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Research article

No impact of filaggrin deficiency on the efficacy of epicutaneous immunotherapy in a murine model

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Abstract: *Background:* Epicutaneous immunotherapy (EPIT®), is currently being investigated for the treatment of food allergy, utilizes intact skin to activate the immune system. Mutations in the gene encoding the key epidermal protein filaggrin (FLG) are risk factors for peanut allergy and disrupt the skin integrity. We investigated the association between FLG deficiency and peanut EPIT® efficacy in a murine model. *Methods: FLG* mutant mice deficient in filaggrin (*FLG*-/-) or wild-type (WT) mice were sensitized with peanut protein extract (peanut protein) and cholera toxin. Sensitized mice received a patch per week during 8 weeks for EPIT®, using Viaskin®, and were then submitted to sustained peanut oral exposure. We assessed blood humoral and cellular responses and evaluated eosinophil infiltration in the gut mucosa. The different steps of allergen capture and transportation following deposition on the skin was also analyzed in sensitized mice. *Results:* Sensitization of mice was confirmed by a significant increase of specific Th2 biased immunological responses. In sensitized mice, EPIT® significantly reduced IgE levels, splenocyte secretion of Th2 cytokines and recruitment of eosinophils in esophagus, compared to sensitized mice without epicutaneous immunotherapy. The allergen applied onto the skin of *FLG-/-* mice did not undergo passive skin passage or systemic delivery. Instead, the allergen was captured by skin CD205^{high} DCs, which migrated to afferent lymph nodes, as already described in WT mice. *Conclusions:* EPIT® was efficient and safe in *FLG*-/- mice, suggesting that in humans, the efficacy and safety observed with EPIT® is maintained in the presence of loss of function of *FLG*.

Keywords: allergy; epicutaneous immunotherapy; filaggrin; skin; atopic dermatitis

Abbreviations:

AD	atopic dermatitis
DCs	dendritic cells
EPIT	epicutaneous immunotherapy
FLG-/-	filaggrin deficient
Ig (E, G1, G2a)	immunoglobulin type E, G1, G2a
RT-qPCR	reverse transcriptase-quantitative-polymerase chain reaction
WT	wild-type

1. Introduction

Mutations in the gene encoding filaggrin (*FLG*), an essential component of the epithelial barrier, are a significant predisposing factor for atopic dermatitis (AD) [1,2] and other allergies, including peanut allergy [3], as well as for the coexistence of atopic asthma or allergic rhinitis and AD [3,4,5]. Interestingly, the association between FLG deficiency and peanut allergy is not fully considered to be associated with coexistent AD. Filaggrin has been demonstrated to play a role in stratum corneum (SC) integrity as a structural protein that organizes keratin filaments. It is of note that lipid composition was aberrant in FLG-/- SC, whereas free fatty acid levels remained normal, and levels of ceramide and cholesterol were increased [6]. It is possible that aberrant lipid composition, as well as SC fragility, might have direct or indirect effects on the penetration of certain materials by affecting SC hydrophobicity. These data suggest that FLG deficiency constitutes a skin barrier defect that facilitates enhanced exposure of peanut allergen to antigen-presenting cells and subsequent sensitization, even in the absence of AD [1,2,4].

Epicutaneous immunotherapy (EPIT®) uses a novel Viaskin® patch technology containing peanut proteins (DBV Technologies, Paris, France) [7] and should be performed on intactskin, in order to ensure the efficacy and safety of the treatment by avoiding a massive free passage of the allergen into the blood stream but also to induce a tolerogenic immune profile [7,9]. EPIT® has proven to be effective in peanut-sensitized mice [8–12] and is safe and well tolerated by subjects with peanut allergy [13]. The efficacy has been investigated in a large DBPC phase II dose-finding trial (VIPES) in Europe and North America, and is currently being investigated in a pivotal Phase III program for the treatment of peanut allergy in children [14].

In view of the strong association of *FLG* mutations with peanut allergy (20% of cases) and the importance of skin integrity for EPIT® safety and tolerance induction, we soughta to investigate the association between FLG deficiency and peanut EPIT® efficacy.

2. Materials and Methods

2.1. Animals

All experiments were performed in the animal facility of the Faculty of Pharmacy (Châtenay-Malabry, France) according to the European Community rules on animal care and with permission

92-305 from the French Veterinary Services and with approval of French Ethic Committee number 26 (authorization 2012-091). For our experiments, we used a transgenic mice produced by Pr. Chambon [unpublished data]. His team generated *FLG* mutant mice in C57BL/6 background that were completely deficient in FLG (*FLG*-/-) and then we proceed to a backcross in a BALB/c background. The total loss of *FLG* expression was confirmed at mRNA level by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Figure 1a) and at protein level by immunohistochemistry in the skin (Figures 1b and 1c), upon methods published by Presland et al. [15] and Stout et al. [16],respectively. Atopic dermatitis (AD) was assessed by macroscopic observation in young *FLG* mutant pups and measurement of transepidermal water loss (TEWL) [Unpublished data]. In adult mice, TEWL is similar to Wild Type (WT) mice (respectively, mean 23.7 ± 0.6 vs 24.9 ± 0.6 $g/m^2/hr$).

