

# Group 1 allergens, transported by mold spores, induce asthma exacerbation in a mouse model

To the Editor

Grass pollen is the most common cause responsible for allergic asthma in Europe, being *Phleum pratense* (Timothy grass) the most frequent associated grass.<sup>1</sup> However, many patients with grass allergy also suffer severe asthmatic exacerbations in early autumn, as was reported in the case of Ciudad Real (Spain) or Canada.<sup>2,3</sup> In the center of Spain, in autumn, grass has undergone senescence and turned to straw, and many mold spores live on their surfaces. Mold spores are usually found in high quantities in the air in that season, and it may be relevant inducer of asthma attacks.<sup>4</sup> Therefore, we hypothesized that some asthma exacerbations suffered by grass allergic patients in autumn could be triggered by the combination of both elicitors: mold spores and grass allergens.

In order to confirm this hypothesis, an asthma murine model was developed (Ref. PROEX 392/15; Appendix S1). Thus, BALB/c mice were sensitized with grass (*Phleum pratense*) pollen proteins (100 µg) for 6 weeks once every week, and in week seven, allergic animals were provoked asthma by inhalation of different challenges: pollen proteins (100 µg), or straw proteins (100 µg) with mold spores (10<sup>6</sup>) (Figure 1A). Mold used as challenge were isolated from grass straw, harvested in September-October in the center of Spain, and deposited directly on PDA medium. *Alternaria* sp was identified by ITS sequencing with over 90% of identity.

Allergic mice suffered from asthma exacerbations, with both challenges, pollen or grass straw and spores. However, these symptoms (temperature drop, Figure 1B, and lung function loss, Figure 1C) were not observed in healthy nonsensitized mice challenged in the same way, or in mice sensitized with pollen and challenged with straw proteins (data not shown).

In accordance, allergic mice showed similar cell profile in their bronchoalveolar lavage (BAL) after challenge with pollen or with grass straw and spores (Figure 1D). Total cell number in BAL was significantly increased in allergic mice challenged compared to control nonsensitized mice (Figure 1D, total). This increase was mainly due to a higher number of CD170<sup>+</sup> cells (Figure 1D, CD170<sup>+</sup>), rather than CD3<sup>+</sup> cells (Figure 1D, CD3<sup>+</sup>).

Histological analysis of lungs revealed that allergic mice developed an asthma (AHR) phenotype, related to their symptoms as evidenced by the general architecture and infiltration. Eosin-hematoxylin staining showed significant differences between allergic and healthy mice (Figure 1E). Pollen allergic mice showed epithelial

disruption, alveoli lumen narrowing, metaplasia, infiltration, and sub-epithelial fibrosis, while healthy control groups revealed a clear parenchyma devoid of inflammatory cells. Detection of CD45<sup>+</sup> cells revealed significant infiltration in pollen sensitized mice compared to nonsensitized control (Figure S1).

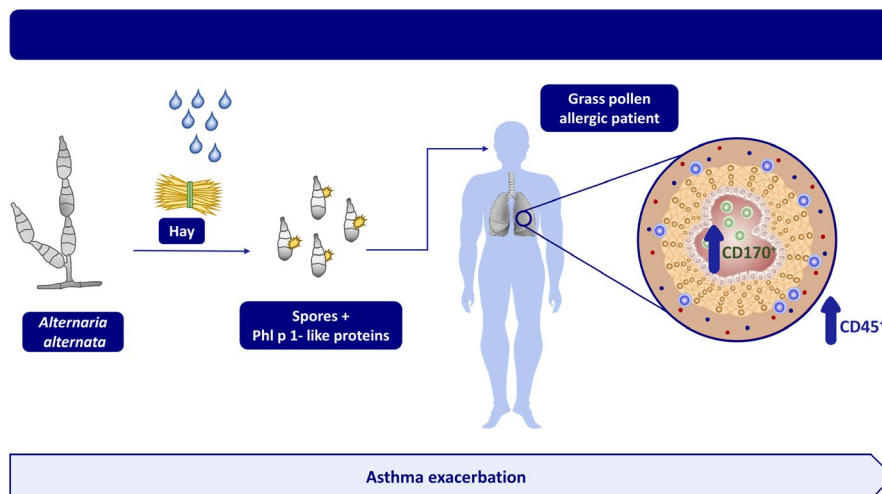
Since no description has been made for the presence of allergens in grass straw, we next set to study how straw proteins could induce the same phenotype as pollen when used as challenge. So, different immunodetection assays were performed using a serum pool from grass pollen patients (n = 32) (Figure 2A), recruited based on their clear natural history to grass pollen allergy. Patients suffered from asthma, mainly caused by grass pollen.

