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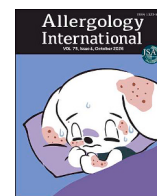
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Original Article

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

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Abbreviations:

AUC, Area under the curve; BALF, Bronchoalveolar 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-

ABSTRACT

Background: Various mouse models are used to study pollen allergies, but a systematic experimental comparison is lacking. We aimed to establish a physiologically relevant adjuvant-free birch pollen allergy model to understand the dynamics of the allergic immune response and to compare the sensitising potential of different self-collected birch pollen extracts in different distinct *in vivo* and *in vitro* routes and models.

Methods: Different intranasal (i. n.) models in BALB/c mice and IL-4 reporter mice (BALB/c:4Get), an intradermal (i. d.) model (BALB/c:4Get), and human dendritic cells (moDCs) were used to investigate the sensitising and inflammatory effects of birch pollen extracts (BPE). A timeseries experiment was performed in the i. n. model to determine the onset of Th2 responses. Bronchioalveolar lavage fluid (BALF), lungs, draining lymph nodes, and serum were analysed for cell infiltrate, cytokines, and antibodies.

Results: Repeated i. n. instillations of adjuvant-free BPE prepared from commercial pollen resulted in BALF Th2 cells, eosinophilic lung inflammation, and specific serum immunoglobulins in BALB/c mice. After 6 i. n. instillations, eosinophils and CD4⁺ T cells peaked in BALF, and CD4⁺IL-4⁺ and CD4⁺IL4R α ⁺ cells peaked in mediastinal lymph nodes. Both, i. n. and i. d. models detected subtle differences in the sensitising potential of BPEs from two self-collected pollen samples. MoDCs showed higher IL-10 release towards the less inflammatory extract.

Conclusions: Adjuvant-free murine sensitisation models, including intranasal and intradermal routes, as well as a human DC model, covered different aspects of the sensitisation route and temporal resolution. The models may be broadly helpful in screening approaches in studying mechanisms of pollen allergy.

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derived dendritic cell; Nitrogen dioxide, NO₂; Ozone, O₃; PAS, Periodic acid-Schiff reaction; RT, Room temperature; sBPE, Self-collected birch pollen extract

Introduction

Birch pollen (*Betula* spp.) is a major cause of respiratory allergy and the most dominant allergenic tree pollen in central and northern Europe. The prevalence of birch pollen sensitisation, depending on the region, ranges between 8% and 54%.¹ Over 95% of birch pollen allergic patients are sensitised to a 17 kDa protein, the major birch allergen Bet v 1.²

A key step in the initiation of allergic sensitisation is the induction of IL-4 in activated CD4⁺ T cells, which is required for allergen-specific B cells to undergo isotype switching to produce IgE. The process of allergic sensitisation depends on host genetic susceptibility as well as on environmental triggers, such as repeated exposure to the allergen via routes that favour type 2 responses (skin, airways). Other cell types involved in allergic immune responses are dendritic cells, professional antigen-presenting cells priming naïve T cells,³ and eosinophils. The latter are recruited through Th2-related cytokines such as IL-5 and IL-13 and are involved in allergic late-phase reactions.⁴ In addition, sensitising co-factors, present either within the allergen structure itself or co-released from the allergen source, are required to activate pattern recognition receptors and initiate an innate response. For birch pollen, various receptors have been identified that are involved in facilitating allergic sensitisation to the otherwise weakly immunogenic Bet v 1, such as TLR-4⁵ or IL4Rα.⁶

As allergic sensitisation is challenging to study in humans, various models in guinea pigs, rats, mice and with human cells are applied to understand the development and pathophysiology of allergies. Today, the induction of an adaptive and humoral immune response can only be investigated *in vivo*, as the mechanisms are too complex to be reproduced in cell culture systems. Historically, mouse models required adjuvants like Alum to induce Th2 responses and IgE production, and immunisation often occurred via non-physiological routes, such as intraperitoneal injection. Intranasal (i. n.) instillations of pollen extracts to induce pollen sensitisation have already been applied for decades, but many models still relied on extrinsic adjuvants.⁷ Intranasal instillation mimics natural pollen exposure via the airways and results in sensitisation and type-2 airway inflammation.^{7–9} Intranasal pollen allergy models have been described for ragweed,¹⁰ timothy grass,¹¹ birch,¹² Japanese cypress,¹³ and Japanese cedar¹⁴ pollen. Adjuvant-free pollen allergy models are less well established, as they typically require multiple repetitive intranasal instillations, but they are important to investigate the auto-adjuvant activity of pollen. Adjuvant-free i. n. allergy models have been described for ragweed^{10,15} and timothy grass pollen.¹⁶ For birch pollen allergy, comparable models are lacking. Only one model with eleven i. n. instillations with commercial birch pollen extract for sensitisation followed by three i. n. booster challenges has been described, where IgE production depended on NLRP3 inflammasome activation downstream and independent of Th2 induction.¹² Studies on the temporal resolution of the sensitisation to birch pollen in an adjuvant-free context are completely lacking.

Most murine allergy models rely on C57BL/6 and BALB/c strains,^{8,17} but BALB/c models are especially common as they readily develop Th2- and IgE-dominated immune responses.⁷ The genetics underlying the increased allergy-susceptibility of BALB/c

mice are poorly defined, but several pathways appear to be involved, among them a fragile skin barrier,¹⁸ hyperresponsiveness of mast cells,¹⁹ altered TLR4 signalling,²⁰ and an impaired Th1 axis.²¹ To study Th2 responses directly without *ex vivo* restimulation, IL-4 reporter mice (4Get) were designed in the BALB/c background. In these mice the IL-4 gene and a gene for the green-fluorescence protein (eGFP) are placed under the control of the same promoter, resulting in bicistronic transcripts.²² Thus, IL-4 expressing cells can readily be identified *in vivo*. These mice are primarily used to study parasitic infections²³ and have never been used for systematic evaluation of sensitisation to pollen before. In our previous study, we successfully used 4Get mice to show increases in Th2 cells (IL-4/eGFP⁺) after stimulation with a high molecular weight fraction of birch pollen extract.⁶

To study mechanisms in allergic immune responses in humans, researchers rely on cells from both human, non-allergic individuals and those with allergies. Monocyte-derived DCs (moDCs) have been successfully used to study mechanisms of the allergic sensitisation phase *in vitro*.^{24–26} As previously shown, the level of IL-10 secretion of moDCs *in vitro* reflected the degree of type-2 inflammation towards different pollen extracts *in vivo* in an adjuvant-free mouse model of ragweed allergy.¹⁰

In this study, we established an adjuvant-free i. n. birch pollen exposure model to investigate, in a near-physiological setting, allergic immune reactions to birch pollen *in vivo*. Additionally, we aimed to understand the dynamics of key events in the allergic immune response by tracing Th2 polarisation, type 2 inflammation, and humoral responses over time. Finally, we compared responses to two natural birch pollen extracts in two mouse models with different routes of sensitisation and in a human monocyte-derived dendritic cell (moDC) model. The different models provided complementary insights, capturing distinct aspects of the allergic sensitisation and inflammation cascade.

