

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Interaction of Alt a 1 with SLC22A17 in the airway mucosa

María Garrido-Arandia¹  | Jaime Tome-Amat¹ | Diego Pazos-Castro¹ |
 Vanesa Esteban² | Maria M. Escribese^{3,4} | Guadalupe Hernández-Ramírez¹ |
 Alma Yuste-Montalvo² | Domingo Barber³  | Luis F. Pacios¹  | Araceli Díaz-Perales¹ 

¹Centre for Plant Biotechnology and Genomics (UPM-INIA), Universidad Politécnica de Madrid, Madrid, Spain

²Department of Allergy and Immunology, FIIS-Fundación Jiménez Díaz, UAM, Madrid, Spain

³IMMA, Facultad de Medicina, Universidad San Pablo CEU, Madrid, Spain

⁴Departamento de Ciencias Médicas Básicas, Universidad San Pablo CEU, Madrid, Spain

Correspondence

Araceli Díaz-Perales, Centre for Plant Biotechnology and Genomics (UPM-INIA), Universidad Politécnica de Madrid, Pozuelo de Alarcón, 28223 Madrid, Spain.
 Email: araceli.diaz@upm.es

Funding information

Spanish Government, MINECO, Grant/Award Number: BIO2017-84548-R; Instituto de Salud Carlos III (ISCIII), Grant/Award Number: PI16/00249, PI15/02256, R, 00, 0006, 0006 and 0003; FEDER Thematic Networks and Cooperative Research Centers: ARADYAL; Allerscreening project, Grant/Award Number: EU SEP-210415617

Abstract

Background: Despite all the efforts made up to now, the reasons that facilitate a protein becoming an allergen have not been elucidated yet. Alt a 1 protein is the major fungal allergen responsible for chronic asthma, but little is known about its immunological activity. Our main purpose was to investigate the ligand-dependent interactions of Alt a 1 in the human airway epithelium.

Methods: Alt a 1 with and without its ligand (holo- and apo- forms) was incubated with the pulmonary epithelial monolayer model, Calu-3 cells. Allergen transport and cytokine production were measured. Pull-down and immunofluorescence assays were employed to identify the receptor of Alt a 1 using the epithelial cell model and mouse tissues. Receptor-allergen-ligand interactions were analyzed by computational modeling.

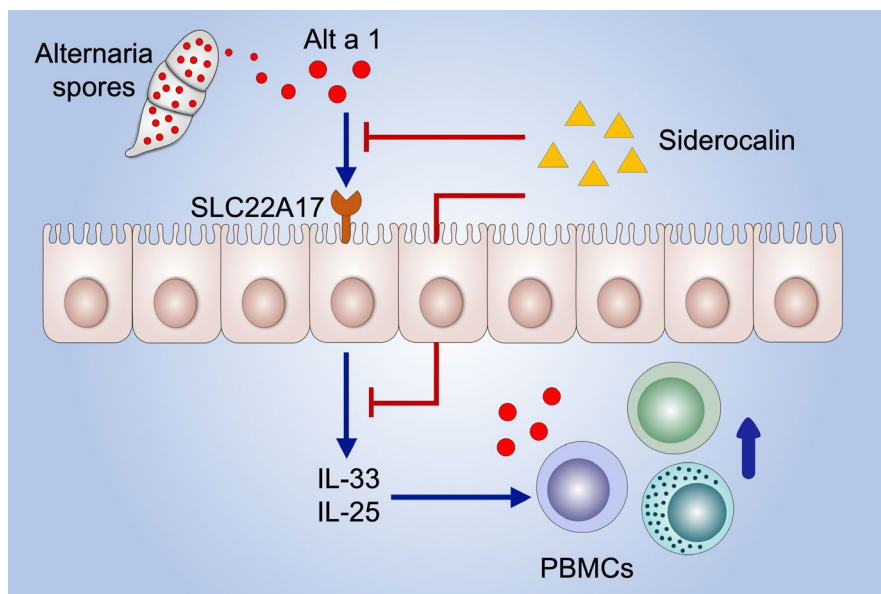
Results: The holo-form could activate human monocytes, PBMCs, and polarized airway epithelial (Calu-3) cell lines. The allergen was also transported through the monolayer, without any alteration of the epithelial integrity (TEER). Alt a 1 also induced the production of proinflammatory IL8 and specific epithelial cytokines (IL33 and IL25) by Calu-3 cells. The interaction between epithelial cells and holo-Alt a 1 was found to be mediated by the SLC22A17 receptor, and its recognition of Alt a 1 was explained in structural terms.

Conclusions: Our findings identified the Alt a 1 ligand as a central player in the interaction of the allergen with airway mucosa, shedding light into its potential role in the immunological response, while unveiling its potential as a new target for therapy intervention.

KEYWORDS

Alt a 1, *Alternaria alternata*, fungal allergen, human airway epithelium, solute carrier family 22 member 17 receptor

Abbreviations: DPBA, Diphenyl boric acid aminoethyl ester; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; HPLC, High-performance liquid chromatography; ITC, Isothermal titration calorimetry; LPS, Lipopolysaccharide; LTI, Lymphocyte transformation test; MST, MicroScale thermophoresis; NMR, Nuclear magnetic resonance; PBMCs, Peripheral blood mononuclear cells; PBS, Phosphate-buffered saline; PDB, Protein Data Bank; SEAP, Secreted embryonic alkaline phosphatase; SLC, Solute carriers; TEER, Transepithelial electrical resistance; TLR, Toll-like receptor.



GRAPHICAL ABSTRACT

Alt a 1 is released from *Alternaria alternata* spores in complex with its native ligand, being recognized by the Solute carrier family 22 member 17 receptor. Production of cytokines IL25 and IL33 occurs only in the presence of the ligand, being abolished with specific antibodies against Solute carrier family 22 member 17. Alt a 1 shows structural relationship with human siderocalin mimicking its airway entry route.

1 | INTRODUCTION

Allergy results from aberrant immune responses to environmental proteins.¹ Although the proteins that induce allergy represent a small fraction of all those to which people are usually exposed, allergy is a common phenomenon. Within a particular extract, there are dominant proteins, major allergens that sensitize most of the allergic patients. There is still no answer to the question of why certain proteins are more prone to become allergens. The nature of the allergens and their interactions with innate immune system should be highly relevant in this process. In fact, only a limited number of protein families are known to be able to induce allergic sensitization.^{2,3} Besides, there is increasing evidence that points to the existence of conserved conformations with similar biological function in allergens which could be relevant in the elicitation of allergic sensitization.^{4,5}

Alternaria alternata has been described as the most important mold agent associated with respiratory allergies and severe asthma,^{6,7} especially in children.⁸⁻¹⁰ The most intense exposure to *A. alternata* allergens is likely to occur outdoors; however, *Alternaria* and other allergenic fungi can colonize indoor environments and thereby increase the fungal aeroallergen exposure levels. A consequence of human exposure to fungal aeroallergens, sensitization to *A. alternata*, has been unequivocally associated with increased asthma severity and persistence.¹⁰⁻¹² In South Europe, over 20% of the patients with a history of respiratory allergy showed sensitization to *Alternaria*,¹³ being Alt a 1 the most prevalent allergen.^{6,7,14,15}

Alt a 1 is a heat-stable, 30 kDa homodimer that dissociates into 14.5- and 16-kDa subunits under reducing conditions.¹⁶ The crystal structure of Alt a 1 shows an all- β architecture composed of 11

β -strands arranged in the form of a single β -barrel which does not have equivalents in the Protein Data Bank (PDB).¹⁴ We have recently demonstrated that the tetramer of Alt a 1 assembles only in the presence of a methylated flavonol ligand produced by *Alternaria*, with similar properties to quercetin, a plant flavonol. Thus, Alt a 1 can change its aggregation state, from a tetramer as holo-form in the presence of its ligand to a dimer as apo-form in the absence of its ligand.^{17,18}

It has been already accepted that ligands carried by allergens, or exposed together, might influence the host response to the specific allergen. Thus, immune-regulatory ligands could significantly contribute to the allergenicity of several proteins.¹⁹ All of these molecules transported by potentially allergenic proteins are suggestive candidates as nonself structures recognized by the innate immune system with potential capability of inducing Th2-skewed responses, which contribute to allergic sensitization in susceptible individuals.⁵

Little is known regarding the immunological properties of Alt a 1 and the putative role played by its catechol ligand in the immunological activity of this protein. Catechol (o-benzenediol) moiety is a constitutive part of a great number of flavonol-type compounds such as the ligand of Alt a 1 whose chemical nature was recently identified by our group.¹⁷ Catechols are instrumental in chelating iron as well as in participating in antioxidant reactions, both types of processes that, as discussed below, may be related with the immunological activity of the *Alternaria* allergen. The mechanisms of Alt a 1 sensitization are still poorly understood, and most data have focused on allergic reactions of the respiratory tract rather than on the initial steps of allergic sensitization. The

principal goal of our study was to investigate the interactions of Alt a 1 with the airway epithelium, testing the transport kinetics of the allergen, and the epithelial response, measuring its integrity by means of transepithelial electrical resistance (TEER) and cytokine production, as well as the pathway for entry of Alt a 1 into cells. Our results indicated that Alt a 1 is prone to induce proinflammatory responses through interaction with the solute carrier family 22 member 17 (SLC22A17) receptor. The SLC22A17-Alt a 1 interaction demonstrated in the current work could provide essential clues to unveil the entry route of this fungal allergen in the initial stages of processes which in turn lead to respiratory allergic responses and severe asthma.

