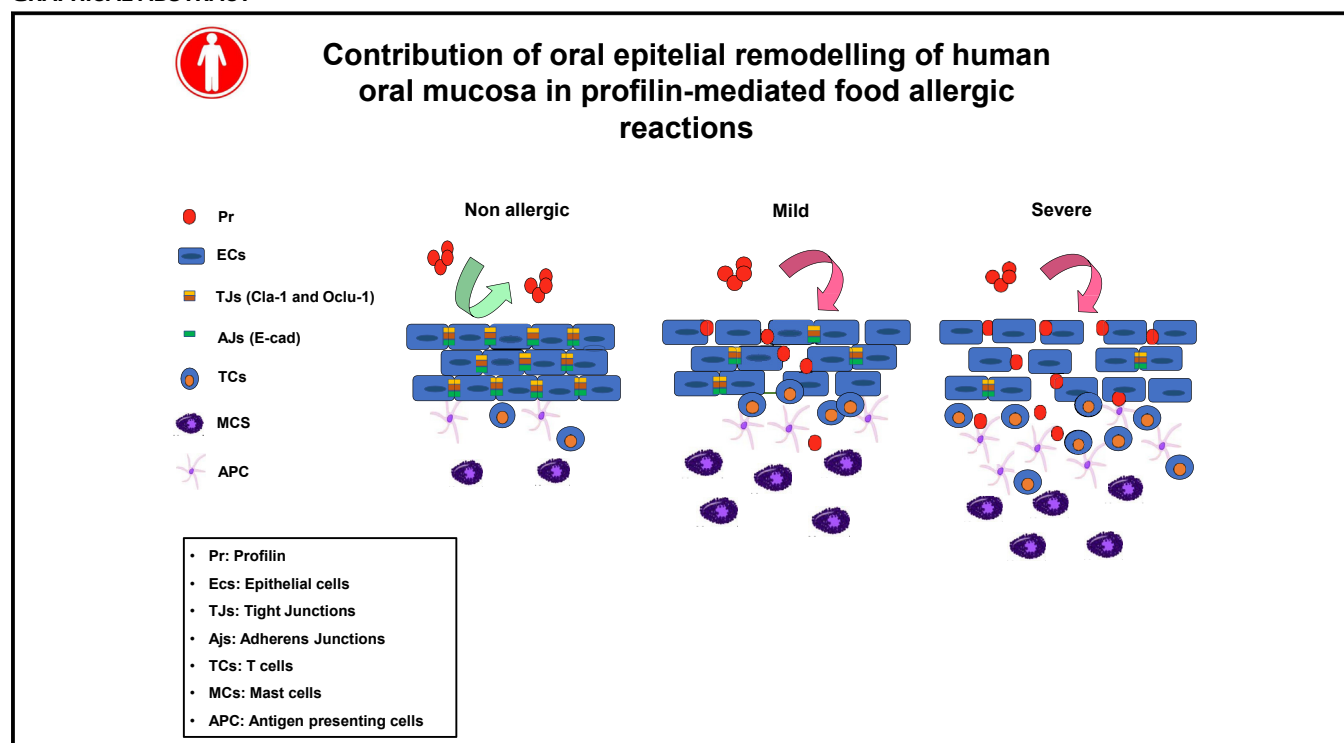


Profilin-mediated food-induced allergic reactions are associated with oral epithelial remodeling



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GRAPHICAL ABSTRACT



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Background: In areas of high exposure to grass pollen, allergic patients are frequently sensitized to profilin, and some experience severe profilin-mediated food-induced reactions. This specific population of patients is ideal to study the relationship between respiratory and food allergies.

Objective: We sought to determine the role of oral mucosal epithelial barrier integrity in profilin-mediated allergic reactions.

Methods: Thirty-eight patients with profilin allergy stratified into mild or severe according to their clinical history and response to a profilin challenge test and 6 nonallergic subjects were recruited. Oral mucosal biopsies were used for measurement of CD11c, CD3, CD4, tryptase, claudin-1, occludin, E-cadherin, and vascular endothelial growth factor A levels; Masson trichrome staining; and *POSTN*, *IL33*, *TPSAB*, *TPSB*, and *CMA* gene expression analysis by using quantitative RT-PCR. Blood samples were used for basophil activation tests. **Results:** Distinct features of the group with severe allergy included the following: (1) impaired epithelial integrity with reduced expression of claudin-1, occludin, and E-cadherin and decreased numbers of epithelial cells, which is indicative of acanthosis, higher collagen deposition, and angiogenesis; (2) inflammatory immune response in the mucosa, with an increased number of CD11c⁺ and CD4⁺ infiltrates and increased expression of the cytokine genes *POSTN* and *IL33*; and (3) a 10-fold increased sensitivity of basophils to profilin. **Conclusions:** Patients with profilin allergy present with significant damage to the oral mucosal epithelial barrier, which might allow profilin penetration into the oral mucosa and induction of local inflammation. Additionally, severely allergic patients presented with increased sensitivity of effector cells. (J Allergy Clin Immunol 2019;143:681-90.)

Key words: Allergy, inflammation, oral mucosa, epithelial remodeling, grass pollen, profilin

Although most prevalent food allergies during early childhood are caused by foods with a high allergenic protein content, such as milk, egg, nuts, or fish, in a later phase, food-induced allergic reactions connected with previously established respiratory allergies progressively lead the food allergy landscape.

Profilin, a pollen-related allergen, plays a relevant role in selected regions, with grass pollen dominating allergy.^{1,2} Its relevance has been demonstrated as an aeroallergen,³ but in general, it plays a limited role as a food allergen because of its low stability and is easily processed by proteases and denatured in acidic conditions. However, it is able to sensitize some allergic patients with pollinosis and is normally associated with disease progression.⁴⁻⁶ Interestingly, an increased prevalence of profilin sensitization has been reported (up to 60% in some regions) in patients with pollen allergy overexposed to grass pollen and subsequent development of severe profilin-mediated, food-induced allergic reactions.⁷

In an attempt to understand this phenomenon, we formulated the hypothesis that the oral mucosa could play a pivotal role in the development of severe food-induced allergic reactions. The oral mucosa lines the inside of the mouth and consists of stratified squamous epithelia, which are primarily nonkeratinized (mucosal lining),⁸ and a highly vascularized connective tissue called the lamina propria. Epithelial integrity is maintained by junctional complexes, such as adherens junctions (AJs) and tight junctions (TJs).⁹ In addition to being a physical barrier, the oral mucosa

Abbreviations used

AJ:	Adherens junction
APC:	Antigen-presenting cell
BAT:	Basophil activation test
DBPCFC:	Double-blind, placebo-controlled food challenge
FFPE:	Formalin-fixed paraffin-embedded
SPT:	Skin prick test
TJ:	Tight junction

contains immune cells that maintain mucosal homeostasis.^{8,10} In fact, the mucosal epithelium is known to play a key role in the immune-protective system of the mucosa.¹¹ Likewise, inflammatory factors, such as IFN- γ and TNF- α , have been reported to disrupt epithelial integrity through downregulation of TJ proteins, increasing epithelial permeability.¹²