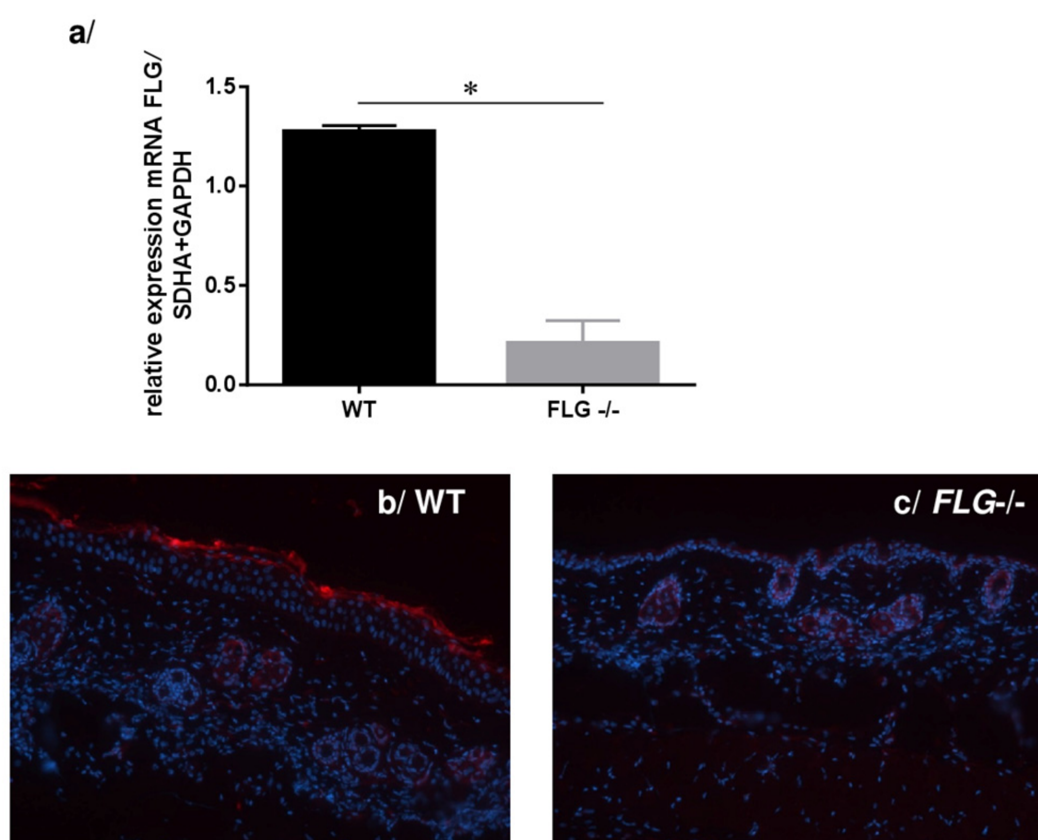

Figure 1. Assessment of Filaggrin deficiency in the skin of mice. Reduced levels of profilaggrin mRNA in the dorsal skin of FLG-deficient mice. (a) RT-qPCR was performed on total RNA isolated from WT and *FLG*-deficient (*FLG*-/-) mouse skins using primer pairs specific for profilaggrin (forward: 5'GAATCCATATTTACAGCAAA GCACCTTG3' and reverse: 5'GGTATGTCCAATGTGATTGCACGATTG3'). SDHA and GAPDH represent housekeeping genes for the relative normalization. Immunohistology of dorsal skin of WT and FLG deficient mice. Skin tissues were stained with polyclonal FLG antibody (PRB-471P, Covance) followed by an anti-rabbit secondary antibody labelled with APC, then analyzed by microscopy (×20). (b) normal and (c) *FLG*-deficient dorsal skin from mice. Note the complete absence of FLG expression in the epidermis of *FLG* deficient mice.