Methods

Sampling of birch pollen

Catkins were collected from birch trees in the region of Augsburg, Germany. Two representative, healthy, reproductively mature trees were sampled over four successive years (2015–2018), with pollen pooled from 100 catkins per tree and year. The sampling took place during the birch flowering season in spring. Catkins were air-dried, and pollen was extracted by sieving as described before.²⁷ Passive sampling of ozone (O₃) and nitrogen dioxide (NO₂) was performed at the trees for a period of 30 days prior to pollination as described before.²⁷

Preparation of the aqueous birch pollen extract (BPE)

European *Betula pendula* pollen from a commercial source (Allergon; batch number: 0000 460464 and 0125 18701) were used to generate an aqueous birch pollen extract (cBPE) with an initial concentration of 180 mg pollen per ml PBS. The suspension was shaken at room temperature (RT) for 1 h, followed by a centrifugation step (13,000×g, 4 °C). The supernatant was collected and centrifuged again. The obtained extract was filtered

with a 0.45 µm pore-size filter (GE Healthcare Bioscience) and stored at –20 °C. Bradford assay (Pierce Coomassie Protein assay, Thermo scientific, Waltham, MA, USA) with bovine serum album (BSA) as standard was used to quantify total protein content. The same extraction protocol was used for aqueous birch pollen extracts made from self-collected birch pollen (sBPE). Due to the limited availability of self-collected pollen, we prepared sBPE from 40 mg of pollen per ml PBS for the i. n. administration. The total protein concentration was determined using the Bradford assay.

Mouse strains

All mice used in this study were of the BALB/c background. IL-4 reporter mice (4Get, JAX stock #004190, The Jackson Laboratory, USA.)²⁸ and wild-type BALB/c mice (Janvier, LeGenest-Saint-Isle, France and Charles River, Sulzfeld, Germany) were maintained in the animal facility of the University of Salzburg or Helmholtz Munich under specific pathogen-free (SPF) conditions. Male and female mice were used in equal numbers for 4Get mice; for wild-type BALB/c mice, only female animals were used. Mouse experiments were conducted according to the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local ethics committee and government authorities (approval numbers: 55.2-1-54-2532-156-12, ROB-55.2-2532.Vet_02-18-94 and BMWF – V/3b; approval number 2021–0.118.574).

Sensitisation protocols

For intranasal (i. n.) sensitisation protocols, 7- to 20-week-old BALB/c mice were used. As established in previous studies,^{10,29,30} mice were lightly anaesthetised and then received daily instillations with BPE or PBS.

For the 6-week i. n. model using cBPE, mice (n = 8) were administered a total of 14 consecutive i. n. instillations and four booster i. n. instillations of 25 µl per nostril (50 µl in total). The total protein amount administered per instillation (BPE) was 100 µg. One week after the last instillation, the mice were euthanised and sera and BAL fluid (BALF) were collected.

For the time-series experiment using cBPE, IL-4 reporter mice were used and received either 4, 6, 8 or 10 daily i. n. instillations of cBPE (100 µg/instillation, n = 4 per group), or PBS (n = 3). Twenty-four hours after the last instillation, mice were euthanised, and sera, BALF and mediastinal lymph nodes were collected. To obtain sera, whole blood was collected in Microtainer® Tubes with Microgard® Closure (BD Biosciences, San Jose, CA, USA), incubated for 30 min at RT and centrifuged (5 min, 14,000 g). The serum was collected, aliquoted and stored at –20 °C until analysis.

For the 12-day i. n. model to compare sBPEs, female BALB/c mice (n = 10 per group, two independent experiments) received 11 i. n. instillations with sBPE #1, sBPE #2, or PBS (15 µl per nostril; 30 µl in total; sBPE: 1 µg). On day 12, mice were sacrificed and sera, BALF and lungs were collected.

For the 5-day intradermal (i. d.) model, IL-4 reporter mice in BALB/c background were immunized with the two different sBPEs (each n = 5) without added adjuvants, or with PBS as control (n = 2). For immunisation, 65 µg of total protein from BPE was injected once into the lower belly region, near the inguinal lymph nodes. The inguinal lymph nodes of immunized and naïve mice were collected on day 5, single cell suspensions prepared in PBS and lymphocytes stained with an APC-conjugated anti-mouse CD4 antibody and analysed for IL-4/eGFP expression by flow cytometry (BD Biosciences, San Jose, CA, USA).³¹

Human cell donors

Isolation, culture, and stimulation of human cells for this study were approved by the Ethics Committee of the Faculty of Medicine at the Technical University of Munich (54/17 S). Blood samples were obtained from healthy, non-atopic volunteers after written informed consent. Donor sensitisation status was determined by serology (ImmunoCAP; ThermoFisher, Massachusetts, USA), which measured total and specific IgE antibodies against a panel of common aeroallergens (Supplementary Table 2). Non-atopic donors were defined by negative clinical history, total IgE <100 kU/L and negative (≤ 0.1 kU/L) IgE against all tested aeroallergens.

See Supplementary Material for details on production of: recombinant Bet v 1 production; Bet v 1-specific immunoglobulin ELISAs; collection of cells from BALF and lymph nodes; cytokine profiling; flow cytometry of cells in BALF, lymph nodes and lung tissue; RBL assay; histology; quantification of compounds in sBPE; and human moDC stimulation.

Statistical analysis

Statistical analysis and graph design were performed using GraphPad Prism 10.3. *In vivo* and *in vitro* data are depicted as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum, or as bar charts, indicating mean $\pm$ SD.

To test for statistical differences between groups, a normality test (Shapiro–Wilk) was performed, followed by parametric or non-parametric tests, as appropriate. When comparing two groups, t-tests or their non-parametric counterparts (unpaired: Mann–Whitney; paired: Wilcoxon) were used. When more than two groups were compared, one-way ANOVA (for time-series experiment) or two-way ANOVA (for serum immunoglobulins) with Tukey's post-hoc test was performed. *In vitro* data were analysed by one-way ANOVA (paired) or Mixed Model ANOVA (paired) with post-hoc pair-wise comparisons (Kruskal–Wallis test). Differences between groups were considered significant at $p < 0.05$.

Results

Establishment of an in vivo, adjuvant-free, intranasal birch pollen exposure model to study allergic sensitisation

To gain a better understanding of the immunological events during birch pollen allergic responses, an intranasal model of exposure was established using cBPE without any extrinsic adjuvant. Female wild-type BALB/c mice were i. n. instilled 14x with cBPE or PBS (control) daily, followed by four additional booster challenges in weeks four and five. The readout was on day 56 (Fig. 1A).

Comparing the BALF composition between the cBPE and the PBS group (Fig. 1B, absolute values Supplementary Fig. 4A), an increase in the percentages (mean $\pm$ SD) of CD4⁺ T cells (5.7 ± 2.6 % cBPE-treated vs. 1.2 ± 0.6 % PBS control; $p < 0.001$), eosinophils (42.4 ± 27.0 % cBPE-treated vs. 2.6 ± 1.1 % PBS control; $p < 0.001$) and CD19⁺ B cells (mean of the group: 1.5 ± 0.6 % in the BPE-treated and 0.2 ± 0.1 % in the PBS control) emerged.