2 | MATERIAL AND METHODS

2.1 | Production of recombinant Alt a 1 and isolation of its ligand

Recombinant Alt a 1 (rAlt a 1) was produced in *Pichia pastoris* and purified from the supernatant media. After dialysis, it was purified by two chromatographic steps: molecular exclusion followed to reverse phase. The protein purity was tested by anti-Alt a 1 antibodies (Roxall Medicina España SA) and MALDI-ToF, and its identity was verified by peptide-mass fingerprint, using standard methods. The presence of endotoxin was tested using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific). The immunological activity of the recombinant protein was compared to native protein by immunodetection by ELISA using allergic patients sera ($n = 10$; kindly selected by Dr Bartra Clinic Hospital of Barcelona, Spain); by quercetin binding and staining with DPBA; and finally by THP1-XBlue-CD14™ activation.

The natural ligand of Alt a 1 was extracted as previously described.¹⁸ Briefly, the ligand was purified from a pulverized mixture of mycelium and spores of *Alternaria*, and dissolved in ethanol 70%. The mixture was left for 1 hour in agitation at room temperature. The catechol ligand was purified by HPLC (Nucleosil 120 C18, 250 × 4 mm) with an acetonitrile gradient (10%-85% in 30 minutes). Spectra were recorded at 254 nm, and the peaks were analyzed by DPBA staining.

Thus, whereas the apo- form of Alt a 1 corresponded to purified recombinant protein, its holo-form resulted from incubation of the apo- form and the Alt a 1 ligand (4:1 mol/mol).

2.2 | Lymphocyte transformation test (LTI) with peripheral blood mononuclear cells (PBMCs)

Buffy coat (cell-enriched plasma) samples were obtained from 4 healthy donors at the Transfusion Centre (Madrid, Spain) for isolation of PBMCs. PBMCs were isolated using a density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) and cultured (2×10^5 cells/well) in the presence of both holo- and apo-forms of Alt a 1 (5 µg/mL) in RPMI media (Invitrogen). Proliferation

was represented as percentage in the increase in the cell number (PBMCs with antigen) referred to the control (PBMCs without antigen).

2.3 | THP1-XBlue-CD14™ activation

The transfected cell line THP1-XBlue-CD14™ (Invivogen, France) derived from the human monocytic THP1 cell line was used according to the manufacturer's instructions. Results were expressed as stimulation index (SI) calculated as the ratio between stimulated and nonstimulated cells.

2.4 | Transport of Alt a 1 through the human airway epithelial cell model

Calu-3 epithelial cell line (ATCC) was grown in 24-well Transwell® inserts (Inc) in supplemented DMEM-F12 medium (Lonza). The monolayer was considered formed when the TEER reached plateau exceeding 300 Ω cm² and was confirmed by anti-tubulin-specific polyclonal antibody (1:100; Invitrogen).

Alt a 1 transport was quantified by ELISA, using the basolateral media and anti-Alt a 1 antibodies. Bio-Plex Pro™ Assays (Bio-Rad 10014905) were used to measure cytokine production (IL1, IL13, IL8, eotaxin, and IFN-γ). Epithelial cytokines (IL25 and IL33) were quantified using Cusabio kits (Wuhan, China).

The inhibition of transport was carried out adding anti-SLC22A17 antibodies (1 µg/mL) 10 minutes before adding holo-Alt a 1.

2.5 | Pull-down immuno-assays

Dynabeads® M-280 Tosylactivated (Invitrogen) was conjugated with holo-Alt a 1 (0.2 µg/µL) or with specific antibodies against SLC22A17 (0.2 µg/µL; PA5-20543; Thermo Fisher). Cell lysates were obtained using RIPA buffer (20 mmol/L Tris-HCl (pH 7.5); 150 mmol/L NaCl; 1 mmol/L Na2EDTA; 1% NP-40; 1% protease inhibitor), and the soluble fraction was quantified by Bradford method and tested by SDS-PAGE.

For pull-down, the functionalized nanoparticles were incubated with Calu-3 cell lysate (0.01 µg/µL) at 4°C overnight. After centrifugation, the pellet was extensively washed and resuspended in denaturing Laemmli buffer. Samples were run into a 15% SDS-PAGE. In the case of Alt a 1 beads, the retained 65 kDa band was cut and identified by peptide fingerprint-mass spectrometry (UCM; <https://www.ucm.es/gyp/proteomica>) and confirmed by immunodetection with specific antibodies against SLC22A17. In the case of anti-SLC22A17 beads, the identity of the retained bands was confirmed by anti-Alt a 1 antibodies.

Lysates from the human cell lines Calu-3 (lung epithelial, ATCC® HTB-54), HaCaT (epidermal keratinocytes, ATCC® HB-241), Caco-2 (epithelial colon, TCC® HTB37™), THP1 (monocytes, TCC® TIB-202™), and human PBMCs ($n = 8$) were also used for immunodetection with specific antibodies against the SLC22A17.

2.6 | Solute carrier family 22 member 17 receptor immunofluorescence

For immunofluorescence assays, Alt a 1 was labeled with Alexa 647 (Alexa Fluor™ 647 Antibody Labeling Kit; Thermo Fisher) and added to apical size of monolayer of Calu-3 epithelial cells, as holo- or apo- forms, for 5 minutes. Then, cells were fixed and incubated with SLC22A17 antibody. The specimens were mounted with ProLong Gold (Thermo Fisher) with DAPI, and images were obtained using a Leica TCS-SP8 confocal microscope using 488-nm and 633-nm laser excitations.

Healthy 8-week-old BALB/c female mice were obtained from the breeding colony (Charles River Laboratories) and used with the permission of the Institutional Animal Care and Use Committee from Community of Madrid (Ref. PROEX 392/15). This research was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the OPRR (Office for Protection against Research Risks) of the US National Institutes of Health, Washington, DC (NIH Publication No. 85-23, revised 1985). Mice were maintained in the IIS-Fundación Jiménez Díaz (Madrid, Spain) until they were killed, which was performed in a suitable CO₂ chamber for rodents, in compliance with the current European legislation. For immunolocalization assays, mice lungs were fixed in 4% PFA and maintained in 0.1% PFA until its inclusion in paraffin. After that, the same protocol explained above was carried out. The inhibition of the Alt a 1-SLC22A17 binding was carried out by adding human siderocalin (SRP6465; 1 µg, Sigma-Aldrich) 10 minutes before adding the holo-form.

2.7 | Structures, docking calculations, and estimates of protein-protein binding affinity

Since no experimental structure is available for the SLC22A17, a model was constructed with I-TASSER.^{20,21} The best C-score (-1.44) model was selected. Complexes of this model structure of SLC22A17 with the tetrameric assembly of the crystal structure of Alt a 1 were obtained in protein-protein docking calculations with RosettaDock.²² The PDB entry 1QQS²³ was used for human siderocalin, and the biological assembly of the PDB entry 3VOR¹⁴ was used for tetrameric Alt a 1. The systems were prepared with VMD²⁴ and minimized at 10 000 conjugate gradient steps with NAMD²⁵

using the CHARMM3.6 proteins force field.²⁶ These water-optimized structures of complexes were then submitted to PRODIGY²⁷ to estimate protein-protein binding ΔG affinities and dissociation constants. Molecular graphics were prepared and rendered with PyMOL-2.2.0 (The PyMOL Molecular Graphics System, version 2.0 Schrödinger, LLC) and ChimeraX-0.6.²⁸

Structural comparison between Alt a 1 and human siderocalin was computed using three reliable structural alignment methods: FATCAT,²⁹ CE,³⁰ and TM-Align.³¹ The structural similarity was thus quantified by their scoring parameters: *P*-value, *Z*-score, and TM-score, respectively.

