Pollen exposure weakens innate defense against respiratory viruses

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Materials and methods, detailed

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References

47 # Materials and Methods, detailed

48

49 **Pollen and pollen extracts**

50 Pollen grains from birch (*Betula pendula*) and timothy grass (*Phleum pratense*) were extracted
51 from male flowers collected in spring/summer of 2014 in Munich and Augsburg as previously
52 described[1]. Ragweed (*Ambrosia artemisiifolia*) pollen used for the RSV mouse model was
53 collected from plants grown in greenhouses. Aqueous pollen extracts (APEs) and a protein-free
54 fraction thereof (APE<3kDa) were prepared as described in [2]. Briefly, 10mg of pollen were
55 suspended in aqueous buffer (PBS for cell stimulations; 0.9% NaCl for nasal challenge) and
56 incubated in a shaking water-bath for 30 minutes at 37°C, with vortexing every 10 minutes to
57 prevent sedimentation of the pollen. The pollen grains were then pelleted by centrifugation (10
58 min, 1,000xg) and the supernatant was sterile-filtered (0.2µm pore-size filter) to remove residual
59 pollen. For generating a protein-free extract, the total APE was ultra-filtered using 3 kilo Dalton
60 (kDa) cutoff filters (Amicon Ultra, Millipore). Total protein concentrations of APEs were
61 100µg/mL (birch), 160µg/mL (timothy grass) and 750µg/mL (ragweed; by Bradford assay). The
62 major allergen concentrations, as determined by ELISA, were 50µg/mL Bet v 1 (birch), 70µg/mL
63 Phl p 5 (timothy grass) and 100µg/mL Amb a 1 (ragweed).

64

65 **Human rhinovirus (RV) 16 propagation and titration**

66 Human rhinovirus (HRV16; ATCC VR-283™, Teddington, UK) was amplified using H1 HeLa
67 cells as previously described [3,4]. Infectivity of stocks and release of infective virions in cell
68 culture supernatants was determined using a HeLa titration assay and 50% tissue culture infective

69 dose assay ($TCID_{50}$/ml). Ultraviolet-irradiated virus controls (UV-RV16) were prepared by
70 exposure of virus stocks to UV light at 1200 mJ/cm^2 on ice for 50 min.
71
72 **RNA extraction from differentiated PBECs and RT-qPCR**
73 Using standard phenol-chloroform extraction, RNA was isolated from cell lysates and reverse
74 transcribed to cDNA using a Precision Reverse Transcription kit (PrimerDesign, Southampton,
75 UK) according to the manufacturer's instructions. cDNA was amplified by qPCR (cycling
76 conditions 95 ºC 10 min, then 50 cycles of 95 ºC 15 s, 60 ºC 1 min) using Perfect Probe assays
77 specific for IFN-β, IL-29, MDA-5 and RIG-I (PrimerDesign). Data were normalised to the
78 geometric mean of the housekeeping genes (ubiquitin C and glyceraldehyde 3-phosphate
79 dehydrogenase, probe-based duplex primer mix, PrimerDesign) and fold change in gene
80 expression relative to controls (without pollen and RV16 stimulation) was determined using the
81 ΔΔCt method.
82
83 **Stimulation of HNEC monolayer cultures with viral mimics, pollen extracts and pollen**
84 Passage 2 HNECs isolated from surgical biopsies (see methods in main manuscript) were seeded
85 into tissue culture plates in complete airway epithelial cell growth medium (PromoCell,
86 Heidelberg, Germany) until 80-90% confluent. One day prior to stimulation, medium was
87 changed to complete medium without hydrocortisone. Control cells were incubated with medium
88 (untreated) or treated cells with PolyIC (10µg/mL), High molecular weight PolyIC-LyoVec
89 (10µg/mL), aqueous pollen extract (APE; corresponding to 10, 50 or 100µg total protein/mL or 5,
90 10 or 50µg of Bet v 1/mL), a protein-free, low MW fraction of aqueous pollen extract
91 (APE<3kDa; at the same concentrations) or whole pollen grains (birch, timothy grass;
92 corresponding to 1 and 5 µg/mL aqueous pollen extract) for 24h. The pollen grains were added

93 drop-wise to the cell cultures from a freshly prepared pollen suspension. Sedimentation of the
94 pollen grains onto the HNEC monolayer occurred within 5 minutes, as observed under the light
95 microscope. After the stimulation, supernatants were recovered, centrifuged briefly to remove
96 pollen grains and cell debris and subjected to ELISA or multiplex assay for detection of type III
97 interferones and pro-inflammatory cytokines and chemokines.
98

99 **Reagents, ELISA and multiplex kits**

100 IFN-λ1 (IL-29) was measured using Human IL-29 (IFN lambda 1) ELISA Ready-SET-Go!®
101 ELISA kit (eBioscience, San Diego, CA, USA); IFN-λ2 (IL-28A) and chemokines were detected
102 by singleplex or multiplex using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic
103 Bead Panel II (Millipore, Darmstadt, Germany); CCL5 release was determined using a Human
104 RANTES Tissue Culture Kit (Mesoscale Discovery, Rockville, MD) according to the
105 manufactures instructions. PolyIC and LyoVec-PolyIC were purchased at Invivogen, Toulouse,
106 France. Inhibitors of PPAR-γ (GW-9662) and adenosine receptors (MRS-1754, SCH-442416)
107 were purchased at Sigma Aldrich, Taufkirchen, Germany.
108

