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ORIGINAL ARTICLE

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Bet v 1 triggers antiviral-type immune signalling in birch-pollen-allergic individuals

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Abstract

Background: In allergic patients, clinical symptoms caused by pollen remind of symptoms triggered by viral respiratory infections, which are also the main cause of asthmatic exacerbations. In patients sensitized to birch pollen, Bet v 1 is the major symptom-causing allergen. Immune mechanisms driving Bet v 1-related responses of human blood cells have not been fully characterized.

Objective: To characterize the immune response to Bet v 1 in peripheral blood in patients allergic to birch pollen.

Methods: The peripheral blood mononuclear cells of birch-allergic (n = 24) and non-allergic (n = 47) adolescents were stimulated ex-vivo followed by transcriptomic profiling. Systems-biology approaches were employed to decipher disease-relevant gene networks and deconvolution of associated cell populations.

Results: Solely in birch-allergic patients, co-expression analysis revealed activation of networks of innate immunity and antiviral signalling as the immediate response to Bet v 1 stimulation. Toll-like receptors and signal transducer transcription were the main drivers of gene expression patterns. Macrophages and dendritic cells were the main cell subsets responding to Bet v 1.

Conclusions and clinical relevance: In birch-pollen-allergic patients, the activated innate immune networks seem to be, in part, the same as those activated during viral infections. This tendency of the immune system to read pollens as viruses may provide new insight to allergy prevention and treatment.

KEYWORDS
Bet v 1, birch allergy, dendritic cells, immune network, macrophages, systems immunology

1 | INTRODUCTION

With an estimated prevalence of approximately 30%, pollen allergy has a tremendous clinical impact, leading to high health care costs, sick leaves and economic losses as well as significantly reduced quality of life of patients.[1,2]

Birch, with the corresponding major allergen Bet v 1, represents a main pollen allergen-producing tree in northern Europe.[3] Bet v 1

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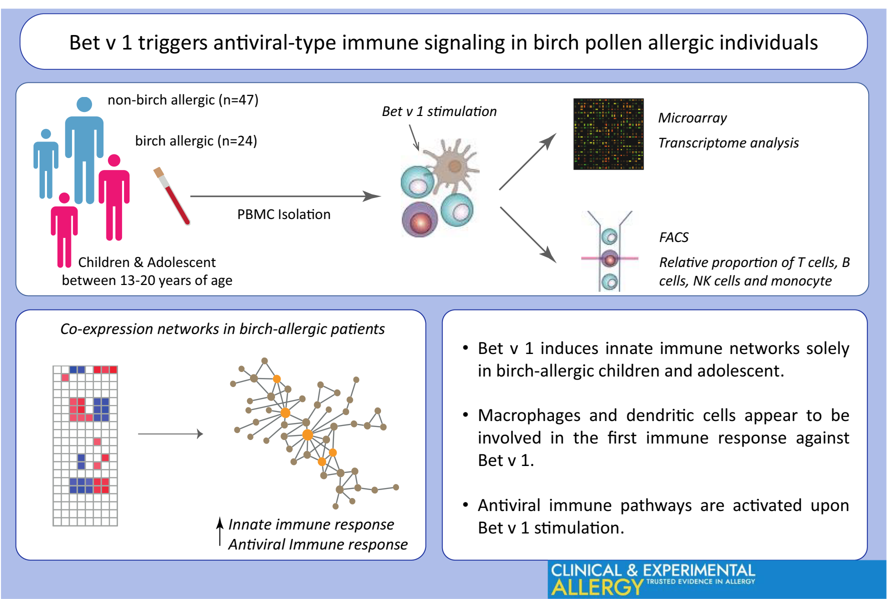

GRAPHICAL ABSTRACT

Systems immunology study to characterize the impact of bet v 1 stimulation of peripheral blood mononuclear cells (PBMC) of birch-allergic and non-birch allergic children and adolescent. Utilizing transcriptomics and FACS analysis, co-expression networks revealed innate and antiviral immune networks as well as associated immune cells in birch-allergic patients. bet v 1 – major birch allergen; PBMC – peripheral blood mononuclear cells, FACS – fluorescence activated cell sorting

and related homologues appear to share the unique capability to sensitize predisposed individuals.[4] Moreover, the majority of birch-allergic patients (>90%) react towards Bet v 1, which is one of the most intensively studied allergens.[5] Various studies investigated structural properties[5,6] as well as the interaction of Bet v 1 with T cells,[7,8] B cells[9,10] and dendritic cells.[11,12]

In recent years, the innate immune branch was recognized as an important regulator of the allergic adaptive immune response.[13] Respiratory viral infections are common triggers of asthma exacerbation among wheezing children and adults.[14-16] Viruses are detected by different classes of pattern recognition receptors, which also detect allergen-associated molecular patterns.[17,18] Thus, allergens may modulate innate immune pathways promoting allergen-specific maladaptive immune responses,[18,19] and viral infections may act by enhancing these signals. It has also been recently reported that pollen exposure modulates antiviral defence of the respiratory epithelium.[20] However, current evidence does not fully establish, how Bet v 1 regulates immune pathways leading to a clinical allergic response.

System-scale data collection and network analysis facilitate an unbiased approach to detect disease- relevant mechanisms.[21] Systems-level studies utilizing genetic and epigenetics,[22,23] transcriptomics,[24-26] proteomics,[27] metabolomics[28] and microbial sequencing techniques[29] have advanced our understanding of complex allergic diseases. Furthermore, transcriptomic studies can be used to predict late-phase asthmatic responses against inhaled allergens.[24] In-depth characterization of allergens using multi-omics studies will help us to develop novel diagnostic tools as well as revealing novel immunotherapeutic targets.[30]

Key Messages

- Birch-allergic individuals display downregulated innate immunity-related genes at baseline.
- Bet v 1 stimulation of PBMCs induces innate and antiviral immune networks in birch-allergic children and adolescents.
- Macrophages and dendritic cells appear to be involved in the primary immune response against bet v 1.

Therefore, we performed a large "birch-omics" study that uses transcriptomic networks in a Finnish adolescent patient cohort. Utilizing cell deconvolution, weighted gene correlation network analysis (WGCNA) and protein–protein interaction (PPI) networks, we searched for important innate immune pathways in the early Bet v 1 blood-derived immune response.

