Cytokine Responses to Rhinovirus and Development of Asthma, Allergic Sensitization, and Respiratory Infections during Childhood

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Abstract

Rationale: Immunophenotypes of antiviral responses, and their relationship with asthma, allergy, and lower respiratory tract infections, are poorly understood.

Objectives: We characterized multiple cytokine responses of peripheral blood mononuclear cells to rhinovirus stimulation, and their relationship with clinical outcomes.

Methods: In a population-based birth cohort, we measured 28 cytokines after stimulation with rhinovirus-16 in 307 children aged 11 years. We used machine learning to identify patterns of cytokine responses, and related these patterns to clinical outcomes, using longitudinal models. We also ascertained phytohemagglutinin-induced T-helper cell type 2 (Th2)-cytokine responses (PHA-Th2).

Measurements and Main Results: We identified six clusters of children based on their rhinovirus-16 responses, which were differentiated by the expression of four cytokine/chemokine groups: interferon-related (IFN), proinflammatory (Inflam), Th2-chemokine (Th2-chem), and regulatory (Reg). Clusters differed in their clinical characteristics. Children with an

IFN^{mod}Inflam^{highest}Th2-chem^{highest}Reg^{highest} rhinovirus-16-induced pattern had a PHA-Th2^{low} response, and a very low asthma risk (odds ratio [OR], 0.08; 95% confidence interval [CI], 0.01–0.81; $P = 0.03$). Two clusters had a high risk of asthma and allergic sensitization, but with different trajectories from infancy to adolescence. The IFN^{lowest}Inflam^{high}Th2-chem^{low}Reg^{mod} cluster exhibited a PHA-Th2^{lowest} response and was associated with early-onset asthma and sensitization, and the highest risk of asthma exacerbations (OR, 1.37; 95% CI, 1.07–1.76; $P = 0.014$) and lower respiratory tract infection hospitalizations (OR, 2.40; 95% CI, 1.26–4.58; $P = 0.008$) throughout childhood. In contrast, the IFN^{highest}Inflam^{mod}Th2-chem^{mod}Reg^{high} cluster with a rhinovirus-16-cytokine pattern was characterized by a PHA-Th2^{highest} response, and a low prevalence of asthma/sensitization in infancy that increased sharply to become the highest among all clusters by adolescence (but with a low risk of asthma exacerbations).

Conclusions: Early-onset troublesome asthma with early-life sensitization, later-onset milder allergic asthma, and disease protection are each associated with different patterns of rhinovirus-induced immune responses.

Keywords: asthma; rhinovirus; cytokines; machine learning

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At a Glance Commentary

Scientific Knowledge on the

Subject: Immune mechanisms governing the relationships between susceptibility to virus infection, lower respiratory tract infections (LRTIs), and asthma pathogenesis, and amongst patients with asthma predisposition to virus-induced exacerbations, are poorly understood.

What This Study Adds to the

Field: By employing machine learning, we identified the architecture of multiple cytokine responses by human blood mononuclear cells to rhinovirus stimulation comprising six response profiles, and observed major differences in trajectories of asthma, allergic sensitization, and LRTIs during childhood between these profiles. Two clusters of rhinovirus-induced cytokine responses were associated with a high risk of asthma but had different disease trajectories from infancy to adolescence. Our findings suggest that there may be two pathways leading to different phenotypes of childhood asthma. One of those pathways may be driven by virus-dependent innate responses and lead to early-onset asthma with early-life allergic sensitization and severe exacerbations, high medication usage, unscheduled asthma consultations, and frequent LRTIs. Early sensitization associated with this troublesome early asthma may in some individuals be a marker of impaired anti-virus immunity, rather than a consequence of the adaptive T-helper cell type 2 (Th2) immunity related to allergen exposure. The other pathway may arise through allergen-dependent adaptive Th2 immunity, and may lead to later-onset mild allergic asthma, with no increase in exacerbation rates or health care utilization.

Asthma is the most common chronic disease in childhood, and severe exacerbations remain one of the commonest reasons for childhood hospital admission in the developed world (1). Allergic sensitization and susceptibility to virus infection are key features of asthma (2, 3), and virus

infections (particularly rhinoviruses) are linked with asthma development (4) and exacerbations (5, 6).

