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Bridging the gap between immunology, virology, genetics, and epigenetics in bronchiolitis: The multiomics pathway to asthma development

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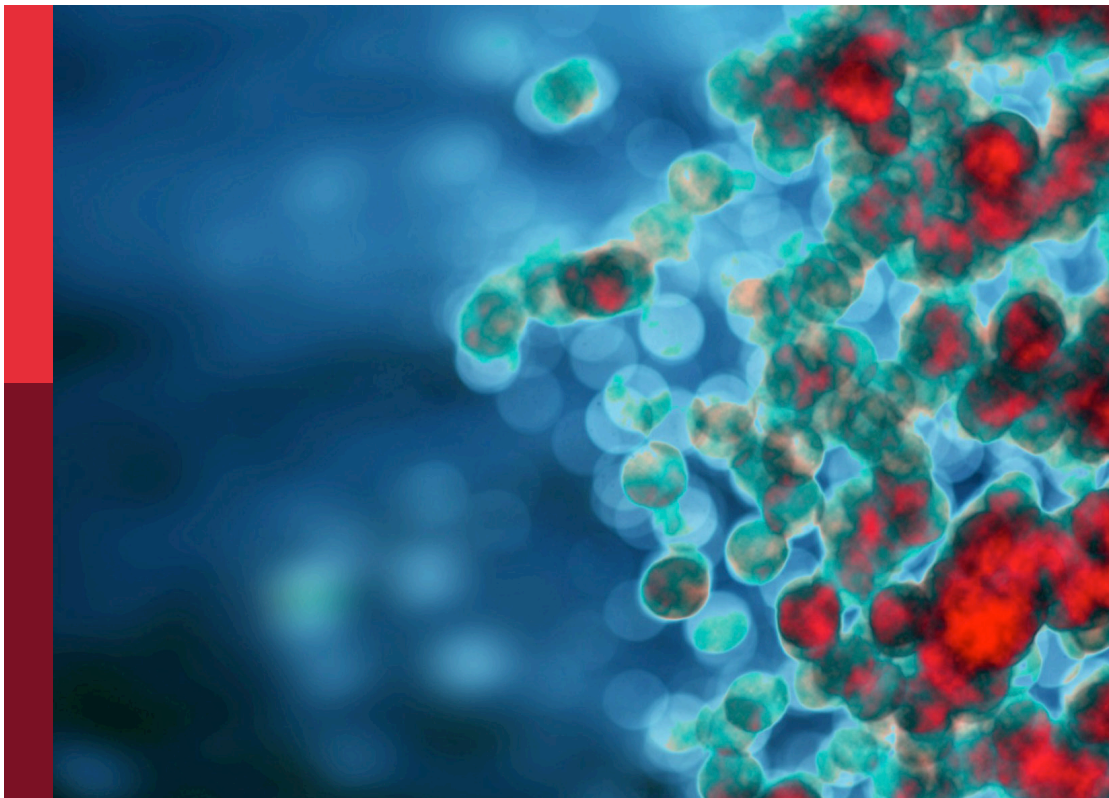

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Bridging the gap between immunology, virology, genetics, and epigenetics in bronchiolitis: The multiomics pathway to asthma development

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Editorial: Bridging the gap between immunology, virology, genetics, and epigenetics in bronchiolitis: The multiomics pathway to asthma development

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Editorial on the Research Topic
Bridging the gap between immunology, virology, genetics, and epigenetics in bronchiolitis: The multiomics pathway to asthma development

Severe bronchiolitis (i.e., bronchiolitis or first episode of wheeze requiring hospitalization) during infancy is a heterogeneous condition associated with an increased risk for childhood asthma development (1, 2). Bronchiolitis cohort studies have identified early-life environmental, genetic and immune risk factors for childhood asthma development by carrying out analysis at single level (e.g., associations with respiratory virus types, host immune response or the microbiome composition of the host) (3, 4). However, severe bronchiolitis pathogenesis involves interaction of factors at multiple levels (e.g., genome, epigenome, transcriptome, metabolome, microbiome). Optimistically, the increasing use of omics methodologies in observational studies allows for a more holistic approach, that can shed light on severe bronchiolitis pathophysiology by identifying distinct biological processes associated with long-term sequelae like asthma (5). In addition, mechanistic studies are required to validate and test identified pathogenetic pathways from omics studies.

To further address the issues outlined above, our Research Topic congregates evidence from observational and interventional studies exploring the severe bronchiolitis to childhood asthma causal pathway with the aim to identify severe bronchiolitis endotypes that can guide predictive (i.e., response to treatment) and prognostic (i.e., association with long-term respiratory sequelae) enrichment strategies (see Figure 1). The current editorial introduces our research collection by discussing the relevant studies.

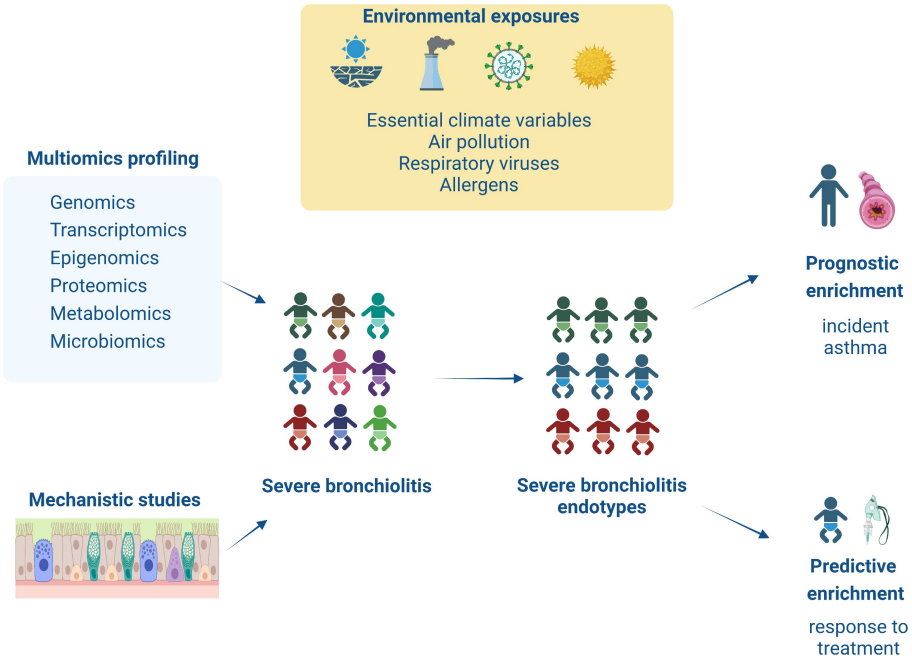

FIGURE 1
This figure summarizes the content of the articles collected on this Research Topic. Infants with severe bronchiolitis are at higher risk of having a severe and prolonged hospitalization and long-term respiratory sequelae (i.e., asthma). However, due to increased heterogeneity in clinical presentation and underlying pathology in severe bronchiolitis, we are still missing targeted treatments and sensitive biomarkers for asthma development. This Research Topic presents novel studies utilizing multiomics (i.e., genomic, transcriptomic, epigenomic, proteomic, and metabolomic) or mechanistic (i.e., *in/ex vivo*, *in vitro*) approaches to explore in-depth severe bronchiolitis pathophysiology. These findings help identify severe bronchiolitis endotypes that respond to targeted treatments (i.e., predictive enrichment) or are associated with increased risk for asthma development (i.e., prognostic enrichment).

Starting with environment-gene interactions in severe bronchiolitis, the role of epigenetic mechanisms as drivers of asthma pathogenesis has been explored (6). Epigenetic mechanisms may underpin a crosstalk between maternal and infant immune systems associated with diverse lung function trajectories (7). However, although the mediating role of epigenetic mechanisms is well understood in viral infections causing latency, further studies are needed to validate findings in viral infections causing acute illness (8). In this Research Topic, Pischedda et al. compare the methylome of children who have been hospitalized with respiratory syncytial virus (RSV)-induced bronchiolitis and are followed-up for three years. Their study assessed differentially methylated positions (DMPs) in genes in peripheral blood samples and associated with risk for recurrent wheeze development. The lead methylation position (cg24509398) identified in this study falls at the gene body of *Eyes absent protein 3* (*EYA3*), a tyrosine phosphatase connected with pulmonary vascular remodeling, a key underlying pathogenetic mechanism in asthma. Interestingly, atopic asthma after rhinovirus-induced severe wheeze has been associated with different type of DNA methylation changes, (i.e., change in *SMAD3 gene* promoter) (9).

Keeping the above findings in mind, an *in vivo* study submitted at this research collection, focused on identifying the role of regulators *Methyl-CpG-binding domain protein 2 (MBD2)* and *Misshapen-like kinase 1 (MINK1)* in T-helper (Th)17-dominant asthma (Chen et al.). The *MBD2* and *MINK1* genes were silenced or overexpressed by small interfering RNA and plasmids and the study showed that *MBD2* and *MINK1* regulate Th17 cell differentiation and IL-17 release. It is of note that other *in vivo* models of chronic airway inflammation have showcased that Th17 responses contribute to airway remodelling too, independent of the Th2 responses (10). Through an analytical assessment of the above findings, we suggest future assessment of *EYA3* methylation patterns in Th17 cells in severe bronchiolitis as possible endotypes of early airway remodelling. The above studies identified a possible severe bronchiolitis endotype at risk of early airway remodelling. However, the question whether epigenetic changes associated with airway remodelling are triggered by specific respiratory viruses still remains unanswered.

To further approach the question around the role of respiratory viruses, two observational studies submitted at this research collection attempt to identify genetic variants and cytokine expression patterns in severe bronchiolitis and relate to subsequent asthma development (Hurme et al.; Dong et al.). The first study shows that infants with decreased expression of a thymus and activation-regulated chemokine (TARC) or increased expression of interleukin 13 (IL-13) in anti-CD3/anti-CD28 stimulated PBMC during a rhinovirus-induced acute respiratory infection are associated with relapses within a 2-month period of follow-up. The second study shows that genetic variants in the interleukin 33 gene in infants with severe bronchiolitis, regardless respiratory virus type etiology, is associated with increased risk for asthma development. Although these studies identify possible biomarkers for asthma development, we still need to understand whether pro-Th2 or Th2 mediators are more strongly associated with subsequent asthma development in comparison to respiratory virus type.

Toward this direction, a systematic review submitted at this research collection explores the potential for use of respiratory virus

testing in guiding acute treatment with corticosteroids and prediction of subsequent long-term respiratory sequela (Ambrozej et al.). However, meta-analysis was not possible due to small number of studies. However, evidence points toward non-RSV viruses being associated with higher risk for asthma and increased effectiveness with corticosteroids and warrants further trials. Possibly, other than respiratory virus exposures (i.e., innate immune responses represented by macrophage differentiation or extracellular vesicles) can act as more sensitive biomarkers too. The relevant evidence is presented in the following reviews (Wang et al. and Ambrożej et al.).

In addition to respiratory virus types as possible exposures associated with increased risk for asthma development, this research collection is bringing to the fore a comprehensive review around the role of microbial dysbiosis in asthma development (Liu et al.). In response to non-protective environmental exposures (air pollutants, antibiotics, allergens), disruption of lung and gut microbiota diversity is associated with the activation of leukocytes and secretion of mediators that further promote Th2 differentiation. Therefore, as discussed at the description of this Research Topic, the interaction between exposures in severe bronchiolitis is complex, and analytical integration of data deriving from these epidemiological studies may be required.

This integrative approach is highlighted through the last study of this research collection presenting the first integration of genomic and metabolomic data in severe bronchiolitis (Ooka et al.). In this study, 749 infants with severe bronchiolitis underwent both genotyping and nasopharyngeal metabolome profiling. Through an integrated analysis, sphingomyelins, genes on chromosome 19p13 (e.g., *MUC16*), and *1,2-dioleoyl-GPG* were associated with increased risk for asthma development. Although these genes are known to be associated with asthma development, their biological functional potential is further enhanced through confirmation of metabolites expression too.

In brief, this Research Topic highlights that severe bronchiolitis is a heterogeneous condition. Different respiratory viruses, in interaction with the host genome, methylome, proteome and microbiome, can possibly drive risk for asthma development. However, the exact mechanisms are still unknown and therefore there is lack of validated predictors for severity in the acute phase as well as risk predictors for asthma. There is no doubt that, with the use of novel sampling methods and novel omics analytical approaches followed by mechanistic studies, we can advance our knowledge on bronchiolitis endotyping and identify novel therapeutic targets and biomarkers for asthma development.

Author contributions

HMa: composed and distributed the first draft of the manuscript, designed the figure. HMo: revised the drafted manuscript and the figure. EN: revised the drafted manuscript and the figure. TJ: revised the drafted manuscript and the figure and provided detailed feedback around the included studies he co-authored. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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ORIGINAL RESEARCH
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Role and Diagnostic Performance of Host Epigenome in Respiratory Morbidity after RSV Infection: The EPIRESVi Study

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Background: Respiratory syncytial virus (RSV) infection has been associated with the subsequent development of recurrent wheezing and asthma, although the mechanisms involved are still unknown. We investigate the role of epigenetics in the respiratory morbidity after infection by comparing methylation patterns from children who develop recurrent wheezing (RW-RSV), subsequent asthma (AS-RVS), and those experiencing complete recovery (CR-RSV).

Methods: Prospective, observational study of infants aged < 2 years with RSV respiratory infection admitted to hospital and followed-up after discharge for at least three years. According to their clinical course, patients were categorized into subgroups: RW-RSV (n = 36), AS-RSV (n = 9), and CR-RSV (n = 32). The DNA genome-wide methylation pattern was analyzed in whole blood samples, collected during the acute phase of the infection, using the Illumina Infinium Methylation EPIC BeadChip (850K CpG sites). Differences in methylation were determined through a linear regression model adjusted for age, gender and cell composition.

Results: Patients who developed respiratory sequelae showed a statistically significant higher proportion of NK and CD8T cells (inferred through a deconvolution approach) than those with complete recovery. We identified 5,097 significant differentially methylated positions (DMPs) when comparing RW-RSV and AS-RVS together against CR-RSV.

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Methylation profiles affect several genes involved in airway inflammation processes. The most significant DMPs were found to be hypomethylated in cases and therefore generally leading to overexpression of affected genes. The lead CpG position (cg24509398) falls at the gene body of *EYA3* (*P*-value = 2.77×10^{-10}), a tyrosine phosphatase connected with pulmonary vascular remodeling, a key process in the asthma pathology. Logistic regression analysis resulted in a diagnostic epigenetic signature of 3-DMPs (involving genes *ZNF2698*, *LOC102723354* and *RPL15/NKIRAS1*) that allows to efficiently differentiate sequelae cases from CR-RSV patients (AUC = 1.00). Enrichment pathway analysis reveals the role of the cell cycle checkpoint (FDR *P*-value = 4.71×10^{-2}), DNA damage (FD*P*-value = 2.53×10^{-2}), and DNA integrity checkpoint (FDR *P*-value = 2.56×10^{-2}) in differentiating sequelae from CR-RSV patients.

Conclusions: Epigenetic mechanisms might play a fundamental role in the long-term sequelae after RSV infection, contributing to explain the different phenotypes observed.

Keywords: RSV, DNA methylation, immune system, respiratory sequelae, recurrent wheezing, asthma

INTRODUCTION

Respiratory syncytial virus (RSV) is a common pathogen that infects children by two years of age and is the leading global cause of hospitalization of infants (1). It is the principal cause of acute lower respiratory infections (ALRI) in young children, and it is associated with morbidity and mortality in childhood (2). Approximately 34 million new ALRI episodes in children worldwide were attributable to RSV (1), a huge number that resulted in 3.2 million hospital admissions and almost 60,000 global childhood deaths each year. No effective vaccines are available to treat RSV yet, although some good candidates are being tested in large-state clinical trials (3, 4). For the time being, palivizumab is the only monoclonal antibody approved to prevent severe RSV in infants and children at high risk for severe disease (5, 6).

Besides the acute burden of RSV, a growing body of evidence from epidemiology data supports that RSV infection in the first three years of life can be directly correlated with long-term respiratory morbidities, such as recurrent wheezing and asthma (7, 8). It has been observed that RSV, like other respiratory viruses, causes a "*hit and run*" phenomenon, characterized by the increased risk of developing recurrent wheezing and asthma in childhood after the infection, as a permanent phenotype that persists long after the virus clearance (9). Wheezing is the typical high-pitched, whistling sound made during breathing. Asthma, on the other hand, is characterized by abnormalities in lung function that include variable airway obstruction and increased bronchial reactivity (10). Recognizing asthma is usually obvious and most of the time asthmatic patients also report wheezing episodes; however, it is very challenging to predict and distinguish which children will present only early-life symptoms, from those whose symptoms persist, and those who may develop definitive wheeze or asthma (11).

The risk of wheezing and/or asthma incidence has been increasingly related to a combination of genetic and environmental factors as well as the severity of the respiratory infection (12–14). Several efforts have been made to establish the ultimate causes underlying the apparition of respiratory sequelae as a consequence of RSV infection, but further complementary studies are still necessary to understand the underlying genetic and molecular mechanisms. It has been hypothesized that prenatal interaction between the maternal and the child immune system as well as the high cytokines production after the infection can affect the lung structure and function inducing changes in the regulation of immune response (15).

The immune system plays a pivotal role in the sequelae reported in patients suffering from respiratory infections. The immune response shows many functional differences in neonates and adults, and these differences might be associated with epigenetic modifications of genes that control inflammation and immune response (16). Thus, the modulation of epigenetic mechanisms governs the immune cell phenotype and function allowing the external environment to influence the immune response outcome (17).

Epigenetic regulation behaves as a dynamic interface between genome and environment; in the case of viral infection, this regulation in host defense cells is directly related to disease development (18). Pieces of evidence support the involvement of epigenetic mechanisms in the modulation of the interaction between host and pathogen (19), as it also occurs as a consequence of vaccination [e.g. rotavirus infection (20)]. For instance, it has been shown that some viral infections (e.g. by herpesvirus, KSHV, EBV) can lead to the modification of host epigenetic marks, and this probably contributes to the establishment of latency and some pathogenic roles (21, 22).

DNA methylation involves the addition of methyl groups to the DNA molecule and is the major epigenetic factor influencing gene activities. It is generally associated with a decrease of gene expression when it occurs in promoters; however, according to recent evidence (23, 24) the effect of DNA methylation occurring in low CpG density regions, such as the gene body, or intergenic regions, might have also great impact on the regulation of gene

expression, with repressive or permissive effects. Recently, Elgislouli and colleagues (25) observed an alteration of the methylation pattern of a particular enhancer region in the perforin 1 gene (*PRF1*), an essential cytotoxic protein for the control of viral infection, occurring after severe RSV infection. Infants hospitalized with exacerbated RSV-induced bronchiolitis exhibited decreased methylation of the perforin-1 enhancer after four years of follow-up, suggesting that immune response changes due to RSV could persist even years after infection (26). Besides, Wang et al. (18) showed that cultured bronchial human epithelial cells (BECs) infected with RSV presented a high expression level of the gene *NODAL* (a member of the TGF-beta superfamily), whose promoter was found to be hypermethylated in normal BECs, resulting in increased Th2 and Th17 skewing of T cells. In murine models, it was observed an overexpression of the demethylase genes *Kdm5b* and *H3K4*, in dendritic cells after RSV infection (27); expression of these genes has the potential to repress transcription of type I IFN and other innate cytokines, causing a decrease of pro-inflammatory cytokines and expression of a Th2 phenotype. Methylation of *H3K4* in regulatory T cells by the histone methyltransferase SMYD3 is necessary to control inflammation in the lungs after RSV infection (28).

The aim of the present study is to analyze the DNA methylation changes related to the development of wheezing and/or asthma induced after RSV infection. To the best of our knowledge, this is the first time that an epigenomic approach is applied to the investigation of epigenetic modifications due to RSV infection and the subsequent long-term respiratory sequelae.

MATERIAL AND METHODS

Study Subjects and Design

EPIRSVI is a prospective, transversal, observational study of children admitted to the Hospital Clínico Universitario de Santiago de Compostela, and the Complejo Hospitalario Universitario de Ourense, part of the GENDRES consortium (www.gendres.org), for a respiratory infection due to RSV (**Supplementary Table 1**). The recruitment of patients was performed from 2010 to 2015, during their acute phase of RSV infection (within seven days from the beginning of symptoms). Patients, independently from their age, were all followed up from their recruitment for a time-lapse of at least three years after discharge to specifically monitor the onset of wheezing or asthma. Patients were categorized according to their clinical course into three different subgroups (n = 77): (a) recurrent wheezing RSV cases (RW-RSV; n = 36), (b) asthma RSV cases (AS-RSV; n = 9); and (c) not-wheezing/asthma RSV cases with complete recovery (CR-RSV; n = 32). The three groups matched perfectly for age, gender, and disease severity at the time of sample collection.

Wheezing was determined here as a respiratory episode occurring with wheezing lasting more than one day. The interval between two episodes was defined as a period of at least seven days without respiratory symptoms. Instead, recurrent wheeze was defined as three or more episodes of wheezing during the first year of life. The diagnosis of asthma in preschool children was carried out by a pediatric pulmonologist based on the reiterated presence of compatible symptoms (several episodes of bronchial obstruction, generally witnessed by the physician), adequate response to regular treatment, and the exclusion of other alternative diagnoses, following the international practice guidelines. From the clinical perspective, the diagnosis of asthma in these children involves a series of peculiarities inherent to patients of this age, such as limitations for performing pulmonary function tests, greater attention to differential diagnoses, limited response to common asthma treatments, and a high probability of symptoms remission during childhood.

DNA Isolation and Methylation Profiling

Blood samples were collected in EDTA tubes, within 24/48 hours from the hospital admission of each patient. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (PROMEGA) QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. After DNA extraction, DNA was purified, and concentration was evaluated using a Nanodrop and Picogreen assay.

Genomic DNA from whole blood samples was bisulfite-treated using the EZ-96 DNA Methylation kit (Zymo Research Corp) following the manufacturer's recommendations for Infinium assays. The technique consists of the conversion of unmethylated cytosine residues to uracil through deamination, while leaving the methylated cytosine (5-mC) unaffected.

Following bisulfite conversion, the samples were hybridized to Illumina Infinium MethylationEPIC BeadChip. This microarray allows the examination of >850K methylation sites quantitatively across the genome at single-nucleotide resolution. The microarray coverage includes 99% of the RefSeq genes, 95% of CpG islands, non-CpG islands, and differentially methylated sites, high coverage of transcription factors binding sites, miRNA promoter regions, and enhancer sequences. We compared the methylome of RW-RSV, AS-RSV, with CR-RSV to evaluate whether the divergent epigenetic marks could be specific for the development of recurrent wheezing and/or asthma.

Data Processing, Cell Deconvolution and Statistical Analysis

DNA methylation quality control, processing, normalization, and statistical analyses were performed in the statistical R software (29), using different Bioconductor packages, and following the workflow of EPIC methylation analysis described recently by Fortin et al. (30). The raw intensity files (IDAT), were imported into R and were preprocessed and transformed into β and M-values using the *minfi* (v1.30.0) package (31, 32). The β-values are defined as the intensity ratio of the methylated signals over the total (methylated and unmethylated) signals for each site. Their values range between 0, illustrating that the position is not methylated at all in all the cells and 1, indicating that all cells in the sample have a given position methylated (33). The M-values were calculated as the $\log_2$ ratio of the intensities of methylated probes *vs.* unmethylated probes; their values range

from −1 to 1, where values close to 0 define a similar intensity between the methylated and unmethylated probes, positive M-values indicate that there are more methylated than unmethylated molecules, while negative M-values mean the opposite (34).

Counting blood cells is a necessary step to adjust for individual differences in cellular heterogeneity in the blood sample from which genomic DNA was extracted. Because of the extreme cell-type specificity of DNA methylation (35), variations in cell-type composition between phenotypes can confound analyses and bring to a wrong interpretation of the results. Therefore, before checking for differentially methylated positions (DMPs), we used the package *FlowSorted.Blood.EPIC* and its modified function *estimateCellCounts2* for the estimation of blood cell type composition. At the base of the function, there is a modification of the Houseman algorithm (36), that allows estimating the relative proportions of white blood cell subtypes, namely, CD4+ T-lymphocytes, CD8+ T-lymphocytes, natural killer (NK) cells, B-lymphocytes, monocytes, and neutrophils.

Infinium 850K has two kinds of probes, Infinium I and Infinium II, that are not directly comparable, therefore, a correction must be applied to control for design bias. The normalization procedures were performed using the function *preprocessQuantile* available in the *minfi* package (37). Moreover, probes underwent several filtering processes, by removing probes with *P*-value > 0.01 and probes located in the sex chromosomes. Additionally, sites containing SNPs or with a minor allele frequency (MAF) < 0.05 were also excluded from the data analysis because probe binding might be affected by genetic variation in the binding area. Finally, we removed probes known to have cross-reaction.

DMPs were identified with *limma* (38) assuming a linear model, where M-values of each probe were used as quantitative dependent variables in all analyses, and including cell composition, age, and gender as covariates in all the models. After running the linear model, we applied the statistical analysis using an empirical Bayes method to moderate standard errors. DMPs were filtered using a significance threshold of adjusted *P*-value < 0.01 [Benjamini–Hochberg method (39)].

We used a Principal Component Analysis (PCA) to compare DNA methylation of children with complete recovery (CR-RVS) and those with respiratory sequelae after an RSV infection (RW-RVS + AS-RVS). The threshold Delta β of 0.1 and adjusted *P*-value < 0.01 were used to identify candidate DMPs among the comparison groups. The diagnostic efficiency of the most significant candidate DMPs was evaluated using receiver operating characteristic (ROC) curve analyses. Furthermore, the candidate DMPs were included in the logistic regression analysis using the Parallel Regularised Regression Model Search (PReMS) (40) method that allows identifying the minimum epigenetic signature with the highest diagnostic performance to differentiate between RW/AS-RSV and CR-RSV groups. This logistic regression model balances small positions number with accurate discrimination, minimizing the number of biomarkers selected in the signatures. The optimal model size (with the lowest out-of-sample log-likelihood) was determined using 20 cross-validation folds. In addition to the best model, results for model sizes with predictive log-likelihood within one-standard error of the best model is also shown. We randomly split the cohort into 75% training and 25% test sets, ensuring equal proportions of RW/AS-RSV and CR-RSV in each set. The epigenetic signature was identified in the training set and afterwards validated in the test set. Wilcoxon test was used to assess statistical significance between patient groups.

To deeply analyze the functional interpretation of our reported differential probes, we performed pathways enrichment analysis on the candidate DMPs applying the *methylglm* and *methylRRA* functions of the R *methylGSA* package (41). To adjust for the number of CpGs, the first method employs a logistic regression model incorporating the number of CpGs as a covariate, while the second approach makes use of a Robust Rank aggregation (RRA) procedure. Indeed, the number of CpGs is variable for genes of similar length and DNA methylation studies give rise to multiple CpG association *P*-values per gene.

To find co-methylated modules related to respiratory sequelae, we built a signed weighted correlation network using the *WGCNA* package (42) with the CpGs showing the most different β-values between samples (top 25% with a higher variance; n = 123,617). We selected a soft-thresholding power of 12 based on the criterion of scale-free topology after testing a set of candidate powers (**Supplementary Figure 1A**). As module detection parameters, we chose a minimum module size of 30, a medium sensitivity for cluster splitting and a 0.25 as dendrogram cut heigh threshold for module merging. We calculated gene significance (GS) to detect significant associations between modules/genes and phenotype. Module membership (MM), as a measurement of intramodular connectivity, was also calculated by correlating the methylation profile with the eigengene of a given module. We explored the correlation between GS and MM and calculated the average absolute gene significance for all genes within a module to find the most important modules. The top hub genes within the most relevant module were selected using both MM > 0.8 and GS ≤ −0.7 as thresholds. We studied the biological significance of the most important module related to the trait by performing an over-representation analysis through the *Clusterprofiler* R package (43), and using Gene Ontology (GO) database as a reference.

RESULTS

Patient Characteristics

Table 1 summarizes the clinical characteristics of the subjects studied and maintained for the downstream statistical analysis after filtering raw data (n = 68), and it provides information regarding the most important risk factors related to the development of asthma in early childhood. For the clinical characteristics of the full cohort see **Supplementary Table 1**. More males than females represented sequelae and complete recovery groups (60.7% and 70.0% for CR-RSV and RW/AS-RSV, respectively). Concerning disease severity, all children were

TABLE 1 | Clinical characteristics of patients left for downstream analysis, classified as recurrent wheezing RSV (RW-RSV), asthma RSV (AS-RSV), and complete recovery RSV cases (CR-RSV).

	CR-RSV (*n* = 28)	RW/AS-RSV (*n* = 40)	*P*-value
Demographic variables			
Sex Male	17 (60.07%)	28 (70.0%)	0.447
Age in months (mean [SD])*	7.14 [5.32]	5.85[3.62]	<0.001
Ethnicity			0.184
Western Europe	24 (85.7%)	36 (90.0%)	–
Southern Europe	2 (7.1%)	1 (2.5%)	–
Southern America	1 (3.6%)	0	–
Roma	0	3 (7.5%)	–
Other	1 (3.6%)	0	–
RSV infection	28 (100.0%)	40 (100.0%)	1.000
Past medical history prior to RSV-A			
Premature	4 (14.3%)	4 (10.0%)	0.717
Atopic Dermatitis*	2 (7.1%)	13 (32.5%)	0.017
Alimentary allergies	1 (3.6%)	5 (12.5%)	0.399
Stational allergies	0	2 (5.0%)	0.508
Asthma	0	0	1.000
Admissions prior to RSV	10 (35.7%)	12 (27.0%)	0.399
Annual bronchitis prior the RSV-A*	3 (10.7%)	21 (52.5%)	0.005
Family history			
Asthma	4 (14.3%)	14 (35.0%)	0.052
Respiratory problems	5 (17.9%)	15 (37.5%)	0.053
Clinical characteristics of the RSV-H			
Respiratory distress			0.147
Mild	8 (28.6%)	4 (10.0%)	
Moderate	16 (57.1%)	29 (72.5%)	
Severe	4 (14.3%)	7 (17.5%)	
Oxygen requirement	23 (82.1%)	25 (62.5%)	0.107
Respiratory support			0.128
Non-invasive	3 (10.7%)	8 (20%)	
Mechanical	2 (7.1%)	0	
Diagnosis			0.093
Bronchiolitis	25 (89.3%)	31 (77.5%)	
Bronchospasm	0	1 (2.5%)	
Pneumonia	2 (7.1%)	2 (5.0%)	
Other	1 (3.6)	6 (15.0%)	
Bacterial superinfection suspected*	20 (71.4%)	12 (30.0%)	<0.001
Follow-up 3 years			
Hospital admission	5 (17.9%)	11 (27.5%)	0.962
Additional episodes of bronchiolitis*	7 (25%)	37 (92.5%)	<0.001

Fisher's exact test is used to assess the association between the different variables. RSV-A, RSV admission; RSV-H, RSV hospitalization.
**Statistically significant variables.*

hospitalized because of RSV infection and their admission lasted > 5 days. Most subjects were diagnosed with bronchiolitis and required oxygen during their hospital admission. Most children were under six months of age; however, a significant difference ($P < 0.001$) was observed in the mean age of both groups, being children with respiratory sequelae slightly younger than patients with complete recovery after RSV. No comorbidities were present in the study cohort apart from prematurity (see **Table 1**). Thus, admissions prior to RSV were due to prematurity, bronchiolitis (mainly), rotavirus gastroenteritis in one case and hypernatremia dehydration in another case. Most of the children admitted below the age of one year were normally more common to have oxygen requirement, suffer apneas or need of intravenous fluids due to lack of appetite, therefore the number of admissions is generally higher compared to older ages. According to the previous medical history of these patients, the only clinical variable found to be statistically significant was the personal history of atopic dermatitis (Fisher exact test; *P*-value = 0.017), while alimentary and stational allergies showed no significant difference between the two groups. The family history of asthma and respiratory problems were not found to be significantly different between groups. Finally, we observed a significant difference (Fisher exact test; *P*-value = 0.005) in the number of bronchitis episode prior to hospitalization in patients with respiratory sequelae, and a higher number of suspected bacterial infection (Fisher exact test; *P*-value <0.001) in children with normal recovery.

DMPs Between Complete Recovered and Sequelae Cases

We interrogated the genome-wide DNA methylation profiles of 77 children during their acute phase of RSV infection that experienced complete recovered or developed respiratory sequelae.

During the data preprocessing nine samples were discarded because they did not pass the initial quality control; therefore, 68

out of 77 samples were included in the statistical analysis. After removing poorly performing probes, namely, those overlapping with SNPs or hybridized to multiple locations in the genome and those located on the X and Y chromosomes, a total of 811,035 probes were preserved for downstream analysis.

Cell deconvolution analysis indicates a significant difference in the number of NK and CD8+T cells between sequelae and recovered cases (*P*-value = 0.03 and *P*-value = 0.04, respectively); **Figure 1A**. When we compared RW/AS-RSV against CR-RSV patients, adjusting the linear model for cell-type composition, age, and gender, we found 5,097 significantly DMPs (FDR *P*-value < 0.01), corresponding to 3,278 unique genes according to the Illumina Human Methylation EPIC manifest annotation file (44) (**Supplementary Table 2**). Among these, 1,155 DMPs were hypomethylated (22.7%) and 3,942 hypermethylated (77.3%) in CR-RSV. PCA of these significant DMPs showed a clear separation of the two groups along with the first principal component (PC1; accounting for 43.2% of the variance) (**Figure 1B**).