2 | METHODS AND MATERIALS

2.1 | Patient recruitment

The study subjects were originally school children selected for a long-term allergy study in 2003.[31] The blood samples were collected in the whole Finnish cohort in September 2010 and 2011, when the children were 13–20-year-old adolescents and

a follow-up study was performed.[32] In total, we collected blood samples from 24 birch-allergic (BA) and 47 non-birch-allergic (NBA) patients. The homes of the study subjects varied from flats in apartment buildings to row and individual houses in the town of Joensuu (a small town of 73,000 inhabitants) to villages of different sizes and to isolated houses in the sparsely populated rural area. For further description of the study cohort, see Hanski et al.[33] The study was approved by the Ethics Committee of the Helsinki University Central Hospital and informed consent was obtained from all participants (202/E7/2003; TY2012202/53/2012; TY2012202/25/2014).

2.2 | Study subjects and atopic sensitization

Total and allergen-specific sIgE measurements are given in Table 1. The clinical data has been originally presented elsewhere.[33] Serum IgE (sIgE) antibodies were measured against eight common inhalant allergens (Phadiatop®: timothy grass, birch, mugwort, Cladosporium herbarum, horse, cat, dog and house dust mite, i.e. *Dermatophagoides pteronyssinus*) and six common food allergens (cow's milk, hen's egg, cod fish, soy, wheat and peanut), and Phadiatop® for inhalant and food allergens were measured using the UniCAP test (UniCAP 1000 version 2; Pharmacia Upjohn). Birch sensitization was defined by the specific IgE to Bet v 1 >0.35 kUA/L; $n = 24$ for Bet v 1 allergic subjects and $n = 47$ for Bet v 1 non-allergic subjects.

2.3 | Cell culture

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using BD Vacutainer CPT tubes (BD Biosciences Pharmingen) and frozen until analysed. Thawed PBMCs were cultured in 24-well plates at 1×10^6/ml in complete RPMI-1640 medium (Gibco, Life Technologies) with 50 U/ml of penicillin, 50 mg/ml of streptomycin (PEST, Life Technologies) and 10% heat-inactivated FBS (Gibco, Life Technologies). PBMCs were left untreated or stimulated with the major birch pollen allergen (Bet v1) at 10 µg/ml (recombinant Bet v 1, Indoor Biotechnologies, Product Code RP-BV1-1) for 6 h at 37°C and 5% CO_2, as previously described.[34]

2.4 | Flow cytometry

The frozen PBMC samples were thawed, the number of total cells was counted (Beckman Coulter AcT/Diff). Next, relative fractions of T cells (CD3+/CD4+ & CD3+/CD8+), B cells (CD19+/CD20+), monocytes (CD14+) and NK cells (CD16+/CD56+) were determined in freshly thawed and unstimulated PBMCs via flow cytometry using standard protocols for FACS surface staining. Briefly, cells were stained with following antibodies using two panels: CD3-Alexa Fluor 488 (clone UCHT1), CD4-PerCP-Cy5.5 (clone RPA-T4), CD8-APC-H7 (clone SK1), CD19-PE-Cy7 (clone SJ25C1), CD20-Alexa Fluor 700 (2H7), CD16-PE (clone 3G8), CD56-PE (clone MY31) and CD14-APC (clone M5E2, all antibodies were purchased from BD Pharmingen). Additionally, both panels were stained with CD45-PE-TexasRed (clone HI30, Invitrogen). In addition, cell viability was checked and percentage of dead cells was determined. Cells were analysed using a BD FACS Canto II flow cytometer and data was analysed using the FlowJo software (BD Biosciences).

2.5 | RNA preparation and microarray analysis

RNA was extracted from PBMCs with the AllPrep DNA/RNA mini kit (Qiagen, Hilden, GER). The purity of RNA was analysed by NanoDrop ND-1000 (Thermo Fisher Scientific), where a ratio A260/A280 >1.8 was considered pure. 300 ng of RNA was reverse transcribed into cDNA and labelled with fluorescent colour for analysis by DNA microarrays (SurePrint G3 Human Gene Expression Microarrays, Agilent). After hybridization of the fluorescence-labelled targets to the microarrays, a fluorescence image of the array was scanned, and fluorescence image data were generated. The fluorescence image was converted into expression level values for each probe on the array. Data pre-processing and differential expression analysis of the gene expression data were done in eUTOPIA.[35] Probe profile expression data were normalized using quantile normalization and corrected for batch processing effects using ComBat.[36] After mapping from probe sets to gene symbols, a differentially expressed gene (DEG) list was identified for each time point using limma.[37] DEGs were defined as a fold change ±1.5 and a p-value <.05 after adjustment for multiple comparisons (Benjamini-Hochberg).

2.6 | Leukocyte deconvolution analysis

The CIBERSORT algorithm (https://cibersort.stanford.edu) is an online computational tool to estimate leukocyte subset proportions in bulk transcriptome data.[38] Using the provided and validated "LM22" gene signature matrix, we are able to identify 22 different immune cells in our bulk transcriptome data.[38] Estimations are based on 1000 permutations. No significance filter has been applied to the estimated cell fractions to include all samples for further analysis.

2.7 | Weighted gene co-expression network analysis

Co-expression networks were calculated using the WGCNA package in R.[39] WGCNA was run on each study group (birch-allergic/non-allergic controls) separately. Soft-thresholding power was

TABLE 1 Clinical symptoms and sensitization characteristics of the 71 teenaged subjects in the Finnish Karelia