2.2. Treatment (Figure 2a)

After oral sensitization by gavages with peanut protein and cholera toxin, WT or *FLG*-/- mice were epicutaneously treated using a Viaskin® loaded with 100 µg peanut protein (EPIT) or a placebo Viaskin® (Sham) followed by sustained oral exposure to peanuts for 10 consecutive days as already published [8]. The application of the Viaskin® was done on the back of mice, 24 h after the removal of hairs as previously described [8,9]. Blood was taken on days 42 (the end of sensitization) and 102 (the end of EPIT and substained peanut oral exposure) to determine peanut-specific IgE and IgG2a levels by in house indirect ELISA [10,11]. After termination by cervical dislocation, spleen and esophagus were collected on day 102 for cellular responses and cell infiltration analysis. Splenocytes were restimulated in vitro in presence of peanut protein (100 µg/mL) for 72 h and cytokine levels were measured using Bioplex® cytokine assay (Biorad, Marnes-la-Coquette, France) according to the manufacturer's instructions. Eosinophil infiltration was assessed by histology using Hematoxylin Erythrosine Safran (HES) staining and eosinophils were blindly counted by a skilled European College of Veterinary Certified Pathologist. Results were expressed as number of eosinophils per mm^2. Negative control animals were naive WT or *FLG*-/- mice without sensitization and immunotherapy protocol.

2.3. Passive passage of allergen in the bloodstream (Figure 2b)

In order to evaluate the free passage to the bloodstream following epicutaneous administration of peanut protein, six-week-old sensitized WT or *FLG*-/- mice received a single application of Viaskin® loaded with 500 µg of peanut protein for 48 h (Viaskin-500). Epicutaneous application on tape-stripped skin (ie. stratum corneum removed by using iterative application on the same area of the skin of 6 adhesive tapes) and subcutaneous injections (200 µl containing 500 µg of peanut protein) were done as positive controls of bloodstream passage. Blood was collected by retro-orbital bleeding at different time points (0, 2, 8, 24, 48 h) to quantify Ara h 1 in serum using a commercial ELISA kit (Indoor Biotechnologies) as previously described [12]. The limit of quantification was determined as 7.8 ng/mL. Results were expressed in ng/mL as means ± SD for each group (8 mice per group).

2.4. Allergen delivery, capture and migration of DCs (Figure 2c)

The allergen delivery through the skin was assessed by microscopy 2 h and 24 h after application of a Viaskin® loaded with 100 µg of Alexa 488-conjugated OVA (OVA*) on intact skin of WT and *FLG*-/- mice. Skin below the OVA*-Viaskin was harvested for fluorescence microscopy. The immune uptake of OVA and migration of DCs were measured after sensitization using OVA*-Viaskin® applied onto intact skin of WT or *FLG*-/- mice (n = 5 per group). Positive control of allergen passive passage through the skin was obtained by applying the OVA* onto tape-stripped skin of WT mice. Negative control animals received an empty Viaskin® (naive WT mice). Inguinal LNs were harvested after 2 h to evaluate the absence of passive passage across the skin into lymphatics and 24 h for cell isolation and flow cytometry analysis of Alexa 488-positive $CD11c^+$ DCs to determine the cell-mediated passage into LNs.