Impaired antiviral immunity may influence the onset, presence, and severity of asthma. A series of studies has shown that antiviral immunity is impaired in some patients with asthma (7, 8), and that these impairments are related to asthma exacerbation severity (8). Furthermore, children with deficient antiviral cytokine responses have increased antibiotic use, wheezing, and asthma exacerbations in early life (9), and it has been proposed that rhinovirus infections could cause asthma in susceptible individuals (10). However, the immune mechanisms governing the relationships between susceptibility to virus infection, lower respiratory tract infections (LRTIs), asthma pathogenesis, and among those with asthma, predisposition to virus-induced exacerbations, are poorly understood.

We hypothesize that there are multiple immunophenotypes of immune responses to rhinovirus that differ in their relationship with asthma and respiratory tract infections, and that data-driven techniques may help identify such immunophenotypes through better understanding of the patterns of cytokine responses. To address our hypotheses, we characterized the architecture of multiple cytokine responses of human peripheral blood mononuclear cells (PBMCs) to rhinovirus stimulation in children participating in a birth cohort study, and then investigated the association of these patterns with clinical outcomes during childhood.

Methods

Detailed methods are presented in the online supplement.

Study Design, Setting, Participants, and Data Sources

The Manchester Asthma and Allergy Study is a population-based birth cohort (11). The study was approved by the local research ethics committee. Written informed consent was obtained from parents, and participants gave their consent/assent when applicable.

Participants attended follow-ups at ages 1, 3, 5, 8, 11, and 16 years. Validated questionnaires were interviewer-administered to collect information on parentally

reported symptoms, physician-diagnosed illnesses, and medication usage. We assessed allergic sensitization by skin prick tests (12). We extracted all data from birth to age 11 years from primary care medical records, including wheeze episodes, LRTIs, asthma medication and oral corticosteroid prescriptions, emergency department admissions, and hospitalizations (9).

Definitions of clinical outcomes (asthma [13], severe asthma/wheeze exacerbations [14], allergic sensitization, hospitalizations, and LRTIs) are provided in the online supplement.

In Vitro Immune Responses of PBMCs

PBMCs were collected from children aged 11 years and cryopreserved (9). PBMCs were thawed and distributed in 96-well plates (2×10^5 cells per well). We used medium control and rhinovirus-16 (at a multiplicity of infection of one) as stimuli (15). Supernatants were harvested 24 hours after stimulation; we measured levels of 28 cytokines and chemokines (see Table E1 in the online supplement) using multi-arrays (Meso Scale Discovery).

To investigate T cell-mediated T-helper cell type 2 (Th2) responses, we measured IL-4, IL-5, and IL-13 levels 24 hours after stimulation of PBMCs with phytohemagglutinin (PHA, 10 μ g/ml; Roche Diagnostics).

Data Analysis

Cluster analysis. We used a Gaussian mixture model to cluster children based on their PBMC cytokine responses to rhinovirus-16. Before clustering, the data were normalized by subtracting the log-transformed medium response for each cytokine from the log-transformed cytokine responses to virus stimulation. The model was fitted by the expectation-maximization algorithm. We chose the optimal number of clusters, using the Bayesian information criterion. We assessed stability to changes in initial conditions and bootstrap resampling of the data set. The divergence between cytokine responses in each cluster and the overall population was measured using Welch's *t* test.

Clinical associations of cytokine profiles. We compared clinical outcomes at each age between different clusters, using logistic regression. We then investigated developmental profiles of clinical outcomes

from infancy (age, 1 yr) to adolescence (age, 16 yr) across clusters, using longitudinal regression models. We compared the characteristics in each cluster with the rest of the population, and investigated whether profiles changed significantly over time by including an interaction term between cluster membership and age.

Results

Participant Flow and Demographic Data

Among 1,184 children born at full-term, 822 attended follow-up at age 11; PBMC stimulation data were obtained for 340. After quality control (see the online supplement), we excluded data for 33 children. Characteristics of the study population are shown in Table E2; there were no significant differences in demographics and clinical outcomes between children included and excluded from this analysis.

Profiles of PBMC Cytokine Responses

A six-cluster model provided an optimal solution for clustering 28 cytokine responses

to rhinovirus-16 in 307 children. The stability of the clustering scheme was good (16) (Figures E1–E3 and Table E3). Figure E4 shows cell viability across all samples, viability across the clusters, and the relationship between cytokine response and viability. Although 4 of 28 cytokine responses correlated significantly with viability, the correlation was weak (IFN- γ , $r = 0.18$; IL-2, $r = 0.22$; IL-16, $r = 0.20$; monocyte chemoattractant protein 4, $r = 0.19$).

