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.

Objective: 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 were positively correlated with airborne birch pollen abundance, and nasal birch pollen challenge led to down-regulation of type I and -III interferons in nasal mucosa. In a large patient cohort, numbers of rhinovirus-positive 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.

Key words

Pollen; nasal symptoms; rhinovirus; respiratory syncytial virus; antiviral response; lambda-interferones; non-allergenic pollen compounds

Abbreviations

ADO	Adenosine
ALI	Air-liquid interphase
APE(s)	Aqueous pollen extract(s)
APE<3kDa	Low molecular weight fraction of aqueous pollen extract
BALF	Bronchioalveolar lavage fluid
HNEC	Human nasal epithelial cell
PPE_1	E_1-Phyotprostane(s)
PBEC	Primary bronchial epithelial cell
HODE	Hexaoctodecadienoic acid
HOTE	Hexaoctodecatrienoic acid
IFN	Interferon
IRF	Interferon regulatory factor
kDa	kilo Dalton
MDA5	Melanoma differentiation-associated protein 5
RIG-I	Retinoic acid inducible gene-I
PALM(s)	Pollen-associated lipid mediator(s)
PolyIC	Polyinosinic:polycytidylic acid
HRV	Human rhinovirus
RSV	Respiratory syncytial virus
TLR	Toll-like receptor

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 non-allergic individuals.

Methods

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.

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

PBECs 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). PBECs were expanded in bronchial epithelial growth medium (BEBM) (Lonza, Basel, Switzerland) up to passage 1 as previously described [18]. Differentiation of PBECs was induced at passage 2. PBECs were plated on transwell permeable supports (diameter 6.5mm, polyester membrane with 0.4μm 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, Aston, UK) and cells with a TER $\geq$330Ωcm^2 on day 21 were used for experiments.

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

HNECs 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, Southend, UK). 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, Cambridge, UK) and expanded for two passages. Passage 2 PNECs were plated onto transwell permeable supports (diameter 6.5mm, polyester membrane with 0.4µm 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, Freiburg, Germany).

Stimulation and viral infection of differentiated human respiratory epithelial cells

Fully differentiated PBECs (21 days post ALI) were starved for 24h before stimulation with Bronchial Epithelial Basal Medium (BEBM, Lonza) supplemented with 1x ITS Liquid Media Supplement (Sigma), 50U/ml penicillin, 50µg/ml streptomycin (Invitrogen, Paisley, UK) and 1.5µg/ml BSA (Sigma). HNECs were stimulated after 28 days of differentiation in PneumaCult ALI Medium (Stemcell Technologies). Cells were apically stimulated with buffer controls or pollen extract (33.4µl of extract equivalent to 50µg of total protein or 1mg of pollen grains) for 24h, 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 6h, washed apically 3x using HBSS and incubated for additional 18h (RNA extraction, 24h in total) or 42h

(basolateral supernatants, 48h 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 3x with HBSS and lysed using TriZol (Invitrogen) for RNA extraction.

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 of the study protocols. RSV 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 $4x10^6$ 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].

Panel study on non-allergic volunteers

In autumn 2015, healthy, non-allergic 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 – 3 in a smartphone-based symptom diary (PHD; Patient´s Hayfever Diary [24]).

Experimental pollen challenge study

Healthy volunteers without allergic diseases were screened for serum IgE against an aeroallergen panel by ImmunoCAP. 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, Stockach, Germany) and deep-frozen until processed. We then challenged the subjects by spraying one puff (100µL) 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. 1 h after the last challenge on day 3, a final curettage was obtained from the challenged nostril.

Patient samples for rhinovirus detection

The retrospective study included clinical naso-pharyngeal 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].

Statistics

(i) 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. (ii) 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, seven-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 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.

Results

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 extract for 48h resulted in an increased release of infective virions indicating enhanced viral replication ($p<0.01$; **fig. 1, A**) and attenuated by trend the virally induced expression of the IFN-λs, IL28A (fig. **1, B**) and IL-29 (**fig. 1, C-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 (**fig. S1**). Exposure of HRV16-infected differentiated HNECs to aqueous grass pollen extract did not result in increased virion release (**fig. 1, E**) and only slightly attenuated the IL-29 response (**fig. 1, F**). Upon co-exposure of HNECs to aqueous birch pollen extract, the virion release of HRV16-infected differentiated HNECs was, by trend, increased ($p=0.08$; **fig. 1, G**), and the IL-29 response was significantly reduced ($p<0.05$; **fig. 1, H**). The attenuation of the HRV16-induced IL-29 secretion was already significant after 24h of exposure with either an aqueous birch pollen extract or with whole birch pollen grains (**fig. S2**).