109 **Nuclear translocation of transcription factors**

110 HNEC submerged monolayer cultures were incubated with indicated stimulants for 5 min in
111 hydrocortisone-free complete medium. The reaction was stopped by placing the cells on ice and
112 washing twice with ice-cold PBS containing phosphatase inhibitors. Nuclear and cytoplasmic
113 extracts were prepared from cells using the Nuclear Extract Kit (Active Motif, Carlsbad, CA,
114 USA) following the manufacturer´s instructions. 10 µg of total proteins were subjected to SDS-
115 PAGE and transferred to a nitrocellulose membrane by Western Blotting. Phosphorylated IRF-3
116 and -7 were probed with specific monoclonal antibodies (Phospho-IRF-3 (Ser396) Rabbit mAb;

117 Phospho-IRF-7 (Ser477) Rabbit mAb, Cell Signaling Technology, Danvers, MA, USA) and
118 secondary HRP-conjugated goat anti-rabbit antibody (Cell Signaling Technologies, Danvers,
119 MA, USA), followed by chemiluminescence (ECL) detection on a ChemoCam imager (Intas,
120 Goettingen, Germany). Intensity of the bands was quantitated using the ChemoStar software
121 (Intas).
122
123 **Gene expression analysis of murine lung tissue**
124 Complementary DNA (cDNA) was prepared by reverse transcription of 0.5 µg of each total RNA
125 using the RevertedAid H Minus First Strand cDNA synthesis kit (Fermentas GmbH/Germany).
126 Quantitative real-time PCR (qPCR) was performed using specific primers and probes for each
127 gene and using the Probes Master 480 (Roche). Reactions were analyzed using the
128 LightCycler480 (Roche) at 50°C for 2 min, 94°C for 10 min and 45 cycles of 94°C for 15 s and
129 60°C for 15 s. For relative quantification, the expression levels relative to the housekeeping gene
130 *18s RNA* were determined. The *CT* (cycle threshold difference) between the target gene and *18s*
131 *RNA* was calculated for each sample and expressed as $2^{-\Delta Ct}$.
132
133 **RSV-specific real-time PCR**
134 Viral load was determined in the bronchoalveolar lavage fluid by real-time PCR as described
135 previously [5]. In short, viral genomic RNA was isolated from the fluid using a MagnaPure LC
136 total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). The isolated viral RNA was
137 reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied
138 Biosystems, Foster City, CA), according to the manufacturer's guidelines. Murine
139 encephalomyocarditis virus (RNA) was used as an internal control. Samples were assayed in a 25
140 µl reaction mixture containing 10 µl of cDNA, TaqMan universal PCR master mix (Applied

141 Biosystems, ABTI), primers (900 nM RSV-A primers), and fluorogenic probes (58.3 nM RSV-A
142 probe) labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye 6-
143 carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an
144 ABTI 7900 HT system for 2 min at 50oC, 10 min at 95°C, and 45 cycles of 15 sec at 95°C and 1
145 min at 60°C. Samples were controlled for the presence of possible inhibitors of the amplification
146 reaction by the indicated internal control, signals of which had to range within a clear-cut
147 interval. Sample cycle threshold values (Ct) were compared with a standard curve of RSV A2.
148

149 **Re-stimulation of murine, lung-infiltrating cells**

150 Isolated lung cells ($1x10^6$) were co-cultured with RSV-infected D1 cells ($2x10^5$ cells). D1 is a
151 non-transformed, growth factor-dependent, myeloid dendritic cell line derived from C57BL/6
152 mice [6]. D1 cells were grown in IMDM, 5% FCS, 1% penicillin/streptomycin and 50µM β-
153 mercapto-ethanol and supplemented with 30% conditioned medium form GM-CSF producing R1
154 cells [7]. Before adding them to lung cells, D1 cells were infected for 48 hours with RSV
155 (multiplicity of infection, m. o. i. 2). Cell suspensions were stimulated for 6 h at 37°C, 5% CO_2 in
156 200 µl IMDM supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 5% FCS,
157 penicillin/streptomycin, 50µM β-mercapto-ethanol and 50 U/ml recombinant IL-2 and 10 µg/ml
158 Brefeldin A. For flow-cytometric analysis, cells were harvested, and T cells were identified by
159 anti-CD3, anti-CD4, and anti-CD8. Fixed and permeabilized cells were then stained with anti-
160 INF-γ, anti-IL-4 and anti-IL-13. Samples were acquired on a FACSCanto II flow cytometer and
161 analyzed by FlowJo software.
162

163 **Airborne pollen concentrations**

164 Airborne pollen concentrations were monitored using Hirst-type volumetric pollen traps in
165 Augsburg, Germany and Gothenburg, Sweden, as described by the European Aerobiology
166 Society [8]. So as to exclude uncommon long-distance pollen transport and pollen re-suspension
167 phenomena, only the main birch pollen season was used. Its start and end were defined by 5%
168 and 95% respectively of the total annual pollen sum [9].
169