	Study subjects (*n* = 71, 30 males, 41 females, aged 13–18 years)			
Clinical disease and allergic symptoms	**BIRCH pollen allergic, birch-specific IgE ≥0.35 kUA/l (*n* = 24)**		**Not allergic to birch pollen, birch-specific IgE <0.35 kUA/l (*n* = 47)**	
Physician-diagnosed asthma, *n* (%)	6 (25.0)		3 (6.4)	
Self-reported symptoms of pollen allergy (hay-fever), *n* (%)	10 (41.7)		2 (4.3)	
Self-reported symptoms of atopic eczema, *n* (%)	10 (41.7)		3 (6.4)	
Response to other allergens than birch	**sIgE ≥0.35 kUA/l (%)**	**CI (95%)**	**sIgE ≥0.35 kUA/l (%)**	**CI (95%)**
Timothy grass	79.2	57.3–91.5	10.6	4.4–23.6
Mugwort	50.0	30.1–69.9	4.3	1.0–16.0
Cladosporium herbarum	12.5	3.8–33.9	0.0	0.0–6.4
Horse	25.0	11.2–46.9	8.5	3.1–21.0
Cat	50.0	30.1–69.9	17.0	8.6–30.9
Dog	70.8	49.0–86.0	17.0	8.6–30.9
House dust mite[a]	25.0	11.2–46.9	17.0	8.6–30.9
Peanut	25.0	11.2–46.9	0.0	0.0–6.4
Cow's milk	0.0	0.0–12.5	4.3	1.0–16.0
Hen's egg white	4.2	0.5–26.5	2.1	0.3–14.3
Soybean	20.8	8.5–42.7	0.0	0.0–6.4
Wheat	25.0	11.2–46.9	0.0	0.0–6.4
Fish (cod)	0.0	0.0–12.5	0.0	0.0–6.4
Response to any allergen[b]	100.0	87.5–100	36.2	23.5–51.1
Any (not house dust mite)[c]	100.0	87.5–100	27.7	16.6–42.4
Response to group testing				
Phadiatop® (inhalation allergens)	100.0	87.5–100	40.4	27.2–55.2
Phadiatop® (foods)	29.2	14.0–51.0	4.3	1.0–16.0
Total IgE ≥110 kUA/l	75.0	53.1–88.8	38.3	25.3–53.2

Note: In the birch-pollen-allergic (*n* = 24) and non-allergic (*n* = 47) subjects, the percentages of allergen-specific IgE levels ≥0.35 kUA/l against the 13 other tested allergens are given. The total IgE levels ≥110 kUA/l in the two groups are also given.

[a]House dust mite =*Dermatophagoides pteronyssinus*.

[b]One or more positive (≥0.35 kU_A/l) responses out of the 14 allergens included.

[c]One or more positive (≥0.35 kU_A/l) responses excluding house dust mite.

determined with the function pickSoftThreshold. Block-wise network construction and module detection was done with the blockwiseModules function of the package. The parameters used were minCorKME =0.7 and minKMEtoStay =0.5, indicating that the correlation of a block of genes within a module have to be at least 0.7 and that all genes within a module have a Pearson correlation of at least 0.5 with the module's eigengene. The module eigengene was calculated and association analysis with the phenotypic trait of each group was performed using the cor and corPvalueStudent function. Data were visualized using the corrplot function of the corrplot package.[40] Additionally, estimated cell fraction numbers from the CIBERSORT algorithm were correlated with modules.

2.8 | Enrichment analysis, PPI network generation and data visualization

Enrichment analysis of DEGs and identified modules were performed using the clusterProfiler[41] and ReactomePA[42] R package with the corresponding functions enrichGo and enrichPathway. PPI networks of DEGs or top 200 genes with the highest module membership were created using STRING database (www.string-db.org, Version 11.5).[43] This database consists of known and predicted PPI and was used to determine networks within gene lists. All interaction sources were used and only interactions with the highest confidence of 0.9 were included into the network analysis. All created networks showed a PPI enrichment *p*-value <.05.

This PPI enrichment p-value is calculated within the STRING-DB and indicates that the observed number of edges is significant and that the nodes are not random. Only networks with more than 5 connected nodes were further analysed. Cytoscape (v3.7.2)[44] was used for visualization of the PPI network. The CytoHubba[45] plug-in was used to identify hub genes using the Maximal Clique Centrality (MCC) with the assumption that essential proteins tend to cluster in a PPI. Further data analysis and graph visualization was conducted using R software (v3.6.1) with the ggplot2,[46] ggpubr[47] and ggfortify[48] package for R. Venn diagrams were generated jvenn (http://jvenn.toulouse.inra.fr).[49]

3 | RESULTS

3.1 | Bet v 1 solely induce major transcriptomic changes in birch-allergic subjects

Gene expression profiles were assessed after six hours Bet v 1 stimulation of PBMCs from verified birch-allergic (BA, $n = 24$) and non-birch-allergic (NBA, $n = 47$) subjects. The study outline is summarized in Figure 1A. Solely, PBMCs from BA subjects displayed major transcriptomic changes (Figure 1B, blue circle) compared with NBA control subjects (Figure 1B, red circle). Unstimulated PBMCs from allergic subjects exhibit 99 down-regulated genes, mainly involving genes of the innate immune branch (Table S1) compared with non-allergic unstimulated control subjects (Figure 1B, yellow circle). However, Bet v 1 engagement in the allergic cohort induced significant gene expression changes, leading to 167 up-regulated and 79 down-regulated genes (Table S2). Bet v 1 stimulation of PBMCs derived from NBA patients responded less with only 32 differently expressed genes (Table S3; Figure 1C). Interestingly, uniquely overlapping genes between the unstimulated conditions and BA Bet v 1 stimulations (Figure 1B, intersection of yellow circle with blue circle) showed 78 genes contrary expressed; down-regulated at unstimulated state, but significantly upregulated upon Bet v 1 stimulation (Figure 1D). Gene expression analysis between the Bet v 1-BA and Bet v 1-NBA groups showed only two differentially expressed genes and were, therefore, excluded in the further downstream analysis. This gene regulation is mirrored in GO biological processes and Reactome pathway enrichment, showing an "enrichment overlap" between down-regulated genes in unstimulated PBMCs (Figure 1E/F, DOWN unstim-BA vs. unstim-NBA) as well as up-regulated genes after Bet v 1 stimulation of allergy-derived PBMCs (Figure 1E/F, UP Bet v 1-BA vs. unstim-BA). Overall, innate immune mechanisms as well as antiviral/interferon-related processes and pathways were enriched in these two conditions. Differentially expressed genes of Bet v 1 stimulated non-allergic PBMCs exhibited no significant biological process enrichment. Therefore, further analysis was focused only on unstimulated samples (referred as "baseline"; unstim-BA vs. unstim-NBA) and Bet v 1 stimulation of PBMCs derived from BA patients (referred as "stimulation"; Bet v 1-BA vs unstim-BA).

