Dysfunctional neutrophil type 1 interferon responses in preschool children with recurrent wheezing and IL-4–mediated aeroallergen sensitization

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Background: The innate mechanisms associated with viral exacerbations in preschool children with recurrent wheezing are not understood.

Objective: We sought to assess differential gene expression in blood neutrophils from preschool children with recurrent wheezing, stratified by aeroallergen sensitization, at baseline and after exposure to polyinosinic:polycytidylic acid (poly(I:C)) and also to examine whether poly(I:C)-stimulated blood neutrophils influenced airway epithelial gene expression.

Methods: Blood neutrophils were purified and cultured overnight with poly(I:C) and underwent next-generation sequencing with Reactome pathway analysis. Primary human small airway epithelial cells were treated with poly(I:C)-treated neutrophil culture supernatants and were analyzed for type 1 interferon gene expression with a targeted array. Symptoms and exacerbations were assessed in participants over 12 months.

Results: A total of 436 genes were differently expressed in neutrophils from children with versus without aeroallergen sensitization at baseline, with significant downregulation of type 1 interferons. These type 1 interferons were significantly upregulated in sensitized children after poly(I:C) stimulation. Confirmatory experiments demonstrated similar upregulation of type 1 interferons in IL-4–treated neutrophils stimulated with poly(I:C). Poly(I:C)-treated neutrophil supernatants from children with aeroallergen sensitization also induced a type 1 interferon response in epithelial cells. Children with aeroallergen sensitization also had higher symptom scores during exacerbations, and these symptom differences persisted for 3 days after prednisolone treatment.

Conclusions: Type 1 interferon responses are dysregulated in preschool children with aeroallergen sensitization, which is in turn associated with exacerbation severity. Given the importance of type 1 interferon signaling in viral resolution, additional studies of neutrophil type 1 interferon responses are needed in this population. (J Allergy Clin Immunol Global 2024;3:100229.)

Key words: *Asthma, wheezing, phenotype, endotype, neutrophil, type 1 interferon, respiratory infection, innate immunity, sensitization, next-generation sequencing*

Preschool children with recurrent wheezing are a heterogeneous group of patients with variable clinical trajectories who are often very difficult to treat.[1-5] Each year, nearly half of these children experience a significant wheezing exacerbation, irrespective of controller medication usage.[6] Furthermore, preschool children, compared with school-age children with asthma, have disproportionately higher rates of emergency department utilization and hospitalization for wheezing exacerbations,[7] resulting in disproportionate and significant costs.[8] Respiratory viruses are the primary trigger of wheezing exacerbations in most preschool children with recurrent wheezing,[9] but the underlying mechanisms associated with viral exacerbations in these children are not well understood.

Neutrophils are the most abundant cell type in the blood and are a fundamental component of the innate immune response. However, it remains unclear whether neutrophils have direct antiviral effects during respiratory viral infections in patients with asthma or whether they are merely a bystander of local inflammation with effector functions that can both help and hinder the host.[10] It is also recognized that neutrophil responses to viral infection, like in patients with asthma, are equally heterogeneous and may reflect differing endotypes of wheezing.[11] For example, recent multiomics studies of nasopharyngeal samples (that are predominantly composed of neutrophils[12]) from young children hospitalized with bronchiolitis have identified discrete endotypes of childhood asthma susceptibility that vary according to viral etiology, underlying type 2–mediated inflammation, and the nature and intensity of the immune response.[13,14] To date, neutrophils have been underappreciated in young children with wheezing illnesses10 and there are very limited studies of neutrophil responses to viruses in preschool children with recurrent wheezing.

To address this gap, we assessed differential gene expression in isolated blood neutrophils from otherwise healthy preschool children with recurrent wheezing, stratified by aeroallergen sensitization. Analyses were conducted at baseline and after exposure to an intracellular viral double-stranded RNA analog, LyoVec low-molecular-weight polyinosinic:polycytidylic acid (poly(I:C)), which preferentially signals through the cytoplasmic retinoic acid–inducible gene 1 receptor inducing an inflammatory

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Abbreviations used
CCL: C-C motif chemokine ligand
CXCL: C-X-C motif chemokine ligand
CX3CL: C-X3-C motif chemokine ligand
FDR: False-discovery rate
JAK: Janus kinase
poly(I:C): Polyinosinic:polycytidylic acid
RNA-seq: RNA sequencing
STAT: Signal transducer and activator of transcription

and antiviral IFN-α response[15] that can be studied *ex vivo* without the limitation of multiple virus types.[10] We hypothesized that blood neutrophil gene expression patterns would differ in children with versus without aeroallergen sensitization at baseline and in response to poly(I:C) stimulation. We also examined whether blood neutrophils stimulated with poly(I:C) *ex vivo* influenced airway epithelial gene expression and whether these findings were also associated with longitudinal respiratory symptom flares and wheezing exacerbations.

METHODS

Preschool children aged 12 to 59 months with recurrent wheezing and at least 1 wheezing episode treated with systemic corticosteroids in the previous 12 months were included in the study. Participants were identified through a review of outpatient clinical encounters at Children's Healthcare of Atlanta (Atlanta, Ga). Caregivers of children meeting inclusion criteria were informed of the study opportunity by e-mail, phone, or in person during clinical encounters. Recurrent wheezing was defined as a lifetime history of 2 or more episodes of wheezing, with each episode lasting at least 24 hours and requiring repeated treatment with albuterol sulfate. Children were excluded if they had comorbid disorders associated with wheezing (such as immune deficiency, cystic fibrosis, pulmonary aspiration, congenital airway anomalies, or premature birth before 35 weeks of gestation) or if they had a significant developmental delay or failure to thrive. Informed written consent was obtained from legal guardians before study participation. All study procedures were performed in accordance with the relevant guidelines and regulations in the Declaration of Helsinki.

Study design and characterization procedures

Preschool children attended a research-only outpatient visit that was rescheduled if the child had a wheezing exacerbation treated with systemic corticosteroids in the preceding 2 weeks. Caregivers completed demographic questionnaires and medical history questionnaires. Respiratory and asthma control were assessed with the 5-item Test for Respiratory and Asthma Control in Kids questionnaire,[16] with scores of 80 or higher reflecting good control.[17] Preschool participants also submitted blood samples for plasma cytokines, total serum IgE and blood eosinophil counts (Children's Healthcare of Atlanta, Atlanta, Ga), and RNA-sequencing (RNA-seq) analyses. Aeroallergen sensitization was determined by specific IgE testing with an inhalant allergen panel specific to the Atlanta region with 12 extracts: *Dermatophagoides farinae*, *D pteronyssinus*, dog dander, cat dander, *Blatella germanica*, *Alternaria tenuis*, *Aspergillus fumagatis*, oak tree, pecan tree, Bermuda grass, Johnson grass, and common ragweed (*Ambrosia artemisiifolia*) (Greer Laboratories, Lenoir, NC). Test results were considered positive if specific IgE values were higher than 0.35 kU/L.