170 **Antiviral gene expression in nasal curettages**

171 Total RNA was extracted from nasal curettages using RNeasy Mini Kit (Qiagen). RNA was
172 treated with DNase and concentrations were determined on a BioDrop spectrophotometer
173 (Harvard Bioscience). 150ng of each RNA was used as a template to synthesize cDNA using
174 iScriptTM cDNA Synthesis Kit (Bio-Rad). Thermal cycling protocol was 25°C for 5 min, 46°C
175 for 20 min and 95°C for 1 min. Per sample A set of antiviral and house-keeping genes were pre-
176 amplified from 5µl cDNA using the SsoAdvancedTM PreAmp Supermix and PrimePCRTM
177 PreAmp for IFNA1, INFB1, IL28A, IL29 and GAPDH (Bio-Rad). 18S and EF1A primer pairs
178 were designed in our lab. Thermal cycling protocol was 95°C for 3 min, followed by 12 cycles of
179 amplification at 95°C for 15 sec and 58°C for 4 min. 1µl of each pre-amplified sample was used
180 to perform qPCR using the iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX384
181 Touch Real-Time PCR Detection System (Bio-Rad). Thermal cycling program was run at 95°C
182 for 30 sec, followed by 40 cycles of amplification at 95°C for 5 sec and 60°C for 30 sec.
183 Quantification cycle (Cq) values were determined by using automatic threshold. GAPDH, 18S
184 and EF1A were used as reference genes. ΔΔCq Values were calculated to obtain relative gene
185 expression.
186

187 **Meteorological data**

188 Data on average weekly outdoor temperature (degrees Celsius), vapor pressure (VP; hPa),
189 relative humidity (RH, %), wind speed (meter/second) and precipitation (mm) for the study
190 period were obtained from the Swedish Meteorological and Hydrological Institute (SMHI)
191 obtained at the local weather station in Gothenburg (5 meters above the sea level, situated at
192 Latitude: 57.7157N, Longitude: 11.9925E).
193
194 **Statistics**
195 (i) In vitro experiments, RSV mouse model, human pollen challenge study (unless stated
196 otherwise in the legends): two-sided Mann Whitney test for simple comparisons between two
197 groups; two-sided Wilcoxon test for pairwise comparisons between treatment and control groups;
198 Friedman test for comparisons of multiple treatment groups with a single control group. (ii)
199 Human cohorts: Relationships of symptoms with airborne pollen concentrations were
200 investigated with simple linear regressions (GLM) and cross-correlations (time series analysis).
201 In all analyses of symptoms, seven-day moving averages of normalized values were used, so as
202 to eliminate periodicity effects (lower hospitalization rates over the weekends). All statistical
203 tests were performed with Prism or Statistica.
204

205 ## Supplementary Text

206 **Pollen diminish the epithelial response to rhinovirus infection.**

207 Human primary bronchial epithelial cells (PBECs) were differentiated at the air-liquid interface,
208 exposed to control medium or timothy grass pollen extract (Phl-APE) and infected with human
209 rhinovirus (HRV16) for 24h. Exposure of infected PBECs to Phl-APE resulted in reduced mRNA
210 levels of several anti-viral and pro-inflammatory response genes, as compared to only HRV16
211 infected cells (**fig. S1**). Levels of IFN-β and CCL-5 gene expression were significantly reduced
212 (**fig. S1**, **A-B**; $p<0.05$), levels IRF-7 ($p=0.06$) and MDA-5 by trend ($p=0.07$) (**fig. S1**, **C-D**).

213

214 **Birch pollen exposure diminishes the IFN-λ response in rhinovirus-infected, differentiated**
215 **human nasal epithelial cells.**

216 To study the kinetics of the IFN-λ response under combined exposure to pollen and rhinovirus,
217 differentiated human nasal epithelial cells were infected with human rhinovirus (HRV16) and co-
218 exposed to aqueous birch pollen extract (Bet-APE) or whole birch pollen grains (BP) for 24 and
219 48h. Basolateral supernatants were then analyzed for IL-29 by ELISA. Bet-APE and whole birch
220 pollen grains significantly attenuated the IL-29 response of HRV-infected HNECs at both time-
221 points (**fig. S2**). UV-irradiated HRV16 was used as stimulation control and did not induce IL-29
222 (data not shown).

