

Supplementary Material

Defining *in vivo* and *in vitro* models for evaluating the sensitising potential of birch pollen extracts

Erica Pelamatti^{†1}, Swetlana Urban^{†2,3}, Denise Rauer^{2,3}, Benjamin Schnautz⁴, Annika Eggstein^{2,3}, Franziska Kolek^{2,3}, Johanna Grosch⁴, André Stapelfeldt³, Athanasios Damialis⁵, Ayse Sener^{2,3}, Carsten B. Schmidt-Weber^{4,6}, Claudia Traidl-Hoffmann^{1,2,7}, Francesca Alessandrini^{‡4}, Lorenz Aglas^{‡3,8}, Stefanie Gilles^{‡2,3}

- ¹ Department of Biosciences and Medical Biology, Paris Lodron University of Salzburg, Salzburg, Austria
- ² Institute of Environmental Medicine and Integrative Health, Environmental Medicine, Faculty of Medicine, University Hospital Augsburg, Augsburg, Germany
- ³ Institute of Environmental Medicine, Helmholtz Munich, German Research Centre of Environmental Health, Augsburg, Germany
- ⁴ Center of Allergy & Environment (ZAUM), Technical University of Munich (TUM) and Helmholtz Munich, German Research Centre for Environmental Health, Germany
- ⁵ Terrestrial Ecology and Climate Change, Department of Ecology, School of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece
- ⁶ Member of the German Centre of Lung Research (DZL), Munich, Germany
- ⁷ Christine Kühne – Center of Allergy Research and Education (CK-Care), Davos, Switzerland
- ⁸ Human Microbiome (HUMI) Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[†]These authors contributed equally to this work.

[‡]These authors contributed equally to this work.

Corresponding author:

Stefanie Gilles, Institute of Environmental Medicine and Integrative Health, Faculty of Medicine, University Hospital Augsburg, Stenglinstrasse 47, D-86156 Augsburg, Germany; phone: +49 821 400-168403; stefanie.gilles@med.uni-augsburg.de

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

SU: Measurement of pollen compounds, manuscript first draft, plotting, analysis, manuscript editing; EP: Experiments and readouts on 6-week i. n. and time-series mouse i. n. sensitisation model, plotting, analysis; DR: DC stimulation and analysis; BS: FACS analysis of lung cells in 12-day i. n. model; AE, ASe: Pollen extract preparation, measurement of pollen compounds; FK: Pollen sampling; JG: Lung histology and serology in 12-day i. n. model; Ast: Pollen extract preparation, experiments on i. d. model; AD: Supervision of pollen-sampling, statistical advice; CSW, CTH: Infrastructure, funding; FA: Experiments and readouts 12-day i. n. model, study design, supervision, funding; LA: Study design, supervision, funding; SG: Study design, supervision, funding, manuscript editing. All: Critical reviewing of manuscript.

Abbreviations

AUC,	Area under the curve;
BALF,	Broncho-alveolar lavage fluid;
BPE,	Birch pollen extract;
cBPE,	Birch pollen extract derived from commercial pollen;
eGFP,	Enhanced green fluorescent protein;
H&E,	Haematoxylin and eosin staining;
HRP,	Horseradish peroxidase;
i. d. ,	Intradermal;
IL-4R α ,	IL-4 receptor alpha chain;
i. n. ,	Intranasal;
MFI,	Mean fluorescence intensity;
moDC,	Monocyte-derived dendritic cell;
Nitrogen dioxide,	NO ₂ ;
Ozone,	O ₃ ;
PAS,	Periodic acid-Schiff reaction;
RT,	Room temperature;
sBPE,	Self-collected birch pollen extract

Supplementary Methods

Measurement of compounds in sBPE

Concentration of Bet v 1, adenosine, eicosanoid-like PALMs in sBPEs were measured by commercially available enzyme immunoassay according to the supplier's protocols (Bet v 1: Inbio, Cardiff, UK; adenosine: my biosource, San Diego, USA; PALMs prostaglandin E₂ and leukotriene B₄: GE Healthcare, Düsseldorf, Germany. Lipopolysaccharide was quantified with the Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher, Darmstadt, Germany) according to the manufacturer's protocol.

Recombinant Bet v 1

In-house produced recombinant Bet v 1.0101, which will be referred to as Bet v 1 in the following, was expressed in *E. coli* BL21 Star (DE3). For purification, protein precipitation with 200 mM sodium chloride was performed, followed by low-pressure chromatography. The purified protein underwent physicochemical characterisation by mass spectrometry, dynamic light scattering, circular dichroism and Fourier transform infrared spectroscopy, as previously described ¹. Lyophilised Bet v 1 was stored at -20°C.

ELISA for Bet v 1-specific immunoglobulin detection

Serum levels of Bet v 1-specific immunoglobulins were measured with an in-house established indirect IgG₁ and IgG_{2a} ELISA protocol as previously described ². In brief, high-binding capacity 96-well plates were coated overnight at 4°C with 2 µg/ml Bet v 1. A titration of sera was performed in blocking buffer, starting with 1:50 or 1:200 for IgG₁ and 1:50 for IgG_{2a}. Blocking buffer was used as blank for background subtraction. HRP-conjugated rat anti- mouse IgG₁ (clone: SB77e, product number: 1144-05, Southern Biotech, USA) or IgG_{2a} (clone: SB84a, product number: 1155-05 Southern Biotech, USA), both diluted 1:6000, were added and incubated 2 hours RT. Plates were developed with TMB substrate solution (KPL, Europe), the reaction was stopped with 1 N HCl, and the absorbance measured at 450 nm. For data analysis, the area-under-the-curve (AUC) was calculated per mouse on log-transformed absorbance data and serum dilutions. Additionally, the level of immunoglobulins was quantified by determining the endpoint titre and using the limit of quantification (LOQ), defined as the mean of the blanks plus ten times the standard deviation of the blanks, as cut-off. In case absorbance values were below the LOQ (e.g. PBS group), the endpoint titre was adjusted to 1.