At the completion of this visit, caregivers received a written action plan for their child, an albuterol sulfate metered-dose inhaler with a valved holding chamber and face mask, a single course of prednisolone (2 mg/kg/d for 2 days followed by 1 mg/kg/d for 2 days), and a paper diary to be completed at home during respiratory symptom flares. The action plan included green, yellow, and red zones defined by symptoms. The yellow zone instructed caregivers to administer 2 inhalations of albuterol sulfate every 4 hours as needed for up to 24 hours. The red zone instructed caregivers to initiate the prednisolone prescription and to seek medical care. Diary cards for each day instructed caregivers to record inhalations of albuterol sulfate and to score the severity of 4 respiratory symptoms (cough, wheezing, trouble breathing, and activity limitation) on a scale from 0 (none) to 5 (very severe). Symptom questions were adapted from the Pediatric Asthma Caregiver Diary[18] and were summed for a total score ranging from 0 to 20, with higher scores reflecting greater respiratory symptoms. The diary cards also questioned whether prednisolone was administered. Diary cards were collected from parents after the respiratory symptom flare had resolved. Parents were also telephoned every 2 months to assess symptom flare and exacerbation occurrence.

Patient blood neutrophil isolation and culture

Blood was collected by venipuncture into an EDTA vacutainer tube during the enrollment visit. Blood was kept at room temperature and centrifuged at 400*g* within 30 minutes of collection to separate cells from platelet-rich plasma. Plasma was stored at −80°C. Pelleted blood cells were resuspended in sterile PBS (ThermoFisher Scientific, Waltham, Mass) with 2.5 mM EDTA up to the original whole blood volume. Neutrophils were purified by negative selection from PBS-EDTA–washed whole blood using the EasySep Direct Human Neutrophil Isolation Kit (StemCell Technologies, Cambridge, Mass) according to the manufacturer's protocol. Neutrophil processing was completed within 30 minutes, and there was more than 99% neutrophil purity on the basis of cytospin staining with Diff-Quik (ThermoFisher Scientific). Two million neutrophils were preserved in 1 mL of RNALater and stored at −80°C. Two million additional neutrophils were resuspended in RPMI medium (ThermoFisher Scientific) without serum and stimulated with 740 ng/mL low-molecular-weight LyoVec poly(I:C) (InvivoGen, San Diego, Calif) resuspended in endotoxin-free water. Neutrophils were incubated with poly(I:C) or vehicle solution for 20 hours at 37°C and 5% CO_2 in a humidified incubator. Viability after culture was assessed with trypan blue staining (ThermoFisher Scientific). After centrifugation at 400*g*, neutrophil pellets were saved in RNALater and frozen at −80°C. Culture supernatants were also stored at −80°C.

Neutrophil RNA isolation and RNA-seq

RNA was isolated from blood neutrophils using the Nucleospin RNA II Kit (Takara, Mountain View, Calif) with on-column

genomic DNA digestion according to the manufacturer's protocol as described previously.[19] RNA was quantified using the NanoDrop Fluorospectrometer (ThermoFisher Scientific). RNA was quality-assessed using the 2100 Bioanalyzer (Agilent, Santa Clara, Calif) in the Emory Integrated Genomics Core. cDNA was generated using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara), and the final RNA-seq library was generated using the Nextera XT Kit (Illumina, San Diego, Calif). RNA-seq libraries were sequenced on the NovaSeq 6000 (Illumina) with a sequencing depth of 30M PE100 reads per sample.

Plasma cytokine measurement

Cytokines in plasma were measured on a Luminex MAGPIX system (Millipore, Burlington, Mass) according to the manufacturer's protocol. A 21-plex human T-cell panel array (HSTCMAG 28SK, T-Cell Panel, Millipore) was used to quantify C-C motif chemokine ligand 3 (CCL3) (ie, macrophage inflammatory protein 1α), CCL4 (ie, macrophage inflammatory protein 1β), CCL20 (ie, macrophage inflammatory protein 3α), C-X-C motif chemokine ligand 11 (CXCL11) (ie, interferon–inducible T-cell alpha chemoattractant), C-X3-C motif chemokine ligand 1 (ie, fractalkine), GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, IL-21, IL-23, and TNF-α.

Isolation and culture of primary human donor neutrophils

Primary human neutrophils were isolated as described previously[20] for confirmatory experiments from donor blood collected in K2-EDTA tubes using the EasySep Direct Human Neutrophil Isolation Kit (StemCell Technologies, Vancouver, BC, Canada). Neutrophils were cultured overnight at 37oC, 5% CO2 in RPMI-1640 media with l-glutamine supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 50 mg/mL gentamicin, with 740 ng/mL low-molecular-weight LyoVec poly(I:C) as noted. Recombinant human IL-4 (Sigma, Burlington, Mass) was added at 50 ng/mL for 2 hours before harvesting.

Nanostring experiments

Differential gene expression of 579 genes of interest from purified neutrophils was measured using the Human Immunology v2 NanoString nCounter Gene Expression CodeSet (NanoString, Seattle, Wash). All NanoString-based measurements were conducted at the Emory University Integrated Genomics Core facility using an amplification step for low abundant RNA applied to all samples as previously described.[21]

Culture of primary human airway epithelial cells

Primary human small airway epithelial cells isolated from normal human lung tissue in the distal portion of the lung (bronchiole area 1 mm) were purchased from Lonza (Basel, Switzerland) and expanded in PneumaCult-Ex Plus Media (StemCell Technologies, Vancouver, BC, Canada) on 6.5-mm Transwell 0.4-μm Pore Polyester membrane inserts (Corning, Lowell, Mass) at a density of 1×10^5 cells/cm^2 and incubated at 37°C, 5% CO_2 until confluent, for approximately 3 to 4 days. Once confluent, cells were transitioned and maintained at air-liquid interface culture with PnuemaCult-ALI-S Maintenance Medium (StemCell Technologies) at 37°C, 5% CO_2 as per the manufacturer's instructions for 28 days until fully differentiated. At differentiation, the small airway epithelial cell cultures contain goblet cells, club cells, basal cells, and cilia. These were visualized by inverted confocal microscopy (Stellaris 8, Leica, Deerfield, Ill) and Imaris imaging software version 10.0 (Oxford Instruments, Abingdon, United Kingdom) after staining for MUC5AC-AF405 (1:200; Novus Biologicals, Centennial, Colo), SCGB3A5-AF555 (1:100; Abcam, Fremont, Calif), cytokeratin 5-AF488 (1:200; Abcam), and acetylated-tubulin-AF647 (1:100; Santa Cruz Biotechnology, Dallas, Tex).

Targeted real-time PCR array

Targeted mRNA gene expression was assessed in primary airway epithelial cells with a commercial array (RT2 Profiler Human Type 1 Interferon Response, GeneGlobe ID PAHS016Z, Qiagen, Germantown, Md). Eight microliters of the first-strand cDNA synthesis reaction was preamplified (RT2 PreAMP PCR Master Mix and RT2 PreAMP Type 1 Interferon Response, Qiagen) and the equivalent of 0.8 μL cDNA was added to each well of the array plate. The cycle threshold values of the target cDNAs for each experimental marker were normalized to 5 housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*).

