ORIGINAL ARTICLE

Experimental Allergy and Immunology

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI **WILEY**

Pollen exposure weakens innate defense against respiratory viruses

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Abstract

Background: Hundreds of plant species release their pollen into the air every year during early spring. During that period, pollen allergic as well as non-allergic patients frequently present to doctors with severe respiratory tract infections. Our objective was therefore to assess whether pollen may interfere with antiviral immunity.

Methods: We combined data from real-life human exposure cohorts, a mouse model and human cell culture to test our hypothesis.

Results: Pollen significantly diminished interferon-λ and pro-inflammatory chemokine responses of airway epithelia to rhinovirus and viral mimics and decreased nuclear translocation of interferon regulatory factors. In mice infected with respiratory syncytial virus, co-exposure to pollen caused attenuated antiviral gene expression and increased pulmonary viral titers. In non-allergic human volunteers, nasal symptoms

Abbreviations: ADO, adenosine; ALI, air-liquid interphase; APE(s), aqueous pollen extract(s); APE < 3 kDa, low-molecular-weight fraction of aqueous pollen extract; BALF, bronchioalveolar lavage fluid; HNEC, human nasal epithelial cell; HODE, hydroxyoctodecadienoic acid; HOTE, hydroxyoctodecatrienoic acid; HRV, human rhinovirus; IFN, interferon; IRF, interferon regulatory factor; kDa, kilo dalton; MDA5, melanoma differentiation-associated protein 5; PALM(s), pollen-associated lipid mediator(s); PBEC, primary bronchial epithelial cell; PolyIC, polyinosinic:polycytidylic acid; PPE$_1$, E$_1$-phyotprostane(s); RIG-I, retinoic acid inducible gene-I; RSV, respiratory syncytial virus; TLR, toll-like receptor.

Stefanie Gilles and Cornelia Blume contributed equally to this work.

were positively correlated with airborne birch pollen abundance, and nasal birch pollen challenge led to downregulation of type I and -III interferons in nasal mucosa. In a large patient cohort, numbers of rhinoviruspositive cases were correlated with airborne birch pollen concentrations.

Conclusion: The ability of pollen to suppress innate antiviral immunity, independent of allergy, suggests that high-risk population groups should avoid extensive outdoor activities when pollen and respiratory virus seasons coincide.

KEYWORDS

antiviral response, lambda-interferones, nasal symptoms, nonallergenic pollen compounds, respiratory syncytial virus, rhinovirus
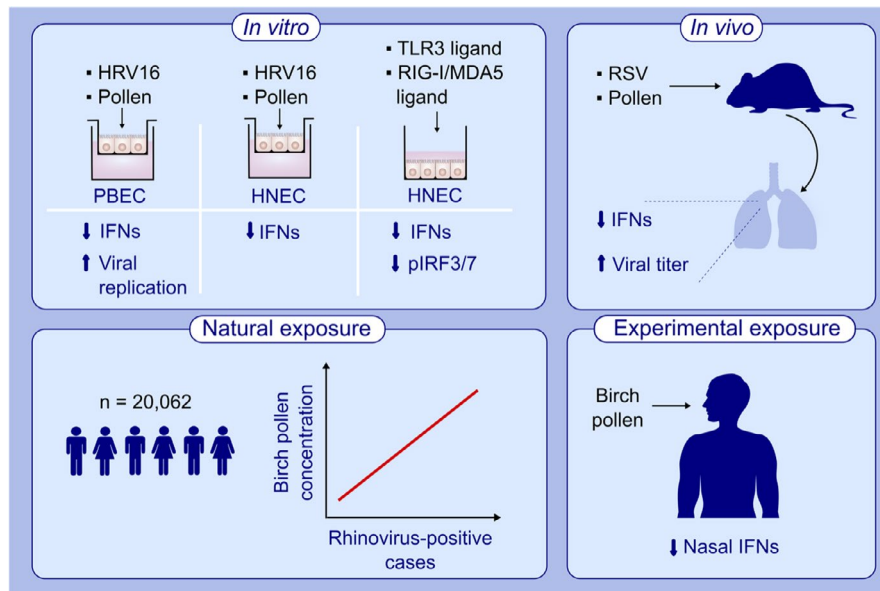
GRAPHICAL ABSTRACT

Pollen significantly diminished the epithelial response to rhinovirus infectionand viral mimics, and decreased nuclear translocation of IRFs. In a murine RSV infection model, pollen increased pulmonary viral load in the absence of allergic sensitization. Evidence from different, independent human cohorts suggests that springtime pollen exposure compromises the respiratory antiviral response, not only in allergic, but also in non-allergic individuals.

Abbreviations: HNEC, human nasal epithelial cells; HRV16, human rhinovirus 16; IRF, interferon regulatory factor; MDA5, melanoma differentiation-associated protein 5; PBEC, primary bronchial epithelial cell; RIG-I, retinoic acid inducible gene-I; RSV, respiratory syncytial virus; TLR3, toll-like receptor 3

1 | INTRODUCTION

Atopic and asthmatic patients are especially susceptible to respiratory viral infections and viral exacerbations.[1-4] The epithelial antiviral response depends on the rapid induction of antiviral type I and type III interferons (IFNs).[5-7] Antiviral IFN signaling is compromised in murine models of allergic airway disease as well as in humans affected by asthma or atopy.[8-12]

During the pollen season, humans are co-exposed to airborne pollen and respiratory viruses, and the respiratory epithelia encounter both environmental factors at the same time. We have previously

shown that pollen grains have potent immune-modulatory effects, which are independent of allergens and are therefore not restricted to sensitized individuals.[13] Specifically, low-molecular-weight pollen components alter the immunological barrier functions of respiratory epithelial cells.[14] In dendritic cells, they interfere with NF-κB signaling, partly via PPAR-γ-dependent pathways.[15] Both pathways are part of the earliest signaling events in virus-infected cells. We therefore assessed in this study whether exposure to pollen might compromise the innate antiviral defense, possibly contributing to increased susceptibility to respiratory viral infections during pollen season not only in allergic patients, but also in nonallergic individuals.

2 | METHODS

2.1 | Pollen and pollen extracts

Pollen grains from birch (*Betula pendula*), timothy grass (*Phleum pratense*) and common ragweed (*Ambrosia artemisiifolia*) were extracted from male flowers as previously described.[16,17] For details, see supplementary methods.

2.2 | Culture and differentiation of human primary bronchial epithelial cells (PBECs)

Primary bronchial epithelial cells were grown from epithelial brushing using fiberoptic bronchoscopy from healthy subjects selected from a volunteer database. All procedures were approved by the Southampton and South West Hampshire Research Ethics Committee and were undertaken following informed consent (05q/1702/165 and 10/H0501/66). Primary bronchial epithelial cells were expanded in bronchial epithelial growth medium (BEBM) (Lonza) up to passage 1 as previously described.[18] Differentiation of PBECs was induced at passage 2. Primary bronchial epithelial cells were plated on transwell permeable supports (diameter 6.5 mm, polyester membrane with 0.4 µm pores, Corning Life Sciences, Amsterdam, the Netherlands) and differentiated at an air-liquid interface (ALI) for 21 days. Transepithelial electrical resistance (TER) was monitored weekly using a EVOM Voltohmmeter (World Precision Instruments), and cells with a TER ≥ 330Ω cm^2 on day 21 were used for experiments.