Generation of epithelium-derived cytokines, such as thymic stromal lymphopoietin, IL-25, and IL-33, leads to T_H2 immunity.¹³ In particular, IL-33 activates group 2 innate lymphoid cells and mast cells¹⁴ during allergic reactions. Another cytokine associated recently with epithelial tissue damage is periostin, which provides signals for tissue development and remodeling.¹⁵⁻¹⁷

Mucosal tissues also host a wide variety of specialized immune cells, the so-called mucosa-associated lymphoid tissue. Although little is known about the immune processes taking place in the oral mucosa, there are a few reports describing the location of intraepithelial dendritic cells in the basal region of the oral epithelium.¹⁸ These cells are located mainly in perivascular spaces in the epithelial layer and are more abundant than in the skin.¹⁹

Additionally, effector cells (ie, mast cells and basophils) in the bloodstream are central to allergic pathologies among others.^{20,21} Moreover, basophils are an essential tool for the basophil activation test (BAT), a diagnostic tool that assesses the sensitivity of a patient to a given allergen.²²

In this study we postulate a role for the oral mucosa in the development of severe food-induced allergic reactions to profilin observed in patients overexposed to grass pollen and sensitized to multiple grass allergens. We provide evidence that extensive remodeling of the oral mucosa associated with epithelial barrier disruption and release of inflammatory signals occurs in patients with severe allergy. In addition, increased effector cell sensitivity was also observed.

METHODS

Patients

Thirty-eight patients (18-58 years old) with a clinical history of suspected food allergies and 6 nonallergic subjects were included in the study. The patients were studied in the allergy departments of 4 hospitals: Hospital Universitario de La Princesa (Madrid, Spain), Hospital Universitario Clínico San Carlos (Madrid, Spain), Hospital HM Sanchinarro (Madrid, Spain), and Hospital Virgen del Puerto de Plasencia (Cáceres, Spain). All patients were subjected to a thorough interview to establish their clinical history. Allergic patients who received any pollen extract immunotherapy in the last 5 years, presented with uncontrolled asthma or diseases, or had received any other treatment making them unsuitable for profilin challenge were excluded. All subjects provided written informed consent, and protocol approval was obtained from the research and ethics committees of the hospitals involved in this study. Allergic patients included in the study displayed a negative skin

prick test (SPT) response to lipid transfer proteins and a positive SPT response to profilin. Results were negative for specific IgE to Pru p 3 and Bet v 1 by using ImmunoCAP and positive for profilin by using ImmunoCAP ISAC (Phadia, Uppsala, Sweden). The clinical features of the studied population are shown in Table I.

SPTs

All subjects underwent SPTs with a battery of commercial pollen and palm tree pollen profilin (Pho d 2; ALK-Abelló S.A, Madrid, Spain). SPTs were performed according to standard procedures.²³ A positive response corresponded to a mean wheal diameter of at least 3 mm greater than that elicited by the negative control 15 minutes after puncture. Data for pollen and food allergen sensitization and sizes of Pho d 2 SPT reactions are compiled in Table I.

Oral allergen challenge

Oral challenges were performed outside the grass pollen season, according to the procedure developed by Alvarado et al,⁷ by using single 2-mL dose vials with increasing concentrations of purified Pho d 2 (0.074, 0.74, 7.4, 74, and 740 µg/mL) and placebo (phosphate buffer, pH 6.5). Placebo and active doses were administered on the same day. Allergic patients received 9 serial doses of profilin, whereas nonallergic subjects received 2 doses. Oral challenge was always started with a placebo dose. A second placebo dose was intercalated with the active dose combined in a randomized table for doses between 2 and 7 to ensure that double-blind conditions were maintained. The double-blind, placebo-controlled food challenge (DBPCFC) was performed according to European Academy of Allergy and Clinical Immunology guidelines.²³ Increasing doses were administered every 20 minutes until a final dose was reached or until patients presented with clinical objective reactions to oral challenge or subjective intense reactions resulting in the patient's refusal to continue. Nonallergic subjects first received a placebo dose, which was followed by an active dose (74 µg/mL).

The oral challenge result was considered positive when at least 1 of the following symptoms was detected: objective symptoms, such as uvula edema, urticaria and/or angioedema, respiratory symptoms with decreased FEV₁, and gastrointestinal symptoms (vomiting or diarrhea), or subjective symptoms (persisting >45 minutes), such as abdominal pain, oral pruritus, and dyspnea without associated FEV₁ decrease. Side reactions during oral challenge were treated with antihistamines, corticosteroids, β-agonist medications, or adrenaline, according to clinical guidelines. Data for oral allergen challenge tests are shown in Table E1 in this article's Online Repository at www.jacionline.org.

Of the 44 subjects recruited (38 allergic patients and 6 nonallergic subjects), 40 were subjected to DBPCFCs with Pho d 2. In the remaining 4 patients, oral challenge was not prescribed because of the severity of the reported reactions in the clinical history.

Classification criteria

Classification criteria were determined based on the patient's clinical history of food allergy and the observed reaction during profilin oral challenge: mild reactions corresponded to objective local reactions, such as oral allergy syndrome, angioedema, and oral pruritus; severe reactions were systemic (eg, urticaria and asthma). Thus patients with a history of mild food-induced reactions and mild reactions to profilin oral challenge were included in the mild group. Patients with a history of severe reactions to food but mild reactions to profilin oral challenge were included in the mild group. Patients with a history of severe reactions to food and severe systemic reactions to profilin on oral challenge were included in the severe group. Patients with a history of severe reaction to food and mild reactions to nonsystemic severe reactions (eg, uvula edema) during profilin oral challenge who did not complete the test were included in the severe group, and patients with a history of profilin-mediated anaphylaxis who were not suited for oral challenge were included in the severe group. Nonallergic subjects were included as control subjects.

Oral mucosal biopsies and histologic analysis

Oral mucosal biopsy specimens were obtained from the cheek lining of 30 allergic patients and 6 nonallergic subjects by using a 5-mm surgical punch. All biopsy specimens were fixed in 4% paraformaldehyde and embedded in paraffin for histologic analysis. All sections were stained with hematoxylin and eosin and Masson trichrome, according to the manufacturer's protocol. Two expert pathologists performed a blind histologic analysis of each sample analyzing epithelial thickness, acanthosis, and collagen fiber deposition. Immunohistochemistry was performed with the Bond Polymer Refine Detection Kit on a BOND-MAX Automated IHC/ISH Stainer (Leica Biosystems, Wetzlar, Germany), according to the manufacturer's protocol. The following antibodies were used: rat anti-human CD3 (MCA1477), mouse monoclonal anti-human CD4 (NCL-L-CD4-368), mouse monoclonal anti-human CD11c (NCL-L-CD11c-563), mouse monoclonal anti-human claudin-1 (ab56417), rabbit recombinant monoclonal (EPR20992) to occludin (ab216327), Bond Ready-To-Use Primary Antibody E-Cadherin (36B5 [PA0387]), rabbit polyclonal to vascular endothelial growth factor A (ab46154), and rabbit monoclonal anti-human mast cell tryptase (134931) antibodies.

Sample scanning was performed with a Leica SCN400 slide scanner (Leica Biosystems). Pictures were captured with a Leica DM5500 microscope and a Leica CTR6000. Image analysis was performed with ImageJ software (version 1.51; National Institutes of Health, Bethesda, Md). For all analyses, a sufficient number of images covering the whole biopsy specimen were captured. Every picture was separated into different colors by using the plugin "Color Deconvolution," choosing the appropriate original staining (eg, Masson trichrome) and selecting the channel of interest. The appropriate threshold value was applied to all pictures from the same biopsy specimen. The final value was calculated as the average of all measurements per sample.