RNA-seq data analyses

The data preprocessing tool fastp (http://github.com/OpenGene/fastp) was used to perform quality control and adapter trimming[22] and the results were filtered by quality. The software program Hierarchical Indexing for Spliced Alignment of Transcripts 2 (https://github.com/DaehwanKimLab/hisat2) was used to map RNA-seq reads before building the index using the human reference genome grch38.[23] Indices were used to map sequential reads to the human genome. FeatureCounts was applied to generate a matrix of row counts of each of the genes in all the samples. The R package Deseq2 was used to determine differential gene expression. Variance-stabilizing transformations were used for raw data transformation. Data were then transformed on the $\log_2$ scale and normalized to library size. Anan empirical Bayes shrinkage methods were used to detect and correct for dispersion and calculate $\log_2$[fold change] for the genes. Differentially expressed genes were defined as having a $\left|\log_2[\text{fold change}]\right|$ of more than 1. The *P* values were corrected for multiple testing using the Benjamini-Hochberg procedure. A false-discovery rate (FDR) of less than 0.05 was considered significant.

RNA-seq pathway analyses

Reactome was used to obtain the relevant biological pathways from the differentially expressed genes. The report generated is an overrepresentation analysis based on a hypergeometric distribution test that determines whether certain Reactome pathways are enriched in the submitted differentially gene expression data compared with that by chance alone. The statistical test produces a probability score corrected for FDR using the Benjamini-Hochberg method. Only pathways with an FDR of less than 0.05 were used for the analysis.[24-29]

Other statistical analyses

Aeroallergen sensitization was defined as 1 or more positive aeroallergen specific IgE tests. Data not involving gene expression were analyzed with IBM SPSS software version 28 (Chicago, Ill). Baseline features of children with aeroallergen sensitization were compared with those of children without aeroallergen sensitization using chi-square tests and *t* tests. Symptom flare and exacerbation outcomes were assessed with negative binomial regression with adjustment for controller medications and tobacco smoke exposure. These analyses used a .05 significance level without adjustment for multiple comparisons.

RESULTS

Seventy-nine children with recurrent wheezing were included, including 25 children with aeroallergen sensitization and 54 children without sensitization. At enrollment, the median (25th, 75th percentile) number of days since the receipt of any systemic corticosteroids was 49 (32, 114) days and was not different between groups (sensitized: 56 (34, 159) days; not sensitized: 45 (31, 83) days; $P = .300$). Sensitization profiles are provided in Table E1 (in the Online Repository available at www.jaci-global.org). Most of the sensitized children (n = 15 [60%]) had multiple sensitizations. Other features of the participants are provided in Table I. There were no differences in age, sex, ethnicity, family history, historical wheezing episodes, current symptom control, or use of respiratory medications between groups. However, children with aeroallergen sensitization were more likely to be Black with lesser daycare attendance during the first 12 months of life, more previous intensive care unit admissions for wheezing, and more features of type 2 inflammation, including physician-diagnosed eczema, higher serum IgE levels, and higher blood eosinophil counts. Children with aeroallergen sensitization also had significantly higher concentrations of the plasma cytokines CXCL11, IL-4, IL-6, and IL-13 (Table I); there were no significant differences in the other plasma cytokines measured (data not shown).

Differential gene expression of freshly isolated blood neutrophils

Fifty-two children (no sensitization, n = 36; aeroallergen sensitization, n = 16) had acceptable RNA samples for RNA-seq analyses. Features of these children were not significantly different from those of the parent sample (data not shown). With an FDR of less than 0.05, 436 genes were differently expressed in isolated blood neutrophils from children with versus without aeroallergen sensitization, including 147 upregulated genes and 289 downregulated genes. Fold change gene expression differences are shown in a volcano plot in Fig 1. The top 50 upregulated and top 50 downregulated genes, ordered by adjusted *P* value, are provided in Tables E2 and E3 (in the Online Repository available at www.jaci-global.org).

Reactome pathway analyses with differentially expressed genes identified 38 significant upregulated pathways in sensitized children, with 4 of these meeting the FDR of less than 0.05. The upregulated pathways were overwhelmingly related to Wnt signaling, which regulates host responses to viral infection,[30] and included repression of Wnt target genes (adjusted $P = .012$), generation of second messenger molecules (adjusted $P = .012$), binding of T-cell factor/lymphoid enhancer factor family (ie, end-point mediators of Wnt signaling) to target gene promoters (adjusted $P = .024$), and runt-related transcription factor 3 (ie, a suppressor of canonical Wnt signaling) regulation of Wnt signaling (adjusted $P = .024$) (Fig 2, *A*). Reactome pathway analyses also identified 50 downregulated pathways in sensitized children, 9 of which met the FDR of less than 0.05. These downregulated pathways were predominantly related to antiviral responses and included interferon signaling, IFN-γ signaling, cytokine signaling in the immune system, IFN-α/β signaling, immune system (for all, adjusted $P = 1.68 \times 10^{-14}$), antiviral mechanism by interferon-stimulated genes (adjusted $P = 4.47 \times 10^{-5}$), neutrophil degranulation (adjusted $P = 8.46 \times 10^{-5}$), 2′-5′-oligoadenylate synthetase antiviral responses (adjusted $P = .003$), and interferon-stimulated gene 15 antiviral mechanisms (adjusted $P = .012$) (Fig 2, *B*).

Responses of isolated blood neutrophils to poly(I:C) stimulation

Given evidence of dysregulated antiviral responses in isolated blood neutrophils from children with versus without aeroallergen sensitization at baseline, neutrophils were then cultured overnight with low-molecular-weight intracellular poly(I:C) as a viral analog stimulus. Viability of the neutrophils was 79% and 81% for untreated versus poly(I:C)-stimulated neutrophils, respectively, after overnight culture. RNA-seq analyses were performed on paired, poly(I:C)-stimulated versus unstimulated neutrophils. Children with aeroallergen sensitization also had differential expression of 317 paired poly(I:C)-stimulated versus unstimulated genes, including 26 downregulated genes and 291 upregulated genes (Fig 3, *A*). The top 50 upregulated and 26 downregulated genes, ordered by adjusted *P* value, are listed in Tables E4 and E5 (in the Online Repository available at www.jaci-global.org). Reactome pathway analyses with differentially expressed genes identified 25 significant upregulated pathways in sensitized children with poly(I:C) stimulation (Fig 3, *B*), including IFN-α/β signaling, interferon signaling, cytokine signaling in the immune system (for all, adjusted $P = 1.90 \times 10^{-14}$), IFN-γ signaling (adjusted $P = 2.39 \times 10^{-12}$), antiviral mechanism by interferon-stimulated genes (adjusted $P = 6.16 \times 10^{-8}$), immune system (adjusted $P = 2.70 \times 10^{-5}$), interferon-stimulated gene 15 antiviral mechanism (adjusted $P = 5.30 \times 10^{-5}$), 2′-5′-oligoadenylate synthetase antiviral response (adjusted $P = 7.49 \times 10^{-4}$), TNF receptor–associated factor 3–dependent interferon regulatory factor activation pathway (adjusted $P = .035$), negative regulators of DEAD Box Protein 58 (also known as the antiviral innate immune response receptor retinoic acid–inducible gene 1)/interferon induced with helicase C domain 1 signaling (adjusted $P = .039$), and DEAD Box Protein 58/interferon induced with helicase C domain 1–mediated induction of IFN-α/β (adjusted $P = .043$). The IFN-α/β signaling pathway is also shown in Fig 3, *B*, but it did not meet the threshold of significance (adjusted $P = .088$). Reactome pathway analyses did not identify any significant downregulated pathways in sensitized children.