There are regional differences between the distribution of statistical different hypomethylated and hypermethylated CpGs when examined in a genomic context. Thus, hypermethylated positions are more concentrated in the gene body (59.2%), while the hypomethylated positions are distributed more homogeneously along the gene body (29.8%), promoter regions (45.6%), and transcriptions start sites [TSS1500 (22.1%), TSS200 (23.5%)], with a lower amount falling at 'UTR (15.4%) and 1st Exon (7.2%) (**Figure 2A**). Island's regions are overrepresented by hypomethylated sites (38.8%), while the OpenSea regions are more characterized by hypermethylated DMPs (61%); the distribution of DMPs at shelf and shore was quite similar between the two comparison groups (**Figure 2B**). Moreover, all chromosomes were more represented by hypermethylated DMPs than the hypomethylated ones (**Figure 2C**).

To narrow down the analysis and reduce the large number of significant methylated positions observed, we focused on finding the most consistent methylation changes by selecting only

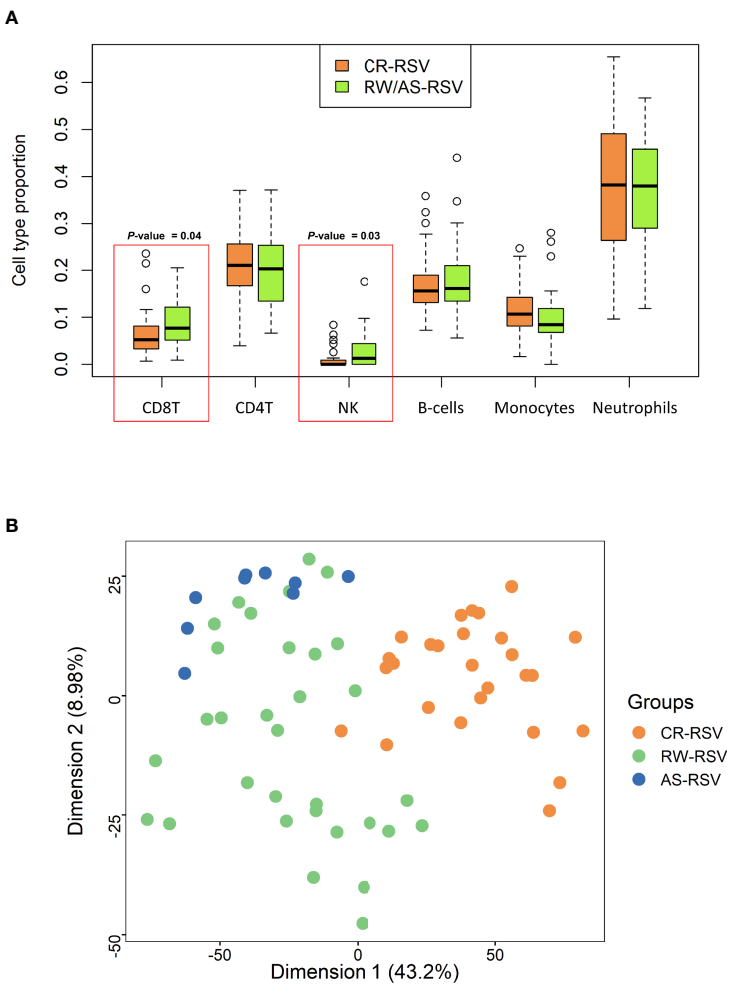

FIGURE 1 | **(A)** Boxplot showing the proportion of leukocyte cell type in RW/AS-RSV and CR-RSV groups. Red rectangles highlight the two types of cells that show statistically significant differences between groups. **(B)** PCA of the significant DMPs (FDR *P*-value < 0.01) between RW/AS-RSV and CR-RSV cases.

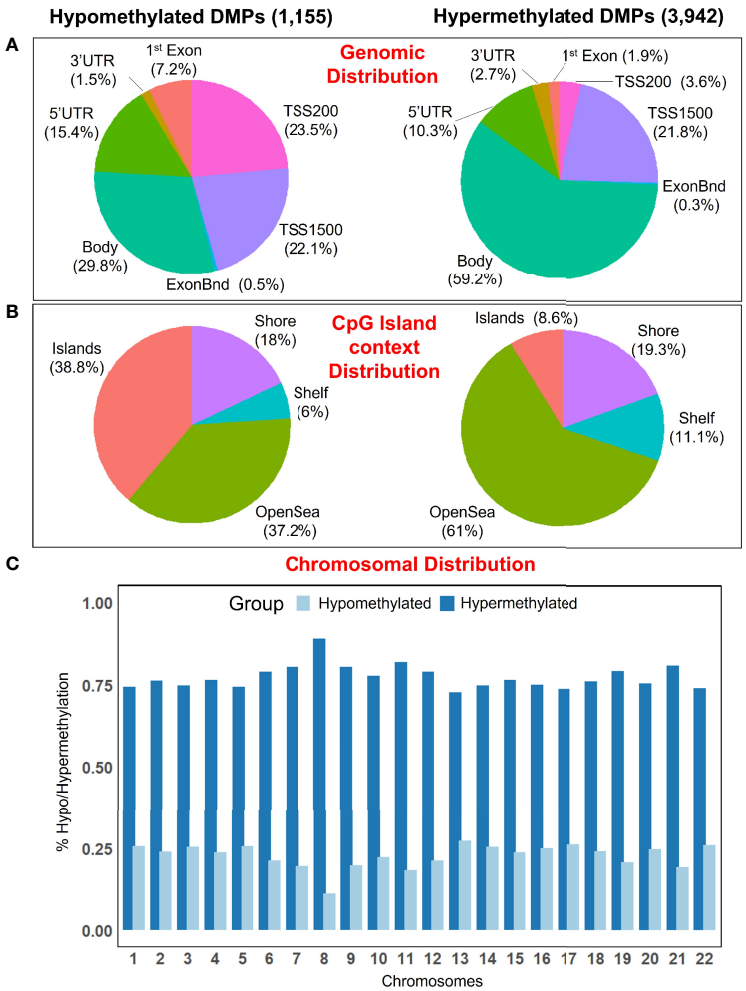

FIGURE 2 | **(A)** Pie chart showing the percentage of the significant DMPs in the genomic context, and **(B)** according to their distribution in the Island context. **(C)** Barplot showing the distribution of hypomethylated and hypermethylated DMPs in the chromosomes.

significant CpGs with an absolute Delta β > 0.10 when comparing CR-RSV against RW/AS-RSV (**Figure 3A**). This analysis found 28 CpGs (**Table 2**) associated with 23 unique genes; five of them were moderately hypermethylated, the remaining showed a lower methylation level for sequelae cases.

Methylation Signature for RSV Sequelae

A total of 9 out of the 28 significant positions with absolute Delta $\beta > 0.10$ showed an FDR *P*-value < 1×10^{-4}, and were selected to evaluate their ability, in terms of methylation levels, to discriminate RW/AS-RSV from CR-RSV groups (**Figure 3B**). All the selected positions exhibit hypomethylation pattern in RW/AS-RSV patients when compared to the CR-RSV group (**Figure 3C**). Two of the nine positions, namely, the cg00044440 (Chromosome 5; OpenSea; FDR *P*-value = 6.77×10^{-6}; Delta β = -0.12), and the cg06579481 (Chromosome 7; North-Shelf; FDR *P*-value = 8.57×10^{-6}; Delta β = -0.12), were not annotated to a gene. The remaining seven DMPs, instead, were associated to eight different genes: cg24509398 (FDR *P*-value = 2.77×10^{-10}; Delta β = –0.14) falls in the body of *EYA3*; cg15619333 (FDR *P*-value = 4.39×10^{-9}; Delta β = -0.116×10^{-1}) locates in the body of *ZNF268*; cg12216772 (FDR *P*-value = 3.33×10^{-8}; Delta β = –0.12) is in the 5'UTR of *ANUBL1*; cg15822108 (FDR *P*-value = 4.28×10^{-7}; Delta β = –0.11) settles in the body of *NAA15*; cg02737727 (FDR *P*-value = 1.84×10^{-9}; Delta β = –0.11) falls in the body of *LOC102723354*; cg14819618 (FDR *P*-value = 2.26×10^{-6}; Delta β = –0.10) resides within the body of *PRAGMIN*; and finally, cg23621438 (FDR *P*-value = 1.84×10^{-9}; Delta β = –0.10), locates within the TSS1500 of *MIR6817* and the body of *CRYBB2P1*.

A logistic regression analysis performed with the 28-CpG lead positions found a minimum epigenetic signature composed of three methylation markers (cg15619333, cg02737727, and cg06259441) as the best diagnostic model to differentiate RW/AS-RSV from CR-RSV in the training set (n = 52) (**Figure 4A**). The cg15619333 and the cg02737727 were previously described

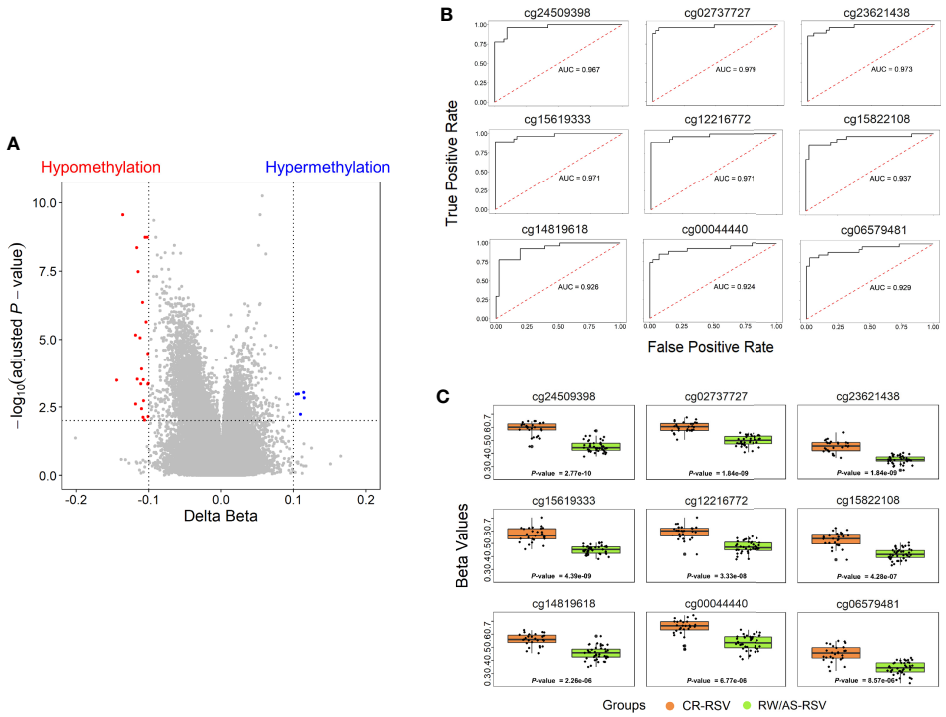

FIGURE 3 | **(A)** Volcano plots for differential DNA methylation status. The x-axis shows the mean DNA methylation (β -value) difference, whereas the y-axis indicates the $-\log_{10}$ of the adjusted *P*-value for each CpG site. The most significant DMPs (n = 28) in children with respiratory sequelae when compared with children complete recovered (threshold: Delta $\beta > 0.10$, FDR *P*-value < 0.01) are plotted in red (hypomethylated sites) and blue (hypermethylated CpGs). **(B)** Receiver operating characteristic (ROC) curves indicate that the accuracy of the test based on the reported CpGs is very high (AUC > 90%) when comparing RW/AS-RSV and CR-RSV groups. **(C)** Boxplot of the nine most significant DMPs with a Delta $\beta > 0.10$ in the comparison RW/AS-RSV vs. CR-RSV cases.

among the candidate CpGs with the lowest FDR *P*-value, while the North Shore cg06259441 resides within two overlapping genes *RPL15* and *NKIRAS1* in chromosome 3. Interestingly, this last position showed an opposite pattern of methylation, being highly methylated in RW/AS-RSV cases.

This 3-position signature showed high diagnostic performance with the training set (*P*-value = 1.00×10^{-14}; **Figure 4B**), with AUC value of 100% (95% CI: 1-1) (**Figure 4C**). When the 3-signature model was applied to the test set (n = 16), we obtained similar prediction results (*P*-value = 2.50×10^{-4}) with an AUC value of 100% (95% CI: 1–1) as in the training set (**Figure 4C**). The diagnosis accuracy of the model was not affected when all samples were analysed together (**Figure 4C**).

Enrichment Pathways Analysis

To address the potential biological significance of DMPs, a gene set enrichment and pathways analysis was carried out. When comparing the sequelae group against CR-RSV cases, many significantly enriched pathways (FDR *P*-value < 0.05) in the GO database was detected (**Figure 5A**). Among the top overrepresented categories, there were those related to several cell cycle processes, such as the cell cycle checkpoint (FDR *P*-value = 4.71×10^{-2}), DNA damage (FDR *P*-value = 2.53×10^{-2}), and DNA integrity checkpoint (FDR *P*-value = 2.56×10^{-2}). The enrichment pathways analysis performed exclusively on positions overlapping promoters (TSS200 and TSS1500), revealed significant enrichment in the GO ubiquitin ligase binding (FDR *P*-value = 1.21×10^{-2}), and response to transforming growth factor beta (FDR p.value =$1.21\text{x}10^{-2}$), among other pathways (**Figure 5B**).

Co-Methylation Modules

We detected 99 co-methylation modules (**Supplementary Figure 1B**) when comparing methylation patterns of RW/AS-RSV against CR-RSV patients. The top one (showing the highest correlation with the RW/AS-RSV phenotype; *P*-value = 1.67×10^{-9}; **Supplementary Table 3**; **Supplementary Figure 1C**) includes 3,147 CpGs (**Supplementary Table 3**). This module shows a negative correlation with the trait (–0.65), pointing to a hypomethylation state in RW/AS-RSV concerning the CR-RSV group. This pattern can also be inferred from the heatmap and samples eigengenes plots, with overall higher methylation values in the CR-RSV cohort (**Supplementary Figures 2A, B**). A highly significant correlation between gene significance (GS) and module membership (MM) was observed for this module, meaning that CpGs strongly associated with RW/AS-RSV were also core elements of the module (**Supplementary Figure 2C**). Interestingly, the vast majority of the main hub CpGs within this

TABLE 2 | The 28 most significant DMPs in the comparison RSV sequelae patients vs. CR-RSV cases (FDR *P*-value < 0.01, absolute average β-value > 0.10).

CpG_ID	Chr	Position	Location	GN	GG	*P*-Value	Delta β
cg21226224	8	55370171	IS	*SOX17*	TSS1500	3.29×10^{-04}	–0.144
cg24509398	**1**	**28416532**	**SS**	***EYA3***	**TSS1500**	$\mathbf{2.77\times10^{-10}}$	**–0.136**
cg11702503	19	6215254	IS	*MLLT1*	Body	2.43×10^{-03}	–0.118
cg00044440	**5**	**138671449**	**OS**	**–**	**–**	$\mathbf{6.77\times10^{-06}}$	**–0.118**
cg15619333	**12**	**133759653**	**SS**	***ZNF268***	**5'UTR; Body**	$\mathbf{4.39\times10^{-09}}$	**–0.116**
cg05800416	8	19460097	IS	*CSGALNACT1*	TSS200; Body; 5'UTR	3.00×10^{-04}	–0.116
cg12216772	**10**	**46164062**	**NE**	***ANUBL1***	**5'UTR**	$\mathbf{3.33\times10^{-08}}$	**–0.115**
cg06579481	**7**	**104621597**	**NE**	**–**	**–**	$\mathbf{8.57\times10^{-06}}$	**–0.112**
cg19977004	11	1482563	IS	*BRSK2*	3'UTR	4.45×10^{-04}	–0.111
cg05116443	20	62562680	IS	*DNAJC5*	Body	1.25×10^{-04}	–0.110
cg08938155	5	77043612	OS	*TBCA*	Body	3.68×10^{-03}	–0.110
cg15822108	**4**	**140306311**	**OS**	***NAA15***	**Body**	$\mathbf{4.28\times10^{-07}}$	**–0.109**
cg19841649	5	4866322	IS	**–**	**–**	7.58×10^{-03}	–0.108
cg26900509	11	127514	IS	*LOC100133161*	Body	3.13×10^{-04}	–0.108
cg13165070	11	2154113	IS	*INS-IGF2; IGF2*	Body; 3'UTR	1.87×10^{-03}	–0.107
cg02077481	16	33939020	IS			9.62×10^{-03}	–0.106
cg02737727	**14**	**105561470**	**SS**	***LOC102723354***	**Body**	$\mathbf{1.84\times10^{-09}}$	**–0.105**
cg14819618	**8**	**8180214**	**SE**	***PRAGMIN***	**Body**	$\mathbf{2.26\times10^{-06}}$	**–0.104**
cg23621438	**22**	**25850271**	**OS**	***MIR6817; CRYBB2P1***	**TSS1500; Body**	$\mathbf{1.84\times10^{-09}}$	**–0.102**
cg05992347	16	33964783	IS	*MIR1826*	TSS1500	4.54×10^{-04}	–0.101
cg16003687	6	168613889	SS	**–**	**–**	3.28×10^{-05}	–0.101
cg04479860	4	190767364	IS	**–**	**–**	7.15×10^{-03}	–0.101
cg06583549	19	46387962	IS	*IRF2BP1*	1st Exon	4.31×10^{-04}	–0.101
cg27552418	4	97598786	OS	**–**	**–**	1.08×10^{-03}	0.104
cg25338438	5	161276341	OS	*GABRA1*	5'UTR; TSS1500	1.04×10^{-03}	0.107
cg09728337	10	32668256	OS	*EPC1*	TSS1500	5.80×10^{-03}	0.110
cg20967739	1	50895827	SE	**–**	**–**	9.25×10^{-04}	0.114
cg06259441	3	23957589	NS	RPL15; NKIRAS1	TSS1500; 5'UTR	1.48×10^{-03}	0.115

The positions are ordered according to their Delta β-value. The first 23 CpGs exhibit a lower methylation level in CR-RSV than in sequelae cases, while the following 5 DMPs have an opposite pattern. In bold, positions with the lowest P-value, represented in ***Figures 5***, ***6****. Chr, chromosome; SE, S Shelf; NE, N Shelf; NS, N Shore; SS, S Shore; IS, island; OS, OpenSea; GN, Gene name; GN; GG, Gene group; P-value, FDR P-value.*

module corresponds with the previously identified 5.097 DMPs (~92%). Three of them are among the 28-CpGs previously described as most remarkable DMPs between sequelae and non-sequelae groups (cg24509398 [*EYA3*], cg15822108 [*NAA15*], cg15619333 [*ZNF268*]; **Supplementary Table 3**). Genes linked to the most significant module positions were mainly involved in pathways related to GTPase activity, epigenetic modifications, and cell cycle; and molecular functions related to actin, cadherin, and calmodulin binding and also, with focal adhesion components (**Supplementary Table 3**).

Identification of DMPs Associated With Sequelae Sub-Phenotypes

We next investigated RW-RSV and AS-RSV separately using the same linear model and thresholds described above for the merged sequelae group. When comparing RW-RSV vs. CR-RSV we detected 3,432 significant DMPs, of which 2,663 were hypermethylated, and 769 were hypomethylated in CR-RSV cases. Likewise, the comparison of AS-RSV patients vs. CR-RSV allowed us to identify 1,358 DMPs, with the vast majority being hypermethylated (n = 1,059), and 299 being hypomethylated in CR-RSV. A total of 512 significant DMPs were shared by the two contrasts, and also overlap with the most significant DMPs found when comparing respiratory sequelae cases vs. CR-RSV.

As shown above, a PCA considering DMPs between sequelae cases vs. CR-RSV patients allows also to separate the two sequelae phenotypic groups in its second component (**Figure 2**: see PC2; accounting for ~9% of the variation). By running a linear model with the contrast AS-RSV vs. RW-RSV and using a less stringent threshold (FDR *P*-value <0.05), a total of 47 significantly DMPs associated with 41 genes could be detected. A PCA was carried out exclusively with these 41 DMPs and the two sequelae phenotypes clearly separate the two groups in its PC1 (49% of the full variance) (**Figure 6**). Of the significant CpGs, 30 positions were found to be hypermethylated in RW-RSV when compared to AS-RSV, while the remaining 17 exhibit the opposite pattern (**Table 3**). The position with the highest difference in β value (FDR *P*-value = 1.8×10^{-2}; Delta β >0.14) was the cg18873878 located within the *TP73* gene, while the most significant CpGs (FDR *P*-value = 0.11×10^{-1}) was the cg05838113 located within the body of *ADAM8* gene.

DISCUSSION

It is known that host factors might impact subsequent respiratory morbidity in RSV infection, but the underlying biological mechanisms through which recurrent wheezing and asthma are prone to emerge after RSV infection are still unknown.

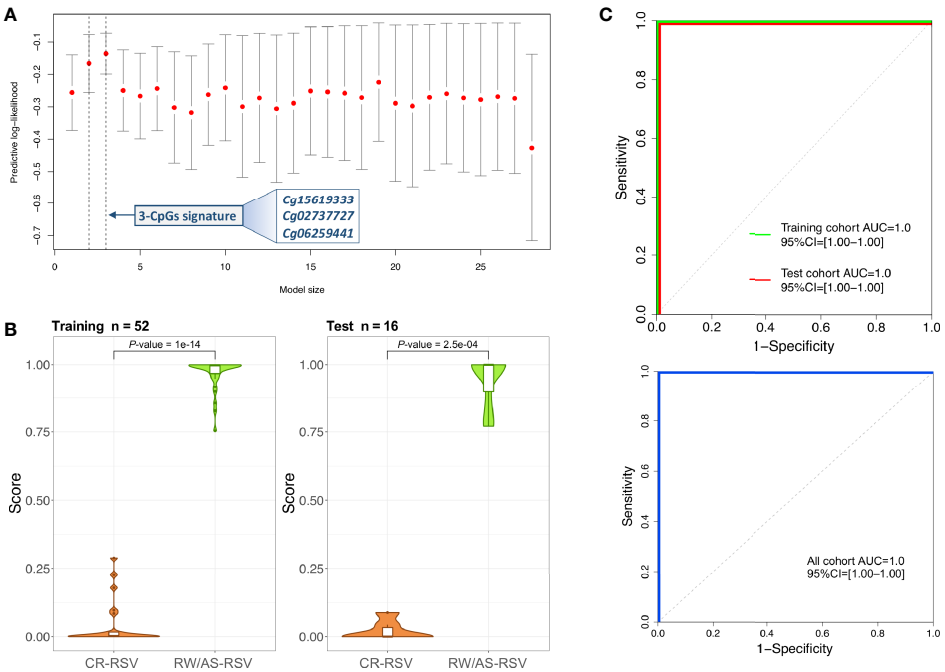

FIGURE 4 | **(A)** Optimal model size according to logistic regression analysis. The x-axis represents the training set predictive log-likelihood, while the y-axis shows the number of genes of the signature. Solid grey bars indicate one standard error (SE) of the predictive log-likelihood. Vertical dashed lines show the model with the best predictive log-likelihood and the model within one-SE of the best model. **(B)** Violin and boxplots of the predicted values from the posterior mean of the optimal model in training and test cohorts. **(C)** ROC curves of the 3-CpGs signature from both training and test cohorts show the area under the curve (AUC) and 95% confidence intervals (CIs). AUC values for the whole cohort are also displayed.

The analysis of the DNA methylome in RSV infected children allowed us to find >5.000 DMPs mainly located in inflammatory genes when comparing patterns in individuals with sequelae from those completely recovered (**Figure 7**). Overall, the findings suggest that epigenetic mechanisms might play a fundamental role in the long-term sequelae after RSV infection. The association between RSV infection and the subsequent development of wheezing and/or asthma in infants has been widely reported (7, 8, 45, 46). This correlation seems to be very complex owed to the several host environmental factors that influence the expression of respiratory sequelae developed after the infection, such as genetics, age, prematurity, RSV bronchiolitis, atopic dermatitis, and maternal asthma. The patients analyzed in the present study had a very similar clinical course, with the particularity that those developing respiratory sequelae were two months younger than those completely recovered and show more incidence of atopic dermatitis; similar findings were already described to be related with respiratory morbidity after RSV infection (47).

Estimation of the relative proportion of cell types in peripheral blood samples of patients revealed a statistically significantly higher proportion of NK and CD8T cells in children with respiratory sequelae than those with complete recovery (**Figure 7**). This is in line with evidences demonstrating that RSV infection can induce severe acute lung immune injury promoting the accumulation of lung NK cells at the early stage of infection in mice, as a consequence of the increased production of IFN-γ (48). Similar characteristics were described for CD8+ T cells, which are activated after RSV infection to produce inflammatory cytokines (49), and seem to enhance airway inflammation and airway dysfunction in mice (50). However, there is growing body of evidence suggesting that NK cells, as well as CD8+T cells are involved in both the promotion and inhibition of allergic lung inflammation and airway diseases (51).

After correcting the linear model for cell composition, age, and gender, the outcomes of the preliminary analysis revealed 5,097 DMPs when comparing CR-RSV vs. RW/AS-RSV. The most significant CpG (FDR *P*-value = 2.77×10^{-10}; Delta β = -0.14) was cg24509398 within the *EYA3* gene, a tyrosine phosphatase involved in DNA repair and in distinguishing apoptotic and repair responses to genotoxicity. Recently, Wang et al. (52) suggested that the over-expression for this gene in patients with pulmonary arterial hypertension (PAH) might stimulate the survival of pulmonary vascular cells in the presence of DNA damage causing vascular remodeling, a typical feature of PAH. Although PAH and asthma are considered two different and unrelated clinical phenomena, there are pieces of evidence demonstrating that they share some pathological features such as inflammation, smooth muscle constriction, and proliferation (53). Although DNA methylation in the CpG selected might not be exerting strong changes in the expression of *EYA3*, the putative

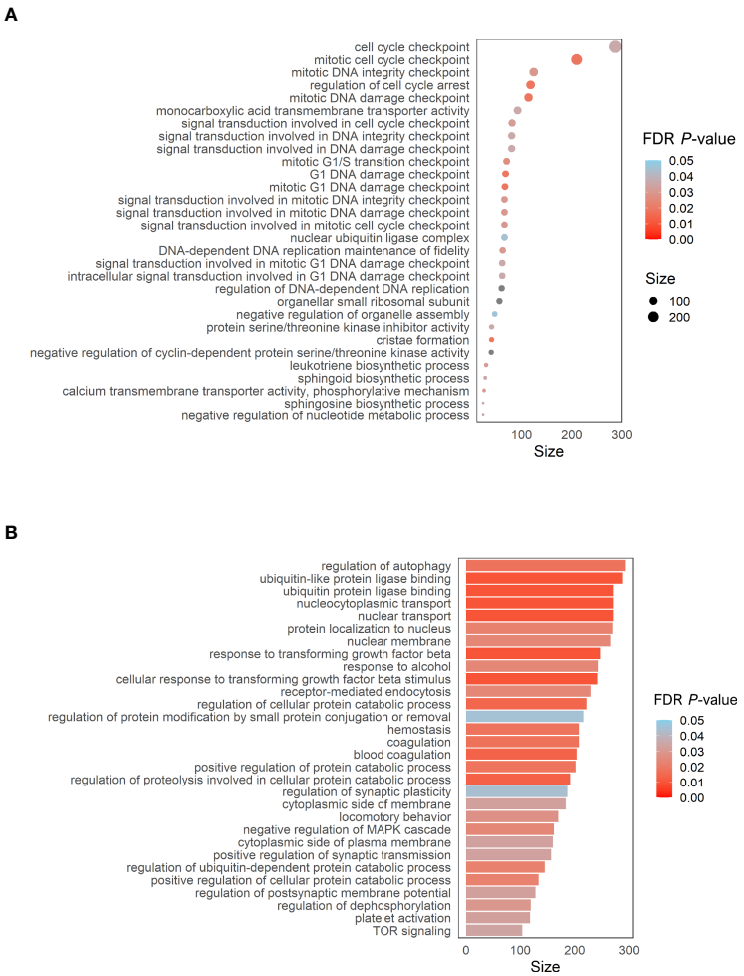

FIGURE 5 | **(A)** Dot plot of the top GO pathways (FDR *P*-value < 0.05) obtained between RW/AS-RSV and CR-RSV (methylglm approach). **(B)** Bar plot of the enrichment pathways analysis performed considering only CpGs within the promoter regions (methylRRA approach). Size along the x-axis indicates the number of genes involved in each pathway. Colors correspond to the different FDR *P*-values associated with the pathways.

involvement of this gene in the vascular remodeling (a key process in the asthma pathology), might indicate a possible connection between the altered methylation status of the gene and its uncontrolled gene expression.

To test the diagnostic potential of epigenetic markers in the context of RSV sequelae, we carried out a logistic regression analysis using the top 28 DMPs. We found a 3- CpGs position model (related to *ZNF2698*, *LOC102723354* and the overlapping *RPL15*/*NKIRAS1* genes; AUC = 1.00) as the best epigenetic signature to distinguish RW/AS-RSV phenotypes from CR-RSV in both training and test sets as well as in the whole cohort (**Figure 7**). While *LOC102723354* is related to an uncharacterized long non-coding RNA (lncRNA), whose function is still completely unknown, *ZNF268* and *NKIRAS1* are known to regulate, at a different level and in a different way, the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) (54, 55), an important transcription factor that plays a critical role in the production of many inflammatory cytokines. NF-κB is involved in activating and coordinating both innate and adaptive immune responses; it is found to be associated with allergic airway diseases, and it is activated in bronchial asthmatic patient biopsies and airway epithelium from mice (56).

Among the pathways found to be significantly enriched from the functional analysis, those involved in the cell cycle checkpoint are the most significantly enriched. These finding might suggest that cell proliferation may play an important role in the pathogenesis of wheezing and asthma after RSV infection. Besides, significant enrichment in pathways involved in DNA damage and integrity checkpoint was also found, probably suggesting that the cellular DNA damage machinery is activated and exploited by viruses which have acquired the ability to manipulate the key regulators of these pathways to promote their own replication. Considering the positions residing within promoters, we identified a significant enrichment for the nuclear ubiquitin ligase complex pathway.

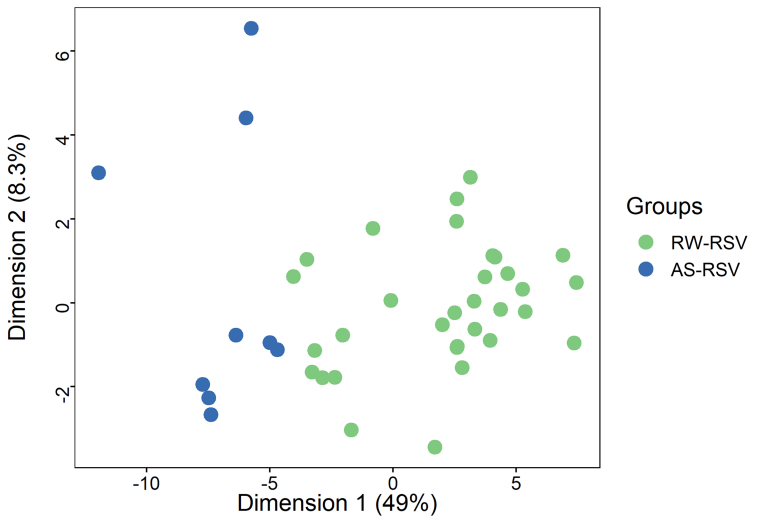

FIGURE 6 | PCA of the most different DMPs (FDR *P*-value < 0.05) shows an almost clear separation between RW-RSV and AS-RSV group.

It has been shown that allergic airway inflammation associated with rhinovirus infection leads to the upregulation of the E3 ubiquitin ligase midline 1 (*MID1*) in mouse bronchial epithelium, thus, the inhibition of *MID1* attenuates rhinovirus-induced airway inflammation and asthma exacerbations (57). In addition, the transforming growth factor-beta (TGF-β) signaling pathway was also found to be significant (FDR *P*-value = $1.21x10^{-2}$) in promoters' enrichment pathways. This cytokine's family seems to play a critical role in the development of airway inflammation and remodeling in asthma (58, 59). In asthmatic airways, TGF-β can induce an antiapoptotic effect in the airway epithelial cells through the SMAD signaling pathway and can trigger apoptosis activating MAPK signaling pathways (60–62). Significant DMPs fall within genes involved in the TFG-β related pathways (e.g. *BMP2*, *FLCN*, *ING2*, *PMEPA1*, *PRDM16*, *TGFB1I1* and *TGFBR3*), in line with previous studies that reported an association between these genes and the increased asthma risk (63–68). All over, these results would suggest that the regulation of the TGF-β could be involved in the induction of a moderate or severe course of asthma, a common respiratory sequela observed in children after RSV infection.

In addition, we found a set of co-methylated positions that were significant negatively correlated with the sequelae phenotype, pointing to a hypomethylation state of this CpGs block in RW/AS-RSV patients. Genes associated with these positions pointed out to GTPase signaling as the most significant route in sequelae after RSV infection. Other related significant pathways were those involved in Ca^{2+} metabolism, actin binding and cell junctions. The Rho family of GTPases has been proposed as a promising therapeutic target for asthma, and it is known to play an important role in the pathophysiology of asthma, including airway smooth muscle contraction, airway hyper-responsiveness and bronchial epithelial barrier dysfunction and recently, in mesenchymal stem cell differentiation and migration for airway remodeling and repairing (69).