RNA isolation from formalin-fixed, paraffin-embedded biopsy specimens and quantitative RT-PCR

RNA isolation from formalin-fixed, paraffin-embedded (FFPE) sections was performed by using several slides with a thickness of 10 µm for each sample. Total RNA and microRNAs were extracted from FFPE sections with the miRNeasy FFPE Kit (217504; Qiagen, Hilden, Germany), according to the manufacturer's protocol. Total RNA was retrotranscribed into cDNA with a High Capacity RNA-to-cDNA kit (4387407; Applied Biosystems, Foster City, Calif). SYBR Premix Ex TaqTli RNaseH Plus (RR420A; Takara Clontech, Mountain View, Calif) was used for quantification of double-stranded DNA by using quantitative RT-PCR. Oligonucleotides for selected genes were designed by using Roche software (Roche, Mannheim, Germany).

BATs

All tests were carried out within 4 hours after blood sampling. Briefly, 100 µL of heparinized whole blood and 100 µL of stimulation buffer were added to all the tubes. Subsequently, 10 µL of increasing profilin concentrations (0.2 µg, 0.02 µg, 2 ng, 0.2 ng, 0.02 ng, and 2 pg) was added to different tubes of the same study subject. Basophil activation was measured as upregulation of CD63 on CD203c⁺ basophils with the BasoFlowEx Kit (EX-BIO, Vestec, Czech Republic) by using flow cytometry according to the manufacturer's procedures. Samples were analyzed with an Attune Acoustic Focusing Cytometer (Applied Biosystems). The half-maximal effective concentration (ie, the concentration of an allergen able to activate 50% of the basophils) was calculated for each subject.

Statistics

GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif) was used for statistical analysis. The Mann-Whitney *U* test and 1-way ANOVA Kruskal-Wallis test, followed by the Dunn multiple comparison test, were used where appropriate to compare data among experimental groups. For correlation analysis, Spearman correlation was applied. A *P* value of less than .05 was considered significant for all analyses.

TABLE I. Detailed information on study population, clinical history of the patients, and SPT results

	Age (y)	Sex	Self-reported food	Self-reported reactions	SPT results		
					Pollen (POS/9)	Food	Pho d 2 (mm)
Control subjects	34	M	None	None	Negative	Negative	Negative
	27	F					
	29	F					
	35	F					
	36	F					
	38	F					
Mild	26	M	M, W, B	OAS, LA	POS7	M, O, T	10
	28	M	M, T, O	OAS, EU	POS8/9	M, T	10
	29	M	P, Co, T, M, W	OAS, UR	POS8	M, T	16.5
	29	M	None	None	POS6	M, B, T	8
	30	M	M, W, S, A, Pe, B, Av, Ch, Co	OAS, NA, VO, OP	POS5	M, T	8
	37	M	None	None	POS5	T	6
	37	M	M	OAS	POS5/9	M	3.5
	38	M	K, Le, Ma, M, Pi, S	OAS	POS7	M, O, T	7
	53	M	None	None	POS8	Negative	9
	18	F	M, W, Al, P, K, B, Wa, H, Ti	OAS, UR, LA	POS8/9	M	6
	19	F	M, Pe, S	OAS	POS6	T	10
	25	F	M, Pi	OAS	POS7	Negative	6
	25	F	Ma, Cu, M	OAS, RESP	POS7	Negative	11
	27	F	M, W	OAS, ERY	POS5/9	M	8
	28	F	M, W, Che	OAS, DY	POS6	M, T	9
	31	F	M, W	OAS	POS7	M, T	15
	33	F	T, P	NA, VO	POS5	Negative	6.5
	33	F	S, Al, K, Av, Pi, Cn, O, Ma, H, Wa, Pn, T, M	OAS, RESP	POS5	Negative	5
	34	F	S, A	ERY	POS8	M, T	9
	37	F	M, Ma, W	OAS, VO	POS7	M, T	10.5
	41	F	P, Pe, Ap, Che, K, B, Av, Pi, Ma, Pn, M, W	ERY, GP, NA, OAS, RESP	POS5	T, K	7
	44	F	L	AS, EP, HY, NA, RHI, UR, VO	POS7	L	5
	44	F	P, Pe, S, Q, K, B, Av, Pi, Cn, O, F, Wa, Au, T, Cu, M	OAS	POS6	M	7
	45	F	C, M, W, Pi, Ch, Wa	OAS	POS3	M, C, T	5
	58	F	M	OAS, CS	POS 8/9	M	9
Severe	18	M	M	DSP, OAS	POS 8/9	M	10
	22	M	M, S	AS, OAS	POS5	M	12
	18	F	M, W, P, A, Ap, S, Al, G, K, B, Pi, O, Ma, Wa, Pn	OAS, DY, EU, EP, PB	POS8/9	M	14.5
	18	F	M, W, P, Pe, Ap, G, B, Pi, O, Ma	OAS, UR, LA, PA	POS8/9	M	10
	20	F	Ma, T	OAS	POS6	M, T	8
	23	F	M, T	OAS, EU	POS8/9	M, T	10
	25	F	M, W	ABP, OAS, DY, DSP, EP, EU, PO, NA, VO	POS8	M	17
	26	F	L, M, T	AS, EU	POS5	M, T	10
	28	F	M, W	CG, DY, EU, OAS, PO	POS7	M	11
	41	F	M	AS, EU, UR	POS7	M, T	7
	43	F	B, T	OAS, GP	POS8	A, M, T	7
	44	F	M	OAS, ERY, CG, PO, EP	POS7	M	6.5
	48	F	M	ERY, DP, PO, TO, VO	POS6	M, T	7

ABP, Abdominal pain; ANA, anaphylaxis; AS, asthma; CG, cough; CS, canker sores; DI, diarrhea; DP, dyspnea; DSP, dysphagia; DY, dysphonia; EP, epigastric pain; ERY, erythema; EU, edema uvula; F, female; GP, gastric pain; HA, headache; HY, hypotension; LA, labial angioedema; M, male; NA, nausea; ON, oral enanthem; OP, oral pruritus; PB, pharyngeal bolus; PO, pharyngeal occupation; RESP, Respiratory; RHI, rhinitis; OAS, oral allergy syndrome; TO, thorax oppression; UR, urticaria; VO, Vomit.

Food: A, Apple; Al, almond; Ap, apricot; Au, aubergine; Av, avocado; B, banana; C, carrot; Ch, chestnut; Che, cherry; Cn, cashew nut; Co, coconut; Cu, cucumber; F, fig; G, grape; H, hazelnut; K, kiwi; L, Lettuce; Le, Lemon; M, melon; Ma, mandarin; P, peach; Pe, pear; Pg, pomegranate; Pi, pineapple; Pn, peanut; Po, potato; Q, quince; S, strawberry; O, orange; T, tomato; Ti, tigernut; W, watermelon; Wa, walnut; Z, zucchini.