To assess whether allergic sensitisation was induced by our protocol, the humoral response was screened in sera (Fig. 1C–E). The presence of functional and specific IgE was analysed using a mediator's release assay, and the AUC was calculated for each curve. BPE-specific IgE was detected in the sera of mice exposed to BPE with a 2.7-fold increase compared to the PBS, where no IgE was detected (Fig. 1C; mean of the groups: 76.6 AUC for BPE-treated mice and 28.2 AUC for PBS control; dashed line at 41.4

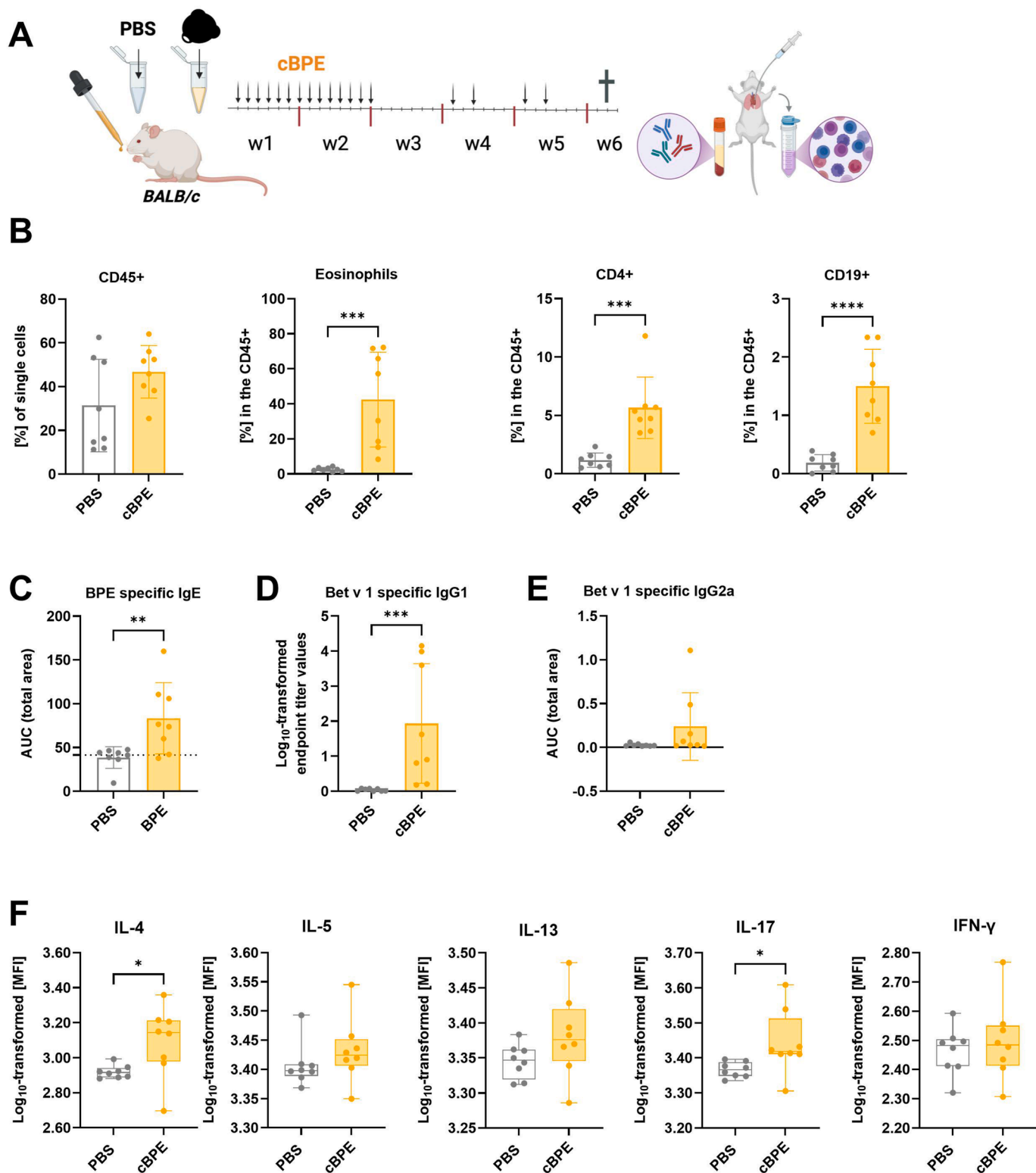


Fig. 1. Adjuvant-free intranasal sensitisation with BPE induces allergic responses in mice. **A:** Experimental design. Each arrow represents an intranasal instillation with BPE or PBS ($n = 8$ per group) in BALB/c mice. **B:** BAL cell composition, showing the frequencies of leukocytes (CD45+), and eosinophils, T helper and B cells. **C:** Mediator release from RBL cells sensitised with individual sera and stimulated with a titration of BPE. Area under the curve (AUC) was calculated. The dashed line indicates the AUC background signal. **D:** Bet v 1-sIgG₁; **E:** sIgG_{2a} in serum, as measured by ELISA. The log₁₀-transformed endpoint titre values for sIgG₁ (PBS values were adjusted to 1) and AUC for IgG_{2a} are shown. **F:** Log₁₀-transformed MFI values for the cytokines measured in BAL supernatant. Data, showing mean $\pm$ SD, were analysed with unpaired t test (B, E and F: IL-4, IL-5, IL-13 and IFN- γ [MFI]) or Mann–Whitney (C, F: IL-17 [MFI]) according to the normality test. Statistical significance is indicated for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

AUC depicts the AUC background signal; mediator release curves [Supplementary Fig. 4B](#)). As shown by ELISA, mice exposed to cBPE developed Bet v 1-specific IgG₁ ([Fig. 1D](#); mean of the endpoint titre values of log₁₀ 1.9). On the other hand, mice that received PBS did not show any Bet v 1-specific IgG₁, and the mean AUC values showed a 50-fold increase between BPE and PBS (IgG₁: $p < 0.001$). A mild IgG_{2a} specific response was developed in the sera of cBPE treated mice ([Fig. 1E](#); mean of the AUC values for BPE group is 0.24 AU and for PBS group is 0.03 AU).

To gain insight into the airway inflammation, BALF cytokines were investigated. A Th2-associated cytokine profile was observed in the BALF of cBPE-treated mice ([Fig. 1F](#) and [Supplementary Fig. 4C](#)), with an increase in the MFI levels (mean $\pm$ SD) of IL-4 (1345 ± 541 ; $p < 0.05$) compared to BALF of PBS-mice (833 ± 72). No significant differences were seen in IL-5 and IL-13 between the groups. In addition, a significant increase in IL-17 (2829 ± 632.9 ; $p < 0.05$) was observed in BALF of cBPE-mice compared to PBS-mice (IL-17: 2325 ± 116.4), whereas INF- γ did not differ significantly between groups.