A moderate number of statistically significant DMPs emerged when analyzing wheezing and asthma phenotypes separately. The highest difference in Delta β (0.148) corresponds to the cg18873878 observed within the TSS200 region of the *TP73* gene being hypermethylated in RW-RSV children in comparison with those with asthma. *TP73* gene encodes a member of the p53 family of transcription factors involved in cellular responses to stress and development; its role in airway epithelium is unknown, even if its homolog, *TP63*, is found to be essential for tracheobronchial epithelium development and homeostasis (70).Another interesting position with a lower methylation pattern in children with asthma was the cg05838113 located within the body of the *ADAM8* gene, an ADAM Metallopeptidase Domain which is linked to asthma. It seems that mice with allergic airway inflammation (AAI) show higher levels of expression of *ADAM8* in airway epithelium and airway inflammatory cells (71). In addition, increased expression of *ADAM8* was observed in the sputum and endobronchial biopsies of patients with moderate and severe asthma (72).

There are a few limitations in the present study. First, the main limitation is the limited number of patients analyzed, and the relative unbalanced in the available samples for the different RSV phenotypes studied. This fact led us to consider a less conservative threshold for the contrast analysis RW-RSV vs. AS-RSV which, in consequence, might increase the probability for false positives. In turns, our study is better powered for the recurrent wheezing outcome. Second, the clinical history of patients is incomplete for some co-variates of interest. Third, the three years of follow up could be not sufficient to discriminate between recurrent wheezing and asthma, being patients with recurrent wheezing at risk to develop asthma in the following years; however, a three-year

TABLE 3 | PDMPs between AS-RSV and RW-RSV groups are ordered by Delta β-values (FDR *P*-value < 0.05).

CpG_ID	Chr	Position	Location	GN	GG	*P*-value	Delta β
cg18873878	**1**	**3607116**	**IS**	***TP73***	**Body; TSS200**	**0.018**	**0.141**
cg19332572	11	65321591	IS	*LTBP3*	Body	0.011	0.118
cg07367519	22	40075288	IS	*CACNA1I*	Body	0.041	0.114
cg11507793	6	29856363	IS	*HLA-H*	Body	0.041	0.113
cg10274606	14	73118334	OS			0.041	0.109
cg14065121	9	77643271	IS	*C9orf41*	1stExon; 5'UTR	0.011	0.106
cg27594116	9	100069897	IS	*CCDC180; C9ORF174*	TSS200; Body	0.031	0.105
cg22371961	1	169132356	OS	*NME7*	Body	0.041	0.091
cg05838113	**10**	**135082349**	**IS**	***ADAM8***	**Body**	**0.010**	**0.086**
cg02478023	19	57351322	IS	*MIMT1; PEG3; ZIM2;*	TSS1500; 5'UTR	0.036	0.082
cg25612428	5	10649867	IS	*ANKRD33B*	Body	0.040	0.082
cg25550913	13	114783665	IS	*RASA3*	Body	0.042	0.077
cg05472874	22	44258179	IS	*SULT4A1*	1stExon	0.030	0.075
cg09519644	12	85401946	OS	–	–	0.011	0.075
cg11946459	6	29911558	SS	*HLA-A*	Body	0.011	0.073
cg01848660	2	68269960	OS	*C1D*	3'UTR	0.041	0.073
cg01979489	16	332603	IS	*ARHGDIG; PDIA2*	Body; TSS1500	0.041	0.072
cg00664920	16	2664747	IS	*LOC652276*	Body	0.021	0.067
cg09046688	9	75621983	OS	–	–	0.041	0.065
cg01997696	20	43374401	IS	*KCNK15*	TSS200	0.036	0.063
cg14377711	16	1384369	IS	*BAIAP3*	5'UTR; TSS200	0.041	0.060
cg16740746	16	78134345	SS	*WWOX*	Body	0.037	0.059
cg23215256	7	631862	IS	*PRKAR1B*	Body	0.041	0.058
cg01077616	6	42017945	NS	*CCND3; TAF8*	TSS1500	0.022	0.056
cg23861120	12	67835705	OS	–	–	0.014	0.046
cg14390580	17	35873008	IS	*DUSP14*	3'UTR	0.041	0.042
cg18560442	1	39174410	IS	–		0.041	0.040
cg15842722	20	23499644	OS	*CSTT*	TSS200	0.041	0.035
cg25326090	19	47197766	SS	*PRKD2*	Body	0.044	0.033
cg05801818	22	23262424	OS	–		0.044	0.024
cg08103551	11	76777993	IS	*CAPN5*	1stExon; 5'UTR	0.041	–0.007
cg10504753	13	100258763	IS	*CLYBL*	TSS200	0.041	–0.010
cg06787731	14	38069079	IS	–	–	0.044	–0.012
cg08806408	16	51185001	IS	*SALL1*	TSS1500; Body	0.021	–0.016
cg14181391	20	33265182	IS	*PIGU*	TSS200	0.041	–0.017
cg09672082	5	271577	IS	*PDCD6*	TSS200	0.036	–0.018
cg25372335	10	82168065	IS	*C10orf58*	TSS200	0.041	–0.020
cg21345913	18	61822270	OS	*LOC284294*	Body	0.041	–0.023
cg07648504	19	21262035	NE	–	–	0.041	–0.034
cg18693345	5	2754148	IS	*C5orf38*	Body	0.041	–0.035

Chr, chromosome; SE, S Shelf; NE, N Shelf; NS, N Shore; SS, S Shore; IS, island; OS, OpenSea; GN, Gene name; GN, GG, Gene group; P-value, FDR P-value. In bold, positions with higest difference in methylation and lowest P-value, respectively.

follow-up should highly capture the diagnosis of asthma in those children with predisposition to develop the disease. Finally, in our cohort, there is higher bacterial superinfection rates in CR-RSV (71.9%) vs. the RSV sequelae cohort (28.9%). The presence of bacteria could have impacted the epigenetics results if the changes observed were due to bacterial presence rather than differences in response to the viral infection. However, it is important to note that bacterial superinfection, as defined in our study, was not always based on a bacterial isolation in a sterile site, but rather frequently based on suggestive clinical symptoms, radiological findings and/or analytical values such as elevated biomarkers (i.e procalcitonin).To account for the mentioned limitations, statistical analyses were carried out considering all possible confounding factors. Even so, further studies will be needed to corroborate the main findings of the present study, as well as other complementary epigenetic studies that takes into account other epigenomic modifications e.g. in the chromatin.

CONCLUSION

In conclusion, there is suggestive evidence in the present study indicating that epigenetic factors might contribute to the susceptibility to develop recurrent wheezing and asthma after RSV infection. Many DMPs associated with respiratory sequelae developed after RSV infection were detected, with remarkable patterns of methylation profiles observed for genes involved in airway inflammation processes. Functional analysis considering all significant CpGs between sequelae and complete recovery patients revealed significant enrichment for pathways involved in cell cycle checkpoint, DNA damage and integrity checkpoint. In addition, we reported a 3-CpGs epigenetic signature that might be of interest as a diagnostic tool for RSV sequelae. DNA methylation might play a fundamental role in the development of asthma and/or wheezing after RSV infection and could explain the different post-infection sequelae observed. Further

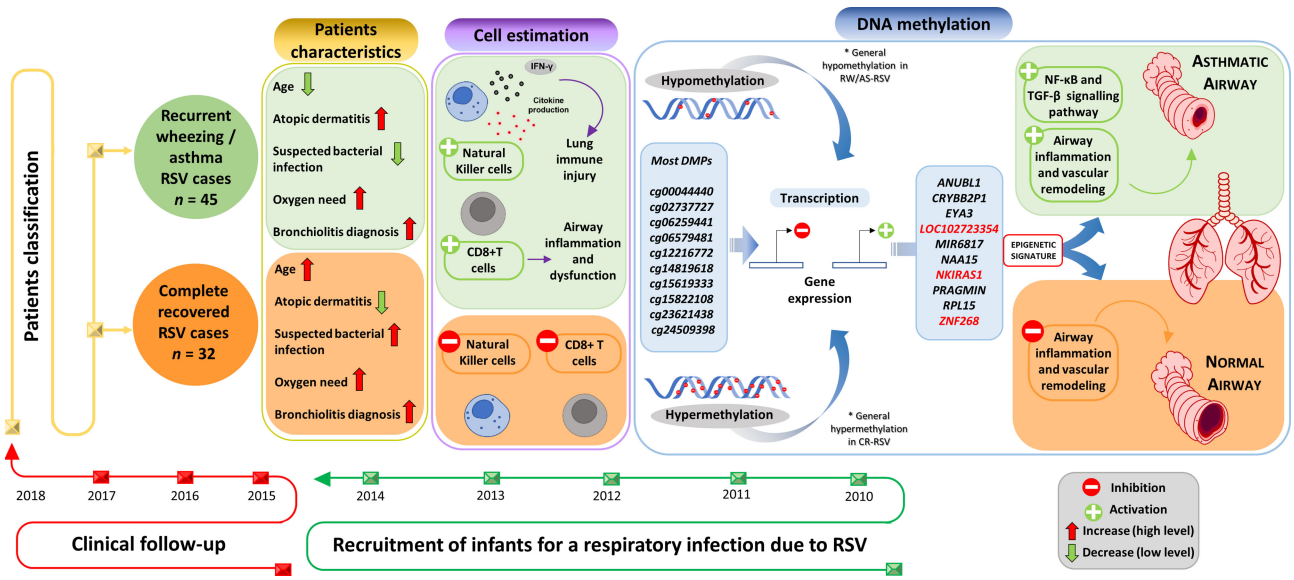

FIGURE 7 | Study design and overview of main results.Supplementary Material Legend.

investigation using larger cohorts, as well as transcriptomic studies, would be needed to further disentangle the role of epigenomics in asthma and other clinical manifestations (e.g. wheezing), as well as to validate epigenetic biomarkers that allow predicting with precision the different respiratory sequelae that can emerge after infection.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited on Gene Expression Omnibus (GEO) repository, Accession number: GSE199334.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Clinical Investigation of Galicia (CEIC 2010/015; updated version 2017/07/26, reg 2017/398). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FM-T, AS, and JG conceived and designed the experiments. IR-C, MC-L and MTG-L were involved in sample recruitment and collection of clinical data. SP, AC, AD-L performed the experiments. SP, and AG-C analyzed the data. SP, AG-C, and AS wrote the first draft of the manuscript, and was contributed by FM-T. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.875691/full#supplementary-material

Supplementary Figure 1 | (A) Selection of the soft-thresholding power. Plots showed a correlation between soft-thresholding powers and both the scale-free fit index (upper) and the mean connectivity (lower). **(B)** Clustering dendrogram of CpGs and co-methylation modules detected represented by different colors. **(C)** Average significance of all CpGs in each module.

Supplementary Figure 2 | (A) β-values heatmap of the CpGs from the pink module. Samples eigengenes values are also represented **(B)** Differences in samples eigengenes values from pink module between RW/AS-RSV and CR-RSV groups. **(C)** Correlation between gene significance (GS) for sequelae phenotype and module membership (MM) in the pink module. **(D)** Over-representation analysis of gene ontology terms using genes in the pink module (BP, biological processes; MF, molecular functions; CC, cellular components).

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Microbial dysbiosis and childhood asthma development: Integrated role of the airway and gut microbiome, environmental exposures, and host metabolic and immune response

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Asthma is a chronic and heterogeneous respiratory disease with many risk factors that typically originate during early childhood. A complex interplay between environmental factors and genetic predisposition is considered to shape the lung and gut microbiome in early life. The growing literature has identified that changes in the relative abundance of microbes (microbial dysbiosis) and reduced microbial diversity, as triggers of the airway-gut axis crosstalk dysregulation, are associated with asthma development. There are several mechanisms underlying microbial dysbiosis to childhood asthma development pathways. For example, a bacterial infection in the airway of infants can lead to the activation and/or dysregulation of inflammatory pathways that contribute to bronchoconstriction and bronchial hyperresponsiveness. In addition, gut microbial dysbiosis in infancy can affect immune development and differentiation, resulting in a suboptimal balance between innate and adaptive immunity. This evolving dysregulation of secretion of pro-inflammatory mediators has been associated with persistent airway inflammation and subsequent asthma development. In this review, we examine current evidence around associations between the airway and gut microbial dysbiosis with childhood asthma development. More specifically, this review focuses on discussing the integrated roles of environmental exposures, host metabolic and immune responses, airway and gut microbial dysbiosis in driving childhood asthma development.

KEYWORDS

microbial dysbiosis, airway microbiome, gut microbiome, immune mechanism, metabolic mechanism, childhood asthma

Introduction

Asthma is one of the most common chronic respiratory diseases affecting more than 300 million individuals worldwide (1). The prevalence of asthma remains high, mainly due to high incidence in the first years of life (2, 3). Asthma is characterized by symptoms of wheeze, shortness of breath, chest tightness, cough, and expiratory airway limitation (1). Epidemiological studies have suggested many risk factors for asthma development, but mainly representing three domains: demographics (e.g., age, sex, and family history), genetics, and environmental exposures (e.g., bacterial and viral infection, air pollution, and diet) (3–6). The interplay between these risk factors underlies the pathobiological mechanisms of asthma and contributes to the variability in pathogenic mechanisms and in response to treatment. It is accepted that asthma consists of a range of subtypes differing in presentation and pathobiological mechanisms (7), such as allergic vs. non-allergic asthma (8–10) and childhood- vs. adult-onset asthma (9, 11–13). As expected, the effects of risk factors on asthma incidence vary between asthma subtypes. Thus, it is critical to identify asthma subtype-specific risk factors. Of the various risk factors for childhood asthma development, microbial dysbiosis in the airway and gut may play a crucial role given their complex interplay with host genetic susceptibility, environmental exposures, and metabolic and immune response (14–16). For several reasons, such complex interplay becomes the most important for asthma during early-life development. First, early life is a crucial period in which the gene-environmental interaction helps to shape immune development (17). Second, most infections occur in infancy, and often are recurrent episodes and are relatively severe, which have long-term respiratory sequela in later life (18). Third, early life is a critical period of airway development, which is crucial in determining lung function and respiratory diseases in later childhood and adulthood (19). This review will examine the current epidemiological evidence about the relationship between airway and gut microbial dysbiosis and childhood asthma development, their integrated roles with environmental exposures, and the metabolic and immune mechanisms underlying airway and gut microbiota diversity and asthma development in childhood.

Human airway and gut microbiota

The human microbiota contains 10-100 trillion microbes harbored by each person (20). The microbes are distributed across several major body sites, including oral cavity, respiratory system, gastrointestinal system, vagina, and skin (21), with the microbial composition varying depending on the body site. Such distinct microbial composition is determined based on genetic susceptibility and environmental exposures (22). The interplay of these factors underlies the pathobiological mechanisms for diseases, such as asthma (16). Growing evidence suggests that microbiota in the respiratory system consists of four major pathogenic bacterial phyla, including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (21). The gastrointestinal system's microbiota consists of six major bacterial phyla, including Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Verrucomicrobia (21). The cross-talk between airway and gut microbiota can have synergistic effects on the development of respiratory diseases. Disturbances in gut microbial composition limit the capacity to modulate adequate immune responses, which not only has been linked to inflammatory conditions in the gastrointestinal tract itself but also in the airway, referred to as the "gut-lung axis" (23–25). The importance of such interaction has become more evident with the identification of microbe-produced metabolites in both the airway and gut. These metabolites can contribute to various respiratory diseases, such as asthma (26–29). Yet, until recently, the mechanisms that underlie the link between microbial dysbiosis in the airway and gut and childhood asthma development remained unclear.

Airway microbiota and childhood asthma development

Growing evidence suggests that the airway microbiota in early life is associated with childhood asthma development (29–41). For example, an early study from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC)—a prospective birth cohort—has reported that pathogenic bacterial colonization (e.g., *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*) of the nasopharynx in infants is associated with asthma development by age 5 years (30). Likewise, a study from Childhood Asthma Study (CAS)—a prospective birth cohort from Australia—has also found that *Streptococcus* colonization in infants' upper airway is strongly associated with asthma development by age 5 years (32). In addition, a recent study from the 35th Multicenter Airway Research Collaboration (MARC-35)—a prospective cohort of infants with severe bronchiolitis—has used a dual-transcriptome (metatranscriptome and transcriptome) approach and found that a higher abundance of *Streptococcus pneumoniae* in the nasopharyngeal airway is associated with a greater risk of developing asthma by age 6 years, particularly in infants with non-rhinovirus infection (29). And the major bacterial species drive the asthma-related microbial functional pathways, such as fatty acid metabolism and glycolysis pathways (29), which have been found to play important roles in asthma pathobiology (9, 42–44). The abundance of major bacterial species is also associated with asthma-related host transcripts (e.g., *DAGLB*)

(29). Furthermore, a recent study from the Steps to the Healthy Development and Well-being of Children (STEPS)—a prospective population-based birth cohort study—has reported that infants with persistent *Moraxella* sparsity have a higher risk of asthma development by age 7 years (34). Finally, a study from COPSAC 2010 ($COPSAC_{2010}$) has reported that an increased α-diversity (i.e., within-sample measures of similarity or dissimilarity) and higher abundance of *Veillonella* and *Prevotella* in the airway at age 1 month are associated with asthma development by age 6 years (33). These studies collectively suggest the important roles of airway microbiota from early life in the development of childhood asthma.

Gut microbiota and childhood asthma development

Compared with the airway microbiota, it seems less intuitive that the gut microbiota would be connected with respiratory diseases since gut and lungs differ anatomically. Yet, alterations in gut microbial composition may have a notable effect on respiratory diseases, such as asthma, by shaping microbial communities and modulating the metabolic and immune response, a so-called "gut-lung axis" concept (23–25). Growing evidence suggests that gut microbiota in early life is associated with childhood asthma development (26–28, 36, 45–50). For example, a study from $COPSAC_{2010}$ has found that immature gut microbial composition (measured by microbiota age) in children at age 1 year is associated with a higher risk of asthma development by age 5 years (47). Such association is stronger in children who have mothers with asthma, indicating the synergistic effect between genetic predisposition and inadequate gut microbial stimulation on asthma development (47). Additionally, a study from the Wayne County Health, Environment, Allergy and Asthma Longitudinal Study (WHEALS)—a prospective birth cohort—has found that lower relative abundance of beneficial bacteria in neonatal gut (e.g., *Akkermansia*, *Bifidobacterium*, and *Faecalibacterium*) are associated with a higher risk of asthma by age 4 years (26). Furthermore, a recent study from the Vitamin D Antenatal Asthma Reduction Trial (VDAART)—a randomized trial on the effects of prenatal vitamin D supplementation on asthma in offspring—has found that a higher level of *Veillonella* and histidine pathway metabolites or a lower level of *Oscillospiraceae* UCG-005 in gut of children at age 3 years are associated with an increased wheeze frequency between ages 3 and 5 years (28). In contrast, a mature gut microbial composition in early life may help to lower the risk of childhood asthma development. For example, a recent study from the Protection against Allergy-Study in Rural Environments (PASTURE)—a prospective birth cohort—has found that a mature gut microbial composition in infants from age 2 to 12 months—consisting of *Bacteroides, Coprococcus*, *Roseburia*, and *Turicibacter*—can produce short-chain fatty acids (SCFAs), such as butyrate, which have a protective effect on asthma development by 6 years (48). Likewise, a recent study from the Canadian Healthy Infant Longitudinal Development (CHILD)—a prospective birth cohort—has found that an increased α-diversity in gut microbiota due to decreased antibiotic use in infancy is associated with a reduced risk of asthma development by age 5 years (49). Taken together, these diverse pieces of evidence provide support for the relation of gut microbiota in early life and development of childhood asthma.

Integrated roles of environmental exposures and microbiota on childhood asthma development

The complex interplay between environmental exposures and microbiota underlies the pathobiological mechanisms for asthma, especially in early life (Figure 1). One prominent example of environmental exposure affecting asthma development is the farm effect. Many epidemiological studies have found living on a farm in early life is associated with reduced risk of childhood asthma development (48, 51–54). For example, an early study has investigated two farm populations (Amish and Hutterites) with similar genetic backgrounds but different farming practices and found a distinct risk for childhood asthma development (52). The Amish follow traditional farming practices (i.e., high microbial exposures to animals) whereas the Hutterites use industrialized farming practices. The study has found the environment from Amish farms protects children against asthma development (52). On the other hand, a recent study from $COPSAC_{2010}$ found that an airway and gut microbial signature due to urbanization (i.e., no farm effect) in infancy increases the risk of asthma, eczema, and allergic sensitization at age 6 years (55). Collectively, these interesting results suggest that early-life microbial farm animal exposures may lower the risk of childhood asthma development, perhaps by shaping the innate immune response.

In addition to farm environmental exposures, other environmental exposures (e.g., antibiotics use, diet) may also have an effect on childhood asthma development by modulating the airway and gut microbiota in children's early life. For example, a recent study from the STEPS study has found that exposures to antibiotics within the first year of life are associated with increased risk of asthma development by age 7 years; and such effect is partially mediated by longitudinal changes in the nasal airway microbiota characterized as a low asthma risk profile with persistent *Moraxella* dominance vs a high asthma risk profile with early *Moraxella* sparsity (56). Also, a study from the Urban Environment and Childhood Asthma (URECA)—a longitudinal birth cohort—has reported that the exposure to

both allergens and the bacterial species (primarily from Bacteroidetes and Firmicutes phyla) within the first year of life can protect from atopy and recurrent wheeze development by age 3 years (57). Additionally, a study from the Infant Susceptibility to Pulmonary Infections and Asthma Following Respiratory Syncytial Virus Exposure (INSPIRE)—a longitudinal observational birth cohort—has found that breastfeeding during infancy has several beneficial effects on childhood asthma development by reducing the dose-response effect of the feeding on the α-diversity of the early-life upper airway and gut microbiota, and protects children from the development of lower respiratory tract infections in infancy, and asthma at age 4 years (41). Moreover, a recent study from $COPSAC_{2010}$ has found that if the gut microbial signature at age 1 year is retained from a cesarean section delivery period, there is a higher risk of asthma development by age 6 years, but not at a higher risk if the gut microbial signature became mature (e.g., a higher abundance of *Akkermansia*, *Bacteroides*, and *Ruminococcus*) (50). This finding indicates the maturation of gut microbial composition may mitigate the effect of cesarean section delivery on childhood asthma development. These studies collectively suggest that it is crucial to consider the integrated roles of environmental exposures and microbiota from early life in the childhood asthma development.

Immune mechanisms for airway microbiota and asthma link

Microorganisms can present in host (e.g., airway) without interaction with the host, i.e., colocalization. Once there is an imbalance in the disease-related microbial community and interaction with host (i.e., microbial dysbiosis), host immune system can respond to the pathogenic microbes and lead to the local or/and systematic inflammation (14). Underlying mechanisms of the airway microbial dysbiosis and asthma link warrant further clarification. Figure 2A illustrates the major host immune mechanisms for airway epithelial cell signaling in response to bacterial pathogens. First, many studies have reported that innate immune responses play direct roles in host defense during the early stages of a respiratory infection, and they also exert a profound influence on the generation of the adaptive immune responses that ensue and on driving long-term respiratory sequela (most commonly asthma) (58, 59). The airway epithelium is a physical barrier and the first point of contact for inhaled pathogens (60). Notably, the airway epithelium contains a wide variety of pattern recognition receptors and antimicrobial compounds (e.g., mucins) that establish innate immunity (60). The pattern recognition receptors in the airway epithelium, such as Toll-like receptors (TLRs), are activated by invading pathogens, bacterial virulence factors, and endogenous mediators released due to airway tissue damage (61). The innate immune recognition of *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*, mainly involves TLR2 and TLR4. These TLRs activate the transcription factor nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 (IRF3) (61), which regulates the expression of inflammatory genes and the release of cytokines and chemokines related to asthma (62, 63), such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α. For example, an *in vivo* study using an ovalbumin (OVA)-induced mouse model of allergic asthma has demonstrated that dysfunction of TLR4-positive innate immune cells including neutrophils and macrophages during *Haemophilus influenzae* infection promotes bacterial persistence and leads to the development of steroid-resistant neutrophilic asthma (64). In addition, an *in vitro* study has also shown that chronic colocalization with *Haemophilus influenzae* can induce neutrophil extracellular trap formation (NETosis) and releases soluble IL-6 receptor (sIL-6R) and IL-6 from neutrophils, which also associated with higher lung epithelium expression of TLR2 and TLR4 (65). The causal relationship between sIL-6R and childhood asthma is also supported by a recent Mendelian randomization study (66).

Second, in addition to the innate immunity, growing evidence suggests that adaptive immunity plays a pivotal role in the underlying mechanisms of the airway microbiota and asthma link (14, 67). For example, a recent *in vivo* study using a house dust mite (HDM)-challenged mouse model of the allergic airway inflammation has found that airway infection of *Streptococcus pneumoniae* leads to increased number of activated T helper 2 (T_H2) cells and elevated level of T_H2 cytokines, such as IL-4, IL-5 and IL-13 (68). Additionally, an *in vivo* study using HDM-challenged mouse model has found that airway infection with *Moraxella catarrhalis* triggers a strong inflammatory response with neutrophilic infiltrates, such as high amounts of IL-6 and TNF-α and moderate levels of CD4+ T-cell-derived interferon (IFN)-γ and IL-17. If bacterial infection occurs during HDM allergen sensitization, the allergic airway response is exacerbated, particularly by the expansion of T helper 17 (T_H17) cells and increased TNF-α levels (69). In addition, a study using human bronchoalveolar lavage samples has demonstrated that a high bacterial load and supraglottic predominant taxa (e.g., *Prevotella* and *Veillonella*) is associated with an increased number of CD4+ IL-17+ T cells, and cytokines (IL-1β and IL-6) or chemokine (fractalkine) related to T_H17 differentiation (70). Finally, an *in vivo* study using a HDM-challenged neonatal mouse model has demostrated that a dynamic change in lung microbiota in the first 2 weeks of life, from a dominance of Gammaproteobacteria and Firmicutes towards Bacteroidetes, is associated with an increased level of regulatory T cells (Tregs) and reduced aeroallergen responsiveness (71). Such findings indicate that the maturation of airway microbiota in early life is crucial to reduce the risk of developing allergic airway inflammation in later life. Notwithstanding the complexity of these mechanisms, the

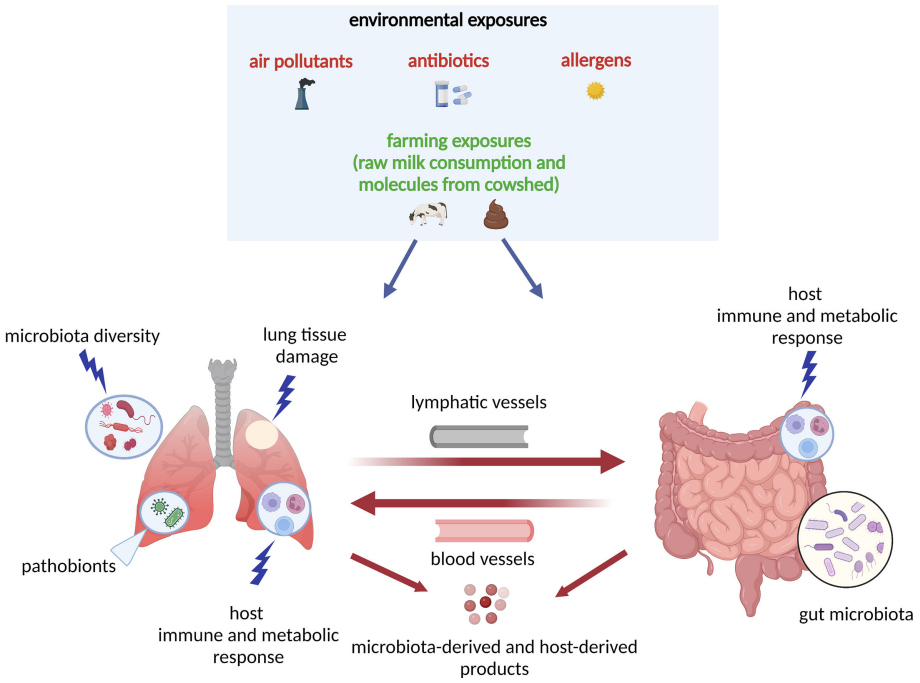

FIGURE 1
Model of the host–microbiota interaction during dysbiosis as a pathogenetic mechanism underlying asthma development: the "gut-lung axis". A variety of environmental exposures, either protective (green color) or non-protective (red color) can trigger microbial dysbiosis (i.e., the alteration of the function and composition of the microbiota). In this model, in response to the non-protective environmental exposures (air pollutants, antibiotics, allergens), there is lung tissue damage and release of molecules that interact with host immune and metabolic pathways. The lung and gut microbiota diversity is perturbed, and there is increase in pathobionts (i.e., potentially disease-causing organisms that under normal circumstances act as symbionts) in both lung and gut tissues. In response to microbial dysbiosis, there is also increased activation of leukocytes and other innate-mediated soluble factors, which circulate through blood and lymphatic vessels and trigger adaptive immune responses with T_H1, T_H2, T_H17, Treg differentiation. All host- and microbiota-derived (e.g., SCFAs, cytokines and chemokines) products act at the local (lung) or distal (gut) levels *via* circulation through blood and lymphatic vessels. The "gut-lung axis" refers to bidirectional crosstalk between these two mucosal sites of the body as described above. SCFAs, short-chain fatty acids; T_H2, T helper 2; T_H17, T helper 17; Treg, regulatory T cell.

identification of the airway microbiome-host immune response interaction and its contribution to asthma pathobiology will likely prove important to future efforts to prevent childhood asthma.

Metabolic and immune mechanisms for gut microbiota and asthma link

The mechanisms underlying a link between gut microbiota and asthma involve complex interactions between microbes, metabolites, and host immune responses (14). Figure 2B illustrates the major metabolic and immune mechanisms for the link between gut commensal bacteria and asthma. In the gut, commensal bacteria help to shape the cellular and physical maturation of both innate and adaptive immunity in early life and have profound effects on asthma pathobiology (72, 73). More specifically, the presence of specific microbial species helps maintain the gut barrier function by preserving tight junction formation at the gut epithelium, and by modulating immune responses to allergens (74). For example, an early *in vivo* study of a mouse model showed polysaccharide A from *Bacteroides fragilis* induces and ligates TLR2 on plasmacytoid dendritic cells, which is priming IL-10 producing T cells with potential anti-inflammatory properties (75). Additionally, another *in vivo* study using an HDM-challenged mouse model has found that high-fiber diet increases the abundance of *Bacteroides* and *Bifidobacterium*, which can digest the fiber and produce SCFAs, such as butyrate (76). Butyrate can decrease excessive inflammation through downregulating the secretion of pro-inflammatory mediators (e.g., IL-6, TNF-a) and activating IL-10 producing T cells and macrophages (77). Consequently, the study has found that mice fed a high-fiber diet have increased concentration of circulating SCFAs and decreased airway hyperresponsiveness (AHR) and allergic airway inflammation (76). A recent study has also demonstrated that infants who are breastfed and given *Bifidobacterium infantis* EVC001, has reduced intestinal T_H2 and T_H17 cytokines and increased IFN-β level (78). Furthermore, HDM-challenged mice given with *Faecalibacterium prausnitzii* have increased SCFAs (e.g., butyrate, propionate) level, which leads to reduced levels of IL-4, IL-

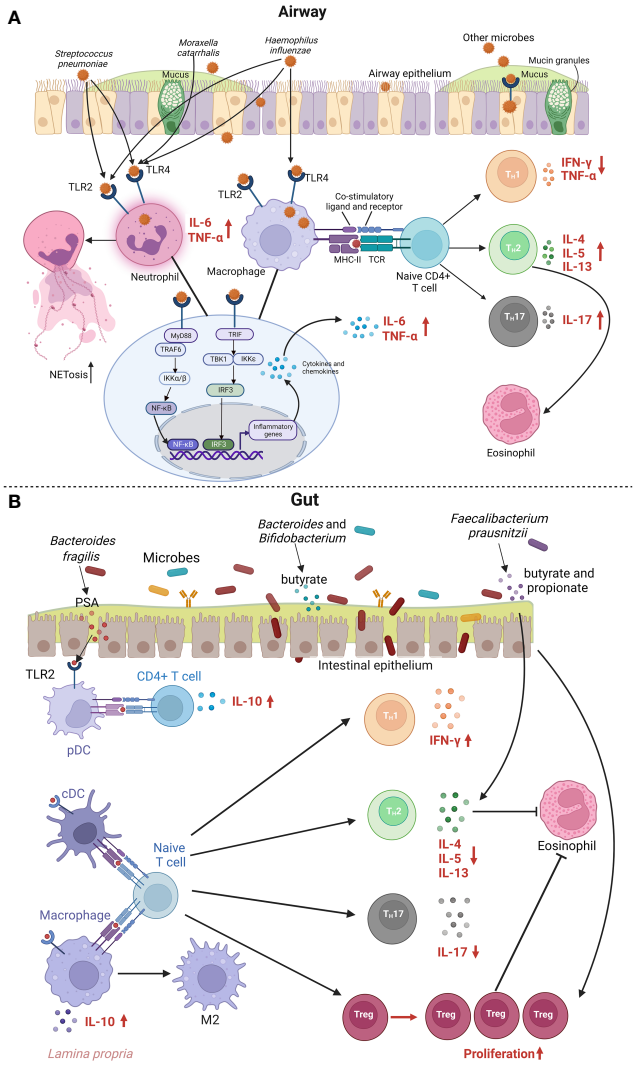

FIGURE 2
Metabolic and immune mechanisms for the link between airway or gut microbiota and asthma. **(A)** shows immune mechanisms for the airway epithelial cell signaling in response to bacterial pathogens. Various bacterial pathogens bind to innate sensors TLR2 and TLR4 and further activate MyD88- and TRIF-dependent pathways. MyD88 recruits TRAF6, which further activates the IKK complex, allows NF-κB to translocate into the nucleus, and leads to the overall production of inflammatory cytokines and chemokines, and activation of T cells. Additionally, recognition of various bacterial pathogens activates the TRIF-dependent signaling pathway, which involves the recruitment of TRIF, that leads to subsequent activation of TBK1 and IKKε along with induction of transcription factor IRF3. This signaling pathway results in interferon-related cytokines, and can potentiate NF-κB gene transcription. Enhanced neutrophil TLR2 and TLR4 signaling by bacterial pathogens promote neutrophils cytokine production and NETosis, a program for formation of NETs. Bacterial pathogens can also be recognized by TLR2 and TLR4 on macrophages, leading to activation of NF-κB and IRF3 signaling pathway and secretion of inflammatory mediators. Macrophages can also function as APC and regulate T cell activation. The T cell is presented an antigen with MHC II by APC. The recognition of the antigen-MHC II complex and the co-stimulatory molecules activates the T cell and leads downstream to differentiation into T_H2 and T_H17 cells, that can release various cytokines such as IL-4, IL-5 and IL-13, which lead to eosinophilic inflammation. APC, antigen-presenting cell; IKK, inhibitory kappa B kinases; IL, interleukin; IRF3, interferon regulatory factor 3; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response protein 88; NETosis, neutrophil extracellular trap formation; NETs, neutrophil extracellular traps; NF-κB, nuclear factor kappa B; TBK1, TANK-binding kinase 1; T_H2, T helper 2; T_H17, T helper 17; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor associated factor 6; TRIF, TIR-domain-containing adapter-inducing interferon-β. **(B)** shows metabolic and immune mechanisms for the link between gut microbiota and asthma. PSA from *Bacteroides fragilis* induces and ligates TLR2 on pDC, which stimulate anti-inflammatory cytokine IL-10 secretion by CD4 + T cells. cDC and macrophage bound by gut microbiota show impaired ability to promote TH2- and TH17-type responses and tend to promote TH1-type responses and Treg proliferation, which lead to decreased eosinophilic inflammation. IL-10 dependent reprogramming of tissue macrophages is also essential for resolving inflammation by promoting M2 macrophage polarization. *Bacteroides* and *Bifidobacterium* can digest the fiber and produce SCFAs, such as butyrate. *Faecalibacterium prausnitzii* increases SCFAs level, such as butyrate and propionate, which leads to reduced levels of IL-4, IL-5 and IL-13, and elevated level of Tregs. cDC, classical dendritic cell; IFN, interferon; IL, interleukin; pDC, plasmacytoid dendritic cell; PSA, polysaccharide A; SCFAs, short-chain fatty acids; T_H2, T helper 2; T_H17, T helper 17; TLR, Toll-like receptor; Treg, regulatory T cell.