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 (10µg/mL). Cells of atopic and non-atopic donors all responded to PolyIC stimulation with release of IFN-λs (**fig. S3, A-B**). Co-incubation of HNECs with PolyIC and whole birch or

grass pollen grains significantly decreased the PolyIC-induced production of the IFN-λs (**fig. 2, A**), 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<3kDa). APE<3kDa, at the highest tested concentration, also significantly decreased the PolyIC-induced release of IFN-λs (**fig. 2, B)**. The effect of APE on the PolyIC-induced IFN-λ1 (IL-29) response did not differ significantly between non-atopic and atopic cells (**fig. 2, C**). 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 ($p<0.05$ for grass pollen), but not IL-29. (**fig. 2, D**). Aqueous birch pollen extract significantly decreased the IFN-λ production of HNECs transfected with PolyIC-LyoVec (**fig. S3, C**).

We also checked for the effect of pollen exposure on the pro-inflammatory cytokine and chemokine response of HNECs to PolyIC stimulation (**fig. S3, D**). 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.

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 non-allergenic 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/-hydroxyoctodecatrienoic acids (HODEs, HOTEs). We tested both phytoprostanes and HODE/HOTEs as candidate active substances because (i) the promoter

regions of type III IFN genes contain PPAR-γ responsive elements (**fig. S4, A**) and (ii) 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 (**fig. S4, B**). A PPAR-γ antagonist, GW-9662, did not abolish the inhibitory effect of APE<3kDa on the IFN-λ response to PolyIC (**fig. S4, C**).

Since adenosine is a major constituent of APE<3kDa 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 (1-10μM), adenosine did not have a pronounced effect on the PolyIC-induced IL-29 production of HNECs (**fig. 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 (**fig. S4, E**).

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

We next assessed whether pollen interfere 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 (**fig. S5**). PALMs (PPE_1 or a mixture of HODEs/HOTEs) were tested along with APE as candidate substances. As shown in **fig. 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 (**fig. 3, B**).

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 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 post infection). We analyzed the antiviral immune response of the mice 4, 6 or 10 days after RSV-infection (**fig. 4, A**). 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 post infection (**fig. S6**); however, weight loss did not differ between the RSV+PBS or RSV+pollen treatment (**fig. 4, B**). On day 6 after infection, RSV-infected pollen-treated mice had a significantly higher virus load in BALF compared to only RSV-infected mice (**fig. 4, C**). On day 4 after infection we determined the expression of antiviral genes in lung tissue. Most measured genes of the antiviral response, with the exception of RIG-I, were by trend decreased in RSV-infected pollen-treated mice as compared to RSV-infected PBS-treated mice (MDA5: $p<0.05$; IFN-λ: $p=0.15$, IFN-β: $p=0.16$) (**fig. 4, D-G**). On day 6 we restimulated total lung cells *in vitro* with RSV infected or uninfected dendritic cells (D1 cells) for 24h 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 towards 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 (**fig. 4, H, I**). Numbers of RSV

specific Th2 cells ($IL4^+$, $IL13^+$) were not changed by pollen co-exposure (data not shown). For gating strategy, see **fig. S7**.

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, e.g. 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=5,782) within this data set 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=0.005). The relationship between rhinovirus and pollen was non-linear and positive, whereas it was negative between rhinovirus/pollen and precipitation (**fig. 5, A**).

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 non-allergic 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<0.001, r=0.76) (**fig. 5, B**). 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 **fig. 5, B**).

We additionally performed a controlled out-of-season pollen challenge experiment on two groups (n=9 each) of non-allergic 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 2,500 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=0.06; IFNB1: p=0.09; IFNL1: p=0.16; IFNL2: p=0.05; **fig. 5, C**).