Confirmatory gene expression experiments

Because IL-4 is a key cytokine associated with secretion of IgE by B lymphocytes and allergic inflammation and was upregulated systemically in children with aeroallergen sensitization at baseline, confirmatory experiments were performed in primary human

TABLE I. Features of the participants

Feature	No sensitization (N = 54)	Sensitization (N = 25)	*P* value
Age (mo)	33.5 ± 13.9	35.0 ± 13.4	.661
Sex: male	32 (59.3)	19 (76.0)	.148
Race			
White	30 (55.6)	5 (20.0)	.010
Black	20 (37.0)	18 (72.0)	
Other	4 (7.4)	2 (8.0)	
Hispanic ethnicity	7 (13.0)	3 (12.0)	.905
Body mass index percentile	66.1 ± 29.0	69.0 ± 25.8	.665
Age of symptom onset (mo)	14.1 ± 9.9	15.3 ± 12.1	.645
Family history			
Mother with asthma	21 (38.9)	14 (56.0)	.154
Father with asthma	21 (38.9)	12 (48.0)	.445
Highest household education			
Did not complete high school	0	1 (4.0)	.452
High school or GED	3 (5.6)	2 (8.0)	
Technical training	1 (1.9)	2 (8.0)	
Some college, no degree	10 (18.5)	4 (16.0)	
Associate degree	7 (13.0)	4 (16.0)	
Bachelor's degree	33 (61.1)	12 (48.0)	
Combined household income			
Don't know	3 (5.6)	1 (4.0)	.308
<$25,000	4 (7.4)	2 (8.0)	
$25,000-$49,999	8 (14.8)	9 (36.0)	
$50,000-$99,999	17 (31.5)	5 (20.0)	
≥$100,000	22 (40.7)	8 (32.0)	
Indoor exposures			
Cat	8 (14.8)	0	.042
Dog	21 (38.9)	7 (28.0)	.347
Tobacco smoke	7 (13.0)	2 (8.0)	.518
Daycare attendance			
Attended during first 12 mo	30 (55.6)	5 (20.0)	.003
Current attendance	44 (81.5)	18 (72.0)	.340
Past health care utilization for wheezing			
Hospitalization (ever)	25 (46.3)	17 (68.0)	.072
Intensive care unit admission (ever)	12 (22.2)	11 (44.0)	.048
Wheezing episodes (past 12 mo)	5.7 ± 5.3	5.9 ± 4.8	.838
With unscheduled visits	3.1 ± 1.4	2.7 ± 1.8	.349
With oral corticosteroid bursts	2.5 ± 1.6	3.1 ± 2.8	.225
TRACK score*	63.0 ± 20.1	60.6 ± 22.1	.639
TRACK score <80	42 (77.8)	18 (72.0)	.576
Current controller medications			
Inhaled corticosteroid	27 (50.0)	13 (52.0)	.869
Long-acting β-agonist	6 (11.1)	2 (8.0)	.670
Montelukast	7 (13.0)	4 (16.0)	.717
No. of controllers	1 (0, 1)	1 (0, 1)	.926
Eczema (physician-diagnosed)	22 (40.7)	18 (72.0)	.010
Serum IgE (kU/L)	29.9 (12, 89)	250 (98, 708)	<.001
Blood eosinophil count (cells/mL)	202 (131, 318)	342 (207, 586)	.014
Plasma cytokines (pg/mL)			
CXCL11	17.0 (11.6, 24.5)	20.5 (11.7, 31.2)	.034
IL-4	25.8 (16.7, 35.6)	30.1 (15.5, 76.2)	<.001
IL-6	1.7 (1.0, 2.5)	3.0 (1.4, 10.8)	<.001
IL-13	1.9 (1.4, 2.9)	3.9 (2.1, 7.5)	<.001

Data are represented as mean ± SD, the median (25th, 75th percentile), or the number of participants (%).
GED, General Equivalency Diploma; *TRACK*, Test for Respiratory and Asthma Control in Kids.
*Scores are summed from 0 to 100, with higher scores reflecting better asthma control. Scores <80 reflect uncontrolled asthma.

neutrophils exposed to IL-4 and poly(I:C). Using a targeted nanostring array, we first compared primary donor neutrophils treated with IL-4 to donor neutrophils treated with vehicle. In these experiments, IL-4 treatment was associated with significant upregulation of *IFITM1*, which suppresses the early stages of viral replication, but downregulation of multiple other type 1 interferon–related genes (Fig 4, *A*). IL-4 exposure also increased expression of the Janus kinase (JAK) gene *JAK1*, *SOCS1*, and the signal transducer and activator of transcription (STAT) gene *STAT6*, which are associated with IL-4 activation, and

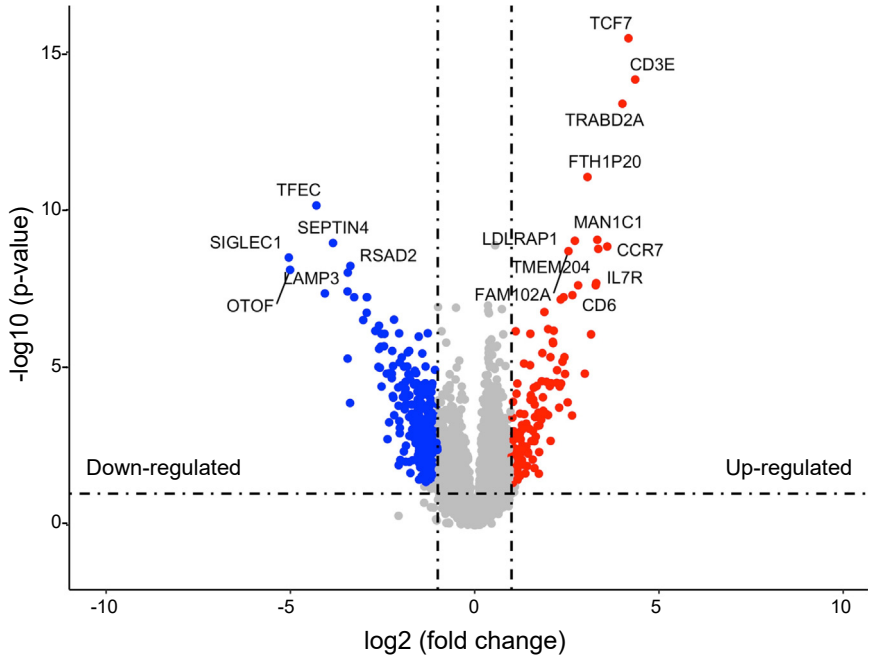

FIG 1. Volcano plot of differentially expressed genes in isolated neutrophils from children with vs without aeroallergen sensitization. Significance thresholds for *P* values and fold change are represented by dashed lines. *P* values were adjusted for an FDR of 0.05.

downregulated *STAT5A*, *STAT3*, *STAT2*, and *MX1* (Fig 4, *B*). However, when primary neutrophils treated with IL-4 were stimulated with poly(I:C), they had significant upregulation of type 1 interferon–related genes (Fig 4, *C*) similar to our observations in poly(I:C)-stimulated neutrophils from children with aeroallergen sensitization, and upregulation of multiple STAT genes, with downregulation of *JAK1* (Fig 4, *D*). The antiviral response to poly(I:C) was exclusive to IL-4–treated neutrophils, because neutrophils treated with vehicle versus those stimulated with poly(I:C) alone showed no significant upregulation of type 1 interferon genes or JAK/STAT genes (data not shown). Similarly, there were no differences in type 1 interferon genes or JAK/STAT genes when neutrophils stimulated with IL-4 plus poly(I:C) were compared with neutrophils stimulated with poly(I:C) alone (data not shown).

