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ORIGINAL ARTICLE

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Whole-blood CD3/CD28-stimulated cytokine responses in children with preschool wheeze, asthma, and healthy controls

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Abstract

Background: Childhood asthma and wheezing disorders are heterogeneous conditions that may be reflected by distinct immune response patterns. In this study, we investigated whether CD3/CD28-stimulated cytokine responses differ between children with asthma, preschool wheeze, and healthy controls.

Methods: Baseline samples from 511 children in the All Age Asthma cohort (ALLIANCE; 196 children with asthma, 181 with preschool wheeze, and 134 healthy controls) were analyzed. Whole blood was stimulated with anti-CD3/CD28 antibodies for 48 h, and 37 cytokines were quantified using multiplex immunoassays. Cytokine co-variation patterns were assessed using principal component analysis (PCA). Co-regulated cytokine modules were identified using CytoMod, and associations with clinical characteristics were examined using linear and logistic regression models.

Results: Two cytokines, sCD30 and sCD163, showed significant age-related decreases ($\beta = -.07$ (95% CI −0.09 to −0.06), padj = 1.2e-14; and $\beta = -.03$ (95% CI −0.04 to −0.01), padj = .018, respectively). After correction for multiple testing, no individual cytokine differed significantly between children with asthma, preschool wheeze, or healthy controls. PCA demonstrated highly conserved cytokine co-variation patterns across groups (Spearman's rho >.9 for the first principal component loadings). Modular analysis identified six distinct co-regulatory modules after adjustment for background cytokine levels. Associations between module expression and clinical characteristics were generally modest. Module 1, comprising cytokines associated with tissue remodeling and IL-6 signaling, was inversely

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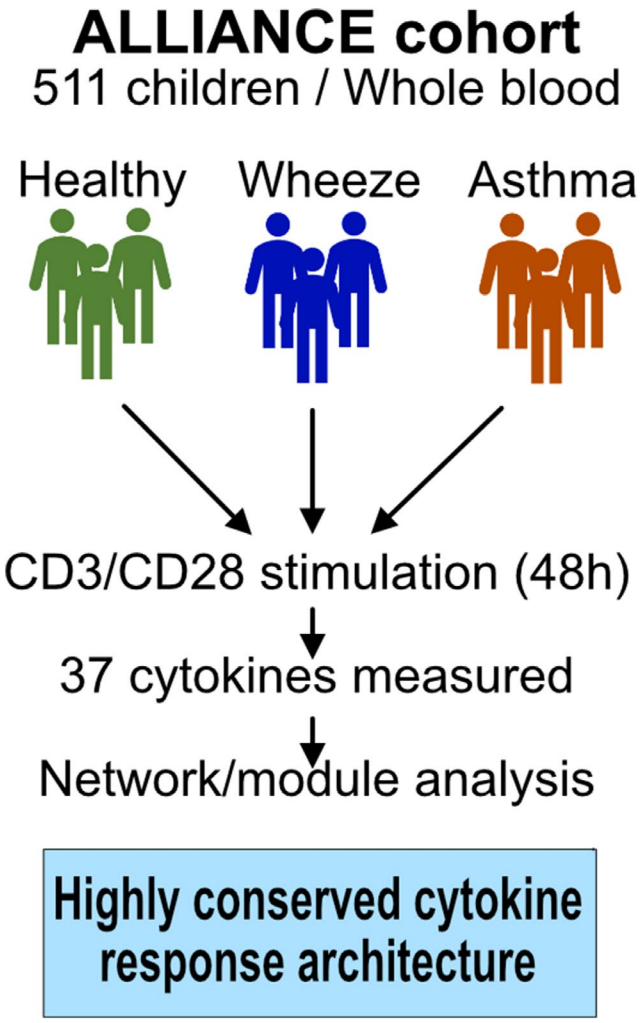
associated with asthma in children aged ≥6 years (OR = 0.63 (95% CI 0.46–0.85), *p*adj = .017). However, this association attenuated in age-matched sensitivity analyses (OR = 0.72 (95% CI 0.51–0.99), *p*adj = .281), suggesting residual age-related confounding.

Conclusions: CD3/CD28-stimulated cytokine responses show highly conserved T-cell activation networks across pediatric asthma, preschool wheeze, and health, with limited ability to distinguish between clinical groups at the single-cytokine level. Nevertheless, modular network analyses identify coordinated immune patterns associated with clinical features, highlighting the potential value of immune profiling in refining biological phenotyping and advancing the understanding of immune heterogeneity in pediatric airway disease.

KEYWORDS
asthma, cytokine modules, cytokines, multiplex, pediatric



GRAPHICAL ABSTRACT

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.

Multi-cytokine profiling of 37 cytokines following 48 h CD3/CD28 stimulation in whole blood from 511 children of the ALLIANCE cohort (healthy, preschool wheeze, asthma) demonstrates a highly conserved cytokine response architecture across clinical groups.

1 | INTRODUCTION

Childhood wheeze and asthma are among the most common airway disorders in children, with varying immunological features, clinical presentations, treatment responses, and long-term outcomes.[1] While preschool wheeze often represents a transient and clinically heterogeneous condition, asthma is typically characterized by chronic airway inflammation and may persist into later childhood and adulthood.[2]

T cells play a central role in the immunopathology of asthma. In asthma, CD4+ T helper cells, particularly those of the Th2 subtype, drive allergic airway inflammation through the production of cytokines such as IL-4, IL-5, and IL-13, which promote IgE class switching, eosinophil recruitment, and airway hyperresponsiveness.[3] Beyond the classical Th2 paradigm, Th1, Th17, and regulatory T cell (Treg) subsets have also been implicated in non-allergic and more severe asthma phenotypes, underscoring the broad involvement of T-cell responses across disease subtypes.[3] In preschool wheeze, much less is known about the role of T cells; however, an important role of Tregs is suspected[4] and Th2 phenotypes are found even in very young children.[5]

CD3/CD28 co-stimulation provides a polyclonal, antigen-independent activation signal that engages the broader T-cell compartment and therefore offers a practical approach to assess overall T-cell cytokine response capacity. In contrast to allergen-specific stimulation, which reflects sensitization to particular environmental triggers, CD3/CD28 stimulation enables the assessment of a broader T-cell effector potential across participants independent of known allergen exposure. Given that asthma involves a broader perturbation of the T cell compartment affecting a range of T cell subsets, we hypothesized that polyclonal T-cell activation may reveal disease-associated differences in the overall cytokine response profile. CD3/CD28 stimulation has previously been used to characterize global adaptive cytokine responses in the context of environmental exposures,[6] healthy population cohorts,[7] and infectious diseases such as tuberculosis.[8] However, its application in pediatric asthma and preschool wheeze remains limited, and it is currently unclear whether global T-cell activation patterns differ across these clinical phenotypes.