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 anti-viral 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 non-atopic donors, and the non-allergenic fraction (APE<3kDa) had similar effects on cells of

both atopic and non-atopic 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 non-allergic 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 challenge study, we observed by trend a down-regulation 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,

401 indicating that pollen exposure enhances the susceptibility to rhinovirus under real-life co-
402 exposure. In a carefully designed case-crossover study on an Australian children and
403 adolescents asthma cohort, hospital admissions occurred most frequently in springtime, but
404 not during the pollen season at large (October-January) [47]. Interestingly, high levels of
405 airborne grass pollen (50 grains/m^3 or more) increased hospital admissions in HRV-infected
406 boys but not in girls [48]. A recent meta-analysis stresses the relevance of outdoor pollen
407 exposure for asthma exacerbations, especially in children and adolescents [49]. Unfortunately,
408 information on demographics, sensitization or asthma status was not available for our
409 Gothenburg rhinovirus cases. Atopy and asthma are traits linked to a defective antiviral
410 response [8], possibly due to chronic exposure of the respiratory epithelium to Th2 and type-2
411 cytokines, e.g. IL-33 [50,51]. Since samples from allergic asthmatics or patients with other
412 respiratory diseases could be over-represented among samples obtained during the birch
413 pollen season, the positive correlation we observed between pollen and rhinovirus is most
414 likely due to a combined effect of atopy or respiratory disease and pollen. Overall, however,
415 we have acquired consistent results in three independent human cohorts from two different
416 geoclimatic regions, despite confounding effects such as rising air temperatures in springtime.
417 Taken together, our results indicate that pollen exposure itself modulates the antiviral defense
418 of the respiratory epithelium. This might be of special relevance for individuals with chronic
419 respiratory diseases where viral infections are a main cause of severe exacerbations.
420 Furthermore, also non-allergic individuals at risk for respiratory infections might benefit from
421 restricting their extensive outdoor activities when pollen and respiratory virus seasons
422 coincide, particularly during days with high pollen counts. However, large-scale clinical trials
423 are needed to confirm these findings and to formulate guidelines for people at risk.
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Figures