2.3 | Culture and differentiation of human primary nasal epithelial cells (HNECs)

Human nasal epithelial cells were obtained from healthy volunteers free from respiratory tract infections for at least 4 weeks as previously described[19] or from patients undergoing conchotomic or turbinectomic surgery. All procedures were undertaken after written consent and approved by the Southampton and South West Hampshire Research Ethics Committee (code: 06/Q1702/109) or the Ethics Committee of the Medical Faculty of Augsburg (code: 2016/7). For HNEC cultures from brushings, two nasal brush biopsies per volunteer were taken by gently brushing the nasal epithelium of the inferior turbinate using a cytology brush (Olympus Keymed Ltd., 2 mm diameter). Nasal epithelial cells for submerged monolayer cultures were prepared from biopsies as recently described.[20] For differentiation, HNECs were plated onto collagen-coated cell culture plates using PneumaCult Ex Medium (Stemcell Technologies) and expanded for two passages. Passage 2 PNECs were plated onto transwell permeable supports (diameter 6.5 mm, polyester membrane with 0.4 µm pores, Corning Life Sciences) and differentiated at an air-liquid interface (ALI) for 28 days using PneumaCult-ALI Medium (Stemcell Technologies). Cultures with visible beating cilia were used for experiments. Sensitization of patients/donors against common aeroallergens (house dust mite, cat/dog dander, pollen) was assessed by immunoCAP (Phadia).

2.4 | Stimulation and viral infection of differentiated human respiratory epithelial cells

Fully differentiated PBECs (21 days post-ALI) were starved for 24 hours before stimulation with Bronchial Epithelial Basal Medium (BEBM, Lonza) supplemented with 1× ITS Liquid Media Supplement (Sigma), 50 U/mL penicillin, 50 µg/mL streptomycin (Invitrogen, Paisley, UK) and 1.5 µg/mL BSA (Sigma). Human nasal epithelial cells were stimulated after 28 days of differentiation in PneumaCult-ALI Medium (Stemcell Technologies). Cells were apically stimulated with buffer controls or pollen extract (33.4 µl of extract equivalent to 50 µg of total protein or 1mg of pollen grains) for 24 hours, a dose that has been previously shown to significantly modulate epithelial barrier responses.[14,21] After removing apical supernatants, cells were infected with human rhinovirus 16 (RV16) at a multiplicity of infection (MOI) of 1 for 6 hours, washed apically 3× using HBSS and incubated for additional 18 hours (RNA extraction, 24 hours in total) or 42 hours (basolateral supernatants, 48 hours in total) at the air-liquid interface. Supernatants were centrifuged to remove pollen grains and cell debris and subjected to ELISA or multiplex assay for detection of type III interferons and pro-inflammatory cytokines and chemokines. Cells were washed 3× with HBSS and lysed using TriZol (Invitrogen) for RNA extraction.

2.5 | Mouse model of RSV infection and intranasal pollen instillation

Pathogen-free 6-week-old female C57BL/6 mice (Charles River Nederland Maastricht, the Netherlands) were housed under standard housing conditions and had ad libitum access to tap water and diet. The Animal Ethics Committee of the Medical Faculty of Utrecht University approved the study protocols. Respiratory syncytial virus strain A2 (VR-1302, ATCC) was grown on HEp-2 cells (CCL-23, ATCC), purified by polyethylene glycol 6000 precipitation and stored in PBS with 10% sucrose in liquid nitrogen until further use. Mice were anesthetized with isoflurane and intranasally infected with 4 × 10^6 plaque-forming units (pfu) RSV in a volume of 40 µl diluted in PBS. Aqueous ragweed pollen extract was instilled intranasally on three successive days (day −1, day 0, day +1) around RSV infection, essentially as described.[22] To determine pulmonary virus load, RSV-specific qPCR was performed essentially as previously described.[23]

2.6 | Panel study on nonallergic volunteers

In autumn 2015, healthy, nonallergic volunteers living in Augsburg, Germany, were enrolled after written-informed consent and screened for sensitizations against seasonal and perennial aeroallergens by ImmunoCAP. The aeroallergen panel consisted of house dust mite; cat/dog dander; pollen (tree mix, grass mix); and fungi (Aspergillus, Cladosporium, Penicillium). The ethical committee of Klinikum Rechts der Isar, Technical University of Munich approved of the study (code no. 19/15). Eight participants without any

aeroallergen sensitizations were included in the study (for details on study participants, see Table 1). Throughout the birch pollen season of 2016 (March-June), they daily entered the strength of their nasal symptoms on a scale from 0 to 3 in a smartphone-based symptom diary (PHD; Patient's Hayfever Diary[24]).

2.7 | Experimental pollen challenge study

Healthy volunteers without allergic diseases were screened for serum IgE against an aeroallergen panel by ImmunoCAP. A total of 18 subjects without sensitizations were enrolled after given written-informed consent and in accordance with the local ethics committee (code no. 2983/10, amendment of 2017). They were randomized into two groups (n = 9 each) and subjected to 3 serial nasal challenges with either a NaCl solution or aqueous birch pollen extract (APE) as recently described.[25] Briefly, on the first day, we performed an initial, bilateral nasal lavage with 20 mL sterile 0.9% NaCl, followed by a unilateral, superficial curettage from the mucosa of the inferior turbinate by means of a sterile plastic curette. The cells obtained thereby were preserved in RNAprotect Cell™ (Qiagen) and deep-frozen until processed. We then challenged the subjects by spraying one puff (100 μL) of sterile 0.9% NaCl or APE (the dose corresponding to 2,500 standard biological units, SBE; Allergopharma) from a pump-spray bottle into one nostril (the one not subjected to curettage before). On days 2 and 3, we repeated lavage and challenge. About 1 hour after the last challenge on day 3, a final curettage was obtained from the challenged nostril.

2.8 | Patient samples for rhinovirus detection

The retrospective study included clinical nasopharyngeal swab samples collected between October 2010 and July 2013 (n = 20 062), which were sent to the Department of Virology at Sahlgrenska University Hospital in Gothenburg, Sweden, for detection of respiratory pathogens by routine multiplex real-time PCR. The study population covered all age groups, including children. Samples were predominantly from hospital inpatients but also from primary health care facilities as well as hospital outpatient clinics. No clinical or demographic information regarding the patients was available.[26]

2.9 | Statistics

(a) In vitro experiments, RSV mouse model, human pollen challenge study (unless stated otherwise in the legends): two-sided Mann-Whitney test for simple comparisons between two groups; two-sided Wilcoxon test for pairwise comparisons between treatment and control groups; Friedman test for comparisons of multiple treatment groups with a single control group. (b) Human cohorts: Relationships of symptoms with airborne pollen concentrations were investigated with simple linear regressions (GLM) and cross-correlations (time series analysis). In all analyses of symptoms, 7-day moving averages of normalized values were used, so as to eliminate periodicity effects (lower hospitalization rates over the weekends). All statistical tests were performed with Prism or Statistica.

See supplementary methods for a detailed description of pollen and pollen extracts; reagents, ELISA and multiplex and kits; stimulation of HNEC monolayer cultures with viral mimics; nuclear translocation of transcription factors.