Impact of neutrophil poly(I:C) exposure on airway epithelial cell gene expression

Because systemic neutrophils are not the primary effector cell in children with allergic asthma, we also examined how poly(I:C)-treated neutrophils from children with versus without aeroallergen sensitization influenced gene expression in primary human small airway epithelial cells. For this proof-of-concept experiment, small epithelial airway cells isolated from the distal portion of the lung containing goblet, club, and basal cells were differentiated to the point of cilia formation (see Fig E1 in this article's Online Repository at www.jaci-global.org). Poly(I:C)-treated neutrophil culture supernatants from 10 children with aeroallergen sensitization and 10 children without aeroallergen sensitization who were representative of the parent sample were pooled for this experiment. Primary airway epithelial cells were cultured overnight in the neutrophil supernatant and were analyzed for expression of type 1 interferon genes with a targeted array. This analysis demonstrated increased epithelial expression of *CAV1*, which encodes the membrane protein caveolin 1; *CCL2* and *CXCL10*, which promote airway hyperresponsiveness and epithelial fibrosis; *HLAG*, which encodes histocompatibility antigen, class I, HLA-G, and has been associated with allergic disorders; the interferon-induced transmembrane proteins *IFITM1* and *IFITM2*; *IFNA1*; *IL6*; *SHB*, an adapter protein involved in regulation of immune responses; *SOCS1*, a STAT-induced suppressor of cytokine signaling; *TIMP1*, which inhibits tissue metalloproteinases; and *VEGFA* in children with versus without aeroallergen sensitization (Fig 5).

Symptom flares and exacerbations in enrolled participants

After enrollment, participants (N = 79) were followed by telephone and with diary cards for 12 months. Nearly all the children had at least 1 respiratory symptom flare (92.0% vs 96.3% occurrence for children with vs without aeroallergen sensitization; $P = .418$), which predominantly occurred in the fall months between September and November (see Fig E2 in this article's Online Repository at www.jaci-global.org). Fifty-two children (65.8%) had an exacerbation treated with prednisolone (64.0% vs 66.7% occurrence for children with vs without aeroallergen sensitization; $P = .816$). The number of reported symptom flares (3.08 ± 1.59 vs 3.02 ± 2.05; $P = .940$) and the number of exacerbations (2.08 ± 2.43 vs 1.91 ± 2.05; $P = .769$) over 12 months was also not different between children with versus without

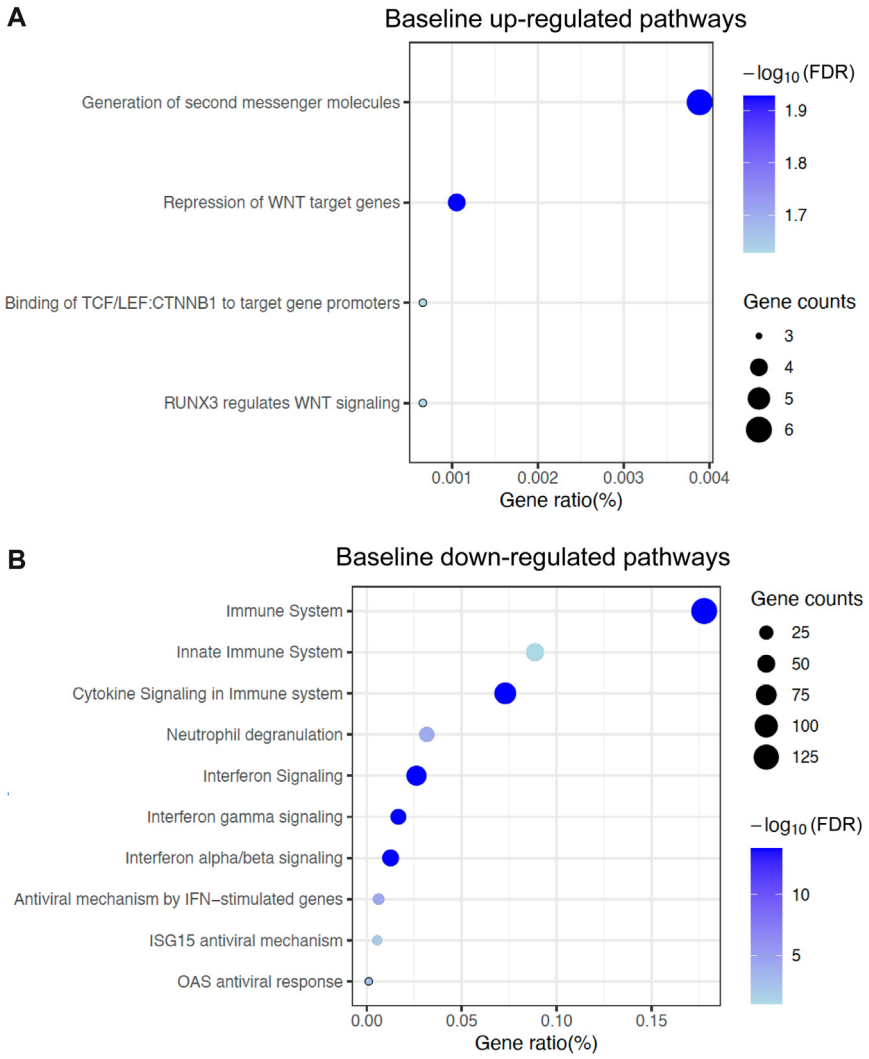

FIG 2. **A** and **B,** Upregulated (Fig 2, *A*) and downregulated (Fig 2, *B*) Reactome pathways listed by gene ratio (%) for the significant genes from isolated blood neutrophils from children with vs without aeroallergen sensitization. Circle size indicates the gene counts in the pathway. *Darker blue* indicates a lower adjusted *P* value. An FDR of less than .05 was considered significant. *ISG15,* Interferon-stimulated gene 15; *OAS,* 2′-5′-oligoadenylate synthetase; *TCF/LEF,* T-cell factor/lymphoid enhancer factor.

aeroallergen sensitization, even after adjustment for controller mediations and tobacco smoke exposure ($P > .05$ for each).