Collection of cells from bronchoalveolar lavage fluid (BALF) and cytokine profiling

To obtain BALF, a cannula (BD Insite™, Peripheral IV cannula, BD Vialon) was inserted into the trachea of euthanised mice, two washes (500 µl for the first and 1 ml for the second lavage) of the lungs were performed with PBS, and the fluid was collected with a syringe. Both washes were centrifuged (5 min, 250 x g). The supernatant from the first wash (150 µl per mouse) was frozen at -80°C for cytokine analysis. The pellets from the

first and second washes were collected on cytospins for differential cell counts ²⁷ or pooled in FACS buffer for subsequent FACS analysis. Cytokine screening was performed on 150 µl BAL supernatant by LEGENDplex™ (MU Th Cytokine Panel 12-plex, Cat. No: 741028, BioLegend, San Diego, USA) according to the manufacturer's protocol. Results were analysed with the BioLegend software, and either MFI or raw concentration values were used. The latter were log-transformed, and zero values were adjusted to 0.01 pg/ml.

Lymph node collection

Inguinal (i. d. model) and mediastinal lymph nodes (i. n. model) were collected in PBS and homogenised to obtain single-cell suspensions. Cells were directly stained and measured using a CytoFlex S (Beckman Coulter, Indianapolis, IN, USA) to gain a better understanding of the cells' composition. 50.000 events were recorded for every sample.

Flow cytometry of BALF, lymph node cells and lung infiltrating cells

BALF cells and single cell suspensions prepared from lymph nodes were processed separately. Cells were pre-incubated with FC-block TruStain FcX (anti-mouse CD16/32, BioLegend, San Diego, USA) for 5 minutes, washed, and stained for 30 minutes with an appropriate antibody mix (**Supplementary Table 1**). After staining, cells were washed twice, resuspended in FACS buffer (PBS, 0.5% BSA, 2 mM EDTA, 1% FCS) and acquired with a CytoFlex S flow cytometer (Beckman Coulter, Indianapolis, IN, USA). Median fluorescence intensity (MFI) values were normalised to the average of the PBS samples. Gating strategies for BALF and lymph nodes are shown in **Supplementary Fig. 1**.

For lung-infiltrating cells, single-cell suspensions were generated from mouse lungs and dissociated using a lung dissociation kit (kit number 130-065-927; Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspensions were first stained with surface markers (**Supplementary Table 1**) and viability dye. Subsequently, staining for transcription factors Foxp3 and GATA3 was performed using Foxp3 Staining Buffer Set (eBioscience, Darmstadt, Germany) according to the manufacturer's protocol. Samples were measured using a BD FACSCanto II flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA), and data were analysed with FlowJo graphics application V9.7.2 (TreeStar Inc., Ashland, OR, USA). The gating strategy for identifying different cell populations is presented in **Supplementary Fig. 2**.

RBL mediator release assay

To indirectly measure the BPE-specific IgE in the sera, the mediator release from RBL-2H3 (ATCC number CRL-2256) cells was measured as previously described ². The cells were passively sensitised with serum from the different mice in a 1:20 dilution. On the following day, the cells were washed and stimulated with a BPE titration series ranging from 10 µg/ml to 0.1 ng/ml for BPE and incubated for 1 hour. The supernatants were

collected and incubated with the substrate 4-methyl umbelliferyl-N-acetyl- β -D-glucosaminide (Sigma, Darmstadt, Germany) for one hour. The reaction was then stopped using a glycine buffer (15 g/l glycine, 11.7 g/l NaCl, pH 10.7) and the fluorescence intensity was measured at an excitation wavelength of 360 nm and emission wavelength of 440 nm. For the analysis, the background signal was provided by unstimulated cells and subtracted from the other values. The obtained values were normalised to the maximum (100%) release from lysed cells treated with 10% Triton X-100. The AUC was calculated per serum sample from log₁₀-transformed concentration values.

Histology

Following BALF collection, the lungs were excised, and the left lobe was fixed in 4% buffered formalin and embedded in paraffin. Sections (3-4 μ m) were stained with H&E and PAS. Mucus hypersecretion and inflammatory cell infiltration were graded in a blinded fashion on a scale from 0 to 4 (0 = none, 1 = mild, 2 = moderate, 3 = marked, 4 = severe), reflecting the degree of the pathological alteration ³.

Human monocyte-derived dendritic cell culture and stimulation

Dendritic cells (DCs) were generated by culture from MACS[®]-sorted (CD14 MicroBeads[®], Miltenyi Biotech, Bergisch Gladbach, Germany) monocytes with rh GM-CSF (500 U/ml; Miltenyi Biotech) and IL-4 (50 U/ml; PeproTech) ⁴. After 5 days of culture, DCs were harvested and analyzed on a CytoFlex LX flow cytometer (Beckman Coulter, Indianapolis, IN, USA) for viability (PromoFluor-840, PromoKine), purity (CD14, FITC; CD1a PE-Cy7) and maturation status (CD80, APC-H7; CD86, Pacific-Orange; CD40, PE; CD83, APC, HLA-DR, PE-CF594). Cells were 94 $\pm$ 3% differentiated (CD14⁻, CD1a⁺), 98 $\pm$ 1% viable (PromoFluor-840^{neg}) and showed an immature DC phenotype (CD80^{low}, CD86^{low}, CD83^{low}, CD40^{int}, HLA-DR⁺; **Supplementary Fig. 3**).