In this study, we therefore aimed to address the following related questions: First, we examined the influence of age and sex on CD3/CD28-induced cytokine responses in healthy children, given the well-documented age dependency of immune maturation during childhood[9,10] and the scarcity of data on cytokine responses following broad T-cell stimulation responses in healthy pediatric populations. Second, we compared cytokine profiles between children with preschool wheeze, asthma, and healthy controls to assess whether global T-cell activation response patterns differ across these phenotypes. Third, because coordinated cytokine responses may provide additional biological insight beyond single-analyte comparisons, we sought to identify co-regulated cytokine modules and evaluate their associations with clinical characteristics in childhood asthma.

Key message

Cytokine profiling of immune responses stimulated by CD3/CD28 reveals largely shared patterns across healthy children and those with preschool wheeze and asthma. This provides insight into T cell activation networks while demonstrating limited differences in cytokine responses across clinical groups, particularly in mild pediatric asthma.

2 | METHODS

2.1 | Study population

The All Age Asthma cohort (ALLIANCE) of the German Center for Lung Research (DZL) is a prospective asthma cohort conducted at five pediatric tertiary centers (Hannover, Lubeck, Munich, Marburg, and Cologne) and two adult specialist centers (LungenClinic Grosshansdorf and Research Center Borstel). The study was registered at ClinicalTrials.gov (pediatric arm NCT02496468, adult arm NCT02419274).[5,11] Details regarding study design and definition of clinical variables have been described elsewhere.[11]

The present study was conducted within the pediatric arm of the ALLIANCE study. The pediatric arm stratified children into three study groups: children with asthma (aged 6–18 years with physician-diagnosed asthma according to GINA guidelines), children with recurrent wheezing (younger than 6 years with at least two wheezing episodes within the preceding 12 months), and healthy controls (no history of asthma or preschool wheezing). All participants underwent comprehensive clinical assessment including medical history, physical examination, lung function testing (if aged 6 years and older), and blood sampling. Written informed consent was obtained from the parents or legal guardians of children younger than 8 years, and from both the parent/guardian and the child for participants aged 8 years or older. The study was conducted in accordance with the Declaration of Helsinki and approved by the relevant local institutional ethics committees.

2.2 | Whole blood stimulation, sample selection, and multiplex cytokine measurements

Whole blood samples were collected into TruCulture tubes (RBM, Austin/Tx, USA) preloaded with anti-CD3/CD28 antibodies. Baseline whole blood samples were sourced from all ALLIANCE study participants with available clinical data at the time of measurement. Of 568 samples, 544 were included; excluded children were mainly siblings of included participants to minimize statistical dependency, as well as those with limited availability of paired biomaterial for other multi-omics analyses. Samples were stratified across plates by study group (healthy, preschool wheeze, and asthma), age group (<6 and ≥6 years), and study site to ensure

balanced plate composition and minimize batch effects. All samples were incubated at 37°C for 48 h according to the manufacturer's instructions. Following incubation, cell culture supernatants were harvested and stored at −80°C until analysis. Cytokine concentrations were quantified using the Bio-Plex Pro™ Human Inflammation Panel (37-plex, Bio-Rad Laboratories, Inc., Hercules/CA, USA) on a Luminex xMAP platform (Luminex, Austin/Tx, USA), following the manufacturer's protocol. The following analytes were analyzed: APRIL (TNFSF13), BAFF (TNFSF13B), sCD30 (TNFRSF8), sCD163, Chitinase-3-like 1, gp130/sIL-6Rβ, IFN-α2, IFN-β, IFN-γ, IL-2, sIL-6Rα, IL-8, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-19, IL-20, IL-22, IL-26, IL-27 (p28), IL-28A/IFN-λ2, IL-29/IFN-λ1, IL-32, IL-34, IL-35, TNFSF14 (LIGHT), MMP-1, MMP-2, MMP-3, Osteocalcin, Osteopontin, Pentraxin-3, sTNF-R1, sTNF-R2, TSLP, and TNFSF12/TWEAK. Detailed information regarding factors such as curve fitting, lower and upper limits of quantification, inter- and intra-assay coefficients of variation, and lot numbers can be found in the Supplement Methods section: Appendix S1 and Tables S1–S3. Median cytokine concentrations for all study groups are provided in Table S4. Principal component analysis stratified by recruitment site did not reveal distinct clustering or systematic site-specific bias (Figure S1A), but was nevertheless included as a covariate in multivariable regression models to ensure robustness of our findings. As the TruCulture whole-blood assay preserves the cellular milieu, the resulting cytokine measurements reflect system-level immune responses, encompassing contributions from cellular abundance, composition, and per-cell functional capacity.