2.8 | Statically analysis

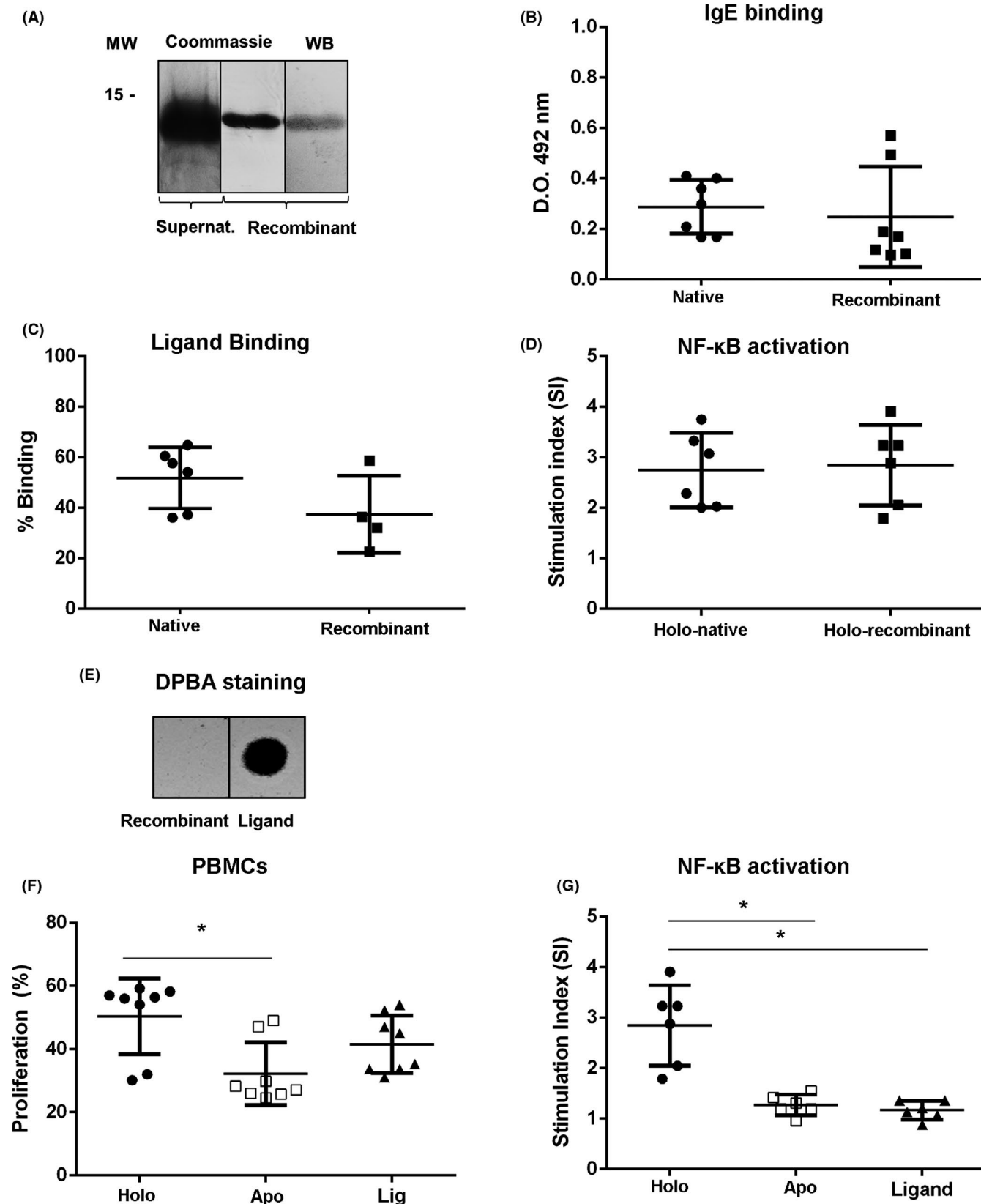
For the analysis of PBMC and THP1 assays, the statistically significant differences were analyzed by GraphPad6 using Kruskal-Wallis test with Dunn's correction for multiple comparisons. *P*-values <0.05 were considered significant for all assays. The difference in the cytokine production was analyzed using the two-way ANOVA with Bonferroni correction for multiple comparisons. *P*-values <0.05 were considered significant.

3 | RESULTS

3.1 | The catechol-like ligand of Alt a 1 is responsible for the activation of antigen-presenting cells

Given the difficulty to obtain native ligand-free Alt a 1, we produced the apo- form of Alt a 1 as a recombinant protein using *Pichia pastoris*. Therefore, we first set to characterize the recombinant protein and compare it to the native one (Figure 1A-D). Different biological and immunological assays were performed: In all cases, the recombinant protein showed the same activity as the native. To confirm the absence pollutants, the purified recombinant protein was dissolved in methanol 70%, and the supernatant was loaded on a silica plate and stained with DPBA (specific staining for flavonols). As seen in Figure 1E, nothing was detected in the supernatant of purified rAlt a 1. The holo-form of Alt a 1 was then obtained by mixing the apo-form with the natural ligand from *Alternaria* (4:1 mol/mol).

FIGURE 1 Immunological activity of rAlt a 1. A, Alt a 1 was produced as recombinant protein in the yeast *Pichia pastoris*. The Coomassie staining (Coomassie) and immunodetection with specific antibodies against Alt a 1 (WB) can be observed. B, IgE binding comparison between native (*native*) and recombinant (*recombinant*) Alt a 1 by ELISA. Sera (*n* = 6) of *Alternaria* patients were kindly provided by Dr J Bartra (H Clinic, Spain). OD to 492 was represented. C, Comparison of binding capacity between native (*native*) and recombinant (*recombinant*). Quercetin (Q4951, Sigma-Aldrich) was used as ligand. The % of the binding was represented as: $100 \times [1 - (\text{OD}_{492} \text{ Alt a 1}) / (\text{OD}_{492} \text{ 0\% binding})]$, being 0% binding the absorbance obtained with free quercetin. D, Comparison of the activation capacity of THP1 cells for native (*holo-native*) and recombinant (*holo-recombinant*) proteins. Activation of NF- κ B/AP-1 was revealed with QUANTI-Blue™. All results expressed as stimulation index calculated as ratio between stimulated and nonstimulated cells. E, Quality control. Dot blot of the recombinant Alt a 1, after dissolved it in methanol 70% (apo- form) and its ligand after staining with DPBA measured as fluorescence at 354 nm. F, Proliferation assays of human PBMCs (*n* = 8, two biological replicates) incubated with catechol-like ligand and both forms of Alt a 1 (holo- and apo- form; 5 µg/mL). After 5 d, the proliferation of PBMCs was counted with a hemocytometer. The % of proliferation is represented. G, NF- κ B/AP-1 activation was measured using the transfected cell line THP1-XBlue-CD14™ (*n* = 6). Cells were incubated with the catechol-like ligand and both forms of Alt a 1 (holo- and apo- forms; 5 µg/mL). Means and SE (bars) are shown. **P* < 0.05



Thus, when human PBMCs ($n = 8$) were cultured with holo- and apo- forms, significant differences were found. PBMCs proliferation was higher in the presence of the ligand (Figure 1F; $P < 0.05$), although apo-form and the ligand could also induce

it. In contrast, only the holo-form was able to activate the THP1 cell line (human monocyte cells) (Figure 1G; $P < 0.05$), whereas this activation was not seen when the ligand or the protein were added separately.

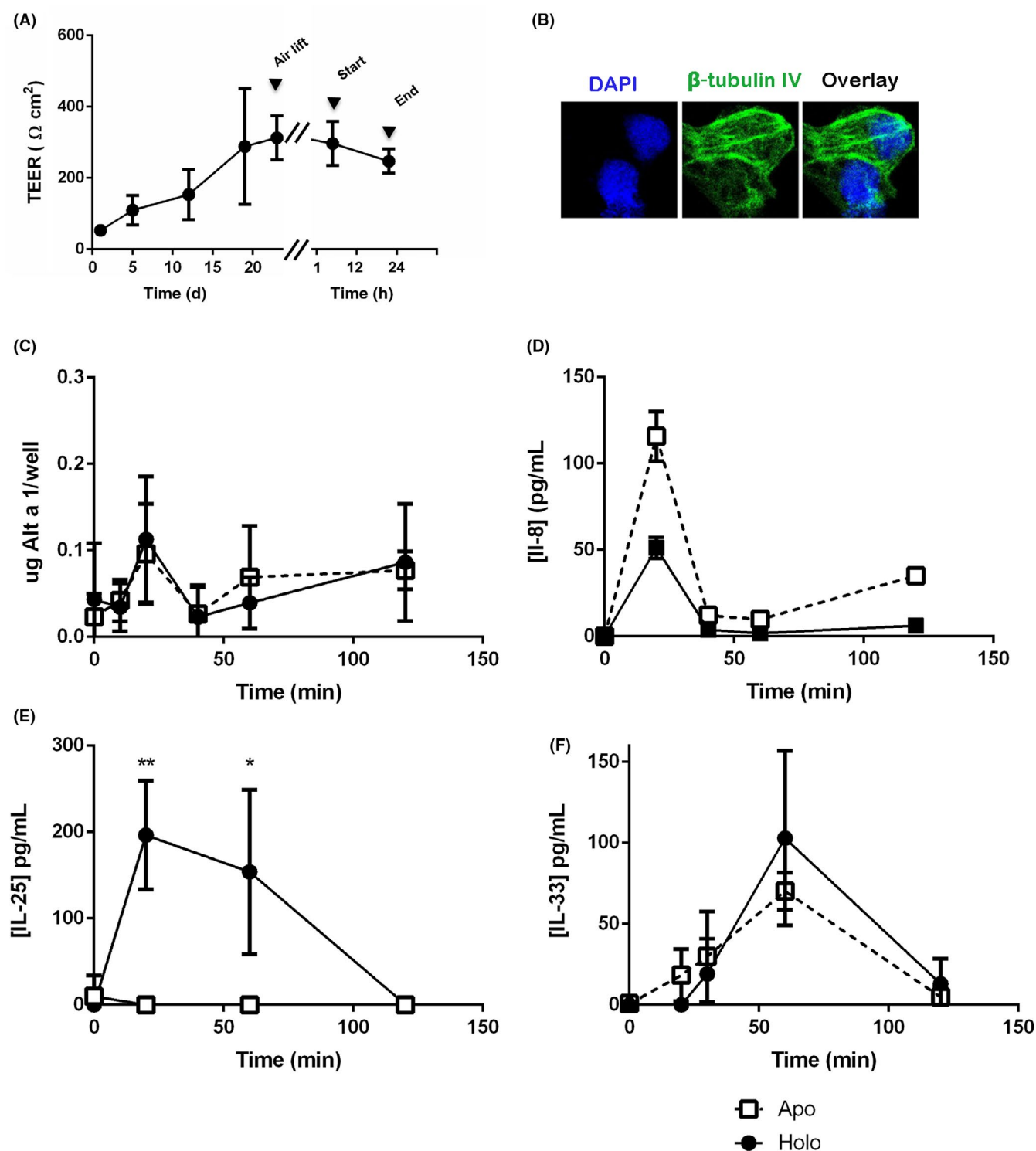


FIGURE 2 Interaction between Alt a 1 with respiratory epithelial cell model (A, B) Calu-3 cells were grown on Transwell®. Stability of the monolayer was tested by TEER measurement (A) and the polarization by staining with anti- β tubulin IV (B). C, Kinetic of transport through polarized monolayer of Alt a 1, in holo- and apo- forms, to basolateral side. D-F, Proinflammatory response of epithelium monolayer incubated with both forms of Alt a 1 as cytokine production measured in the basolateral side: IL8 (D); IL25 (E), and IL33 (F). Means and SE (bars) are shown. Statistics shows the significant differences between the holo- and apo- forms in cytokine production. * $P < 0.05$; ** $P < 0.001$