Figure 1A shows the patterns of cytokine responses to rhinovirus-16 across the six clusters in terms of the magnitude of divergence of the response in each cluster from the population mean, and Figure E5 shows the mean fold induction. Cytokine responses for each individual child across the six clusters are shown in Figure E6, and cytokine levels (in pg/ml) are presented in Table E4.

To facilitate interpretation of cytokine function in each cluster, we designated cytokines that were significantly induced into four distinct biological/functional groups: 1) interferons, Th1 and interferon-induced cytokines/chemokines (IFN); 2) proinflammatory cytokines/chemokines (Inflam); 3) Th2-associated chemokines

(Th2-chem); 4) regulatory cytokines (Reg). The rationale for this grouping is presented in the online supplement, and the group allocation of each cytokine/chemokine is shown in Table E5. We assigned the relative cytokine group expression in clusters as “highest,” “high,” “moderate,” “low,” or “lowest.” Cluster 1 was the smallest (C1, $n = 18$, 5.9%), and was characterized by moderate induction of interferons, and the highest induction of proinflammatory cytokines/chemokines and IL-10 across all clusters (IFN^{mod}Inflam^{highest}Th2-chem^{highest}Reg^{highest}). Children in cluster 2 (C2, $n = 28$, 9.1%) and cluster 6 (C6, $n = 78$, 25.4%) had low induction across most cytokines and chemokines, with subtle differences in a few cytokines accounting for their separation (IFN^{low}Inflam^{lowest}Th2-chem^{low}Reg^{lowest} and IFN^{low}Inflam^{lowest}Th2-chem^{low}Reg^{low}, respectively). Cluster 3 (C3, $n = 46$, 15.0%) was characterized by high interferon induction, with low or moderate induction of other cytokine groups (IFN^{high}Inflam^{low}Th2-chem^{mod}Reg^{mod}). Cluster 4 (C4, $n = 63$, 20.5%) had the lowest interferon induction, and high induction of proinflammatory cytokines (IFN^{lowest}Inflam^{high}Th2-chem^{low}Reg^{mod}).

Figure 1. Cytokine responses across the six clusters. Shown is the divergence between the mean responses within each cluster and the population mean, using t statistics from Welch's t test. Heatmap of t statistics: red (positive t value) indicates the cluster mean is greater than the population mean, and blue (negative t value) indicates that the cluster mean is less than the population mean. (A) All cytokine responses to rhinovirus-16. (B) T-helper cell type 2 (Th2) cytokine responses to phytohemagglutinin in rhinovirus-16 response clusters.

Cluster 5 (C5, $n = 74$, 24.1%) had the highest induction of interferons across all clusters, and high IL-10 induction (IFN^{highest}Inflam^{mod}Th2-chem^{mod}Reg^{high}).

We also performed the unsupervised clustering of cytokines, which determined three cytokine groups that largely coincided with our predefined groups described above (Figure E7).

PBMC Th2 cytokine responses to PHA.

Th2 cytokines were not significantly induced to biologically relevant levels in response to rhinovirus-16 (Table E4). Therefore, to investigate characteristics of Th2 responses in rhinovirus-16 response clusters, we measured IL-4, IL-5, and IL-13 after stimulation of PBMCs with PHA (PHA-Th2). Levels of Th2 cytokines (in pg/ml) are shown in Table E6. PHA-Th2 differed significantly between the clusters: children in C4 were the lowest producers of Th2 cytokines, whereas those in C5 were the highest (Figure 1B).

Characteristics of Rhinovirus-16 Cytokine Response Clusters

Demographic and early-life features.

Children in C2 were significantly less likely, and those in C5 were significantly more likely, to have atopic parents (Table E7). There was no significant association between cluster membership and gestational age, sex, birth weight, maternal smoking during pregnancy, number of older siblings, pet ownership, or socioeconomic status.

Asthma and allergic sensitization.

Results of cross-sectional analyses for various outcomes (current wheeze, asthma, allergic sensitization, and dust mite sensitization) at each time point from age 1 to age 16 are shown in Table E8. Asthma was rare in C1 (IFN^{mod}Inflam^{highest}Th2-chem^{highest}Reg^{highest}, PHA-Th2^{low}), and common in C5 (IFN^{highest}Inflam^{mod}Th2-chem^{mod}Reg^{high}, PHA-Th2^{highest}). At age 16, asthma prevalence was significantly higher in C5 (37.25%; 95% confidence interval [CI], 23.78–50.73%) compared with all other clusters. The proportion of children with allergic sensitization and dust mite sensitization was also significantly higher in C5 compared with others.