2.3 | Data analysis and statistics

Statistical analyses followed the order of the study objectives: age and sex effects were first characterized in healthy controls to investigate reference patterns, followed by cross-group comparisons, network-based modular analysis, and finally association testing of module scores with clinical traits. Cytokine values were log-transformed prior to analysis to approximate a normal distribution. Age and sex effects within healthy controls were assessed using linear regression models, with age, sex, and recruitment site as independent variables and log-transformed cytokines levels as dependent variables. The covariate structure and rationale is depicted in Figure S1B. Study group effects (asthma and preschool wheeze vs. age-stratified healthy controls, respectively) were assessed using linear regression models adjusted for age, sex, and recruitment site. To assess multivariate predictive utility, LASSO logistic regression models were trained using all 37 cytokine analytes and evaluated via nested 5-fold cross-validation. Discriminatory performance was reported as AUROC with 95% CI; calibration was assessed using the Brier score relative to a null reference. Principal component analyses examined patterns of cytokine variation across study groups to ensure that common cytokine modules in the following analysis were meaningful (see also[12]). The similarity of PCA axes across groups was quantified using Spearman correlation between principal component loadings. CytoMod was used to identify modules of co-regulated cytokines based on pairwise Spearman correlation matrices.[13] Bootstrapping assessed the stability of identified modules, and the optimal number of clusters was determined using the Tibshirani gap statistic. Module scores were calculated as the mean of cytokine concentrations within each module. Analysis was conducted on both absolute cytokine levels and relative levels. Relative levels were derived as residuals from a linear regression of each cytokine's concentration against the participant's mean global cytokine burden, effectively obtaining cytokine levels adjusted for overall background cytokine levels within each participant.[13] Specifically, for each individual, the mean concentration across all log-transformed cytokines was calculated to serve as the predictor in a linear regression model for each cytokine j: $\text{Cytokine}_{j,\text{expected}} = \beta_{0j} + \beta_{1j}{}^{*}\ \text{Mean} + e_j$. The background-adjusted level was defined as the residual from this model (i.e., $\text{Cytokine}_{j,\text{adjusted}} = \text{Cytokine}_{j,\text{measured}} - \text{Cytokine}_{j,\text{expected}}$), representing the unexplained deviation of that cytokine from the expected level, given the average cytokine level in this individual (see also reference[13] for details). Residuals were standardized to mean zero and unit variance prior to calculating module scores. Associations between module scores and clinical traits were tested using linear regression for continuous variables and logistic regression for binary variables. As sensitivity analyses, age-matched analyses as well as analysis with models incorporating natural cubic spline terms for age (df = 3) and an age-by-module interaction terms were performed. Analyses were performed both unadjusted and adjusted for age, sex, and recruitment site. In all calculations, multiple testing correction was applied using the Benjamini-Hochberg procedure. Analyses were performed in R (version 4.4) and Python (version 3.10).[14,15]

3 | RESULTS

3.1 | Study design and patient characteristics

For this analysis, we included 511 baseline whole blood samples with matching clinical data: from 134 healthy controls, 181 children with recurrent wheezing, and 196 children with asthma (Table 1). As expected from the study design, the median age differed substantially between groups, with the preschool wheeze group by definition being younger than the asthma group and the healthy control group as well. The asthma group had the highest prevalence of atopic characteristics and elevated eosinophil counts, followed by the wheezing group, and the lowest prevalence was observed in the healthy control group. Similarly, lung function (FEV1, z score) was reduced in the asthma group compared to the control group, and FeNO values were elevated. Samples for this specific study were recruited at three centers within the ALLIANCE consortium (Hannover, Lübeck, Munich), with the proportion of healthy controls, children with preschool wheeze, and children with asthma varying across sites (see Table S5 for demographics by site).

TABLE 1 Clinical characteristics of the study population.

	Healthy ($n=134$)	Preschool Wheeze ($n=181$)	Asthma ($n=196$)	p Value
Age (years)	8.2 (IQR 5.0, 12.2)	3.1 (IQR 1.9, 4.3)	11.5 (IQR 8.7, 13.6)	<.001
Female sex	62 (47%)	53 (31%)	63 (33)	.011
Asthma family history	39 (29%)	79 (45%)	116 (59%)	<.001
Allergic rhinitis	7 (5%)	17 (10%)	93 (48%)	<.001
Atopic eczema	8 (6%)	37 (21%)	78 (40%)	<.001
Food allergy	2 (2%)	24 (13%)	51 (27%)	<.001
Atopy	33 (25%)	62 (36%)	153 (79%)	<.001
Eosinophils (/μL)	185 (IQR 117, 321)	336 (IQR 166, 575)	404 (IQR 236, 632)	<.001
Neutrophils (/μL)	3291 (IQR 2499, 4353)	3104 (IQR 2380, 3881)	3212 (IQR 2518, 4284)	.431
Basophils (/μL)	21 (IQR 0, 39)	40 (IQR 0, 39)	36 (IQR 7, 62)	<.001
Total IgE (U/ML)	26 (IQR 10, 100)	35 (IQR 10, 141)	211 (IQR 90, 486)	<.001
Salbutamol intake	—	120 (67%)	93 (48%)	—
ICS intake	—	37 (21%)	57 (30%)	—
FEV1 z score (ppm)[a]	0.01 (IQR −0.78, 0.65)	−0.31 (IQR −1.13, 0.14)	−0.61 (IQR −1.25, 0.14)	<.001
FeNO[b]	11.0 (IQR 6.4, 17.5)	12.9 (IQR 8.3, 26.0)	15.8 (IQR 9.5, 32.2)	.003
GINA control status				<.001
Healhty	134 (100%)	0 (0%)	0 (0%)	
Controlled	0 (0%)	67 (37%)	107 (55%)	
Partly controlled	0 (0%)	58 (32%)	70 (36%)	
Uncontrolled	0 (0%)	56 (31%)	19 (10%)	

[a]Lung function tests were performed in all participants aged ≥6 years. They were available in $n=34$ children (6 years old) with preschool wheeze and $n=10$ healthy controls.

[b]FENO measurements were generally only performed from 6 years, and therefore only available in a minority of the children with preschool wheeze ($n=13$) and healthy controls ($n=76$, with only 5 individuals below 6 years). p Values are derived from overall comparisons across the three study groups.