3 | RESULTS

3.1 | Pollen diminish the epithelial response to rhinovirus infection

To test whether pollen exposure affects the antiviral response of respiratory epithelia, we used 3D models of differentiated human primary bronchial epithelial cells (PBECs) and nasal epithelial cells (HNECs) incubated with pollen extracts or whole pollen grains and subsequently infected with human rhinovirus (HRV16). Exposure of HRV16-infected differentiated PBECs to aqueous grass pollen

TABLE 1 Overview of the nonatopic panel cohort

| SUB_ID	Total IgE (IU/mL)	HDM d1	Cat dander e1	Wheat f1	Timothy grass g6	Rye g12	Birch t3	Hazel t4	Mugwort w6
PAB_NA_01	46.80	0.01	0.00	0.06	0.02	0.02	0.00	0.00	0.00
PAB_NA_02[a]	21.60	0.00	0.00	0.05	0.04	0.05	0.03	0.03	0.03
PAB_NA_03	7.44	0.02	0.02	0.02	0.00	0.01	0.00	0.00	0.02
PAB_NA_04[a]	8.51	0.01	0.01	0.05	0.03	0.03	0.01	0.01	0.01
PAB_NA_05	5.62	0.01	0.01	0.02	0.13	0.10	0.00	0.00	0.00
PAB_NA_06	37.80	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.00
PAB_NA_07	4.47	0.00	0.00	0.05	0.01	0.01	0.00	0.00	0.01
PAB_NA_08	17.90	0.00	0.00	0.05	0.01	0.02	0.00	0.00	0.00
PAB_NA_09	12.20	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
PAB_NA_10	152.00	0.00	0.00	0.03	0.01	0.00	0.00	0.00	0.01

Note: Shown are serum levels of total IgE (IU/mL) and IgE specific for common perennial and seasonal aeroallergens (HDM, house dust mite).
[a]Subjects PAB_NA_2 and PAB_NA_4 were excluded from analysis because they were abroad during parts of the Augsburg birch pollen season.

extract for 48 hours resulted in an increased release of infective virions indicating enhanced viral replication ($P < .01$; Figure 1A) and attenuated by trend the virally induced expression of the IFN-λs, IL28A (Figure 1B) and IL-29 (Figure 1C,D). Grass pollen exposure also reduced the expression of several antiviral genes (IFN-β, IRF-7, MDA-5) and of the gene for the pro-inflammatory chemokine CCL5 (Figure S1). Exposure of HRV16-infected differentiated HNECs to aqueous grass pollen extract did not result in increased virion release (Figure 1E) and only slightly attenuated the IL-29 response (Figure 1F). Upon co-exposure of HNECs to aqueous birch pollen extract, the virion release of HRV16-infected differentiated HNECs was, by trend, increased ($P = .08$; Figure 1G), and the IL-29 response was significantly reduced ($P < .05$; Figure 1H). The attenuation of the HRV16-induced IL-29 secretion was already significant after 24 hours of exposure with either an aqueous birch pollen extract or with whole birch pollen grains (Figure S2).

3.2 | Pollen modulate TLR3, RIG-I and MDA5 signaling in nasal epithelial cells

To characterize how pollen interferes with antiviral pathways, we carried out further experiments using submerged monolayer cultures of primary human nasal epithelial cells (HNECs) stimulated with viral mimics. We first tested the effect of a TLR3 ligand, PolyIC (10 μg/mL). Cells of atopic and nonatopic donors all responded to PolyIC stimulation with the release of IFN-λs (Figure S3A,B). Co-incubation of HNECs with PolyIC and whole birch or grass pollen grains significantly decreased the PolyIC-induced production of the IFN-λs (Figure 2A), with birch pollen being slightly more potent than grass pollen. Following our hypothesis of an allergen-independent effect of pollen, we tested an allergen-free fraction of aqueous birch pollen extract (APE < 3 kDa). APE < 3 kDa, at the highest tested concentration,
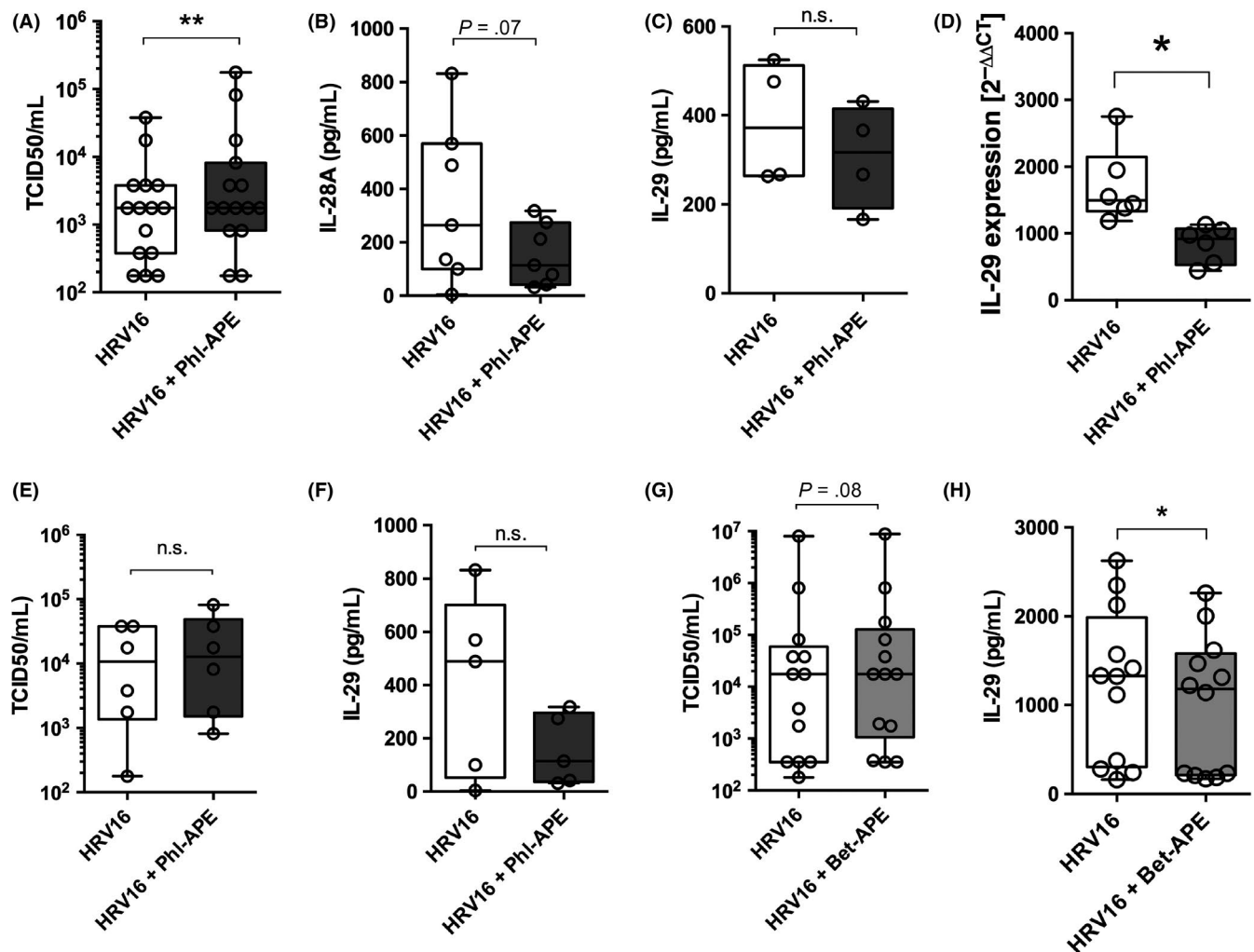
FIGURE 1 Viral replication and antiviral IFN-λ expression in rhinovirus-infected human airway epithelium upon co-exposure to pollen. Differentiated human primary bronchial (PBEC; A-D) and nasal epithelial cells (HNEC; E-H) were infected for 48 h with HRV16 and co-exposed with 100 μg/mL aqueous extracts of birch (Bet-APE) or timothy grass pollen (Phl-APE). A: HRV16 virion release in apical washes of PBEC cultures 48 h after infection. **:$P < .005$ (n = 15). B-D: Release of antiviral FN-λs 48 h after infection. *: $P < .05$ (n = 4-6). E, G: HRV16 virion release of HNECs 48 h after infection, with or without co-exposure to 100 μg/mL aqueous grass (E: Phl-APE) or birch pollen extract (G: Bet-APE). F, H: IFN-λ response of HRV16-infected HNECs with or without co-exposure to grass (F) or birch pollen extract (H; $P < .05$; n = 5-13). As control, UV-irradiated RV16 was used (data not shown). All experiments were carried out with cultures of n different, nonatopic donors