To assess the DC's cytokine response, the cells were seeded into 96-well flat-bottom plates at 100,000 cells per well (final volume 200 μ l) in Hu-DC medium (RPMI-1650, 10% low endotoxin FCS, 2 mM L-glutamine, 20 μ g/ml gentamycin, 50 μ M 2-mercaptoethanol) and stimulated with 10, 30 and 100 μ g/ml BPE (concentrations referring to the total protein concentration of the original extract). Medium-treated cells served as unstimulated controls. After 24h, supernatants were harvested and frozen at -70 °C for further use. Supernatants were analyzed by ELISA for the secretion of IL-10 (BD OptEIA, Becton Dickinson, Heidelberg, Germany). For analysis cytokine secretion of unstimulated cells were set as 100%.

Supplementary Figures and Tables

Antigen	Fluorochrome	Supplier
cBPE: BALF		
CD45	APC-eFluor 780	Invitrogen
Gr1	APC	BioLegend
Siglec-f/CD170	PE	BioLegend
CD4	Brilliant Violet (BV) 421	BioLegend
IL-4R/CD124	PE/dazzle	BioLegend
CCR3/CD193	PE/Cy7	BioLegend
CD3	PerCP/Cy5.5	BioLegend
CD19	BV605	BioLegend
cBPE: lymph nodes		
CD4	BV421	BioLegend
IL-4R/CD124	PE/dazzle	BioLegend
CCR3/CD193	PE	BioLegend
CD19	PE/Cy7	BioLegend
ST2	BV605	BioLegend
sBPE: skin draining lymph nodes		
CD4	APC	BioLegend
sBPE lung infiltrating cells: T Cell Panel		
I/d	Zombie Aqua	BioLegend
CD45	APCe780	eBioscience
CD3	FITC	Becton Dickinson
CD4	a700	Becton Dickinson
Foxp3	PerCP Cy5.5	eBioscience
GATA3	e660 (=APC)	eBioscience
sBPE lung infiltrating cells: DC Panel		
CD45	PerCP Cy5.5	BioLegend
CD11c	PE Cy7	BioLegend
MHC II	BV711	Becton Dickinson
CD11b	APC	eBioscience

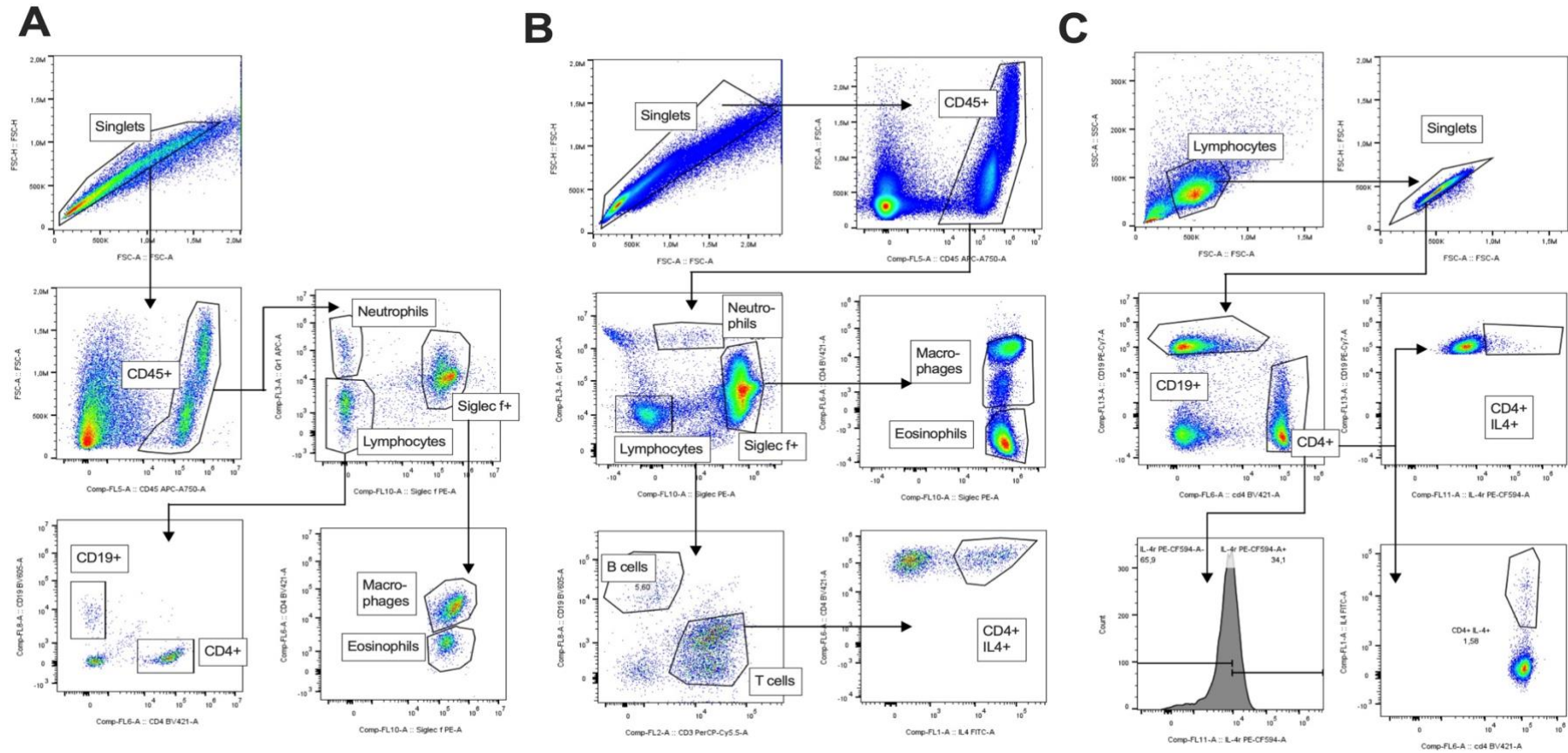
Supplementary Table 1. Antibodies used for FACS analysis of BALF, lymph nodes and lungs.