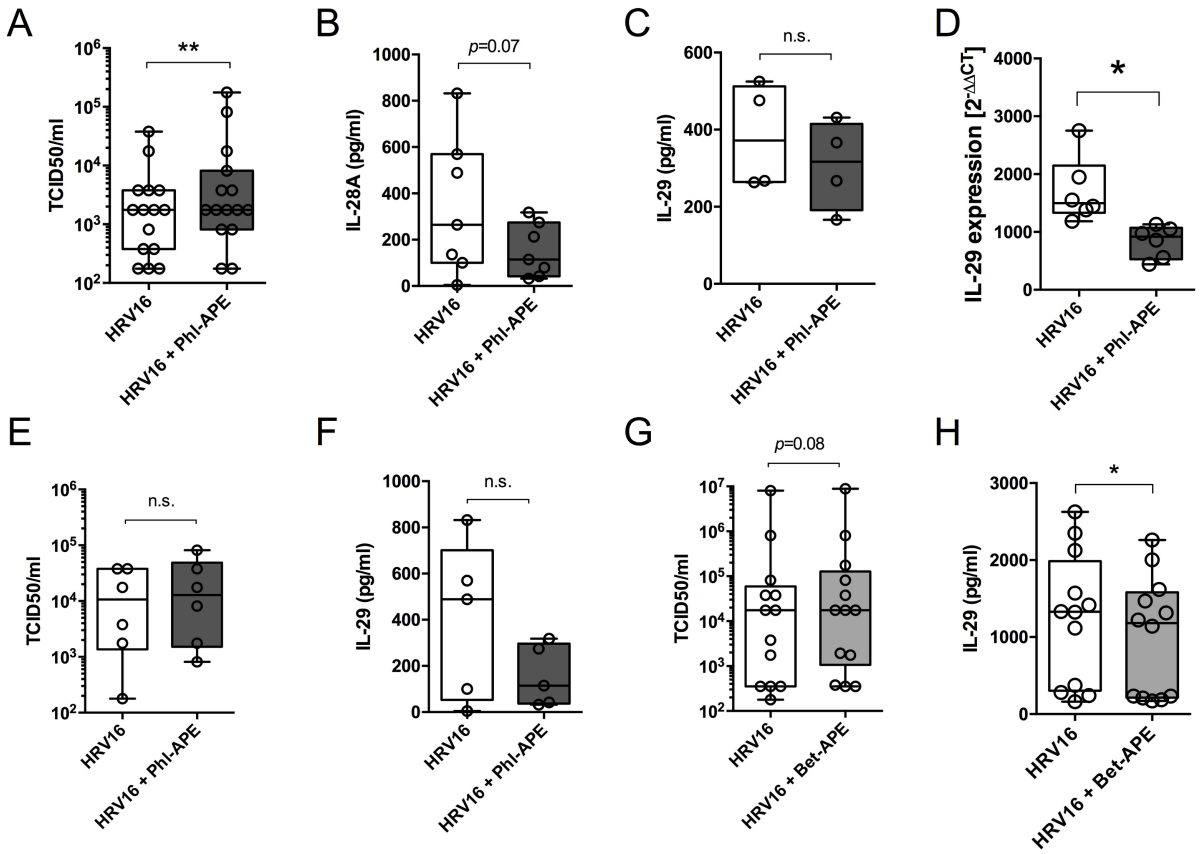

Figure 1: Viral replication and antiviral IFN-λ expression in rhinovirus-infected human airway epithelium upon co-exposure to pollen. Differentiated human primary bronchial (PBEC; **A-D**) and nasal epithelial cells (HNEC; **E-H**) were infected for 48h with HRV16 and co-exposed with 100µg/mL aqueous extracts of birch (Bet-APE) or timothy grass pollen (Phl-APE). **A:** HRV16 virion release in apical washes of PBEC cultures 48h after infection. **:p<0.005 (n=15). **B-D:** Release of antiviral FN-λs 48h after infection. *: $p<0.05$ (n=4-6). **E, G:** HRV16 virion release of HNECs 48h after infection, with or without co-exposure to 100µg/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<0.05; n=5-13). As control, UV-irradiated RV16 was used (data not shown). All experiments were carried out with cultures of n different, non-atopic donors.

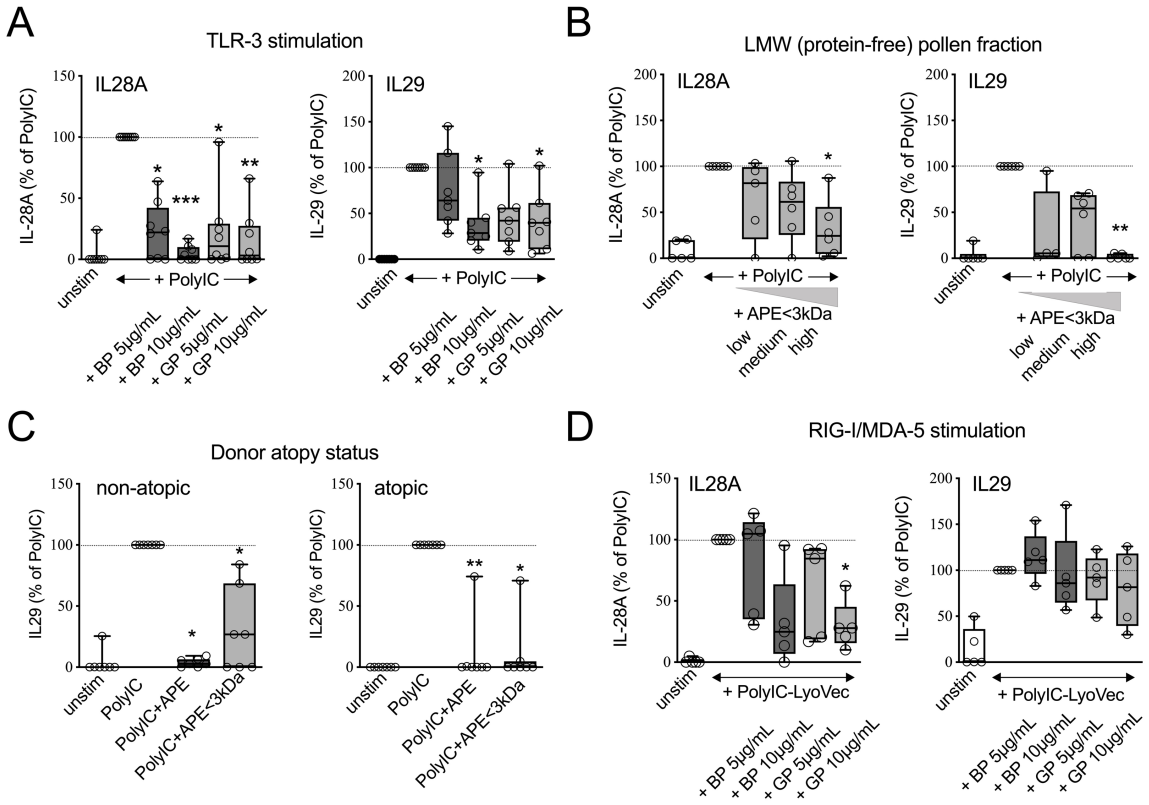

Figure 2: Antiviral response in primary human nasal epithelial cells stimulated with viral mimics.