223

224 **Pollen modulate signaling of nasal epithelial cells to viral mimics.**

225 To characterize in detail how pollen interfered with antiviral pathways, we carried out
226 experiments using monocultures of primary human nasal epithelial cells (HNECs) stimulated
227 with synthetic viral mimics. First we tested a TLR3 ligand, PolyIC, in the absence or presence of

228 pollen grains. Whereas unstimulated HNECs did not release significant amounts of IFN-λs,
229 PolyIC-stimulated cells showed a robust IFN-λ response (**fig. S3, A**). Mean IFN-λ levels in
230 supernatants of PolyIC-stimulated cells were 774 ± 490 pg/mL (IL-28A) and 1159 ± 310 pg/mL
231 (IL-29). Since defective IFN-λ responses have been described for atopic asthmatics, we
232 compared IFN-λ responses in HNECs from atopic and non-atopic donors (**fig. S3, B)**. Mean
233 levels of IL-28A in PolyIC-stimulated HNECs were 1520 ± 316 pg/mL (non-atopic) and 971 ±
234 426 pg/mL (atopic donors); mean IL-29 levels 656 ± 94 pg/mL (non-atopic) and 727 ± 164
235 pg/mL (atopic donors). IFN-λ production did not differ significantly between cells of non-atopic
236 and atopic donors (IL-28A: p=0.08; IL-29: p=0.96; atopic vs. non-atopic). Aqueous birch pollen
237 extracts also significantly decreased both IL-28A and IL-29 production of cells transfected with
238 the RIG-I/MDA-5 ligand (**fig. S3, C**). We also checked for the effect of pollen exposure on the
239 pro-inflammatory chemokine response of HNECs to PolyIC stimulation. Pollen grains
240 significantly decreased the release in several chemokines, such as G-CSF, CCL2, CCL3, CCL4,
241 CCL5 and CXCL10, whereas they increased levels of IL-1β (**fig. S3, D**).
242

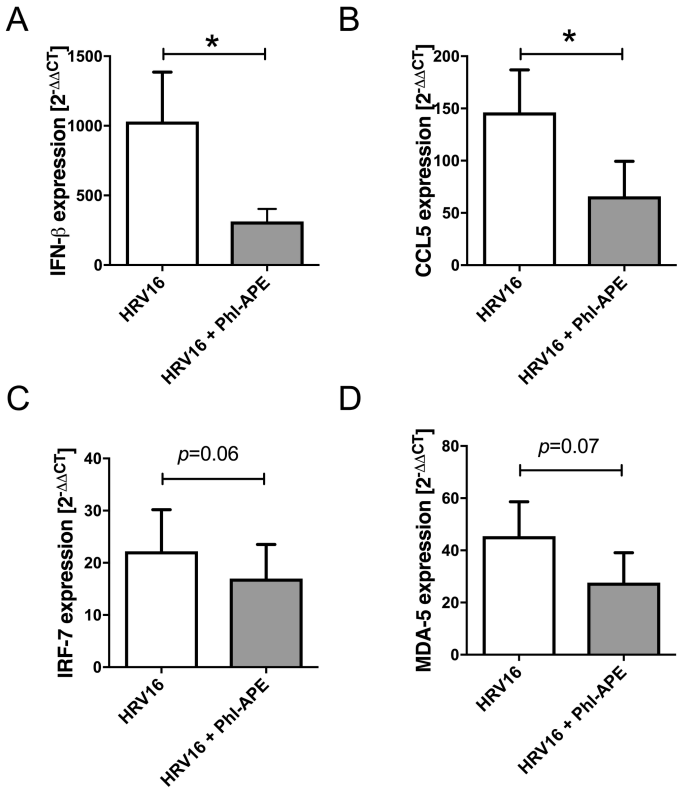
243 **The PolyIC-induced IFN-λ response of HNECs is not mediated by known immune-**
244 **modulatory substance in pollen.**

245 We previously reported immune-modulatory effects, such as PPAR-γ dependent inhibition of IL-
246 12 and blocking of nuclear translocation of NF-κB, by non-allergenic pollen substances [10]. Two
247 classes of PALMs were previously identified and discussed as potential PPAR-γ ligands, the
248 phytoprostanes and the phyto-hydroxyoctodecadienoic/-hydroxyoctodecatrienoic acids (HODEs,
249 HOTEs). We tested both phytoprostanes and HODE/HOTEs as candidate active substances
250 because (i) the promoter regions of type I and III IFN genes contain PPAR-γ responsive elements
251 (**fig. S4, A**) and (ii) PPAR-γ agonists were previously shown to inhibit IRF-3 translocation to the

252 IFN-β promoter in LPS- and PolyIC-stimulated murine peritoneal macrophages [4]. However, none
253 of the tested lipid mediators had any significant effect on the PolyIC-induced IFN-λ secretion
254 (**fig. S4, B**). A PPAR-γ antagonist, GW-9662, did not abolish the inhibitory effect of APE<3kDa
255 on the IFN-λ response to PolyIC (**fig. S4, C**).
256 Since adenosine is a major constituent of APE<3kDa and can exert immune-modulatory effects
257 on human dendritic cells [2], we assessed whether the effect of pollen on the PolyIC-induced IFN-λ
258 response of HNECs could be mediated in part by adenosine. At concentrations as contained in
259 aqueous pollen extracts (1-10μM), did not have a pronounced effect on the PolyIC-induced IL-29
260 production of HNECs (**fig. S4, D**). However, in the presence of an A2a inhibitor, the IL-29
261 response to PolyIC stimulation was approximately 4-fold increased as compared to cells not
262 treated with the inhibitor (**fig. S4, E**).
263

264 **Supplementary figures and legends**

265



266

267 **Figure S1: Expression of antiviral genes in virally infected human bronchial epithelial cells**

268 **during exposure to pollen.**

269 Human primary bronchial epithelial cells (PBECs) were differentiated at the air-liquid interface,

270 exposed to control medium or timothy grass pollen extract (Phl-APE) and infected with human

271 rhinovirus (HRV16) for 24h. Mean + SEM; *: $p<0.05$; n=5-6. Experiments were carried out with

272 cultures of n independent donors.