Trajectories of Asthma, Sensitization, and LRTIs in Rhinovirus-16 Response Clusters

Results of longitudinal regression models to investigate the developmental profiles of

different clinical outcomes from early life (age, 1 yr) to adolescence (age, 16 yr) in the various clusters are shown in Table 1. Longitudinal trajectories of asthma and allergic sensitization in six rhinovirus-16 response clusters are presented in Figure 2.

Asthma. Children in C1 (IFN^{mod}Inflam^{highest}Th2-chem^{highest}Reg^{highest}) had significantly lower risk of asthma (OR [95% CI], 0.08 [0.009–0.81]; $P = 0.03$), and were significantly less likely to receive asthma medication (OR [95% CI], 0.09 [0.01–0.89]; $P = 0.04$) throughout childhood (Table 1). The risk of asthma from infancy to adolescence was significantly higher in C4 (IFN^{lowest}Inflam^{high}Th2-chem^{low}Reg^{mod}, PHA-Th2^{lowest}) compared with other clusters (OR [95% CI]: 3.89 [1.24–12.19]; $P = 0.02$). Children in C4 also had significantly increased risk of being prescribed asthma medication during childhood (OR [95% CI]: 3.46 [1.07–11.17]; $P = 0.04$), and had significantly higher number of severe asthma exacerbations (OR [95% CI]: 1.37 [1.07–1.76]; $P = 0.01$) and unscheduled visits to primary health care providers for wheeze (OR [95% CI]: 1.14 [1.02–1.29]; $P = 0.02$; all Table 1). Early-life wheeze and asthma were significantly more common in C4 compared with other clusters (Table 2); these children were 2.1 times more likely to wheeze (95% CI, 1.10–3.91; $P = 0.02$), and 6.5 times more likely to be prescribed asthma medication in the first year of life (95% CI, 1.34–31.16; $P = 0.02$) (Table E9). The proportion of children with asthma in C4 remained high throughout childhood, with some reduction over time (Figure 2A). After infancy, the rate of change in asthma over time significantly differed in C4 compared with other clusters (interaction with time, OR [95% CI]: 0.89 [0.81–0.97]; $P = 0.01$) (Table 1). In contrast, in C5 the proportion of children with wheezing/asthma was lower in infancy but increased during childhood, so that by age 16 years asthma prevalence was highest in this cluster (Table E8 and Figure 2A). However, there was no increased risk of asthma exacerbations in C5 (Table 1).

To ascertain whether the effect of rhinovirus-16 cytokine response clusters on asthma was modified by allergic sensitization, we extended our longitudinal models to test for interaction between the

cluster and sensitization. The association between asthma and C4 was independent of sensitization (OR for C4, 3.09; 95% CI, 1.00–9.59; $P = 0.05$), and the effect of C4 cluster membership on asthma was not modified by sensitization (P value for interaction = 0.24). In C5, we were unable to test for the interaction, as there were no children in this cluster who had asthma and were not sensitized.

Allergic sensitization. The risk of sensitization from age 1 to age 16 years was significantly higher in C5 compared with other clusters (OR [95% CI], 3.21 [1.02–10.07]; $P = 0.04$). The risk was also high in C4 (OR [95% CI], 3.62 [0.87–15.13]; $P = 0.08$) (both in Table 1). However, the slopes of longitudinal sensitization trajectories of these two clusters differed significantly: the proportion of sensitized children at age 1 year was significantly higher in C4 than C5 (30.43 vs. 11.11%; Table 2), but the rate of increase thereafter was significantly lower in C4 (interaction with time, OR [95% CI]: 0.89 [0.80–0.98]; $P = 0.02$) (Table 2 and Figure 2B). Consequently, by middle-school age, the highest prevalence of sensitization was found in C5 (51.35%; 95% CI, 39.84–62.86 at age 11 yr) (Table E8). Children in C5 had the highest risk of mite sensitization during childhood (OR [95% CI], 5.0 [1.44–17.35]; $P = 0.01$) (Table 1).