3.2 | Impact of age, sex, and disease group on CD3/CD28-induced cytokine responses

Since developmental changes in the immune system during childhood may substantially influence cytokine responses, we first aimed to characterize the effects of age and sex on CD3/CD28-induced cytokine responses in healthy controls, before comparing responses across clinical groups. Among the 37 cytokine analytes tested, two showed significant associations with age after correction for multiple testing: sCD30 declined markedly with increasing age ($\beta=-.07$ (95% CI −0.09 to −0.06), padj=1.2e-14), and sCD163 decreased significantly with age, albeit more moderately ($\beta=-.03$ (95% CI −0.04 to −0.01), padj=.018, Figure 1A, left panel, and Figure 1B). No statistically significant differences in cytokine levels were observed between sexes (Figure 1A, right panel). When comparing cytokine levels in children with preschool wheeze and asthma with those in age-stratified healthy controls, uncorrected comparisons yielded significant differences for chitinase-3-like-1, gp130, IL-8, IFN-ß, MMP-1, osteocalcin, and sIL-6Ra, but no difference in individual cytokine levels reached statistical significance after correction for multiple testing in models adjusted for age, sex, and site (Figure 1C). This indicates that CD3/CD28-induced cytokine responses measured after 48 h were largely similar across study groups. To evaluate whether multivariate cytokine signatures carried predictive utility, we trained a cross-validated LASSO logistic regression model using all 37 cytokine analytes to discriminate between children with preschool wheeze or asthma, respectively, and age-stratified healthy controls. Neither the model predicting preschool wheeze (AUC=0.57, 95% CI 0.47–0.67; Brier score 0.16, null=0.16) nor that predicting asthma (AUC=0.53, 95% CI 0.45–0.59; Brier score 0.22, null=0.22) demonstrated meaningful discriminatory performance, suggesting that the cytokine signature does not provide sufficient signal to reliably classify children with preschool wheeze or asthma in this cohort.

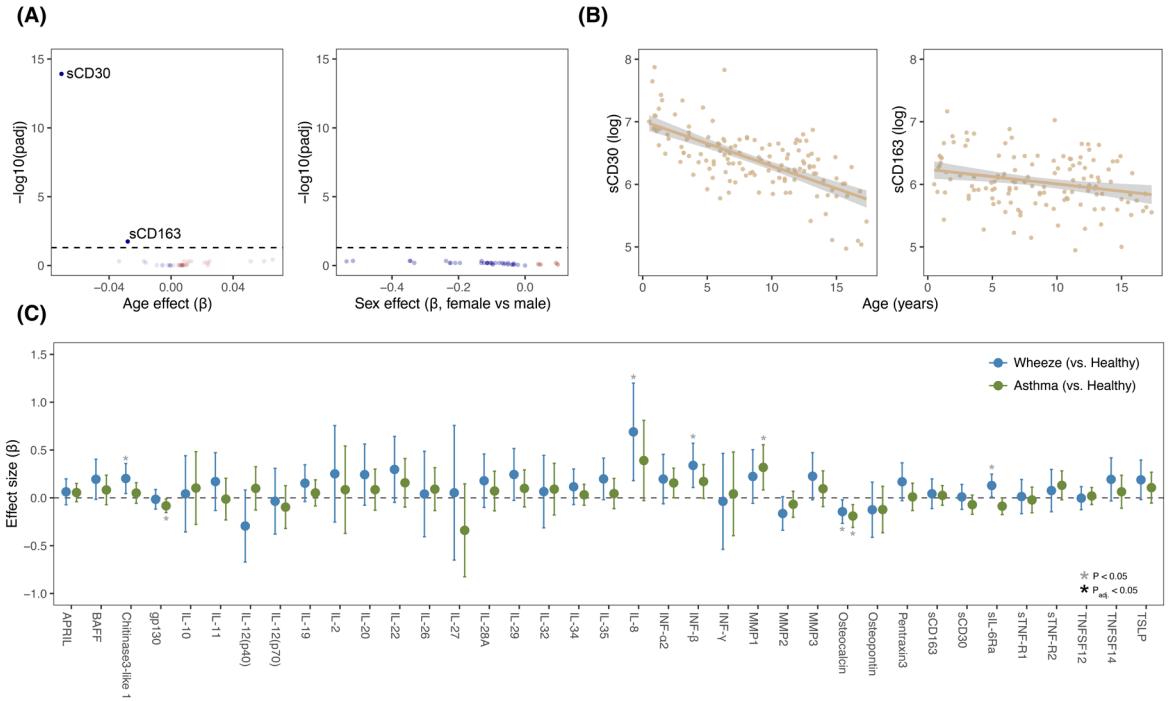


FIGURE 1 Age, sex, and disease group effects on CD3/CD28-induced cytokine responses. (A) Volcano plots showing age effects (left) and sex effects (right) on cytokine levels in healthy controls. The *x*-axis represents effect size (β coefficient) and *y*-axis shows −log10(adjusted *p*-value). Horizontal dashed line indicates significance threshold (padj <.05). (B) Scatter plots illustrating the relationship between age and log-transformed levels of sCD30 (left) and sCD163 (right) in healthy controls, with fitted regression lines and 95% confidence intervals. (C) Forest plot comparing cytokine levels between preschool wheeze versus healthy controls (blue) and asthma versus healthy controls (green). Effect sizes (β coefficients) with 95% confidence intervals from models adjusted for age, sex, and study site are shown for each cytokine. Gray asterisks indicate cytokines with unadjusted $p < .05$. Unadjusted *p* values refer to individual model tests prior to multiple testing correction, while adjusted *p*-values (padj) reflect multiple-testing correction across all 37 cytokines. No cytokines reached statistical significance after correction for multiple testing (padj <.05), indicating similar CD3/CD28-induced responses across study groups.