Overall, 63 children (79.7%) submitted diary cards. Diary cards were completed during a respiratory symptom flare for 45 (58.4%) children (not sensitized, n = 31; sensitized, n = 14) and during an acute exacerbation requiring prednisolone for 28 (44.2%) children (not sensitized, n = 18; sensitized, n = 10). Demographic features including age, sex, race, ethnicity, socioeconomic features, Test for Respiratory and Asthma Control in Kids scores, controller medications, exposures, historical wheezing episodes, and health care utilization did not differ between children who completed the diary cards while symptomatic versus those who did not (data not shown). Despite similar occurrences, symptom scores during flares and exacerbations were significantly higher (ie, worse/more burdensome) in children with versus without aeroallergen sensitization (Fig 6, *A* and *B*). These symptom differences persisted for 3 days in sensitized children. Children with aeroallergen sensitization also received more daily inhalations of albuterol sulfate during flares and exacerbations (Fig 6, *C* and *D*), suggesting they were not undertreated.

DISCUSSION

In this study, we quantified gene expression in isolated blood neutrophils from preschool children with recurrent wheezing at baseline and after *ex vivo* stimulation with low-molecular-weight poly(I:C) as a viral analog stimulus. Consistent with our hypothesis, we observed different neutrophil gene expression patterns, namely, dysregulated type 1 interferon responses, in children with versus without aeroallergen sensitization who also had evidence of systemic type 2 inflammation. Although children with aeroallergen sensitization had *impaired* neutrophil type 1 interferon gene expression at baseline, these same children had *more robust* neutrophil type 1 interferon responses after *ex vivo* poly(I:C) stimulation. In confirmatory experiments, this dysregulation of type 1 interferon responses was related to IL-4.

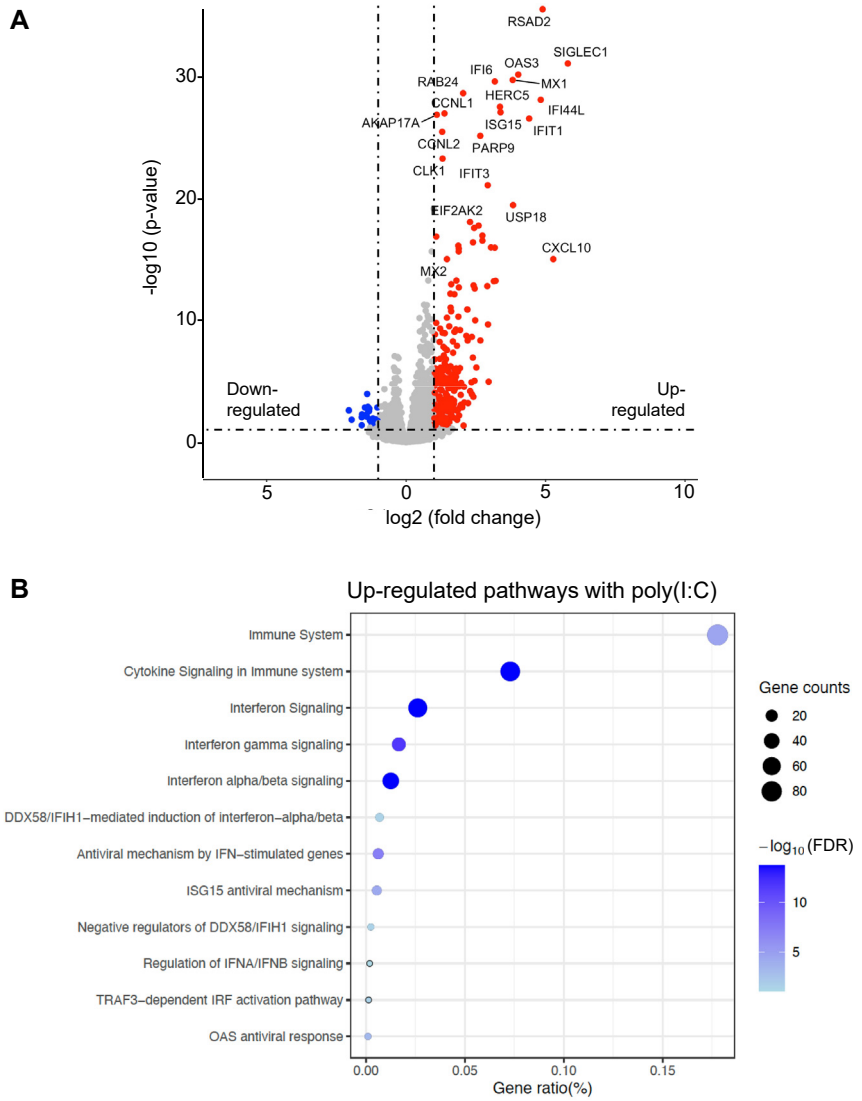

FIG 3. A and **B,** Volcano plot of differentially expressed genes (Fig 3, *A*) and upregulated Reactome pathways (Fig 3, *B*) listed by gene ratio (%) for the significant genes from paired, poly(I:C)-stimulated neutrophils from children with vs without aeroallergen sensitization. Circle size indicates the gene counts in the pathway. *Darker blue* indicates a lower adjusted *P* value. An FDR of less than 0.05 was considered significant. *DDX58,* DEAD Box Protein 58; *IFIH1,* interferon induced with helicase C domain 1; *ISG15,* interferon-stimulated gene 15; *OAS,* 2′-5′-oligoadenylate synthetase; *TRAF,* TNF receptor–associated factor 3.

Furthermore, neutrophils stimulated with poly(I:C) from children with aeroallergen sensitization also influenced gene expression in primary small airway epithelial cells and upregulated genes associated with airway inflammation and type 1 interferons. Children with aeroallergen sensitization also reported more severe and prolonged symptoms during respiratory flares and acute exacerbations. Together, these findings suggest that neutrophil type 1 interferon responses to respiratory viruses, while present, are dysfunctional in sensitized preschool children with recurrent wheezing and may also promote airway tissue damage and inflammation in these children.

Type 1 interferons are ubiquitously expressed[31] and provide the first line of defense against respiratory viruses by inducing hundreds of interferon-stimulated genes through the JAK/STAT signaling cascade,[32] which induce neutrophil activation and recruitment to the site of infection.[33] Although type 1 interferon responses have been extensively studied in asthma, understanding of type 1 interferon responses has evolved considerably over the past 2 decades. For example, several older studies from otherwise healthy patients with asthma previously demonstrated decreased or impaired type 1 interferon mRNA or protein in PBMCs, dendritic cells, nasal and bronchial epithelial cells, and bronchoalveolar lavage cells compared with healthy controls.[34] This led to the conclusion that type 1 interferon responses were impaired in patients with asthma, yet clinical trials of on-demand inhaled IFN-β administration failed to improve exacerbation-related outcomes in asthmatic adults.[35,36] More recent studies have instead focused on type 1 interferon responses after viral stimuli. In contrast to previous work, these studies have demonstrated robust epithelial type 1 interferon responses in adult patients with asthma 3 to 6 days after experimental rhinovirus infection, which correlated with viral loads, clearance,