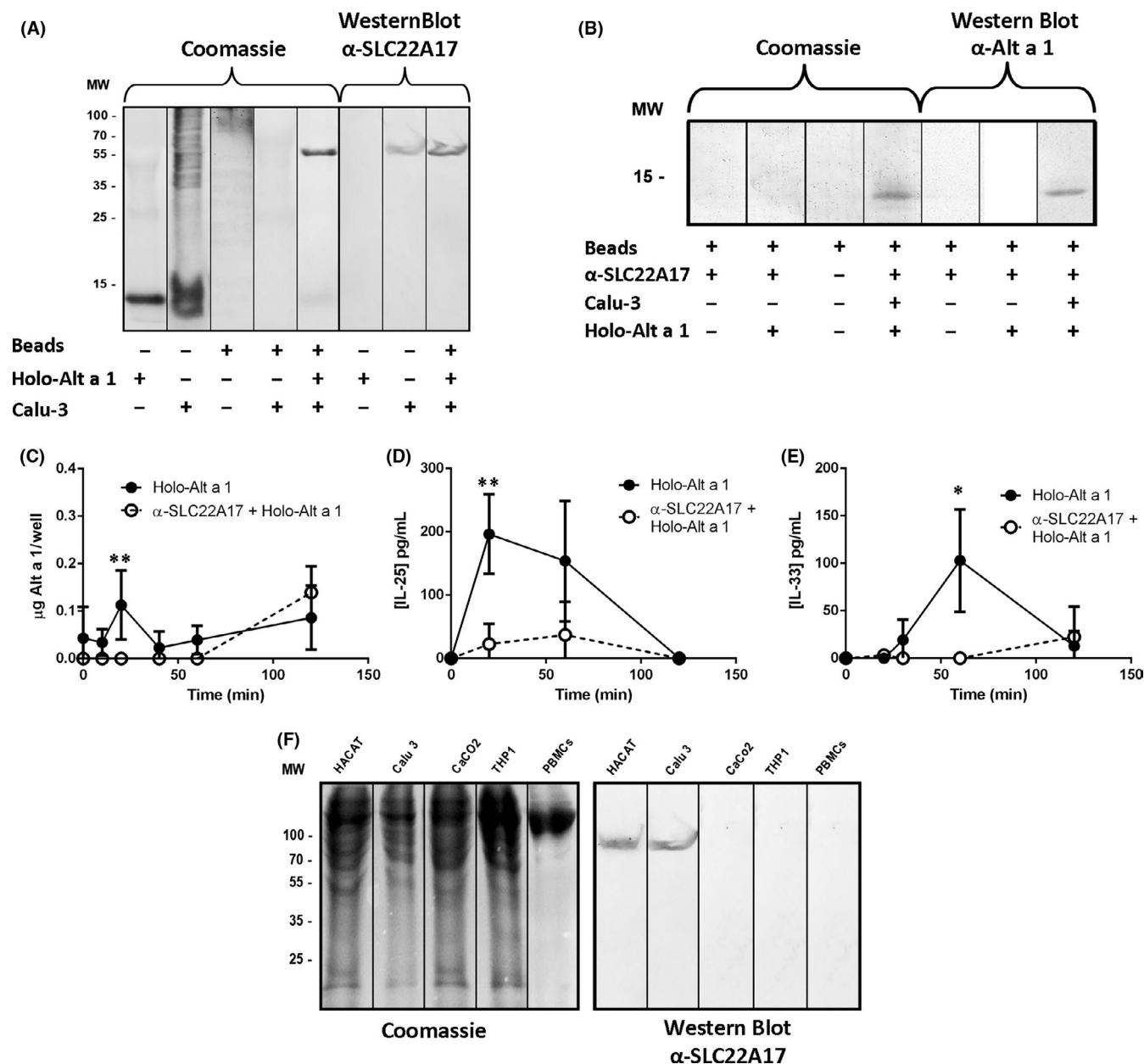


FIGURE 3 Alt a 1 interacts with the SLC22A17 in the presence of its catechol-like ligand. A, Pull-down assays. Alt a 1 fixed to M-280 Tosyl Dynabeads was incubated with Calu-3 cell lysate. After extensive washes, beads were loaded into SDS-PAGE, stained with Coomassie (first 5 lanes), and immunodetected with antibodies against SLC22A17 (last 3 lanes). Holo-Alt a 1 and Calu-3 lysate can be seen stained by Coomassie. As control of pull-down, beads without incubation and beads incubated to Calu-3 lysate without Alt a 1 can be observed. B, Pull-down assay by fixed anti-SLC22A17 antibodies to M-280 Tosyl Dynabeads and incubated with Calu-3 lysate. After that, beads were incubated with holo-Alt a 1. The result of pull-down was separated by SDS-PAGE under denaturing conditions, visualized with Coomassie (Coomassie) staining, and the presence of Alt a 1 was confirmed using specific polyclonal antibodies (Western Blot). C, Kinetics of transport of holo-Alt a 1 to basolateral side, through Calu-3-polarized monolayer pre-incubated with antibodies against SLC22A17. D and E, Proinflammatory response of epithelium monolayer pre-incubated with antibodies against SLC22A17 and holo-Alt a 1, measured as production of IL25 (D) and IL33 (E). Means and SE (bars) are shown. Statistics shows the significant differences between the holo- and apo-forms in cytokine production. * $P < 0.05$; ** $P < 0.001$. F, Immunodetection of SLC22A17. Confluent cultures of different cell lines (HaCaT, Calu-3, CaCo-2, and THP1) and human PBMCs were lysed and separated by SDS-PAGE. The lysed extracts were tested by staining with Coomassie (Coomassie) and the presence of the receptor by immunodetection with specific antibodies against the SLC22A17 (Western blot)

So, the presence of the catechol-like ligand of Alt a 1 is essential for its immunological activity.

3.2 | Alt a 1 induces a proinflammatory response in an epithelial cell model

To study the interaction between airway epithelium and Alt a 1, Calu-3 cells were grown in transwell to form a polarized monolayer with a stable TEER measurement and ciliogenesis development (Figure 2A, B). Holo- or apo-Alt a 1 was added on the apical side of the monolayer and TEER was measured (Figure 2A, Start-End) during this time, observing that the integrity of the monolayer was not affected by the presence of the allergen.

On the other hand, both apo- and holo-forms of Alt a 1 were able to pass through the monolayer (Figure 2C) reaching the basolateral side also inducing the production of proinflammatory cytokines such as

IL8 (Figure 2D) with no differences between both forms. In contrast, the presence of the catechol-like ligand increased the expression of IL25, but not IL33 (Figure 2E, F; $P < 0.05$). Therefore, the binding of Alt a 1 significantly promotes a proinflammatory environment.

3.3 | The holo-form of Alt a 1 interacts with SLC22A17

To study the interaction between Alt a 1 and the airway epithelium, immunoprecipitation assays were carried out in two different ways. Alt a 1 was immobilized on magnetic beads and incubated with Calu-3 cell lysates. As shown in Figure 3A, a band of 60 kDa was detected. The band was identified and confirmed by specific polyclonal antibodies (Figure 3A), as SLC22A17 (Q8WUG5), the receptor of the human siderocalin protein.³² In order to assure the specific binding