5 and IL-13, and elevated level of Tregs, consequently alleviating the symptoms of allergic asthma (79). Finally, intervention studies have provided mechanistic evidence on gut commensal bacteria and immunoregulation. For example, a recent *in vivo* study of OVA-induced murine allergic airways disease has showed that probiotic administration (e.g., *Akkermansia*) alleviate the airway inflammation in mice with a genetic predisposition for airway inflammation (80). The study has found that probiotic bacteria—such as *Akkermansia*—can produce acetate, another SCFA that can inhibit NF-κB activity (80). The inhibition of NF-κB is known to alleviate airway inflammation in asthma by reducing T_H2 cytokine production, such as IL-5 and IL-13 (81). The biodiversity intervention (i.e., more nature-oriented environment) may modify the gut microbiota in children (e.g., *Faecalibacterium*), which was associated with changes in plasma cytokine and Tregs levels (82). This finding suggests the biodiversity intervention improved immunoregulatory pathways in children and can potentially lower the risk of immune-mediated diseases (e.g., asthma) in urban societies (82).

Conclusion and future directions

In this review, we have examined a broad range of major epidemiological and mechanistic studies and summarized the current evidence for the link between airway or gut microbiota and childhood asthma development. We also discussed some of the metabolic and immunological mechanisms underlying the link between microbiome exposure in early life and childhood asthma development. Nearly all microbiome-asthma studies have investigated either airway or gut microbiota – but not both. Thus, the synergistic effect of the airway and gut microbiota on childhood asthma development remains largely unclear (36, 83). We suggest future research shall examine the integrated effect of airway and gut microbiota on childhood asthma development in the same individual. Also, majority of mechanistic studies are in murine models, and often not early life models. The next step is to consider intervention studies with mechanisms built in. Additionally, we believe that integrating microbiome with other omics data—such as genomics, epigenomics, transcriptomics, proteomics, and metabolomics—will provide further insights into the pathobiology of asthma and its subtypes. Taken together, these efforts will facilitate the development of an early life microbiome-targeted prevention and intervention strategies for the primary prevention of childhood asthma (84).

Author contributions

CL conducted the literature review, drafted the initial manuscript, designed the figure, and approved the final manuscript as submitted. HM designed the figure, reviewed the initial manuscript, and approved the final manuscript as submitted. SS, MB, and L-LL reviewed the initial manuscript and approved the final manuscript as submitted. CC and KH conceptualized the study, reviewed the initial manuscript, and approved the final manuscript as submitted. ZZ conceptualized and supervised the study, obtained funding, conducted the literature review, drafted the initial manuscript, designed the figure, and approved the final manuscript as submitted.

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Conflict of interest

CL, MB, and L-LL are employees of Sanofi US and may hold shares and/or stock options in the company. CC and KH report grants from National Institutes of Health outside the submitted work. ZZ reports grants from National Institutes of Health and Harvard University during the conduct of the study.

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

Publisher's note

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MBD2 mediates Th17 cell differentiation by regulating MINK1 in Th17-dominant asthma

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Objectives: .Asthma is a highly heterogeneous disease, and T-helper cell type 17 (Th17) cells play a pathogenic role in the development of non-T2 severe asthma. Misshapen like kinase 1 (MINK1) is involved in the regulation of Th17 cell differentiation, but its effect on severe asthma remains unclear. Our previous studies showed that methyl-CpG binding domain protein 2 (MBD2) expression was significantly increased in patients with Th17 severe asthma and could regulate Th17 cell differentiation. The aim of this study was to investigate how MBD2 interacts with MINK1 to regulate Th17 cell differentiation in Th17-dominant asthma.

Materials and methods: Female C57BL/6 mice and bronchial epithelial cells (BECs) were used to establish mouse and cell models of Th17-dominant asthma, respectively. Flow cytometry was used to detect Th17 cell differentiation, and the level of IL-17 was detected by enzyme-linked immunosorbent assay (ELISA). Western blot and quantitative real-time PCR (qRT-PCR) were used to detect MBD2 and MINK1 expression. To investigate the role of MBD2 and MINK1 in Th17 cell differentiation in Th17-dominant asthma, the MBD2 and MINK1 genes were silenced or overexpressed by small interfering RNA and plasmid transfection.

Results: Mouse and BEC models of Th17-dominant asthma were established successfully. The main manifestations were increased neutrophils in BALF, airway hyperresponsiveness (AHR), activated Th17 cell differentiation, and high IL-17 levels. The expression of MBD2 in lung tissues and BECs from the Th17-dominant asthma group was significantly increased, while the corresponding expression of MINK1 was significantly impaired. Through overexpression or silencing of MBD2 and MINK1 genes, we have concluded

that MBD2 and MINK1 regulate Th17 cell differentiation and IL-17 release. Interestingly, MBD2 was also found to negatively regulate the expression of MINK1.

Conclusion: Our findings have revealed new roles for MBD2 and MINK1, and provide new insights into epigenetic regulation of Th17-dominant asthma, which is dominated by neutrophils and Th17 cells. This study could lead to new therapeutic targets for patients with Th17-dominant asthma.

KEYWORDS

Th17-dominant asthma, misshapen like kinase 1, methyl-cpg binding domain protein 2, t-helper cell type 17 cells, bronchial epithelial cells

1 Introduction

Asthma is an epidemic and highly heterogeneous chronic inflammatory clinical disease that affects all age groups (Stern et al., 2020). Distinct asthma endotypes are used to describe the inflammatory pathways that participate in the pathogenesis of asthma at the cellular and molecular levels (Kuruvilla et al., 2019). The inflammatory pathways of asthma are driven by multiple immune mechanisms. $CD4^+$ T cells differentiate into a variety of subtypes, including T helper type 2 (Th2) cells and T helper type 17 (Th17) cells, both of which play an important role in the immune response (Muhammad Yusoff et al., 2020). Currently, most studies classify asthma into type 2 (T2) and non-T2 endotypes (Kuo et al., 2017). Sputum eosinophil counts have been identified as biomarkers for classic allergic asthma, which is thought to be driven by a Th2-mediated pathway, known as T2 asthma (asthma) (Peters et al., 2014). However, studies have found that almost 50% of asthma cases are primarily infiltrated by neutrophils rather than eosinophils (a non-T2 endotype) and respond poorly to glucocorticoids. This pathway is driven mainly by Th17 cells, known as Th17-dominant asthma (or neutrophil-dominant asthma) (Douwes et al., 2002; Manni et al., 2016). Th17 cells recruit neutrophils into the airway by secreting the cytokine interleukin-17 (IL-17), which is less susceptible to glucocorticoid inhibition than IL-4 and IL-5 produced by Th2 cells (Newcomb and Peebles, 2013).

Asthma can be triggered by allergies, house dust mites (HDM), oxidative stress, smoking and infections. These risk factors induce an immune response to the initial initiation of T cells by antigen presenting cells (APCs), mainly dendritic cells and bronchial epithelial cells (BECs) (Liu et al., 2020). Several studies have found that BECs can present antigen to T cells and promote the antigen presentation process and subsequent T cell proliferation and differentiation under the action of certain genes, playing a key role in the sensitization and pathogenesis of asthma (Lambrecht and Hammad, 2012; Qu et al., 2013; Lee et al., 2017; Liu et al., 2018).

The germinal center kinase family regulates a variety of cellular processes, including cell growth and differentiation, gene transcription, and immune and stress responses, through the mitogen-activated protein kinase pathways (Fu et al., 1999; Yin et al., 2012). As a member of the germinal center kinase family, misshapen like kinase 1 (MINK1), is a serine-threonine kinase (Dan et al., 2000). MINK1 negatively regulates Th17 differentiation through direct phosphorylation of SMAD2 at T324 residues (Fu et al., 2017). In addition, in animal models of experimental autoimmune encephalomyelitis (EAE), MINK1 deficiency increases IL-17 levels, promoting Th17-dependent inflammation and exacerbating the severity of EAE (Fu et al., 2017). Therefore, we hypothesized that MINK1 is involved in asthma by regulating Th17 cell differentiation and may be a therapeutic target for Th17-dominant asthma.

In addition to genetics, environmental influences also play a role in asthma development, which may be regulated by epigenetic mechanisms (Reese et al., 2019). The epigenetic regulatory mechanisms of asthma include DNA methylation, and the methyl-CpG binding domain (MBD) family of proteins play a "reader" and regulatory role as a key bridge in the process of DNA methylation, and MBD2 is a member of the family (Horsburgh et al., 2015; Shen et al., 2020). MBD2 has been reported to be involved in the pathogenesis of experimental colitis, rheumatoid arthritis, lupus nephritis and other immune diseases (Liu et al., 2011; Zhang et al., 2017; Jones et al., 2020). Zhong et al. found that MBD2 deletion leads to failure to read methylation information, which disrupts T-bet/Hlx homeostasis and leads to reduced differentiation of Th17 cells, thereby playing a protective role in EAE (Zhong et al., 2014). Previous studies have shown that MBD2 is involved in neutrophil-dominant asthma and positively regulates Th17 cell differentiation (Jia et al., 2017; Sun et al., 2018). At present, Th17-mediated asthma is more likely to develop into severe asthma due to poor response to glucocorticoids, and MBD2 may also have a potential therapeutic effect on severe asthma.

A previous study showed that MBD2 was significantly increased in patients with Th17 severe asthma compared to healthy controls and patients with asthma (Chen et al., 2021). In this study, we aimed to determine the role of MBD2 and MINK1 in Th17-dominant asthma. We induced Th17-dominant

asthma mouse and BECs models and found that MBD2 was significantly increased *in vivo* and *in vitro*, while MINK1 was decreased, and there was a reverse expression between MBD2 and MINK1. In addition, we also investigated the effect of MBD2 intervention on MINK1 expression and Th17 cell differentiation and found that MBD2 deletion promoted MINK1 expression and inhibited Th17 cell differentiation and IL-17 release. Taken together, these data suggested that MBD2 might promote Th17 cell differentiation by inhibiting MINK1, thereby exacerbating the severity of asthma.

2 Materials and methods

2.1 Animal model

Female C57BL/6 mice (6–7 weeks old, 18–20 g) were provided by the animal center of the Second Xiangya Hospital of Central South University (Changsha, China) and maintained under specific pathogen-free conditions. All experimental protocols were approved by the Animal Care and Use Committee of the Second Xiangya Hospital of Central South University and were conducted in accordance with the guiding principles of the institution. The establishment of a Th17-dominant asthma mouse model was consistent with previous reports (Daan de Boer et al., 2013; Jia et al., 2017). Animals in the Th17-dominant asthma group (n = 6/group) were given an intraperitoneal sensitization injection containing 100 μg of ovalbumin (1 mg/ml, OVA, Grade V, Sigma Aldrich), 100 μg of HDM (10 mg/ml, Greer Laboratories, United States) and 15 μg of lipopolysaccharide (1 mg/ml, LPS, Sigma Aldrich) with 2 mg of aluminum hydroxide (Sigma Aldrich) dissolved in 200 μL of saline on days 0,1 and 2. On days 14, 15, 18, and 19, the animals were challenged with 6% OVA solution atomized for 30 min before 100 μg/10 μL HDM was administered intranasally. The normal control group was sensitized and atomized with saline only, but the injection time, location, dose and atomization time were the same as those in the Th17-dominant asthma group.

The T2 asthma group was sensitized by intraperitoneal injection of 100 μg OVA (1 mg/ml) and 2 mg aluminum hydroxide dissolved in 200 μL saline on days 0–7, followed by atomization with 6% OVA solution on days 14–20 for 30 min (Hoffman et al., 2013; Ni et al., 2018). Mice were sacrificed on day 21 for analysis.

2.2 Assessment of airway hyperresponsiveness (AHR)

Methacholine (Mch)-induced airway resistance was measured with direct plethysmography (Buxco Electronics, RC System, Wilmington, United States) on day 21 (Locke et al., 2007). First, baseline lung resistance (RL) was measured for 1 min, then the airways of mice were stimulated with 10 μL of aerosolized saline and 10 μL of Mch at increasing doses (0.39 mg/ml, 0.78 mg/ml, 1.56 mg/ml, 3.12 mg/ml), and RL was recorded again.

2.3 Bronchoalveolar lavage fluid processing

Bronchoalveolar lavage fluid (BALF) was collected by injecting 0.5 ml of saline into the lungs 3 times through an endotracheal tube (37°C). BALF inflammatory cells were centrifuged and resuspended in cold phosphate-buffered saline (PBS, 1500 rpm, 5 min, 4°C, Eppendorf Centrifuge Configurator, Hamburg, Germany), then the cells were fixed and stained with Wright-Giemsa, and 200 cells were counted and differentially counted under a light microscope using a counting chamber.

2.4 Histopathology

The lungs were fixed with 10% formalin *via* the trachea, then removed and stored in 10% formalin. Fixed lung tissues were paraffinized and sectioned (5 μm) for hematoxylin and eosin (H&E) staining and immunohistochemistry [MBD2 antibody (Abcam, Cambridge, United States), MINK1 antibody (Proteintech, Wuhan, China), eosinophil antibody (anti-ECP, Biorbyt, Cambridge, United Kingdom) and neutrophil antibody (anti-Gr-1, Biolegend, San Diego, United States)]. Select stained sections per group were collected and assessed for MBD2, MINK1, eosinophil, and neutrophil protein expression.

2.5 Bronchial epithelial cell isolation and culture

Bronchial epithelial cells (BECs) were isolated using an improved version of the protocol previously reported (Davidson et al., 2000; Cong et al., 2020). Briefly, bronchi were removed from the gross anatomy of mice, and the tracheae were dissected lengthways, washed with PBS, and transferred to minimal essential medium (MEM, 11095–080, Fisher Scientific International) preheated to 37°C containing 0.1 mg/ml DNase and 1.4 mg/ml pronase (Roche Diagnostics). After incubation at 37°C for 1h, the tube containing the tracheae was carefully inverted 12 times to separate the epithelial cells from the airways. One milliliter of sterile fetal bovine serum (FBS, Gibco, Australia) was added to stop enzyme digestion. After that, a 150-mesh cell sieve (Biosharp, BS-100-XBS, China) was used to remove undigested excess tissue. After centrifugation for 5 min at

800 × *g*, the supernatant was discarded and the cells were resuspended with MEM containing 10% FBS. The cells were then inoculated in culture bottles and left standing at 37°C with 5% CO_2 for 2 h to remove contaminated non-epithelial cells. Subsequently, the culture medium (including suspended cells) was collected, and the supernatant was removed after centrifugation at 800 *g* for 5 min. BECs were cultured with bronchial epithelial growth medium (Procell, CM-M007, China) in a humidified incubator at 37°C with 5% CO_2.

Generally, studies have shown that 95% of epithelial cells are positive for cytokeratin, an epithelial marker that is not expressed by lymphocytes (Tryphonopoulos et al., 2004). The BECs were centrifuged onto slides and stained with DAPI and cytokeratin-specific monoclonal antibody (pan-Cytokeratin, SantaCruz, sc-8018, United States).

2.6 Cell asthma model and transfection

To induce Th17-dominant asthma, BECs were treated with 100 μg/ml HDM and 100 ng/ml LPS for 24 h. BECs were treated with 100 μg/ml HDM for 24 h to establish T2 asthma or PBS to establish normal controls (Liu et al., 2019).

Small interfering RNA targeting MBD2, MINK1 (siR-MBD2, siR-MINK1), and negative control (siR-NC) were purchased from RiboBo (Guangzhou, China). The MBD2 interfering sequence was 5′-GCAAGATGATGCCTAGTAA-3′ and the MINK1 interfering sequence was 5′-GCAAGTACA AGAAGCGATT-3′. Mouse MBD2, MINK1 over-expression plasmids (OE-MBD2, OE-MINK1), and negative control (OE-NC) were purchased from HonorGene (Hunan, China). Small interfering RNA and plasmid were transfected in BECs for 48 h using Lipofectamine 3000 (Invitrogen, United States) according to the manufacturer's protocols. After 2 days, the cells were treated with 100 μg/ml HDM and 100 ng/ml LPS for 24 h.

2.7 $CD4^+$ T cell isolation and cocultivation

Mouse spleen $CD4^+$ T cells were isolated by magnetic bead separation (130–117-043, Miltenyi Biotec, Germany). The established asthma model and transfected BECs were cocultured with $CD4^+$ T cells (TCs) at a ratio of 10:1 (TCs: BECs) for 24 h, respectively, in complete RPMI 1640 culture medium (Gibco, Australia) supplemented with 10% FBS, 1% penicillin and streptomycin, soluble anti-CD28 (1.0 μg/ml, eBioscience), soluble anti-CD3e (0.5 μg/ml, eBioscience), and IL-2 (20 ng/ml, eBioscience). For analysis of T cell subsets, suspended cells were collected 24 h later, and the concentration of IL-4 and IL-17 A was determined by flow cytometry to obtain the ratio of Th2 to Th17 cells. Total protein of BECs was extracted for western blotting.

2.8 Flow cytometry

$CD4^+$ T cells were stimulated with 2 μL/ml (1×10^6 cells/ml) leukocyte activation cocktail (550583, BD Biosciences, United States), and cultured at 37°C with 5% CO_2 for 6 h, then collected for flow cytometry analysis. After a 6-h incubation, cells were stained with a marker of cell viability (Fixable Viability Stain 510 antibody, BD Pharmingen) for 15 min at room temperature in the dark. Then, cells were stained for surface markers with FITC-anti-CD4 antibody (Biolegend) followed by fixation and permeabilization using the Cytofix/Cytoperm Soln Kit (BD Pharmingen) for 30 min at 4°C in the dark. After washing with permeabilization buffer, cells were stained for intracellular markers with APC-anti-IL-17 A (Biolegend) and PE-anti-IL-4 (BD Pharmingen) antibodies in permeabilization buffer for 30 min at 4°C in the dark. Isotype controls were employed in the control group. Flow cytometry was performed, and data were analyzed using FACS CantoII(Becton Dickinson) and FlowJo version X software.

2.9 Western blot

After dissection, the right lung lobes were flash frozen for protein analysis. The lungs were crushed and lysed in a radio immunoprecipitation (RIPA) buffer containing 1% protease inhibitors (Beyotime, Shanghai, China). Proteins in cells were prepared in the same buffer. The protein concentration was measured by using the bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China) according to the manufacturer's instructions. A 30-μg sample of protein was transferred to the membrane after 1–1.5 h of sodium salt dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was washed with Tris-buffered saline-Tween 20 (TBST) for 5 min and sealed with 5% skim milk powder at room temperature for 1.5 h. After that, the membrane was incubated with appropriately diluted MBD2, GAPDH (Proteintech, Wuhan, China), and MINK1 antibodies at 4°C overnight. The membrane was incubated with the secondary antibody at room temperature for 1 h. Images were obtained using a chemiluminescence gel imaging system, and the band intensities were measured using ImageJ software (National Institutes of Health). The relative protein expression level was calculated as the ratio of the gray value of target bands to that of GAPDH.

2.10 Quantitative real-time PCR

Total RNA from lung tissues and BECs was extracted using TRIzol regent (Invitrogen). The first-strand cDNA was synthesized with the PrimeScript RT Reverse transcriptase Reagent Kit (Takara, Japan). Quantitative real-time PCR

(qRT-PCR) was performed using the SYBR Premix Ex Taq kit (Takara, Japan) in the CFX96 Real-Time PCR Detection System (Bio-Rad). β-actin was used as the internal reference. According to the cycle threshold (Ct) value of the samples, the expression volume of related genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers for target genes were generated by Sangon Biotechnology (Shanghai, China). The primer sequences were: MBD2-forward, 5′-AGTGCTGGCAAGAGCGA-3′ and MBD2-reverse, 5′-GCCGGTCCTGAAGTCAAA-3′;

MINK1-forward, 5′-CCACCTACTATGGGGCCTTTA-3′ and MINK1-reverse, 5′-AGCACCGCAGAACTCCATC-3′; β-actin-forward, 5′-GTGCTATGTTGCTCTAGACTTCG-3′ and β-actin-reverse, 5′-ATGCCACAGGATTCCATACC-3′.

2.11 Enzyme-linked immunosorbent assay

The levels of IL-17 and IL-4 in mouse serum and cell supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using the Mouse IL-17 ELISA Kit (CSB-E04608m, Cusabio, China) and Mouse IL-4 ELISA Kit (CSB-E04634m, Cusabio, China). All experiments were performed according to the manufacturers' instructions.

2.12 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted by using the ChIP assay kit (Millipore, Cat. NO. 17–371) according to the manufacturer's instructions. First, 37% formaldehyde was added to the medium to fix the cross-linking of protein and DNA. The cells were then collected and washed three times in cold PBS, centrifuged and resuspended in SDS lysis buffer. The cell lysates were then sonicated to crosslink DNA fragments between 200 and 1000 base pairs in length. To obtain input, 10 μL of supernatant was prewashed with protein G agarose and centrifuged at 4000 × *g* for 1 min, and then the supernatant was stored at 4°C. Next, we added anti-MBD2 antibody and rabbit IgG to the remaining supernatant and incubated it overnight at 4°C with rotation to pull down the immunoprecipitation (IP) products. To collect antibody/antigen/DNA complexes, 60 μL protein G agarose was added to each IP and incubated for 1 h at 4°C by rotation. Then, 100 μL of elution buffer was added to each tube containing the antibody/agarose complex (including the input) for elution; this was incubated at room temperature for 15 min. Subsequently, crosslinking of the eluted protein/DNA complex was reversed by the addition of 5 M NaCl and incubated overnight at 65°C. Then 1 ml of binding reagent A was added, and the DNA flow-through was collected with a spin filter, separated, and purified. The isolated DNA was detected by ChIP-PCR using the following primers: F, 5′-ACG GCGGCAGCGGAGT-3′ and R, 5′-AGGTCGATGTCGTCC AGGCT-3′. The value of quantitative PCR was normalized with input DNA for comparison.

2.13 Statistics analysis

All experiments were performed at least 3 times, and all data were expressed as mean ± standard deviation (M ± SD). The differences among different groups were analyzed by one-way analysis of variance (ANOVA) or Kruskal–Wallis test followed by Dunn's multiple comparisons test. All statistical analyses and graph generation were performed using GraphPad Prism 8.0.1 software (GraphPad Software Inc.). A p-value < 0.05 indicated a statistically significant difference.

3 Results

3.1 A Th17-dominant asthma mouse model was established

To assess whether Th17-dominant asthma was established, airway resistance and BALF cells induced by Mch were measured on day 21. Compared to the normal control and T2 asthma mice, mice with Th17-dominant asthma had higher baseline RL. After Mch challenge, the RL of these mice increased significantly, especially in the mice with Th17-dominant asthma (Figure 1A). In BALF of the three groups, we found that the mice with Th17-dominant asthma had the highest total cell counts and neutrophil counts, while the mice with T2 asthma had the highest eosinophil counts (Figure 1B). Histological analysis of the lungs showed significantly increased peribronchial inflammatory cell infiltration in the mice with Th17-dominant asthma compared to the normal control and T2 asthmatic mice (Figure 1C). Immunohistochemistry of Gr-1 (neutrophil-specific antibody) confirmed that neutrophil infiltration in the lung was significantly increased in the Th17-dominant asthma group compared to the T2 asthma group. ECP (eosinophil-specific antibody) immunohistochemistry indicated that eosinophil infiltration was higher in both the Th17-dominant asthma and T2 asthma groups than that in the normal control group, but increased significantly in the T2 asthma group (Figure 1C).

3.2 Th17-dominant asthma mediated by Th17 cells

IL-4 and IL-17 are representative cytokines of Th2 and Th17 cells, respectively. To evaluate whether Th17-dominant asthma is mainly driven by Th17 cells, Th2 and Th17 cells in mouse spleen $CD4^+$ T cells were detected by flow cytometry. Compared to the normal and T2 asthma groups, both Th2 and

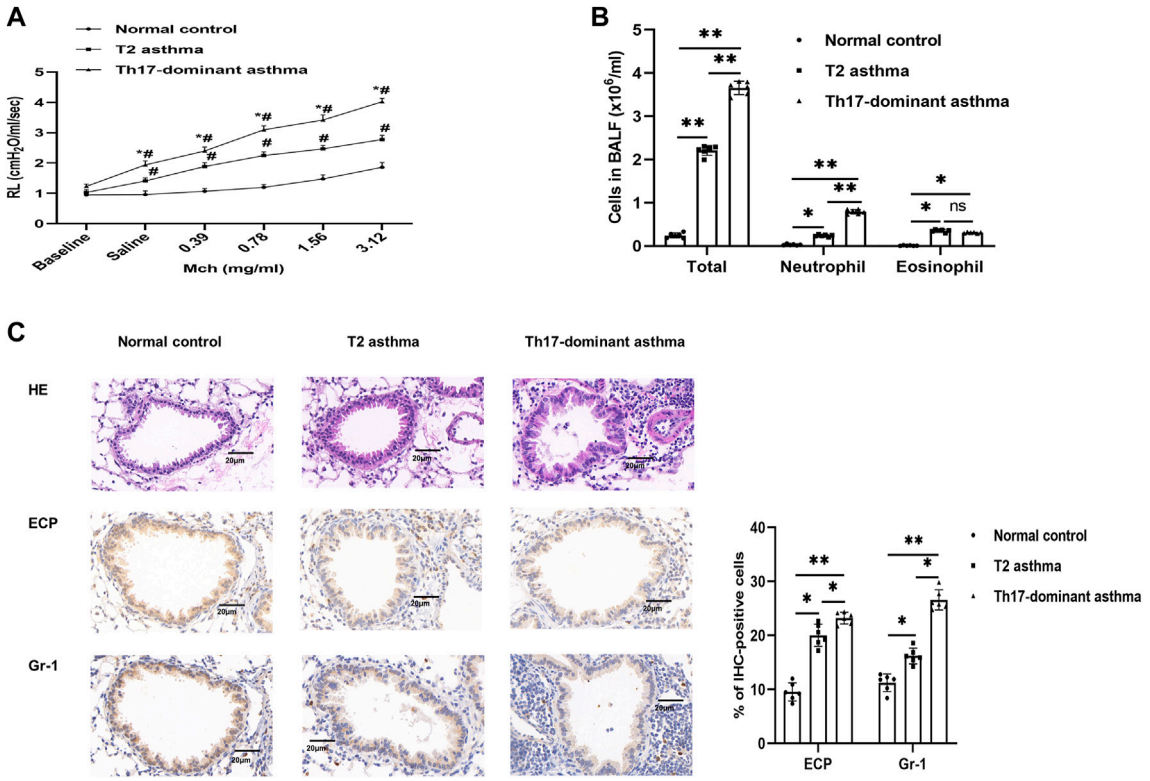

FIGURE 1
A Th17-dominant asthma mouse model was established **(A)** Lung resistance in the normal control, T2 asthma, and Th17-dominant asthma groups. #$p < 0.05$ as compared to the normal control group. *$p < 0.05$ as compared to the T2 asthma group. **(B)** Three groups of BALF cells: total, neutrophil, and eosinophil cells **(C)** Lung tissues of the three groups were stained with H&E and immunohistochemical staining performed with [neutrophil-specific antibody (anti-Gr-1) and eosinophil antibody (anti-ECP)]. Scale bar, 20 μm *$p < 0.05$. **$p < 0.01$. IHC, immunohistochemistry.

Th17 cells were increased in the Th17-dominant asthma group, especially Th17 cells, while Th2 cells were mainly increased in the T2 asthma group (Figure 2A). The blood serum IL-17 level in the Th17-dominant asthma group was significantly higher than that in the T2 asthma group, while the serum IL-4 level was significantly higher in the T2 asthma group (Figure 2B).

BECs could be used as antigen presenting cells to initiate an immune response. After isolation, immunofluorescence identification results showed that the rate of cytokeratin positive cell population of BECs in mice was more than 90% (Figure 2C). Then, BECs were treated with HDM + LPS, HDM or PBS for 24 h and cocultured with mouse spleen $CD4^+$ T cells. Flow cytometry was used to detect the differentiation of Th2 and Th17 cells to evaluate the establishment of cellular asthma model. The results showed that compared to the PBS and HDM groups, Th2 and particularly Th17 cells were increased in the HDM + LPS group, while Th2 cells were mainly increased in the HDM group (Figure 2D). The level of IL-17 in the cell supernatant was significantly higher in the Th17-dominant asthma group than in the T2 asthma group, while that of IL-4 was significantly higher in cell supernatant of the T2 asthma group (Figure 2E). These results suggested that Th17-dominant asthma was mainly mediated by Th17 cells, while Th2 cells were mainly mediated by T2 asthma. In addition, BECs could be used as APCs to initiate the immune response and mainly induced Th17-dominant asthma in the HDM + LPS-exposed group and T2 asthma in the HDM-exposed group.