A: Inhibition of the PolyIC-induced IFN-λ response of HNECs by whole birch pollen (BP) and timothy grass pollen (GP) grains. *: $p<0.05$, **: $p<0.01$, ***: $p<0.005$; n=7. **B:** IFN-λ production in cells stimulated with PolyIC (10µg/mL) in the absence or presence low molecular weight aqueous birch pollen extracts (APE<3kDa; 10, 30 and 100µg/mL). *: $p<0.05$, **: $p<0.01$; n=6. **C:** IFN-λ inhibition by aqueous birch pollen extract (APE; 100µg/mL) and the low molecular weight fraction (APE<3kDa; 100µg/mL) in cells from non-atopic and atopic donors. *: $p<0.05$, **: $p<0.01$; n=7. **D:** IFN- λ response in cells transfected with PolyIC-LyoVec in the absence and presence of whole pollen grains. *: $p<0.05$; n=5. All experiments were carried out with cells of n independent donors.

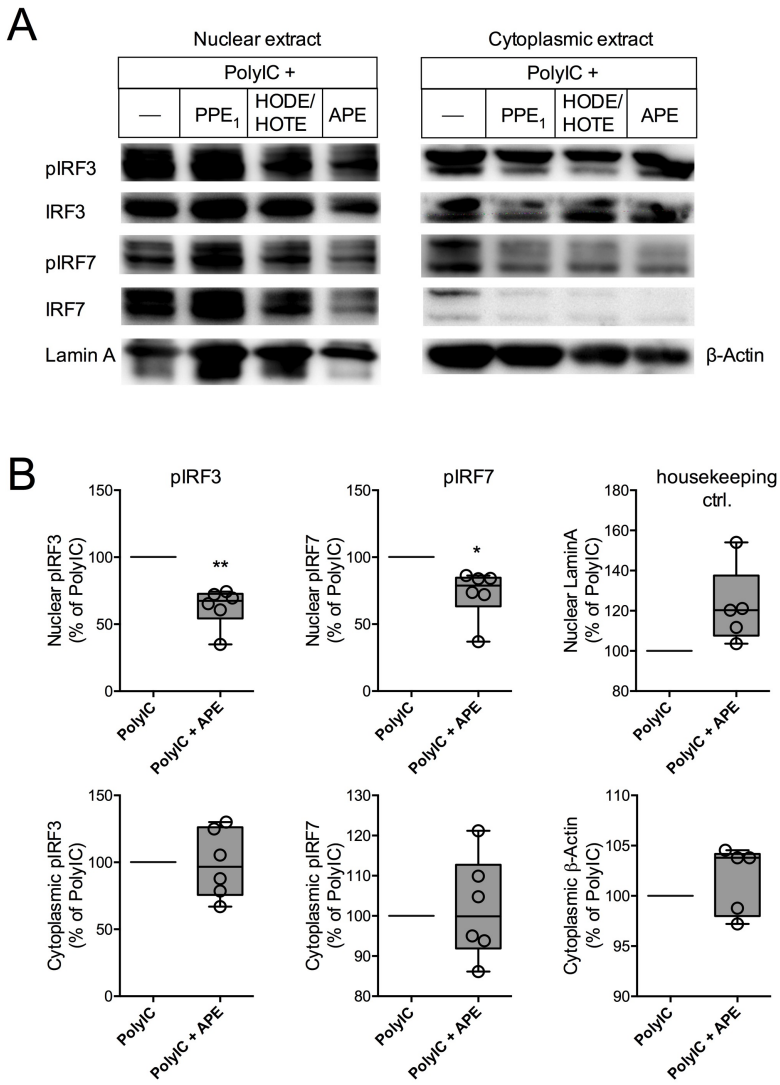

Figure 3: Nuclear translocation of phosphorylated transcription factors IRF-3 and IRF-7 in PolyIC- and pollen-stimulated nasal epithelial cells.