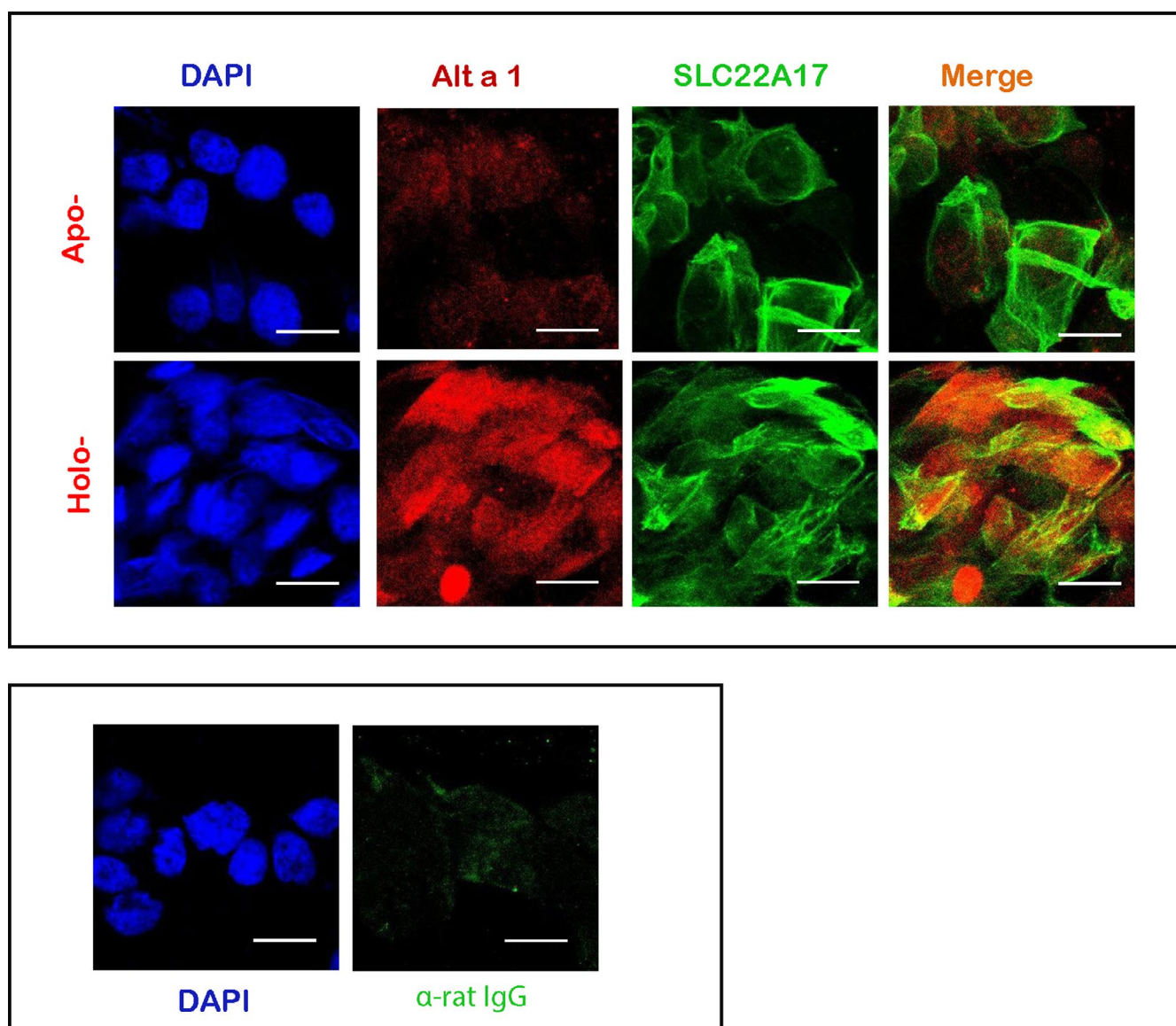


FIGURE 4 Immunolocalization of Alt a 1 in airway mucosa. Alexa 647-labeled Alt a 1 (1 μ g; red), in holo- and apo- forms, was added to polarized Calu-3 monolayer. After 5 min, cells were fixed with paraformaldehyde 4% and the location of the SLC22A17 was revealed by specific antibodies (green). DAPI (blue) and 488-labeled anti-rat IgG (1 μ g) was used as control. Bar: 20 microns

of the receptor and Alt a 1, the immunoprecipitation was repeated with anti-SLC22A17 bound to magnetic beads and incubation with cell extract and Alt a 1. The interaction was confirmed using specific antibodies against Alt a 1 (Figure 3B).

To better characterize the specificity of the interaction between Alt a 1 and SLC22A17, we used inhibition assays aiming to block Alt a 1 transport through the monolayer and analyzed the inhibition in cytokines release (Figure 3C-E). As it can be seen, the absence of

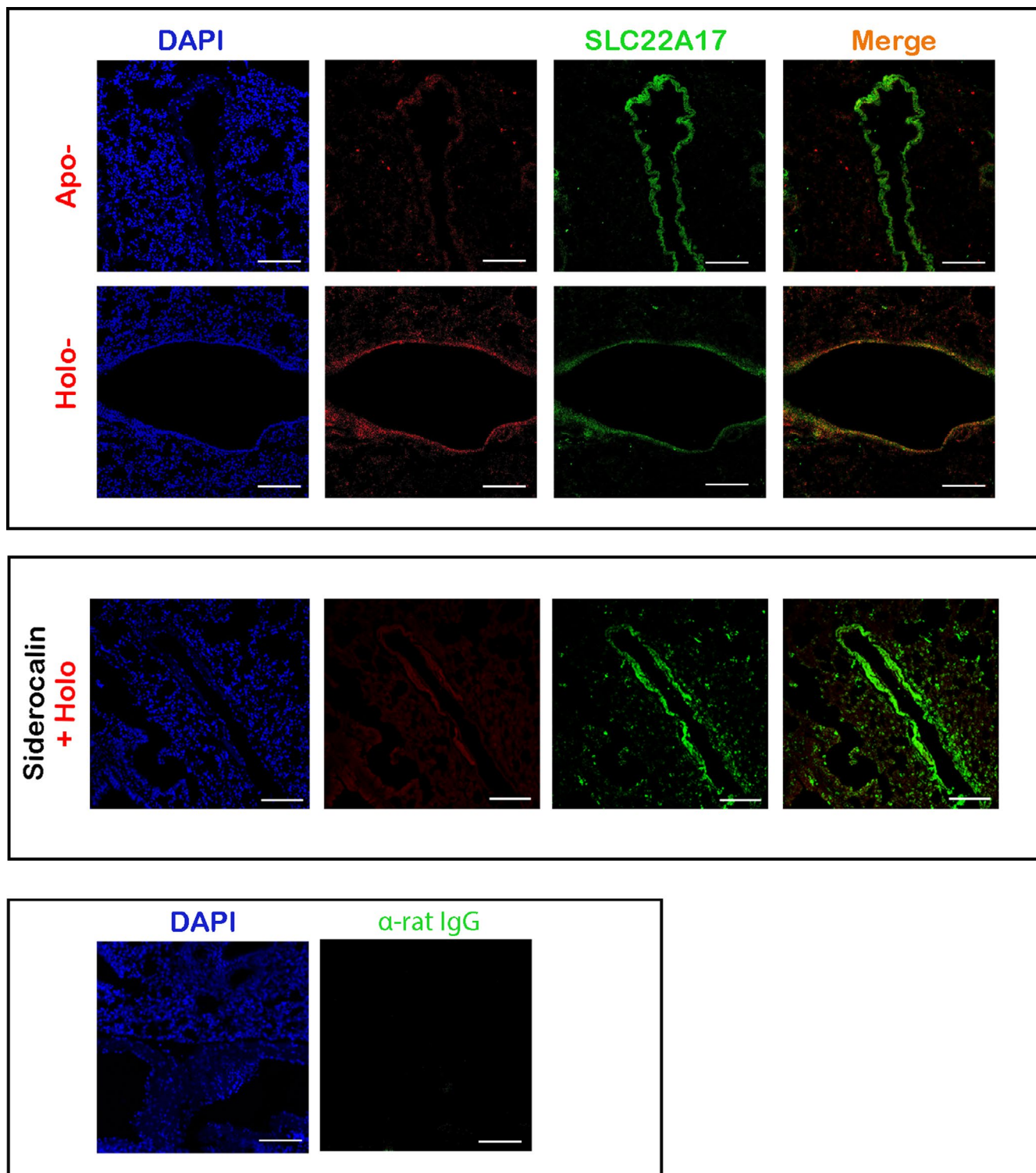


FIGURE 5 Immunolocalization of Alt a 1 in mouse lung. A, Lung sections from naïve mice were fixed with paraformaldehyde 4% and incubated with Alexa 647-labeled Alt a 1, apo- and holo-forms (red). SLC22A17 antibody (green) was added. B, Inhibition assay by pre-incubating with siderocalin and following the same protocol as in a). C, DAPI (blue) and 488-labeled anti-rat IgG (1 µg) was used as control. Bar: 50 microns

active SLC22A17 reduced, even abolished, the transport and the production of IL25 and IL33.

Finally, we wanted to study the presence of SLC22A17 using different epithelial cell types. As it can be noticed in Figure 3F, the presence of SLC22A17 bands was found in HaCaT and Calu-3 cell lines, but it was observed neither in intestinal Caco-2 cells nor in immune-derived cells such as THP1 and PBMCs.

3.4 | The presence of the catechol-like ligand of Alt a 1 is necessary for interaction with SLC22A17 in epithelial cells

The interaction of Alt a 1 with SLC22A17 was also confirmed by means of immunohistochemistry. In the airway epithelial Calu-3 cell model, Alexa 647-labeled holo-Alt a 1 (Figure 4) co-localized with SLC22A17 (Figure 4; Alexa 488), whereas no co-localization was observed in the absence of the ligand (Alexa 647-apo- form; Figure 4, "Apo-").

The same result was observed using mouse lung sections (Figure 5A). Holo-Alt a 1 then immunolocalized in the alveolar regions, co-localizing with the SLC22A17. This co-localization was not observed when lung sections were pre-incubated with siderocalin, as the natural ligand of the receptor competes with the allergen and acts as an inhibitor of this union. Thus, this feature demonstrates the specific binding of Alt a 1 to the SLC22A17 receptor (Figure 5B).

3.5 | In silico analysis suggests an interaction model of holo-Alt a 1 with the SLC22A17

The preceding assays were complemented with in silico studies on the optimized structures of SLC22A17-Alt a 1 and SLC22A17-siderocalin complexes (Figure 6). Our model structure of the receptor shows an all- α architecture with a large transmembrane central helical bundle, an intracellular small helical domain (residues ~230-290), and two extracellular loop regions that span residues ~70-100 and ~380-400 (marked "1" and "2," respectively, in Figure 6B) and locate at opposite sides of the extracellular surface (Figure 6A, B).