Tracing the development of a type 2 response in a longitudinal in vivo model

To investigate the onset of Th2 differentiation as well as the temporal dynamics of the cellular and humoral immune response,

time-series experiments were performed with in 4Get:BALB/c mice with i. n. instillations of cBPE or PBS for 4, 6, 8 and 10 subsequent days ([Fig. 2A](#)). We first assessed the infiltrating cells in BALF ([Fig. 2B](#), absolute number [Supplementary Fig. 5D](#)). After 6 i. n. instillations, BALF eosinophil numbers (mean $\pm$ SD) were significantly increased in the BALF of cBPE-mice with a 24.7-fold increase compared to PBS-mice (0.8 ± 0.6 %; vs. 20.1 ± 16.3 %; $p < 0.05$). The infiltration peak was reached after 10 i. n. instillations with a 47.7-fold increase compared to the PBS (38.8 ± 16.0 %). In contrast, BALF neutrophils steadily decreased after an initial peak at day 4 (3.4 ± 2.0 %). Regarding the induction of an adaptive response, the percentage of B cells (CD19⁺) present in the bronchoalveolar compartment increased in a time-dependent manner, reaching a plateau between days 8 and 10 (0.38 ± 0.15 % vs 0.08 ± 0.1 % for PBS). BALF CD4⁺ T cells peaked on day 6 (5.5 ± 3.2 %), followed by a decline at later time points (3.5 ± 2.3 % at 8 days and 3.5 ± 1.6 % at 10 days), but generally remained higher than the PBS group (1.0 ± 1.0 %). The Th2 response (IL-4/eGFP⁺) was increased in the BALF of cBPE-mice at all investigated time points compared to PBS, with a significant peak ($p < 0.05$) at day 6 (mean of the groups and SD, day 4: 13.28 ± 5.6 %, day 6: 26.38 ± 7.1 %, day 8: 14.81 ± 7.1 %, day 10: 19.0 ± 6.4 % and PBS: 7.0 ± 6.0 %).

The BALF cytokine profile ([Fig. 2C](#) and [Supplementary Fig. 5A](#)) showed elevated concentrations of all investigated cytokines,

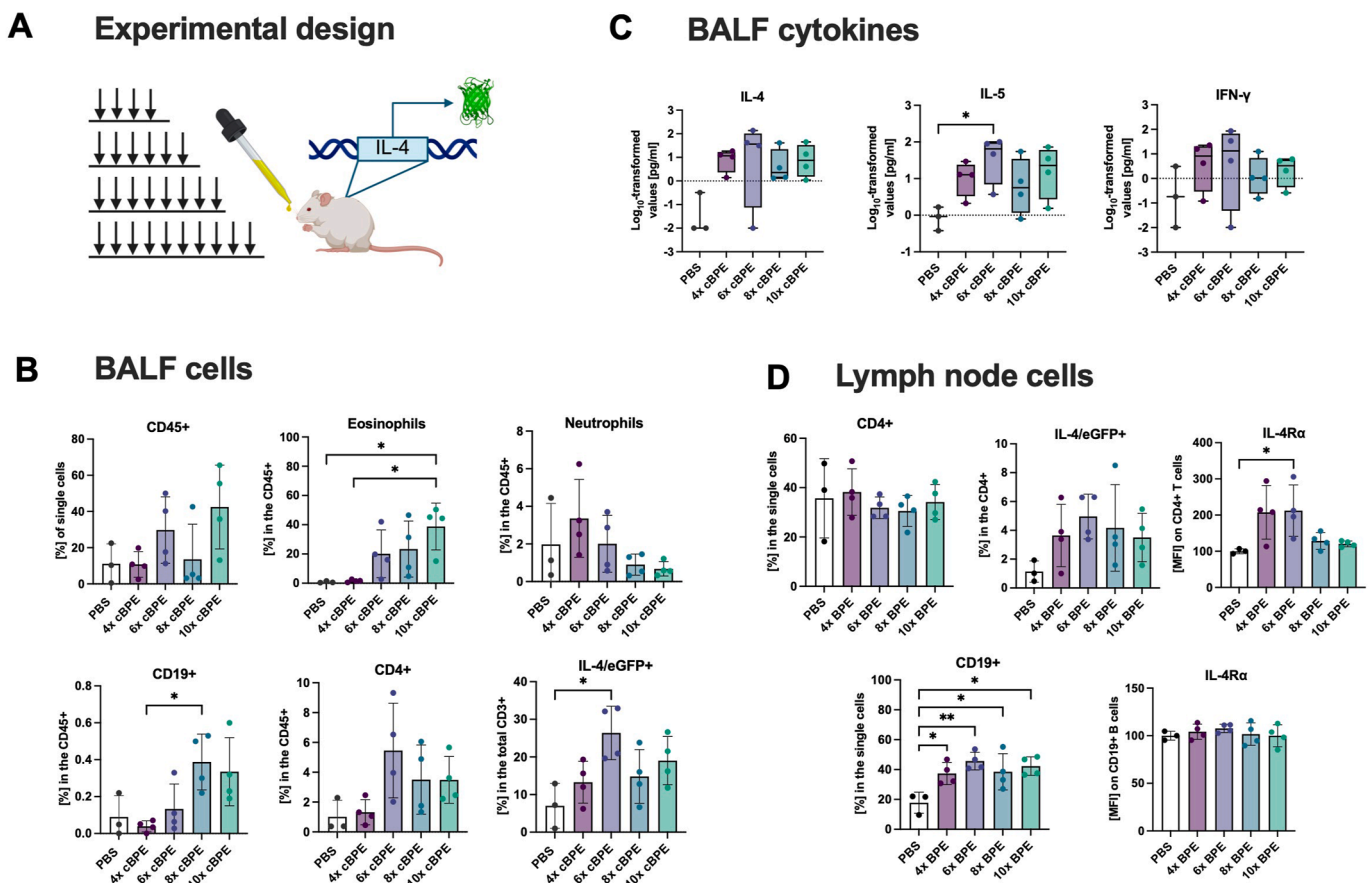


Fig. 2. Monitoring early events and Th2 polarization upon birch pollen exposure. **A:** Schedule of the *in vivo* procedure, where each arrow represents a daily intranasal instillation with BPE ($n = 4$ per group) or PBS ($n = 3$), in 4Get:BALB/c mice. **B:** BAL cell composition, showing the frequencies of leukocytes (CD45⁺), eosinophils, neutrophils, T helper, B cells and IL-4/eGFP⁺ cells. **C:** Log₁₀-transformed concentrations (pg/ml) for cytokines were measured in BAL supernatant. **D:** Immunophenotyping of lymphocytes. The percentage of CD4⁺ T helper, CD19⁺ B cells and IL-4/eGFP⁺ CD4⁺ cells are shown. The upregulation of IL-4R α on CD19⁺ and CD4⁺ cells was calculated as the MFI. Data were analysed with One way-ANOVA (C: IL-5 and IFN- γ , D: IL-4R α on the CD4 [MFI] and IL-4/eGFP⁺) or Kruskal–Wallis test (C: IL-4, D: IL-4R α on the CD4⁺ [MFI]) according to the normality test. Statistical significance is indicated for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). These are combined results of two independently performed experiments.

including type 2 cytokines (IL-4, IL-5, and IL-13), in cBPE-mice compared to PBS on day 6, but no significant differences were observed between the time points. Presence of IL-4 (log10-transformed pg/ml values) was observed at all time points compared to PBS (mean of non-transformed values, BPE 4 days: 10.83 ± 7.1 pg/ml, day 6: 52.8 ± 59.3 pg/ml, day 8: 11.8 ± 19.4 pg/ml, day 10: 15.9 ± 20.0 pg/ml and PBS: 0.11 ± 0.17 pg/ml). IL-5 showed a significant ($p < 0.05$) 60-fold increase after 6 cBPE instillations compared to PBS (1.0 ± 0.6 vs 60.0 ± 44.0 pg/ml), but no further differences were observed over time. No significant differences were observed in BALF IFN- γ levels.

