in comparison with control subjects.
Conclusion: This study provides new insights into our understanding of the atopic march, with innate immunity initiating dermatitis and the adaptive immunity required for subsequent development of compromised lung function. (J Allergy Clin Immunol 2016;137:482-91.)

Key words: *Allergy, asthma, atopic dermatitis, atopy, eczema, filaggrin, flaky tail, type 2 innate lymphoid cells, innate immunity, mouse, mutation*

There has been a profound increase in the incidence of atopic disease morbidity in developed societies in recent decades. Atopic individuals, who are characterized by increased serum IgE levels, are predisposed to having allergies such as atopic dermatitis (AD) and asthma. AD is heritable and characterized by pruritic eczematous lesions, with approximately 20% of children affected in the developed world.[1] The cause of AD is multifactorial, with interplay between genetic predisposition and environmental factors initiating aberrant inflammation.[2] The term atopic march encapsulates the predisposition of patients with AD in infancy to progress to secondary allergic disorders, such as asthma.[3] Although AD as the first manifestation of atopic diathesis in early life is well established, how AD development primes progression to secondary allergies is not known.

Loss-of-function mutations in the human filaggrin gene *(FLG)* have been identified as the major genetic predisposing factor for AD development,[4-6] and in the context of the atopic march, patients with AD with *FLG* mutations are predisposed to the development of asthma.[7,8] We previously identified a mutation in the murine filaggrin gene *(Flg)* in the "flaky tail" double-mutant *($Matt^{ma/ma}Flg^{ft/ft}$)* mouse strain, resulting in a lack of filaggrin protein in the skin.[9] We recently separated the matted and filaggrin mutations present in *$Matt^{ma/ma}Flg^{ft/ft}$* flaky tail mice.[10] We now show that filaggrin-deficient mice, analogous to *FLG* mutations in human subjects, have spontaneous dermatitis, become atopic and progress to lung

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Abbreviations used
AD: Atopic dermatitis
AHR: Airway hyperresponsiveness
C_{dyn}: Dynamic lung compliance
CFP: Cerulean fluorescent protein
dLN: Draining lymph node
eGFP: Enhanced green fluorescent protein
FLG: Human filaggrin gene
Flg: Murine filaggrin gene
iILC2: Inflammatory type 2 innate lymphoid cells
ILC2: Type 2 innate lymphoid cell
IL-7Rα: IL-7 receptor α
NBNT: Non-B/non-T cell
NF-κB: Nuclear factor κB
nILC2: Natural type 2 innate lymphoid cell
R_L: Lung resistance
ROR: Retinoic acid–related orphan receptor
TSLP: Thymic stromal lymphopoietin
WT: Wild-type

inflammation with age. By using a mouse with a mutation in a gene implicated in the atopic march in human subjects, the roles of innate versus adaptive immunity are shown in the initial development of dermatitis and progression to aberrant lung inflammation. Filaggrin-deficient mice on a BALB/c background have a spontaneous expansion of IL-5–producing type 2 innate lymphoid cells (ILC2s) into the skin, with an increase in skin ILC2 numbers also seen in patients with *FLG* mutations, reinforcing the role of innate immunity in the development of AD.

METHODS

Mice

All mice were congenic BALB/c strain, with BALB/c mice used as wild-type (WT) control animals. The Flg^{ft} and *ma* mutations in flaky tail ($Matt^{ma/ma}Flg^{ft/ft}$) mice (Stock a/a ma ft/ma ft/JSun; JR#9078; Jackson Laboratories, Bar Harbor, Me) were separated, and the Flg^{ft} mutation was backcrossed to the congenic C57BL/6J background in accordance with previously published methods.[10] $Flg^{ft/ft}$ C57BL/6J congenic mice were subsequently backcrossed to the congenic BALB/c background, and these mice were used in this study. $Il4^{KN2}$,[11] *Il5CFP*, *Il13eGFP*,[12] *Il17eGFP* (Biocytogen, Worcester, Mass), and *Rag1*-deficient (Jackson Laboratories) mice were crossed with $Flg^{ft/ft}$ mice in house. Mice expressing the luciferase transgene under the control of a nuclear factor κB (NF-κB) promoter (*NF-κB-Luc*; Caliper Life Sciences, Hopkinton, Mass) were crossed to $Flg^{ft/ft}$ mice.

Mice were housed in specific pathogen-free conditions, with irradiated diet and bedding and water *ad libitum*. All animal experiments were performed in compliance with the Irish Department of Health and Children regulations and approved by Trinity College Dublin's BioResources ethical review board.