As said above, the holo-form of Alt a 1 is a stable tetramer.^{17,18} For its interaction with SLC22A17, computational modeling provided a nearly symmetrical arrangement of the tetramer on top of the receptor whose extracellular domains make two interface regions with two subunits of Alt a 1 (Figure 6A). Interestingly, these contact regions are located just below the binding sites of the ligand in tetrameric Alt a 1 (Figure 6A). PRODIGY calculations predicted a SLC22A17-Alt a 1 tetramer binding affinity $\Delta G = -6.9$ kcal/mol, which corresponds to a dissociation constant $K_d = 8.6$ μ mol/L.

The same computational procedure applied to the SLC22A17-siderocalin complex predicted a geometry in which the monomeric lipocalin binds to the N-terminal extracellular domain of SLC22A17 (Figure 6B). This optimized structure shows that the interaction occurs at the calyx of siderocalin. PRODIGY results gave for this complex a binding affinity $\Delta G = -9.3$ kcal/mol, that is, a dissociation constant $K_d = 0.16$ μ mol/L. Our structural model confirms recent experimental results obtained with different biophysical techniques for the interaction between the 105-residue N-terminal domain of SLC22A17 and siderocalin.³² Although no structure of SLC22A17 was considered in that report, solution state nuclear magnetic resonance (NMR) indicated that SLC22A17 binds in the calyx of siderocalin, and thermodynamic isothermal titration calorimetry (ITC) and microscale thermophoresis (MST) data gave a 1:1 stoichiometry with affinity $K_d = 10 \pm 2$ μ mol/L (ITC) and 6.6 ± 1.3 μ mol/L (MST).³¹ It should be emphasized that these K_d values were obtained for a SLC22A17-siderocalin complex in which only the N-terminal fragment composed of the first 105 amino acids of SLC22A17 was present. In contrast, our computational K_d values refer to models of the complete structure of SLC22A17 and therefore include binding contributions arising from other surface regions in the extracellular domain of the SLC22A17 (segments marked "1" in Figure 6B) such as the segment composed of polar residues S150, S151, and T152 (data not shown).

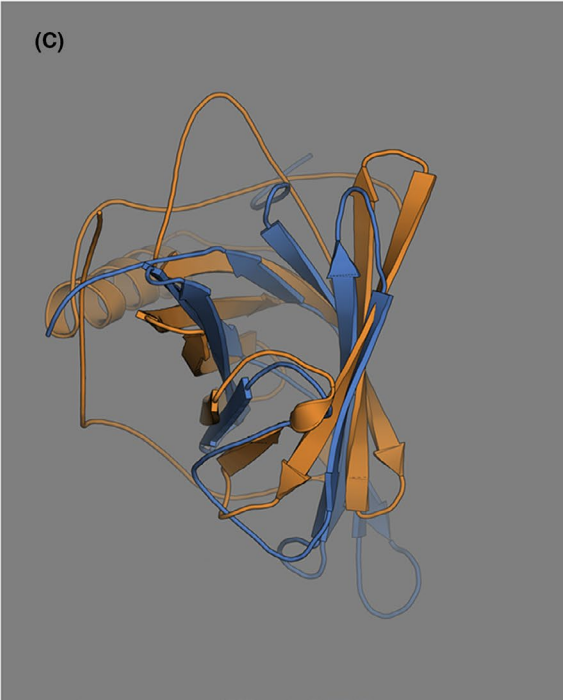
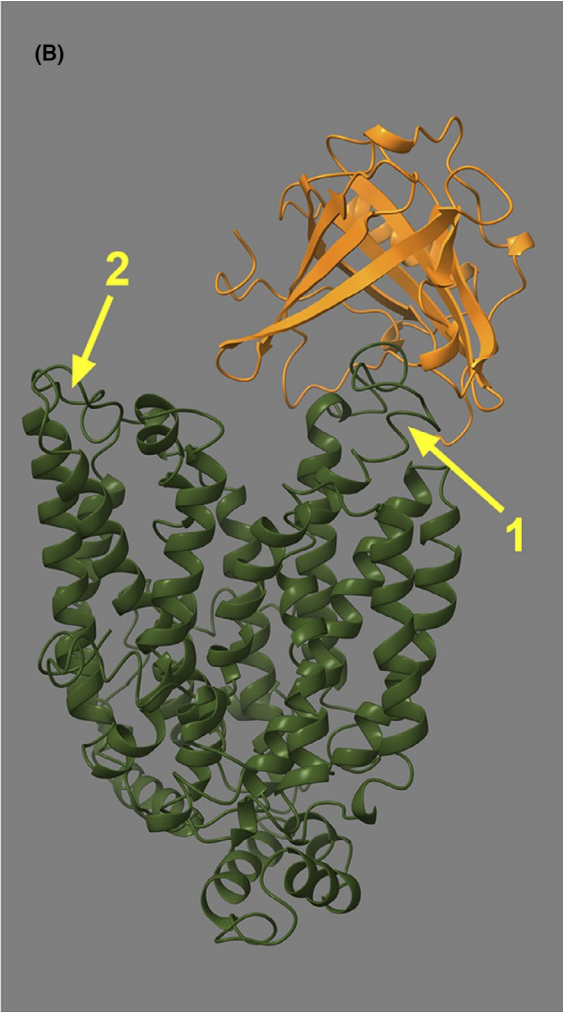
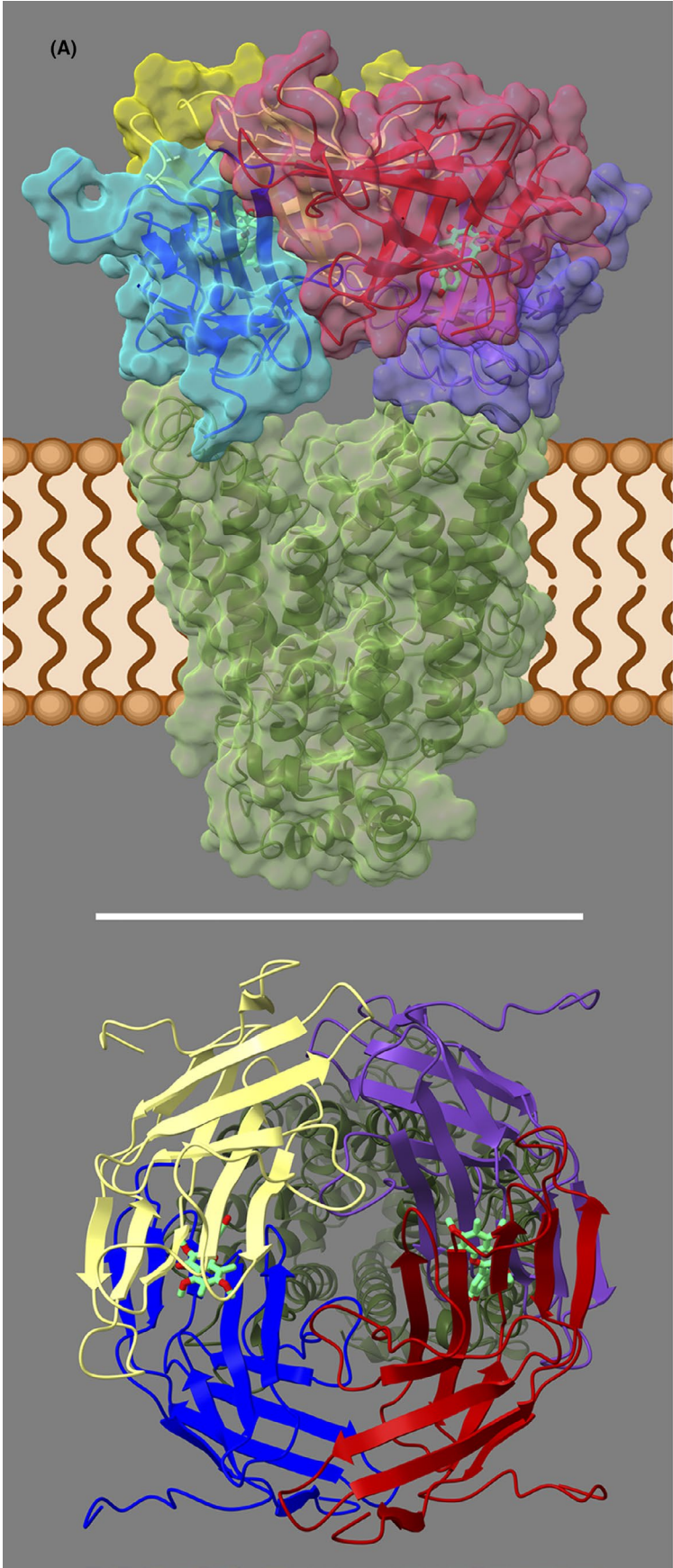
3.6 | Alt a 1 bears structural resemblance to siderocalin

The interaction between SLC22A17 and Alt a 1 might hint at some structural relationship between the allergen and siderocalin. Using the three different algorithms indicated in Material and Methods that implement completely different approaches to the structural alignment problem, the comparison between the structures of both proteins yielded the following scores: FATCAT P -value = 0.01, CE Z-score = 3.70, and TM-Align TM-score = 0.400. According to their significance ranges,^{30,31,33} the three methods therefore predict a weak yet statistically significant resemblance. The superposition of Alt a 1 and siderocalin (CE result, Figure 6C) shows that both proteins share the arrangement of β -strands in the β -barrel, which is very likely a key structural feature in the common recognition of both proteins by SLC22A17.