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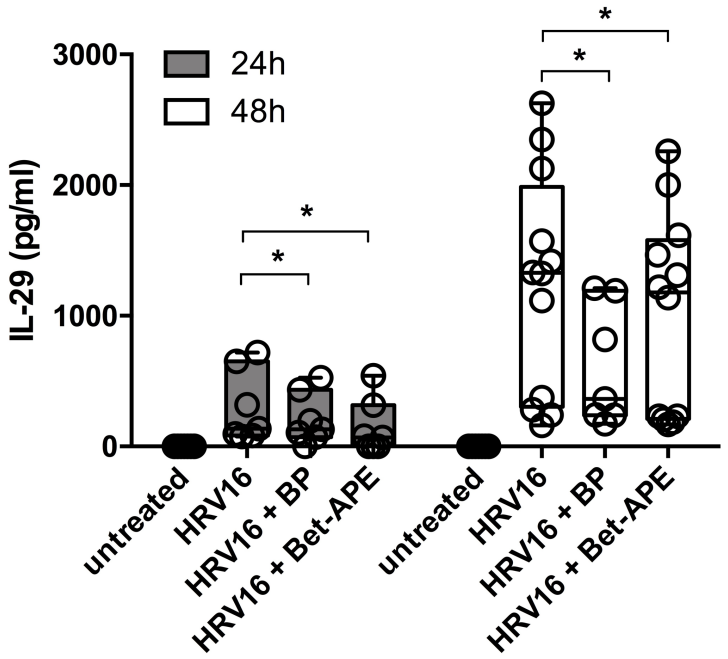

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276 **Figure S2: Time-course of the IL-29 response of human nasal epithelial cells to rhinovirus**
277 **infection and birch pollen co-exposure**.

278 Differentiated primary human nasal epithelial cells (HNECs) were either left untreated or
279 infected with human rhinovirus (HRV16), either alone or under co-exposure with whole birch
280 pollen grains (BP, 10µg/mL) or aqueous birch pollen extract (Bet-APE; 100µg/mL). Basolateral
281 release of IL-29 was measured after 24h and 48h of incubation. UV-irradiated HRV16 was used
282 as stimulation control and did not induce IL-29 (not shown). *: $p<0.05$; n=6-12. All experiments
283 were carried out with cells of independent, non-atopic donors.