Donor ID	Total Serum IgE (KU/l)	e5 Dog	m2 <i>Cladosporium herbarum</i>	w9 Ribwort	w6 Mugwort	t15 Ash	t4 Hazel	t3 Birch	t2 Alder	m7 <i>Botrytis cinerea</i>	d1 <i>Dermatoph. pteronyssinus</i>	m3 <i>Aspergillus fumigatus</i>	m1 <i>Penicillium chrysogenum</i>	g6 Timothy	e1 Cat	m6 <i>Alternaria tenuis</i>
1	20	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2	52	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3	18	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
4	5.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
5	38	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
6	22	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

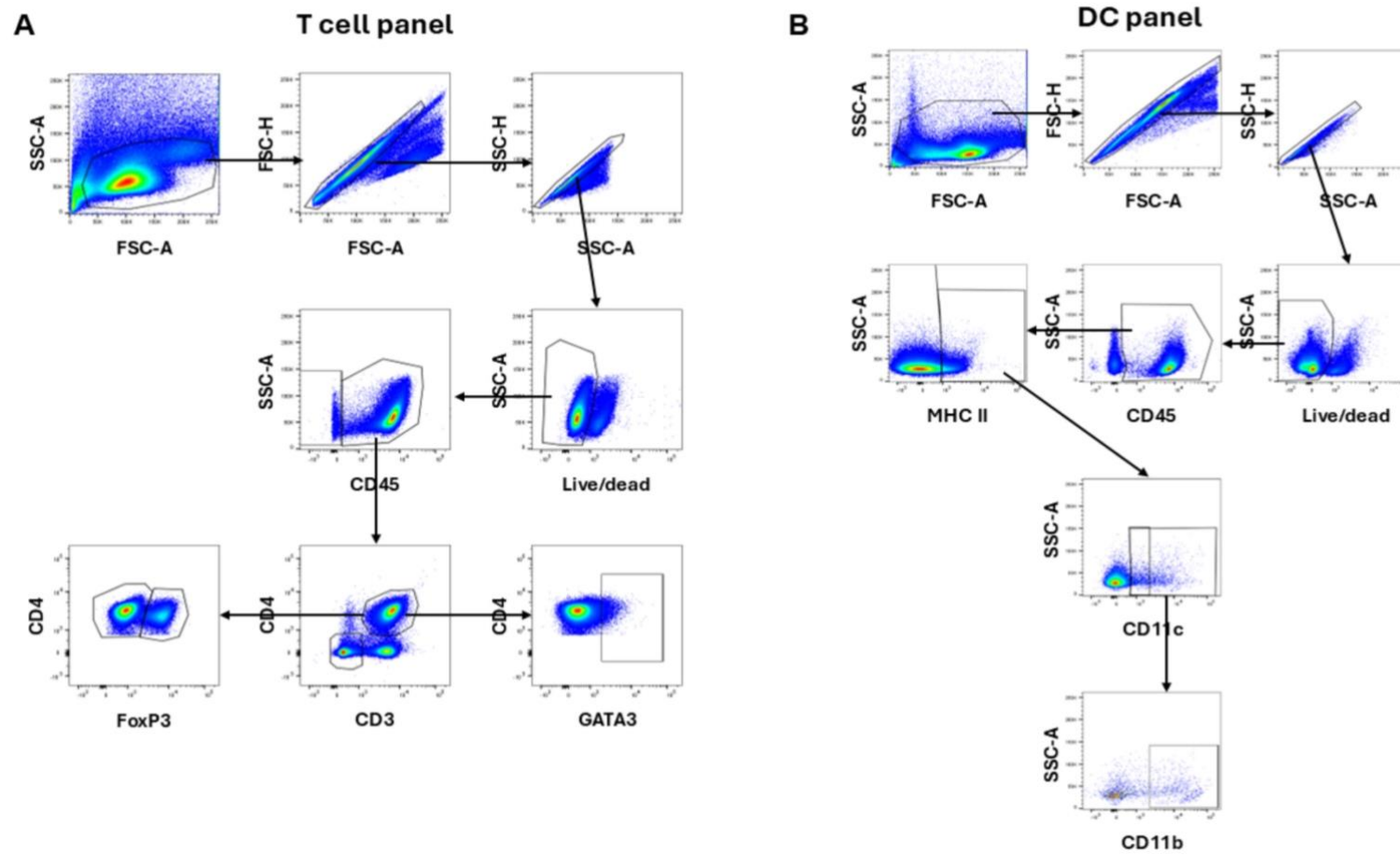
Supplementary Table 2. IgE profiles of cell donors. IgE against an aeroallergen panel was measured in serum by ImmunoCAP (ThermoFisher/Phadia).