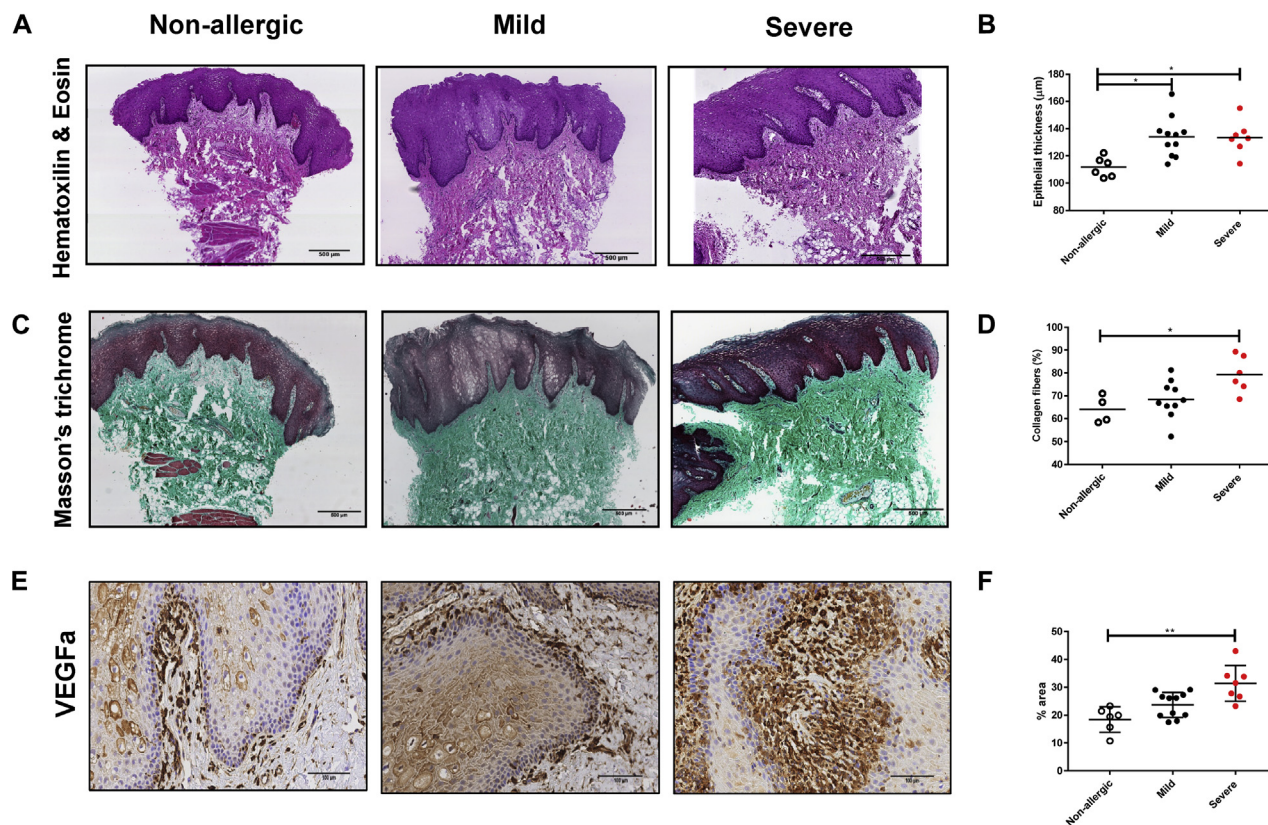


FIG 1. Histologic analysis of oral mucosal biopsy specimens. FFPE biopsy specimens from cheek lining of the oral mucosa from nonallergic subjects and patients with mild or severe profilin allergy were used. **A**, Representative oral mucosal section of each experimental group is shown. **A**, Hematoxylin and eosin staining. Images were captured at $\times 3$ magnification. **B**, Quantification of epithelial thickness in oral mucosal biopsy specimens from the 3 experimental groups. For quantification, multiple measurements were made in each sample and normalized in accordance with the number of epithelial layers. The final value was calculated as the average of all measurements per sample. **C**, Masson trichrome staining. Images were captured at $\times 3$ magnification. **D**, Quantification of collagen fiber deposition measured in Masson trichrome-stained samples. **E**, Vascular endothelial growth factor A (VEGF-A) staining. Images were captured at $\times 20$ magnification. **F**, Angiogenesis quantification based on VEGF-A staining. Data are presented as scatter plots for nonallergic subjects and patients with mild or severe allergy. * $P < .05$ and ** $P < .01$.

RESULTS

Patients with severe profilin allergy show epithelial acanthosis, higher collagen fiber deposition, and increased angiogenesis in the oral mucosa

We aimed to analyze whether the oral mucosal barrier might be compromised, thus potentially facilitating access to the immunologic system of profilin and accounting for increased sensitivity to profilin. Hematoxylin and eosin staining, together with blind evaluation by 2 independent pathologists (Fig 1, A), showed significant thickening of the epithelium in the mild and severe allergy groups compared with nonallergic subjects (Fig 1, B). This epithelial deformation is called acanthosis, a cellular hyperplasia in the intermediate layers of the stratified epithelium. Masson trichrome staining (Fig 1, C) revealed a significantly greater density of collagen fibers in the connective tissue of biopsy specimens from the severe group, whereas nonallergic subjects and those with mild allergy presented a loose connective tissue (Fig 1, D).

We also confirmed the increased angiogenesis observed by the pathologists using vascular endothelial growth factor A staining (Fig 1, E) in patients with severe profilin allergy compared with nonallergic subjects (Fig 1, F).

These results demonstrate remarkable histologic differences in the oral mucosa from profilin-sensitized patients compared with nonallergic subjects, which is indicative of epithelial remodeling.

Epithelial integrity in the oral mucosa of patients with severe profilin allergy is severely damaged

We aimed to analyze whether the oral mucosal barrier might be compromised, thus potentially facilitating access to the immunologic system of profilin, accounting for an increased sensitivity to it. The results in Fig 2, A, showed that although claudin-1, a TJ protein, was expressed homogeneously in the oral mucosal epithelium of nonallergic subjects, in patients with mild and severe allergy, claudin-1 was distributed in patches throughout the epithelium and showed reduced expression. Staining quantification revealed that claudin-1 expression was significantly less in patients with mild allergy compared with that in nonallergic subjects (Fig 2, B).

Because claudin-1 expression is not exclusive of epithelial cells, to further investigate the integrity of the TJ, we also