4 | DISCUSSION

According to the results gathered by our group^{17,18,34,35} and the evidence reported in the literature in recent years,³⁶ it can be hypothesized that the presence of ligands carried by some allergen proteins

FIGURE 6 A, Model structure of SLC22A17-tetrameric Alt a 1 complex. Top: Surface representation with transparency to show backbones in the cartoon representation. The ligand of Alt a 1 is depicted under the surfaces as sticks with light green carbons. SLC22A17 is colored green, and the membrane scheme indicates approximately the extent of its transmembrane helical domain. Bottom: Upper view of the same complex in cartoon representation. The receptor is shown below the four chains of the allergen, and the ligand of Alt a 1 is depicted as in a. B, Model structure of SLC22A17-siderocalin complex in cartoon representation. Loops' regions marked with yellow arrows are N-term ("1") and C-term ("2") extracellular recognition domains of the receptor. C, Cartoon representation of the structural superposition of human siderocalin (orange) and Alt a 1 (blue) proteins computed with the CE method



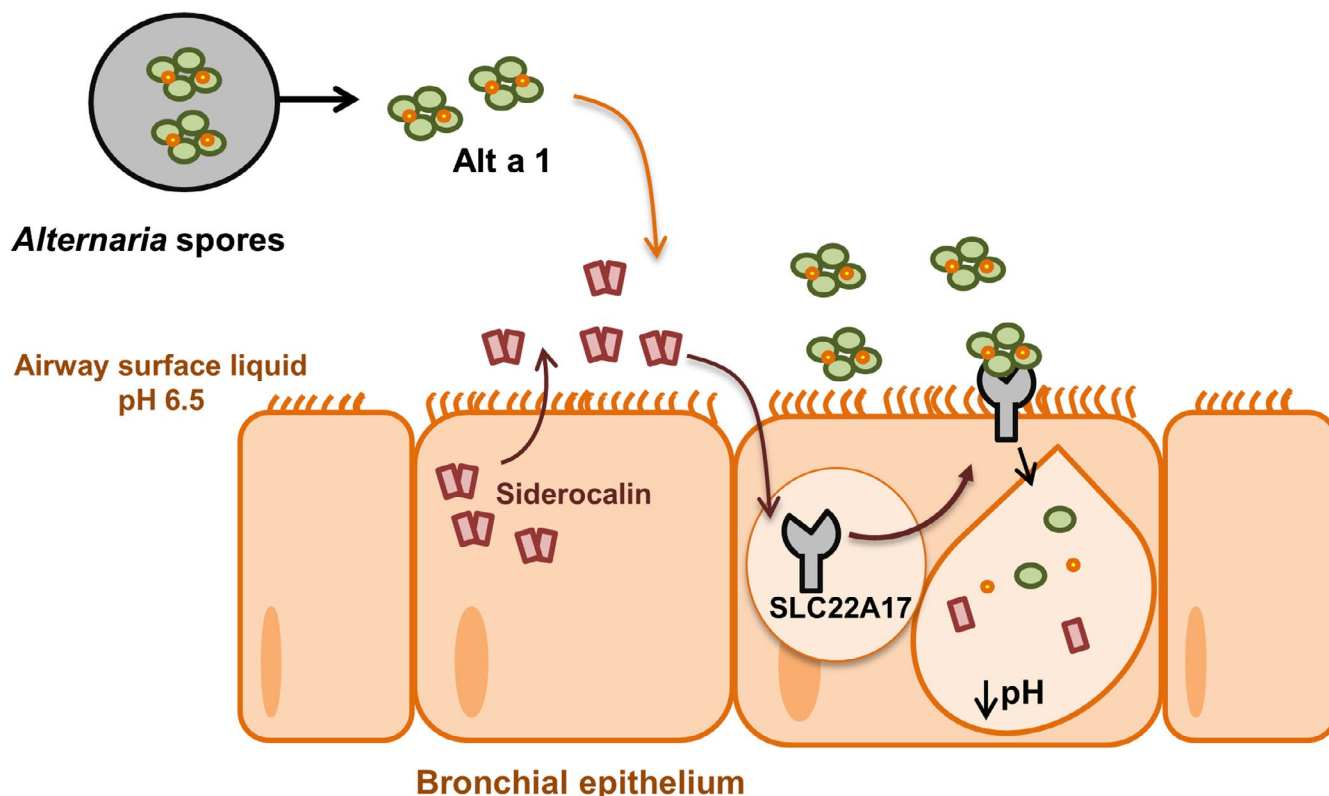


FIGURE 7 Proposed model for the molecular mechanism followed by Alt a 1 to cross through the airway epithelium. See text in Discussion 3.2

may be of paramount importance in the allergic sensitization. Those ligands, despite of having different structures, can be recognized by specific epithelial receptors, activating the innate immune system, thus leading to allergic sensitization in susceptible individuals. In the case of Alt a 1, the major fungal allergen from *A. alternata* responsible for a number of respiratory allergies and severe asthma,³⁷ the protein is released from fungus spores in a tetrameric state that only is assembled in the presence of a flavonol ligand that includes a catechol group.^{17,18}

In this work, we aimed to elucidate the interactions between the *Alternaria* allergen and human airway epithelium. Our data show that when Alt a 1 was added on the apical side of a polarized epithelial monolayer, it was transported, regardless of the presence of its catechol-like ligand. In contrast, Alt a 1 interacted with a cellular receptor only in the presence of its ligand, that is, only in its holo-form. Our study identified this receptor as the solute carrier family 22 member 17, the receptor of human siderocalin protein.^{32,38} Therefore, a first essential point in the interaction between the allergen and airway epithelium is that Alt a 1 in its holo-form is tetrameric and is primarily recognized by the SLC22A17, whereas when the ligand is absent, Alt a 1 in its apo- form is mostly dimeric. These changes in the aggregation state could be the explanation of the differential recognition of Alt a 1 depending on the presence or absence of its ligand.¹⁸

Solute carrier family 22 member 17 receptor was identified as a surface receptor expressed on epithelial cells,³⁹ mediating protein

endocytosis⁴⁰ as well as inducing an innate immune response.^{39,41} SLC22A17 can specifically recognize human siderocalin, also known as uterocalin, or 24p3 protein.^{42,43} Siderocalin is particularly important in protecting against bacterial infection as it can prevent iron acquisition by pathogenic bacteria by binding iron-binding chelators commonly bearing catechol groups called siderophores produced by microorganisms.⁴⁴ Some of the roles in disease are associated with the ability of siderocalin to modulate oxidative stress.

Our findings reveal that SLC22A17 confers the ability to discriminate between free (apo-) and ligand bound (holo-) forms of the Alt a 1 allergen. Our in silico study also suggested that Alt a 1 shares some structural relationship with siderocalin in their similar β -barrel fold and overall size, and that the recognition of both proteins by SLC22A17 involves exactly the same loop region in the extracellular domain of SLC22A17. In contrast, their interaction with the receptor presents some relevant differences. While siderocalin in monomeric form binds at its calyx, the N-terminal region of the extracellular domains of the SLC22A17,³² tetrameric holo-Alt a 1 interacts with extracellular domains of SLC22A17 that interface with two Alt a 1 subunits in the tetramer at a nearly symmetrical arrangement.

The interaction of the holo-allergen with SLC22A17 must thus be the key to activate the epithelial mucosa. This has also been described for other allergens^{35,45} in which the presence of a transported ligand is essential to the activation of the mucosa and cells of the immune system. However, this could not be the only route to activate immune cells. In fact, we show in the current work that

SLC22A17 was not present in the human monocyte cell line THP1 and human PBMCs, although Alt a 1 was capable of activating or inducing their proliferation. Therefore, other recognition pathways should be possible and SLC22A17 would only be one although very active in airway mucosa.

Alt a 1 might be mimicking human siderocalin as the airway entry route, and thus, based on the results of our study, the following model could be proposed (Figure 7). Tetrameric holo-Alt a 1 is released by *Alternaria* spores in the presence of a flavone-catechol ligand and arrives at the airway epithelium. Spores induce the activation of the immune system by increasing the release of siderocalin (data not shown), and holo-Alt a 1 is recognized by SLC22A17 on the cell surface. The subsequent endocytosis promoted by SLC22A17³⁸ moves their target proteins to inner cell compartments where the environment is acidic. At low pH, Alt a 1 tetramer breaks down releasing its constituent monomers and the ligand as well.^{17,18} The catechol groups of the free flavonol ligand could then participate in antioxidant processes, and free Alt a 1 monomers should arrange in their most stable association which, in the absence of the ligand, is a highly symmetric dimer¹⁴ with epitope surface regions located at proper orientation for cross-linking in further recognition by IgE.¹⁸

Nevertheless, new studies are needed to understand the whole picture as well as to clarify the sequence of events that occur in airway cells leading to allergic sensitization. The work here presented offers a relevant piece of information to understand the interplay between innate and adaptive immunity in allergic airway diseases. Elucidating the details of the Alt a 1 sensitization, mechanisms will ultimately lead to a better understanding of the pathophysiology that governs one of the most prevalent and costly chronic diseases in industrialized countries.

ACKNOWLEDGMENTS

This research was funded by the Spanish Government, MINECO (grant BIO2017-84548-R), and ISCIII (Grants PI16/00249 and PI15/02256). JTA was granted from Instituto de Salud Carlos III (ISCIII) co-founded by FEDER Thematic Networks and Cooperative Research Centers: ARADYAL (RD16/0006/0003, RD16/0006/0013, RD16/0006/0015). MGA, DPC, and GHR received fund support from Allerscreening project (EU SEP-210415617). Geometry optimizations were performed on the Magerit supercomputer of Universidad Politécnica de Madrid. The authors thankfully acknowledge the computer resources, technical expertise, and assistance provided by the Supercomputing and Visualization Center of Madrid (CeSViMa). The study was approved by the ethics committees of the Transfusion Centre (PO.DIS.09; Madrid, Spain) and the Universidad Politécnica de Madrid (PIC 52-2013; Madrid, Spain).