also significantly decreased the PolyIC-induced release of IFN-λs (Figure 2B). The effect of APE on the PolyIC-induced IFN-λ1 (IL-29) response did not differ significantly between nonatopic and atopic cells (Figure 2C). We then tested whether pollen also had an impact on the cytosolic antiviral defense pathway and transfected HNECs with a RIG-I/MDA5 ligand (PolyIC-LyoVec) in the absence or presence of birch or grass pollen grains. Transfection with PolyIC-LyoVec led to the production of IFN-λs, and co-exposure to pollen grains decreased the PolyIC-LyoVec induced production of IL-28A ($P < .05$ for grass pollen), but not IL-29. (Figure 2D). Aqueous birch pollen extract significantly decreased the IFN-λ production of HNECs transfected with PolyIC-LyoVec (Figure S3C).

We also checked for the effect of pollen exposure on the pro-inflammatory cytokine and chemokine response of HNECs to PolyIC stimulation (Figure S3D). Pollen grains, mainly of birch, significantly decreased the release of several chemokines, such as G-CSF, CCL2, CCL3, CCL4, CCL5 and CXCL10, whereas they increased levels of IL-1β.

3.3 | The PolyIC-induced IFN-λ response of HNECs is not mediated by known immune-modulatory substances in pollen

We previously reported immune-modulatory effects, such as PPAR-γ dependent inhibition of IL-12 and blocking of nuclear translocation of NF-κB, by nonallergenic pollen substances.[15] Two classes of pollen-associated lipid mediators (PALMs) were previously identified and discussed as potential PPAR-γ ligands, the phytoprostanes and the phyto-hydroxyoctodecadienoic/phyto-hydroxyoctodecatrienoic acids (HODEs, HOTEs). We tested both phytoprostanes and HODE/HOTEs as candidate active substances because (a) the promoter regions of type III IFN genes contain PPAR-γ responsive elements (Figure S4A) and (b) PPAR-γ agonists were previously shown to inhibit IRF-3 translocation to the IFN-β promoter in LPS- and PolyIC-stimulated murine peritoneal macrophages.[27] However, none of the tested PALMs had any significant effect on the PolyIC-induced IFN-λ secretion (Figure S4B). A PPAR-γ antagonist, GW-9662, did not abolish the inhibitory effect of APE < 3 kDa on the IFN-λ response to PolyIC (Figure S4C).
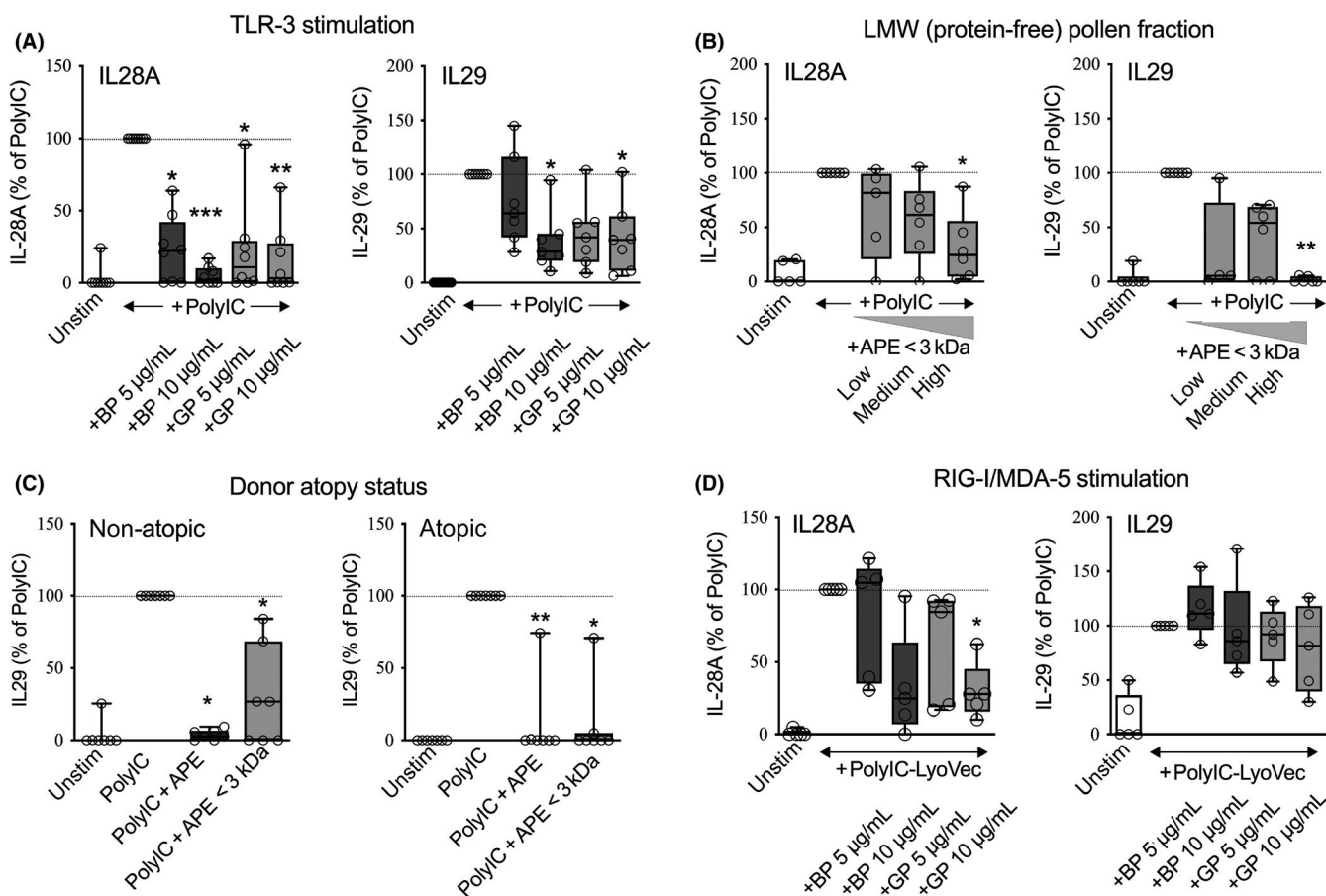
FIGURE 2 Antiviral response in primary human nasal epithelial cells stimulated with viral mimics. A: Inhibition of the PolyIC-induced IFN-λ response of HNECs by whole birch pollen (BP) and timothy grass pollen (GP) grains. *: $P < .05$, **: $P < .01$, ***: $P < .005$; n = 7. B: IFN-λ production in cells stimulated with PolyIC (10 μg/mL) in the absence or presence low-molecular-weight aqueous birch pollen extracts (APE < 3 kDa; 10, 30 and 100 μg/mL). *: $P < .05$, **: $P < .01$; n = 6. C: IFN-λ inhibition by aqueous birch pollen extract (APE; 100 μg/mL) and the low-molecular-weight fraction (APE < 3 kDa; 100 μg/mL) in cells from nonatopic and atopic donors. *: $P < .05$, **: $P < .01$; n = 7. D: IFN-λ response in cells transfected with PolyIC-LyoVec in the absence and presence of whole pollen grains. *: $P < .05$; n = 5. All experiments were carried out with cells of n independent donors