3.3 Expression of MBD2 and MINK1 in Th17-dominant asthma

It has been reported that MBD2 is significantly increased in neutrophil-dominant asthma and positively regulates Th17 differentiation, while MINK1 negatively regulates Th17 cell differentiation, which has not been reported in Th17 dominant asthma (Fu et al., 2017; Sun et al., 2018). In the present study, the results showed that compared to the asthma group, the histological analyses of lungs in the Th17-dominant asthma group showed significantly increased

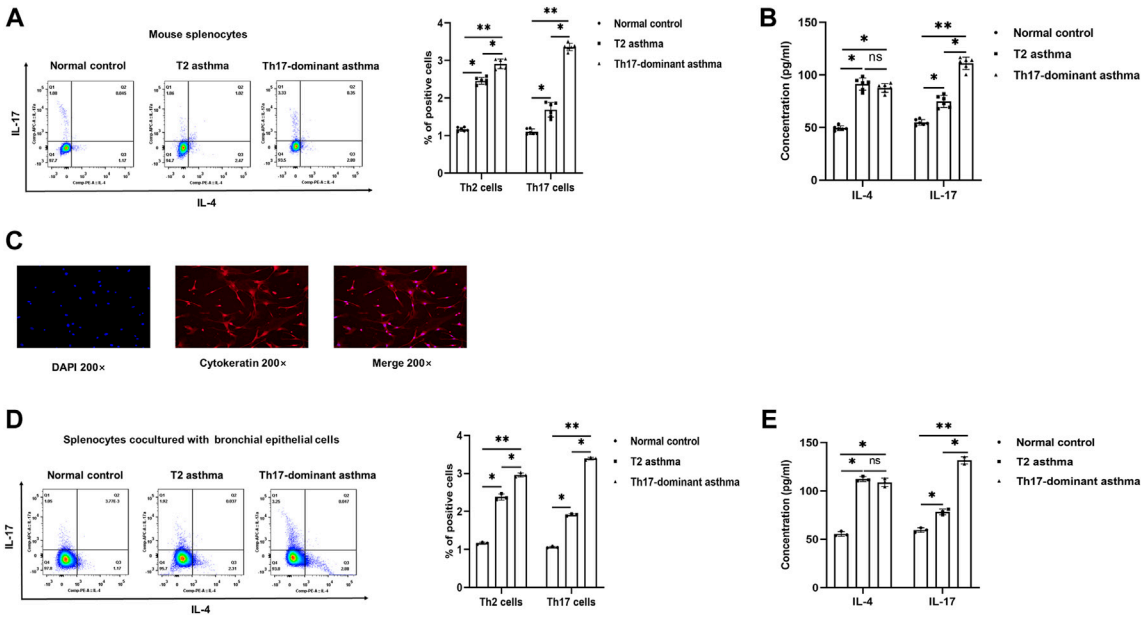

FIGURE 2
Th17-dominant asthma mediated by Th17 cells **(A)** Th17 and Th2 cells were detected by flow cytometry in splenocytes. **(B)** Serum levels of IL-17 and IL-4 were detected by ELISA **(C)** The positive rate of cytokeratin in BECs was determined by immunofluorescence. **(D and E)** After BECs were stimulated with 100 μg/ml HDM, 100 μg/ml HDM +100 ng/ml LPS or PBS for 24 h and cocultured with $CD4^+$ T cells for 24 h, the expression of Th2 and Th17 cells was detected by flow cytometry, and the levels of IL-17 and IL-4 in the cell supernatant were detected by ELISA. *$p < 0.05$. **$p < 0.01$.

MBD2 staining and significantly decreased MINK1 staining (Figure 3A). Compared to the T2 asthma group, the expression of MBD2 protein and mRNA in lung tissues and BECs from the Th17-dominant asthma group was significantly increased, while the corresponding expression of MINK1 mRNA and protein was significantly impaired (Figures 3B–D,F). These data suggested that MBD2 and MINK1 expression were associated with asthma phenotype and severity, and that both were involved in the pathogenesis of Th17-dominant asthma.

3.4 MBD2 is needed to maintain MINK1 silencing

Next, to further understand the molecular mechanism of MBD2 involvement in Th17-dominant asthma, we attempted to determine whether there was any relationship between MBD2 levels and MINK1 expression in BECs exposed or not exposed to HDM + LPS. First, we confirmed by using western blot and qRT-PCR analysis that MINK1 gene silencing or overexpression in BECs with or without HDM + LPS exposure was successful. Then, we detected the expression of MBD2 in each group. There was no significant difference in MBD2 mRNA and protein expression when the MINK1 gene was silenced or overexpressed (Figures 4A,B,E,F). We then used western blot and qRT-PCR analysis to confirm the successful silencing or overexpression of the MBD2 gene in BECs with or without HDM + LPS exposure. Then, we detected the expression of MINK1 in each group. The results showed that the expression of MINK1 mRNA and protein was increased after MBD2 gene silencing compared to the control group. As expected, when the MBD2 gene was overexpressed, MINK1 mRNA and protein expression were significantly reduced compared to the blank and control groups (Figures 4C–F). In addition, we performed a search using the online software MethPrimer (http://www.urogene. org/methp Rimer 2/) to analyze the promoter region of MINK1. The prediction of CpG islands is shown in Figure 4G. ChIP assay showed that HDM + LPS exposure significantly increased MBD2 binding to the MINK1 gene promoter region (Figure 4H). These results suggest that MBD2 is involved in maintaining MINK1 silencing.

3.5 Th17 cell differentiation under MBD2 and MINK1 gene silencing or overexpression

Th17-dominant asthma is mainly mediated by Th17 cells, and both MBD2 and MINK1 genes can affect the differentiation of Th17 cells. In order to further understand the regulatory

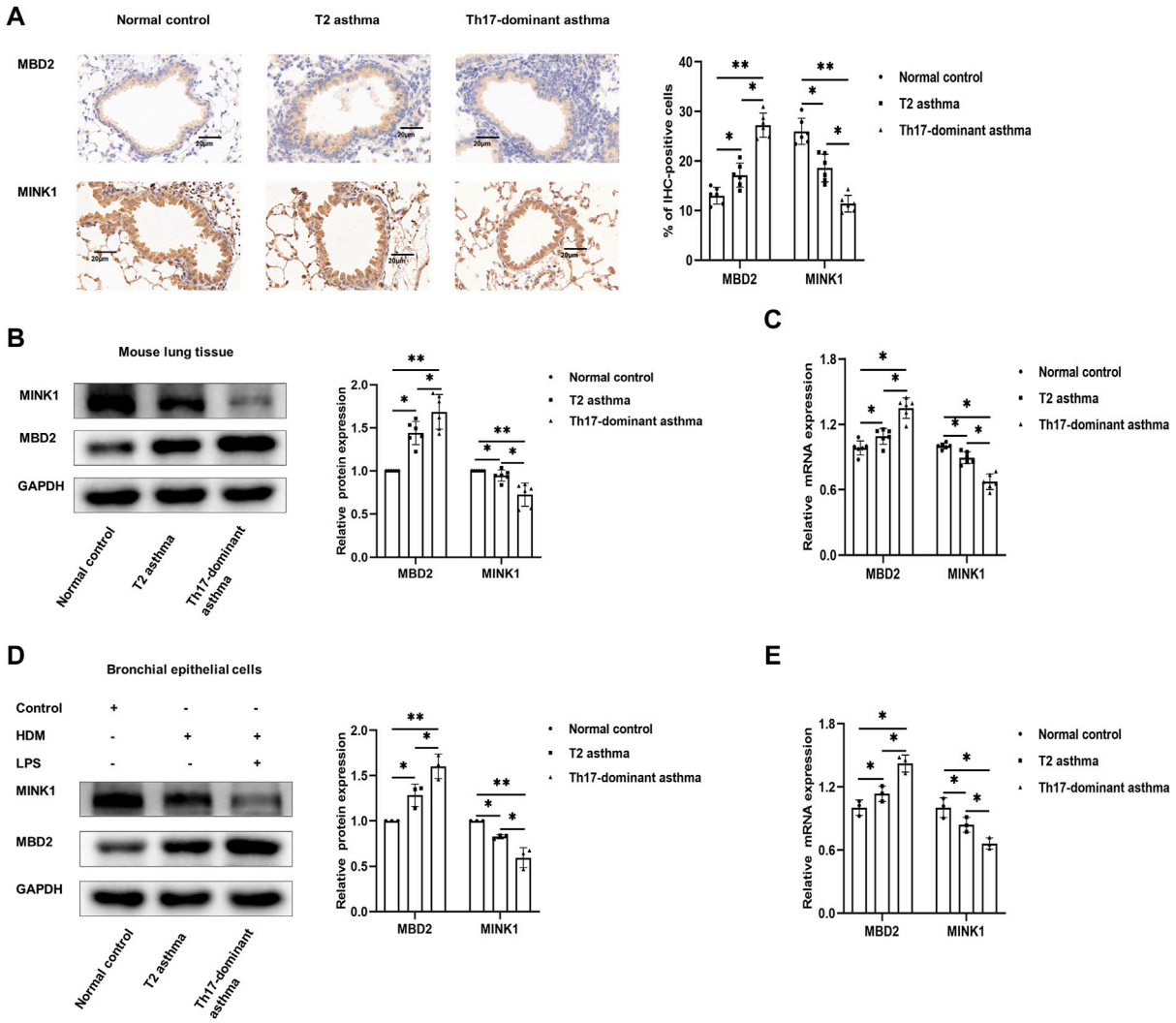

FIGURE 3
Expression of MBD2 and MINK1 in Th17-dominant asthma **(A)** Lung tissues of each group were stained for anti-MINK1 and anti-MBD2. Scale bar, 20 μm. **(B and C)** The expression of MINK1 and MBD2 protein and mRNA in lung tissues of each group was detected by western blot and qRT-PCR. **(D and E)** The expression of MINK1 and MBD2 protein and mRNA in BECs of each group was detected by western blot and qRT-PCR. *$p < 0.05$. **$p < 0.01$. IHC, immunohistochemistry.

effects of MBD2 and MINK1 on Th17 cells, after silencing or overexpression of MINK1 and MBD2 genes in BECs with or without HDM + LPS exposure, the cells were cocultured with spleen $CD4^+$ T cells. Then the differentiation of Th17 cells was analyzed by flow cytometry, and the level of IL-17 was detected by ELISA. The results showed that Th17 cells and the cytokine IL-17 expressed the same trend when MBD2 was silenced or overexpressed. However, when MINK1 was silenced or overexpressed, Th17 cell differentiation and IL-17 showed the opposite trend, increasing or decreasing, respectively. Together, these data indicated that MBD2 induced Th17 cell differentiation by maintaining MINK1 silencing, thereby contributing to the development of Th17-dominant asthma (Figures 5A–D).

4 Discussion

Asthma is a highly heterogeneous airway inflammatory disease. Some patients with asthma have a poor response to glucocorticoid therapy and are prone to developing severe asthma. This type of glucocorticoid-resistant severe asthma has been reported to be associated with Th17 cell-mediated neutrophil inflammation (Manni et al., 2016). In order to better study the pathogenesis of severe asthma, it is necessary to establish a Th17-dominant asthma model. OVA is the classic allergen to induce T2 asthma, and HDM can also induce allergic asthma mainly with Th2 cells (Yasuda et al., 2020). LPS is used as an auxiliary agent in the induction of an asthma model, which is

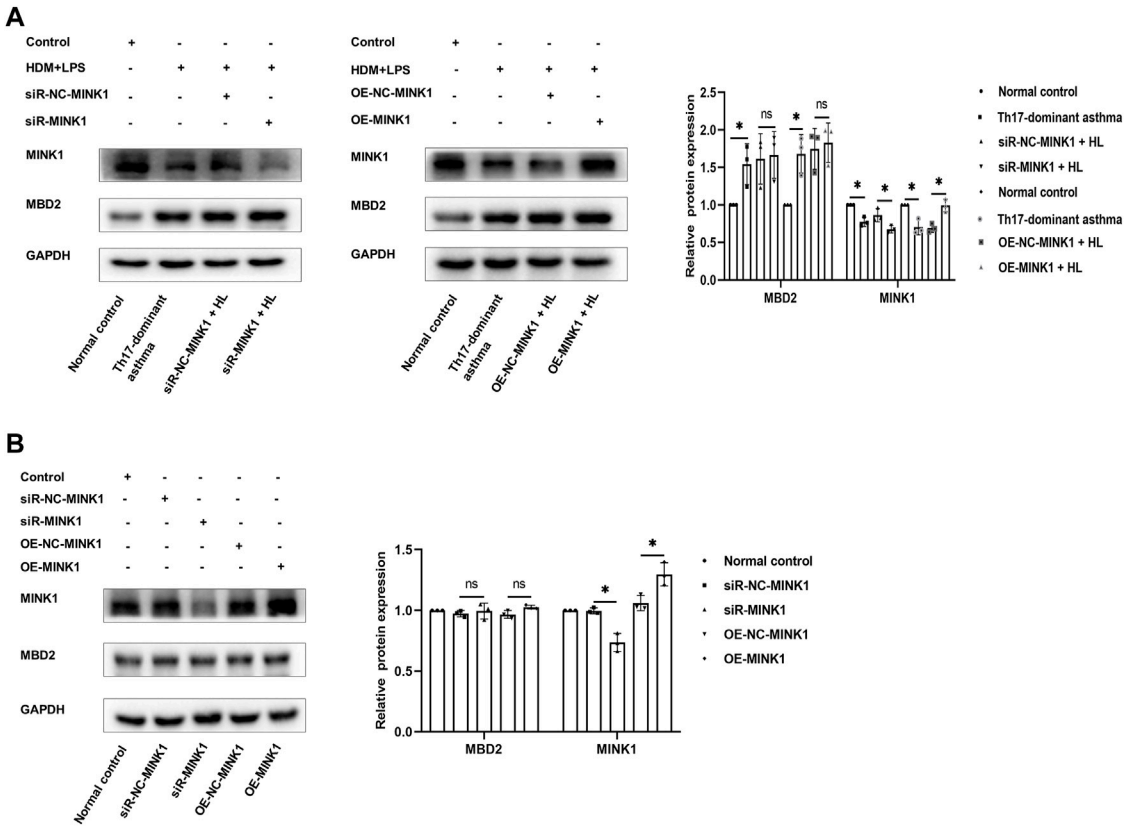

FIGURE 4
MBD2 is needed to maintain MINK1 silencing **(A, B, E, and F)** Western blot and qRT-PCR were used to verify the transfection of the MINK1 gene with or without HDM + LPS exposure. (A, B, E, and **(F)** Western blot and qRT-PCR were used to detect the expression of MBD2 protein and mRNA in MINK1 gene silencing or overexpression with or without HDM + LPS exposure (C, D, E, and **(F)** Western blot and qRT-PCR were used to verify the transfection of the MBD2 gene with or without HDM + LPS exposure. **(C,D,E and F)** Western blot and qRT-PCR were used to detect the expression of MINK1 protein and mRNA in MBD2 gene silencing or overexpression with or without HDM + LPS exposure **(G)** The patterns of CpG islands of the MINK1 promoter and one pair of the primers were predicted by MethPrimer Promoter 2.0 software. **(H)** Quantitative PCR results of MBD2-bound MINK1 promoter region. *$p < 0.05$. **$p < 0.01$. HL, HDM + LPS.

associated with increased severity of asthma. In addition to inhibiting Th2 cell differentiation and eosinophil inflammation, the addition of LPS during HDM induction also transformed to neutrophil inflammation (Daan de Boer et al., 2013; Jia et al., 2017). According to previous reports, in this study, 100 μg OVA +100 μg HDM +15 μg LPS was used to successfully establish a Th17-dominant asthma mouse model. HDM + LPS + OVA-exposed mice with Th17-dominant asthma had greater AHR, higher total BALF cell and neutrophil counts, more lung inflammatory cells, higher IL-17 levels and Th17 cells in splenocytes compared to OVA-exposed asthma mice. At the same time, higher differentiation of Th17 cells and IL-17 levels were found in the Th17-dominant asthma induced by HDM + LPS exposure in BECs compared to HDM-induced T2 asthma. These results suggest that the dominant differentiation of Th17 cells and recruitment of neutrophils are the main manifestations of Th17-dominant asthma, thus promoting the development of asthma. In addition, BECs could be used as APCs to initiate an immune response. Liu et al. found that bombesin receptor subtype-3 (BRS-3) has a protective effect on BECs with oxidative damage, and the activation of BRS-3 could increase the uptake of antigen by BECs and T cell proliferation (Liu et al., 2018).

As a reader of DNA methylation, MBD2 plays a role in regulating Th17 cell differentiation through the T-bet/Hlx axis (Zhong et al., 2014). Considering the role of Th17 cells in glucocorticoid-insensitive asthma, MBD2 may be involved in the pathogenesis of Th17-dominant asthma. In this study, MBD2 was significantly increased in lung tissues and BECs in the Th17-dominant asthma groups compared to that in T2 asthma groups. To verify that MBD2 is involved in Th17-dominant asthma, we performed an *in vitro* study on BECs. The MBD2 gene was silenced or overexpressed in BECs with or without HDM + LPS-exposed, and Th17 cells and the

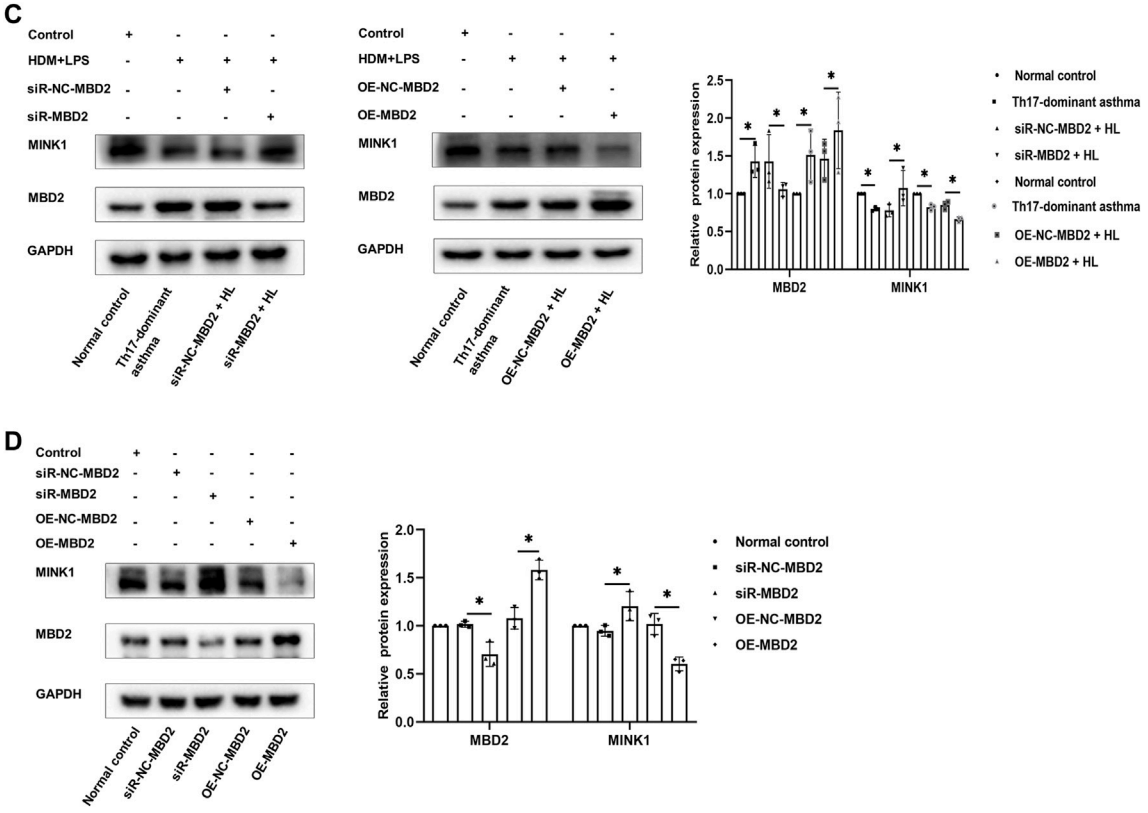

FIGURE 4
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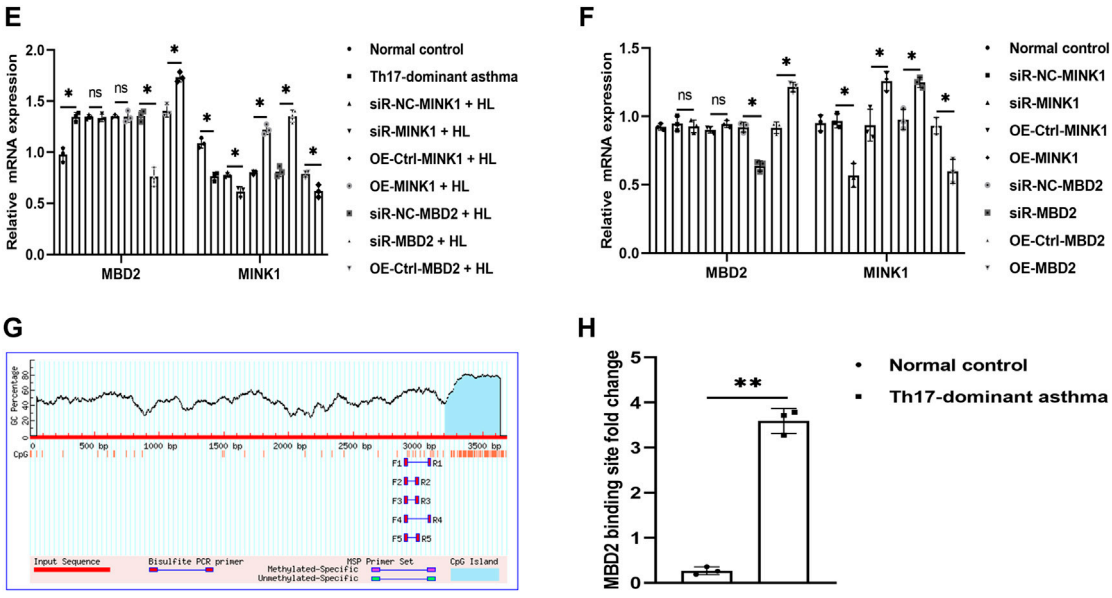

FIGURE 4
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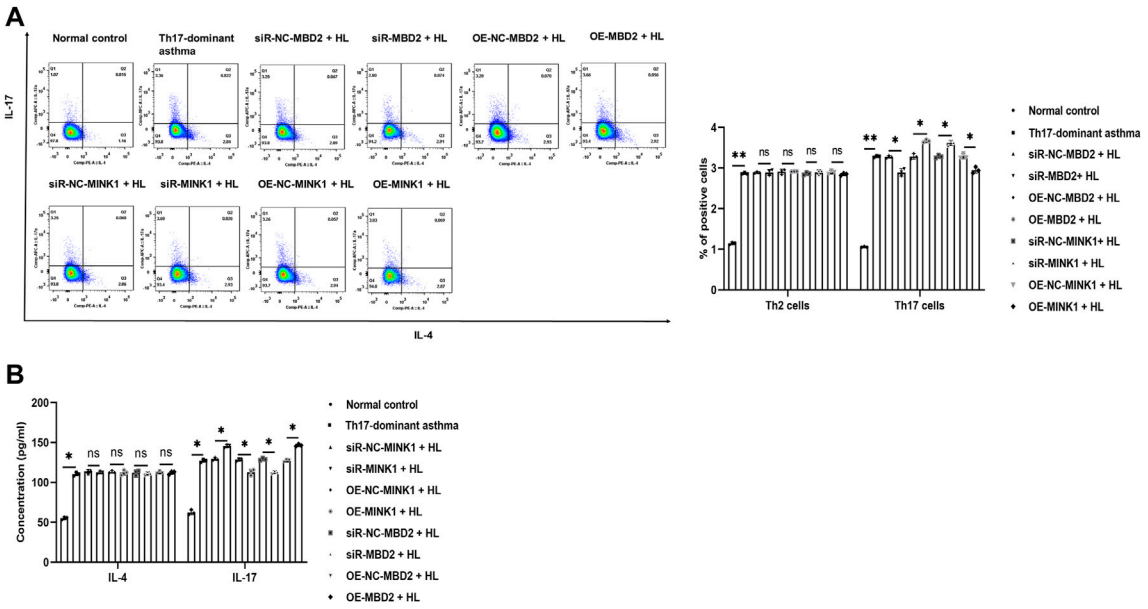

FIGURE 5
Th17 cell differentiation under MBD2 and MINK1 gene silencing or overexpression **(A and B)** After the MBD2 or MINK1 gene was transfected in BECs with HDM + LPS exposure and cocultured with $CD4^+$ T cells for 24 h, the expression of Th2 and Th17 cells was detected by flow cytometry, and the levels of IL-17 and IL-4 in the cell supernatant were detected by ELISA. **(C and D)** After the transfection of the MBD2 or MINK1 gene in BECs without HDM + LPS exposure and cocultured with $CD4^+$ T cells for 24 h, the expression of Th2 and Th17 cells was detected by flow cytometry, and the levels of IL-17 and IL-4 in the cell supernatant were detected by ELISA. *$p < 0.05$. **$p < 0.01$. HL, HDM + LPS.

cytokine IL-17 showed the same changes with MBD2 gene silencing or overexpression. These results suggested that MBD2 is involved in the development of severe asthma by influencing the differentiation of Th17 cells. Xu et al. found that MBD2 could affect the differentiation of Th17 cells by regulating the key transcription factor retinoid-related orphan nuclear receptor γt of Th17 cells (Xu et al., 2018). MBD2 is widely expressed and plays a role in inflammatory pathogenesis, resulting in transcriptional silencing by interacting with nucleosome remodeling and histone deacetylase complexes as inhibitors (Wood et al., 2016). It was found that elevated MBD2 mRNA levels in $CD4^+$ T cells of systemic lupus erythematosus (SLE) patients were positively correlated with the SLE disease activity index (Qin et al., 2013).

MINK1, a serine-threonine kinase, is involved in gene transcription, the inflammatory response, and T cell differentiation. The nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain-containing 3 (NLRP3) inflammasome plays a pathogenic role in inflammatory diseases, and MINK1 positively regulates NLRP3 inflammasome. In the mouse model of acute sepsis, MINK1 deficiency reduces the NLRP3 activation and inhibits the inflammatory response (Zhu et al., 2021). MINK1 can also affect tumor immunity. Studies have found that the Hippo pathway kinase LATS1/2 in tumor cells can affect tumor growth, and MINK1 is involved in the expression of Hippo pathway kinase. Combined deletion of MINK1 and MST1/2 could inhibit the activation of LATS1/2, improve tumor immunogenicity, and inhibit tumor growth (Meng et al., 2015; Moroishi et al., 2016). It has been reported that MINK1 negatively regulates Th17 cell differentiation and has a protective effect on EAE (Fu et al., 2017). In this study, we found that MINK1 was significantly reduced in lung tissues and BECs in the Th17-dominant asthma groups compared to that in T2 asthma groups. We also carried out *in vitro* experiments on the MINK1 gene and found that Th17 cell differentiation and the IL-17 level increased when the MINK1 gene was silenced but decreased when the MINK1 gene was overexpressed, demonstrating the inverse expression trend. At the same time, there was no significant difference in MBD2 expression when the MINK1 gene was silenced or overexpressed. *In vitro* experiments, we further found that the expression of MINK1 was increased when the MBD2 gene was silenced and decreased when the MBD2 gene was overexpressed, confirming that MBD2 could negatively regulate the expression of MINK1. In addition, ChIP assay revealed that MBD2 could directly bind to CpG islands in the MINK1 promoter region. These results suggested that MINK1 plays a protective role in asthma by negatively regulating Th17 cell differentiation and is negatively regulated by MBD2. In addition, these results indicated that Th17 cells are not directly downstream of MBD2, and MBD2 regulates Th17 cell differentiation through negative regulation of MINK1 expression, thus mediating the onset of Th17-dominant asthma.

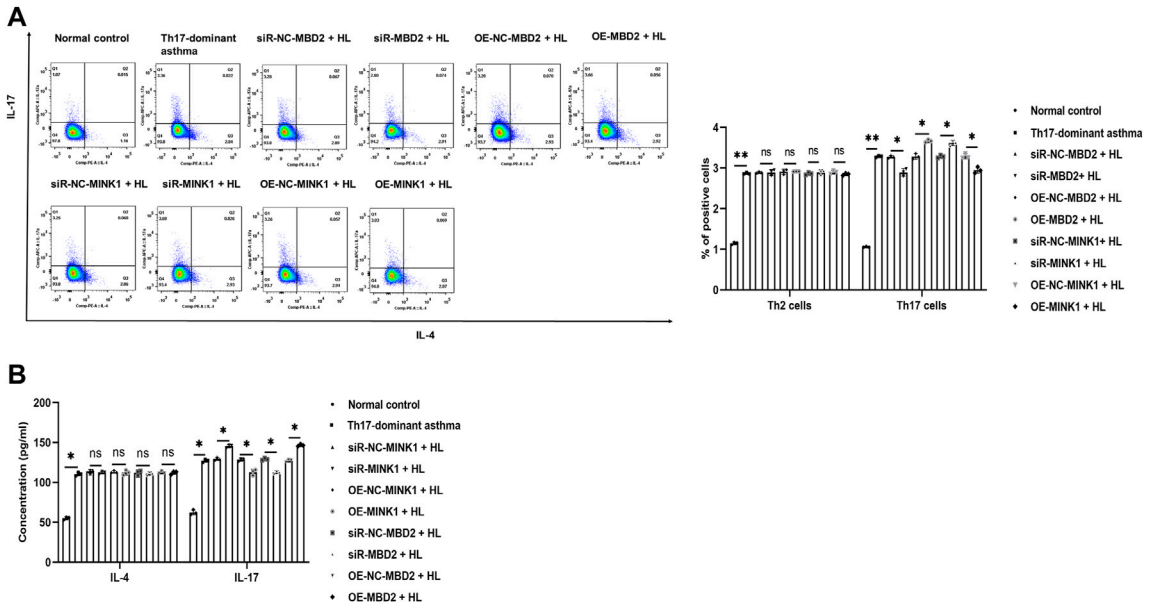

FIGURE 5
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5 Conclusion

In conclusion, the data from this study demonstrated that MBD2-mediated Th17 cell differentiation is associated with reduced MINK1 expression in Th17-dominant asthma. Our findings have revealed new roles for MBD2 and MINK1 and provide new insights into epigenetic regulation of Th17-dominant asthma, which is dominated by neutrophils and Th17 cells. This study could lead to new therapeutic targets for patients with severe asthma.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization and design: ZC, LM, SL, and XX; Methodology: ZC, YS, BX, JL, BC, and XJ; Data management: ZC, RO, YY, YH, WD, and XZ; Statistical analysis and interpretation: ZC, BW, JJ, QZ, DZ, and YL; All authors contributed to drafting the original manuscript of important intellectual content and final approval of the manuscript.

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Conflict of interest

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

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Alveolar macrophages and airway hyperresponsiveness associated with respiratory syncytial virus infection

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Respiratory syncytial virus (RSV) is a ubiquitous pathogen of viral bronchiolitis and pneumonia in children younger than 2 years of age, which is closely associated with recurrent wheezing and airway hyperresponsiveness (AHR). Alveolar macrophages (AMs) located on the surface of the alveoli cavity are the important innate immune barrier in the respiratory tract. AMs are recognized as recruited airspace macrophages (RecAMs) and resident airspace macrophages (RAMs) based on their origins and roaming traits. AMs are polarized in the case of RSV infection, forming two macrophage phenotypes termed as M1-like and M2-like macrophages. Both M1 macrophages and M2 macrophages are involved in the modulation of inflammatory responses, among which M1 macrophages are capable of pro-inflammatory responses and M2 macrophages are capable of anti-proinflammatory responses and repair damaged tissues in the acute and convalescent phases of RSV infection. Polarized AMs affect disease progression through the alteration of immune cell surface phenotypes as well as participate in the regulation of T lymphocyte differentiation and the type of inflammatory response, which are closely associated with long-term AHR. In recent years, some progress have been made in the regulatory mechanism of AM polarization caused by RSV infection, which participates in acute respiratory inflammatory response and mediating AHR in infants. Here we summarized the role of RSV-infection-mediated AM polarization associated with AHR in infants.

KEYWORDS

respiratory syncytial virus, alveolar macrophages, polarization, immune regulation, airway hyperresponsiveness

Introduction

Respiratory syncytial virus (RSV) is the dominant cause of lower respiratory tract infection in children younger than 2 years of age worldwide. It is estimated that 4 million children are admitted to hospitals for RSV infection and 200,000 of the hospitalized children die each year (1, 2). Due to the immature composition and functions of their immune cells and molecules, infants infected with RSV often progress to lower respiratory tract inflammation, and some of them can develop a chronic lung disease (3, 4). When re-infected or exposed to allergens, this infection in infants can manifest as recurrent wheezing. The pandemic of the coronavirus disease 2019 (COVID-19) has changed the epidemic pattern of RSV; it is estimated that the recurrence of RSV will be more intense in the future and may become a major economic burden to society (3, 4).

Alveolar macrophages (AMs) are the important part of the respiratory tract's innate immune barriers and play a key role in engulfing pathogens and antigen presentation (5, 6), and together with epithelial cells, contribute to setting the threshold and the quality of the innate immune response in the acute and convalescent phases of RSV infection. It has been reported that AM polarization is driven by RSV in a variety of microenvironments to exert multiple biological effects (7). Polarized AMs participate in local inflammatory responses and in mediating intercellular communication to stimulate naive lymphocyte differentiation (8, 9), thus regulating the intensity of the inflammatory response, which is associated with immunosensitization and the pathology of airway hyperresponsiveness (AHR) in the late life of infants infected with RSV (10–12). Therefore, immunomodulatory therapy targeting AMs may be one of the approaches to further explore effective treatment strategies. In this paper, we summarize the potential association between AM polarization and AHR after RSV infection in infants.

RSV infection and host response

RSV is a single-stranded negative-sense RNA virus belonging to the Pneumovirus genus of the Paramyxoviridae family (13). Its genome can encode 11 proteins that play roles in mediating viral replication, packaging, and assisting the virus to escape immune surveillance. Glycoprotein binds to glycosaminoglycans on the cell surface, interfering in immune cell recruitment and various cytokine production. Fusion protein mediates the fusion between the virus and the cell membranes of the host to form syncytia. Non-structural protein 1 and 2 inhibit interferon (IFN) production and its signaling conduction (14). Phosphoprotein inhibits exogenous apoptotic signals and contributes to persistent RSV infection in macrophage-like cells (15). By disrupting the host gene transcription and interfering with the synthesis of mitochondrial proteins, matrix protein weakens the body's immune recognition of RSV (16).

In host cells, RSV activates pathogen-associated molecular patterns (PAMPs), which promotes the maturation of antigen-presenting cells (APCs) to express pattern recognition receptors, toll-like receptors (TLRs), and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) (17–19). RSV can also invade lung macrophages directly, is recognized by mitochondrial antiviral signaling protein (MAVS)-coupled RLR (12), and can activate nuclear transcription to regulate innate immune responses (Figure 1). The expression of pro-inflammatory mediators and the recruitment of inflammatory cells to the infected or injured tissue and their migration across the endothelium are crucial events in early immune extravasation defense against RSV infection (20).