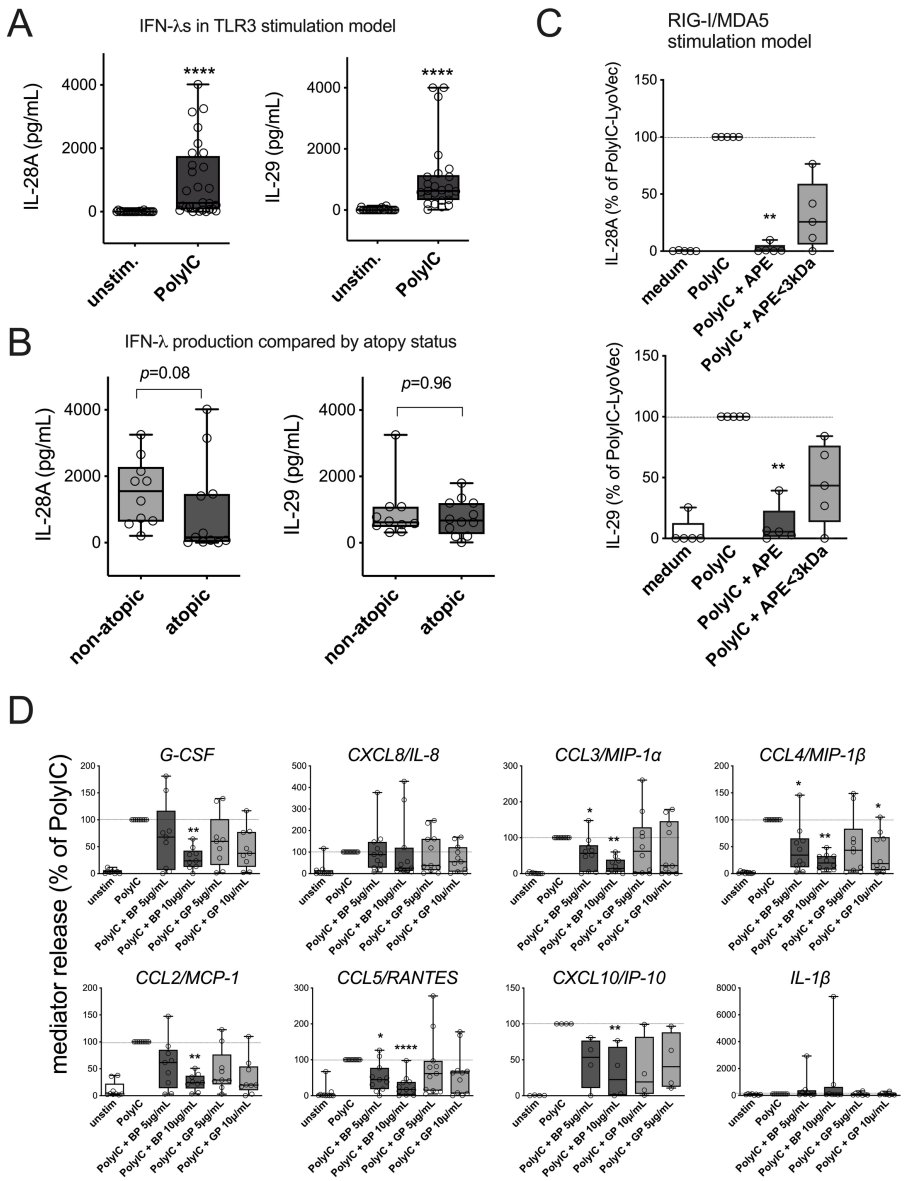

Figure S3: Antiviral response in primary human nasal epithelial cells stimulated with viral mimics.

A: Induction of IFN-λs after PolyIC stimulation. ****: $p<0.001$; n=28. **B:** Comparison of IFN-λ levels between HNECs of non-atopic (n=10) and atopic donors (n=11) stimulated with PolyIC. **C:** IFN- λ response in cells transfected with PolyIC-LyoVec, for RIG-I and MDA-5 stimulation, in the absence and presence of aqueous birch pollen extract (APE) and or low a molecular weight

292 fraction thereof (APE<3kDa). **: $p<0.01$; n=5. **D:** Chemokine and cytokine response of HNECs
293 treated with PolyIC in the absence or presence of whole pollen grains of birch (BP) and timothy
294 grass (GP). The concentration of pollen grains in the cell culture corresponded to 5 and 10µg/mL
295 total protein as contained in aqueous pollen extract. *: $p<0.05$; **: $p<0.01$; ****: $p<0.001$; n=7-
296 14. All experiments were carried out with cells of independent donors.
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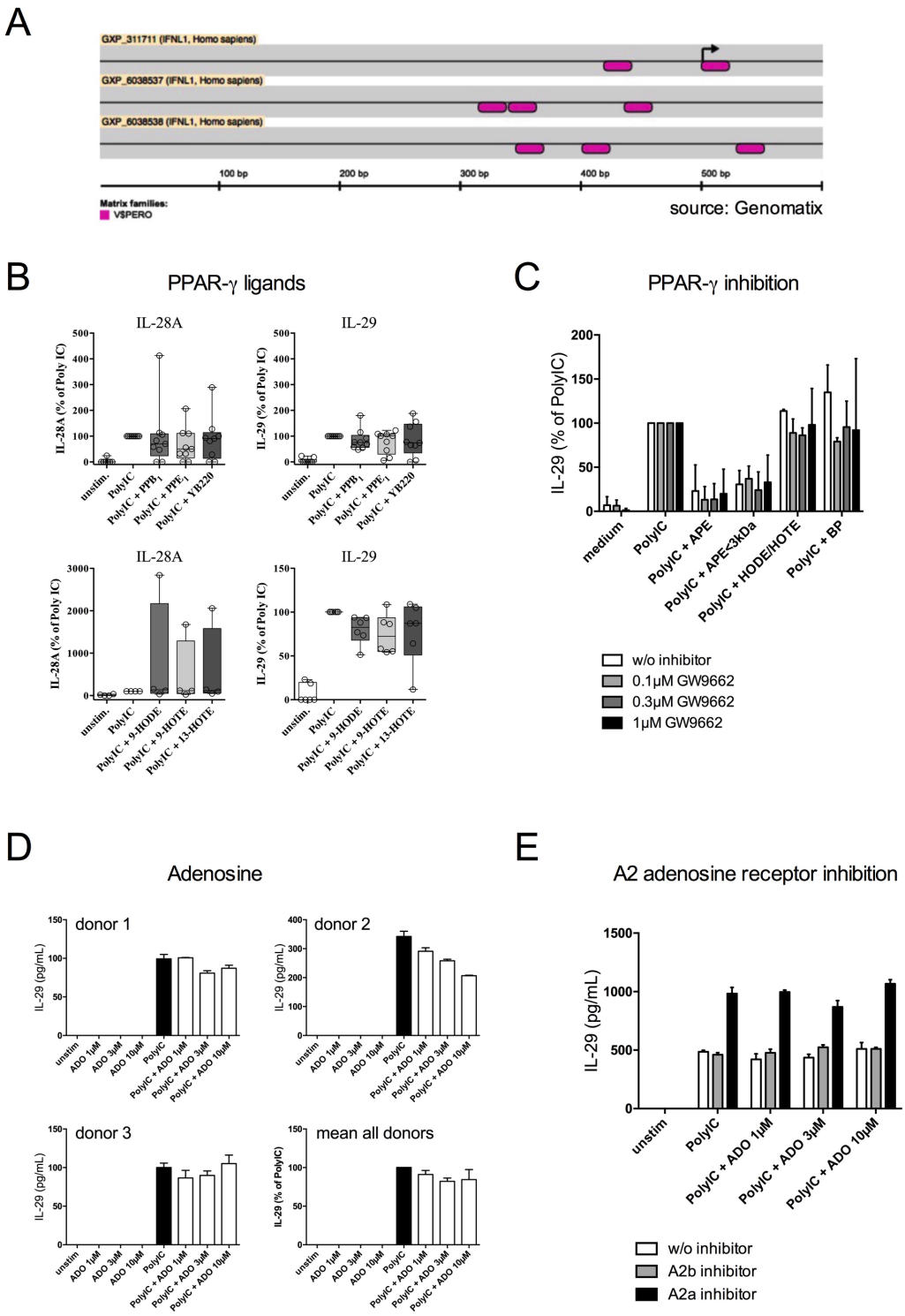