3.3 | Shared axes of cytokine variation across groups

In order to assess whether the underlying structure of cytokine co-variation was similar across study groups—a prerequisite for meaningful cross-group module comparisons—we performed independent principal component analyses (PCA) separately for healthy controls, children with preschool wheeze and children with asthma. PCA biplots revealed similar cytokine loading patterns across all three groups, with most cytokines clustering together and showing comparable vector orientations, particularly among the main contributors to the first principal component (see Figure 2A–C; Table S6). Notably, the loadings of the first two principal components were highly correlated between healthy controls and children with preschool wheeze (Spearman's rho = .94; $p = 2.2e\text{-}16$), as well as between healthy controls and children with asthma (Spearman's rho = .96; $p = 2.2e\text{-}16$). This indicated that the dominant patterns of cytokine co-variation are largely preserved across these groups (see Figure 2B). The first component explained most of the variance (>40%), whereas subsequent components captured less than 10% (Figure 2F).

These findings demonstrate a high degree of shared cytokine responses across groups and provide a rationale for network-based analyses across all study groups.

3.4 | Distinct co-regulatory networks under CD3/CD28 stimulation

To this end, we applied the CytoMod framework with the aim to identify coordinated cytokine modules. Using absolute cytokine values, most cytokines clustered into a single large module, reflecting a strong shared response signal under CD3/CD28 stimulation (Figure S2A). This global signal likely represented overall T cell activation but may mask finer co-regulatory patterns.

To uncover these more subtle networks, we adjusted cytokine values by the overall mean within each participant, as implemented in the CytoMod framework.[13] Using these adjusted values, six distinct modules emerged, highlighting coordinated patterns of cytokine co-variation beyond overall activation. Module sizes ranged from 2 to 9 cytokines (Table S7).

Certain modules showed particularly high reliability across bootstrapped datasets, including Module 1 (osteopontin, MMP2, osteocalcin, gp130, TNFSF12, sCD163, sCD30, and sIL-6Ra), Module 4 (Pentraxin3, IL-11, IL-29, TSLP, IL-20, MMP3, and IFN-β), and Module 5 (sTNFR1 and sTNFR2, Figure 3A). Several significant inverse module-module correlations were observed, such as negative correlations between Module 1 and Module 4/5, while Module 4 correlated with Module 6 in a positive direction (Figure 3B).

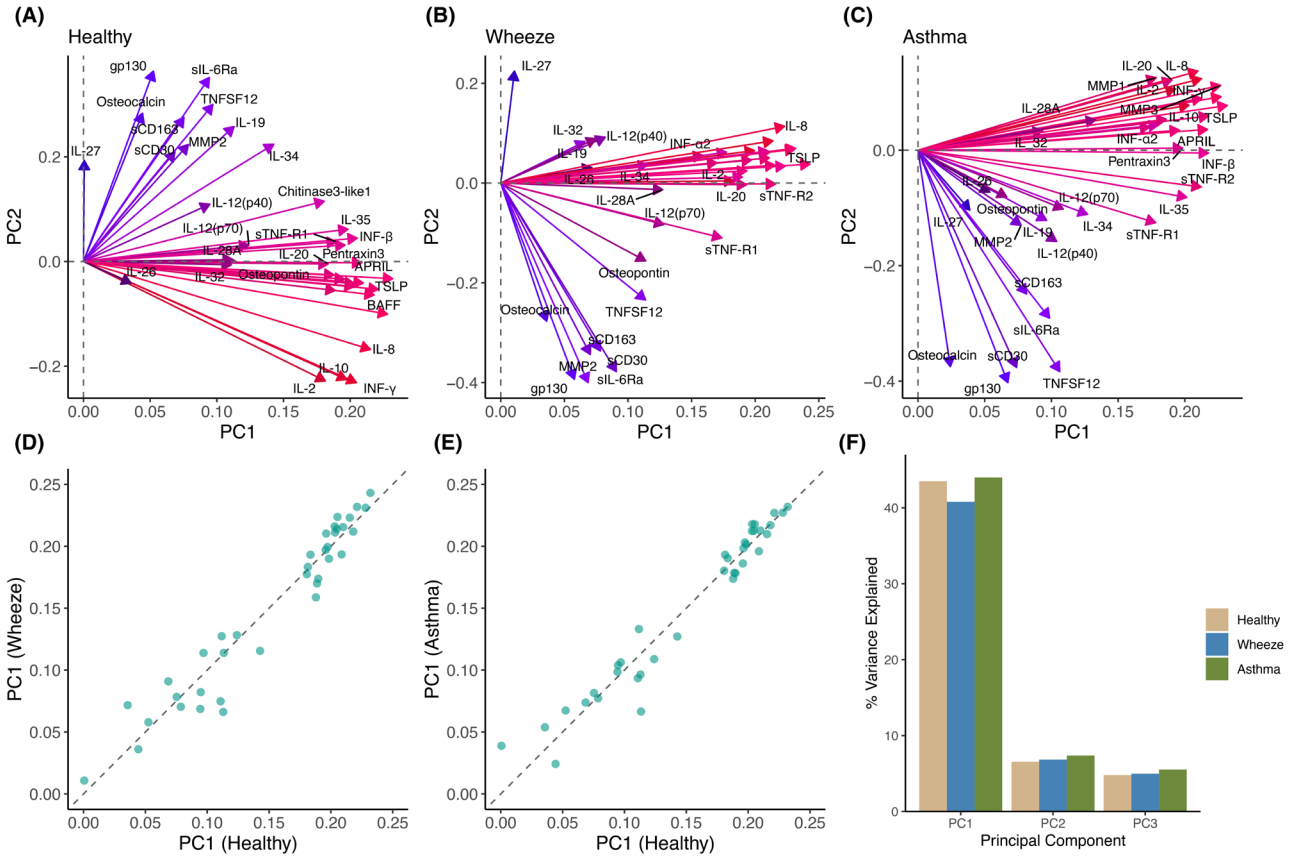


FIGURE 2 Shared axes of cytokine variation across healthy, preschool wheeze, and asthma groups. (A–C) Principal component analysis (PCA) biplots showing the first two principal components for healthy controls (A), preschool wheeze (B), and asthma (C) groups. Individual cytokines are represented as vectors, with vector length indicating contribution to each component. Inverse directions, as observed for some loadings in PC2 across groups, represent the same underlying pattern of co-variation. Arrow colors are kept consistent with A, with red indicating PC1, purple PC2, and black no association; similar color patterns thus reflect shared axis structures (see also[12]). (D, E) Correlation plots comparing PC1 loadings between healthy controls and preschool wheeze (D), and between healthy controls and asthma (E). (F) Bar chart showing percentage variance explained by the first three principal components across all study groups.