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 100µg/mL total protein); PPE_1: E_1-phytoprostane, 1µM; HODE/HOTE: equimolar mixture of hydroxyoctodecadienoic- and hydroxyoctodecatrienoic acids, 1µM. **B:** Quantitation of proteins in nuclear and cytoplasmic extracts of HNECs stimulated with PolyIC (10µg/mL) vs. PolyIC plus APE. *: $p<0.05$, **: $p<0.01$; n=6 independent experiments, each using different donors.

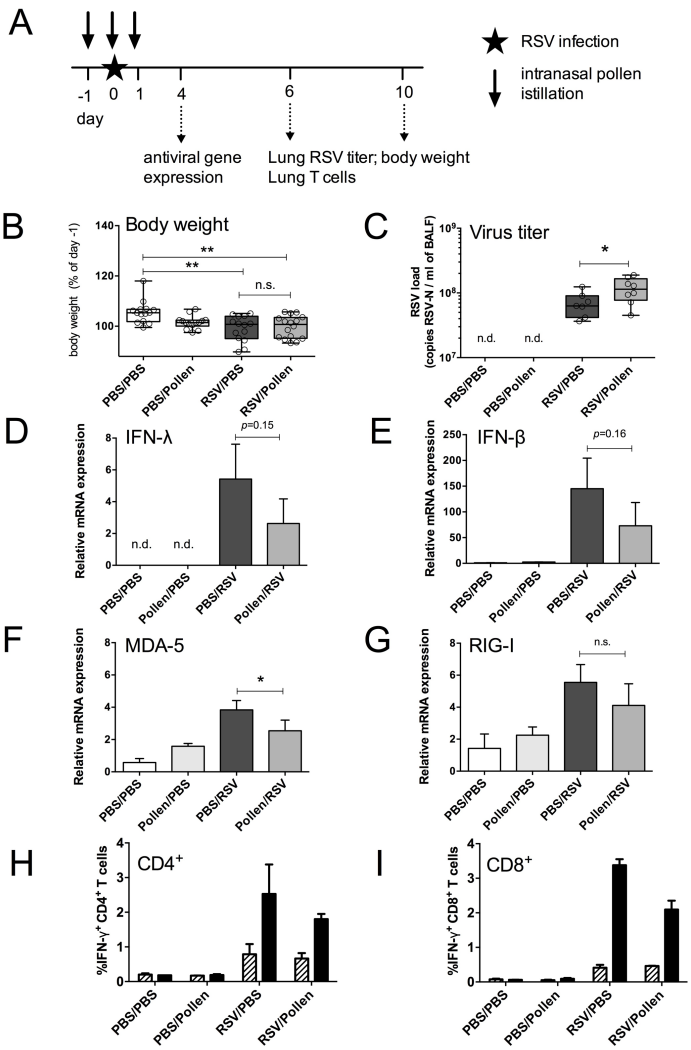

Figure 4: Intranasal pollen exposure increases viral load and diminishes expression of MDA-5 in a murine respiratory syncytial virus model.

A: Experimental setup. **B:** Body weight of mice days after intranasal treatment with buffer (PBS/PBS), ragweed pollen extract (PBS/pollen; 10µg/mL), respiratory syncytial virus (RSV) (RSV/PBS) or a combination of RSV and pollen extract (RSV/pollen). **: $p<0.01$; n=15 mice per group **C**: Viral load in BALF of mice 6 days after indicated treatment. *: $p<0.05$; n=8-9 **D-G:** Antiviral gene expression in the lungs of mice 4 days after indicated treatment. *: $p<0.05$ vs. PBS/RSV; n=3-4. **H-I:** Percentages of virus-specific IFN-γ^+ $CD4^+$ and $CD8^+$ T cells in re-stimulated spleens of mice 6 days after indicated treatment. Clear bars: Re-stimulation with uninfected D1 cells. Grey bars: Re-stimulation with RSV-infected D1 cells.