LRTIs. Children in C4 were at significantly higher risk of being hospitalized with an LRTI during childhood (OR [95% CI], 2.40 [1.26–4.58]; $P = 0.008$), had 8% more primary care consultations for LRTIs (95% CI, 3–14%; $P = 0.001$), and were 3.85 times (95% CI, 1.41–10.47; $P = 0.008$) more likely to have physician-confirmed bronchiolitis (Table 1). Survival analysis revealed a significantly higher hazard rate of LRTI hospital admission from birth to middle-school age in C4 (hazard ratio, 2.17; 95% CI, 1.17–4.03; $P = 0.01$) (Figure 3). The proportion of children with LRTI hospitalization and physician-confirmed bronchiolitis in early life (26.3%, 95% CI [14.73–37.9] and 14%, 95% CI [4.9–23.17]) (Table 2) was significantly higher in C4 compared with other clusters. The risk of LRTI hospital admissions during childhood was significantly lower (OR [95% CI], 0.25 [0.08–0.75]; $P = 0.014$) in C6 (IFN^{low}Inflam^{lowest}Th2-chem^{low}Reg^{low}, PHA-Th2^{low}), with a similar trend for bronchiolitis (Table 1).

Table 1. Longitudinal Regression Models to Investigate the Developmental Profiles of Clinical Outcomes from Early Life (Age, 1 yr) to Adolescence (Age, 16 yr) in Various Clusters Derived from Peripheral Blood Mononuclear Cell Cytokine Responses to Rhinovirus-16

	Cluster 1 (<i>n</i> = 18; 5.9%)	Cluster 2 (<i>n</i> = 28; 8.5%)	Cluster 3 (<i>n</i> = 46; 15.1%)	Cluster 4 (<i>n</i> = 63; 20.3%)	Cluster 5 (<i>n</i> = 74; 24.3%)	Cluster 6 (<i>n</i> = 78; 25.9%)
Asthma						
OR (compared with all other groups)	0.084	1.049	0.518	3.89	2.159	0.75
95% CI	0.009 to 0.814	0.260 to 4.227	0.167 to 1.606	1.241 to 12.195	0.877 to 5.314	0.300 to 1.879
<i>P</i> value	0.033	0.946	0.207	0.02	0.094	0.539
Interaction with time				0.885		
OR (compared with all other groups)				0.806 to 0.971		
95% CI				0.01		
<i>P</i> value						
Prescription of asthma medication						
OR (compared with all other groups)	0.093	0.987	0.475	3.464	2.024	0.834
95% CI	0.010 to 0.893	0.238 to 4.095	0.149 to 1.509	1.074 to 11.172	−0.069 to 0.034	0.333 to 2.089
<i>P</i> value	0.04	0.986	0.207	0.038	0.511	0.699
Interaction with time				0.906		
OR (compared with all other groups)				0.823 to 0.997		
95% CI				0.043		
<i>P</i> value						
Number of severe asthma exacerbations						
OR	0.903	0.650	0.715	1.372	0.977	0.940
95% CI	0.494 to 1.652	0.291 to 1.453	0.410 to 1.245	1.066 to 1.765	0.737 to 1.294	0.707 to 1.249
<i>P</i> value	0.74	0.29	0.23	0.014	0.87	0.67
Number of unscheduled visits for wheeze						
OR	0.792	0.957	1.042	1.144	0.929	0.966
95% CI	0.554 to 1.133	0.784 to 1.168	0.904 to 1.200	1.017 to 1.286	0.811 to 1.064	0.853 to 1.094
<i>P</i> value	0.20	0.66	0.57	0.025	0.28	0.58
Allergic sensitization						
OR (compared with all other groups)	0.559	0.1	0.53	3.619	3.213	0.514
95% CI	0.072 to 4.369	0.009 to 1.162	0.136 to 2.066	0.866 to 15.133	1.024 to 10.075	0.168 to 1.576
<i>P</i> value	0.579	0.066	0.36	0.078	0.045	0.245
Interaction with time				0.888		
OR (compared with all other groups)		1.258		0.801 to 0.984		
95% CI		1.018 to 1.554		0.023		
<i>P</i> value		0.033				
Sensitization to mite						
OR (compared with all other groups)	0.169	1.308	0.32	1.237	4.997	0.492
95% CI	0.016 to 1.821	0.201 to 8.517	0.072 to 1.428	0.330 to 4.641	1.439 to 17.351	0.146 to 1.660
<i>P</i> value	0.143	0.779	0.135	0.753	0.011	0.253