In the mediastinal lymph nodes (Fig. 2D, absolute numbers in Supplementary Fig. 5E), a Th2 response ($CD4^+$ IL-4/eGFP $^+$) was prominent from day 4 on, with no significant differences between the time points. Numbers of Th2 cells were on average 3.57-fold higher in the cBPE group compared to the PBS (mean of each group for $CD4^+$ IL-4/eGFP $^+$, day 4: 3.6 ± 2.1 %, day 6: 5.0 ± 1.6 %, day 8: 4.2 ± 3.0 %, day 10: 3.5 ± 1.7 % and 1.1 ± 0.7 % for the PBS group), whereas the total $CD4^+$ remained constant over time (ranging between 30.6 ± 6.2 % and 38.2 ± 9.4 %). The IL-4R α expression of lymph node resident $CD4^+$ T cells was 2-fold higher after 4 (mean MFI: 207.4 ± 74.0) and 6 (mean MFI: 212.1 ± 71.0 ; $p < 0.05$) cBPE instillations compared to PBS-treatment (normalised mean MFI: 100 ± 6.9), while at the later time points (days 8 and 10) this increase was not present anymore. Compared to PBS, a significant increase was observed in the $CD19^+$ B cells, but no differences were detected between the different time points (mean of the groups: 37.4 ± 7.4 % on day 4, 45.7 ± 5.9 % at day 6, 38.5 ± 12.1 % at day 8, 42.3 ± 6.2 % at day 10 and 17.8 ± 7.0 % in the PBS). On the other hand, at all the time points, similar levels of IL-4R α expression were observed on the lymph node B cells.

Regarding the humoral response, both allergen-specific IgE (Supplementary Fig. 5B) and specific IgG $_1$ antibodies in serum (Supplementary Fig. 5C) were below the ELISA detection limit at any of the investigated time points, except of one mouse in the 8x installation group with showed low levels of specific IgE.

BPEs from self-collected pollen are characterised by distinct combinations of site- and pollen-specific factors

We aimed to test whether we can detect differences in the sensitising potential of native birch pollen samples. To increase the likelihood of the pollen samples eliciting different levels of response,²⁷ we selected trees at two sites that differed in their environmental exposure regimes. Pollen for sBPE #1 was from a rural tree with significantly lower ambient NO $_2$ ($p < 0.0001$) (Supplementary Fig. 6A) than sBPE #2 but comparable O $_3$ levels (Supplementary Fig. 6B). The content of total protein, pollen-associated lipid mediators (PALM-PGE $_2$, PALM-LTB $_4$) and adenosine did not differ significantly between both sBPEs (Supplementary Fig. 6C–F). The levels of Bet v 1 were significantly ($p < 0.05$), LPS-levels marginally ($p = 0.09$) higher in sBPE #1 than in sBPE #2 (Supplementary Fig. 6G, H).

Quantitative differences in the sensitising potential of different BPEs across different mouse models and in a human DC model

To validate the results obtained with the commercial pollen extract (cBPE) in the established mouse models, we compared the sensitising potential of two different self-collected birch pollen extracts (sBPE) in an adjuvant-free 12-day i. n. model in BALB/c mice (Fig. 3A). Both sBPEs induced focal mucus hypersecretion and goblet cell hyperplasia (Fig. 3B), as well as perivascular and peribronchial cell infiltration (Fig. 3C) compared to the PBS control. A quantification of the histology results of all treated mice confirmed

a mild, but significant mucus hypersecretion (Fig. 4A) and inflammatory cell infiltration (Fig. 4B) in the lungs of mice treated with sBPE #1 and sBPE #2 compared to PBS, with inflammatory cell infiltration being higher in mouse lungs treated with sBPE #2 compared to sBPE #1 ($p < 0.05$). Serum total IgE levels (Fig. 4C) were increased in mice treated with both sBPEs, with a more pronounced significance in the sBPE #2 than in the sBPE #1 group (PBS vs. sBPE #1: $p < 0.01$; PBS vs. sBPE #2: $p < 0.001$) but without statistical significance between both sBPEs in a direct comparison. The percentages of eosinophils (Fig. 4D, absolute numbers in Supplementary Fig. 7A) and lymphocytes in BALF (Fig. 4F, absolute numbers in Supplementary Fig. 7C) were also significantly increased in both sBPE groups compared to PBS, with a more pronounced significance in the sBPE #2 than in the sBPE #1 group (sBPE #1 vs. PBS: $p < 0.01$; sBPE #2 vs. PBS: $p < 0.001$ for eosinophils; sBPE #1 vs. PBS: $p < 0.05$; sBPE #2 vs. PBS: $p < 0.01$ for lymphocytes) but again, with no significant difference between the two sBPEs. The percentage of BALF neutrophils (Fig. 4E, absolute numbers in Supplementary Fig. 7B) did not differ between the experimental groups. A trend-wise decrease of the percentage of macrophages was observed in sBPE compared to the PBS control, which was slightly more pronounced after sBPE #2 than after sBPE #1 treatment (Fig. 4G, absolute numbers in Supplementary Fig. 7D). In lung tissue, the numbers of Th2 cells (Fig. 4H) were significantly increased only in mice treated with sBPE #2, not with sBPE #1 (PBS vs. sBPE #2: $p < 0.05$). Numbers of Tregs (Fig. 4I) in the lungs did not differ between the groups, but the ratio of Th2/Tregs (Fig. 4J) was significantly increased only after treatment with sBPE #2. Numbers of $CD11b^+$ dendritic cells (Fig. 4K) were significantly increased ($p < 0.05$) in both sBPE groups compared to PBS.

To assess whether differences in the Th2 induction potential of various pollen samples can be tracked using a fast sensitisation protocol, we subjected 4Get: BALB/c mice to a single i. d. injection with our different sBPEs (Fig. 5A). Increases in IL-4/eGFP $^+$ $CD4^+$ T cells were observed after i. d. BPE sensitisation compared to the PBS group, and the numbers of IL-4/eGFP $^+$ $CD4^+$ T cells (mean $\pm$ SD) in inguinal lymph nodes were slightly higher after immunisation with sBPE #2 ($10,87 \pm 3344$) than with sBPE #1 (9318 ± 3913) (Fig. 5B). In previous work, where cBPE was used, we observed an increase of IL-4/eGFP $^+$ $CD4^+$ T cells of 11.5 %.⁶ A negative control was not used explicitly as it was shown in previous work that Bet v 1 not the sensitizing compound in BPE, and no GFP signal is detected in mice treated only with purified recombinant Bet v 1.³²

As the IL-10 release of DCs had previously been shown to be a sensitive marker reflecting different inflammatory potencies of different ragweed pollen extracts,¹⁰ we decided to analyse the IL-10 release of moDCs after stimulation with our two sBPEs (Fig. 5C). We observed a substantial IL-10 release from DCs stimulated with sBPE #1 (681 ± 877 pg/ml), whereas the IL-10 production of DCs stimulated with sBPE #2 was significantly lower than that of DCs stimulated with sBPE#1 (111.6 ± 69.8 pg/ml; $p < 0.001$) and did not differ significantly from the PBS control (Fig. 5D).