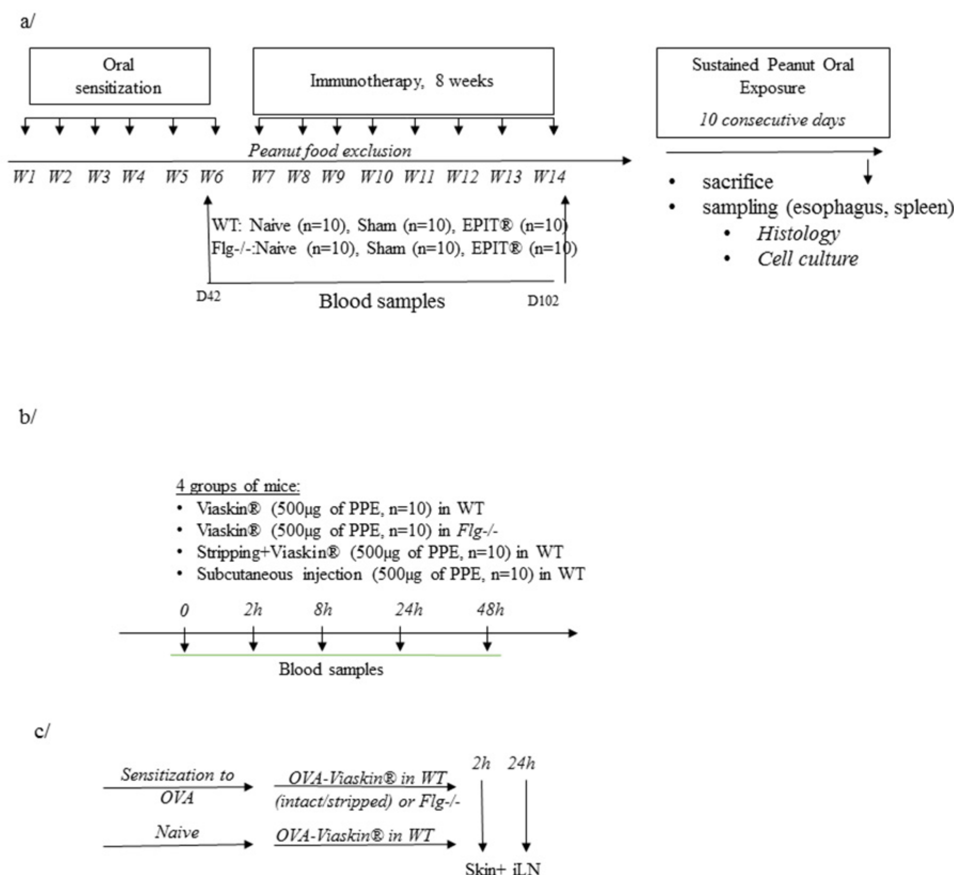

Figure 2. Study design. (a) Epicutaneous immunotherapy evaluated in Flg deficient mice; (b) Passive passage of major peanut allergen in bloodstream after peanut Viaskin® application; (c) Allergen (OVA*) delivery by Viaskin® patch, capture and migration of DCs.

2.5. Phenotype of migrating DCs

To characterize the phenotype of migrating DCs in LNs of WT or *FLG*-/- mice, the OVA*-Viaskin® was applied onto the back of sensitized mice (n = 5 per group) for 24 h. Control received OVA*-Viaskin® on stripped skin (stripped WT mice). Inguinal LNs were harvested and the expression of DC marker CD205^{high} and CD205^{low} was analyzed on Alexa 488-positive CD11c^{+} DCs.

2.6. Ancillary methods

Reverse-transcription quantitative PCR (RT-qPCR): Total RNA from skin was extracted using the RNeasy Mini Kit (Qiagen, France) following the manufacturer's instructions. cDNA was synthetized by reverse transcription (SuperScript II RNase H reverse transcription reagents, Invitrogen, France) containing 500 ng of RNA from the experimental samples. Quantitative PCR analyses in real time were performed with CFX (Biorad, France) for quantification. The thermal cycling conditions were: 95 °C for 5 min and then 45 cycles at 95 °C (5 s), 55° C (5 s) and 72 °C (10 s).

This was followed by the standard denaturation curve. The murine primers sequence for *Flg* is 5’ GAATCCATATATTTACAGCAAAGCACCTTG3’ (forward) and 5’GGTATGTCCAATGTGATT GCACGACGATTG3’ (reverse). Expression of results was normalized to housekeeping genes (GAPDH and SDHA).

Fluorescence microscopy: Skin tissue was harvested in Shandon cryomatrix (Thermo, France) and frozen in liquid nitrogen immediately after removing the Viaskin®. Cryostat sections (7 µm) mounted with Vectashield DAPI (Vactor lab, UK) were analyzed using a DMR microscope (Leica, France). Immunostaining for Flg was done with polyclonal antibodies (PRB-417P, Covance, UK) followed by an Alexa Fluor 555 anti-rabbit secondary antibody (A10042, Life Technologies, USA).

Flow cytometry: cell suspensions wereincubated with anti-mouse CD16/CD32 Fc Block (BD Biosciences) and then stained with CD11c, CD3, MHC II and B220 (BD Biosciences) and CD205 (eBioscience, UK) and their corresponding isotype control were also used. Flow cytometry was performed on FACSCalibur and analyzed using CellQuest software.

2.7. Statistical analysis

All statistical analysis were performed using the GraphPad Prism Software 6 (San Diego, CA, USA). Results are expressed as mean±standard deviation (SD). For histological analyses, cytokine responses and humoral responses, statistical analyses were determined by Kruskall Wallis and post-Dunn test when comparing all groups.

3. Results

3.1. Absence of allergen passive passage into bloodstream after EPIT application in FLG-deficient mice

We first evaluated whether a passive passage of Ara h 1 to the bloodstream could appear following application of a Viaskin® patch loaded with peanut protein on sensitized mice. When Viaskin-500 was applied on intact skin of sensitized *FLG*-/- or WT mice, no Ara h 1 was detected in the serum from 0 to 48 h (with a limit of quantification of the ELISA at 7.8 ng/ml). In contrast, when Viaskin-500 was applied on stripped skin, Ara h 1 was detected in the serum 2 h (39.5 ± 21.2 ng/mL) and 8 h (10.8 ± 5.4 ng/mL) after the application, in *FLG*-/- as well as in WT mice. In the positive control group (ie. mice treated by subcutaneous injection of 500 µg of peanut protein), a high quantity of Ara h 1 was detected from 2 h to 48 h, with a peak at 8 h (147.5 ± 20.6 ng/mL).