			absolute count				percentage total		percentage parent		percentage grandparent	
Group	Mouse	Recorded events	Lymphocytes	single cells	CD4+	CD4+ IL-4+	CD4+	CD4+ IL-4+	CD4+	IL-4+	CD4+	IL-4+
sBPE #2	M1	50000	34501	29926	9038	550	18,1	1,1	30,2	6,09	26,2	1,84
sBPE #2	M2	50000	35692	28142	10864	967	21,7	1,93	38,6	8,9	30,4	3,44
sBPE #2	M3	50000	34540	28412	11041	1274	22,1	2,55	38,9	11,5	32	4,48
sBPE #2	M4	50000	34828	30826	10518	1270	21,0	2,54	34,1	12,1	30,2	4,12
sBPE #2	M5	50000	35587	28369	7552	1157	15,1	2,31	26,6	15,3	21,2	4,08
sBPE #1	M1	50000	34433	27414	9327	1011	18,7	2,02	34	10,8	27,1	3,69
sBPE #1	M2	50000	35003	28321	8246	1081	16,5	2,16	29,1	13,1	23,6	3,82
sBPE #1	M3	50000	26691	24043	11148	371	22,3	0,742	46,4	3,33	41,8	1,54
sBPE #1	M4	50000	33054	27303	12061	885	24,1	1,77	44,2	7,34	36,5	3,24
sBPE #1	M5	50000	31210	28670	10561	961	21,1	1,92	36,8	9,1	33,8	3,35
PBS	M1	50000	30161	28576	14981	89	30	0,178	52,4	0,594	49,7	0,311
PBS	M2	50000	33455	31781	15836	93	31,7	0,186	49,8	0,587	47,3	0,293

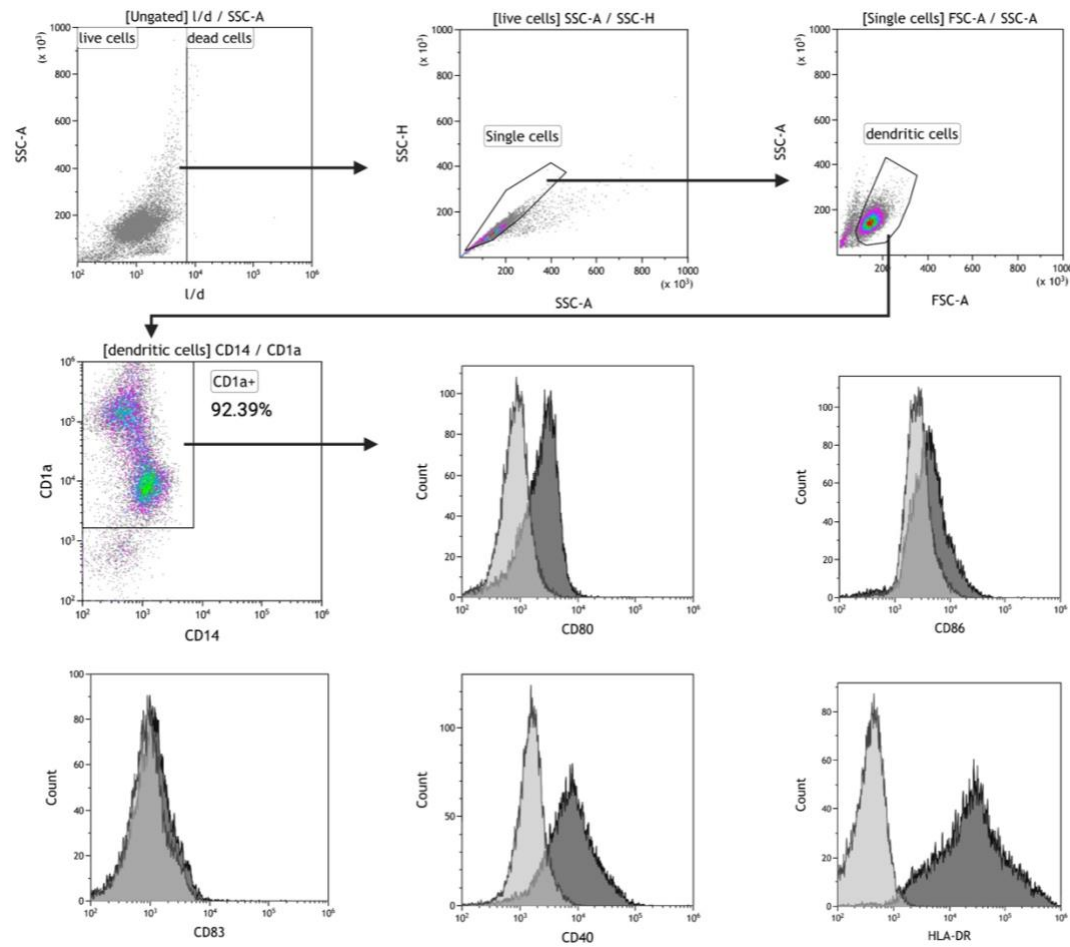
Supplementary Table 3. Absolute counts of CD4+ and CD4+IL4+ cells from draining lymph nodes in the i.d. model.