Figure S4: Effects of known candidate compounds from pollen on the PolyIC-stimulated IFN-λ response of HNECs.

301 **A:** Localization of consensus PPAR-γ binding sequence elements (depicted in pink) in the
302 putative core promoter region of the human IFNL1 gene, coding for the IL-29 protein. The
303 promoter analysis was performed using an online promoter analysis tool (Genomatix Suite,
304 release 3.0; www.genomatix.com), using three different genebank reference sequences for the
305 IFNL1 core promoter. **B:** IFN-λ response of HNECs to PolyIC (10µg/mL) and co-stimulated with
306 pollen-associated lipid mediators (1µmol/L each). n=4-9. PPB_1: equimolar mixture of type-I and
307 type-II B_1-phytoprostanes; PPE_1: equimolar mixture of type-I and type-II E_1-phytoprostanes;
308 YB220: 15-E_{2t}-Isoprostane; HODE: Hydroxy-octodecadienoic acid; HOTE: Hydroxy-
309 oxtodecatrienoic acid. **C:** IL-29 response of HNECs treated with PolyIC in combination with
310 aqueous birch pollen extract (APE), a low molecular-weight fraction (APE<3kDa), birch pollen
311 grains (BP) or HODE/HOTE. Cells were pre-incubated for 1h with or without a PPAR-γ
312 inhibitor, GW9662 (0.1-3µmol/L). Mean + SEM; n=3. **D:** IFN-λ response in PolyIC treated nasal
313 epithelial cells co-stimulated with adenosine (ADO; 1-10µmol/L). **E:** IFN-λ response of HNECs
314 stimulated with PolyIC (10µg/mL) and adenosine (ADO; 1-10µmol/L) in the absence or presence
315 of inhibitors for A2a (SCH-442416; 1µmol/L) and A2b (MRS-1754; 1µmol/L). Mean + SEM;
316 n=2. Except for indicated single donor results in (**D**), all results are shown as averages of
317 experiments carried out with cells of independent donors.
318
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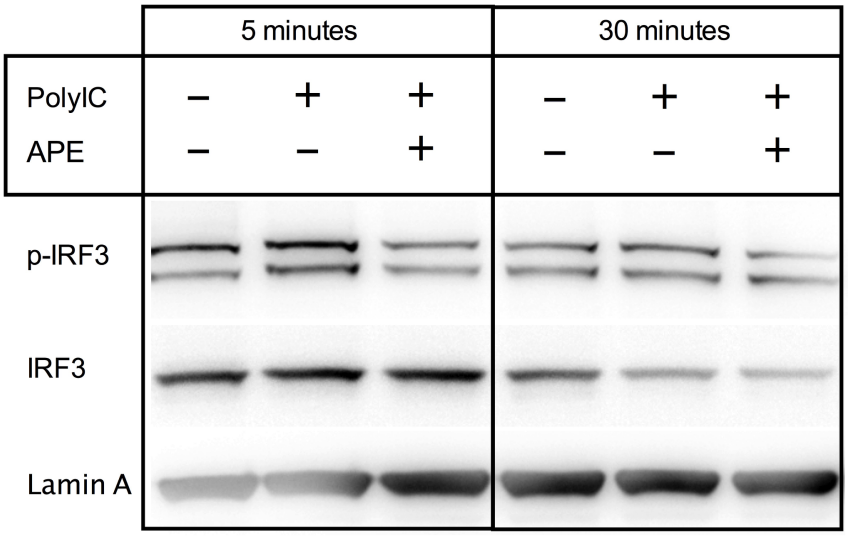

Figure S5: Phosphorylated and total IRF-3 in nuclear extracts of human nasal epithelial cells after co-stimulation of PolyIC and aqueous birch pollen extract.

HNEC submerged monolayer cultures were incubated for 5 and 30 minutes with medium, PolyIC (10µg/mL) or PolyIC plus aqueous birch pollen extract (100µg/mL; APE). Nuclear extracts were subjected to Western blotting and blots were probed with antibodies against phosphorylated total IRF-3. Lamin A served as loading control.