Since adenosine is a major constituent of APE < 3 kDa and can exert immune-modulatory effects on human dendritic cells,[17] we assessed whether the effect of pollen on the PolyIC-induced IFN-λ response of HNECs could be mediated by adenosine. At concentrations as contained in aqueous pollen extracts (1-10 µM), adenosine did not have a pronounced effect on the PolyIC-induced IL-29 production of HNECs (Figure S4, D). However, in the presence of an A2a inhibitor, the IL-29 response to PolyIC was approximately 4-fold increased as compared to cells not treated with the inhibitor (Figure S4E).

3.4 | Pollen reduce the PolyIC-induced nuclear translocation of IRF-3 and IRF-7

We next assessed whether pollen interferes with the activation of interferon regulatory factors downstream of TLR-3. We prepared nuclear and cytoplasmic extracts of HNECs, subjected them to Western blotting and probed the blots with antibodies against IRF-3 and -7. Phosphorylation of IRF-3 was maximally induced

after only 5 minutes of PolyIC stimulation (Figure S5). Pollen-associated lipid mediators (PPE$_1$ or a mixture of HODEs/HOTEs) were tested along with APE as candidate substances. As shown in Figure 3, A, co-exposure to PolyIC and APE or HODE/HOTE diminished nuclear phosphorylated IRF (p-IRF) 3 as compared to PolyIC alone. In contrast, co-incubation with PolyIC and PPE$_1$ resulted in increased nuclear localization of p-IRF-3. Phospho-IRF-7 was present at low amounts in nuclear extracts of PolyIC-stimulated cells and was further reduced by APE, whereas it was induced under HODE/HOTE co-stimulation. PolyIC-induced levels of nuclear, but not of cytoplasmic p-IRF-3 and -7 were significantly reduced by APE co-stimulation (Figure 3B).

## 3.5 | Pollen diminish the antiviral response in a murine RSV infection model

In order to study the consequence of pollen on viral respiratory infections in vivo, we used a respiratory syncytial virus (RSV) mouse
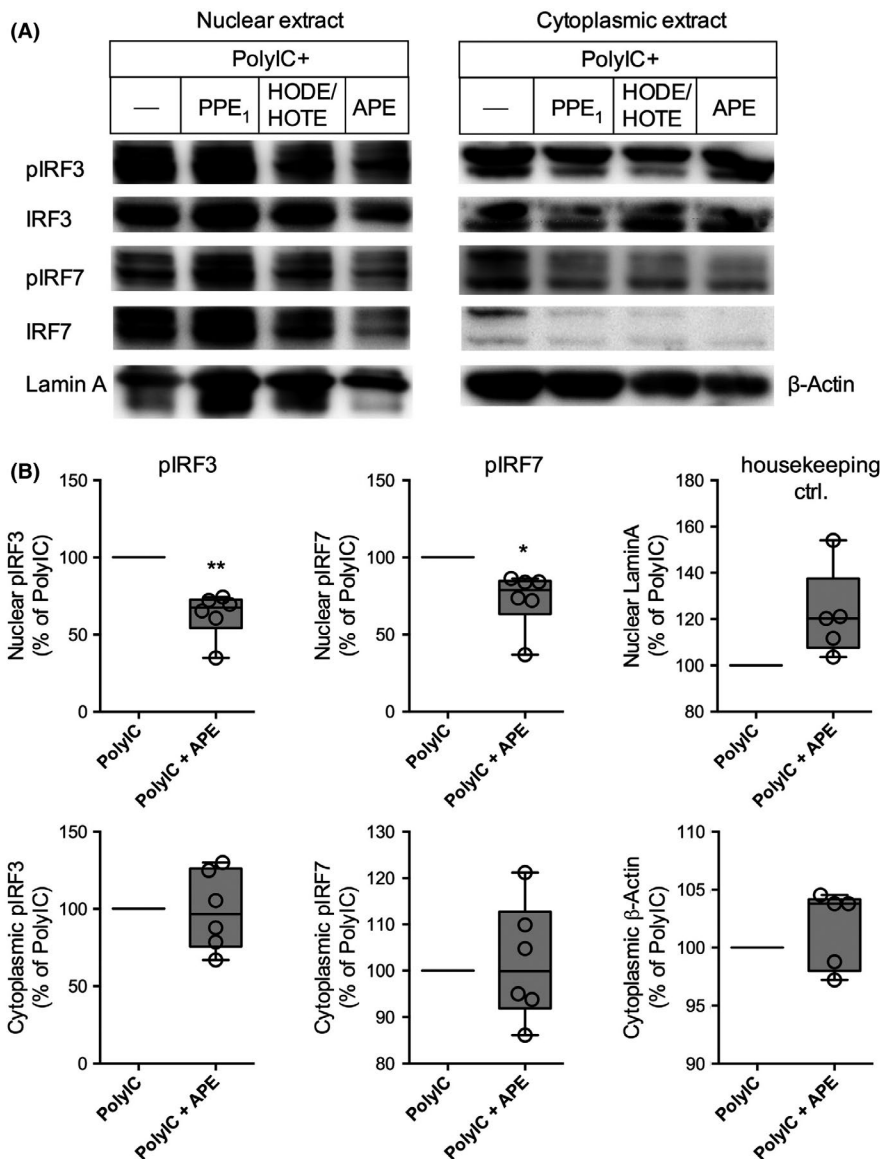
FIGURE 3 Nuclear translocation of phosphorylated transcription factors IRF-3 and IRF-7 in PolyIC- and pollen-stimulated nasal epithelial cells. A: Nuclear and cytoplasmic extracts of HNECs stimulated as indicated were subjected to SDS-PAGE and Western blot analysis using antibodies against total and phosphorylated IRF-3 and IRF-7. β-actin and lamin A served as loading controls. APE: aqueous birch pollen extract (corresponding to 100 µg/mL total protein); PPE$_1$: E$_1$-phytoprostane, 1 µM; HODE/HOTE: equimolar mixture of hydroxyoctodecadienoic and hydroxyoctodecatrienoic acids, 1 µM. B: Quantitation of proteins in nuclear and cytoplasmic extracts of HNECs stimulated with PolyIC (10 µg/mL) vs. PolyIC plus APE. *: $P < .05$, **: $P < .01$; n = 6 independent experiments, each using different donors