3.2 | Protein–Protein interaction networks of differentially expressed genes identify clusters of genes related to innate immune and antiviral/interferon response

Aiming to identify important hub genes within these regulatory networks, the STRING online database (V11.5) was used to uncover underlying protein relationships between the proteins encoded by differentially expressed genes at baseline and after Bet v 1 stimulation. At baseline, the PPI revealed IL1A, IL1B, IL6, IL10 and TNF as important hub genes, further highlighting the importance of innate-related pathways (Figure 2A). However, the Bet v 1 stimulation PPI underline the importance of 2 different networks within the differentially expressed genes. These networks seem to represent different pathways within the differently expressed genes (Figure 2B; turquoise and cyan nodes represent 2 different detected networks). Network 1 mainly involves chemokines, cytokines and pattern recognition receptors and network 2 includes antiviral/interferon-related genes. Utilizing the ARCHS4 tissue database, genes of network 1 were associated with macrophages and network 2 with plasmacytoid dendritic cells (Figure 2B).

3.3 | Bet v 1 stimulation affects blood leukocyte composition in allergic, but not in non-allergic individuals

To further determine the cellular involvement in birch allergy as well as in the allergic immune response, we employed the gene expression-based leukocyte deconvolution algorithm – Cibersort (CS). This algorithm resolves the involved immune cell subsets based on the transcriptomic profile in a deeper and detailed fashion. First, to validate the CS algorithm on our samples, we utilized flow cytometry analysis to determine the relative fraction of CD4+ T cells, CD8+ T cells, CD14+ monocytes and CD56+ natural killer cells and correlated them with the estimated cell fractions from the respective leukocyte subsets of the CS algorithm. Indeed, the estimated cell fraction numbers of the CS algorithm correlated with the relative fractions of CD4+ T cells, CD8+ T cells and CD56+ natural killer cells as determined by flow cytometry. The CS results were further used for downstream analysis (Figure 3A/B). Overall, the cellular fractions were marginally affected at baseline (Figure 3C). Sensitized patients displayed higher fractions of CD4+ and CD8+ T cells but lower estimated fractions of M1 macrophages and activated mast cells (Suppl. Figure 1).

CD4 T cells are known to be major players in allergic immune reactions, eliciting a pronounced Th2-related immune response. Indeed, major Th2 cytokines, IL4, IL13, were elevated after Bet v 1 stimulation in the BA patients. In contrast, Th1 cytokine IFNG and regulatory IL10 were down-regulated at the baseline level in the BA subjects compared with the NBA control subjects (Suppl. Figure 2). Nevertheless, Bet v 1-dependent stimulation led to higher estimated numbers of activated dendritic cells and monocytes in birch-allergic