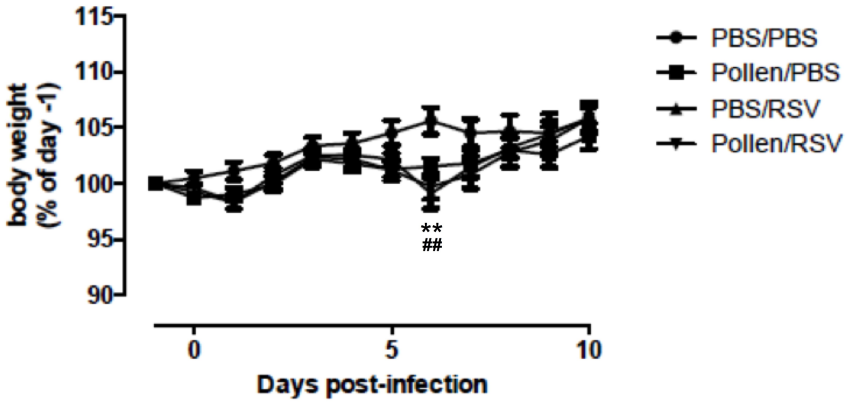

Figure S6: Body weight of animals in the RSV infection experiment. Body weight gain was slowed down in all treatment groups with exception of the PBS/PBS group. The effect was most pronounced at day 6 post infection. **: $p<0.01$ (PBS/RSV vs. PBS/PBS); ##: $p<0.01$ (Pollen/RSV vs. PBS/PBS); One-way ANOVA for repeated measures; n=6 mice per group.

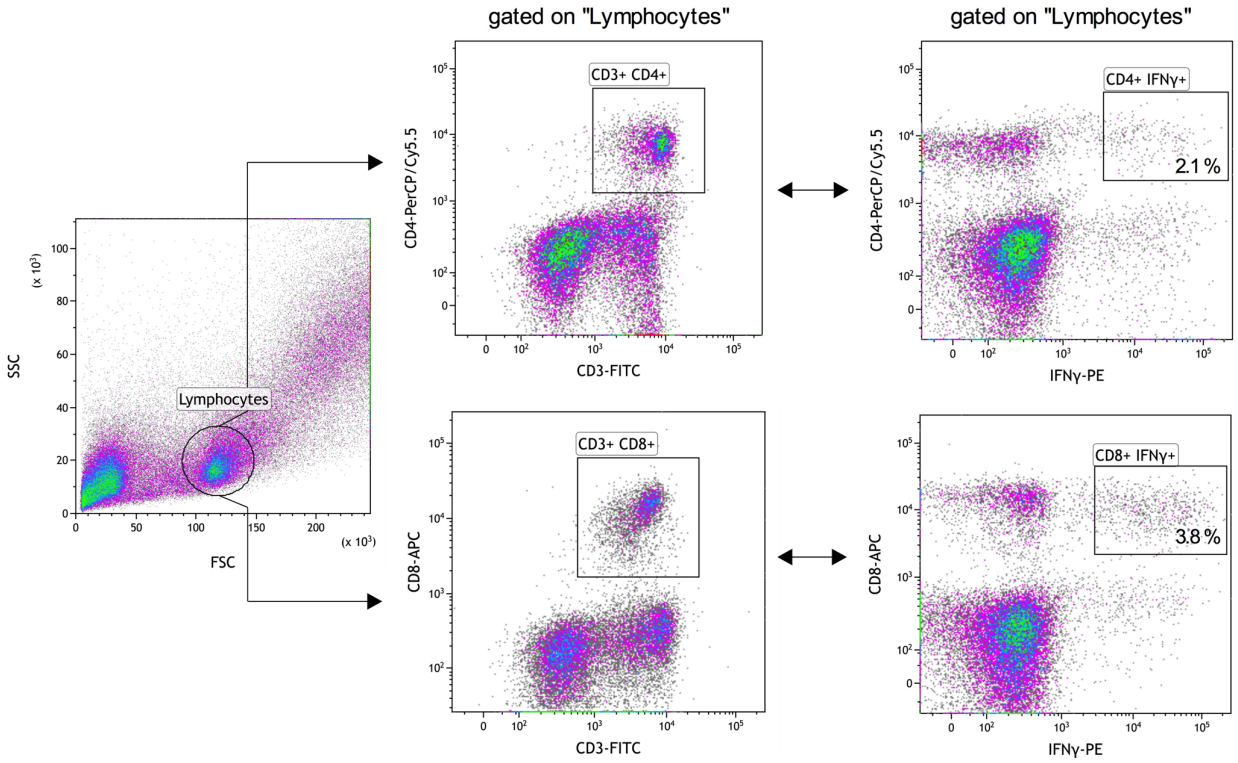

Figure S7: FACS plot depicting the gating strategy for the experiment plotted in fig. 4, H-I. Lung infiltrating cells were re-stimulated with RSV-infected D1 cells. Plotted are IFN-γ^+ $CD4^+$ and $CD8^+$T cells as percentages of all lymphocytes.

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340

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