model and investigated the effect of experimental pollen exposure on the primary immune response. C57BL/6 mice were infected with RSV-A2 and treated them intranasally with either PBS or an aqueous ragweed pollen extract on three successive days (one day prior to infection, the same day and one day postinfection). We analyzed the antiviral immune response of the mice 4, 6 or 10 days after RSV infection (Figure 4A). The clinical phenotype of RSV-infected mice includes increased pulmonary viral load and weight loss,[28,29] the latter typically being less pronounced in C57BL/6

than in BALB/c mice. In our experiment, although we used the C57BL/6 model, both RSV-infected groups lost weight, and weight loss was significant at day 6 postinfection (Figure S6); however, weight loss did not differ between the RSV + PBS or RSV + pollen treatment (Figure 4B). On day 6 after infection, RSV-infected pollen-treated mice had a significantly higher virus load in BALF compared to only RSV-infected mice (Figure 4C). On day 4 after infection, we determined the expression of antiviral genes in lung tissue. Most measured genes of the antiviral response, with the
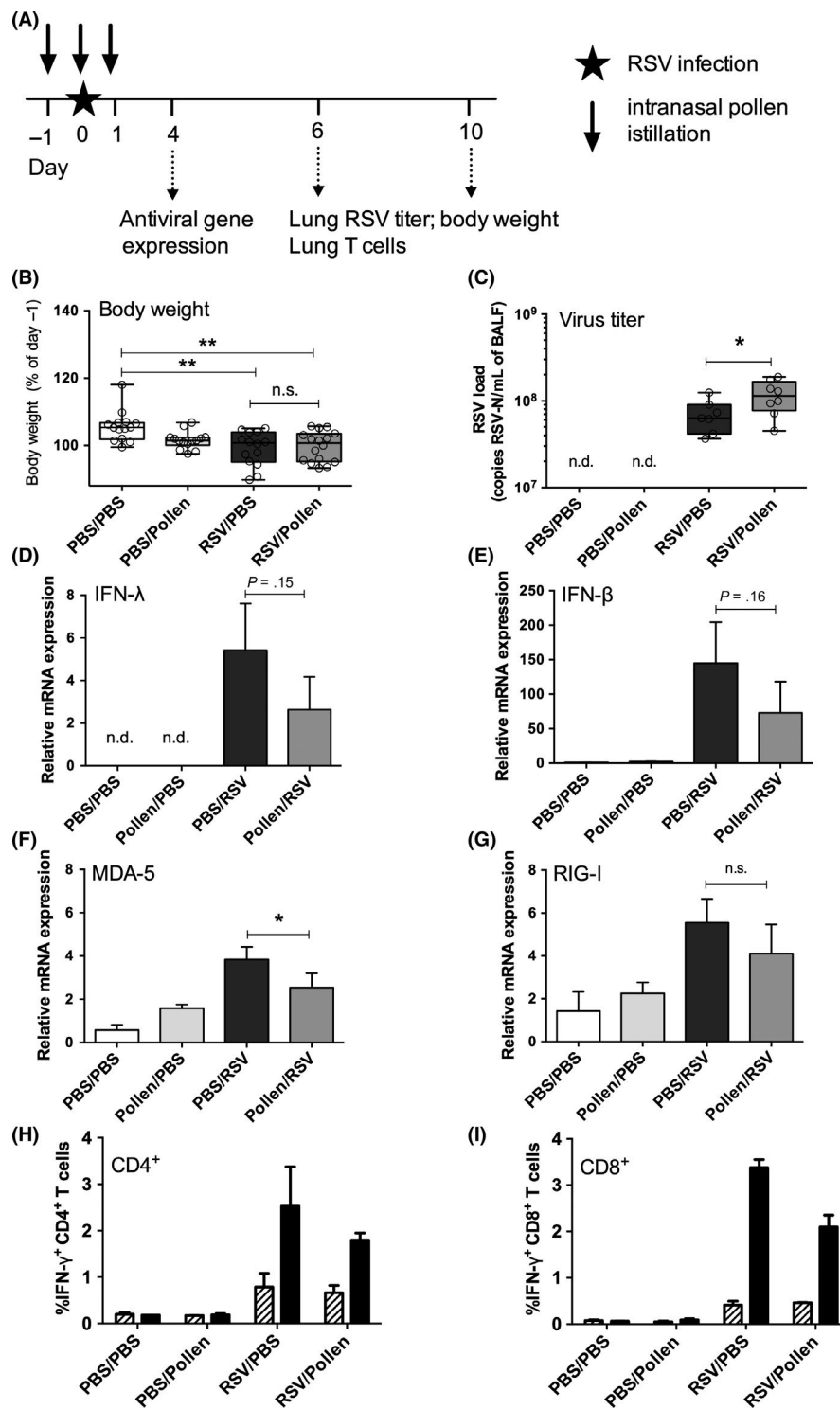
FIGURE 4 Intranasal pollen exposure increases viral load and diminishes expression of MDA-5 in a murine respiratory syncytial virus model. A: Experimental setup. B: Body weight of mice days after intranasal treatment with buffer (PBS/PBS), ragweed pollen extract (PBS/pollen; 10 μg/mL), respiratory syncytial virus (RSV) (RSV/PBS) or a combination of RSV and pollen extract (RSV/pollen). **: $P < .01$; n = 15 mice per group C: Viral load in BALF of mice 6 d after indicated treatment. *: $P < .05$; n = 8-9 D-G: Antiviral gene expression in the lungs of mice 4 d after indicated treatment. *: $P < .05$ vs. PBS/RSV; n = 3-4. H-I: Percentages of virus-specific IFN-γ^{+} CD4^{+} and CD8^{+} T cells in restimulated spleens of mice 6 d after indicated treatment. Clear bars: Re-stimulation with uninfected D1 cells. Gray bars: Re-stimulation with RSV-infected D1 cells

exception of RIG-I, were by trend decreased in RSV-infected pollen-treated mice as compared to RSV-infected PBS-treated mice (MDA5: $P < .05$; IFN-λ: $P = .15$, IFN-β: $P = .16$) (Figure 4D-G). On day 6, we restimulated total lung cells in vitro with RSV-infected or uninfected dendritic cells (D1 cells) for 24 hours and measured antiviral T-cell response by intracellular staining of IFN-γ (Th1) and IL-13- or IL-4 (Th2)-producing lung cells. We observed a slight trend toward decreased numbers in IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the lungs of pollen-treated RSV-infected animals compared to only RSV-infected animals, but differences were not statistically significant (Figure 4H,I). Numbers of RSV-specific Th2 cells ($IL4^+$, $IL13^+$) were not changed by pollen co-exposure (data not shown). For gating strategy, see Figure S7.