AM-mediated lung pathological lesions are usually not invaded by RSV directly, but mainly immune-mediated inflammatory responses (21). The acute infection phase is dominated by airway inflammation such as bronchiolitis, and the convalescent phase is characterized by airway hypersensitivity. Both of them belong to airway hyperresponsiveness. A variety of molecules are involved in the acute phase across epithelial cells (ECs), including interleukin (IL)-6, tumor necrosis factor-α (TNF-α), granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor (GM-CSF), chemokines (CXCL8, CXCL10, and CCL5), antibacterial factors including nitric oxide (NO), β-defensins, lysozyme, and lactoferrin (10, 17), which might cause tracheal smooth muscle spasm, hyperemia, edema, inflammatory cell aggregation, secretion, and cell shedding to block the airway (22–25). Reinfection or inhalation of allergens during the convalescent period can both trigger the overexpression of CD8 T and Th2-like cytokines involved in triggering wheezing.

Classification and characteristics of AMs

Lung macrophages are usually divided into two subpopulations depending on their distinct locations: AMs located on the surface of the alveoli cavity and interstitial macrophages (IMs) located in the interstitial pulmonary stromata (26, 27). In inflammatory states, AMs are recognized as the resident airspace macrophages (RAMs) and the recruited airspace macrophages (RecAMs), depending on their origins and wandering characteristics (Figure 2) (28, 29).

RAMs are steady-state "AMs" that derive mainly from embryonic yolk sacs and fetal liver cells (30), which reside on the surface of the alveoli cavity for a long time. RAMs are not evenly distributed in each alveolus, and notably only 30–40% of alveoli contain RAMs. Most of the RAMs crawl in and between alveoli through the pores of Kohn to monitor the

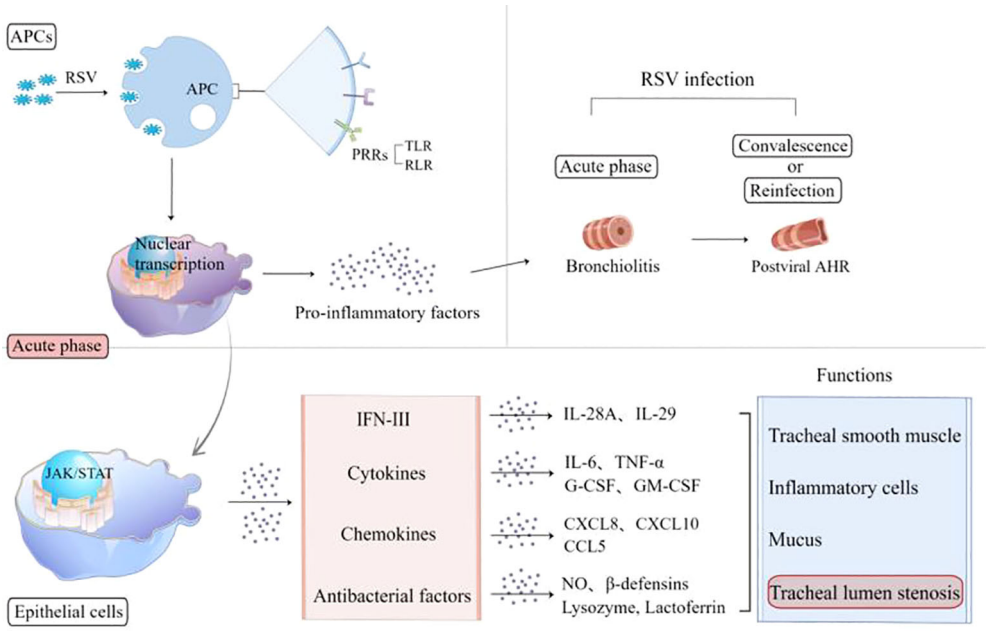

FIGURE 1
RSV activates APCs to express PRRs, including TLR and RLR, and release pro-inflammatory factors. In the acute phase, RSV-infected epithelia express interferons, cytokines, chemokines, and antimicrobial factors involved in the airway inflammation reaction. The pathological lesions caused by these inflammatory mediators experience two stages: bronchiolitis and post-viral AHR. APCs, antigen-presenting cells; RSV, respiratory syncytial virus; PRRs, pattern recognition receptors; TLR, Toll-like receptor; RLR, retinoic acid-inducible gene 1 (RIG-I)-like receptor; AHR, airway hyperresponsiveness; IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor-α; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; NO, nitric oxide.

microenvironment, while the remaining 10% of RAMs are entirely sessile (5). In the physiological environment, there is contact inhibition between RAMs, which contributes to preventing RAMs from accumulating in the alveoli. This distribution characteristics are regulated in part by IL-34 and macrophage-colony stimulating factor (M-CSF) in the alveoli (31). Through the regulation of GM-CSF and the mechanistic target of rapamycin complex 1, RAMs, as long-lived cells, can proliferate *in situ* to replenish themselves without the need for mononuclear macrophages from circulating blood as supplement or replacement, with an annual renewal rate of about 40% (32). GM-CSF have been confirmed to upregulate the expression of anti-apoptotic genes in RAMs, which is necessary to promote maturation and prolong their lifespan (5, 33).

RAMs, being capable of engulfing foreign particles and endogenous proteins (including surfactants and cell debris) to initiate an immune response, play a key role in regulating the innate immunity of the respiratory system and preventing infection from inhaled pathogens. Moreover, together with alveoli ECs, RAMs can also contribute to maintaining lung tissue homeostasis and the intensity of the inflammatory response (34). The distributions of RAMs in the steady-state microenvironment are in the dynamic equilibrium of "self-sufficiency". During endotoxin-induced acute inflammation or exposure to a large number of pathogens, RAMs are the first sentinel of the respiratory tree and constitute the dominant immune cell in the steady state to metabolize pro-inflammatory effectors, including the recruitment of platelets, neutrophils, and other inflammatory cells, which contribute to co-participating in and regulating the onset and development of the disease (35).

RecAMs belong to the subpopulation of IMs that travel towards the site of inflammation in the alveolar cavity in pathological conditions. IMs originate in bone marrow monocytes, circulating through the bloodstream into the interstitial tissues of the lungs and being in transitional states. IMs can patrol in the interstitium of different alveoli, where they identify different inflammatory or necrotic and exfoliated cells and exert a phagocytic effect, which, in turn, release IL-10 to maintain microenvironment homeostasis (9, 36). In the acute phase of infection, IMs will be chemotactic to the alveoli cavity and recruited to become RecAMs (37). In addition, RNA gene sequencing showed that the immunoprogramming of RecAMs was dynamic (32, 35) and could develop into the same phenotype and provide the same functionality as RAMs during the peak inflammatory periods (38, 39), including the production of pro-inflammatory cytokines and elimination of pathogens. RecAMs release anti-inflammatory factors to repair pathologically damaged tissues when the inflammation is

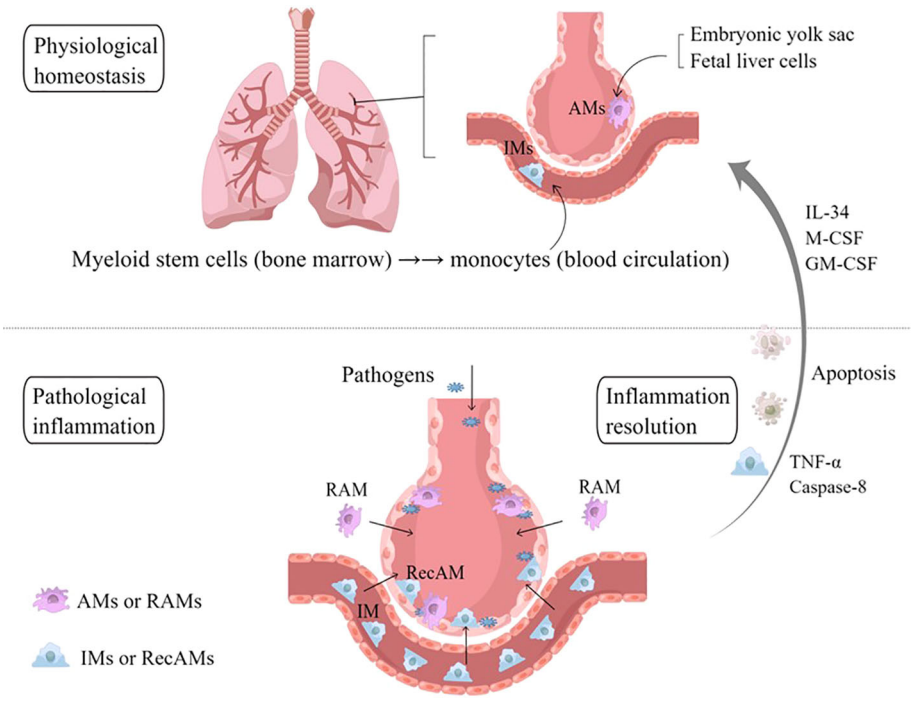

FIGURE 2
Sources and classification of AMs. In physiological homeostasis, AMs are equivalent to RAMs, which originate from embryonic yolk sacs and fetal liver cells. In the event of a large number of microbial invasion or inhalation of allergens, IMs are recruited into the alveoli, known as RecAMs. After the inflammation subsides, RecAMs coming from IMs will undergo programmed apoptosis, while RAMs maintain their original distribution characteristics under the action of IL-34, M-CSF, and GM-CSF. AMs, alveolar macrophages; IMs, interstitial macrophages; RAMs, resident airspace macrophages; RecAMs, recruited airspace macrophages; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; TNF-α, tumor necrosis factor-α.

subsiding. RecAMs program apoptosis after the inflammation is gone, whereas RAMs will continue to survive and sustainably replenish themselves. This causes the amount of AMs to form an emergency dynamic cycle between the homeostasis phase and the inflammatory phase (32).

Inflammation-activated AM polarization

Both RAMs and RecAMs can be activated to divide into M1 and M2 phenotypes according to the microenvironment changes (5). Conventional studies label nitric oxide synthase (NOS) and arginase (Arg) to determine the activation states of M1 and M2, respectively. However, recent studies have shown that both NOS and Arg can be co-expressed within the same cell (32), and AM polarization is not a distinct "dichotomy" but is multidimensional, dynamic, and complex (40). Moreover, the classic "M1 and M2" classification remains representative. M1-like macrophages exacerbate the airway inflammatory response that may be associated with long-term airway sensitization (41). In contrast, M2-like macrophages are capable of anti-inflammatory responses and repairing damaged tissues to maintain immunity balance (5). Once the microenvironment of the alveolars changes, the phenotypes and the functions of M1 and M2 could be reversed.

Based on single-cell RNA sequencing, AMs can be identified as five clusters with unique transcriptome characteristics and presumed functions at three different stages (32): physiological homeostasis, acute inflammatory phase, and convalescent phase. The transcripts of clusters 1 and 2 are mainly upregulated in RAMs, while clusters 3, 4, and 5 are predominantly characteristics of RecAMs. Clusters 1 and 2 are dominated by M2 gene expression profiles, while clusters 3 and 4 transcriptomes are dominated by M1 gene expression profiles. RecAM-labeled cells at peak inflammation are dominated by M1 gene expression, while RAM-tagged cells are predominantly expressing the M2 gene at the homeostasis and inflammation phases. The expression of both M1 and M2 genes in cluster 5 is relatively low. RAMs are dominated by M2-like functions in the steady-state phase and convalescent phase, while RecAMs are mainly characterized by M1-like function in the inflammatory phase only (Figure 3).

RSV infection and AM polarization

The mechanism by which RSV triggers AM polarization is through promoting a regulatory immune mediator response in three pathways (Figure 4): cytokines, intercellular communication signaling (including epithelia–macrophages as well as macrophages–lymphocytes), and RSV invades AMs and directly triggers AM polarization.

Cytokines

It is well known that IFN-γ is the classic pathway to cause macrophage polarization. RSV infection might stimulate the secretion of IFN-γ from CD8 T cells and NK cells in lung tissues (42–45), which, in turn, regulates inflammatory responses and promotes immunopathology by initiating AM polarization (46). AM polarization activated by IFN-γ is age-related, with significant differences among adults and infants. There is a high level of expression of sialic acid-binding immunoglobulin agglutinin (Siglec-1) ligand CD43 on the membranes of CD4 T cells in adults through antagonizing signals from monocytes and inhibiting the release of IFN-γ by CD4 T cells, thus preventing AMs from polarizing into M1 phenotype. In contrast, due to the lower CD43 expression on CD4 T cell membranes in infants, the IFN-γ secreted by monocyte-mediated CD4 T cells is not affected by Siglec-1 signaling in RSV infection (47). Although infants lack specific memory T cells and their IFN-γ expression is delayed, the role of IFN-γ on AMs gradually dominates as the RSV infection progresses, with the increased CD43 expression being age-related. Therefore, IFN-γ has significant gradual age differences in M1-like polarization effects (11, 48, 49), which is one of the main reasons why the inflammatory response and pathological damage by RSV are different from those of adults (12). GM-CSF also promotes AM polarization in RSV infection, but it plays a secondary role (50).

RSV can also induce the production of pro-inflammatory factors that mediate the expression of macrophage migration inhibitor factor (MIF) through reactive oxygen species, 5-lipoxygenase, cyclooxygenase, and PI3K signaling channels, driving AM polarization to produce TNF-α, monocyte chemoattractant protein-1, and IL-10 (51).

Intercellular communication

RSV-infected airway ECs might activate AM polarization through intercellular communication such as the Notch–Jagged pathway (24, 52–54). Notch is a ligand–receptor interaction that

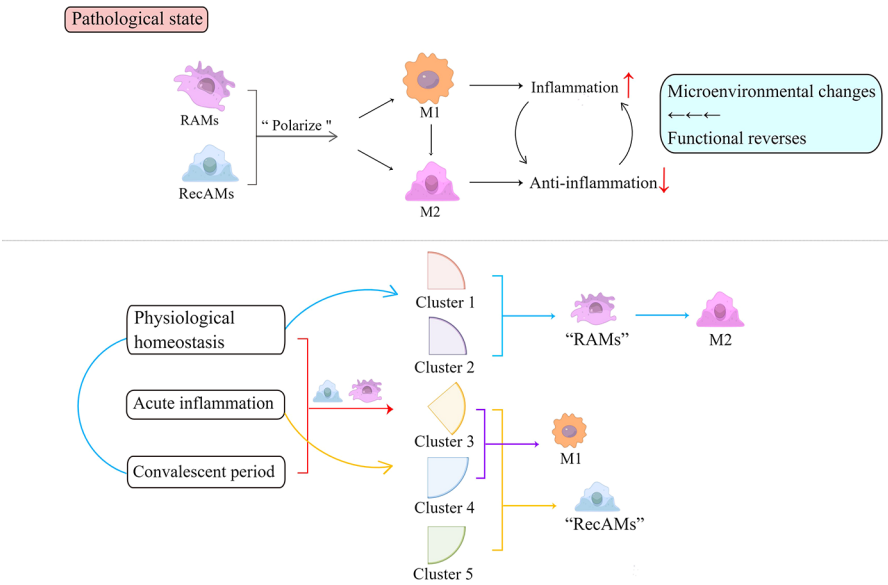

FIGURE 3
Inflammation activates the polarization of AMs. Depending on functions, polarized AMs are roughly divided into pro-inflammatory M1 and anti-inflammatory M2. Based on their single-cell RNA sequencing analysis, AMs can be identified as five clusters at three time points throughout the inflammatory phase, indicated by the red square bracket and arrow. Clusters 1 and 2 contain cells with RAM markers that are present during both homeostasis and inflammation and are dominated by M2-like functions in the homeostasis and convalescent phase, marked by blue parentheses and arrows. Clusters 3, 4, and 5 exist only during inflammation and are predominantly characteristic of RecAMs (herein noted by yellow arrows and square brackets). Among them, clusters 3 and 4 are dominated by M1 gene expression (herein annotated by the purple square bracket and arrow). Cluster 5 has a relatively low expression of both M1 and M2 genes. Each cluster has corresponding cellular characteristics that reflect the cell-derived sources and exhibits different functions. RAMs, resident airspace macrophages; RecAMs, recruited airspace macrophages; M, macrophage.

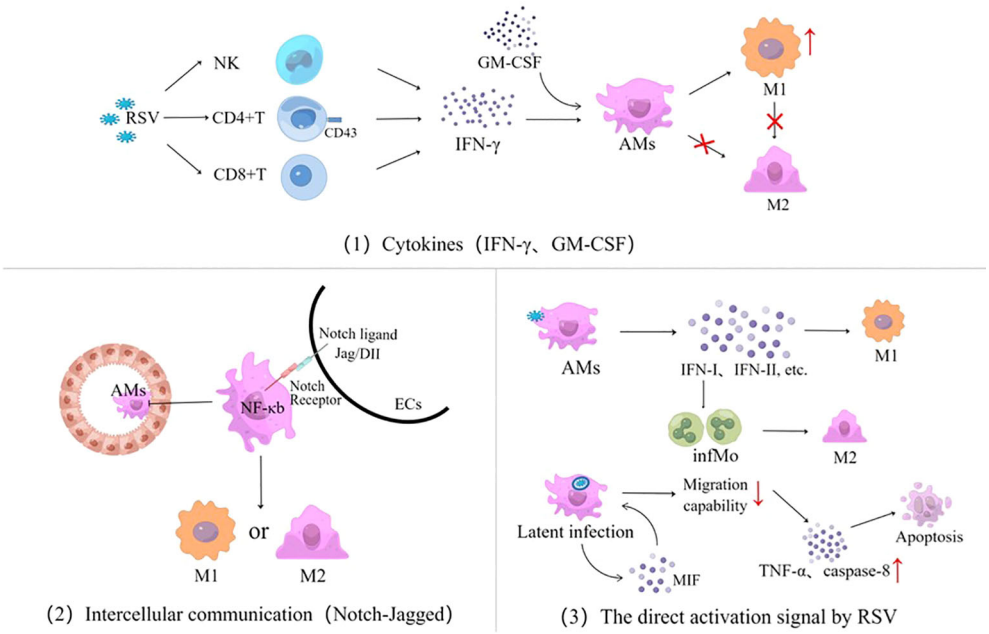

FIGURE 4
Three signaling pathways for AM polarization activated by RSV infection, cytokines represented by IFN and GM-CSF, intercellular communication using the Notch-Jagged pathway as an example, and the direct activation signal by RSV. RSV, respiratory syncytial virus; NK, natural killer cells; IFN, interferon; GM-CSF, granulocyte–macrophage colony-stimulating factor; AMs, alveolar macrophages; ECs, epithelial cells; M, macrophage; infMo, inflammatory monocytes; MIF, macrophage migration inhibitory factor; TNF-α, tumor necrosis factor-α.

triggers a highly conserved signaling cascade with a family of four members (Notch 1–4) (55). Notch–Jagged intercellular communication initiates intracellular digestion and modification of the Notch family, by forming a cross-nuclear complex, to initiate AM polarization in coordination with NF-κb signaling and regulates the development of lymphatic lines such as thymus cells, NK cells, and regulatory T cells (Tregs) in the thymus (56, 57). It has been shown that the signal exchange between infected ECs and AMs not only affects the polarization of AMs directly but also further regulates the differentiation and functions of T cell subsets. In addition, ECs can also interact with AMs through the ligand–receptor of CD200 and program death-ligand-1 (24).

RSV direct activation

AMs can engulf RSV particles directly and recognize viral RNA sequences by PAMPs. *Via* MAVS and RIG-I-like receptors, RSV replication activates AM nuclear transcription to release type I and type II interferons and recruits inflammatory cells (12, 58). RSV infection can maintain inefficient replication within macrophages, forming latent infections (15, 59). By inducing immune cells to express MIF (51), it contributes to weakening the migration of AMs subsequently (5). Through receptor-interacting protein kinase 1 and 3 and mixed-lineage kinase domain-like, RSV upregulates TNF-α and the apoptotic-related gene caspase-8 from the AMs' autocrine pathway, thereby exacerbating necrotizing apoptosis and lung tissue damage in airway histiocytes (53). RSV invades AMs through inducing the expression of type I IFN to promote the aggregation of inflammatory monocytes (infMo) (12), which can drive M2-like macrophages to express high matrix metalloproteinase-12 and thus exacerbating airway hyperresponsivity (60).

AM polarization in the different stages of inflammation

To maintain homeostasis, AMs exert mainly immuno suppressive effects by inhibiting the antigen presentation functions of lung dendritic cells or inducing CD4 T cells to be unresponsive (61). It can also secrete a variety of immunomodulatory molecules such as IL-10, TGF-β, NO, and prostaglandin to reduce lung inflammation. Polarized AMs have a dual effect of pro-inflammatory and immune tolerance in the different phases of RSV infection to maintain the intensity of the inflammatory response and the stability of the internal environment and promote tissue repair (34).

Inflammatory period

Airway ECs and AMs, as the first defense cells of the respiratory tract, can recruit neutrophils through the secretion of molecules to synergistically eliminate pathogens. Damaged lung ECs can induce the loss of the immunosuppressive ligand expression of AMs *via* direct cell–cell contact, which may regulate polarized AMs to M1 phenotype (24). M1 produces pro-inflammatory functions in the acute phase of infection and exhibits a stronger phagocytic activity (62, 63). RSV-mediated AM polarization is mainly through cytokine activation pathways, consisting of IFN-γ, TLR-2, -4, and -9 ligands, lipopolysaccharide, and GM-CSF, manifested as M1-like functions. While inhibiting IL-10 receptor signaling, polarized AMs activate NF-kb nuclear transcription by JAK-STAT1/2 phosphorylation signal to express CD16, to release pro-inflammatory cytokines TNF-α, IL-6, IL-1β, IL-12, and IL-23, and to secrete inducible nitric oxide synthase, which can promote the development of inflammation and upregulate the Th1-like response (64–66). Moreover, in the mitogen-activated protein kinase-dependent pathway, polarized AMs express IL-33 and are capable of activating NF-κB signaling by the production of Th2-related cytokines (13).

AMs are important effector cells to secrete IFN-I, and their secretion levels are age dependent. RSV induces the overexpression of IFN-I in adults but, contrarily, inhibits its production in infants (58). IFN-I inhibits RSV replication by upregulating antiviral gene expression and can also recruit monocytes to differentiate into infMo to exert an antiviral activity (12). Immaturity in the production of IFN-I by infants is one of the molecular bases for their susceptibility to develop severe lung inflammation after an RSV infection. During the acute inflammatory phase, RecAMs are rapidly recruited into the alveoli to participate in the removal of pathogens, promoting inflammation, while RAMs inhibit this inflammation. During the period of inflammation regression, most RecAMs are programmed cell death, while RAMs persist. Within 2 months of infection, the phenotypes and functions of some RecAMs are gradually similar to those of RAMs to supplement the RAMs consumed (67). The increased expressions of IFN-I receptor alpha chain, IFN-induced GTP-binding protein Mx2, 2′–5′-oligoadenylate synthetase 1 (OAS1), OAS2, ribonuclease L, and IFN-induced transmembrane protein 3 in AMs also enhance RSV clearance (68). This phenotype exists in the acute phase of other respiratory virus infections, such as influenza virus (69–72).

Convalescent period

In the convalescent phase of infection, the AM phenotype is more inclined to M2, which is manifested by the secretion of IL-10 to modulate the Th17-mediated inflammatory response (9), such as upregulating Tregs, inhibiting lung inflammation driven by inflammatory cells (including neutrophils), and promoting tissue repair (68, 73).

AMs are polarized into M2 phenotype mainly under M-CSF stimulation. According to the different cytokine expression profiles, M2 can be divided into three subtypes: M2a, M2b, and M2c. M2a releases a small amount of IL-10, the decoy receptor IL-1RII, and the IL-1 receptor antagonist (IL1ra), predominated by the inflammatory responses of type Th2, which might be associated with airway sensitization. M2b releases the pro-inflammatory factors TNF-α, IL-1, and IL-6 and a large number of IL-10. Dominated by a high level of IL-10, M2b regulates the signals of inactivated immunity and inflammation through inhibiting the proliferation and differentiation of T cells to exert anti-inflammatory and immune-regulating effects. As an anti-inflammator, IL-10 regulates immune and inflammatory signals, including inhibiting the proliferation and differentiation of T cells to exert anti-inflammatory and immune-regulating effects. M2c is activated by autocrine IL-10 and TGF-β, modulating the immune response and assisting in tissue remodeling (65, 74–76).

Thus, during the convalescent phase of lung tissue inflammation, the functions of RAMs and RecAMs gradually switch to the phenotype of different M2 subtypes, promoting tissue repair and pathogen clearance.

Post-viral AHR

The functional transformation of IMs in the transition from the inflammation period to convalescence is a major intrinsic factor in tissue repair. Early in the convalescent phase of inflammation, M2a is dominated by IL-4 secretion, which, in turn, upregulate the Th2 type immune response leading to AHR, which is associated with wheezing. In the middle and late phases of convalescence, AMs are gradually converted to M2b, mainly secreting IL-10 and TGF-β, regulating the Th17-like immune response negatively, which may promote the production of functional Treg cells, form a positive feedback loop, and inhibit the tolerance of effector T cells to aspiration antigens. IL-10 is mostly secreted by activated IMs by the TLR4/MyD88 pathway. IMs account for about 55% of CD45+ cells that secrete IL-10, compared with less than 5% of CD4 T cells. Activated IMs can impair neutrophil inflammation, mucus production, and the expression of neutrophil-activated cytokines (IL-17, GM-CSF, and TNF-α) in alveoli, negatively regulating the Th2- and Th17-mediated responses (9). In contact with harmless antigens, AMs co-express TGF-β and retinal dehydrogenase 1/2 (77), inducing the production of nTreg cells to maintain immune tolerance (78).

The responses caused by RSV have shown antithesis in immune inflammation and immune tolerance as well as in viral clearance (78). A moderate inflammatory response helps the host defend against pathological harm caused by harmful microorganisms. Decreased immune tolerance can lead to

chronic inflammation such as asthma. When infants are re-infected with RSV, the Th1-type immune response might produce IFN-γ, TNF-α, IL-1β, and IL-22 (68, 79), thereby activating CTL and NK cells to clear the virus (10). However, infants are mainly characterized by the Th2- and Th17-like response (80), and the Th2-type immune memory expresses IL-4, IL-5, and IL-13, which down-regulate Th1, leading to reduce the virus clearance rate and increase the inflammation (9, 81). It means that the pathological basis of AHR may be closely related to an excessively unbalanced immune response. During convalescence or RSV re-infection, infants fail to develop airway immune tolerance due to the formation of Th2 immune memory and the down-regulation of Treg cells, which may induce eosinophilic asthma.

In addition, platelets are also involved in the recruitment of immune cells in the regulation of the conversion of AMs' functions. Stimulated by sCD40L of CD4 T cells, platelets-expressed P-selectin binds to PSGL-1 on the Treg cell membrane to form platelet–Treg aggregates. It is one of the keys to promoting the recruitment of Treg cells to the lungs and releasing anti-inflammatory factors IL-10 and TGF-β. The interaction of platelets with Treg cells is involved in regulating the transcriptional reprogramming of AMs and initiating the polarization of AMs towards anti-inflammatory phenotypes, which effectively relieve lung inflammation (82). At different stages of RSV infection, the phenotypes and functions of AMs change to play a pro-inflammatory and steady-state role, balance and protect the local alveolar microenvironment, and avoid excessive immunopathological damage (59).

AM-mediated T cell differentiation

Intercellular signaling interactions between airway epithelial cells, AMs, and T lymphocytes may be associated with airway sensitization. RSV might upregulate the expression of Notch signaling protein ligand Dll4 in APCs and lung ECs. The blockade of Dll4 (Notch–Jagged ligand of the signaling pathway) might promote the production of Th2-like cytokines (IL-5 and IL-13), mainly through inducing IL-17A+CD4+T cells to differentiation and IL-17A expression. Thus, it might result in excessive immunopathological damage (57). Upregulated Dll4 promotes T cells to express SET and MYDN domain containing protein 3 through the classic Notch signaling pathway, which contributes to Foxp3 gene methylation and Treg cell differentiation and promotes IL-10 expression (83). Furthermore, RSV promotes the upregulation of Jagged-1 and the downregulation of Jagged-2 in bronchial epithelial cells, which is beneficial to the differentiation of Th2 cells. Besides this, if the expression of Jagged-1 is inhibited, it promotes Th1 and inhibits the differentiation of Th2 cells (54). Thus, the species activity of Notch ligands affects the direction of differentiation of T cells. Whether there are differences in the expression levels of different Notch ligands and whether they are age-related are still unclear.

Polarized AMs affect T cell differentiation in many ways—for example, ultra-fine particles induce AMs to express Jagged-1 and promote allergen-specific T cell differentiation into Th2 and Th17 through the Jagged 1–Notch 4 pathway (84). Lung damage caused by mechanical ventilation upregulates the expression of Notch signal-related proteins and promotes the polarization of AM to M1 phenotype, which, in turn, aggravates airway inflammation (85). Therefore, given the important role of AM polarization and T cell differentiation, experimental evidence is still needed to confirm if RSV infection regulates T cell differentiation through AM polarization, of which it is involved in the later body's sensitization state. However, after RSV infection, conclusive evidence is needed on how AM polarization affects the imbalance differentiation of T cell associated with the formation of AHR.

Prospect of AMs as target for the treatment of AHR-related viral infection

Immunomodulatory therapies target AMs that exist in multiple potential sites during a viral infection of the respiratory tract. In the case of rhinovirus infection, AMs can be M1/M2 polarized by GM-CSF/M-CSF or IFN-γ/IL-4 stimulation (86, 87). M1/M2 herein can be likewise classified by their functions and origins rather than dichotomy. In rhinovirus-induced asthma exacerbations, M1-like monocyte-derived macrophages (MDMs) can produce antiviral IFN, while M2-like MDMs significantly enhance the production of Th2-type chemokines (88), where MDMs are commonly classified as RecAMs (89). Furthermore, the inception of rhinovirus-induced AHR may share the analogical pathways with RSV-induced AHR in adaptive immunity—for example, the synergistic interactions between Th2 and Th17 immune responses, in which cytokines (including but not limited to IL-33, IL-13, and IL-17A) are released, mediate eosinophilic and neutrophilic aggregation, jointly inducing AHR (90, 91). After inflammation is controlled, AHR is often characterized by eosinophilic AHR mediated by Th2-like cytokines (IL-5 and IL-13) mediated by immune memory (92). Whether associated with viral infections or the inflammatory cascade, immunomodulatory therapies for AMs will be quite promising and potential.

In the case of homeostasis or M-CSF stimulation, AMs produce anti-inflammatory factors such as IL-10, which result in tissue repair and remodeling similar to those of M2-like functions (93, 94). The current clinical studies of GM-CSF and its receptors are relatively numerous (95)—for instance, the outcomes of severe COVID-19 patients who received a single intravenous dose of mavlimumab to inhibit GM-CSF signaling were relatively better compared with the normal controls (96). However, most of these preclinical research models that inhibit GM-CSF signaling to control inflammation are used in adults

and few for infants. Therefore, for RSV infection in infants, a large amount of experimental data is required to prove that GM-CSF and M-CSF signals can target AM polarization. Considering that AMs' functions in different microenvironments can be reversed, it is necessary to be cautious when using cytokines such as M-CSF to promote the proliferation and polarization of AMs. In homeostasis and convalescence, most AMs are RAMs with M2-like characteristics. Perhaps it is possible to try to obtain RAM-like cells *in vitro* from embryonic liver cells, which have been reported to have similar functions to primary RAMs (97, 98). This may be clinically applied to alveolar lavage therapy (replenishing RAMs) to promote lung repair. In addition, in intercellular signalings, AMs, as APCs, can regulate immune response types that follow through Notch signaling. Combined with Part-6, upregulating Dll4 and Jagged-2 and blocking or downregulating Jagged-1 may inhibit the production of Th2 and Th17-like cytokines and promote Treg cell differentiation.

The desired scenario is to increase virus clearance while maintaining the stability of the lung microenvironment to avoid excessive immune damage. Further studies may be considered from the perspective of IL-10 modulating the adaptive immune response (99, 100). There are currently reports of a hydrogel-based approach to deliver IL-10 to the lung locally without bleeding or other complications (101). This may be a promising clinical treatment strategy.

Conclusion

In conclusion, RSV infection can affect the polarization of AMs in a variety of ways. At different stages, AMs can regulate the differentiation of T cell by expressing different cytokines to maintain a moderate inflammatory response and homeostasis (102, 103). AMs manifest as M1-like functions, perform pro-inflammatory functions during the early phase of RSV infection, and gradually change to M2. Immunomodulatory therapy targeting AMs is a potential direction for preventing wheezing associated with RSV infection.