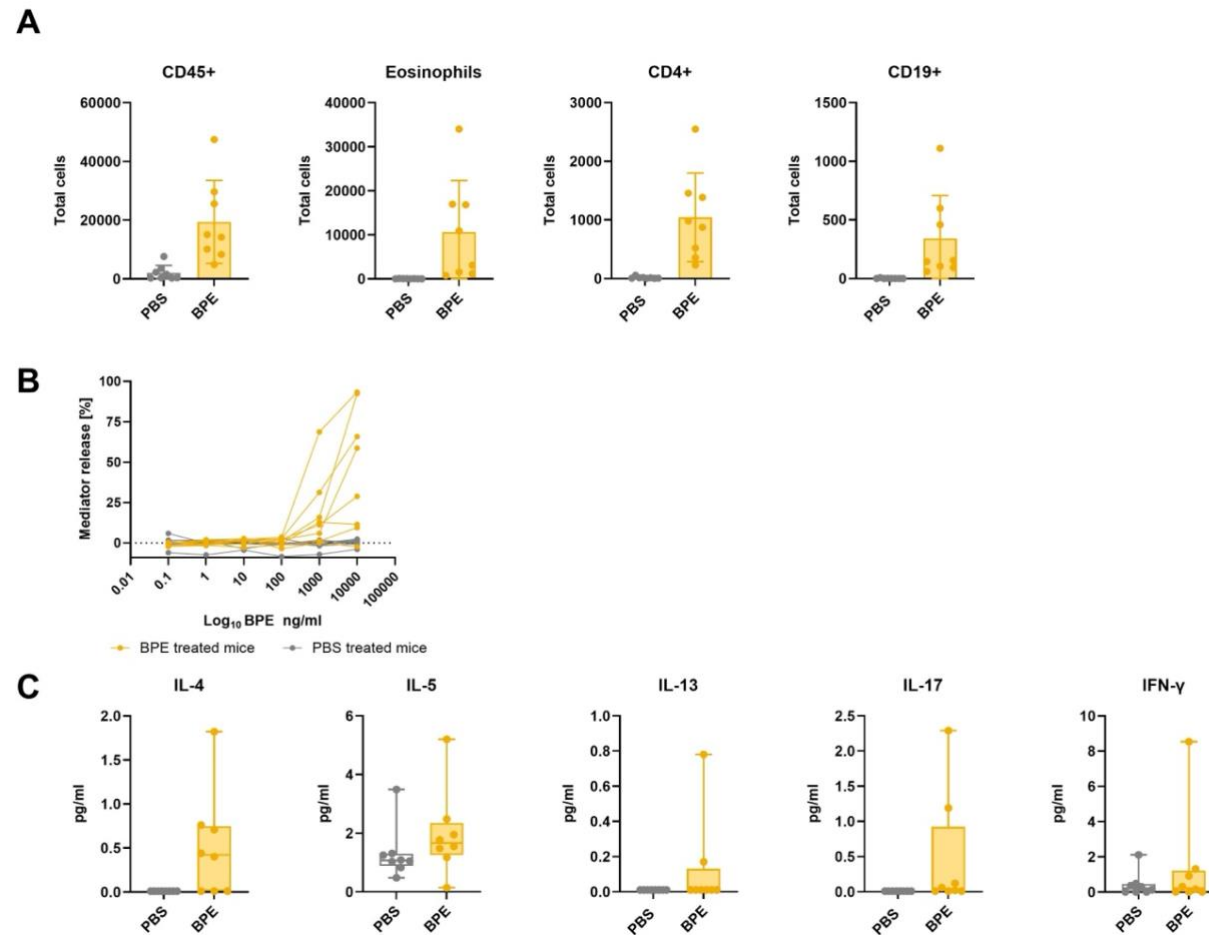
Supplementary Fig. 1: Flow cytometry gating strategy of murine cells. A: BALF of the 6-weeks intranasal model. **B:** BALF in the intranasal time series experiment. **C:** mediastinal lymph nodes in the 5-days intradermal model.