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

MGA, JTA, and ADP performed the experimental design. MGA and GHR performed the protein assays. JTA, DPC, VE, and AMY performed the IHQ assays. MGA and LFP performed the computational analysis. DB, MME, LFP, and ADP wrote, edited, and revised the manuscript.

ORCID

María Garrido-Arandia  <https://orcid.org/0000-0001-6114-5754>

Domingo Barber  <https://orcid.org/0000-0002-5488-5700>

Luis F. Pacios  <https://orcid.org/0000-0002-0585-4289>

Araceli Díaz-Perales  <https://orcid.org/0000-0002-1093-3627>

REFERENCES

1. Thomas WR. Molecular mimicry as the key to the dominance of the house dust mite allergen Der p 2. *Expert Rev Clin Immunol*. 2009;5(3):233–237.
2. Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, Nadeau KC. Molecular and cellular mechanisms of food allergy and food tolerance. *J Allergy Clin Immunol*. 2016;137(4):984–997.
3. Hoffmann-Sommergruber K, Mills E. Food allergen protein families and their structural characteristics and application in component-resolved diagnosis: new data from the EuroPrevall project. *Anal Bioanal Chem*. 2009;395(1):25–35.
4. del Moral MG, Martínez-Naves E. The role of lipids in development of allergic responses. *Immune Netw*. 2017;17(3):133.
5. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol*. 2014;134(3):521–529.
6. Knutsen AP, Bush RK, Demain JG, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol*. 2012;129(2):280–291.
7. Bush RK, Prochnau JJ. *Alternaria*-induced asthma. *J Allergy Clin Immunol*. 2004;113(2):227–234.
8. Hiranrattana A, Stern DA, Guerra S et al. *Alternaria* sensitisation at age 6 years is associated with subsequent airway hyper-responsiveness in non-asthmatics. *Thorax*. 2018;73(12):1170–1173.
9. Soffer N, Green BJ, Acosta L, et al. *Alternaria* is associated with asthma symptoms and exhaled NO among NYC children. *J Allergy Clin Immunol*. 2018;142(4):1366–1368.
10. Lyons TW, Wakefield DB, Cloutier MM. Mold and *Alternaria* skin test reactivity and asthma in children in Connecticut. *Ann Allergy, Asthma Immunol*. 2011;106(4):301–307.
11. Gabriel MF, Postigo I, Tomaz CT, Martínez J. *Alternaria alternata* allergens: markers of exposure, phylogeny and risk of fungi-induced respiratory allergy. *Environ Int*. 2016;89–90:71–80.
12. Peng X, Madany AM, Jang JC, et al. Continuous inhalation exposure to fungal allergen particulates induces lung inflammation while reducing innate immune molecule expression in the brainstem. *ASN Neuro*. 2018;10:175909141878230.
13. Feo Brito F, Alonso AM, Carnés J et al. Correlation between Alt a 1 levels and clinical symptoms in *Alternaria alternata*-monosensitized patients. *J Investig Allergol Clin Immunol*. 2012;22:154–159.
14. Chruszcz M, Chapman MD, Osinski T, et al. *Alternaria alternata* allergen Alt a 1: a unique β -barrel protein dimer found exclusively in fungi. *J Allergy Clin Immunol*. 2012;130(1):241–247.e9.
15. Asturias JA, Ibarrola I, Ferrer A, et al. Diagnosis of *Alternaria alternata* sensitization with natural and recombinant Alt a 1 allergens. *J Allergy Clin Immunol*. 2005;115(6):1210–1217.

16. Morín M, Asturias JA, Domínguez A. Expression of Alt a 1 allergen from *Alternaria alternata* in the yeast *Yarrowia lipolytica*. *FEMS Microbiol Lett*. 2012;333:121-128.
17. Garrido-Arandia M, Silva-Navas J, Ramírez-Castillejo C, et al. Characterisation of a flavonoid ligand of the fungal protein Alt a 1. *Sci Rep*. 2016;6: Article ID: 33468.
18. Garrido-Arandia M, Bretónes J, Gómez-Casado C, Cubells N, Díaz-Perales A, Pacios LF. Computational study of pH-dependent oligomerization and ligand binding in Alt a 1, a highly allergenic protein with a unique fold. *J Comput Aided Mol Des*. 2016;30(5):365-379.
19. Thomas WR. Allergen ligands in the initiation of allergic sensitization topical collection on allergens. *Curr Allergy Asthma Rep*. 2014;14(5). Article ID: 432.
20. Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc*. 2010;5(4):725-738.
21. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. The I-TASSER suite: protein structure and function prediction. *Nat Methods*. 2015;12(1):7-8.
22. Lyskov S, Chou F-C, Conchúir SÓ, et al. Serverification of molecular modeling applications: the rosetta online server that includes everyone (ROSIE). *PLoS One*. 2013;8(5):e63906.
23. Goetz DH, Willie ST, Armen RS, Bratt T, Borregaard N, Strong RK. Ligand preference inferred from the structure of neutrophil gelatinase associated lipocalin. *Biochemistry*. 2000;39(8):1935-1941.
24. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996;14(1):33-38.
25. Phillips JC, Braun R, Wang W, et al. Scalable molecular dynamics with NAMD. *J Comput Chem*. 2005;26(16):1781-1802.
26. Best RB, Zhu X, Shim J, et al. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone ϕ , ψ and side-chain χ_1 and χ_2 Dihedral Angles. *J Chem Theory Comput*. 2012;8:3257-3273.
27. Xue LC, Rodrigues JP, Kastiris PL, Bonvin AM, Vangone A. PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. *Bioinformatics*. 2016;32:3676-3678.
28. Goddard TD, Huang CC, Meng EC, et al. UCSF ChimeraX: meeting modern challenges in visualization and analysis. *Protein Sci*. 2018;27(1):14-25.
29. Ye Y, Godzik A. Flexible structure alignment by chaining aligned fragment pairs allowing twists. *Bioinformatics*. 2003;19(Suppl 2):ii246-ii255.
30. Shindyalov IN, Bourne PE. Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng Des Sel*. 1998;11(9):739-747.
31. Zhang Y, Skolnick J. TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res*. 2005;33(7):2302-2309.
32. Cabedo Martínez A-I, Weinhäupl K, Lee W-K, et al. Biochemical and structural characterization of the interaction between the siderocalin NGAL/LCN2 (Neutrophil Gelatinase-associated lipocalin/lipocalin 2) and the N-terminal domain of its endocytic receptor SLC22A17. *J Biol Chem*. 2016;291(6):2917-2930.
33. Ye Y, Godzik A. FATCAT: a web server for flexible structure comparison and structure similarity searching. *Nucleic Acids Res*. 2004;32(Web Server):W582-W585.
34. Cubells-Baeza N, Gómez-Casado C, Tordesillas L, et al. Identification of the ligand of Pru p 3, a peach LTP. *Plant Mol Biol*. 2017;94(1-2):33-44.
35. Tordesillas L, Cubells-Baeza N, Gómez-Casado C, et al. Mechanisms underlying induction of allergic sensitization by Pru p 3. *Clin Exp Allergy*. 2017;47(11):1398-1408.
36. Trompette A, Divanovic S, Visintin A, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature*. 2009;457(7229):585-588.
37. Ojeda P, Ibáñez MD, Olaguibel JM, Sastre J. A national survey on allergic diseases in the Spanish pediatric population. *J Investig Allergol Clin Immunol*. 2018;28(5):321-329.
38. Langelueddecke C, Roussa E, Fenton RA, Wolff NA, Lee WK, Thévenod F. Lipocalin-2 (24p3/neutrophil gelatinase-associated lipocalin (NGAL)) receptor is expressed in distal nephron and mediates protein endocytosis. *J Biol Chem*. 2012;287(1):159-169.
39. Langelueddecke C, Roussa E, Fenton RA, Thévenod F. Expression and function of the Lipocalin-2 (24p3/NGAL) receptor in rodent and human intestinal epithelia. *PLoS One*. 2013;8(8):e71586.
40. Fang W-K, Xu L-Y, Lu X-F, et al. A novel alternative spliced variant of neutrophil gelatinase-associated lipocalin receptor in oesophageal carcinoma cells. *Biochem J*. 2007;403(2):297-303.
41. Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature*. 2004;432(7019):917-921.
42. Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. *Cell*. 2005;123(7):1293-1305.
43. Chakraborty S, Kaur S, Guha S, Batra SK. The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. *Biochim Biophys Acta - Rev Cancer*. 2012;1826(1):129-169.
44. Correnti C, Richardson V, Sia AK, et al. Siderocalin/Lcn2/NGAL/24p3 does not drive apoptosis through gentisic acid mediated iron withdrawal in hematopoietic cell lines. *PLoS One*. 2012;7(8):e43696.
45. Asam C, Batista AL, Moraes AH, et al. Bet v 1 - a Trojan horse for small ligands boosting allergic sensitization? *Clin Exp Allergy*. 2014;44(8):1083-1093.

How to cite this article: Garrido-Arandia M, Tome-Amat J, Pazos-Castro D, et al. Interaction of Alt a 1 with SLC22A17 in the airway mucosa. *Allergy*. 2019;74:2167-2180. <https://doi.org/10.1111/all.13877>