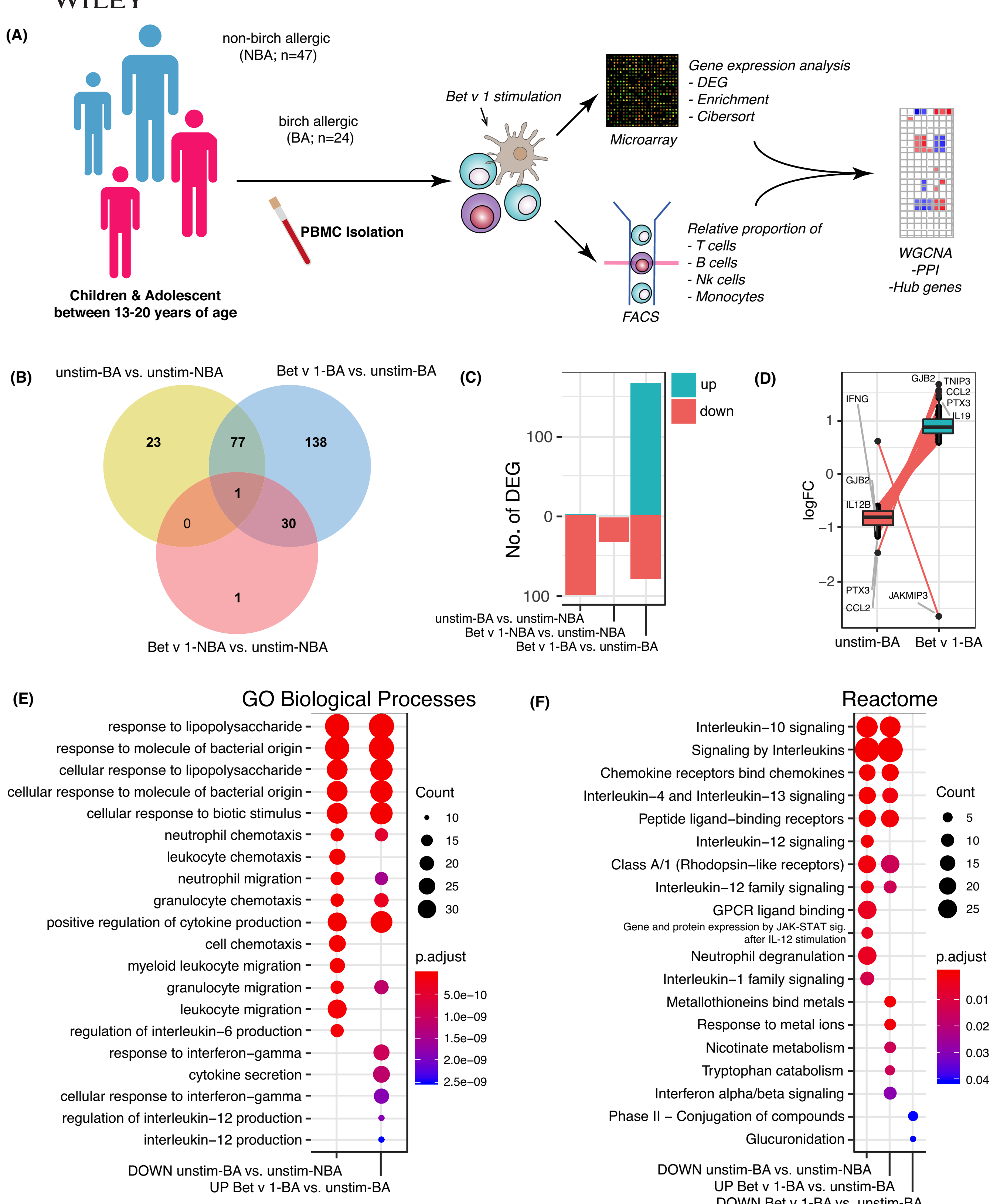

FIGURE 1 Differentially expressed genes of PBMCs from birch-allergic and non-birch-allergic subjects at baseline and six hours after Bet v 1 stimulation. (A) Study outline and graphical summary of the analysis pipeline. (B) Venn diagram representation to identify uniquely and shared DEGs between unstimulated non-birch-allergic (unstim-NBA), unstimulated birch-allergic (unstim-BA), Bet v 1 non-birch-allergic (Bet v 1-NBA) and Bet v 1 birch-allergic (Bet v 1-BA) conditions. (C) Significantly up- (green) and downregulated (red) genes between different conditions. (D) Fold change of shared genes between unstim-BA vs. unstim-NBA and Bet v 1-BA vs. unstim-BA. Red lines indicate changes from unique genes. (E) ClusterProfiler and ReactomePA was used to identify GO biological processes and (F) Reactome pathway enrichment of differently expressed genes

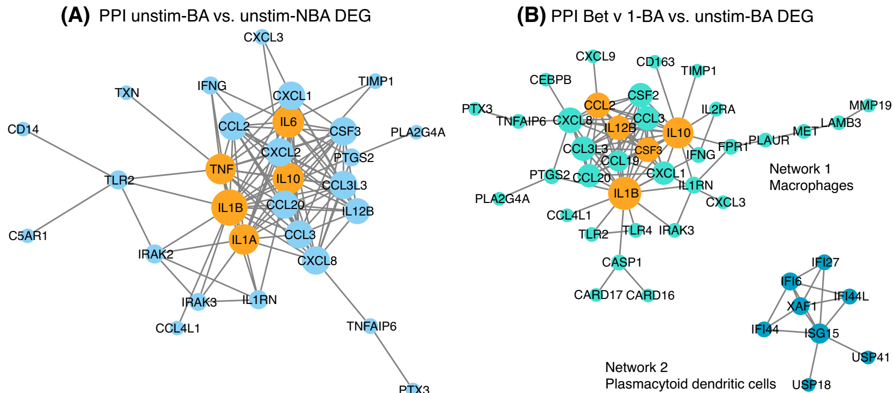

FIGURE 2 Protein–Protein interaction (PPI) networks of differentially expressed genes. PPI networks were built employing the STRING online database (V11.5) and visualized using Cytoscape. (A) PPI of differentially expressed genes between baseline expression of non-birch-allergic (NBA) and birch-allergic (BA) patients. Hub genes are highlighted in orange. (B) Differentially expressed genes of Bet v 1-stimulated PBMCs of BA patients form 2 distinct networks (Network 1= turquoise, Network 2= cyan). The ARCHS4 Tissue database was used to associate networks with cell types. All PPI networks showed an PPI enrichment p-value of $p < 1 \times 10^{-16}$

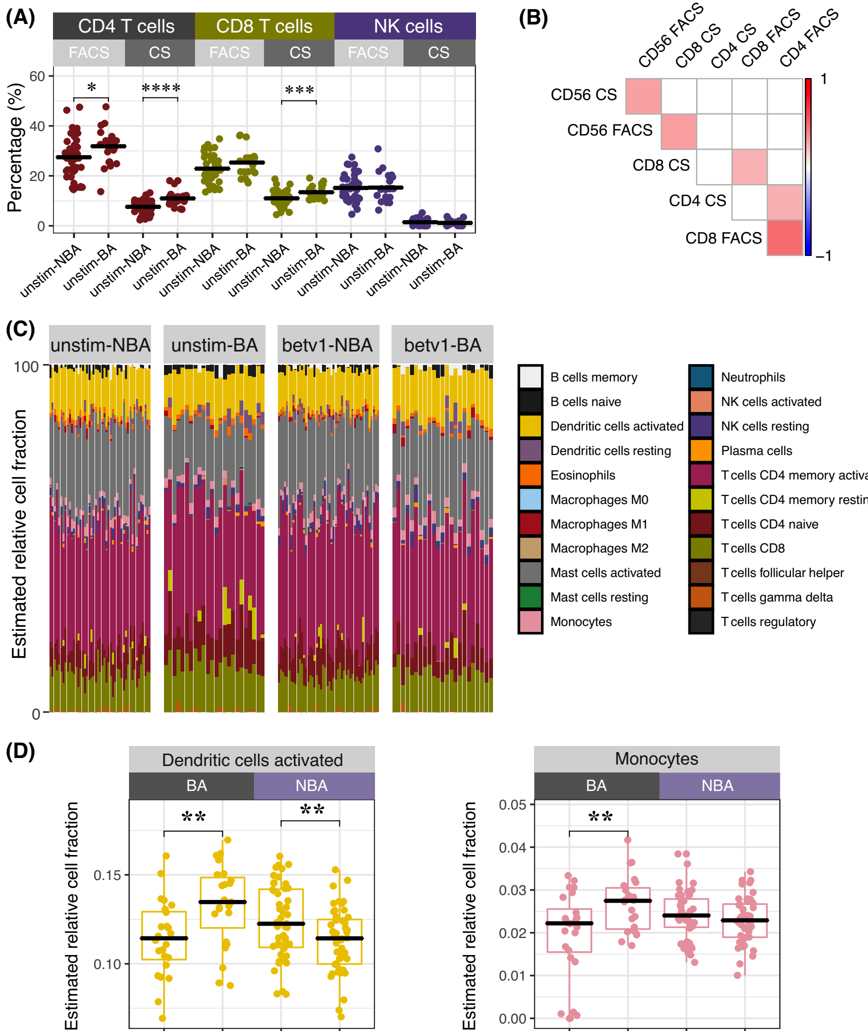

FIGURE 3 Leukocyte composition in non-allergic and allergic patients determined by leukocyte deconvolution algorithm and flow cytometry. (A) Relative cell numbers of flow cytometry experiments and estimated cell fractions of the Cibersort (CS) algorithm were compared to assess congruency among the two methods. (B) Furthermore, estimated cell fractions were correlated with measured relative cell fractions of $CD4^{+}$, $CD8^{+}$ and $CD56^{+}$ cells via flow cytometry. Colour represents the correlation coefficient. Only positive correlations with a p-value <.01 are shown. (C) Estimated cell fractions from transcriptome wide gene expression signatures using the Cibersort (CS) algorithm. (D) Significant leukocyte changes at baseline and after Bet v 1 stimulation in birch-allergic patients and non-allergic controls. *p<.05, **p<.01, ***p<.001, ****p<.0001

compared with NBA subjects, further indicating an important role of these leukocyte subsets during Bet v 1 engagement in allergic individuals (Figure 3D).