(Continued)

Table 1. (Continued)

	Cluster 1 (n = 18; 5.9%)	Cluster 2 (n = 28; 8.5%)	Cluster 3 (n = 46; 15.1%)	Cluster 4 (n = 63; 20.3%)	Cluster 5 (n = 74; 24.3%)	Cluster 6 (n = 78; 25.9%)
Hospital admissions for LRTI						
OR (compared with all other groups)	1.016	0.601	0.615	2.399	0.827	0.245
95% CI	0.271 to 3.808	0.168 to 2.146	0.224 to 1.689	1.258 to 4.577	0.390 to 1.750	0.080 to 0.748
P value	0.981	0.433	0.346	0.008	0.619	0.014
Interaction with time						
OR (compared with all other groups)						1.837
95% CI						1.279 to 2.638
P value						0.001
Number of unscheduled consultations for LRTIs						
OR	0.966	0.977	1.002	1.083	0.972	0.958
95% CI	0.867 to 1.077	0.899 to 1.062	0.943 to 1.065	1.032 to 1.136	0.920 to 1.027	0.906 to 1.013
P value	0.53	0.58	0.95	0.001	0.31	0.13
Bronchiolitis						
OR	2.352	1.38	0.782	3.846	0.402	0.164
95% CI	0.489 to 11.314	0.297 to 6.412	0.172 to 3.560	1.412 to 10.472	0.090 to 1.805	0.021 to 1.260
P value	0.28	0.68	0.75	0.008	0.23	0.082

Definition of abbreviations: CI = confidence interval; LRTI = lower respiratory tract infection; OR = odds ratio.

Values represent odds ratios for binary characteristics and mean differences for continuous measures, with 95% confidence intervals for average effects of each cluster compared with all other. P values assess whether the distribution of characteristics within a given cluster is significantly different from characteristics in all other clusters in a longitudinal regression model (logistic or linear). Where the effect of cluster assignment on a clinical characteristic was significantly modified as the child became older, these values are presented as models with a covariate representing interaction with time. Numbers highlighted in bold represent clusters that had significantly different characteristics ($P < 0.05$) from characteristics in all other clusters.

Figure 2. Trajectories (age, 1–16 yr) and 95% confidence intervals of asthma and allergic sensitization in six rhinovirus-16 cytokine response clusters. (A) Asthma. (B) Allergic sensitization. Th2 = T-helper cell type 2.

Discussion

By employing machine learning techniques, we identified the architecture of multiple cytokine responses by PBMCs to rhinovirus stimulation in school-age children. We observed major differences between the six rhinovirus-16 cytokine response profiles in their trajectories of asthma, allergic

sensitization, and LRTIs during childhood. Two clusters were associated with a high risk of asthma, but had different disease trajectories from infancy to adolescence. We observed the highest risk of asthma and severe asthma exacerbations during childhood among children whose rhinovirus cytokine responses were characterized by the lowest interferon induction, and

high proinflammatory cytokines. This cluster (C4) was also at greatest risk for hospitalization for LRTI, and for bronchiolitis. In this cluster, the proportion of children with early-life wheezing who were prescribed asthma medication was high (at around one-third), after which asthma prevalence gradually declined to reach 21% at age 16 years. Despite having the highest risk of early-life allergic sensitization, children in this cluster produced by far the lowest levels of Th2 cytokines in response to PHA. In contrast, among children in the cluster with robust interferon responses to rhinovirus, but the highest production of Th2 cytokines after PHA stimulation (C5), the proportion of children with asthma and sensitization was low in early life, but increased during childhood to become the highest among all clusters in adolescence. However, asthma exacerbations were rare in this cluster. We also identified a small cluster (C1) characterized by moderate induction of interferons and the highest induction of proinflammatory and regulatory cytokines in response to rhinovirus, and with below-average Th2-cytokine induction by PHA, in which the risk of asthma was lower than in all other clusters.

One of the limitations of our study is that we assessed cytokine responses in PBMCs collected at age 11 years, and do not have samples from early life, which predate the onset of symptoms. We therefore cannot exclude the possibility of reverse causation, for example, by allergy/asthma and/or early-life LRTIs altering immune responses to viruses. Also, little is known about the stability of PBMC responses to viruses throughout life, and how the patterns we observed in school-age subjects relate to those in early life (17). However, our previous findings that impaired antiviral immunity and increased risk of early-life antibiotic prescription are in part genetically determined and associated with variants in chromosomal locus 17q21 (9), which is also the most consistent genetic risk factor for asthma (18), suggest that it is unlikely that the current results have arisen due to residual confounding.