3.6 | Relationships of pollen exposure with rhinovirus, nasal symptoms, and antiviral gene expression in human cohorts

Especially in springtime, co-exposure occurs with pollen and respiratory viruses, for example, rhinovirus. We therefore obtained data from a large set of human clinical samples (n = 20 062) from Gothenburg, Sweden, spanning a total of three successive years, and regressed the numbers of rhinovirus-positive cases (n = 5782) within this dataset against local airborne pollen concentrations and meteorological factors. The datasets were limited to periods within the main pollen season. Time series analysis revealed a significant correlation between rhinovirus-positive cases, airborne birch pollen concentrations and precipitation ($P = .005$). The relationship between rhinovirus and pollen was nonlinear and positive, whereas it was negative between rhinovirus/pollen and precipitation (Figure 5A).

As information on patient demographics or sensitization status was lacking in the Gothenburg patient cohort, which might have an impact, we cannot exclude the possibility of a predominant response by allergic patients. Therefore, we conducted a panel study on 8 well-characterized nonallergic volunteers who reported their daily symptoms during the main birch pollen season of 2016 in Augsburg, Germany. Nasal symptoms, although overall low, coincided with local airborne pollen concentrations ($P < .001$, $r = .76$) (Figure 5B). Time series analysis revealed a significant cross-correlation of nasal symptoms and birch pollen which exhibited a lag effect of up to 9 days (data not shown). The strongest cross-correlations of symptoms were observed with the airborne birch pollen concentrations of the previous day (plotted in Figure 5B).

We additionally performed a controlled out-of-season pollen challenge experiment on two groups (n = 9 each) of nonallergic volunteers and measured the antiviral gene expression in nasal curettages before and after challenge. Three repetitive challenges with aqueous birch pollen extract (each single one corresponding to 2500 SBE) decreased, by trend, the relative mRNA expression of all type I and type III IFNs in nasal samples as compared to saline challenge (IFNA1: $P = .06$; IFNB1: $P = .09$; IFNL1: $P = .16$; IFNL2: $P = .05$; Figure 5C).

4 | DISCUSSION

This is the first study combining evidence from human cohorts, a mouse model and human primary cell culture, showing that pollen compromise the defense against respiratory viruses. Infection of respiratory epithelial cells with rhinovirus, a single-stranded RNA virus, activates an innate antiviral response involving TLR3, RIG-I and MDA5.[30-33] Downstream signaling pathways result in the activation of members of IRF and NF-κB transcription factors families[34] which play a central role in regulating innate antiviral immune responses.[32,35] IRF3 and IRF7 are phosphorylated, dimerize and translocate into the nucleus where they are part of enhanceosome multiprotein complexes regulating the expression of antiviral genes.[36] IRF3 and IRF7 have been shown to be centrally involved in the regulation of the expression of type I and III interferons and pro-inflammatory chemokine CCL5/RANTES.[35,37,38] Using human in vitro models of rhinovirus infection, we showed that exposure to pollen during viral infections reduces the release of pro-inflammatory chemokines and type I and III interferons and increases viral replication. Moreover, pollen exposure reduces the expression of MDA5, and MDA5 deficiency can predispose to recurrent rhinovirus infections.[39] Furthermore, exposure to pollen resulted in a reduced translocation of phosphorylated IRF3 and IRF7 into the nucleus following activation by double-stranded RNA suggesting a mechanistic link between pollen exposure, reduction in enhanceosome complexes and reduced CCL5 and IFN-λ release. IRF3 has also been shown to play a role in triggering apoptosis, a defense mechanism of virally infected cells to reduce viral load and replication and thus prevent spreading of the infection.[40] Reduced phosphorylation and translocation of IRF3 by exposure to pollen during viral infections can result in decreased expression of pro-apoptotic genes, which results in enhanced viral replication during exposure to pollen that we observed in this study. Of note, pollen exposure reduced the IFN-λ production in cells of atopic and nonatopic donors, and the nonallergenic fraction (APE < 3 kDa) had similar effects on cells of both atopic and nonatopic donors. This argues for a mechanism independent of the atopy trait.

Our in vitro findings were reemphasized in vivo. The murine RSV infection model was characterized by a mild clinical phenotype, which is typical for C57BL/6 mice.[23] The immune response to RSV infection differs between mouse strains.[41] Within C57BL/6 mice, there is a clear role for Type I IFNs as well as IFNAR in RSV infection.[42-44] We also chose the C57BL/6 background to exclude any pollen effect mediated by allergic sensitization. Of note, three successive intranasal pollen instillations did not lead to allergic sensitization even in the allergy-prone Balb/c strain.[22] The difference in viral load as well as pulmonary antiviral gene expression when co-treated with pollen supports our hypothesis that pollen exposure compromises innate antiviral immune responses.