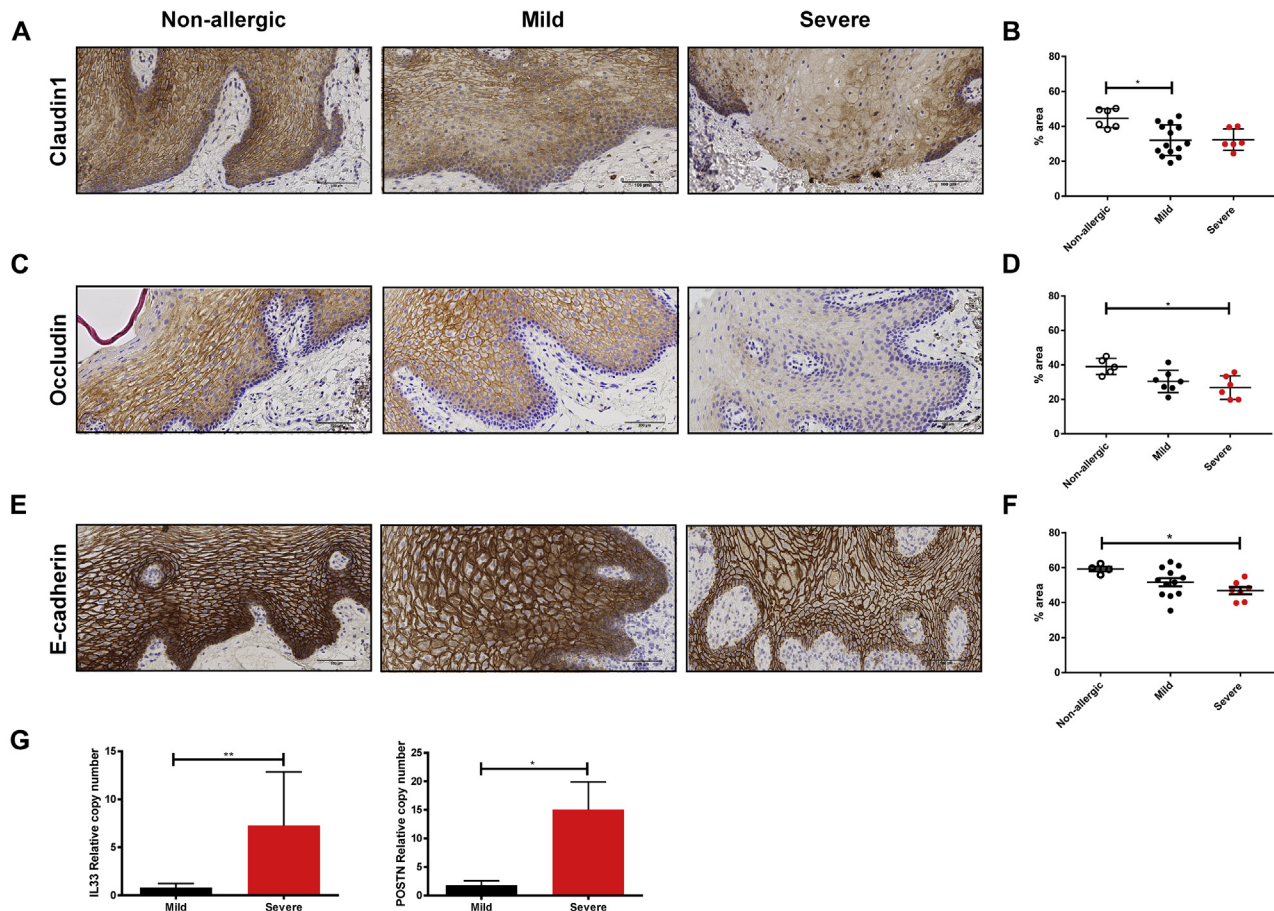


FIG 2. Analysis of epithelial integrity in oral mucosal biopsy specimens. Representative images are shown at $\times 20$ magnification from all experimental groups. Quantification of staining intensity (color deconvolution plugin from ImageJ software) is represented as the percentage area (pixels) for all analyses. Data are presented as scatter plots for nonallergic subjects and patients with mild or severe allergy. **A** and **B**, Claudin-1 staining (Fig 2, A) and claudin-1 (Fig 2, B) quantification. **C** and **D**, Occludin staining (Fig 2, C) and occludin quantification (Fig 2, D). **E** and **F**, E-cadherin staining (Fig 2, E) and E-cadherin quantification (Fig 2, F). **G**, Relative expression of *POSTN* and *IL33* genes in total RNA extracted from FFPE oral mucosal biopsy specimens measured by using quantitative RT-PCR. Assays were done in triplicates, and results were normalized according to expression levels of *BACT*. Results were obtained by using the $\Delta\Delta$ cycle threshold method for quantitation. Data are presented as means $\pm$ SDs. * $P < .05$ and ** $P < .01$.

determined occludin expression (Fig 2, C). Results showed significantly reduced expression of occludin in the group with severe allergy (Fig 2, D), whereas non-statistically significant differences were observed in patients with mild allergy compared with nonallergic subjects. To also test AJ components, we analyzed the expression of E-cadherin (Fig 2, E) and observed lower levels of expression in the group with severe allergy (Fig 2, F).

Here the decreased expression of claudin-1 and occludin, as well as that of E-cadherin, along with their differential distribution throughout the epithelium in allergic patients, suggests an association between epithelial remodeling and an allergic sensitization clinical response to profilin on oral provocation.

Next, we aimed to identify gene expression modifications that could account for the observed structural changes. The results (Fig 2, G) showed that expression of *POSTN* and *IL33* was significantly increased in the oral mucosa of patients with severe allergy. These results, associated with the epithelial remodeling observed in the oral mucosa of patients with severe allergy, are

indicative of a progressive inflammatory phenotype related to the severity of the allergic reaction.

Oral mucosal inflammation in profilin-sensitized patients is characterized by increased immune infiltrates

Next, we analyzed recruitment of immune inflammatory cells into the oral mucosa.

CD11c staining (Fig 3, A) revealed differential distribution between the epithelium and the connective tissue in the experimental groups. Quantification of CD11c⁺ cells (Fig 3, B) showed that in the groups with mild and severe allergy, the number of CD11c⁺ cells was greater in the connective tissue than in the epithelium. Moreover, in the severe allergy group the number of CD11c⁺ cells located in the connective tissue was significantly greater than in the other groups.

We next studied the location of CD3-expressing T cells. CD3 staining (Fig 3, C) showed that T cells were mainly distributed in patches throughout the mucosa of allergic