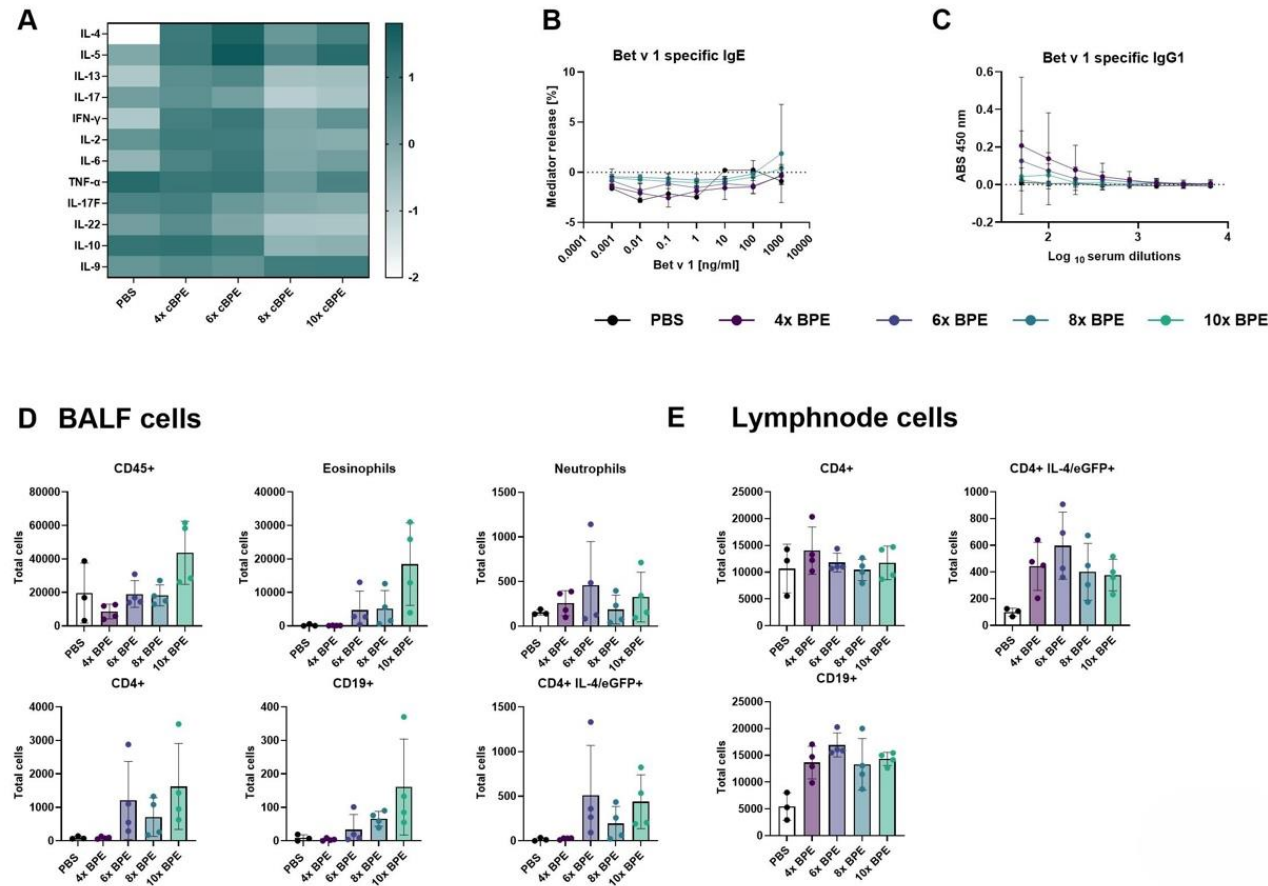
Supplementary Fig. 2: Flow cytometry gating strategy of murine cells. A: Gating of lung infiltrating T cell panel. **B:** Gating of lung infiltrating DC panel.



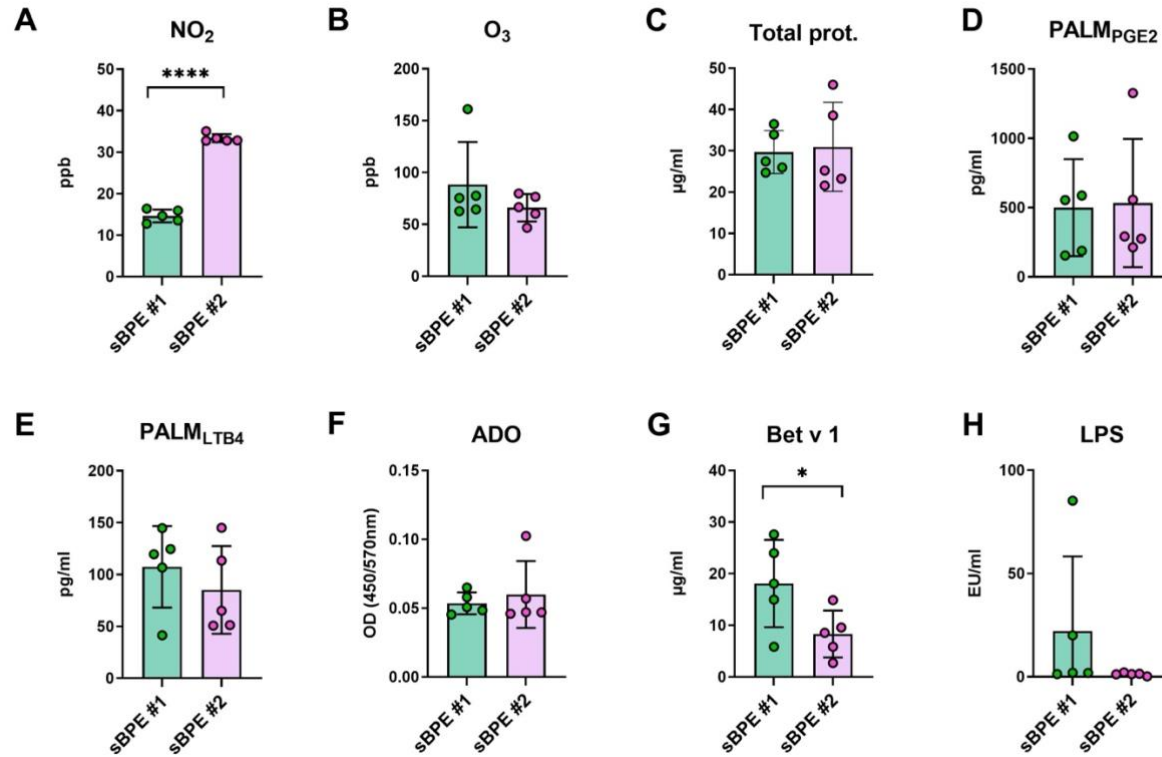
Supplementary Fig. 3: Phenotype of human moDC by FACS on day 5.