The immunodetection assays showed a 30 kDa positive IgE-binding protein in pollen extract (Figure 2A, Sera), in contrast with a IgE positive band of 70 kDa in the straw extract. Both bands were sliced from SDS-PAGE gel and identified as Group 1 allergen by peptide mass fingerprinting at the Proteomic Service Facility from the Complutense University of Madrid.

To confirm that both IgE-binding bands corresponded to Group 1 allergens, an inhibition assay with pollen proteins was carried out. Serum pool was preincubated with pollen proteins, before performing immunoblotting. As shown in Figure 2A (Sera + pollen), both IgE bands were inhibited. In addition, both were confirmed as Group 1 allergens using an specific anti-Phl p 1 antibody (Figure 2A, α-Phl p 1).

Group 1 proteins belong to the β-expansin family which are responsible for cell-wall loosening activity and their expansion.<sup>5</sup> Among all grass pollen allergens, Group 1 are the most prevalent, being recognized by 90% of grass pollen allergic patients in the center of Spain, and showing high cross-reactive allergens in the majority of grass species, including temperate and tropical grasses. It has been described in molecular epidemiological studies in Europe,<sup>6</sup> as well as in molecular sensitization screening of North-American grass allergic patients,<sup>7</sup> that Group 1 allergen prevalence is significantly higher than other grass allergens, despite its lower content in the pollen.<sup>8</sup> Strikingly, almost all children started their sensitization toward the major Group 1 grass pollen allergen, some even years prior to the occurrence of clinical symptoms.<sup>9</sup>

To study how Group 1 allergen reaches the patients' airway, *Alternaria* spores (10<sup>6</sup>) were incubated with straw proteins (100 µg), and retained proteins were analyzed using a serum pool from selected patients (Figure 2C). IgE-reactive proteins showed a similar



### GRAPHICAL ABSTRACT

Grass allergic patients recognize group 1 allergens in grass straw. Group 1 allergens from grass straw are able to interact with spores. Spores bound to Phl p 1-like proteins from grass straw can induce an asthma exacerbation in mouse model.

profile as obtained after incubation with anti-Phl p 1 (Figure 2D). Group 1 allergens, present in pollen and straw, are capable of physically bind to spores. So, mold spores present onto grass might act as air-carriers of pollen-like allergens.

Studies have shown that molds occupy a prominent place in the development of asthma. An increase in the number of hospitalizations due to asthma exacerbations during the main spore counts in hot, humid climates has been well documented, as well as in the Mediterranean region. That is only in South Europe, over 20% of the patients with a history of respiratory allergy showed sensitization to *Alternaria*.<sup>9</sup> Besides the importance of molds as sensitizing agents, this work addresses and adds a potential role within asthma exacerbation acting as allergen carriers.

In this study, it has been shown that the asthmatic phenotype of a grass mouse model can only be reproduced in the presence of

straw allergens carried by *Alternaria alternata* spores, which could be acting as carriers/haptens and promote the onset of the allergic reaction. In conclusion, asthma exacerbations in autumn among grass pollen patients could be mainly due to the inhalation of mold spores carrying grass Group 1 allergens. The presence of this mixture is favored in autumn and during storms, since they increase the amount of spores carrying allergens in the air. In addition, the high degree of co-sensitization between grass and *Alternaria sp* allergens could increase the symptomatology of those patients. Several questions related to the role of molds within asthma are yet to be solved, but it is clear that molds are very relevant players within allergic diseases in very different ways. Better understanding of this key role of molds in asthma would improve both diagnostic and therapeutic tools available for handling this prevalent disease that deteriorates millions of people's quality of life.