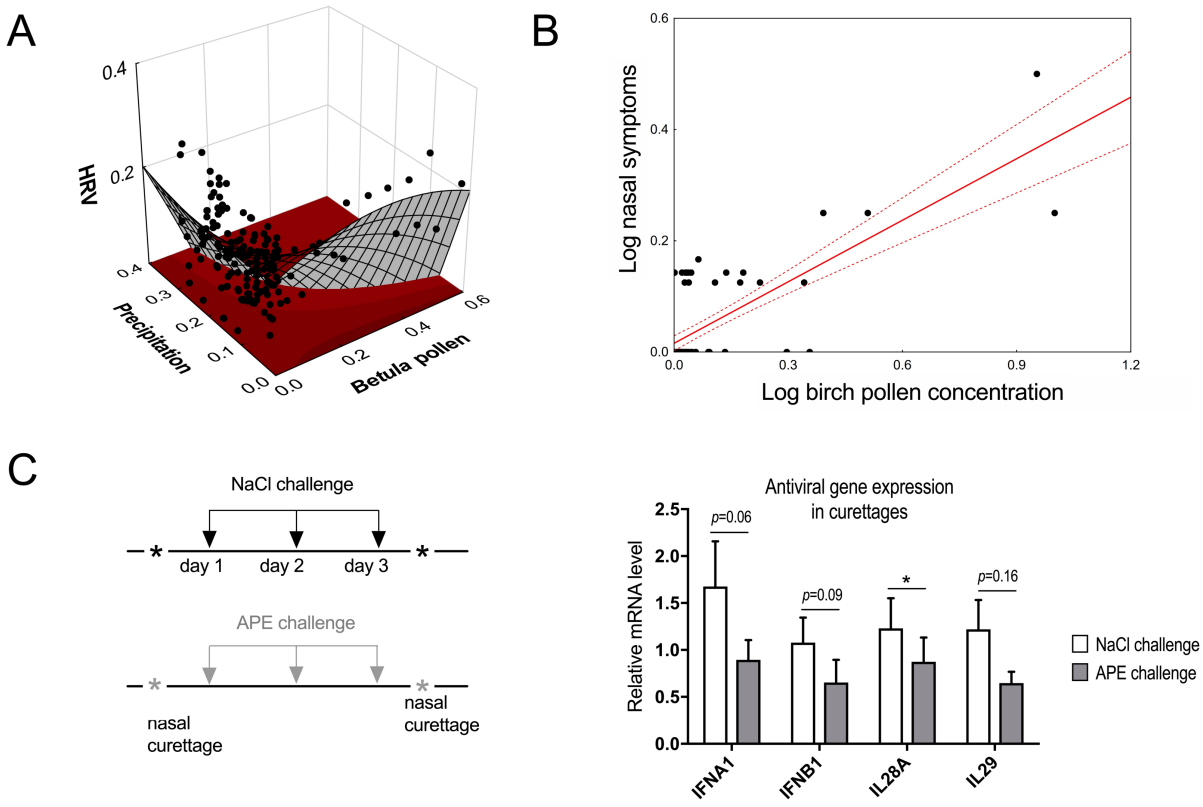

Figure 5: Relationships of rhinovirus, nasal symptoms and expression of IFN genes in three different human cohorts.

A: Correlation (GLM) between rhinovirus positive cases, airborne birch pollen concentrations and precipitation in Gothenburg, Sweden. Nasopharyngeal swabs (n = 5,782) tested positive for rhinovirus (*z*-axis) were regressed against airborne birch pollen concentrations (*y*-axis) and precipitation (*x*-axis); p=0.005. All values are 7-day moving averages of normalized original values. **B:** Time series analysis (cross-correlation) of nasal symptoms in a well-characterized cohort of non-allergic 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 non-allergic volunteers subjected to 3 successive intranasal challenges with saline (NaCl challenge; n=9) or 2,500 SBE of aqueous birch pollen extract (APE challenge; n=9). Mean + SEM. *: p<0.05.

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*	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*	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

Table 1: Overview of the non-atopic panel cohort. Shown are serum levels of total IgE (IU/mL) and IgE specific for common perennial and seasonal aeroallergens (HDM: house dust mite). * Subjects PAB_NA_2 and PAB_NA_4 were excluded from analysis because they were abroad during parts of the Augsburg birch pollen season.

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Study design and main manuscript writing: SG, CB

In vitro experiments: SG (TLR3, RIG-I, MDA5 model); CB (PBEC HRV infection model); SS (Western Blots, signal transduction)

CBG and SE (HNEC HRV infection model)

ENT related expertise, specimens from nasal surgery: AC

Murine RSV infection model: MW, LM, BvtL, JG

Nasal pollen challenge study: MG

Data on airborne pollen concentrations: AD, FH (Augsburg), ÅD (Gothenburg)

Data on rhinovirus samples in human cohort: NS, ML, LMA, JW

Statistical analysis: AD, AUN

Project and manuscript discussions: SG, CB, CAA, DED, CTH

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