3.5 | Clinical associations of cytokine modules

Having identified co-varying cytokine modules, we next tested whether module expression scores were associated with study group membership and, within the asthma group, with specific clinical characteristics. No modules were significantly associated with preschool wheeze status in the younger cohort (<6 years, Figure 4A). Among children ≥6 years, Module 1 was inversely associated with asthma diagnosis compared with age-stratified healthy controls (OR = 0.63, p = .003, padj = .017) after adjustment for age, sex, and recruitment site (Figure 4B). This module included analytes such as sCD30 and sCD163, which showed strong age-related variation in previous analyses. However, although the asthma group was only slightly older than the corresponding control group of healthy children ≥6 years (median 11.5 years [IQR: 8.6–13.6] vs. 11.1 years [IQR: 8.1–13.1], Wilcoxon p = .324), residual confounding by age remained possible despite covariable adjustment. In a sensitivity analysis restricted to only age-matched subsets (n = 284), the association persisted at the nominal level (OR = 0.72 (95% CI 0.51–0.99), p = .047) but did not withstand correction for multiple testing (padj = .281). To assess whether non-linear age effects may have influenced this association, we additionally fitted a model incorporating natural cubic splines for age (df = 3), in which Module 1 remained significantly associated with asthma (OR = 0.62, 95% CI 0.45–0.84, p = .003) while none of the spline terms reached significance, suggesting that age operates approximately linearly within our cohort. An age-by-module interaction term was likewise non-significant, indicating that the Module 1–asthma association did not vary meaningfully across age. Nevertheless, the attenuation of this association in age-matched sensitivity analyses suggests residual age-related confounding cannot be fully excluded. Within the asthma group, we tested associations with binary clinical characteristics such as atopy, environmental smoke exposure, emergency visits and hospitalizations, and inhaled corticosteroid use, but none reached statistical significance after correction for multiple testing (Figure 4C). As shown in Figure 4D, when testing associations of Modules with continuous variables, Module 2 was significantly associated with increased body mass index (BMI), and Module 5 with obstructive lung function values (reduced forced expiratory volume in 1 s, FEV1) at last available

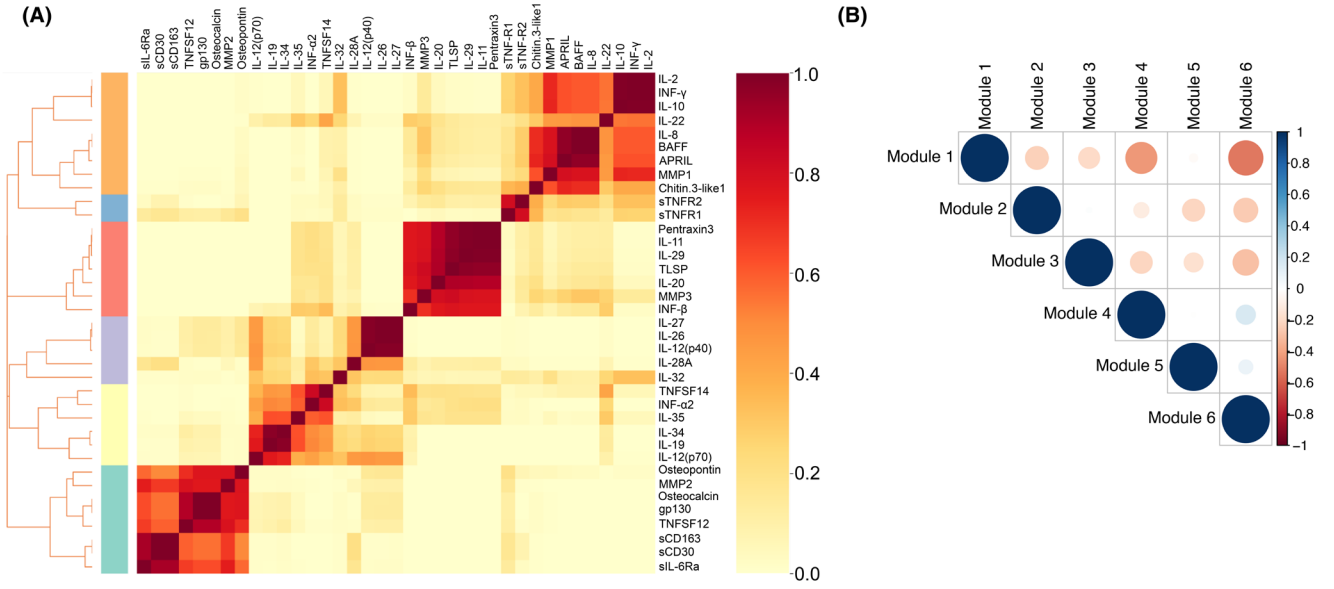


FIGURE 3 Cytokine co-regulatory modules identified by CytoMod analysis. (A) Hierarchical clustering dendrogram and heatmap showing cytokine modules identified using background-adjusted cytokine levels. Six distinct modules were identified, with cytokines grouped by co-regulatory patterns. The color scale represents the reliability of cytokines being clustered together into the same module across 1000 bootstrapped datasets. (B) Module-module correlation matrix showing relationships between the six identified modules. Circle size and color intensity indicate correlation strength, with blue representing negative correlations and red representing positive correlations. Notable inverse correlations were observed between Module 1 and Modules 4/5, while Module 4 showed positive correlation with Module 6.

follow up (median follow-up period: 7 (IQR: 4–8) years). However, no association was observed with FEV1/FVC values, suggesting that the relationship with lung function should be interpreted cautiously.