Discussion

Herein we describe a toolbox of *in vivo* and *in vitro* models addressing various stages of the allergic immune response to birch pollen, from the early (Th2 differentiation) and late (tissue effector cell infiltration) cellular events to the development of a full-blown humoral immune response (specific immunoglobulin production). The 4Get reporter model we used is novel in the context of pollen-induced allergy and, to our knowledge, has not previously been

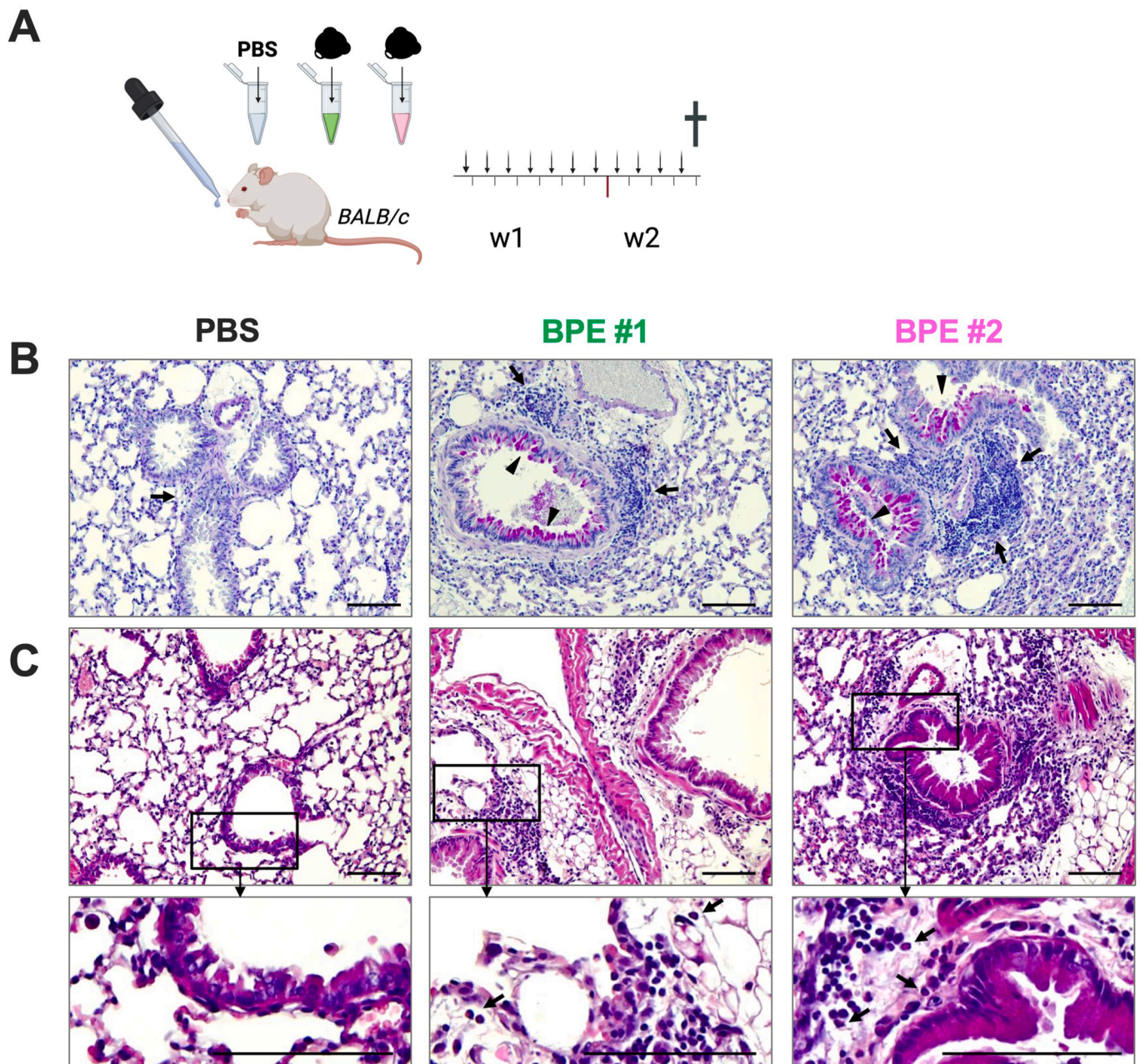


Fig. 3. 12-day intranasal birch pollen sensitisation model with self-collected birch pollen extracts. **A:** Experimental design with 11 i. n. instillations in BALB/c mice, followed by analysis on day 12. **B, C:** Lung histological sections of mice instilled with PBS, sBPE #1 or sBPE #2. **B:** Periodic-acid-Schiff (PAS) staining. Arrows: inflammatory cell infiltrate; arrowheads: mucus hypersecretion. **C:** Haematoxylin/eosin staining. Inserts show magnified infiltrate; arrows: eosinophils. Scale bar: 100 μ m. Representative areas of focal inflammatory infiltrate are shown.

used for the systematic evaluation of allergic sensitisation to pollen; existing studies employing this model are rare and have predominantly focused on parasitic infections. Our work exclusively investigates allergic sensitisation under adjuvant-free conditions, an approach that remains largely underexplored despite its relevance to natural exposure scenarios. Additionally, we directly compare distinct routes of sensitisation (intradermal versus intranasal) within the same experimental framework. Finally, we assess different real-life pollen extracts rather than commercially processed samples, thereby enhancing the physiological relevance and broader applicability of our findings.

This is particularly important when studying the impact of environmental stressors on the allergenicity of birch trees and

their pollen, a topic that has gained increasing importance due to anthropogenic air pollution, urbanisation, and climate change.

As i.n. sensitisation of mice leads to lung inflammation, BALF is important for monitoring effector cytokines and the recruitment of inflammatory cells. Allergic reactions are characterised by the Th2 cytokines IL-4, IL-5, and IL-13, which were also increased in our study. An increase in tissue eosinophils is typically associated with the late phase cellular response, and it was consistently observed in our i. n. models. The hallmark of humoral allergic immune reactions is the systemic presence of antigen-specific IgE and IgG₁ (in human: IgG₄) antibodies. Bet v 1-specific IgG₁ was detected in sera in our adjuvant-free i. n. birch pollen models, albeit only after repeated booster immunisations. Using the highly