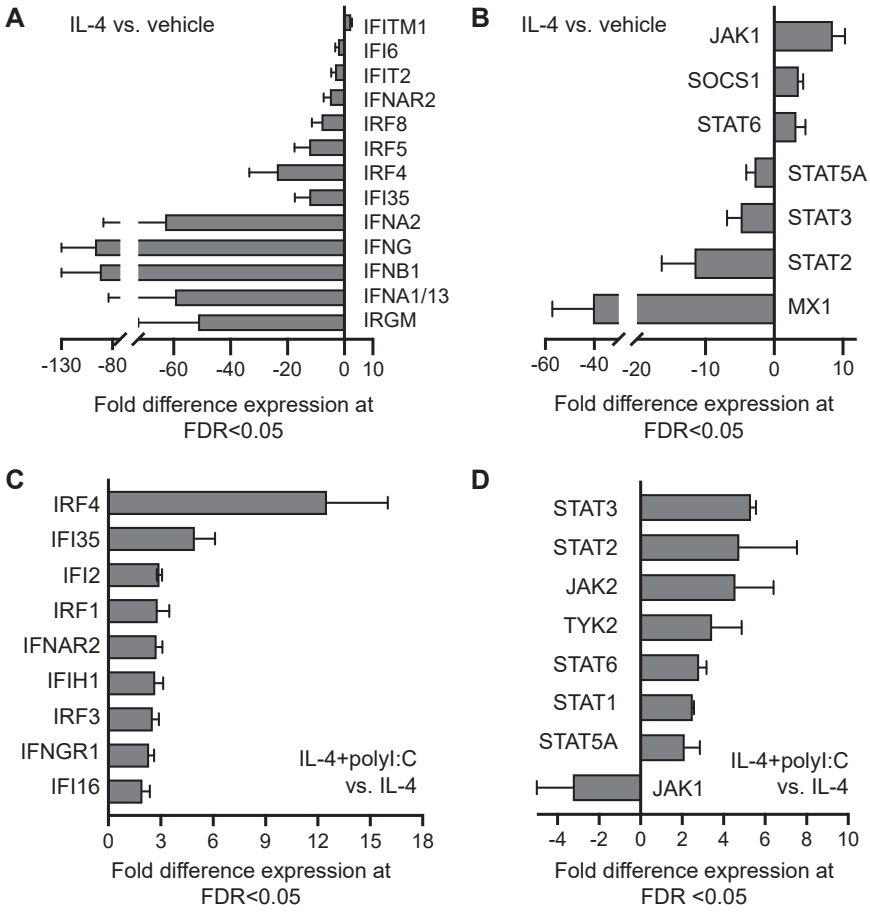

FIG 4. Nanostring expression of type 1 interferon genes and JAK/STAT genes (fold difference expression, mean ± SEM) from primary donor neutrophils treated with IL-4 (**A** and **B**, respectively) and neutrophils treated with IL-4 plus poly(I:C) vs IL-4 (**C** and **D**, respectively). The genes shown are significantly different at an FDR of less than 0.05.

and worsening of airflow obstruction.[37-39] Similarly, *ex vivo* infection of bronchial epithelial cells with respiratory syncytial virus likewise resulted in greater type 1 interferon responses in asthmatic children with versus without airflow obstruction.[40] In a separate study using a rhinovirus model of differentiated primary epithelial cells, the induction of type 1 interferon gene expression was evident at 24 hours and peaked at 48 hours in healthy adults, but peaked in adults with asthma at 72 to 96 hours.[41] Similar observations of increased type 1 interferon production have been made in isolated PBMCs from asthmatic children after *ex vivo* stimulation with rhinovirus[42] and poly(I:C)[43] and in PBMCs[44] and nasal epithelial cells[45,46] from children during acute viral wheezing episodes, although heterogeneity in interferon responses was noted.[45,46] Together, these findings suggest that type 1 interferon responses during viral exacerbations of asthma are delayed, rather than deficient, and may promote a more inflammatory immune response. Our observations of impaired type 1 interferon gene expression in neutrophils at baseline and robust type 1 interferon gene expression after *ex vivo* poly(I:C) stimulation support this hypothesis and extend knowledge of type 1 interferon responses to neutrophils, which have not been previously studied in asthma.

Preschool children with recurrent wheezing are remarkably heterogeneous. Other unsupervised multiomics studies have identified endotypes of children with acute wheezing illnesses that were differentiated by aeroallergen sensitization and type 2–mediated inflammation.[13,14] Recognizing that these preschool children with aeroallergen sensitization also have differing longitudinal wheezing outcomes and treatment responses than children without aeroallergen sensitization,[47,48] we elected to perform hypothesis testing between these 2 predefined clinical groups. Our confirmatory gene expression experiments suggest that the exaggerated interferon responses in neutrophils from children with aeroallergen sensitization are associated with IL-4. This observation is consistent with other literature demonstrating induction of interferon genes in mouse lung cells after intratracheal administration of IL-4.[49] Similarly, IL-4 treatment of human mast cells has also been shown to increase type 1 interferon responses to mucosal viral infection.[50] The mechanisms associated with the initial delay in type 1 interferons are unclear, but could be related to other type 2 inflammatory features such as IgE and blood eosinophilia. Indeed, others have shown IgE-mediated[51] and eosinophil-mediated[52] suppression of interferon responses in plasmacytoid dendritic cells, which typically release significant concentrations of IFN-α on viral encounter.[53] Treatment with anti-IgE (ie, omalizumab) and anti–IL-5/5Rα (ie, mepolizumab and benralizumab) antibodies has been shown to enhance plasmacytoid dendritic cell antiviral IFN-α responses in patients with