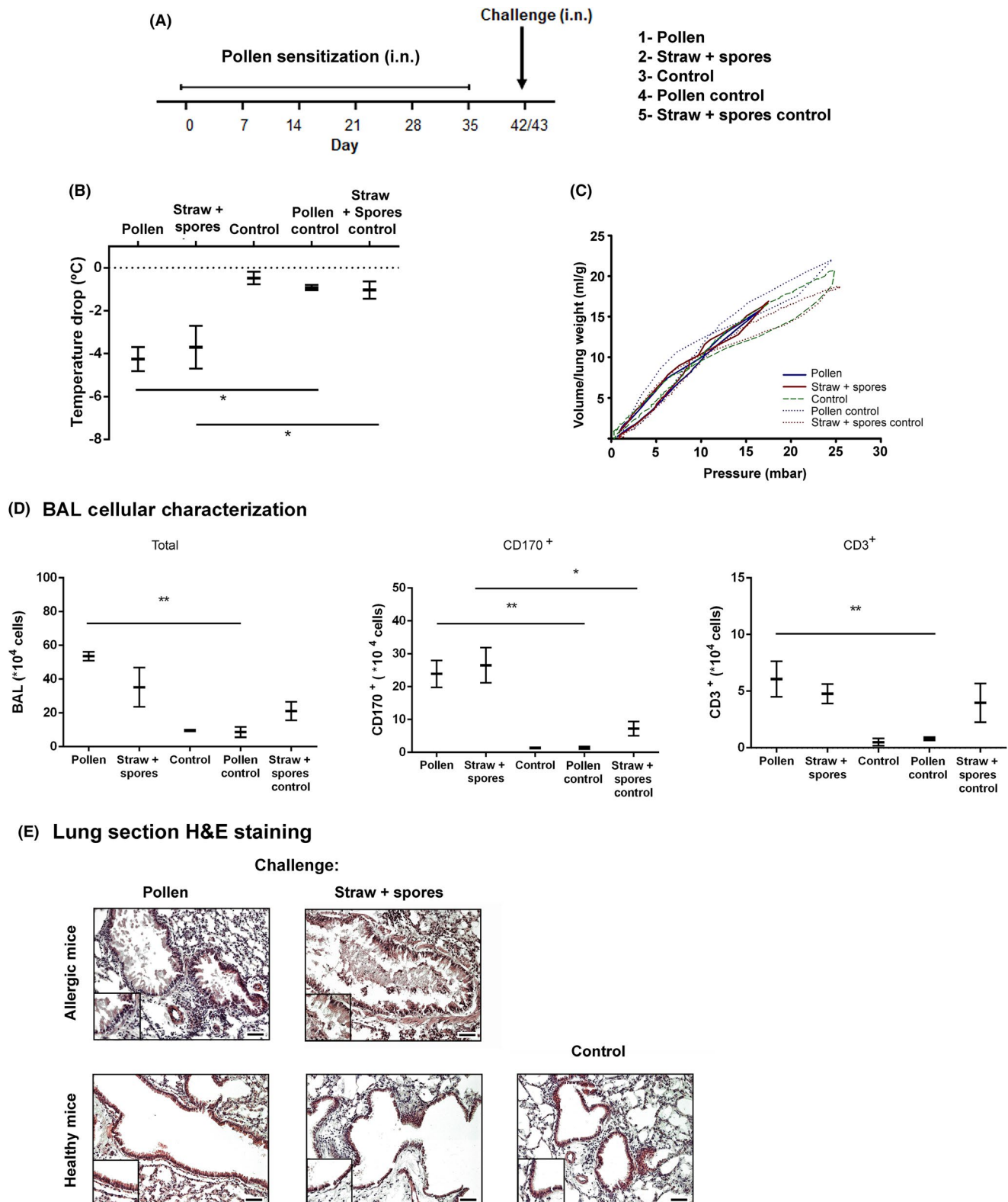


**FIGURE 1** Grass allergic mouse model. (A) BALB/c mice were intranasally (i.n.) exposed to grass pollen protein extract (100 µg) once per week for 6 wk. After sensitization, i.n. challenge was performed with pollen proteins (Pollen; 100 µg pollen), or with straw proteins and spores (Straw + spores; 10<sup>6</sup> spores preincubated with 100 µg of grass-straw proteins). Healthy (nonsensitized) mice were used as control and challenged similarly to allergic mice. (B) Drop in the body temperature after challenge was measured as anaphylactic marker by rectal thermometer. (C) Lung resistance graph was analyzed as the variation in volume capacity corresponding with the area between inspiration/expiration plots for average of each group after challenge (n = 3). Data are mean-SEM. \*\**P* < .05, \**P* < .01. (D) BAL fluid was obtained from mice after challenge. An average of total cells in BAL has been represented; average of CD170<sup>+</sup> cells and average of CD3<sup>+</sup> cells. Data are presented as mean of n = 5 (SEM. \*\**P* < .05, \**P* < .01). (E) Lung sections stained with Hematoxylin-Eosin. Bar = 50 µm