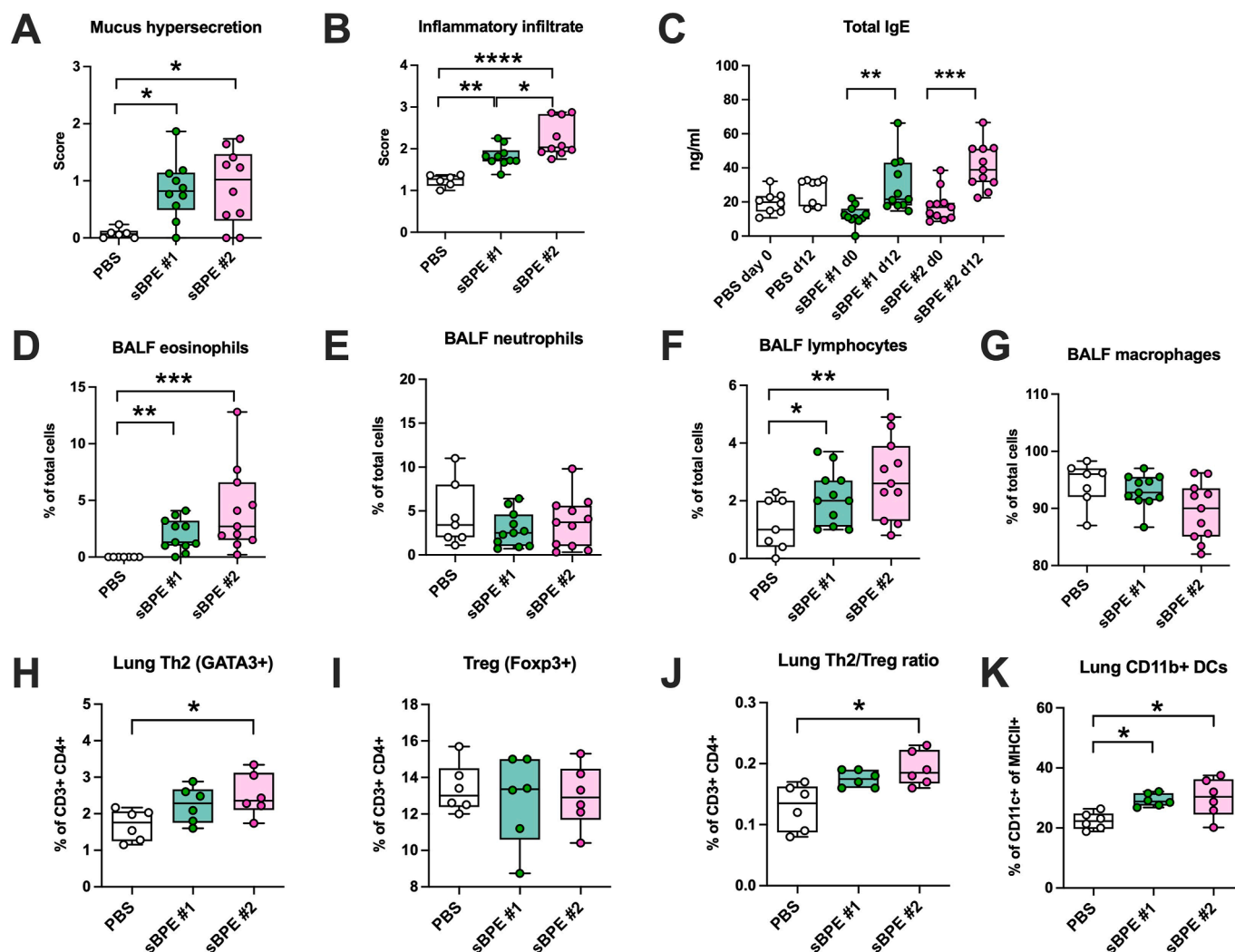


Fig. 4. Intranasal birch pollen sensitisation model with self-collected birch pollen extracts. **A:** Mucus hypersecretion. **B:** Lung inflammatory cell infiltrate. **C:** Serum total IgE. **D–G:** BALF infiltrating immune cells: **D:** Eosinophils; **E:** neutrophils; **F:** lymphocytes; **G:** alveolar macrophages. **H–K:** Immune cells in lung tissue. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$, Kruskal Wallis or Šidák's test.

sensitive RBL assay,³¹ we were able to detect BPE-specific IgE responses indirectly in our 6-weeks i. n. model. Thus, we successfully established a robust, adjuvant-free model of i. n. sensitisation to birch pollen using cBPE.

To better understand the kinetics of the allergic immune reaction to birch pollen in the i. n. model, we performed a time-series experiment with 4–10 instillations. T helper cells peaked on day 6 and decreased afterwards. Eosinophils started to increase in BALF after six and peaked after ten instillations, whereas neutrophils peaked after four instillations and decreased afterwards, which was also described previously for i. n. sensitisation to ragweed pollen.¹⁰ Neutrophil recruitment is mediated by the secretion of IL-8/CXCL-8, which is induced as the first line of defence by epithelia. Neutrophil chemotaxis towards pollen-exposed bronchial epithelium has previously been shown to rely on IL-8/CXCL-8 secretion.²⁹ Additionally, direct chemotaxis of neutrophils and eosinophils to pollen-derived adenosine or PALMs³² could theoretically contribute to granulocyte infiltration in pollen-exposed tissues. Whether the induction of a Th2 response to pollen requires early neutrophil recruitment is currently not known. Neutrophil recruitment could be dampened by the eosinophil and Th2 response, as a study on human samples

demonstrated that IL-4 and IL-13 inhibited IL-8-mediated neutrophil chemotaxis.³³ Our data suggests an immediate neutrophil influx, followed by early Th2 induction and later eosinophil recruitment in the airways, whereas a humoral response needs more time to develop. This was not shown before for birch pollen and is well in line with previous findings from other pollen sensitisation models like ragweed and cedar pollen.^{10,29,34}

We used a 5-day i. d. reporter model and a 12-day i. n. model to compare the immune responses to different self-collected pollen samples (sBPE), which differed in their biochemical composition. The i. d. 4Get model can easily be standardised to the protein concentration of the sensitising extract, its readouts are simple, and it requires only a single injection, avoiding cumulative exposure effects. Thus, the i. d. model is especially suited to study adjuvant-free sensitisation. On the other hand, Th2 polarisation occurs only locally in the draining lymph nodes, and systemic effects are not detectable.³¹ The i. n. model requires multiple repetitive instillations and consequently higher amounts of pollen material, and it is labour-intensive, but it reflects more the natural route of exposure and shows relevant endpoints, such as airway inflammation and specific IgG₁ production.^{9,12} LPS was previously