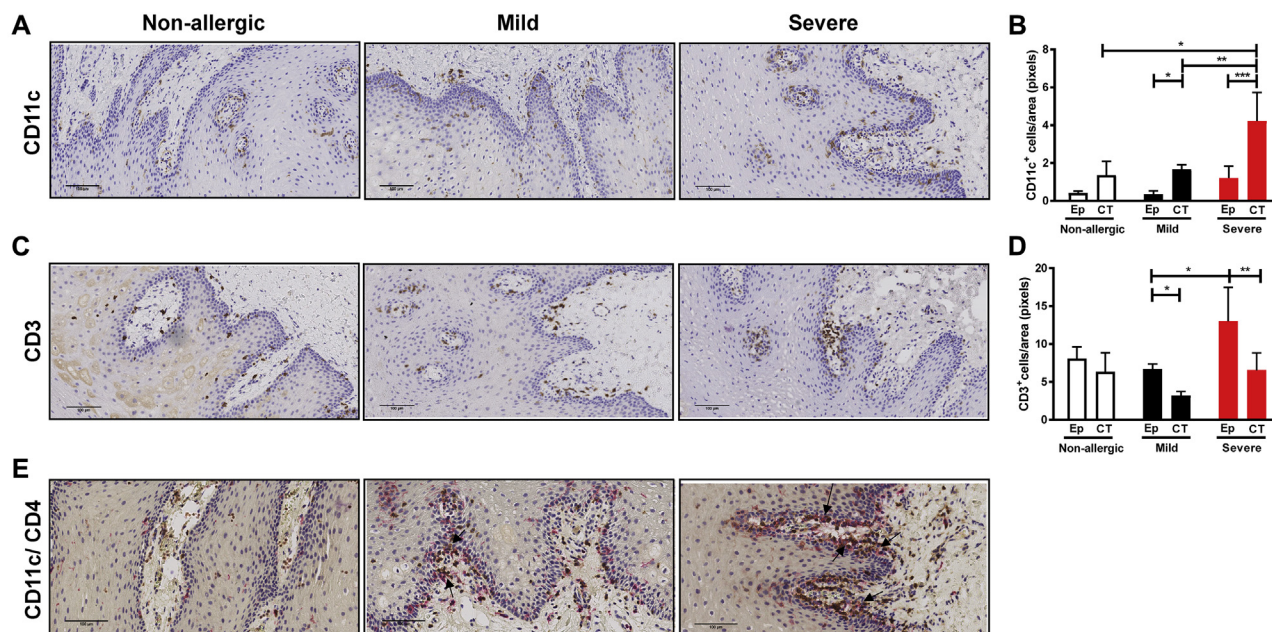


FIG 3. Analysis of immune infiltrates in the oral mucosa. Biopsy specimens from cheek lining of the oral mucosa from nonallergic subjects and patients with mild or severe allergy were stained with CD3 and CD11c, and quantification of positive cells per area (pixels) in the epithelium and connective tissue of the oral mucosa was performed with the freehand selection command in ImageJ software. **A**, CD11c expression in oral mucosal biopsy specimens. Representative images are shown at $\times 15$ magnification. **B**, Number of CD11c⁺ cells per area (pixels) in the epithelium and connective tissue. **C**, CD3 expression in the oral mucosa. Representative images are shown at $\times 15$ magnification. **D**, Number of CD3⁺ cells per area (pixels) in the epithelium and connective tissue. Data are presented as scatter plots. * $P < .05$, ** $P < .01$, and *** $P < .001$. **E**, Double staining for CD11c (in red) and CD4 (in brown). Arrows point to sites of colocalization. Representative images are shown at $\times 20$ magnification. CT, Connective tissue; Ep, epithelium.

patients. Quantitative analysis for CD3 did not show differences between the epithelium and connective tissue of nonallergic subjects. In contrast, CD3⁺ cells were located mainly in the epithelium of patients with mild and severe allergy, being greater in the latter (Fig 3, D).

Next, we examined possible colocalization between antigen-presenting cells (APCs) and T cells in the oral mucosa by double staining the oral biopsy specimens for CD11c⁺ (in red) and CD4⁺ (in brown) cells. We detected colocalization of both cell types mainly at the interface between the epithelium and connective tissue in allergic patients (Fig 3, C). The presence and colocalization of CD4⁺ T cells and APCs in allergic subjects indicates that T-cell priming is taking place in the oral mucosa.

Finally, we analyzed the expression of mast cell tryptase in the oral mucosa (Fig 4, A). The analysis showed no significant differences among groups in distribution of mast cells, which were more abundant in the connective tissue than in the epithelium in all groups (Fig 4, B). The number of tryptase-positive cells was roughly the same in all experimental conditions, as was the expression of tryptase- and chymase-encoding genes (*TPSAB*, *TPSB*, and *CMA*; Fig 4, C), although significant differences were observed in *CMA* expression between the allergic and nonallergic groups (data not shown).

Altogether, these data verify the presence of inflammatory infiltrates of CD4⁺ T cells and APCs in the oral mucosa of allergic patients and establish a link between epithelial remodeling and local inflammatory cell recruitment into the oral mucosa.

Sensitivity of effector cells from patients with severe allergy is lower than in mild profilin reactors

We have shown here significant epithelial remodeling in the oral mucosa in patients with severe profilin allergy. However, this might not be sufficient to account for the augmented severity of profilin-mediated reactions among these patients. Therefore we further explored the potential mechanisms underlying this differential response by performing BATs with pure profilin.

The results presented in Fig 5, A, showed that basophils from patients with severe reactions to profilin presented significantly lower half-maximal effective concentration values (almost 10-fold lower) than basophils from patients with mild reactions to profilin. Moreover, a significant correlation between decreased expression of occludin and higher sensitivity of basophils to profilin was found (Fig 5, B). These results confirm a previously unrecognized role for the oral mucosal barrier in the progression of respiratory allergy to severe food allergy phenotypes.

DISCUSSION

Several lines of evidence show that the interaction between epithelial and immune cells in the mucosa is critical in the onset and maintenance of allergic inflammation.²³ An intact functional mucosal barrier is considered crucial in the maintenance of airway homeostasis because it protects the host immune system from exposure to allergens and noxious environmental triggers.²⁴ Epithelial cells in the mucosa, which were initially thought to be