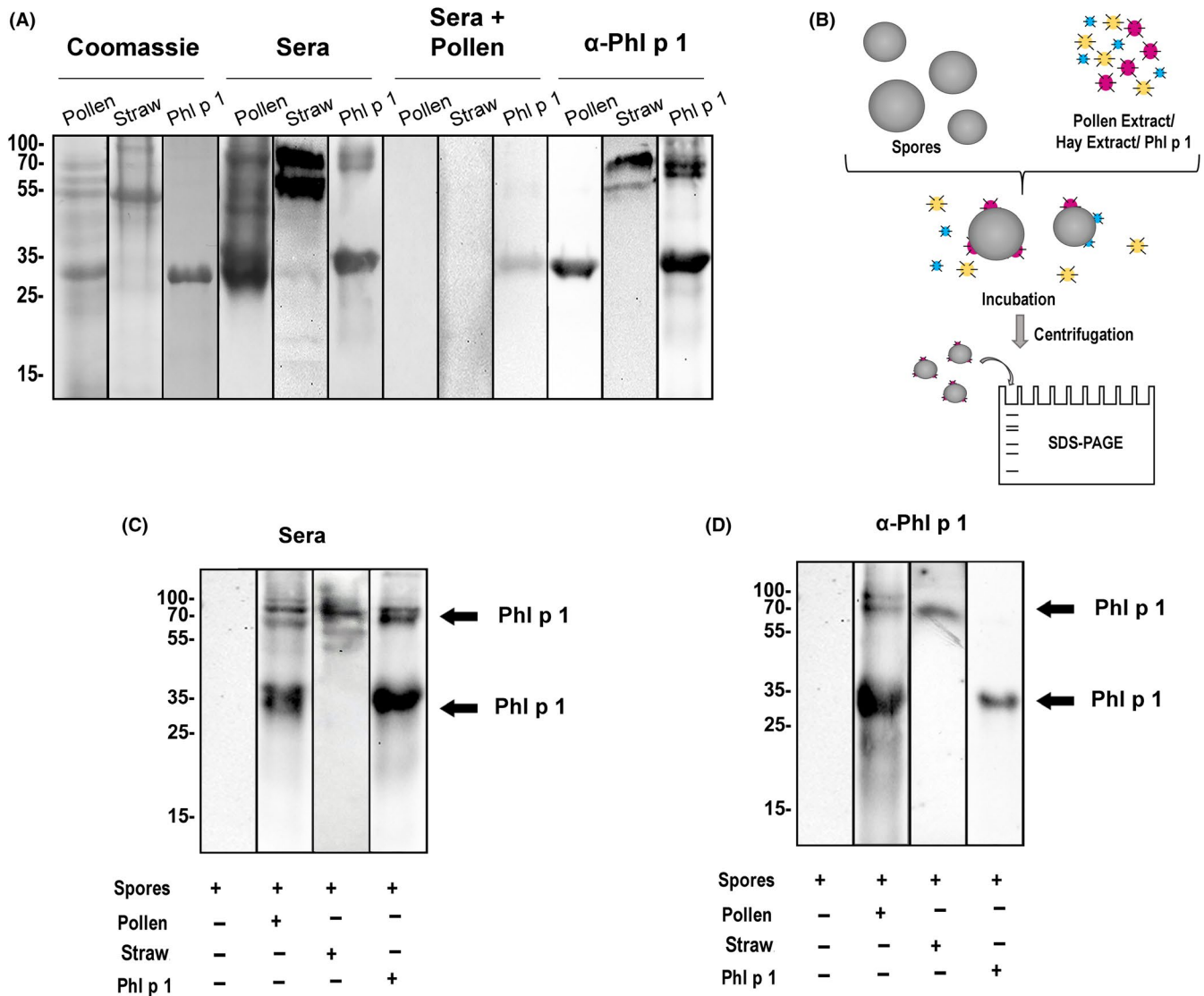


**FIGURE 2** Identification of Group 1 allergens in straw. (A) Grass pollen (Pollen) and straw protein extract (Straw) were separated by SDS-PAGE, and Coomassie stained or electrotransferred onto PVDF and incubated with a serum pool from grass pollen patients ( $n = 32$ ). The serum pool was used directly (Sera) or after preincubation with: pollen proteins (Sera + pollen; 30  $\mu$ g) for inhibition assay. Also, similar PVDF membranes were incubated with specific polyclonal antibody against Phl p 1 (anti-Phl p 1). (B) Scheme of pull-down assays (C-D). Pull-down assay performed with  $10^6$  spores and pollen proteins, straw proteins and purified Phl p 1. The retained fractions were separated by SDS-PAGE, transferred onto PVDF, and incubated with a serum pool from pollen allergic patients ( $n = 32$ , C) or with specific polyclonal antibody against Phl p 1 (D)

#### CONFLICT OF INTEREST

There are no conflicts of interest.

#### FUNDING INFORMATION

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

## SUPPORTING INFORMATION

### **Supplementary Material and Methods.**

#### Grass pollen and straw extracts

Grass pollen and straws from common grasses (*Lolium perenne* and *Phleum pratense*) were defatted with acetone (1:5 [w/v]; 1 h; 4°C). Secondly, once dried, proteins were extracted with PBS (1:5 (w/v), 1 h, 4°C). After centrifugation (5000 rpm; 15 min; 4°C), the supernatants were dialyzed against distilled H<sub>2</sub>O (cut-off point 3.5 kDa). Protein concentration was quantified according to Bradford's method<sup>11</sup>.

#### Experimental mouse model of anaphylaxis.

Female BALB/c mice (6-8 week old) from the Charles River breeding colony (France) were 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, D.C. (NIH Publication No. 85-23, revised 1985). Mice were maintained in the IIS-Fundación Jiménez Díaz (Madrid, Spain) under standard laboratory conditions.

To obtain grass pollen mice (pollen allergic model), pollen extract (100 µg protein) were administered intranasally (i.n.) once a week for 6 weeks. A week after the last exposure, mice were challenged through the same route. To carry out samples administration, mice were slightly anaesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and once unresponsive but breathing comfortably, 50 µl of challenge solution was directly applied on the nostrils. Animals were allowed to slowly inhale the samples and then recover in a lateral position. Rectal temperature was measured 10 min before and 10 min after challenge. A temperature drop higher than 3.5 °C was considered as indicative of anaphylactic reaction.

The different challenges tested were: pollen extract (100 µg protein); hay-straw extract (100 µg protein) mixed with *Alternaria alternata* spores (10<sup>6</sup>). As control groups, healthy mice without challenges (Control) and challenged under the same conditions were included. Each group corresponded to 5 animals (n=5).