Author contributions

YW and DZ conceptualized the study design. YW, JZ, XW, PY, and DZ wrote the initial drafts of the manuscript. XW and PY revised the text and participated in the modification diagram. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Respiratory virus type to guide predictive enrichment approaches in the management of the first episode of bronchiolitis: A systematic review

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It has become clear that severe bronchiolitis is a heterogeneous disease; even so, current bronchiolitis management guidelines rely on the one-size-fits-all approach regarding achieving both short-term and chronic outcomes. It has been speculated that the use of molecular markers could guide more effective pharmacological management and achieve the prevention of chronic respiratory sequelae. Existing data suggest that asthma-like treatment (systemic corticosteroids and beta2-agonists) in infants with rhinovirus-induced bronchiolitis is associated with improved short-term and chronic outcomes, but robust data is still lacking. We performed a systematic search of PubMed, Embase, Web of Science, and the Cochrane's Library to identify eligible randomized controlled trials to determine the efficacy of a personalized, virus-dependent application of systemic corticosteroids in children with severe bronchiolitis. Twelve studies with heterogeneous methodology were included. The analysis of the available results comparing the respiratory syncytial virus (RSV)-positive and RSV-negative children did not reveal significant differences in the associatons between systemic

corticosteroid use in acute episode and duration of hospitalization (short-term outcome). However, this systematic review identified a trend of the positive association between the use of systematic corticosteroids and duration of hospitalization in RSV-negative infants hospitalized with the first episode of bronchiolitis (two studies). This evidence is not conclusive. Taken together, we suggest the design for future studies to assess the respiratory virus type in guiding predictive enrichment approaches in infants presenting with the first episode of bronchiolitis.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42020173686

KEYWORDS

precision medicine, infant, bronchiolitis, viruses, corticosteroids, asthma, rhinovirus

Introduction

It is believed that the first severe episode of wheezing, or severe bronchiolitis, may be the first sign of developing asthma; therefore, immunological methods for its early detection are being sought to implement efficient management early (1, 2). In addition, mechanistic studies have shown that viral respiratory infections can contribute to type 2 inflammation (3, 4). The restriction to access to airway samples in infants has complicated the investigation of host immune responses to respiratory viruses, limiting our understanding of these processes and, thus, impacting the appropriate choice of therapeutic strategies that can benefit children presenting with the first episode of bronchiolitis.

The current conservative bronchiolitis management relies on the one-size-fits-all approach regardless of short-term and chronic outcomes. However, the common clinical practice based on the use of systemic corticosteroids in bronchiolitis has not been supported by any systematic review. Thus, systemic corticosteroids are not recommended as the first-line treatment in any international guidelines for managing bronchiolitis and mainstream bronchiolitis care is solely supportive (5–9).

On the other hand, increasing evidence demonstrates that bronchiolitis is a heterogeneous disease and that a viral trigger may be one of the key exposures and part of the underlying pathobiology, very important in identifying endotypes (2, 10, 11). Although the clinical features of bronchiolitis attributed to different viruses are usually indistinguishable, the recently recognized bronchiolitis profiles are associated with various risks for recurrent wheeze and asthma, some differences in disease severity, and, potentially, different therapeutic responses to systemic corticosteroids (10–13). For example, it has been shown that human rhinovirus (HRV)-associated bronchiolitis can result in shorter hospitalization times than bronchiolitis caused by the respiratory syncytial virus (RSV); however, the evidence around associations between virus type and severity is still unclear (14, 15). While most children hospitalized due to bronchiolitis have an uneventful course, it should be remembered that approximately 2-6% require admission to pediatric intensive care units and invasive mechanical ventilation, or even occasionally be fatal (16–18).

Likewise, current studies evaluated the efficacy of systemic corticosteroid therapy in severe bronchiolitis (19–21). In evaluating these studies, one should remember that the sample size was not homogeneous. Therefore, other confounding factors, such as respiratory virus type or peripheral blood eosinophilia or parental history of asthma/allergy, were not evaluated. Furthermore, the effectiveness of the systemic corticosteroids may not depend on the virus type per se but potentially on the eosinophilic airway inflammation, which is often present in atopic children and/or accompanied by a HRV-induced respiratory infection (22, 23).

Recognition of the variability of bronchiolitis, coupled with the failure to identify effective therapies, has provided incentives to establish a precision medicine approach in bronchiolitis management (24). Precision medicine refers to the customization of diagnostic and therapeutic processes based on the unique features of an individual patient (25, 26), and this concept is called the “concept of enrichment” (27). Prognostic enrichment reflects the selection of patients more prone to a disease-related event, such as mortality. Meanwhile, predictive enrichment refers to selecting patients more likely to respond to therapy based on the biological mechanism.

Currently, no clear evidence suggests using respiratory virus testing as a guide for systemic corticosteroids in the first episode of severe bronchiolitis. Therefore, we aimed to systematically revise the literature on whether the respiratory virus type can

guide predictive enrichment approaches in the first episode of bronchiolitis.

Methods

Search strategy

The study followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (28) and was registered in the National Institute for Health Research's PROSPERO (CRD42020173686).

Four bibliographic databases were searched (PubMed, Web of Science, Embase, and Cochrane's Library) from inception to September 23, 2019. Additionally, the updated search was conducted closer to submission (January 04, 2022). The search term (*steroid* OR *predniso* OR dexamethasone) AND (wheez* OR bronchiolitis) was followed using a Boolean methodology. All extracted citations were imported into EndNote® reference manager (Version X8, Clarivate Analytics, 2016). After removing the duplicates, two reviewers (DA and HM) working independently screened the retrieved titles and abstracts.

Subsequently, all potentially relevant publications were assessed in full text. At each stage, uncertainty about the eligibility of studies for the review was resolved through discussion and through obtaining consensus by other reviewers (AW, NP, WF, TJ), if necessary.

Eligibility criteria

Main eligibility criteria included publication in English language, randomized controlled trial design (RCTs), age group under two years old, the clinical presentation with the first episode of bronchiolitis or wheezing episode at the hospital setting (emergency department (ED) and/or ward), intervention with the administration of systemic corticosteroids, that assessed viral etiology of the illness.

Exclusion criteria included duplicate publications, non-human studies, RCTs that did not involve management at the ED or admission to the paediatric ward, RCTs including children presenting with confirmed one viral agent (e.g., only RSV-positive cases), RCTs including children who received inhaled corticosteroids as the trial intervention, study protocols, editorials or review papers, and conference abstracts.

Actions were taken to contact corresponding authors when additional clarification and further data were required.

Our primary outcomes were viral-dependent short-term outcomes, such as change in the baseline clinical severity scores (i.e., the Respiratory Distress Assessment Instrument, RDAI), need for oxygen therapy, hospitalization rate in the ED studies, and length of hospitalization in the inpatient studies. The secondary outcomes of our review included viral-dependent long-term outcomes (two months or longer after the study enrollment) such as re-admission to medical center due to respiratory symptoms or initiation of regular controller medication for asthma symptoms.

Study extraction and synthesis

Data were extracted from each included study for the following parameters: (a) study origin, (b) participant details, (c) diagnostic intervention(s) and the type of systemic corticosteroids used, (d) administered control, (e) short-term and long-term outcomes, (f) details on results assessed per viral etiology, (g) overall risk of bias assessment, and (h) potential confounding factor(s).

Given the small number of studies utilizing similar methodology and describing virus detection data, meta-analysis was not possible, and consequently, a narrative synthesis method was used.

Risk of bias assessment

Two independent reviewers (DA, HM) assessed the risk of bias in each of the included studies without being blinded to the authors or journal. A revised Cochrane risk-of-bias tool for randomized trials was used (RoB 2) (29). Encountered discrepancies were resolved through a discussion of all the reviewers. The RoB 2 tool is structured into five bias domains, which enables to judge the randomization process, deviations from intended interventions, any missing outcome data, measurement of the outcome, selection of the reported result.

The judgments for each domain were to choose between "low risk of bias", "some concerns", or "high risk of bias". In conclusion, the overall bias was determined by reflecting the individual marks. The graphical summary of the risk of bias assessment was performed using the robvis online tool (30).

Results

Description of the studies

Following the systematic search, 6831 records were obtained, including 5947 unique citations. The PRISMA flow chart is shown in Figure 1. The screening of titles and abstracts excluded 5788 articles, rendering 159 to full-text assessment. Finally, twelve studies fulfilling the inclusion criteria for systemic application of corticosteroids in the first episode (corticosteroid-naïve) of bronchiolitis and details on the performed viral detection were included (n=1931, see Table 1) (31–41, 43). The included studies were conducted in different parts of the world – three studies in Europe (Ireland, Finland) (33, 38, 43), one study in South America (Paraguay) (41), six in North America (USA, Canada) (31, 34, 36,

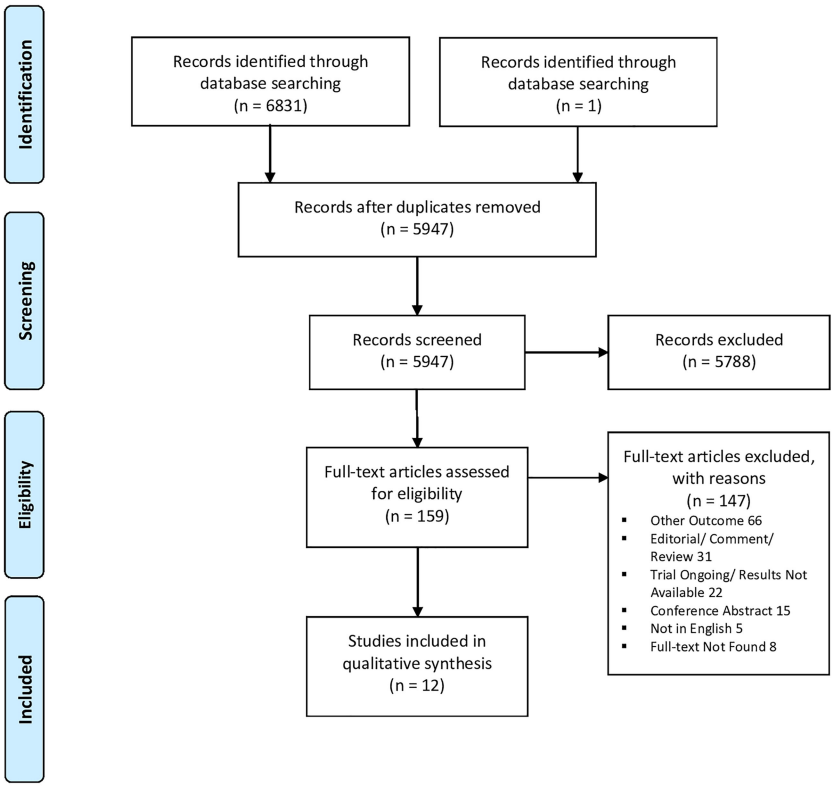

FIGURE 1
Prisma flow diagram of the study.

37, 39, 40), and two in Asia (32, 35). Two of the studies were multicenter (39, 40).

Among the included studies, the overall risk of bias was considered low (33–38) in six, or only with some concerns in three of them (31, 32, 41) (Figure 2). However, one of the studies were judged as with a high risk of bias (43). Their methodological concerns included poor data availability on randomization and blinding process, deviations from the intended intervention, potentially missing outcome data, and some discrepancies in reporting results. Notably, most of the papers presented the results according to the per-protocol analysis (31–36, 38, 41, 43), which might have led to the increased bias. The attempt was made to perform the quantitative synthesis of the short-term effectiveness of corticosteroids in the first episode of bronchiolitis compared between RSV-positive and RSV-negative groups; however, we encountered a significant heterogeneity that heterogeneity (I^2>75%) that any potential subgroup analysis could not have avoided.

Population

The age of eligible participants differed between the included studies. Three studies, all based in the USA, recruited infants up to 12 months of age (31, 39, 40). The remaining studies included children up to 15 (34), 18 (32, 35) or 24 (33, 36, 37, 41, 43) months of age. One of the RCTs initially analyzed children under the age of 3 years; however, according to predefined inclusion criteria, we decided to selectively assess only the patients under the age of 2 years (38). All studies included patients with wheezing as one of the compulsory criteria apart from one (32), in which crackles or wheezing were needed on inspection. Six studies were conducted in the ED units and the pediatrics wards (35–37, 39–41), while six of the studies recruited solely hospitalized children (31–34, 38, 43).

Viral detection

Most of the identified studies performed viral detection tests only on the presence of RSV (31, 32, 34–37, 39, 40). The other additionally detected viruses included HRV, bocavirus (33, 38), influenza virus (33, 38, 41, 43), parainfluenza virus (33, 38, 43), and adenovirus (33, 38, 41, 43). Moreover, the viral detection techniques varied across the studies, i.e., virus isolation, serology testing, rapid immunoassay method of nasopharyngeal swabs, and PCR tests. Two out of twelve studies did not provide the results with an adjustment to the performed viral testing (34, 35).

TABLE 1 Characteristics of the included studies in the magnitude of short-term outcome assessment.

Study ID, country	Study design and analysis method	Population and randomized sample size (n), attrition (%)	RSV-positive vs. RSV-negative	Intervention/ Comparison groups	Short-term outcome(s) measured	Outcomes assessed as per viral etiology
Connolly et al. (1969) (31), Ireland	RCT, PP	Children up to 24 months of age hospitalized with bronchiolitis (n=100)	RSV-positive (n=78) RSV-negative (n=15)	Oral prednisolone (6 days)/Placebo	Duration of hospitalization Duration of illness from symptoms onset	No significant difference in the duration of illness between compared the RSV-positive and RSV-negative groups
Roosevelt et al. (1996) (32), USA	RCT, PP	Infants up to 12 months of age hospitalized with the first wheezing episode (n=122)	RSV-positive (n=79) RSV-negative (n=39)	Intramuscular DM (max. 3 days)/Placebo	Time to resolution of respiratory symptoms Duration of oxygen therapy	No significant differences in the time to resolution of symptoms (HR (95% CI): 1.2 (0.8–1.9), 1.1 (0.5–2.1), respectively), and the duration of oxygen therapy (HR (95% CI): 0.9 (0.6–1.5), 0.7 (0.3–1.4), respectively) compared the RSV-positive and RSV-negative groups
Klassen et al. (1997) (33), Canada	RCT, PP	Infants up to 15 months of age hospitalized with first wheezing episode (n=72)	RSV-positive (n=58) RSV-negative (n=9)	Oral DM (min. 3 days)/Placebo	Change in the RDAI score at 24 hours after enrollment Time until ready for discharge Medical visits after a 1-week follow-up Medication at discharge	NA
Berger et al. (1998) (34), Israel	RCT, PP	Children 2-18 months of age with the first episode of bronchiolitis (n=42)	RSV-positive (n=19) RSV-negative (n=19)	Oral prednisolone (3 days)/Placebo	Clinical evaluation after 3 days of enrollment Clinical evaluation after 7 days of enrollment	NA
Goebel et al. (2000) (35), USA	RCT, PP	Children <24 months of age with the first wheezing LRTI (n=51)	RSV-positive (n=26) RSV-negative (n=22)	Oral prednisolone (5 days)/Placebo	Bronchiolitis clinical severity score at study days 0, 2, 3, 6 according to the enrollment Hospitalization rate	No significant difference in the clinical improvement between the study's measurement timepoints compared RSV-positive and RSV-negative
Schuh et al. (2002) (36), Canada	RCT, ITT	Children <24 months of age with the first wheezing bronchiolitis (n=71)	RSV-positive (n=30) RSV-negative (n=28)	Single dose of oral DM/Placebo	RACS after the 4-hour observation period Differences in hospitalization rates after the 4-hour observational period Changes in transcutaneous oxygen saturation Difference in RACS from baseline to day 7	No significant difference in the hospitalization rate between the RSV-positive and RSV-negative groups
Jartti et al. (2006) (37), Finland *the Vinku study*	RCT, PP	Children up to 24 months of age, with the first wheezing bronchiolitis* (n=113)	RSV-positive (n=48) RSV-negative (n=65)	Oral prednisolone (3 days)/Placebo	Time until ready for discharge Oxygen saturation, wheeze, and cough during 2-week follow-up Blood eosinophil counts at dis- charge and at 2-week follow-up	The mean LOS shorter in the RSV-negative children receiving intervention vs. placebo (mean ± SD: 19,7 ± 31,5 vs. 40,0 ± 31,3 hours, P=.002) No significant difference in the mean LOS in the RSV-positive children between the intervention and placebo groups (mean ± SD: 57,2 ± 39,9 vs. 47,6 ± 38,7 hours, P>.05)
Corneli et al. (2007) (38), USA	RCT, ITT (hospital admissions) and PP (RACS)	Infants 2-12 months of age with the first episode of bronchiolitis (n= 600)	RSV-positive (n=166) RSV-negative (n=103)	Single dose of oral DM/Placebo	Hospitalization rate after a 4-hour observation period RACS after a 4-hour observation period	No significant difference in the hospitalization rate and the RACS after 4 hours compared the RSV-positive and RSV-negative groups

(Continued)

TABLE 1 Continued

Study ID, country	Study design and analysis method	Population and randomized sample size (n), attrition (%)	RSV-positive vs. RSV-negative	Intervention/ Comparison groups	Short-term outcome(s) measured	Outcomes assessed as per viral etiology
Plint et al. (2009) (39), Canada	RCT, ITT	Infants 6 weeks – 12 months of age with the first episode of bronchiolitis (n=401)	RSV-positive (n=263) RSV-negative (n=138)	Two of four study treatment groups: Oral DM plus nebulized placebo (6 days)/ Oral and inhaled placebo	Hospitalization rate after a 7-day follow-up Change in heart and respiratory rate, RDAI score, and oxygen saturation from baseline to 30, 60, 120, and 240 minutes after the intervention Time until ready to discharge	No significant difference in the hospitalization rate between the RSV-positive and RSV-negative groups
Mesquita et al. (2009) (40), Paraguay	RCT, PP	Children 2-24 months of age with the first wheezing bronchiolitis (n=80)	RSV-positive (n=36) RSV-negative (n=29)	Single dose of oral DM/Placebo	Change in the RDAI score at the 4-hour observation period Hospitalization rate	No significant difference in the hospitalization rate and the RDAI score at 4-hour observation compared the RSV-positive and RSV-negative groups
Alansari et al. (2013) (41), Qatar	RCT, PP	Infants up to 18 months of age hospitalized the first episode of bronchiolitis with a history of eczema or with a family history of asthma (n=200)	RSV-positive (n=77) RSV-negative (n=123)	Oral DM (5 days)/Placebo	Early discharge within a 46-hour period Medical re-presentations within a 1-week follow-up	Readiness for discharge at 48 hours higher in the RSV-negative vs. the RSV-positive group (OR 0.28, CI95% 0.09-0.92, P=.03)
Jartti et al. (2015) (42), Finland *the Vinku2 study*	RCT, PP	Children 3-23 months of age with the first wheezing episode due to a HRV confirmed infection, other viral co-infections included (n=79)	RSV-positive (n=10) RSV-negative (n=69)	Oral prednisolone (for 3 days)/Placebo	Post-episode respiratory symptoms a 2-week follow-up Use of bronchodilators a 2-week follow-up Medical re-presentations within a 2-week follow-up	No significant differences in any of the assessed outcomes compared the RSV-positive and RSV-negative groups The prednisolone HRV-positive group had less cough, rhinitis, noisy breathing, severe breathing difficulties, and nocturnal respiratory symptoms within 2 weeks (all P<.05)

PP, Per Protocol; ITT, Intention to Treat; RSV, Respiratory Syncytial Virus; HRV, Rhinovirus; NA, Not Available; DM, Dexamethasone; Respiratory Assessment Change Score, RACS; Respiratory Distress Assessment Instrument, RDAI; LOS, Length of hospital Stay; ED, Emergency Department.
*Detailed data on subgroup analysis of children under the age of 2 years and with the first wheezing episode received from the study investigators.

Interventions

The systemic corticosteroids were administered orally in all the included studies, apart from one in which intramuscular injections were used (31) (Table 1). Prednisolone served as the intervention drug in five studies (33, 35, 36, 38, 43), while dexamethasone was used in the remaining seven (31, 32, 34, 37, 39–41). Usually, prednisolone was given orally at 2 mg/kg/day (33, 35, 36, 38), while in one study decreasing dosages starting from 15 mg to 2,5 mg were administered (43). Furthermore, the heterogeneity across the duration of the interventions was noticed. A single dose of corticosteroids was administered in three studies (37, 39, 41). The 3-day intervention course was introduced in five RCTs (31, 33–35, 38). The 5-, 6-, or 7-day treatment with corticosteroids was tested in four studies (32, 36, 40, 43).

Short-term outcomes of corticosteroid treatment in severe bronchiolitis per viral etiology

Only two studies showed significant differences in the short-term efficacy of systemic corticosteroids compared between RSV-positive and RSV-negative groups (n=313) (32, 38).

In one of these studies, the mean duration of hospitalization of neither RSV-positive nor HRV-positive patients administered corticosteroids varied from the ones receiving a placebo (mean ± SD [hours]: 57,2 ± 39,9 vs. 47,6 ± 38,7 for RSV-positive and 14,6 ± 32,5 vs. 19,5 ± 31,9 for HRV-positive, both P>.05) (38). However, in the RSV-negative group, oral prednisolone almost by one day shortened the mean length of hospital stay compared to the placebo group (mean ± SD [hours]: 19,7 ± 31,5 vs. 40,0 ± 31,3, P=.002). Furthermore, the mean

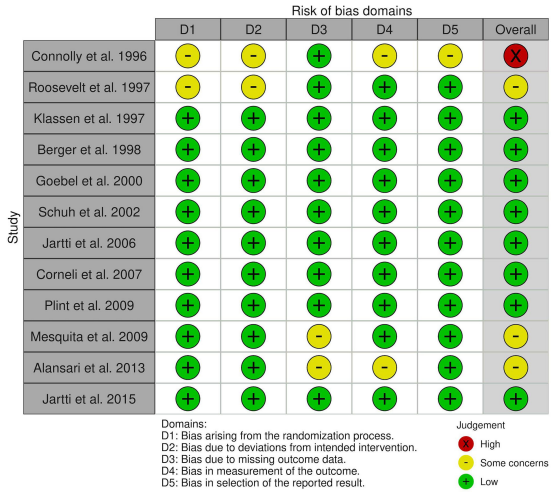

FIGURE 2
Summary of risk of bias assessment of the eligible studies.

hospitalization time significantly differed between RSV-positive and HRV-positive children in the intervention group (mean ± SD [hours]: 57,2 ± 39,9 vs. 19,7 ± 31,5, P=.002) (38).

In the second study, oral dexamethasone's efficacy, regarded as the readiness rate for discharge at 48 hours, was higher in the RSV-negative group than in the RSV-positive group (OR 0.28, CI95% 0.09-0.92, P=.03) (32).

Four studies reported no difference in the hospital admission rate after the initial visit to ED between RSV-positive and RSV-negative children who were administered systemic corticosteroids (37, 39–41).

Chronic outcomes of corticosteroid treatment in severe bronchiolitis per viral etiology

The long-term follow-up – herein defined as two-month or more extended time – was evaluated only in three studies (33, 35, 38). The prevalence of respiratory symptoms during the two-year follow-up by Berger et al. (35) was not reported according to the RSV infection status. Solely Jartti et al. reassessed their participants two (38) and 12 months (33) after their studies' enrollment. Although in their report, the number of wheezing episodes and initiation of regular controller medication for asthma symptoms within 12 months was not significantly reduced in the HRV-positive intervention group, in HRV(+) children less risk of physician-confirmed recurrence within 2 and 12 months was shown in the prednisolone group compared with placebo (both P<.05) (33). Moreover, they observed fewer new wheezing episodes during the 12-month follow-up in the corticosteroid-treated group compared with the placebo (P=.04) (33).

Discussion

Although this systematic review failed to provide evidence in favor of the use of systemic corticosteroids in children with severe bronchiolitis, it has identified a trend of the positive association between the use of systematic corticosteroids and duration of hospitalization in RSV-negative infants in hospitalized bronchiolitis (32, 38).

The scarcity of expected effect may be attributed: (i) to the considerable diversity in methodology among the analyzed studies, (ii) under-reporting the full panel of respiratory viruses in study subjects, (iii) baseline inflammatory endotype of the child rather than only the viral agent, and (iv) that most studies focused on RSV etiology. Only two studies from the same study center reported the details on HRV detection – the second most common cause of bronchiolitis in infants above six months old. These studies showed oral prednisolone as an effective modality in managing the first HRV-induced wheezing episode. This effectiveness was defined as clinical improvement (no escalation to non-invasive ventilation) and reduced relapses during the first months of follow-up (33, 38). Therefore, our analysis highlights the lack of predictive enrichment strategies in trials investigating corticosteroid treatment in bronchiolitis, which is in line with a recent meta-analysis by Elliott et al. (6).

On the other hand, the strengths of this study are in the identification of the existing evidence gaps, in the innovative view on the corticosteroid's treatment in bronchiolitis, including focusing on both the first episode and its viral etiology, and efforts made to contact the authors to address the missing data. Also, there were attempts to synthesize the results quantitatively with subgroup analyses; however, the significant heterogeneity could not have been omitted.

We attempted to make the results of this systematic review as clinically relevant as possible. Hence, we dichotomized the data into RSV and non-RSV (another respiratory virus) data to accommodate the current clinical practice protocols that do not include mandatory testing for all respiratory viruses. Moreover, many confounding factors across the studies undermined the synthesis of the available data. The performed viral testing was obsolete, including viral isolation and serology tests (43). In some studies, most patients were RSV-positive (34, 43), or a noticeable proportion of participants did not undergo viral detection. A considerable loss to follow-up was found to be another confounder (35, 36, 41). Regarding the two Finnish studies, there was a delay in initiating the study's drug administration due to the completion of HRV detection in the second study (33) compared to the first one (38) (45 vs. 0 h, respectively).

RSV remains the leading cause of severe bronchiolitis and, proportion-wise is the most prominent risk factor for future asthma development (42). The possible lack of action of systemic corticosteroids in RSV bronchiolitis may result from at least two reasons: firstly, several studies have shown that RSV can inhibit

the immunosuppressive activity of corticosteroids *via* the glucocorticoid receptor (44). Secondly, dexamethasone was shown to have a favorable inhibitory impact on RSV-driven mucus production yet prevent immune responses that limit RSV infection *in vitro* and *in vivo* (45). On the other hand, one may expect a beneficial effect of systemic corticosteroid therapy in HRV-induced bronchiolitis due to the well-known Th2-skewing, a typical feature for atopic children infected with the HRV-C virus (46). Also, over the last decade, several cohort studies have associated an early life HRV infection with recurrent wheeze and asthma in the following years (11, 47–49).

Recent evolution in high-throughput sequencing technology offers an opportunity for personalized guidance in pharmacological management and assessing long-term respiratory sequelae in infants with bronchiolitis (11, 50, 51). A relatively small number of epidemiologic studies have investigated associations between biological (e.g., viral etiology, proteins), genetic and environmental factors in infants with bronchiolitis. Even fewer intervention studies in bronchiolitis incorporated a predictive enrichment approach using an interplay of such factors in their methodology.

In 2020-2021, we witnessed an unprecedented revolution in respiratory virus testing on a mass scale. A wide distribution of the mass PCR testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections has contributed to the increased use of the multiplex technique for other respiratory viruses (52). Therefore one cannot exclude the notion that in the coming years, studies evaluating a panel of respiratory viruses will provide more evidence around the role of HRV as a potential biomarker for the use of selected medications, including corticosteroids, during the first episode of bronchiolitis (53).

According to our recent meta-analysis on the association between infant bronchiolitis and recurrent wheeze, it has been shown that HRV-bronchiolitis children were more likely to develop recurrent wheeze (OR 4.11) and asthma (OR 2.72) than RSV-bronchiolitis group ($P < 0.01$) (54). Furthermore, it has been proposed that the impact of rhinoviral infection may be species-specific. Of three species (A, B, and C), HRV-C-induced bronchiolitis has been linked to the highest risk for preschool wheeze and asthma in children (10). Thus, future studies evaluating the treatment efficacy according to virus subtypes are eagerly anticipated.

We are aware of several limitations of our review. The heterogeneity of the severity of assessed bronchiolitis episode introduced interventions, pre-specified outcomes, and follow-up time of analyzed studies makes our results less precise. Even though the search strategy was inclusive, within the initially identified studies recruiting only first wheezing bronchiolitis, viral tests were rarely performed and mainly focused on RSV etiology. Hence, the number of finally included studies was sparse.

Severe bronchiolitis remains a heterogeneous disease ideally suited for a precision medicine approach. This systematic review shows that there is currently insufficient data to recommend using systemic corticosteroids for short-term beneficial effects in treating the first episode of bronchiolitis. Nevertheless, an identified trend of the positive association between the use of systematic corticosteroids and shorter duration of hospitalization in RSV-negative infants hospitalized with the first episode of bronchiolitis should be further elucidated.

In conclusion, our study points out the need to identify groups of infants who would benefit from systemic corticosteroid treatment. A precise definition of the group of patients who benefit from a treatment is associated with decreased use of non-specific treatment. Therefore, this approach could ultimately result in decreased overall usage of systemic corticosteroids in bronchiolitis. Predictive enrichment approaches are guided by integration multi-omic data on HRV infection status (with its subtyping). Ongoing research in this area should focus on elucidating the complex interactions between pathogenetic factors in viral bronchiolitis with the aim to increase effectiveness and prevent the development of chronic outcomes.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

DA, HM, WF, TJ, and AW contributed to the study design. DA and HM performed the systematic search, analyzed the data, and wrote the manuscript. WF, AW, NP, TJ reviewed the first draft of the manuscript, gave directions around the table and the figure design, and worked on the editing of manuscript to help reach the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Cytokine expression in rhinovirus- vs. respiratory syncytial virus-induced first wheezing episode and its relation to clinical course

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Rhinovirus (RV) and respiratory syncytial virus (RSV) are common causes of bronchiolitis. Unlike an RSV etiology, an RV etiology is associated with a markedly increased risk of asthma. We investigated the cytokine profiles of RV- and RSV-induced first wheezing episode and their correlation with prognosis. We recruited 52 sole RV- and 11 sole RSV-affected children with a severe first wheezing episode. Peripheral blood mononuclear cells (PBMCs) were isolated during acute illness and 2 weeks later and stimulated *in vitro* with anti-CD3/anti-CD28. Culture medium samples were analyzed for 56 different cytokines by multiplex ELISA. Recurrences were prospectively followed for 4 years. In adjusted analyses, the cytokine response from PBMCs in the RV group was characterized by decreased expression of interleukin 1 receptor antagonist (IL-1RA), interleukin 1 beta (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) and increased expression of eosinophil chemotactic protein 2 (eotaxin-2), thymus- and activation-regulated chemokine (TARC), and epithelial-derived neutrophil-activating peptide 78 (ENA-78) in the acute phase and increased expression of fractalkine in the convalescent phase compared to those in the RSV group. An analysis of the change in cytokine expression between study points revealed an increased expression of fractalkine and IL-1β and decreased expression of I-309 (CCL1) and TARC in the RV group compared to those in the RSV group.. Considering hospitalization time, a significant non-adjusted group × cytokine interaction was observed in the levels of interferon gamma (IFN-γ), macrophage-derived chemokine (MDC), IL-1RA, and vascular endothelial growth factor (VEGF), indicating that a higher expression of cytokine was associated with shorter hospitalization

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time in the RSV group but not in the RV group. A significant interaction was also found in interleukin 6 (IL-6), but the cytokine response was not associated with hospitalization time in the RSV or RV group. In the RV group, increased expression of I-309 (CCL1) and TARC was associated with fewer relapses within 2 months, and decreased expression of interleukin 13 (IL-13) and increased expression of I-309 (CCL1) were associated with less relapses within 12 months. Differences in cytokine response from PBMCs were observed between RV- and RSV-induced first severe wheezing episode. Our findings also reveal new biomarkers for short- and medium-term prognosis in first-time wheezing children infected with RV or RSV.

KEYWORDS

bronchiolitis, cytokine, respiratory syncytial virus, rhinovirus, wheezing

Introduction

Up to a third of all children suffer from bronchiolitis during the first 2 years of life, and it is the most common cause for hospitalization in children. Respiratory syncytial virus (RSV) and rhinovirus (RV) are the most common etiologic agents (1). RSV is most commonly found in children under 12 months of age, but RV starts to dominate thereafter (1, 2). The "common" bronchiolitis diagnosis has been criticized as too obscure, and more specific classification according to virus entities has been anticipated (3).

Both RSV and RV target and replicate in epithelial cells of the airways that result in innate immune activation and a rapid burst of type I/III interferons (IFNs) (1). This is followed by the induction of several cytokines and chemokines, leading to epithelial cell apoptosis, necrosis, epithelial sloughing, and mucus overproduction. Typically, cytopathic effects are more severe in RSV infection. In contrast to RSV, atopic predisposition and a distinct single polymorphism in the *CDHR3* gene or 17q locus increase the risk for more severe RV-induced illness and a more compromised long-term prognosis (4–6). Studies in human and murine models have shown that RV infections of airway epithelial cells are inducers of type 2 innate cytokines, such as interleukin (IL)-25 and IL-33, which subsequently initiate or boost type 2 immunity in the lungs *via* IL-5- and IL-13-producing group 2 innate lymphoid cells (ILC2) and T helper 2 (Th2) cells (7–9).

Although RSV typically causes more severe bronchiolitis, an RV etiology is associated with a higher risk of recurrent wheezing and asthma than does an RSV etiology (1, 2, 6, 10–12). The exact mechanism for these differences is not known, and there are no precise data about the immunopathologic differences between the two major etiologic agents of bronchiolitis, RSV and RV. Therefore, we aimed to investigate the cytokine profiles of RV- vs. RSV-induced first severe wheezing episode and their relation to short- and long-term outcomes. We hypothesized that potential differences in the cytokine response from peripheral blood mononuclear cells (PBMCs) of children with virus-induced acute wheezing due to RV compared to RSV may be linked to prognosis.