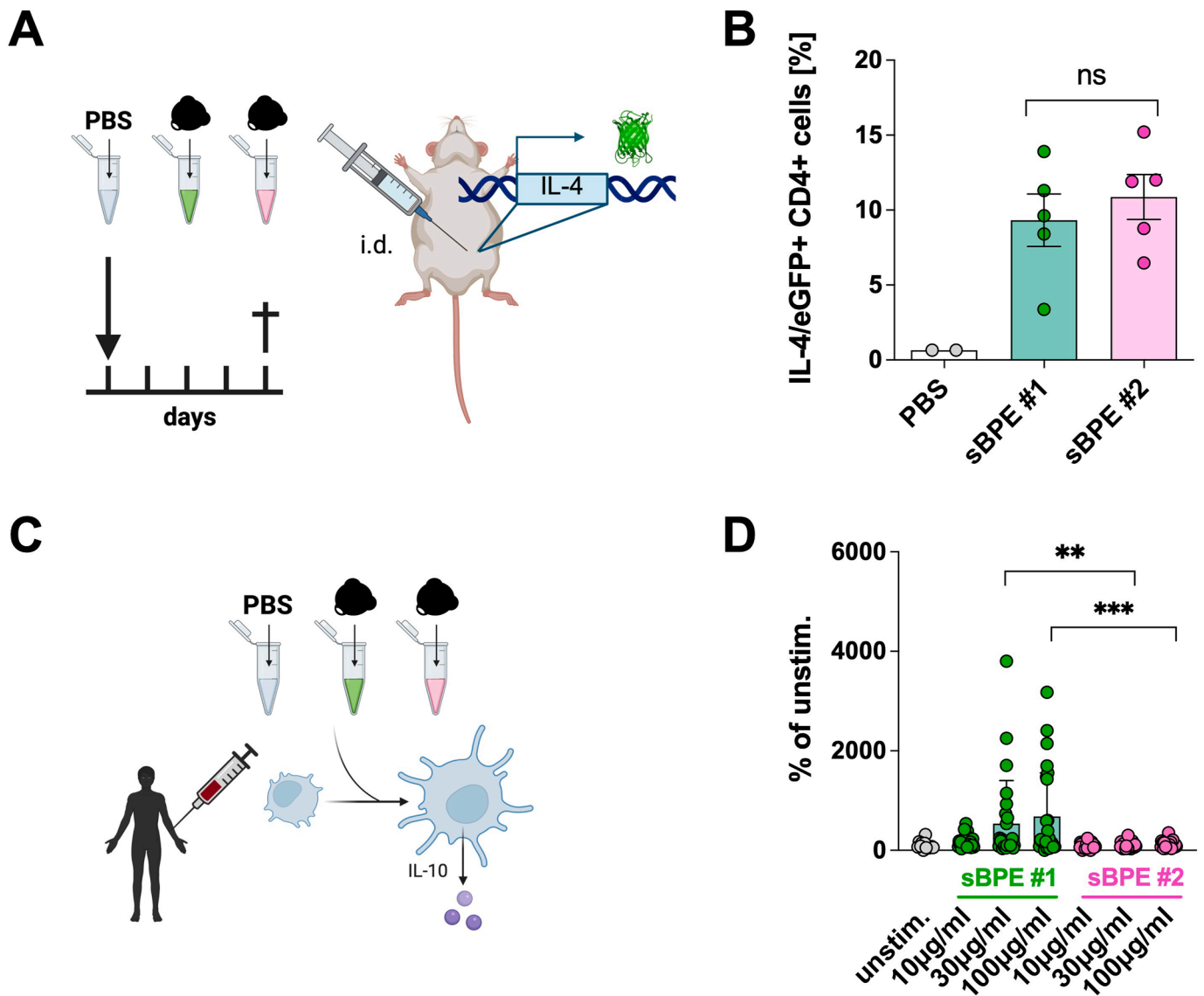


Fig. 5. Intradermal sensitisation model and human moDC model with self-collected birch pollen extracts. **A:** Experimental setup of the murine intradermal sensitisation model. **B:** IL-4/eGFP⁺ CD4⁺ T cells in the inguinal lymph nodes. **C:** experimental setup of the human moDC model. **D:** IL-10 production of human moDCs. **: $p < 0.01$; ***: $p < 0.001$, one-way ANOVA.

reported to induce more PAS + areas,³⁵ but in the i.n. model, low endotoxin contaminations of pollen should only play a minor role, as the upper airways, oral and nasal mucosal surfaces are exposed to endotoxin and other TLR-4 ligands due to their microbial colonisation. In this regard it has been reported that IL-4 signalling bypasses TLR4-and MyD88-responses.³⁶ In the i.d. model different cBPE were tested previously^{6,32} and in another study cBPE and sBPE performed similar in a direct comparison.⁶ Here, the i. d. model did not clearly reveal the subtle differences between the natural birch pollen extracts, whereas in the i. n. model sBPE #2 induced a stronger inflammatory cell infiltration than sBPE #1, suggesting that the i. n. model is more sensitive due to cumulative exposure effects. Here the limitation of our study is the lack of a direct side-by-side comparison of the two sBPEs within the i.n. 4Get model.

Human DCs can be used to compare the sensitising potential of pollen samples.¹⁰ The benefits of this *in vitro* model are that it accounts for the genetic variability of human cell donors and allows for the comparison of innate responses between cells of

donors with different atopy status. On the downside, DCs do not capture the entire allergic sensitisation cascade, and *in vitro* responses of primary human cells show high donor-to-donor variability. In our moDC model, sBPE #1, which had been less inflammatory in the mouse, induced a significantly stronger IL-10 response than sBPE#2. IL-10 is a hallmark for DC-induced Treg differentiation^{37,38} and is required for Treg induction during successful allergen-specific immunotherapy.^{39,40} In older literature, IL-10 had sometimes been associated with type-2 inflammation due to its co-expression in highly activated lymphocytes during acute allergic responses.⁴¹ Nowadays, this paradigm has largely shifted and IL-10 is recognised as a key immunoregulatory cytokine.⁴² In line with our moDC results, the 12-day i. n. model revealed a higher Th2/Treg ratio in murine lungs after exposure to sBPE #2 compared to sBPE #1, supporting a regulatory role of DC-derived IL-10. The connection between high IL-10 levels in a human moDC model and reduced allergic inflammation in mice was also seen in a study using different ragweed pollen.¹⁰ It is well known that dendritic cells exposed to high doses of LPS do not only

secrete pro-inflammatory cytokines but also respond with IL-10 secretion to down-regulate overshooting adaptive immune responses.⁴³ In the future, it would be valuable to define DC responses to allergen stimulation more holistically by combining cytokine and surface marker expression with metabolic and epigenetic signatures in order to capture potential effects of trained immunity.⁴⁴ Such an improved DC model could reduce the need for experimental animals in the future.

The pollen matrix contains a host of plant- and microbe-derived molecules that could serve as potential sensitizers or Th2 adjuvants.³² Here, we quantified a set of known substances, i. e. LPS, PALMs, adenosine, and Bet v 1 in our two natural birch pollen samples. Interestingly, the Bet v 1 concentration was higher in BPE #1 which had proven less inflammatory in the mouse models. This aligns with recent findings that allergic sensitisation *in vivo* required constituents of the pollen matrix, whereas highly purified recombinant Bet v 1 was unable to elicit an allergic phenotype.⁴⁵ The LPS content in sBPE #1 was higher than in sBPE #2. Since high concentrations of LPS are known to induce IL-10 in myeloid cells,⁴⁶ the higher LPS content of sBPE #1 might explain why it caused a stronger IL-10 response in moDCs. The role of LPS in Th2 response and sensitisation should be systematically evaluated in the future.

In conclusion, we report a battery of *in vivo* and *in vitro* immunological assays to assess the sensitising potential of birch pollen extracts from different angles, which could be used in the future to develop a pipeline for characterising extracts for therapeutic and diagnostic purposes. The adjuvant-free i. n. model reflected best the physiological exposure route, but additional effects of liquid in the nose or repeated anaesthesia cannot be ruled out, and the method requires high amounts of pollen material. The i. d. model provides a rapid Th2 polarizing protocol and stably reflects single-exposure effects, while failing to detect long-term systemic effects like IgE production. With our assays we were able to detect even subtle differences in the sensitising potential of birch pollen extracts from different sources like two self-collected and one commercial BPE. We established the IL-4 reporter mouse model to study systematically allergic sensitisation. Additionally, our time-series experiment enabled us to study the kinetics of the allergic immune response and assess which cellular and humoral immune responses are activated when. This will be useful for further research in the field of birch pollen sensitisation. Finally, in the human DC model differences between two environmental birch pollen extracts became evident, a finding which could pave the way for future cell-based allergenicity assays that might replace the need of experimental animals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2026.03.006>.

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, AS: 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.

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