It is also important to acknowledge the limitations in studying PBMCs only, as there is an important role of the airway epithelium in asthma and allergic sensitization (19), in particular epithelium-derived pro-Th2 cytokines IL-25 and IL-33 (20, 21), which our study cannot address, as these cytokines were not induced by

Table 2. Early-Life Clinical Characteristics according to Cluster Assignment

	Cluster 1 (n = 18; 5.9%)	Cluster 2 (n = 28; 8.5%)	Cluster 3 (n = 46; 15.1%)	Cluster 4 (n = 63; 20.3%)	Cluster 5 (n = 74; 24.3%)	Cluster 6 (n = 78; 25.9%)
Current wheeze, age 1 yr						
Mean	17.65%	15.38%	22.22%	35.09%	22.54%	20.27%
95% CI	−1.11 to 36.41%	1.18 to 29.59%	9.88 to 34.56%	22.54 to 47.64%	12.71 to 32.36%	11.01 to 29.53%
Asthma, age 1 yr						
Mean	0	8.33%	0	31.58%	17.39%	9.38%
95% CI		−8.19 to 24.85%		9.86 to 53.30%	1.37 to 33.41%	−1.00 to 19.75%
Sensitization, age 1 yr						
Mean	37.50%	0	5.82%	30.43%	11.11%	8.82%
95% CI	1.28 to 73.72%		−5.76 to 17.53%	11.01 to 49.85%	−1.09 to 3.31%	−0.95 to 18.60%
Bronchiolitis						
Mean	12.5%	8.00%	5.00%	14.04%	2.99%	1.37%
95% CI	−4.31 to 29.31%	−2.90 to 18.90%	−1.87 to 11.87%	4.90 to 23.17%	−1.14 to 7.11%	−1.33 to 4.07%
LRTI hospitalizations						
Mean	18.75%	8.00%	7.50%	17.54%	7.46%	4.11%
95% CI	−1.09 to 38.59%	−2.90 to 18.90%	−0.80 to 15.80%	7.54 to 27.55%	1.09 to 13.83%	−0.50 to 8.72%

Definition of abbreviations: CI = confidence interval; LRTI = lower respiratory tract infection.

Current wheeze, asthma, and sensitization ascertained at follow-up at age 1 year; bronchiolitis and hospitalizations with LRTIs within the first year of life confirmed from health care records.

Numbers highlighted in boldface represent clusters that had significantly different characteristics ($P < 0.05$) from characteristics in all other clusters in a regression model.

rhinovirus stimulation of PBMCs. It would be advantageous to have studied epithelial responses in the same subjects, but sampling lower airways in a birth cohort would be ethically unacceptable.

We acknowledge that cell viability may have differed among the study samples. However, major components of PBMCs (T and B cells, NK cells, monocytes, dendritic cells, etc.) all survive well after cryopreservation (22, 23). In terms of functionality, comparisons of fresh cell subsets with their cryopreserved counterparts also show that cell functionality is largely intact in cryopreserved cells (22), although some studies have reported some differences in cytokine responses (24). As all samples in the current study were subjected to identical procedures, it is unlikely that differential survival of cell subsets could have influenced the clustering results, and the differences between fresh and frozen cells are also unlikely to be of relevance here. Furthermore, studies have shown that the cell composition and immune responses in PBMCs show great heterogeneity within the human population, and in the wider picture, that differences in cell composition are unlikely to influence overall immunity or susceptibility to disease (25, 26).

Although we clustered children only on the basis of their PBMC responses to rhinovirus, the clinical associates of the

various clusters point toward possibly different mechanisms giving rise to different phenotypes of asthma and allergic sensitization (12, 27, 28). The predominant phenotype among children in the lowest interferon-producing cluster with high proinflammatory cytokines (C4) was that of early-onset asthma with severe exacerbations, high medication usage, unscheduled asthma consultations, frequent LRTIs, and early-life sensitization.