3.4 | Co-expression networks identify Bet v 1-stimulation-induced innate immunity and antiviral gene modules in birch-allergic patients

To further investigate transcriptional changes in BA subjects, weighted gene correlation network analysis (WGCNA) was utilized to cluster highly correlating genes, based on their expression into respective modules. Additionally, the resulting modules can be correlated to external traits as well as used for further downstream analysis. At baseline, WGCNA detected a total of 7 modules; 3 related to the BA and 1 related to the NBA phenotype. Furthermore, estimated cell fractions from the CS algorithm were used to identify associated cell subsets with each module. BA-related modules (turquoise, brown, yellow, Figure 4A) were mainly associated with naïve and resting memory CD4+ T cells as well as CD8+ T cells. Conversely, the NBA phenotype-related blue module was associated with innate immune cells such as dendritic cells, monocytes, macrophages and activated mast cells (Figure 4A). Pathway analysis of the individual modules revealed distinct functional properties as summarized in Figure 4B. Modules were annotated according to the related genes and functional enrichment. The Top200 genes of each module, ranked by the module membership, were used to create PPI networks to recognize hub genes as described previously. The blue module contained innate-related genes, forming a dense network around IL1B, IL1R1, IL1RN, IL10 and IRAK2 as hub genes (Figure 4C). In BA subjects, WGCNA identified a translational (turquoise) and epigenetic (yellow) module, associated with naïve and resting memory CD4+ T cells as well as CD8+ T cells (Figure 4D/E).

In parallel, WGCNA of Bet v 1-stimulated BA PBMCs revealed a total of 8 modules; 3 related to Bet v 1 stimulation and 2 related to the unstimulated BA phenotype. Bet v 1-related modules (brown, yellow, green) were associated with monocytes, activated dendritic cells and activated mast cells (Figure 5A). Unstimulated modules showed similar pathway enrichment as described above. Hence, the stimulation associated brown and green modules displayed enrichment for innate-related immune responses as well as antiviral-immune responses, respectively (Figure 5B). Thus, the brown module represents the “innate response module” with IL1R1, IL1RN, IL10, SRC and LYN as hub genes (Figure 5C) and the green module is denoted as “viral response module” with ISG15, IFIT1, IFIT3, MX1 and RSAD2 as hub genes (Figure 5D). Overall, we found no association of detected gene modules with total serum or birch-specific IgE levels in the investigated subjects (Figure 4A/Figure 5A).

4 | DISCUSSION

Determining related pathways by which the major birch pollen allergen, Bet v 1, is uniquely triggering the immune systems in birch-allergic individuals is crucial to pinpoint immune checkpoints. Therefore, we conducted transcriptome network analysis of PBMCs from a cohort of BA and non-allergic individuals after Bet v 1 stimulation to dissect related immune responses. Previous transcriptomic studies mainly focused on the nasal or bronchial epithelial cell responses in allergic asthma patients.[25,50,51] Although these studies provide novel insights in disease mechanisms and progression, we were interested how Bet v 1 directly influence peripheral blood immune cells. Our results provide a systems-level perspective on the molecular network response after Bet v 1 encountering of PBMCs from birch-allergic individuals.

Our findings highlight the critical role of innate immune pathways in allergic diseases, adding novel information into the pathogenesis of the elicitation phase of allergen encounter. One key result is the involvement of innate immune pathways associated with dendritic cells, monocytes/macrophages and mast cells. Due to their location in mucous membranes, skin, respiratory tract and blood, they encounter allergens first-line initiating and augmenting consequent immune responses.[13,52-54] Antigen-presenting cells (APC), especially DCs, play a pivotal role during the sensitization and elicitation phase by propagation of Th2-biased immune responses.[55] Based on the transcriptomic profile, we estimated higher DC and monocyte fractions after Bet v 1 stimulation in allergic individuals but not in non-allergic probands, highlighting significant differences in the cellular response between those groups. Furthermore, those observed cell fractions were linked to innate response modules in allergic patients. This distinctive feature of APCs in allergic diseases is underlined in recent in-vitro studies, highlighting the unique property of DCs from allergic individuals, but not of DCs from non-allergic individuals, to elicit a response against Bet v 1.[12] Additionally, only DCs from allergic individuals were able to induce a Th2 response in autologous T helper cells compared to non-allergic controls.[11] Furthermore, a recent proteomics study of human monocyte-derived dendritic cells showed similar activation patterns of important regulatory proteins such as MX1 or IFIT3,[27] underlining the importance of our detected hub genes.

In recent years, the role of TLRs in allergic diseases received considerable attention. The “hygiene hypothesis” indicates that microbial structures interrogate with their corresponding TLR that, in turn, might exert protective effects against allergic diseases. In our study, we identified important TLRs (TLR2, TLR4) and related pathways to be associated with the allergic phenotype. Recently, the lipopolysaccharide (LPS) – TLR4 axis has gained particular interest, since pathway activation can have a negative or positive impact on the development of allergic diseases.[56-58] Additionally, genetic polymorphisms of CD14, TLR2 and TLR4 are linked towards atopy, asthma and IgE levels, highlighting the high relevance of those pathways in allergic responses.[59-61]

It has been long known that respiratory virus infections are associated with asthma exacerbation.[14-16] Recent study by Gilles et al[20] also suggests a role for pollen allergens in the modulation of respiratory epithelia-mediated antiviral immunity.[20] Authors reported that reduced phosphorylation and translocation of interferon-related