In a temperate climate, airborne pollen as well as many viruses show a high degree of seasonality.[26,45] Airborne pollen concentrations correlated with nasal symptoms in the nonallergic volunteers of our dedicated panel study. The strongest correlation was observed between symptoms and pollen counts of the previous day, indicating a direct, fast-acting effect of pollen. In our off-season pollen
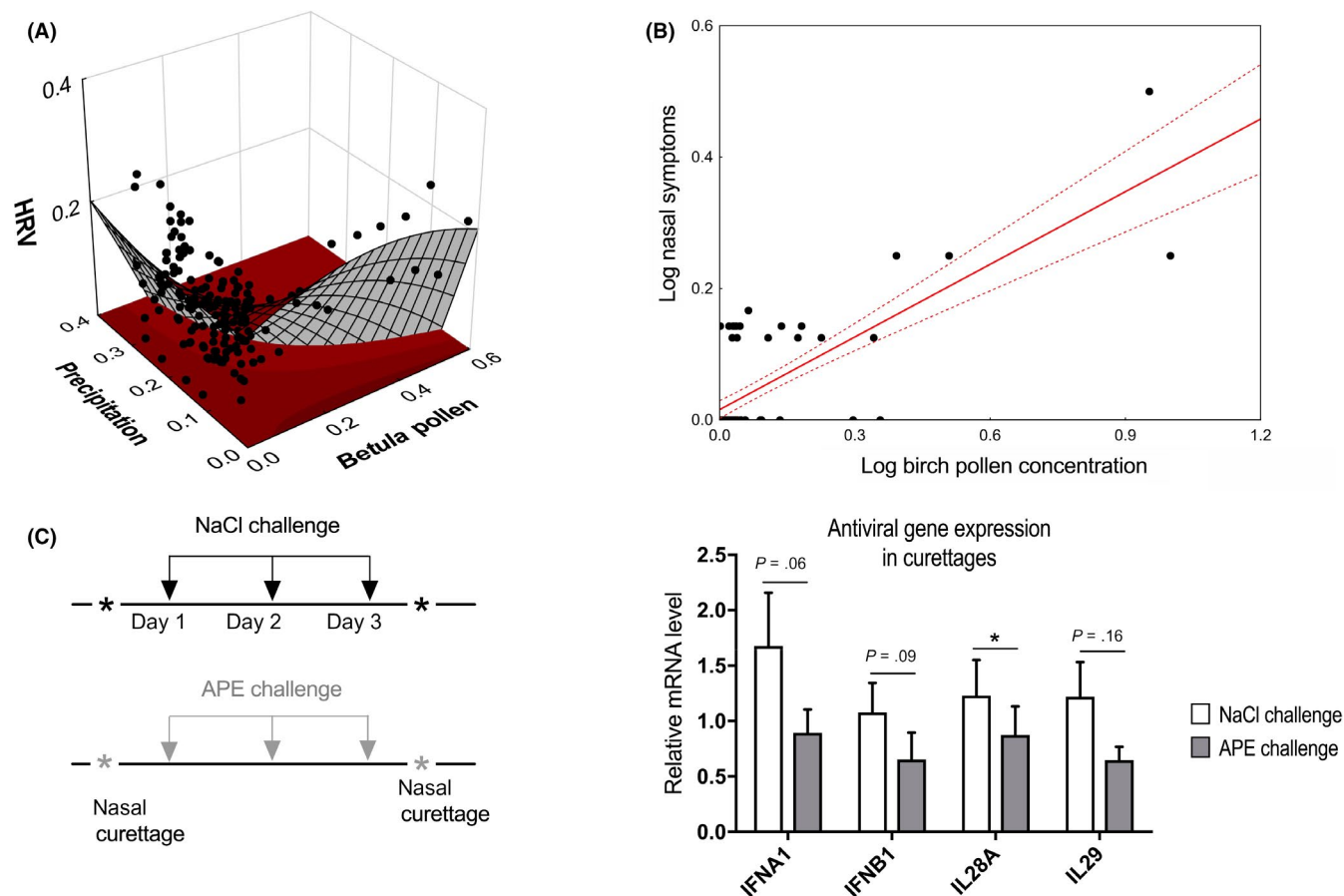
FIGURE 5 Relationships of rhinovirus, nasal symptoms and expression of IFN genes in three different human cohorts. A: Correlation (GLM) between rhinovirus-positive cases, airborne birch pollen concentrations and precipitation in Gothenburg, Sweden. Nasopharyngeal swabs (n = 5782) tested positive for rhinovirus (z-axis) were regressed against airborne birch pollen concentrations (y-axis) and precipitation (x-axis); P = .005. All values are 7-d moving averages of normalized original values. B: Time series analysis (cross-correlation) of nasal symptoms in a well-characterized cohort of nonallergic volunteers (n = 8) from Augsburg, Germany, with local airborne birch pollen concentrations of the previous day (GLM, simple regression). C: Type I and type III IFN gene expression in nasal samples of nonallergic volunteers subjected to three successive intranasal challenges with saline (NaCl challenge; n = 9) or 2500 SBE of aqueous birch pollen extract (APE challenge; n = 9). Mean + SEM. *: P < .05

challenge study, we observed by trend a downregulation of all nasal type I- and type III IFN genes after only three repetitive challenges with APE, supporting an inhibitory effect of pollen on the early antiviral response of the epithelium. Since the cohorts were small and the study could not be conducted on virus-infected individuals, baseline transcript levels of antiviral IFNs were initially rather variable. The only controlled allergen challenge study on virus-infected patients published so far reports diminished cold symptoms and rhinovirus titers after priming by repetitive nasal allergen challenges.[46] However, only 5/10 patients in the allergen treated group were challenged with pollen extract. Of note, the timing of pollen exposure relative to virus challenge could be relevant to the outcome. In our large Gothenburg cohort numbers of rhinovirus-positive patients positively correlated with airborne birch pollen concentrations, indicating that pollen exposure enhances the susceptibility to rhinovirus under real-life co-exposure. In a carefully designed case-crossover study on an Australian children and adolescents asthma cohort, hospital admissions occurred most frequently in springtime, but not

during the pollen season at large (October-January).[47] Interestingly, high levels of airborne grass pollen (50 grains/m^{3} or more) increased hospital admissions in HRV-infected boys but not in girls.[48] A recent meta-analysis stresses the relevance of outdoor pollen exposure for asthma exacerbations, especially in children and adolescents.[49] Unfortunately, information on demographics, sensitization or asthma status was not available for our Gothenburg rhinovirus cases. Atopy and asthma are traits linked to a defective antiviral response,[8] possibly due to chronic exposure of the respiratory epithelium to Th2 and type-2 cytokines, for example, IL-33.[50,51] Since samples from allergic asthmatics or patients with other respiratory diseases could be over-represented among samples obtained during the birch pollen season, the positive correlation we observed between pollen and rhinovirus is most likely due to a combined effect of atopy or respiratory disease and pollen. Overall, however, we have acquired consistent results in three independent human cohorts from two different geoclimatic regions, despite confounding effects such as rising air temperatures in springtime.

Taken together, our results indicate that pollen exposure itself modulates the antiviral defense of the respiratory epithelium. This might be of special relevance for individuals with chronic respiratory diseases where viral infections are a main cause of severe exacerbations. Furthermore, also nonallergic individuals at risk for respiratory infections might benefit from restricting their extensive outdoor activities when pollen and respiratory virus seasons coincide, particularly during days with high pollen counts. However, large-scale clinical trials are needed to confirm these findings and to formulate guidelines for people at risk.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

Dr Akdis reports grants from Allergopharma, grants from Idorsia, grants from Swiss National Science Foundation, grants from Christine Kühne-Center for Allergy Research and Education, grants from European Commission's Horison's 2020 Framework Programme, Cure, other from Sanofi-Aventis_Regeneron, grants from Novartis Research Institutes, grants from Astra Zeneca, grants from Scibase, outside the submitted work. Dr Chaker reports research grants and other from Allergopharma, ALK Abello, ASIT Biotech, Bencard/Allergen Therapeutics, GSK, HAL Allergy, LETI, LOFARMA, Novartis, Phadia/ Thermo Fisher, Zeller, Circassia; further EIT (European Institute of Technology) and DZL: Deutsches Zentrum Lungenforschung (BMBF, governmental), outside the submitted work. Dr Davies reports personal fees from Synairgen, outside the submitted work; In addition, Dr Davies has a patent with royalties paid. Dr Garssen reports and Two (2) affiliations: (1) Danone/Nutricia Research. Function: Director Research & Innovation Immunology and (2) Utrecht University/Utrecht Institute for Pharmaceutical Sciences. Function: Head Division Pharmacology. The remaining authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

CTH, SG, CB, and DED made the original idea. SG and CB designed the study and writing of the main manuscript. SG (TLR3, RIG-I, MDA5 model), CB (PBEC HRV infection model), SS (Western blots, signal transduction), and CBG and SE (HNEC HRV infection model) involved in in vitro experiments. AC contributed to ENT-related expertise and specimens from nasal surgery. MW, LM, BvtL, and JG involved in Murine RSV infection model. MG prepared the Nasal pollen challenge study. AD, FH (Augsburg), and ÅD (Gothenburg) collected data on airborne pollen concentrations. NS, ML, LMA, and JW collected data on rhinovirus samples in human cohort. AD and AUN involved in statistical analysis. SG, CB, CAA, DED, and CTH helped in project and manuscript discussions.

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SUPPORTING INFORMATION

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