These are characteristics similar to clinically defined problematic severe asthma (29, 30), and to persistent troublesome wheezing (31), exacerbation-prone asthma (32, 33), and early-onset severe asthma (32, 34), which were identified by data-driven techniques. The sensitization pattern in this cluster resembles that of a multiple early sensitization class, which was uncovered using machine learning, and shown to be associated with severe

Figure 3. Risk of hospital admission with lower respiratory tract infection in children according to the membership of the six rhinovirus-16 cytokine response clusters. Th2 = T-helper cell type 2.

childhood asthma (12, 35). Children in C5, who had robust interferon responses to rhinovirus, but by far the strongest production of PHA-induced Th2 cytokines, also had a high risk of asthma and sensitization, but mostly in school-age and adolescence. Asthma in these children was predominantly mild, with no increase in exacerbation rates or health care utilization. This cluster was associated with sensitization in parents. These features are consistent with later-onset atopic asthma (36).

The observed differences in trajectories of asthma and sensitization between these two clusters are intriguing. We wish to emphasize that the machine learning techniques we used to identify clusters can lead to the generation of new hypotheses, but that we do not have direct mechanistic data to provide evidence of causality. C4 was dominated by the lack of type I (IFN- α) and type II (IFN- γ) interferon responses to rhinovirus, and high proinflammatory cytokines. We propose that lack of robust antiviral immunity driven by type I and II interferons, coupled with a high inflammatory response, could explain the increased risk of LRTIs and wheeze in early life in C4, which drives the phenotype with frequent asthma exacerbations (7, 37–39). Although children in this cluster developed sensitization in early life (and a high proportion remained sensitized in school-age), they had the weakest induction of Th2 cytokines in response to PHA of all clusters. We hypothesize that this apparent paradox could be explained by the role of viral infection-induced local pro-Th2 responses, including the epithelial-derived IL-33 and IL-25, and type 2 innate lymphoid cells (ILC2s) (20). The lack of a robust type I/II interferon response in this cluster may be important in determining

not only wheezing-related illnesses but also early-life allergic sensitization, which in these children may be a marker of impaired anti-virus immunity, rather than a consequence of the adaptive Th2 immunity related to allergen exposure. We propose that IgE in these patients may be produced locally in the absence of cognate T-cell help (40) (e.g., by B cells, which undergo clonal selection and affinity maturation in the respiratory mucosa [41]). ILC2s may directly activate B cells to produce specific IgE locally but not systemically (42, 43), and IgE measured in serum or by skin prick tests may have escaped from the site of disease (44). Locally produced IgE may interact with virus infection to further increase the risk of exacerbation by suppressing anti-viral immunity (37). Our findings may suggest that multiple early sensitization associated with the troublesome early asthma could be a marker of impaired anti-viral immunity and increased susceptibility to virus infection, and not only a consequence of the adaptive Th2 immunity related to allergen exposure. Therefore, in this cluster, both asthma and early-life sensitization may be driven by virus-dependent innate responses. However, this does not exclude the possibility that among other children, early-life aeroallergen sensitization may modulate host antiviral responses, and predispose children to rhinovirus-induced wheezing illnesses (4).

In contrast, in C5, type I–II interferon responses were robust, and the increased risk of asthma was not apparent until later childhood, along with a later development of mite sensitization. We observed very high Th2 cytokine responses to PHA in this cluster. This expression pattern is consistent with a Th2 cell-driven adaptive response to allergens (44). We propose that

in this group of genetically susceptible children with a family history of atopy, allergen exposure may lead to the development of allergen-dependent adaptive Th2 immunity, and asthma is driven mostly by allergic mechanisms (but is comparably milder, with fewer exacerbations). This cluster likely includes children who would benefit from inhaled corticosteroids. Children in C4, however, lack both interferons and adaptive Th2 responses, and may have relatively poorer response to inhaled corticosteroids, and would possibly benefit from antivirals, interferon therapy, or interferon-boosting therapies.

Our data suggest that there are two different pathways of childhood asthma development, and that apparently shared phenotypic traits, such as allergic sensitization in school age, may arise through different mechanisms. However, it is unlikely that these mechanisms are mutually exclusive, and it is possible that individuals in whom both mechanisms are operating may have the most severe disease.

In conclusion, clearly different patterns of multiple cytokine responses to rhinovirus are associated with protection from asthma, sensitization, and LRTIs; and increased risk of early-life wheezing, early-life sensitization, severe asthma exacerbations and hospitalizations for LRTIs, and late-onset allergic asthma and dust mite sensitization, but without increased exacerbation risk. Identification of these novel immunophenotypes will aid better understanding of disease mechanisms and development of personalized approaches to therapy (45). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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