3.2. No modification of antigen capture and migration of DCs in LNs after EPIT application in filaggrin deficient mice

When the OVA*-Viaskin® was applied onto intact skin of WT and *FLG*-/- for 2 h and 24 h to evaluate the delivery of allergens through intact skin after Viaskin application, fluorescence was mainly observed in epidermis (Figure 3 a–d).

Two hours after application of OVA*-Viaskin® applied on intact skin of sensitized *FLG*-/- or WT mice, the proportion of Alexa 488-positive resident CD11c$^+$ DCs in LNs was not different from the background fluorescence in negative control naive WT mice, indicating that no passive passage

of OVA was observed in LNs and likely the immune uptake of OVA applied by the Viaskin® and migration of DCs would be the same. In contrast, the proportion of Alexa 488-positive resident CD11c^{+} DCs in LNs increased markedly when OVA*-Viaskin® was applied onto stripped skin (positive control, $p < 0.01$) (Figure 3e, Passive passage of allergens to LNs) in *FLG*-/- and WT mice.

After 24 h application of OVA*-Viaskin® on intact skin, aiming to evaluate the antigen capture and processing prior to entering the LNs, fluorescence was detected in cells from all skin layers in both *FLG*-/- and WT mice. MHC-II staining indicated that OVA was specifically captured by CD11c^{+} DCs (data not shown). The migration of these cells from the skin was confirmed by the appearance of Alexa 488-positive CD11c^{+} DCs in LNs of both *FLG*-/- and WT mice after 24 h. This was also seen when OVA* was applied onto stripped skin. In contrast, Alexa 488-positive CD11c^{+} DCs remained low in negative control naive WT mice (Figure 3e, Allergen uptake in the skin and migration of A488+ cells into LNs).

3.3. No difference of phenotype of migrating DCs after EPIT application in filaggrin deficient mice

Migrating DCs in sensitized WT mice consisted of two populations according to CD205 staining intensity, such as previously described: CD205^{low} DCs, which expressed low levels of CD86 and CD83 and CD205^{high}, CD86^{high}, and CD83^{high} migrating DCs [6]. We observed no difference of migrating DCs between *FLG*-/- and WT mice, i.e CD205^{high} DCs (Figure 3**,** Phenotype of migrating DCs). In contrast, migrating DCs of stripped WT mice were CD205^{low}.

3.4. Efficacy of EPIT in FLG-deficient mice

We then investigated the association between FLG deficiency and EPIT efficacy. On day 42, peanut protein sensitized *FLG*-/- mice, as well as peanut protein sensitized WT, a significant increase in specific IgE (Table 1) together with IL-5, IL-13 and IFN-γ secretion of in vitro restimulated splenocytes (data not shown), compared to naive *FLG*-/- or WT mice. Sensitization of *FLG*-/- mice was thus as efficient as that of WT mice using a standard procedure.

After 8 weeks of EPIT in *FLG*-/- and in WT mice, specific IgE significantly decreased ($p < 0.05$) while specific IgG2a significantly increased compared to sham and negative control groups ($p < 0.01$) as previously described [10]. Peanut stimulated splenocytes from naive *FLG*-/- or WT mice did not secrete cytokines. Peanut stimulated splenocytes from sham *FLG*-/- and WT mice reactivated in vitro with peanut for 72 h secreted high levels of IL-5 ($p < 0.05$), IL-13 ($p < 0.05$) and IFN-γ ($p < 0.05$) compared to naive mice. EPIT significantly decreased Th2 cytokines secretion, both in EPIT *FLG*-/- and WT mice, respectively, IL-5 ($p < 0.05$) and IL-13 ($p < 0.05$). No cytokine secretion was detected by restimulation in medium alone (data not shown).

To investigate the potential protection of EPIT in *FLG*-/- mice against allergen exposure, all treated and non treated mice were subjected to peanut oral exposure which induced inflammation and eosinophil infiltration in the esophagus. The eosinophil infiltration in the esophagus of EPIT treated *FLG*-/- and WT mice was significantly lower than in sham treated *FLG*-/- and WT mice ($p < 0.01$) and reached similar values compared to naive mice.