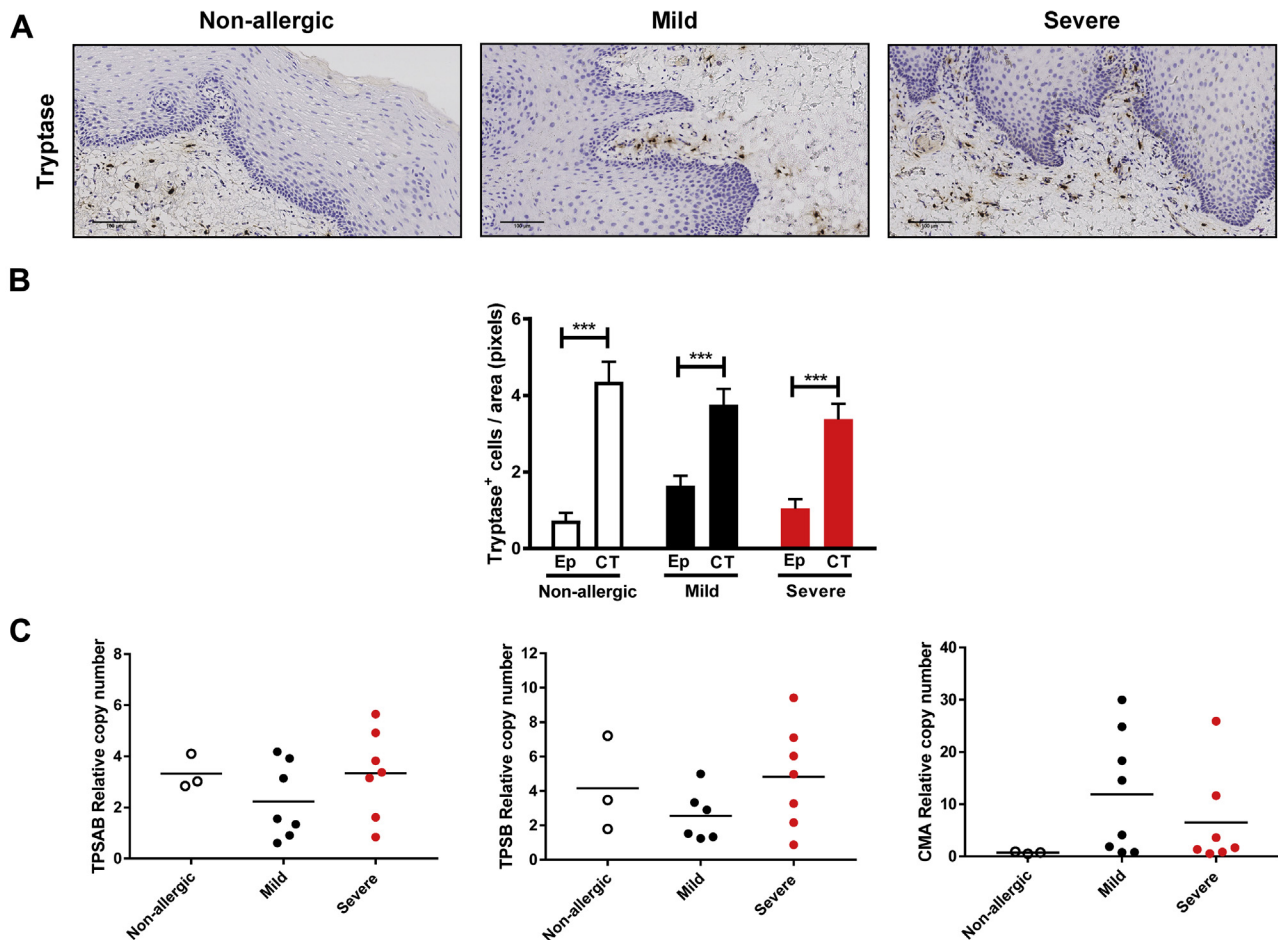


FIG 4. Mast cell characterization in the oral mucosa. **A**, FFPE biopsy specimens from cheek lining of the oral mucosa from nonallergic subjects and patients with mild or severe profilin allergy were stained with mast cell tryptase. Representative images are shown at $\times 15$ magnification are shown. **B**, Number of tryptase-positive cells per area (pixels) in the epithelium (Ep) and connective tissue (CT) of the oral mucosa. Data are presented as means $\pm$ SDs. *** $P < .001$. **C**, Expression of *TPSAB*, *TPSB*, and *CMA* in total RNA extracted from FFPE oral mucosal biopsy specimens. Results were normalized according to expression levels of *BACT*. Results were obtained by using the $\Delta\Delta$ cycle threshold method for quantitation.

just inert barriers, are now considered part of the innate immune response.²⁴⁻²⁶

In this study we hypothesize that a disruption in oral mucosal epithelial barrier integrity is central to understanding severe allergic reactions to profilin, an allergen that is quickly destroyed in acidic media, and was traditionally considered an incomplete food allergen.⁷ Patients with severe reactions induced by profilin accumulate in areas with very high grass pollen counts and oral sensitization to minor aeroallergens, such as profilin. Under these conditions, a significant percentage of patients will have a severe phenotype and represent a perfect model to analyze the progression of respiratory allergic disease to food allergy and the role of the oral mucosa.

To test this hypothesis, patients were selected based on their clinical history and response to pure profiling during a DBPCFC and stratified into mild or severe reactors. We considered severe reactors to be patients with a clear clinical history of severe reactions to profilin who were not sensitized to other food allergens. Moreover, they presented with either an intense local reaction or a systemic non-life-threatening reaction during profilin challenge.

Allergic patients had histologic features of progressive oral mucosal remodeling, with increased acanthosis and angiogenesis. These histologic features were comparable with those previously described in the oral mucosa from patients with inflammatory pathologies.^{27,28} Moreover, the oral mucosa in allergic patients also presented a greater density of collagen fibers, a feature associated with epithelial remodeling.²⁹⁻³¹

These patients also had significant structural changes characteristic of a remodeling process, including decreased expression of TJ (claudin-1 and occludin) and AJ (E-cadherin) proteins. These features have been described previously in polarized cell cultures of airway epithelium from patients with house dust mite-induced airway hyperresponsiveness.²⁴ Even though our results were obtained from human tissue biopsy specimens of complete oral mucosal tissue, we propose for future experiments to perform electron microscopic analysis and quantitative RT-PCR experiments using tissue sections to analyze in detail the architecture of the TJ structures in the human oral mucosal epithelium.

The observed epithelial damage might be associated with the IL-33-dependent group 2 innate lymphoid cell population located in the mucosal epithelium.^{13,32,33} Moreover, we observed recently

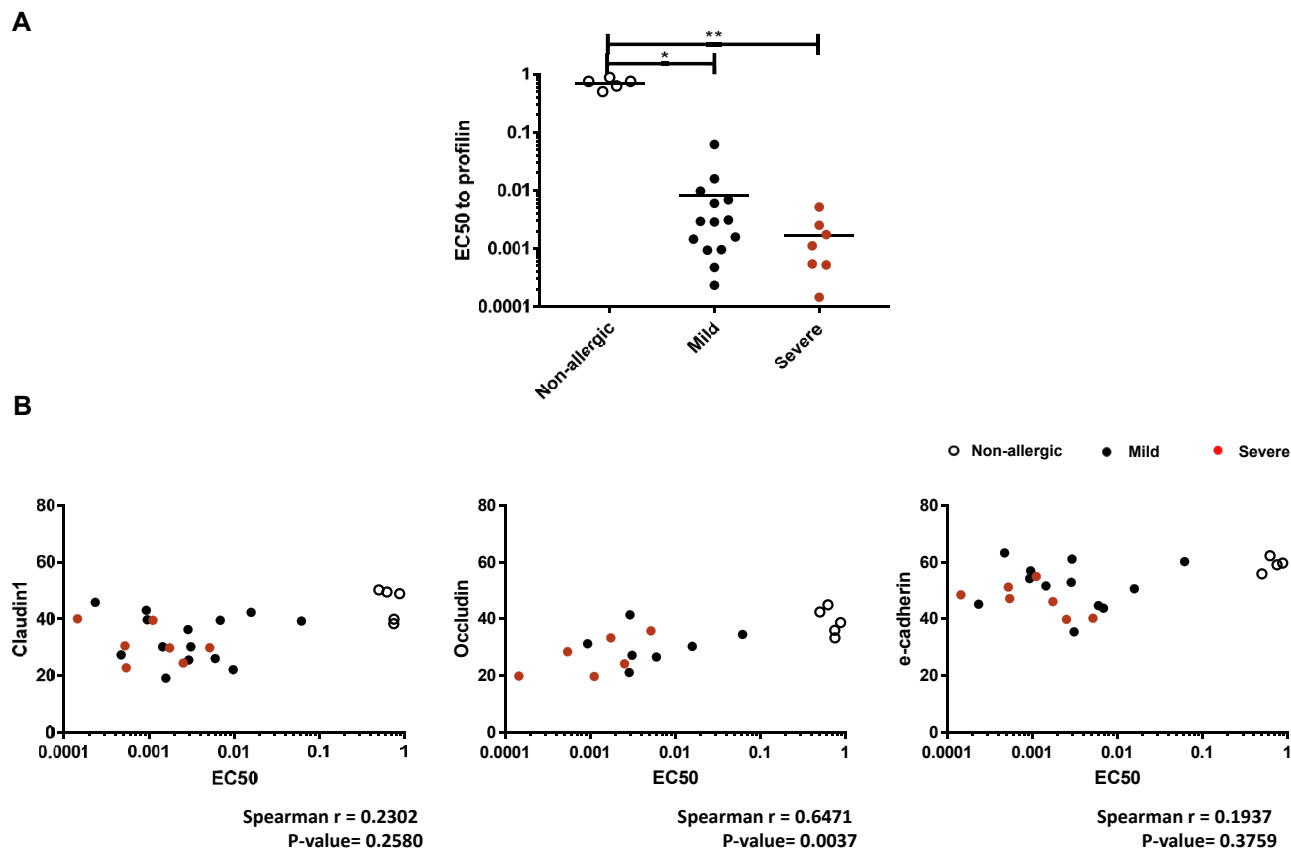


FIG 5. Basophil sensitivity to profilin. Whole blood from patients from all experimental groups was used to perform BATs to profilin. **A**, Half-maximal effective concentration (*EC*₅₀) was used to compare sensitivity to profilin among the experimental groups. Data are presented as scatter plots. **P* < .05 and ***P* < .01. **B**, Spearman correlation of expression of epithelial proteins in the oral mucosa (claudin-1, occludin, and E-cadherin) with basophil *EC*₅₀ values. Data are presented as scatter plots.

that increased *IL33* expression in the epithelium is also involved in impairment of the epithelial barrier, with significant downregulation of occludin and zonula occludens 1 (unpublished data).