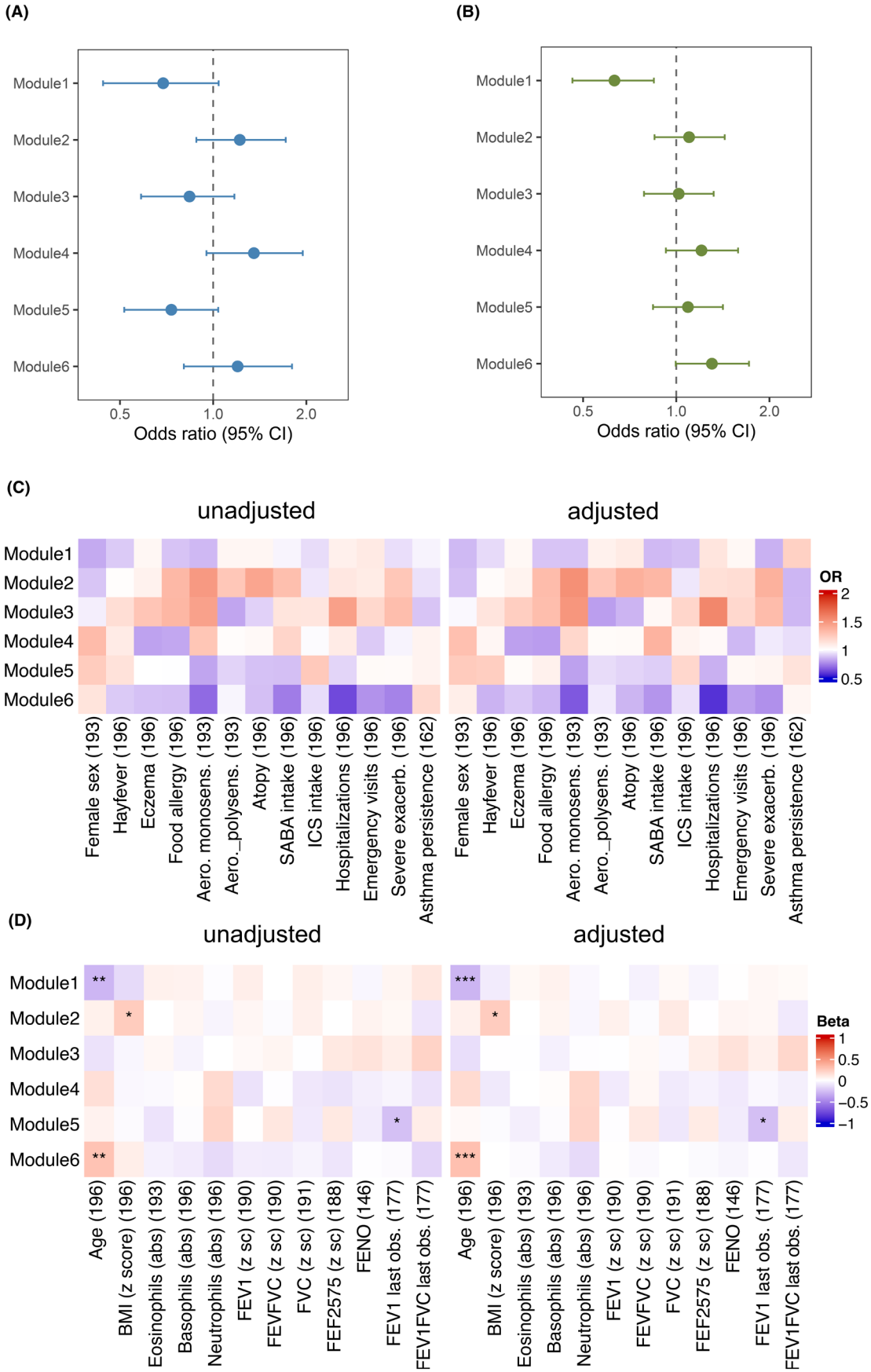
4 | DISCUSSION

This study provides a comprehensive analysis of CD3/CD28-stimulated cytokine responses in a well-characterized pediatric cohort of children with asthma, preschool wheeze, and healthy controls. Our analyses were intended to characterize the total phenotype-associated differences in cytokine responses between predefined clinical groups, reflecting the overall immune landscape in a real-world pediatric cohort, inclusive of contributions from disease biology, atopic background, treatment exposure, and other factors.

The most notable observation was the remarkable similarity of cytokine co-variation patterns across all study groups, as reflected by highly correlated principal component loadings. This suggests that the fundamental architecture of T cell responses to CD3/CD28 stimulation is largely preserved in healthy children and those with preschool wheeze or asthma and likely reflects the potent, to some extent non-specific nature of CD3/CD28 stimulation, which may in part override subtle disease-related differences in immune responsiveness. Importantly, cytokine responses were measured in whole blood without adjustment for cellular composition or leukocyte counts. Therefore, the findings in this study should be interpreted as system-level functional responses rather than cell-intrinsic immune differences. Inter-individual variation in blood cell composition may have contributed to observed cytokine variability.

We observed age-related changes in sCD30 and sCD163 in healthy controls, underscoring the importance of considering developmental changes in immune function when analyzing pediatric cohorts.[16–18] These findings highlight the need to account for age-dependent maturation of the immune system when interpreting cytokine profiles in both healthy and diseased children.