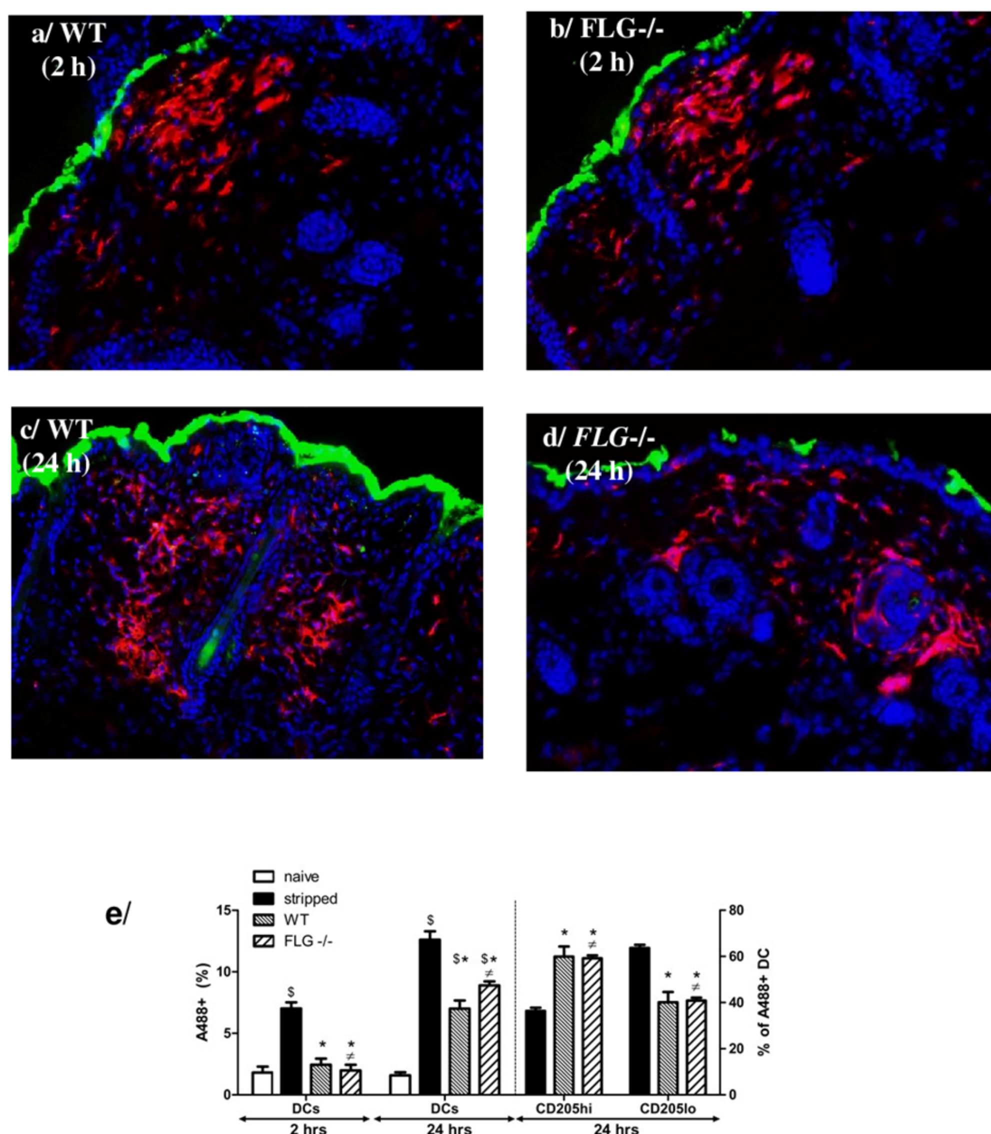

Figure 3. Study of allergen uptake in the skin. a–d, Fluorescence microscopy after 2 h and 24 h application of OVA-A-488-Viaskin patch respectively on (a and c) normal and (b and d) *FLG*-deficient dorsal skin from mice (original magnification ×20; green: OVA; blue: DAPI and red: MHC-II). e, Passive passage of allergens to LNs (left part of the graph, 2 h) and allergen uptake in the skin and migration of $A488^+$ cells into LNs (left part of the graph, 24 h). OVA*-Viaskin® were applied onto intact skin of either WT or *FLG-/-* mice and the presence of Alexa 488-positive resident $CD11c^+$ DCs in inguinal LNs was analyzed by flow cytometry 2 h later. Positive control was obtained by applying the OVA*-Viaskin® onto stripped skin of WT mice (n = 5). Negative control received a placeboViaskin® (naive WT mice, n = 5). Data represent mean ± SD and are representative of three independent experiments. $: stripped vs naive, $p < 0.01$; * WT or FLG vs stripped, $p < 0.01$; ≠ FLG vs WT, ns.Phenotype of migrating DCs (right part of the graph, 24 h). Cells, isolated from the inguinal LNs of WT or *FLG-/-* mice that received OVA*-Viaskin® for 24 h, were gated on Alexa 488-positive $CD11c^+$ DCs, and expression of CD205 was analyzed. Control received OVA*-Viaskin® on stripped skin (stripped WT mice). Data represent mean ± SD and are representative of three independent experiments. * $p < 0.01$, ** $p < 0.001$.