#### Measurement of lung resistance

Lung resistance was measured within 15 min after challenge using a Numiotec ventilator system (Numio Technologies, SL, Madrid, Spain) following provider's instructions. To this end, inspiration/expiration plots were registered for each mouse for 10 min (3 cycles). Control measurements were obtained over a period of 5 min directly after administration of rocuronium. For each mouse the average plot was registered and the plot here presented is the average for each group treated. The maximum point of volume/pressure represents the dynamic compliance and the space between the lower line (inspiration) and upper line (expiration), which is directly related to lung expansion.

#### Bronchoalveolar lavage cell analysis

Tracheas from each mouse were exposed and incised fifteen minutes after the challenge. Bronchoalveolar lavage (BAL) was obtained by rinsing the lungs twice with 1 ml PBS. Total cells counts were determined with a hemocytometer. Cellular profile was also determined by flow cytometry using specific antibodies. Briefly, after fixation with PBS-PFA 4% (10 min, 25°C), cells were stained with the following fluorochrome-conjugated antibodies: anti-mouse CD170 Alexa Fluor<sup>TM</sup> 647 (clon A7R34, eBioscience<sup>TM</sup>, Thermo Fisher) and anti-mouse CD3 Alexa Fluor<sup>TM</sup> 555 (clon 17A2, eBioscience<sup>TM</sup>, Thermo Fisher) for 1 h. at room temperature. After washing, samples were analyzed using a BD Accuri Cytometer (BD, California, USA). Unstained controls were processed to correct compensation.

### Histological analysis

Mice lungs were removed, fixed in 4% PBS-formaldehyde (PFA; ThermoFisher, Waltham, MA, USA) and included in paraffin. Thin sections (7-10 µm) were cut with a microtome (Leica RM1235, Wetzlar, Germany), and stained with Haematoxylin/ Eosin (HE) following provider's instructions (Sigma-Aldrich, Spain). Stained sections were mounted using DPX (Sigma-Aldrich) and analyzed using light microscopy. For immunofluorescence staining, sections were blocked with Casein Blocking Buffer (Sigma-Aldrich). After washing with PBS, samples were incubated for 1 h at room temperature with specific antibodies against the leukocyte marker CD45 (1:100; AF114; R&D Systems, Minneapolis, MN, USA) followed by secondary antibodies conjugated with Alexa Fluor™ 488 (Thermo Fisher) for 1 h at room temperature. Nuclei were stained with DAPI and specimens were mounted with ProLong™ Gold (Thermo Fisher). Images were obtained with a Zeiss LSM 880 confocal microscope, using 405 and 488 nm laser excitations and 40X amplification. Graphical material was analyzed with ZEN 2.6 and Image-Pro Plus software.

### Patient's profile

Patients with a clear clinical history to grass pollen allergy and who suffered symptoms during the mold season (autumn) were recruited by the Allergy Service of the Hospital General Universitario de Ciudad Real (Ciudad Real, Spain). The study population comprised 32 patients (19 women and 13 men) with a mean (SD) age of 32.4 years (range 18-57 years). They were all sensitized to grass pollen and had allergic upper respiratory tract symptoms. 19 of them presented lower tract respiratory symptoms (asthma).

Allergy was also assessed according to the clinical history and SPT responses based on a panel of common commercial pollen allergen extracts. The study was approved by the Ethics Committees from Hospital Universitario de Ciudad Real and Universidad Politécnica of Madrid (BIO2017-84548-R) and written informed consent was obtained from every patient.

### Immunodetection and immunoblot inhibition

Protein extracts were separated by SDS-PAGE and electrotransferred to PVDF membranes. Membranes were washed, blocked and incubated with serum pool from allergic patients (1:5 dilutions) and developed with anti-human IgE-HRP (Sigma-Aldrich). When immunoblot inhibition was carried out, serum pool was incubated (prior incubation over membrane) with pollen extract (15 µg/line). Similar membranes were also incubated with anti-Phl p 1 antibody (RoxallMedicinaEspaña SA, Spain) and the binding was developed with anti-rabbit IgG-HRP.