FIGURE 4 Clinical associations of cytokine modules. (A, B) Forest plots showing associations between cytokine modules in preschool wheeze versus healthy controls (A) and asthma versus healthy controls (B), based on logistic regression models adjusted for age, sex, and recruitment site. (C, D) Heatmaps illustrating associations between cytokine modules and binary (C) and continuous (D) clinical characteristics within the asthma group. Logistic regression models were adjusted for age, sex, and recruitment site; in C and D, age was only adjusted only for sex and site, while sex was only adjusted for age and site. In A and B, plots display odds ratios with 95% confidence intervals; in C and D, color intensity represents odds ratios for binary variables and beta coefficients for continuous variables, with red indicating positive associations and blue indicating negative associations. Statistical significance is indicated as *padj. < .05, **padj. < .01, ***padj. < .001. Asthma persistence refers to asthma symptoms and pathologic lung function at the last available follow-up. Numbers in brackets after the variable name indicate the sample size in that specific analysis.



When adjusting for overall cytokine background levels, six co-regulatory modules emerged, indicating that meaningful biological networks can be detected despite the dominant global activation signal across study groups. Module 1 was associated with asthma status and includes cytokines involved in extracellular matrix damage, tissue remodeling and repair, and IL-6 signaling, which have been implicated in asthma pathophysiology,[19–35] suggesting a biologically plausible but hypothesis-generating link that requires confirmation in independent cohorts. However, several analytes within this module, including sCD30 and sCD163, are strongly age-dependent, and sensitivity analyses suggest the observed association may be influenced by the slightly older age of children with asthma in our cohort. Furthermore, Module 1 and other cytokine modules showed limited associations with clinical features within the asthma group. Taken together, these results indicate that CD3/CD28-stimulated cytokine responses predominantly reflect shared T-cell activation rather than disease-specific immune patterns.

Also, the observed associations within the group of asthmatic children of specific clinical features with cytokine Module 2 and 5 need further research. Several cytokines within Module 2, which were associated with increased BMI, were previously linked to asthma and obesity-related inflammation,[36–42] raising the hypothesis that this module may reflect obesity-related immune dysregulation. The analytes contained in Module 5, which showed an association with reduced FEV1 (but not FEV1/FVC) at last follow-up in our cohort, may also be biologically plausible, given the association of TNF receptors and neutrophilic asthma (a severe, often treatment-resistant phenotype) and the contribution of the TNF-α pathway to airway inflammation and hyperresponsiveness.[43,44] However, all these associations should be seen as hypothesis-generating and warrant further research, and important limitations need to be taken into account when interpreting our data.

The generally limited number of associations between cytokine modules and clinical features may reflect several factors. Children in our cohort predominantly exhibit mild asthma phenotypes, which may limit phenotypic contrast and reduce the likelihood of detecting strong differences in systemic cytokine responses. Additionally, the 48-h stimulation captures responses following prolonged T cell activation, potentially masking earlier or transient differences between groups. Finally, while the Bio-Rad 37-plex was chosen for its broad coverage of inflammatory, regulatory, and tissue remodeling mediators—several of which have not been investigated in the context of asthma yet—it does, however, not include canonical Th2 cytokines. This might in particular constrain the ability to detect Th2-driven asthma endotypes. The substantial differences in total IgE observed between study groups underscore this limitation and suggest that biologically relevant Th2 variation exists in our cohort that was not fully captured by this assay. The potent and broad CD3/CD28 activation might dilute phenotype-specific Th2 signatures and to capture this Th2-driven variation, alternative experimental approaches might be better suited. These could include antigen-specific stimulation with common aeroallergens (e.g., house dust mite or grass pollen) to reveal allergen-specific T cell responses that are otherwise masked by global activation, or more targeted profiling methods such as single-cell RNA sequencing to resolve the composition of T helper subsets at high resolution.

Despite these limitations, the network-based approach adjusting for overall cytokine levels proved valuable in identifying co-varying immune response modules that would otherwise be obscured in the global activation signal. This methodology may be useful in future studies examining complex immune responses and understanding the coordinated regulation of cytokines in asthma and diseases with dysregulated immune responses.

In conclusion, CD3/CD28-stimulated cytokine profiling revealed largely preserved immune response architecture across pediatric populations, with limited differences in cytokine responses between clinical phenotypes within asthma. The association of Module 1 with asthma and the observed associations of further Modules with BMI and lung function outcomes warrant further investigation. Although cytokine profiling as performed in this project warrants only limited clinical relevance, it may set the foundation for future studies dissecting immune heterogeneity in childhood asthma more precisely.

AUTHOR CONTRIBUTIONS

Bianca Schaub: Investigation; writing – review and editing; data curation. **Folke Brinkmann:** Investigation; writing – review and editing; data curation. **Bin Liu:** Investigation; writing – review and editing. **Ruth Grychtol:** Data curation; writing – review and editing; investigation. **Thomas Bahmer:** Investigation; writing – review and editing; data curation. **Klaus F. Rabe:** Writing – review and editing; investigation; data curation. **Nicole Maison:** Investigation; writing – review and editing; data curation. **David DeLuca:** Investigation; writing – review and editing. **Markus Weckmann:** Investigation; writing – review and editing; data curation. **Matthias V. Kopp:** Investigation; writing – review and editing; data curation. **Erika von Mutius:** Writing – review and editing; investigation; data curation. **Anna-Maria Dittrich:** Investigation; writing – review and editing; data curation. **Christine Happle:** Conceptualization; methodology; data curation; resources; supervision; formal analysis; validation; investigation; funding acquisition; visualization; project administration; writing – review and editing; writing – original draft. **Gesine Hansen:** Supervision; data curation; resources; project administration; writing – review and editing; conceptualization; funding acquisition. **Lennart Riemann:** Methodology; writing – original draft; writing – review and editing; investigation; visualization; formal analysis; validation; software. **Chrysanthi Skevaki:** Investigation; writing – review and editing; data curation.

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CONFLICT OF INTEREST STATEMENT

CS received consultancy and research funding from Bencard Allergie and declares consultancy for Novartis Pharma GmbH. The other authors state no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study cannot be made publicly available due to ALLIANCE consortium regulations. Researchers interested in accessing individual-level data may submit a formal proposal to the ALLIANCE consortium, where requests will be reviewed in accordance with consortium governance and ethical requirements. Analysis scripts are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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