4. Discussion

Several immunotherapeutic options are being investigated and EPIT® appears promising to treat peanut allergic patients in Phase II clinical trials [14]. Filaggrin mutations have been implicated in the development of peanut allergy, with filaggrin-deficient peanut allergic subjects representing 20% of cases [3]. Since disruption of the skin barrier might interfere with EPIT® [12], we took advantage of preclinical models of peanut-sensitized mice to test whether FLG loss of function might alter the efficacy of EPIT® [8–12] and showed that the absence of FLG does not reduce the desensitizing capacity of EPIT® in this model.

Filaggrin is a major structural protein in the stratum corneum layer of the epidermis and is produced as the precursor profilaggrin. Mutations in the *FLG* gene are the most significant known genetic risk factor for the development of AD [1,2]. More interestingly, Brown et al. [3] identified that *FLG* mutations confer a risk for peanut allergy, even in the absence of AD. These data suggest a barrier defect that facilitates enhanced exposure of peanut allergen to antigen-presenting cells and subsequent sensitization in FLG-deficient human subjects, even in the absence of AD.

We first confirmed that oral sensitization with peanut protein and cholera toxin in the selected animal model, ie. FLG-null (*FLG*-/-) mice, was similar to WT mice using a standard procedures [10]: the significant increase of peanut protein-specific Th2 biased immunological responses is is in accordance with the observation that general immunity through intraperitoneal sensitization with OVA was comparable between *ft/ma* and control mice [18].

This mouse model among other ones has been selected based on the proposed rationale. Flaky tail/matted (*ft/ma*) mice display a matted hair phenotype due to a loss-of-function mutation in Tmem79 [18] and have spontaneous dermatitis with increased IgE levels under specific pathogen-free conditions [15]. They also express truncated profilaggrin and significantly reduced mature FLG [18] suggesting that FLG deficiency might explain the propensity of *ft/ma* mice to spontaneously have dermatitis in analogy with the situation in human subjects affected with AD [19,20]. Therefore,they have been widely used over the years to investigate pathogenic mechanisms of AD in the context of FLG deficiency [21,22]. However, two recently published studies report that the Tmem79 mutation, rather than the deletion of *FLG*, was found to be associated with the spontaneous dermatitis phenotype in flaky tail mice [19,22]. Furthermore, these mice do not show complete loss of FLG [23]. In this context, we used genetically engineered *FLG*-null mice that were characterized by a total loss of *FLG* expression at mRNA and protein levels. These *FLG*-/- mice have altered stratum corneum barrier function but do not develop spontaneous dermatitis under specific pathogen-free conditions as already described [24]. Skin lesions are possible in young mutant pups (up to 3 weeks of age) and in older ones (after one year of age).

In contrast, *ft/ma* mice are predisposed to develop an allergen-specific immune response after epicutaneous sensitization with the clinically relevant allergen OVA: the topical application of OVA to *ft/ma* mice resulted in cutaneous inflammation and enhanced cutaneous allergen priming with development of systemic Th2 immune responses [17,21]. In *FLG*-/- generated by Kawasaki et al. [23], antigens also penetrated the stratum corneum more efficiently, leading to enhanced responses in hapten-induced contact hypersensitivity and higher serum levels of anti-OVA IgG1 and IgE [23]. In the same way, application of allergen on disrupted skin of naive mice induced sensitization [24,25,26]. These data provide experimental evidence that antigen transfer through a defective epidermal barrier is a key mechanism underlying increased IgE sensitization and initiation

Table 1. Humoral, cellular responses and cell infiltration after oral exposure to peanuts.

		Humoral responses						**Cellular responses**			**Cell Infiltration**
		Specific IgE (µg/mL)		**Specific IgG1 (µg/mL)**		**Specific IgG2a (µg/mL)**		**IL-5 (ng/mL)**	**IL-13 (ng/mL)**	**IFN-g (ng/mL)**	**Eosinophil infiltration in esophagus/mm^2**
		D42	***D102***	***D42***	***D102***	***D42***	***D102***	***D102***	***D102***	***D102***	***D102***
WT	EPIT	0.29 ± 0.04	0.17 ± 0.01*	231 ± 66	351 ± 88	1.5 ± 0.06	4.3 ± 0.18**	137.8 ± 81.6*	204.8 ± 71.6*	40.3 ± 12.1	14.5 ± 2.8**
	Sham	0.25 ± 0.03	0.23 ± 0.02	225 ± 85	226 ± 49	1.1 ± 0.13	1.2 ± 0.23	408.5 ± 144.1	596.5 ± 167.6	30.5 ± 21.3	49.7 ± 8.4
	naive	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	11.3 ± 1.7
FLG -/-	EPIT	0.28 ± 0.02	0.19 ± 0.03*	298 ± 22	315 ± 56*	0.95 ± 0.14	3.8 ± 1.1**	215.4 ± 44.2*	492.3 ± 83.8*	87.1 ± 29.3	16.9 ± 2.7**
	Sham	0.27 ± 0.03	0.26 ± 0.08	323 ± 52	176 ± 63	1.1 ± 0.16	1.2 ± 0.51	718.8 ± 140.6	957.8 ± 208.6	68.9 ± 36.9	50.8 ± 14.5
	naive	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	11.2 ± 3.2

LOD: IgE = 0,024 µg/mL, IgG1 = à 0,004 µg/mL et IgG2a = 0,004 µg/mL.