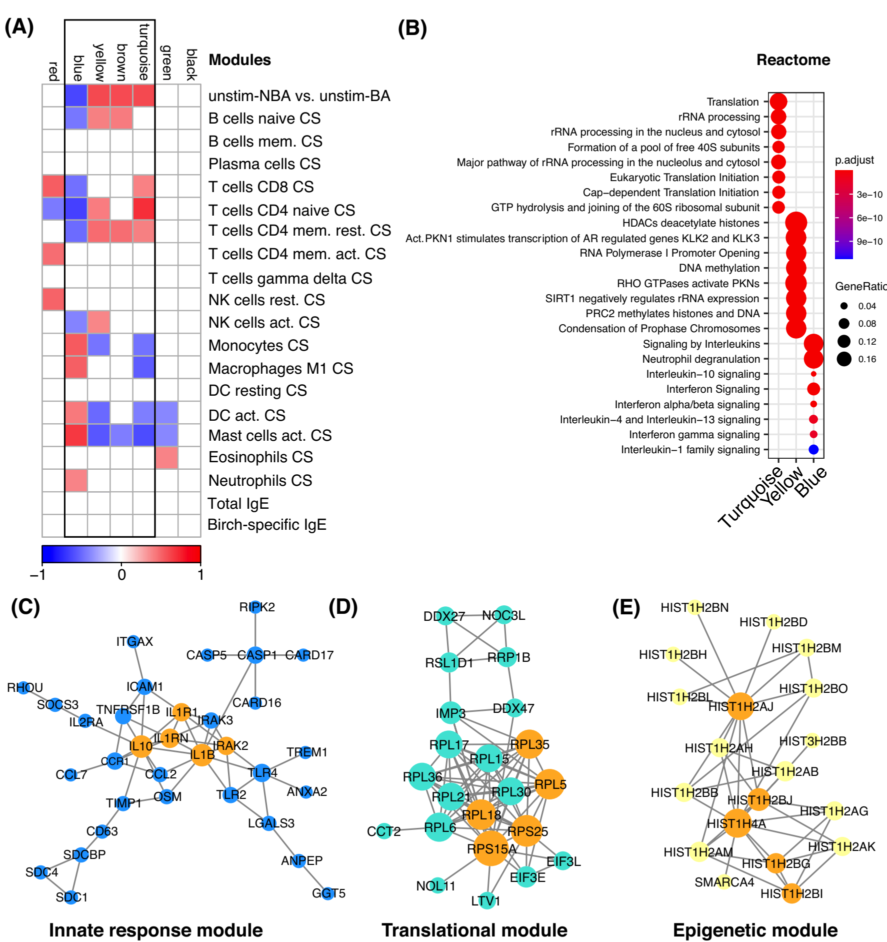

FIGURE 4 Co-expression networks of non-birch-allergic and birch-allergic patients at baseline. (A) Co-expression network modules were identified using WGCNA between unstimulated non-birch-allergic (unstim-NBA) and unstimulated birch-allergic (unstim-BA) patients. Modules were linked to the estimated cell fractions determined by the Cibersort (CS) leukocyte deconvolution algorithm. Colour represents the correlation coefficient. Only correlations with a *p*-value<.001 are shown. (B) Modules correlating with the phenotype were further analysed for pathway enrichment using ReactomePA. (C/D/E) Protein–Protein Interaction (PPI) networks of enriched modules were built with STRING database (V11.5) and visualized using Cytoscape. The CytoHubba plug-in for Cytoscape was used to determine hub genes within each module, which are highlighted in orange. Networks were built with the top 200 genes with the highest module membership. Module names have been chosen according to involved genes, enriched pathways and hub genes. All PPI networks showed an enrichment value of $p < 1 \times 10^{-10}$

genes by the exposure to pollen during viral infections reduces the release of pro-inflammatory chemokines and type I and III interferons. However, pollen-mediated effect was similar both in atopic and nonatopic-derived donors arguing that a mechanism is independent of the atopy trait. In this study, we identified exaggerated antiviral response related to Bet v 1 stimulation in the blood immune cells of birch-allergic patients. Contrary to Gilles et al. Bet v 1-mediated induction of antiviral-type response was clearly restricted to birch sensitization since no response was seen in subjects without birch pollen sensitization. In line with this, we have previously reported in patients with seasonal allergic rhinitis using proteomics that an exaggerated antiviral response is triggered in respiratory epithelia upon seasonal exposure to environmental stimuli.[62] We may speculate that over-activated antiviral-type response in birch-allergic patients during pollen season may lead to exaggerated inflammatory reactions that contribute to the disease phenotype.

The involved antiviral genes in this study were linked to plasmacytoid dendritic cells (pDCs) according to the ARCHS4 tissue database. Different clinical and in vitro studies have shown a significant role of pDCs in allergic diseases. Overall, those studies indicate the crucial role of pDCs to fine-tune immunological tolerance in allergy.[52,63] For example, pDC numbers in infancy seem to inversely correlate with asthma development in the first years of life.[64] Furthermore, it seems that a certain pDC subset expresses functional FcεRI, and that those receptor expression levels depend on the atopic state and IgE serum heights of the patient. IgE-Bet v 1 complex-loaded pDCs induce a Th2-biased immune response in T helper cells, leading to reduced type I IFN production, IL-10 production and pDC apoptosis.[65] Although we found no correlation of IgE serum value to functional and cellular responses, our data suggest that initial pDC activation might be involved in the early allergic immune response, but we cannot conclude the “fate” of pDCs after