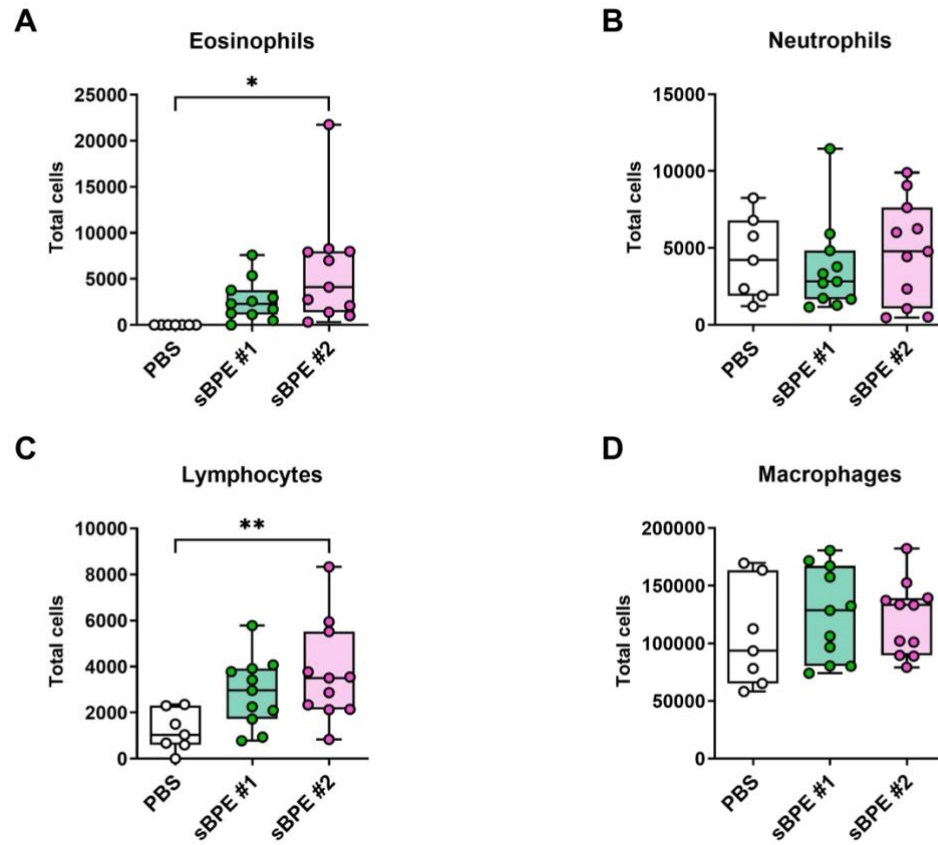
Supplementary Fig. 4: Adjuvant-free intranasal sensitisation with BPE induces allergic responses in mice. A: BAL cell composition, showing the absolute numbers of leukocytes (CD45+), and eosinophils, T helper and B cells. **B:** Mediator curves for AUC calculation of the long i.n. model. **C:** Cytokine concentrations in pg/ml measured in BAL supernatant.



Supplementary Fig. 5: Monitoring early events and Th2 polarization upon birch pollen exposure. **A:** Cytokines screened in the BAL supernatants of mice exposed to BPE. Heat-map with the Log10-transformed pg/ml values for all the cytokines screened. The median of each group is represented. **B:** Mediator release curves from RBL cells sensitised with individual sera and stimulated with a titration of Bet v 1. **C:** Bet v 1-sIgG1 measured via ELISA in each individual serum. Each curve represents a group, and the error bars show the SD. **D:** Absolute numbers of BAL cell composition, showing the cell counts of leukocytes (CD45⁺), eosinophils, neutrophils, T helper, B cells and IL-4/eGFP⁺ cells. **E:** Absolute numbers of CD4⁺ T helper, CD19⁺ B cells and IL-4/eGFP⁺ CD4⁺ cells are shown.



Supplementary Fig. 6: Characterization of BPEs prepared from self-collected pollen of two different trees. Two trees in the region of Augsburg were sampled each year from 2015 to 2018. **A:** NO₂ and **B:** O₃ were measured by passive sampling at the tree. From each sample, 10 mg pollen were extracted in 1 ml PBS and the concentration of **C:** total protein, **D:** PALM-PGE₂, **E:** PALM-LTB₄, **F:** adenosine, **G:** Bet v 1, and **H:** LPS was quantified. *: $p < 0.05$; ****: $p < 0.0001$; Wilcoxon test.



Supplementary Fig. 7: Intranasal birch pollen sensitisation model with self-collected birch pollen extracts. A-D: absolute numbers of BALF infiltrating immune cells: A: Eosinophils; B: neutrophils; C: lymphocytes; D: alveolar macrophages. *: $p < 0.05$; **: $p < 0.01$, Kruskal Wallis test.

References

1. Beck I, Jochner S, Gilles S, McIntyre M, Buters JT, Schmidt-Weber C, et al. High environmental ozone levels lead to enhanced allergenicity of birch pollen. *PLoS One*. 2013;8(11):e80147.
2. Kraiem A, Pelamatti E, Grosse-Kathoefer S, Demir H, Vollmann U, Ehgartner C, et al. Reducing the solubility of the major birch pollen allergen Bet v 1 by particle-loading mitigates Th2 responses. *Allergol Int*. 2025;74(1):126-35.
3. Alessandrini F, Schulz H, Takenaka S, Lentner B, Karg E, Behrendt H, et al. Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung. *J Allergy Clin Immunol*. 2006;117(4):824-30.
4. Gilles S, Fekete A, Zhang X, Beck I, Blume C, Ring J, et al. Pollen metabolome analysis reveals adenosine as a major regulator of dendritic cell-primed T(H) cell responses. *J Allergy Clin Immunol*. 2011;127(2):454-61 e1-9.