We also found a significant increase in *POSTN* expression in the mucosa in severe reactors. Although little is known about the exact role of this protein in allergic patients, it has been described as a marker for asthma severity and been associated with epithelial remodeling processes in airway mucosa.^{15-17,34}

Regarding immunologic characteristics, allergic patients had a progressive inflammatory profile. Although allergic responses in the gut and lung mucosa have been investigated,³⁵ allergic inflammation in the oral mucosa remains largely unexplored. Our data demonstrate an inflammatory process taking place in the lamina propria of patients with severe allergy with colocalization of CD4⁺ and CD11c⁺ cells, suggesting that the oral mucosa is an immunocompetent site, as occurs in other mucosa, such as the gut.³⁵ Moreover, this has also been suggested for the oral mucosa.³⁶ Recently, profilin has been demonstrated to induce a similar T-cell response to major pollen allergens in profilin-sensitized patients.³⁷ These data strengthen the clinical relevance of profilin sensitization and its importance in disease progression. Therefore daily exposure to profilin, which is present in all vegetables, could contribute to maintaining the inflammatory allergy phenotype, even in the absence of immediate relations.

Regarding effector cells, mast cells from allergic patients were localized in the submucosa, where tryptase- and chymase-positive mast cells have been found.³⁸ However, cell counts were not increased, indicating that mastocytosis is not occurring in these patients regardless of their symptom severity. Nevertheless, we detected greater *CMA* expression in allergic patients than in the control group. This might indicate activation of defense mechanisms in the oral epithelium, as described by Waern et al³⁹ in the airways, where *CMA* modulates *IL-33* levels, as well as activation of procollagenase and cleavage of type I procollagen, to initiate collagen fiber formation.⁴⁰⁻⁴²

Additionally, we found a 10-fold greater sensitivity of basophils from severe reactors when compared with nonallergic subjects that correlated with decreased expression of occludin present in the epithelial TJs. This might indicate that an impaired oral mucosal barrier can facilitate antigen access, hence explaining the severe reactions to profilin experienced by these patients. The significant increase in *IL33* expression observed in the severe allergy group is consistent with the concomitant increase in effector cell sensitivity.

In summary, our data suggest a novel role of the oral mucosal barrier in progression of respiratory to food-induced allergic reactions. Oral epithelial remodeling could be a key process for acquisition of a severe allergic phenotype in patients with profilin-mediated food allergy. Disruption of the epithelial barrier

probably allows profilin penetration into the oral mucosa. This triggers a local inflammatory immune response characterized by an increased number of CD11c⁺ and CD3⁺ infiltrates and increased expression of *POSTN* and *IL33*. Additionally, greater sensitivity of basophils to profilin explains the severe systemic reactions experienced by these patients. The finding of the present work could also help us better understand disease progression in complex syndromes, such as pathogenesis-related proteins group 10- or lipid transfer protein-mediated food allergy, or to understand the immunologic basis of sublingual immunotherapy.

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Key message

- Profilin-mediated food reactions can be caused by 2 factors: disruption in the epithelial integrity of the oral mucosa, which results in recruitment of inflammatory cells, and significantly increased sensitivity of effector cells.

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TABLE E1. Results of DBPCFCs with palm tree profilin (Pho d 2)

	Age (y)	Sex	Tolerated profilin dose	Reaction	Rescue medication
Control	34	M	740 µg	None	None
	27	F			
	29	F			
	35	F			
	36	F			
	38	F			
Mild	26	M	740 µg	None	None
	28	M	740 µg	SAO, LA	Antihistamine
	29	M	740 µg	None	None
	29	M	74 µg	OP	Cetirizine, 10 mg
	30	M	740 µg	OAS	None
	37	M	740 µg	None	None
	37	M	740 µg	OAS	Antihistamine
	38	M	7.4 µg	OP, CG, DSP	Cetirizine, 10 mg
	53	M	740 µg	OP (2/10)	None
	18	F	740 µg	SAO, LA	Antihistamine
	19	F	740 µg	OAS	None
	25	F	740 µg	OAS	None
	25	F	740 µg	OAS, RESP	Cetirizine, 20 mg
	27	F	7.4 µg	OAS, LA	Antihistamine, corticosteroid IM
	28	F	740 µg	CS, OAS	None
	31	F	740 µg	LA	None
	33	F	0.74-740 µg	CG, OAS	None
	33	F	740 µg	OAS, RESP	Salbutamol, 4 INH
	34	F	740 µg	SAO	None
	37	F	740 µg	None	None
	41	F	Placebo	RESP	Salbutamol, 4 INH
	44	F	R-74 µg	DSP, OAS	Antihistamine, corticosteroid IV
	44	F	740 µg	OP (8/10)	None
	45	F	740 µg	OAS	None
	58	F	Placebo	OAS, ON	Antihistamine, CC IM
Severe	18	M	0.074 µg	OAS, DSP, PB	Antihistamine, CC IM
	22	M	R-74 µg	AS, EU, DSP	Adrenaline, Actocortina 200, polaramine
	18	F	Not carried out		
	18	F	Not carried out		
	20	F	740 µg	EU, DSP	Adrenaline, Actocortina 200, polaramine
	23	F	R-74 µg	SAO, LA, PO	Antihistamine
	25	F	R-74 µg	OAS, DSP, LA, NA	Antihistamine, corticosteroid IV, adrenaline
	26	F	Not carried out		
	28	F	R-74 µg	OAS, LA, DSP	Antihistamine, corticosteroid IV
	41	F	Not carried out		
	43	F	Placebo	ERY, PO, CG	Adrenaline IM, corticosteroid IV, polaramine IV
	44	F	R-74 µg	LA, OAS, DSP, NA	Adrenaline IM, antihistamine IV
	48	F	740 µg	CS, ERY	None

ABP, Abdominal pain; ANA, anaphylaxis; AS, asthma; CG, cough; CS, canker sores; DI, diarrhea; DP, dyspnea; DSP, dysphagia; DY, dysphonia; EP, epigastric pain; ERY, Erythema; EU, edema uvula; F, female; GP, gastric pain; HA, headache; HY, hypotension; IM, intramuscular; INH, inhalation; IV, intravenous; LA, labial angioedema; M, male; NA, nausea; ON, oral enanthem; OP, oral pruritus; PB, pharyngeal bolus; PO, pharyngeal occupation; RESP, respiratory; RHI, rhinitis; OAS, oral allergy syndrome; TO, thorax oppression; UR, urticaria; VO, vomit.