IgE and IgG2a responses to peanut protein. Blood was harvested at the end of sensitization (D42) and at the end of EPIT and sustained peanut oral exposure (D102) of naive, sham-treated and EPIT WT or *FLG*-/- mice. IgE and IgG2a antibodies reactive to peanut protein were measured by ELISA. Data are expressed as means ± SD for each group of 8 mice. * $p < 0.05$, naive vs sham at D42 for WT or FLG-/- mice.* $p < 0.05$, ** $p < 0.01$ EPIT vs sham at D102 for WT or FLG-/- mice.

Cytokine production by peanut protein-restimulated splenocytes.Spleen was harvested at the end of EPIT of naive, sham-treated and EPIT WT or *FLG*-/- mice. Cytokines production in splenocytes in presence of peanut protein for 72 h were measured by the Bioplex cytokine assay. Data are represented as means ± SD for each group of 8 mice. * $p < 0.05$, EPIT vs Sham at D102 for WT or FLG-/- mice.

Eosinophil infiltration. Esophagus was collected for histological analysis of eosinophil infiltration using HES staining at D102.Results are expressed as number of eosinophils per mm^2 and data are represented as means ± SD for each group of 8 mice. ** $p < 0.01$.

of cutaneous inflammation. This has been illustrated in humans by Lack et al. [27], showing that the exposure of the skin to peanut proteins may facilitate the sensitization process in very young children, when the normal architecture of the skin is altered by local or generalized eczema.

On the other hand, EPIT® needs the integrity of the superficial layers of the stratum corneum to ensure safety and to induce a tolerogenic immune profile. Indeed, application of a Viaskin® on intact skin, in contrast with stripped skin, enhances the skin hydration and results in allergen diffusion through the superficial layers of the epidermis by preserving its architecture [7,9]. We used the model of peanut specific EPIT® in sensitized WT mice, with well characterized decrease of specific IgE, increase of specific IgG2a, lower production of Th2 cytokines and decrease of local eosinophil recruitment using various allergens (peanut, pollen, ovalbumin, house dust mites) [8–12]. *FLG*-/- mice showed similar EPIT® efficacy, thus strongly suggesting that EPIT® can be used effectively to treat allergic patient with FLG deficiency.

We next demonstrated that *FLG*-/- mice show no difference with WT mice for free passage of allergen (i.e absence of passive passage of Ara h1 to bloodstream) following EPIT® application of peanut protein by Viaskin®. This contrasts with skin stripping, which leads to passive and increased passage of allergen through the skin and the lymphatics [9] resulting in systemic spread in vivo, completely modifying the biodistribution of allergen. FLG deficiency, although altering the skin barrier, thus appears different from skin stripping, which alters the activation of Langerhans cells, triggers mechanical injuries, activates keratinocytes and upregulates thymic stromal lymphopoietin expression by keratinocytes and mRNA expression of inflammatory cytokines. All these processes, observed after skin stripping, are involved in the polarization of skin DCs to elicit a Th2 response. In *FLG*-/- mice, the allergen is captured by $CD205^{high}$ DCs in the superficial layers of the stratum corneum of the epidermis, which migrate through the dermis into afferent LNs, as it was previously shown in WT mice [8].These results suggest that *FLG*-/- mice are not different than WT mice in terms of (i) absence of passive passage of allergen, (ii) antigen uptake by DCs and (iii) migration of DCs to draining LNs.

5. Conclusion

This study demonstrates for the first time the efficacy of EPIT® in *FLG*-deficient mice and suggests that EPIT® may be a safe and effective treatment for allergic patients with a FLG deficiency.

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Declarations

Ethics approval

All experiments on mice were performed according to the European Community rules on animal care and with permission 92-305 from the French Veterinary Services and with approval of french Ethic Committee number 26 (authorization 2012-091).

Consent for publication

All co-authors gave their consents for data publication..

Availability of data and material

The datasets supporting the conclusions of this article are included within the article. For more detailed information, please contact author for data requests.

Competing interests and funding

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Christophe Dupont and Pierre-Henri Benhamou received honoraria and/or compensation in regards to the study, as investigators, coordinators or experts, in relation with the time spent on the study.

The funders had a role in study design, decision to publish, and preparation of the manuscript. This does not alter our adherence to AIMS policies on sharing data and materials.

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