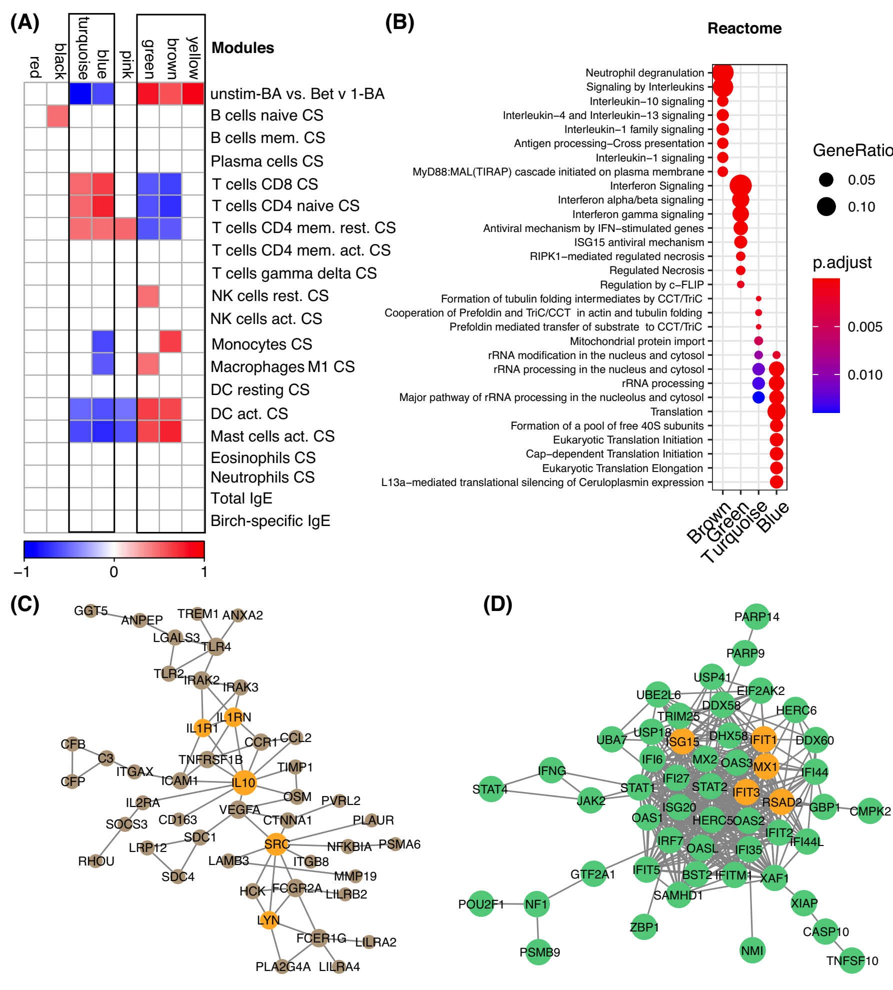

FIGURE 5 Co-expression networks of birch-allergic patients at baseline and after Bet v 1 stimulation. (A) Co-expression network modules were identified using WGCNA between unstimulated birch-allergic (unstim-BA) and Bet v 1-stimulated BA (Bet v 1-BA) patients. Modules were linked to the estimated cell fractions determined by the Cibersort (CS) leukocyte deconvolution. Colour represents the correlation coefficient. Only correlations with a p-value<.001 are shown. (B) Modules correlating with the phenotype were further analysed for pathway enrichment using ReactomePA. (C/D) Protein–Protein Interaction (PPI) networks of enriched modules were built with STRING database (V11.5) and visualized using Cytoscape. The CytoHubba plug-in for Cytoscape was used to determine hub genes within each module, which are highlighted in orange. Networks were built with the top 200 genes with the highest module membership. Module names have been chosen according to involved genes, enriched pathways and hub genes. All PPI networks showed an enrichment value of $p < 1 \times 10^{-12}$

prolonged allergen stimulation. However, we underline the potential role of pDCs and associated viral immune responses in birch-allergic patients. Furthermore, some.

Memory T cells are key players in allergic diseases. In case of allergic asthma, tissue-resident T memory cells primarily respond to inhaled allergens resulting in eosinophilic airway inflammation and mucus hypersecretion.[66] This specific T cell subset seems to be distinct from circulating T memory cells in sense of cell surface markers and the inability to recirculate into the blood stream.[67] Furthermore, tissue-resident T memory cells reside for a lifetime in lung tissue and exert "allergenic memory" upon re-stimulation in mice.[68] In our dataset, stimulation of the PBMC with Bet v 1 induced expression of IL4 and IL13 only in birch-pollen-allergic subjects. In line with this, Th1 response (IFNG) and regulatory response (IL10) were suppressed at the baseline level of BA patients. However, birch-allergic individuals showed higher IL-10 expression after Bet v 1 stimulation compared to non-allergic individuals. If this observation is due to epigenetic changes or a counter-balancing immune response is currently unclear und needs to be elucidated in further studies. Thus, classical allergen-induced Th2 response was restricted to individuals with specific IgE antibodies to birch pollen allergens, which is in line with previous studies.[12,62] Interestingly, estimated cell fractions of CD4 helper and memory cells were associated with translational and epigenetic modules in BA patients. Nevertheless, a longer stimulation time would have given us different insides into the "later" T cell-associated responses, but this was not the focus of our study.

Epigenetic mechanisms in allergic disorders have been elucidated in recent years. In asthmatic children, several genes involved in T cell immunity and maturation, including RUNX3, IL4 and IL13, showed different methylation patterns.[69] Another study identified epigenetic variations of NF-kB-related MAPK signalling cascades in CD4 T cells in children with IgE-mediated food allergy.[70] An interesting hypothesis in allergic diseases is the potential role of epigenetic modification of innate immune cells via "trained immunity," resulting in distorted immune responses after antigen encounter.[71] Another example of epigenetic modification of innate immune checkpoint might be the influence of CD14 promoter region in placental tissue, showing a relationship between environmental factors (e.g. farm-live) on CD14 receptor methylation and mRNA expression.[72] Thus, underlying epigenetic mechanisms might indicate the differential expression as well as upregulation of a specific gene set in BA patients at baseline as well as after Bet v 1 encounter. Nevertheless, epigenetic modifications in BA patients needs to be further elucidated.

Taken together, we comprehensively profiled transcriptomic changes during *ex vivo* Bet v 1 stimulation of PBMCs derived from

a random Finnish cohort including BA and non-allergic individuals. Employing an integrative data-driven approach, we identified important hub genes, corresponding pathways and involved cell types associated with the acute Bet v 1 response. Our results highlight important gene networks related to macrophage and dendritic cell responses during the early Bet v 1 response in birch-allergic individuals. Those allergic innate immune and antiviral networks reveal interesting targets to study further roles of these key players in sensitization and clinical course, with the potential to unravel new strategies for allergy prevention and treatment.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

L.W. contributed in study design, data-analysis, data interpretation, figures and writing; N.F. contributed in data generation and writing; T.L. contributed in cohort design, data collection and manuscript commenting; A.B. contributed the manuscript commenting; P.K. contributed in data generation and manuscript commenting; J.N. contributed in data interpretation and manuscript commenting; L.P contributed clinical data analysis and manuscript commenting T.H. contributed in study design, and writing; H.A. contributed in study design, data collection, data-analysis, data interpretation, figures and writing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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