Clinical scoring

The severity of skin inflammation was clinically scored longitudinally by using a system based on the macroscopic diagnostic criteria described by Saunders et al[10] and adapted from assessment of skin inflammation in the Nc/Nga mouse model.[13] In brief, a scoring system (0, none; 1, mild; 2, moderate; and 3, severe) was applied to the signs of edema, erythema, scaling, and erosion. The total score for each mouse was calculated from the sum of individual scores for each parameter.

Analysis of airway hyperresponsiveness

Lung function or airway hyperresponsiveness (AHR) was analyzed in 32-week-old mice by using an invasive method in which mice were tracheostomized and ventilated with whole-body plethysmography[14] by using a pneumotachograph linked to a transducer (EMMS, Hants, United Kingdom). Changes in lung resistance (R_L) and dynamic lung compliance (C_{dyn}) in response to increasing doses of nebulized and inhaled methacholine (10, 30, 60, and 100 mg/mL; Sigma-Aldrich, St Louis, Mo) were recorded, as previously described.[9,15]

Flow cytometric and cytokine analyses of human suction blisters

Suction blistering was performed on patient donors after obtaining informed written consent, and sample use was given ethical approval from the NRES Committee South Central, United Kingdom. Patients with moderate-to-severe AD were recruited and genotyped for *FLG* mutations (see this article's Online Repository at www.jacionline.org).[5] Patients with WT, heterozygous, and compound heterozygous *FLG* status were included in the study. Suction blister cups were applied to the skin of patients with a vacuum pressure of 200 to 400 mm Hg, as previously described.[16] Blisters were formed within 60 to 90 minutes, and suction was then removed. Twenty-four hours later, fluid was aspirated with a 30-gauge needle. Fluids were centrifuged at 1500 rpm for 5 minutes at 4°C, and cell pellets were resuspended in RPMI 1640 supplemented with 10% human serum.

For surface staining, single-cell suspensions were prepared in flow cytometry buffer. Live/dead violet (Invitrogen, Carlsbad, Calif) was used to determine cell viability. Directly conjugated antibodies with fluorescein isothiocyanate, phycoerythrin, phycoerythrin–Texas Red, peridinin-chlorophyll-protein complex, peridinin-chlorophyll-protein complex–Cy5.5, PeCy7, V450, allophycocyanin, and allophycocyanin-Cy7 were used. Human cells were stained with the BioLegend (San Diego, Calif) mAbs CD4 (MEM-241), CD8 (RPA-T8), CD11b (DCIS1/18), CD45 (H130), CD56 (B159), FcεRI (AER-37 [CRA-1]), and IL-7 receptor α (IL-7Rα; A019D5); the BD Biosciences (San Jose, Calif) mAbs CD3 (SK7), CD19 (SJ25C1), and CD14 (MφP9); the Abcam (Cambridge, United Kingdom) mAb CD11c (BU15); the Miltenyi Biotec (Bergisch Gladbach, Germany) mAb chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (BM16); and the R&D Systems (Minneapolis, Minn) mAb CD123 (FAB301C). Cells were acquired by using FACSDiva (BD Biosciences) or Summit software (Beckman Coulter, High Wycombe, United Kingdom) on an LSRFortessa or CyAn flow Cytometer, respectively. Lineage gating included CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, FcεRI, and CD123. ILC2s were defined as $Lin^-CD45^+IL\text{-}7R\alpha^+$ chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes positive. FlowJo (TreeStar, Ashland, Ore) and Summit software were used for further data analysis. Blister fluid was analyzed with the MAGPIX Multiplex Array (Luminex, Austin, Tex), according to the manufacturer's instructions. Quantification of ILC2s and IL-1β levels in patient samples was performed in a blinded manner.

Statistical analyses

Data are expressed as means ± SEMs and analyzed by using 2-way ANOVA or the unpaired Student *t* tests (Prism 6; GraphPad Software, La Jolla, Calif).

RESULTS

Filaggrin deficiency leads to spontaneous dermatitis and atopy

Single mutant Flg^{ft} congenic mice without the $Matt^{ma}$ mutation were generated (see Fig E1 in this article's Online Repository at www.jacionline.org) on the proallergic BALB/c background.[17-19] $Flg^{ft/ft}$ mice have attenuated profilaggrin expression in the epidermis and absent functional filaggrin monomer (see Fig E2 in this article's Online Repository at www.jacionline.org), which is similar to what is seen in *FLG*-null patients.[5] As neonates, $Flg^{ft/ft}$ mice spontaneously have marked ichthyosis-like dermatitis with edema, erythema, hyperlinearity, and scaling compared with