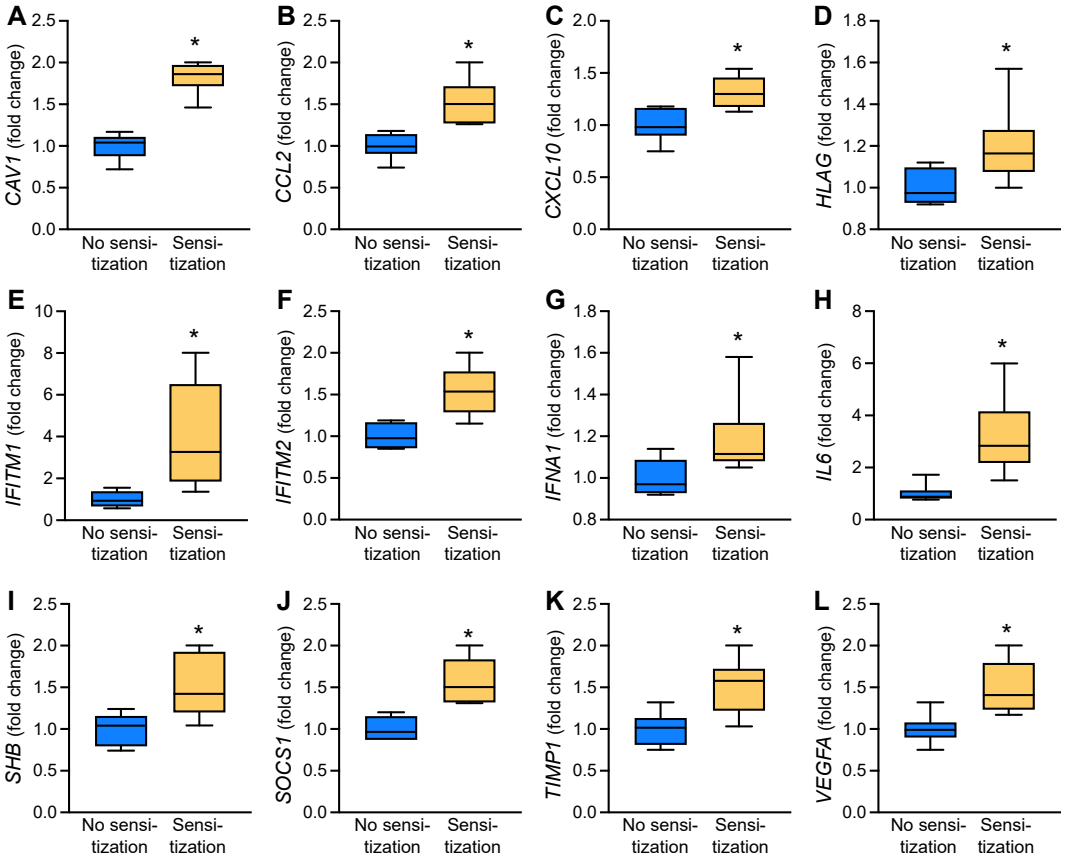

FIG 5. A-L, Fold change mRNA gene expression of *CAV1* (Fig 5, *A*), *CCL2* (Fig 5, *B*), *CXCL10* (Fig 5, *C*), *HLAG* (Fig 5, *D*), *IFITM1* (Fig 5, *E*), *IFITM2* (Fig 5, *F*), *IFNA1* (Fig 5, *G*), *IL6* (Fig 5, *H*), *SHB* (Fig 5, *I*), *SOCS1* (Fig 5, *J*), *TIMP1* (Fig 5, *K*), and *VEGFA* (Fig 5, *L*) in primary airway epithelial cells cultured overnight with poly(I:C)-treated neutrophil culture supernatants (pooled from 10 children per group). Boxplots reflect the median and 5th to 95th percentile from 6 independent experiments. *$P < .05$.

asthma,[54] which may account for the improvements in exacerbation rates seen with these therapies.[55]

To our knowledge, this is the first study to characterize type 1 interferon responses in neutrophils from children with recurrent wheezing. Strengths of the study include the careful phenotyping and stratification of enrolled children, application of next-generation sequencing methods, and the characterization of exacerbation responses. Nonetheless, this study does have limitations. First, we used poly(I:C), a double-stranded RNA, to evaluate the immune responsiveness to viral infection. Therefore, the responses of blood neutrophils to poly(I:C) that we observed in this study may differ from the responses to other viruses such as human rhinoviruses.[56] We also examined differential gene expression in blood neutrophils, which may have a different phenotype from that of the extravasated neutrophils in the airway. The sample size was also small, with blood neutrophil experimentation performed at a single time point when participants were otherwise healthy. Because respiratory viral testing was not performed, we were unable to assess whether intercurrent viral infections not contributing to wheezing were present. We also cannot comment on the developmental regulation of type 1 interferons, which has been previously reported in the literature,[57] or how these type 1 interferon responses change or persist during acute viral illness. The lack of healthy controls is also a limitation that prohibits complete understanding of the type 1 interferon responses observed in our study population. Finally, although our groups were well balanced with regard to asthma features, we cannot rule out unmeasured sources of confounding, including confounding by asthma treatment or inhaled corticosteroids.

This study demonstrates dysregulated type 1 interferon responses in children with versus without aeroallergen sensitization, with impaired gene expression at baseline yet robust neutrophil type 1 interferon responses after *ex vivo* poly(I:C) stimulation. This robust type 1 interferon response also promoted type 1 interferon gene expression in primary airway epithelial cells and was associated with more severe and prolonged symptoms during respiratory flares and acute exacerbations. Given the importance of type 1 interferon signaling in viral resolution, these findings argue for additional studies of the role of neutrophil type 1 interferons in this population, particularly *in vivo* studies during acute wheezing exacerbations.

DISCLOSURE STATEMENT

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

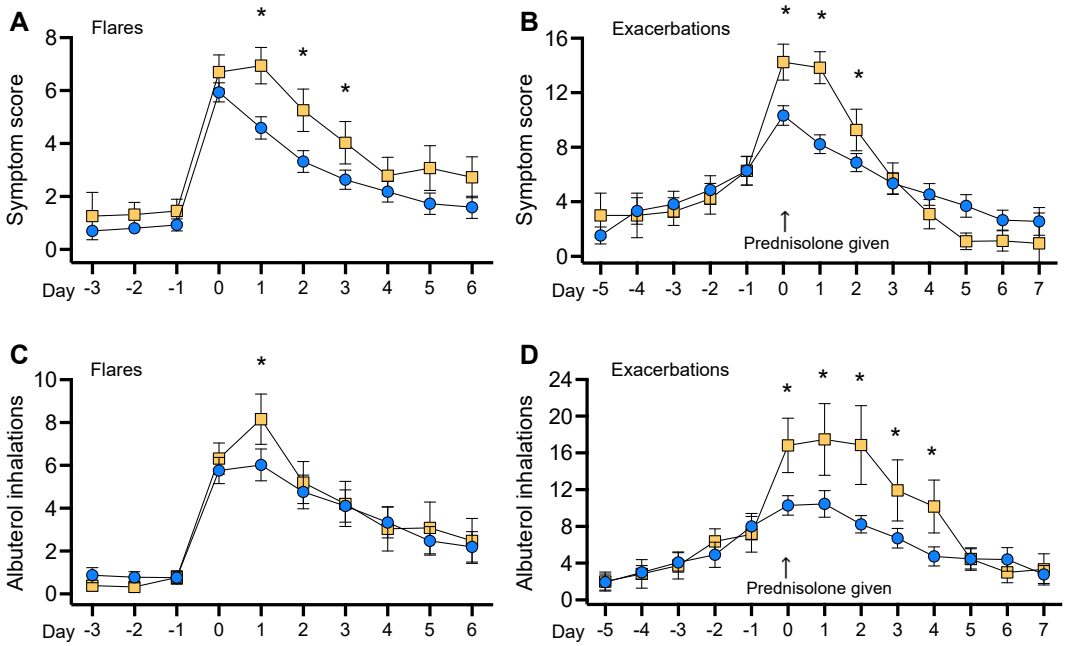

FIG 6. A-D, Diary card reported symptom scores (mean ± SEM) during respiratory symptom flares (Fig 6, *A*) and exacerbations (Fig 6, *B*) and albuterol sulfate inhalations (mean ± SEM) during respiratory flares (Fig 6, *C*) and exacerbations (Fig 6, *D*) in children with (*orange*) vs without (*blue*) aeroallergen sensitization. **P* < .05.

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Key messages

- **Preschool children with recurrent wheezing and aeroallergen sensitization, compared with children without aeroallergen sensitization, have impairment of type 1 interferons at baseline, yet robust type 1 interferon responses after viral stimulation, which may be due to increased circulating IL-4 concentrations.**
- **Poly(I:C)-stimulated blood neutrophils from children with aeroallergen sensitization also increased type 1 interferon responses in primary human small airway epithelial cells.**
- **Children with aeroallergen sensitization also had more severe and prolonged symptoms during acute exacerbations. Together, these findings suggest that neutrophil type 1 interferon responses are dysregulated in preschool children with recurrent wheezing and aeroallergen sensitization and could contribute to exacerbation severity in these children.**

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