### Identification of molds presented in hay straws

Grass straws were recovered in September-October 2017 in the central region of Spain. Associated molds were cultured and isolated from straws by directly placing them onto potato dextrose agar medium (PDA; Fisher Scientific, USA) and incubation for 2-3 days at 28°C. Isolated fungal colonies were transferred to a new plate and incubated for 3-10 days at 28°C repeatedly until obtaining a pure culture. Spores were scrapped off from plates into sterile distilled water containing 0.1% Triton X-100 and counted using a hemocytometer. They were then stored at -20°C in 20% glycerol until further use. Fungal genomic DNA was isolated following the manufacturer's instructions of Norgen DNA extraction kit (NorgenBiotek Corp, Ontario, Canada). Molecular identification of the isolates was performed by amplification of the internal transcribed spacer (ITS) region using the ITS1 5'-TCCGTAGGTGAACCTGCGG -3' and ITS4 5'- TCCTCCGCTTATTGATATGC-3' primers. Sequence analysis was carried out at Macrogen Corp. (USA) and the identification was performed by alignment/comparison with NCBI database.

### Isolation of spores-binding proteins

Grass pollen and grass straw extracts (70 µg) were incubated with *Alternaria* sp. spores (10<sup>6</sup>)

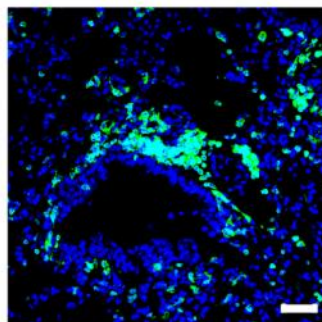
for 1 hour at 25 °C, washed and resuspended in Laemmli buffer. After centrifugation, supernatants were separated in 15% SDS-PAGE using pollen and straws extracts (10 µg) and spores ( $10^6$ ) as controls. The retained proteins were identified by peptide mass fingerprinting at the Proteomic Service facility from the Universidad Complutense de Madrid (Spain, <https://www.ucm.es/gyp/proteomica>).

#### Statistical analysis

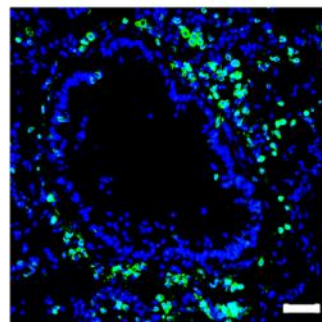
All data were reported as mean  $\pm$  SEM. Data were compared by one-way analysis of variance (ANOVA) with Kruskal-Wallis with Dunn's correction for multiple comparison test using GraphPad Prism 6 (GraphPad Software). p Values  $<0.1$  ( $p < 0.1$ ) were considered significant between two groups.

*Supplementary figure:* Thin sections of paraffin-embedded lungs incubated with specific antibody anti-CD45. CD45 infiltration in lung sections was determined for grass pollen mice, after challenge **(A, B)** compared with healthy mice without or with challenged **(C-E)**. Images were obtained by confocal microscopy. CD45<sup>+</sup> cells are colored in green and nucleus (DAPI staining) in blue. Bar 100  $\mu\text{m}$ .

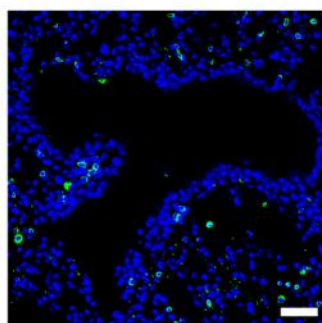
(A)



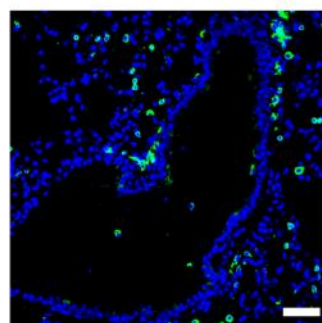
(B)



(C)



(D)



(E)