Abbreviations: ENA-78, epithelial-derived neutrophil-activating peptide 78, CXCL5; eotaxin-2, eosinophil chemotactic protein 2, CCL24; eotaxin-3, eosinophil chemotactic protein 3, CCL26; fractalkine, CX3CL1; I-309, CCL1; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; IL-1RA, interleukin 1 receptor antagonist; IL-6, interleukin 6; IL-13, interleukin 13; MCP-1, monocyte chemoattractant protein-1, CCL2; MDC, macrophage-derived chemokine, CCL22; MIP-1α, macrophage inflammatory protein-1 alpha, CCL4; TARC, thymus- and activation-regulated chemokine, CCL17; VEGF, vascular endothelial growth factor.

Materials and methods

Subjects

The study population was part of the Vinku2 study in which RV-affected first-time wheezing children were randomized to receive oral prednisolone (2 mg/kg/day for 3 days) or placebo (updated version for 7-year follow-up NCT00731575, original version EudraCT 2006-007100-42) (13). Its recruitment was carried out in 2007–2010 in the Department of Pediatrics, Turku University Hospital (Turku, Finland). The main inclusion criteria for the current analysis were age 3–23 months, delivery at ≥36 gestational weeks, first wheezy episode (parental report and confirmed from medical records), sole steroid-naive RV or RSV infection detected in a nasopharyngeal aspirate sample by polymerase chain reaction (PCR), and written informed consent from a parent or guardian. The exact PCR procedure has been

previously described in detail (14). The main exclusion criteria consisted of chronic non-atopic illness, previous systemic or inhaled corticosteroid treatment, or the need for intensive care unit treatment (Figure 1). The study was approved by the Ethics Committee of Turku University Hospital and commenced only after obtaining written informed consent from the guardians.

Study protocol

The need for hospitalization was decided by an on-duty study physician independent of the study. Recruitment to the study was done by the study physician. At study entry, the guardian filled in a standard questionnaire on host and environmental risk factors for asthma. The child was then physically examined by the study physician, a nasopharyngeal aspirate sample was obtained for viral diagnostics using a standardized procedure (15), and a baseline blood sample was drawn. The children were randomized to be given either oral prednisolone or a placebo after a positive RV PCR test (prednisolone receivers were excluded from subsequent analyses). The second blood sample was drawn at the convalescent phase, 2 weeks after study entry. The Vinku2 study used follow-up protocols, including daily symptom diaries, for the first 2 months. Thereafter, new breathing difficulties were prospectively recorded in a diary, and follow-up visits at 2 weeks, 2 months, 12 months, and 4 years were scheduled by the study physicians. Furthermore, the guardian was asked to bring the child to the study physician each time the child had breathing difficulties.

Study aims

We had several aims in the study:

1. To compare the cytokine response from anti-CD3/anti-CD28-stimulated PBMCs between RV- and RSV-affected severe first-time wheezing children (80% hospitalized and 20% treated in the emergency room of a tertiary hospital) in the acute phase and convalescent phase 2 weeks later.
2. To compare the cytokine expression between RV species, that is, *A*, *B*, and *C* species.
3. To investigate whether the cytokine expression is associated with the virus genome load (RV).
4. To assess whether the cytokine expression is associated with recurrences (i.e., new physician-confirmed wheezing episodes within the subsequent 2 months and 12 months) and asthma at 4 years.

Definitions

Asthma refers to recurring airway obstruction and intermittent symptoms of increased airway responsiveness to triggering factors, such as exercise, allergen exposure, and viral infection. Wheezing refers to expiratory breathing difficulty with bilateral high-pitched sounds during expiration. Wheezing episodes accompanied by sole

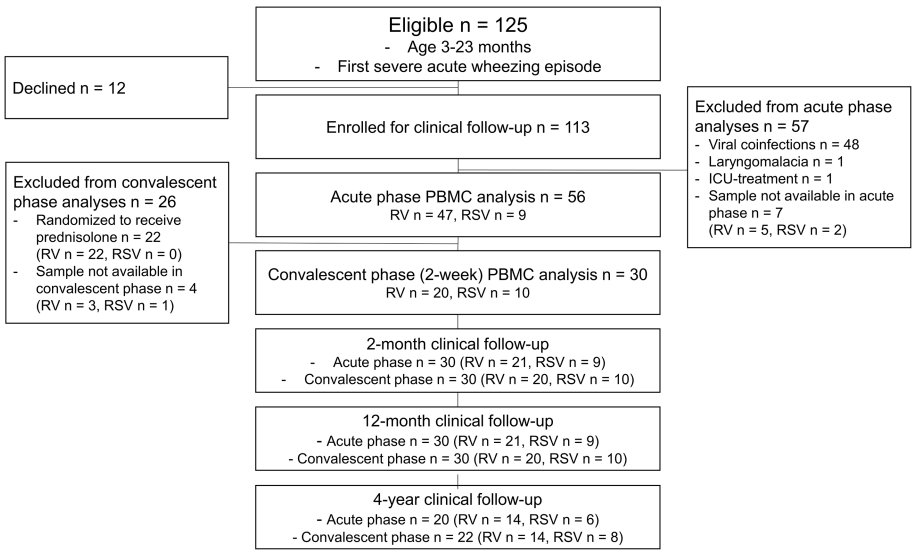

FIGURE 1
Study flowchart. Patients with cytology data were included. ICU, intensive care unit; PBMC, peripheral blood mononuclear cell; RSV, respiratory syncytial virus; RV, rhinovirus.

RV or RSV detection by PCR were called RV- or RSV-induced wheezing episodes, respectively. Atopy was defined as a positive immunoglobulin (Ig) E antibody (≥.35 kU/L) to any of the following allergens: codfish, cow's milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum*, and *Dermatophagoides pteronyssinus* (Phadiatop Combi®, Phadia, Uppsala, Sweden). Aeroallergen sensitization was defined as a positive IgE antibody to any of the latter eight allergens. Perennial aeroallergen sensitization was defined as positive IgE antibodies to dog, cat, or *D. pteronyssinus*. Birch, mugwort, timothy, and *C. herbarum* were considered seasonal aeroallergens. Eczema was defined as a physician diagnosis according to typical symptoms that included pruritus, typical morphology, and chronicity of the disease. Eczema was defined as atopic eczema if a child had atopy (defined above). Type 1 immunity refers to the activity of T helper 1 (Th1) cells, group 1 innate lymphoid cells (ILC1), neutrophils, and classically activated macrophages. Type 2 immunity refers to the activity of Th2 cells, ILC2, eosinophils, mast cells, basophils, and IL-4- and IL-13-activated macrophages.

Virus detection

Nasal swabs (nylon flocked dry swab, 520CS01, Copan, Brescia, Italy) were dipped into the nasopharyngeal aspirate and stored at -70°C until analyzed. Virus analyses were performed as described previously (16, 17). Briefly, the swab was diluted in 1 ml phosphate-buffered saline, and respiratory viruses and RV genome copy numbers were analyzed from extracted nucleic acids. RV A, B, and C, enteroviruses, and RSV A and B were detected by RT-PCR using in-house reverse transcriptase PCR at the Virus Diagnostic Laboratory, Department of Virology, University of Turku (18, 19). A multiplex PCR test (Seeplex RV12 ACE Detection, Seegene, Seoul, Korea) was used for detection of RV A and B, RSV A and B, parainfluenza virus types 1–3, human metapneumovirus, adenovirus, coronavirus (229E, NL63, OC43, and HKU1), and influenza A and B viruses. Human bocavirus-1 was analyzed using PCR and serology, as previously described (20). The blood eosinophil count (B-Eos) and serum levels of allergen-specific IgE were analyzed using routine diagnostics of the Central Laboratory of Turku University Hospital. Serum 25-hydroxyvitamin D measurements were performed by liquid chromatography-tandem mass spectrometry at Massachusetts General Hospital (Boston, MA, USA).

Peripheral blood mononuclear cell processing (isolation, cell cultures, and stimulation) and cytokine analyses

Blood samples for PBMCs were collected during the acute illness and convalescent phase (2-week follow-up). On each time point, the collected blood was stored on a rocking shaker in room temperature, and PBMC isolation of the samples was performed on the same day. PBMCs were separated from the blood using Ficoll-Paque™ PLUS (GE Healthcare, Amersham, UK) density gradient centrifugation according to the manufacturer's protocol. Further processing of the samples was done within the same day. PBMCs (>95% live cells) were then stimulated with anti-CD3/anti-CD28 for 24 h, which was selected as a polyclonal stimulant due to its T-cell activation capabilities (21, 22). Supernatants were collected, centrifuged, and stored in a -80°C refrigerator until analysis. Later, the supernatants were shipped inside dry ice containers to the Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland. Upon arrival, the samples were still frozen and stored in -80°C until analysis. Samples were defrosted right before the analyses and analyzed with Millipore HCYTOMAG-60K-36 and HCYP2MAG-62K-20 assay (Merck KGaA, Darmstadt, Germany) using the Bio-Plex 200 System utilizing the Bio-Plex Manager 6.0 Software (Bio-Rad, Cressier, Switzerland) to perform profiling of 56 different cytokines (Supplementary Tables S1–S3). Internal quality controls for all analytes were satisfactory. However, due to the limitations of quantitative multiplex ELISA profiling, a few cytokines did not reach the quantitative limit of detection (i.e., fluorescence was under or exceeded the quantification limit of the assay) (Supplementary Table S3). Each cytokine found in more than 50% of the patient samples within the limit of quantification was included for analyses (29/56, 52%), thus ensuring that conclusions would not be based on a minority of samples. Samples under the limit of detection were assigned half the value of the lower threshold of the assay (23, 24), and samples exceeding the limit of detection were set to the upper threshold of the assay (Supplementary Tables S1, S2) (25). A more detailed version of PBMC processing and cytokine analyses is provided in the Supplementary Material. The minimum and maximum values of the cytokines are shown in Supplementary Tables S1, S2.

Statistics

The normality of the data distribution was tested using the Kolmogorov–Smirnov test. Due to the skewness of the data, cytokine levels were $\log_{10}$ or x^2 transformed when appropriate. For other statistics, when appropriate, we used the two-sample t-test, Mann–Whitney U test, χ^2 test, Fisher exact test (when cell counts were <5), multivariable linear model analysis [in using the backward stepwise method to adjust for baseline differences, only statistically significant variables (P <.05) were included in the final model], Kruskal–Wallis H test, and negative binominal regression (JMP version 13.1.0, SAS Institute, Cary, NC, USA). A more detailed version of the statistics is provided in the Supplementary Material.

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Results

Study population

Originally, 125 children were eligible for the Vinku2 study, among whom 12 declined to continue and 113 were enrolled for clinical follow-up. For the current study, all other than sole RV- or RSV-affected children were excluded before further analyses. Of the 113 enrolled children, 63 were eligible for PBMC analysis in the acute phase, and ultimately, cytology was done in 56 children. In the convalescent phase, 22 were excluded from further analyses due to randomization to prednisolone. Thus, 34 children were eligible in the convalescent phase at 2 weeks, and ultimately, cytology was done in 30 children. On further study points at 2 and 12 months, 30 children had clinical data available (Figure 1).

Patient characteristics

The mean age of the study subjects was 12.5 months [interquartile range (IQR) 7.4–15.9], 69% were boys, 80% were treated as inpatients, 29% were atopic, and 20% had atopic eczema. Children infected with RV were older and heavier, had a higher blood eosinophil count, and had fewer preceding symptoms (wheezing, cough, rhinitis, fever) (all P <.05, Table 1). Due to these differences, the analyses comparing cytokine response from PBMCs were adjusted to the aforementioned variables using backward stepwise regression.

Differences in cytokine expression at study entry

Marked differences were observed in the cytokine response from PBMCs between the RV and RSV groups in response to

TABLE 1 Patient characteristics at study entry.

Characteristics	Rhinovirus (n = 47)	RSV (n = 9)	*P*-value
Age, months	13.5 (8.8–16.8)	6.1 (4.3–12.4)	**.006**
Male sex, no.	35 (74%)	4 (44%)	.11
Weight, kg	10.3 (2.1)	7.9 (1.2)	**.001**
Preceding wheezing, days	1 (1–1)	2 (1–3)	**.003**
Preceding cough, days	2 (2–3)	6 (3–7)	**.002**
Preceding rhinitis, days	3 (2–5)	5 (5–7)	**.005**
Preceding temperature over 37.5°C	1 (0–2)	2 (1–4)	**.002**
Clinical score, points	5 (4–8)	4 (2–8)	.31
Oxygen saturation, %	97 (95–98)	98 (96–98)	.27
Temperature, °C	37.4 (37.0–37.8)	37.1 (36.8–38.0)	.29
CRP, mg/L	13 (6–21)	4 (0–39)	.16
Eczema, no.	10 (22%)	1 (11%)	.53
Dr-dg atopic eczema, no.	10 (22%)	1 (12%)	.53
B-Eos (1×10^9/L)	0.52 (0.35–0.73)	0.08 (0.04–0.17)	**<.001**
B-Eos >0.4 $\times 10^9$/L	29 (64%)	1 (11%)	**<.001**
Sensitization, no.	15 (33%)	1 (13%)	.41
Food, no.	14 (30%)	1 (13%)	.42
Aero, no.	10 (22%)	0 (0%)	.33
Perennial, no.	9 (20%)	0 (0%)	.33
Parental asthma, no.	9 (19%)	0 (0%)	.33
Parental allergy, no.	29 (62%)	3 (33%)	.15
Parental smoking, no.	22 (47%)	3 (33%)	.72
Virus load, copies/ml	5,100 (590–21,000)	No data	
S-25-OHD, nmol/L	84 (72–99)	78 (73–103)	0.94
S-25-OHD_2, nmol/L	16 (0–30)	33 (18–56)	**0.04**
S-25-OHD_3, nmol/L	65 (43–80)	57 (26–69)	.18

RSV, respiratory syncytial virus; Dr-dg, doctor-diagnosed; B-Eos, blood eosinophil count; S-25-OHD, serum 25-hydroxyvitamin D.
Values are shown as mean (SD), median (interquartile range), or number (%).
Data were analyzed by two-sample t-test, Mann–Whitney U-test, χ^2 test, or Fisher exact test.
Bold text, statistical significance P <.05.

anti-CD3/anti-CD28 stimulation. In adjusted analyses, when compared to the RSV group, the RV group was characterized by a lower expression of interleukin 1 receptor antagonist (IL-1RA) (median 240 vs. 97 pg/ml), interleukin 1 beta (IL-1β) (30 vs. 3.5), and monocyte chemoattractant protein-1 (MCP-1) (7,500 vs. 6,900) and a higher expression of eotaxin-2 (350 vs. 740), thymus- and activation-regulated chemokine (TARC) (1.8 vs. 3.9), and epithelial-derived neutrophil-activating peptide 78 (ENA-78) (210 vs. 900) during the acute phase (all P <.05; Figure 2, Table 2 and Supplementary Table S4). Differences were also found in the expression of IL-6, I-309, and eotaxin-3, but significance was not reached (all.05 < P <.08; Figure 2, Table 2 and Supplementary Table S4). The biological mechanisms of these cytokines are described in Supplementary Table S5.

Differences in cytokine expression at the convalescent phase

In the convalescent phase (2 weeks later), in adjusted analyses, the cytokine profile of the RV group was characterized by a higher expression of fractalkine when compared to the RSV group (median 15 vs. 8.3 pg/ml, P = .02; Table 2, Figure 2 and Supplementary Table S4). An analysis of the change in cytokine expression between the acute and convalescent phases revealed an increased expression of fractalkine (median 1.1 vs. -4.6 pg/ml) and IL-1β (5.0 vs. -6.8, respectively) in the RV group, whereas in the RSV group, the expression was decreased (all P <.03). Moreover, the RV group was characterized by a decreased expression of I-309 (median -8.4 vs. 10 pg/ml) and TARC (-0.96 vs. 1.4), whereas in the RSV group, the expression was increased (all P <.05; Figure 2, Table 2 and Supplementary Table S4). A difference in the change in cytokine secretion was also observed with IL-6, but it did not reach significance (P = .06).

The association between the cytokine expression and the severity of acute illness

Considering the duration of hospitalization, several statistically significant interactions between the virus group and cytokine response were observed (all P <.04; Table 3 and Supplementary Table S6), indicating that the effect of cytokine response from PBMCs was different in the RV and RSV groups in hospitalization time. Increased expression of interferon gamma (IFN-γ), macrophage-derived chemokine (MDC), IL-1RA, and vascular endothelial growth factor (VEGF) was associated with shorter hospitalization times in the RSV group (all P <.02), but in the RV group, this difference was not significant (all P >.69; Table 3 and Supplementary Table S6). A significant virus group × cytokine expression interaction was observed with IL-6, but the expression of IL-6 was not associated with the duration of hospitalization in the RSV or RV group (all P >.08).

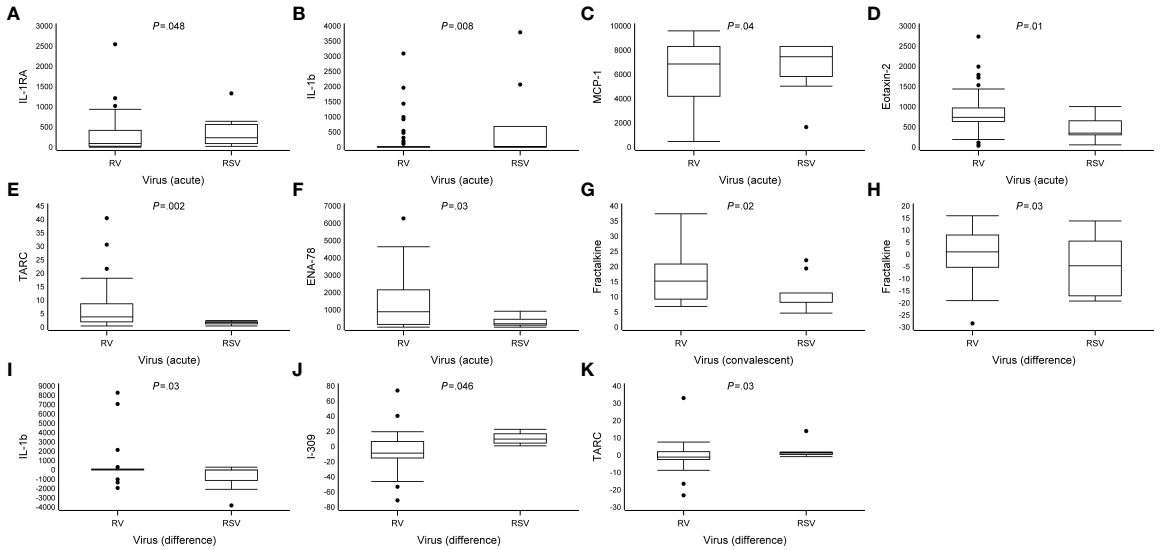

FIGURE 2
Differences in cytokine expression levels at study entry and convalescent phases. Data are presented as median [the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers]. Cytokine concentrations are presented as pg/ml. In the difference in the cytokine expression, multiple significant differences were observed between virus groups (RV vs. RSV, all P <.05) **(A–K)**.

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TABLE 2 Differences in cytokine expression levels at study entry and the convalescent phase.

Cytokine	Timing	RV n (acute) = 47 n (convalescent) = 20 n (difference) = 17	RSV n (acute) = 9 n (convalescent) = 10 n (difference) = 8	*P*-value univariate	*P*-value multivariate	Adjustments
Fractalkine	Acute	14 (8.3–28)	13 (8.3–24)	.46	.73	4
	Convalescent	15 (9.3–21)	8.3 (7.4–13)	**.03**	**.02**	-
	Difference	1.1 (-6.4–8.2)	-4.6 (-17–8.4)	.35	**.03**	1,3
IL-1RA	Acute	97 (28–420)	240 (78–600)	.23	**.048**	1
	Convalescent	170 (50–340)	140 (15–320)	.57	.98	3
	Difference	24 (-280–200)	-56 (-560–280)	.82	.36	-
IL-1β	Acute	3.5 (1.6–25)	30 (1.6–1,400)	.13	**.008**	1
	Convalescent	25 (1.6–270)	4.2 (1.6–120)	.32	.27	-
	Difference	5.0 (0–220)	-6.8 (-1,600–24)	.13	**.03**	-
IL-6	Acute	32 (6.8–380)	110 (17–2,100)	.28	.056	1
	Convalescent	66 (4.2–2,000)	29 (9.5–960)	.68	.70	-
	Difference	2.4 (-31–2,000)	-61 (-2,100–28)	.10	.06	-
MCP-1	Acute	6,900 (4,200–8,300)	7,500 (5,400–8,300)	.60	**.04**	1
	Convalescent	6,700 (4,300–8,300)	6,700 (4,900–7,700)	.93	.09	1,2,5
	Difference	0 (-3,800–2,700)	-160 (-2,200–1,100)	.68	.55	1,2
Eotaxin-2	Acute	740 (640–980)	350 (180–740)	**.01**	**.01**	-
	Convalescent	770 (450–1,100)	520 (330–980)	.25	.81	1,2,5
	Difference	-52 (-340–320)	37 (-140–290)	.56	.36	1,2
I-309	Acute	32 (19–67)	12 (7.8–21)	**.001**	.08	2
	Convalescent	24 (14–41)	29 (11–34)	.68	.58	5
	Difference	-8.4 (-20–7.8)	10 (3.8–17)	**.02**	**.046**	-
TARC	Acute	3.9 (2.0–8.9)	1.8 (0.88–2.1)	**.002**	**.002**	-
	Convalescent	4.4 (2.4–7.8)	2.9 (1.9–3.6)	.31	.64	6,7
	Difference	-0.96 (-2.4–2.5)	1.4 (0.28–1.9)	.11	**.03**	-
Eotaxin-3	Acute	110 (110–220)	110 (110–160)	.28	.07	7
	Convalescent	-	-	-	-	-
	Difference	-	-	-	-	-
ENA-78	Acute	900 (170–2,200)	210 (73–470)	**.02**	**.03**	-
	Convalescent	190 (65–1,800)	230 (50–1,100)	.86	.65	1,2,5
	Difference	-160 (-890–68)	-30 (-200–440)	.20	.32	2

Acute sample, samples drawn at study entry; Convalescent sample, samples drawn at the 2-week follow-up; Difference, difference in cytokine expression when comparing samples drawn at the 2-week follow-up and at study entry.
Values are shown as medians (interquartile range).
Data were analyzed by Mann–Whitney U-test, and by multivariable linear model analysis (after log- or x^2-transformation). The adjustments for immunologic analyses included baseline characteristics that significantly differed between the groups [Age = 1, weight = 2, duration of previous symptoms (rhinitis = 3, cough = 4, wheezing = 5, fever = 6), and B-Eos = 7 at entry]. A backward stepwise method was used for the final adjustment model separately for each cytokine. Only statistically significant variables (P <.05) were included in the final model.
All data are shown in Supplementary Table S4.
Bold text, statistical significance P <.05.

The association between the cytokine expression and recurrences and asthma

Although the incidence of relapses within 2 and 12 months differed between the RV and RSV groups [52% vs. 11% (P = .02) and 81% vs. 22% (P = .002), respectively], the exact cytokine response for this difference remained concealed because of the scarcity of the occurrence of relapses in the RSV group. However, in the RV group, a decreased expression of I-309 (CCL1) and TARC during the acute phase was associated with the occurrence of a new physician-confirmed wheezing episode within 2 months (median, relapse vs. no relapse, 21 vs. 48, P = .049, and 3.0 vs. 7.0, P = .03, respectively). Moreover, in the acute phase, an increased expression of IL-13 (6.0 vs. 1.5) and a decreased expression of I-309 (CCL1, 24 vs. 65) were associated with the occurrence of a new physician-confirmed wheezing episode within 12 months in the RV group (all P <.05; Figure 3, Table 4 and Supplementary Table S7). Overall, in the RV group and in the RSV group, due to the limited number of children, the association of the cytokine expression with 4-year asthma could not be assessed (data not shown).

The association between the cytokine expression and Rhinovirus species and genome load

No statistically significant differences were found in the cytokine expression between different RV species or according to the RV genome load (data not shown).

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TABLE 3 Association between cytokine expression and severity of acute illness (duration of hospitalization).

Cytokine	Group effect RV vs. RSV		Cytokine effect Expression of cytokine		Group × cytokine interaction effect
	Estimate (95% CI)	*P*	Estimate (95% CI)	*P*	*P*
IFN-γ	‡	‡	1.033‡ (0.878, 1.216) 0.651† (0.460, 0.922)	.69[§] **.02**[#]	**.03**
MDC	‡	‡	1.047‡ (0.841, 1.304) 0.609† (0.428, 0.868)	.68[§] **.001**[#]	**.02**
IL-1RA	‡	‡	1.026‡ (0.825, 1.276) 0.481† (0.289, 0.801)	.82[§] **.005**[#]	**.02**
IL-6	‡	‡	1.088‡ (0.938, 1.262) 0.764† (0.566, 1.032)	.26[§] .08[#]	**.04**
VEGF	‡	‡	1.190* (0.940, 1.510) 0.385† (0.290, 0.530)	.16[§] <**.0001**[#]	**.0004**

Data were analyzed by negative binominal regression with log-transformed cytokine level.
CI, confidence interval.
*Relative risk, RV group negative binomial regression.
†Relative risk, RSV group negative binomial regression.
[§]Group effect in the RV treatment arm.
[#]Group effect in the placebo treatment arm.
‡Due to the significant interaction, the cytokine effect was not estimated using all data. The effect of cytokine is presented separately in the RV and RSV groups.
All data are shown in Supplementary Table S6.
Bold text, statistical significance P <.05.

Discussion

To our knowledge, this study is the first to simultaneously analyze multiple cytokines from stimulated PBMCs in young first-time wheezing children infected with sole RV or sole RSV. Our main findings were that 1) there are distinct differences between cytokine responses from PBMCs and RV- and RSV-induced first wheezing episode, especially in the acute phase; 2) there is an association between the cytokine response from PBMCs and the severity of acute illness between the two virus groups; and 3) specific cytokine responses from PBMCs were associated with medium-term prognosis in the RV group.

Although the RV and RSV groups shared similarities in overall cytokine expression, acute-phase samples from the RV

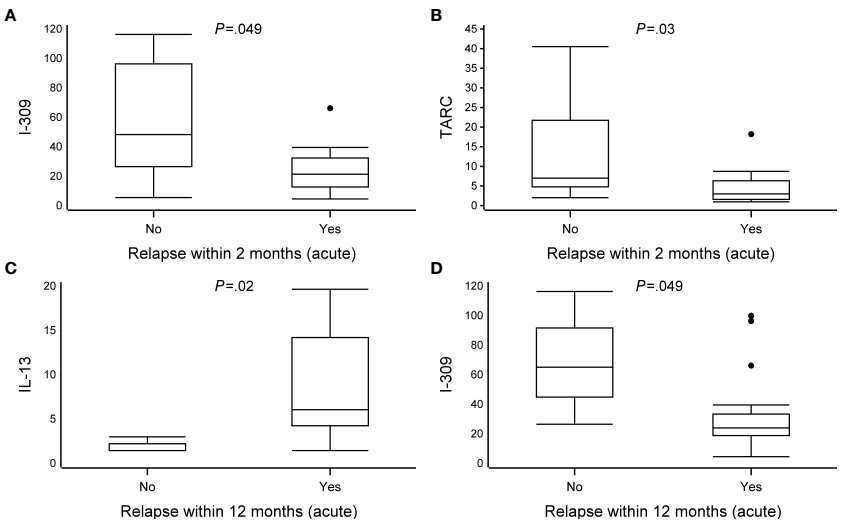

FIGURE 3
The association between cytokine expression and recurrences at 2 and 12 months. Data are presented as median [the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers]. Cytokine concentrations are presented as pg/ml. Due to scarcity of the occurrence of relapses in the RSV group, only patients from the RV group were included in the analyses. In the association between cytokine expression and recurrences at 2 and 12 months, multiple significant differences were observed between virus groups (relapse vs. no-relapse, all *P* <.05) **(A–D)**. An analysis of difference was excluded due to the small number of patients in the no-relapse group (n = 3). All data are shown in Supplementary Table S7.

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TABLE 4 The association between cytokine expression and recurrences.

Cytokine	Timing	2 months			12 months		
		No relapse n (acute) = 10 n (convalescent) = 10	Relapse n (acute) = 11 n (convalescent) = 10	*P*-value	No relapse n (acute) = 4 n (convalescent) = 4	Relapse n (acute) = 17 n (convalescent) = 16	*P*-value
Fractalkine	Acute	14 (8.3–30)	14 (8.3–28)	.89	9.5 (5.9–25)	17 (8.3–28)	.28
	Convalescent	16 (8.0–24)	13 (9.3–20)	.73	570 (120–1,850)	13 (8.5–20)	.07
IFN-γ	Acute	9.3 (1.5–51)	14 (1.5–81)	.91	1.5 (1.5–100)	14 (1.5–53)	.39
	Convalescent	63 (1.6–400)	8.0 (1.6–410)	.54	320 (32–1,400)	8.0 (1.6–350)	.07
IL-13	Acute	5.3 (1.5–16)	5.0 (1.5–9.4)	.97	1.5 (1.5–2.6)	6.0 (3.6–17)	**.02**
	Convalescent	4.7 (1.5–24)	3.2 (1.5–33)	.85	12 (3.5–42)	3.0 (1.5–7.6)	.31
IL-1β	Acute	1.6 (1.6–1,100)	1.6 (1.6–25)	.74	1.6 (1.6–2,300)	1.6 (1.6–170)	.84
	Convalescent	63 (1.6–2,000)	20 (1.6–500)	.51	220 (40–6,300)	12 (1.6–92)	.09
IL-6	Acute	13 (3.7–2,000)	55 (5.4–280)	.89	7.8 (2.3–1,500)	55 (5.9–1,100)	.42
	Convalescent	300 (3.4–2,000)	66 (4.1–2,400)	.82	2,000 (510–7,200)	54 (2.2–1,300)	.09
MCP-1	Acute	6,400 (3,900–8,300)	7,500 (4,900–8,300)	.47	4,200 (2,200–7,500)	7,500 (5,300–8,300)	.11
	Convalescent	4,700 (2,500–8,300)	7,900 (6,200–8,300)	.09	6,500 (4,700–8,300)	6,700 (3,200–8,300)	.70
MIP-1α	Acute	70 (27–900)	210 (120–1,400)	.12	27 (19–1,000)	200 (70–1,200)	.06
	Convalescent	430 (48–1,100)	450 (41–1,400)	.94	430 (390–1,920)	430 (32–1,300)	.45
I-309	Acute	48 (26–97)	21 (13–32)	**.049**	65 (36–100)	24 (16–36)	**.049**
	Convalescent	23 (12–43)	24 (16–42)	.88	16 (11–42)	26 (17–41)	.40
IL-16	Acute	78 (48–130)	70 (49–81)	.52	79 (49–140)	70 (48–87)	.57
	Convalescent	44 (33–75)	53 (38–77)	.60	34 (30–44)	54 (42–83)	.07
TARC	Acute	7.0 (4.1–26)	3.0 (1.6–6.3)	**.03**	5.9 (2.7–32)	4.0 (1.9–8.7)	.45
	Convalescent	4.0 (2.2–12)	4.1 (2.0–7.4)	.65	2.6 (2.4–56)	5.2 (2.0–7.8)	.85

Acute, samples drawn at study entry; Convalescent, samples drawn at the 2-week follow-up.
Values are shown as medians (interquartile range).
Data were analyzed by Mann–Whitney U-test.
Analysis of difference was excluded from the table due to the small number of patients in the no-relapse group (n = 3). All data are shown in Supplementary Table S7.
Bold text, statistical significance P <.05.

group were more of the Type 2 subtype, whereas in comparison, acute-phase samples from the RSV group were more of the Type 1 subtype as well as proinflammatory-associated cytokine profiles. Different types of cell-mediated immunity have been previously described in detail (26). At study entry, children infected with RV were characterized by a higher expression of eotaxin-2 and TARC; the former promotes the migration of eosinophils into the lungs (27), and the latter selectively binds to CCR4, leading to the activation of a type 2 immune response *via*, e.g., Th2 cells, ILC2, and airway eosinophils (28). The expression of ENA-78, although counterintuitive is primarily a neutrophil chemoattractant, was higher in children infected with RV (29). Children infected with RSV were characterized by a higher activity of proinflammatory-associated cytokines IL-1β and its antagonist IL-1RA. Of note, RSV-affected children were characterized by a higher expression of MCP-1, which is induced *via* alveolar epithelial damage and has a wide range of immunological functions such as immediate neutrophil recruitment and recruitment of fibrocytes and profibrotic macrophages as well as Th cell polarization (30, 31). However, MCP-1 is associated with both type 1 and type 2 immunity depending on environmental factors, such as tissue site, type of pathogen, and induction timing (32). Moreover, the macrophage polarization is regulated by the MCP-1–CCR2 axis, and blocking MCP-1 might lead to the upregulation of M1 polarization-associated genes (33).

In the convalescent phase, the former difference was more balanced. Only the expression of fractalkine was increased in the RV group when compared to the RSV group. These results are in line with findings from previous studies on nasopharyngeal aspirates and serum samples (34–36). Furthermore, regarding the change in the cytokine response from PBMCs, the RV group was characterized by a decreasing trend of Type 2-associated profile (I-309 and TARC) and an increasing trend of Type 1- and Th17-associated profiles (fractalkine and IL-1β, respectively). Although previous studies (from nasal swabs) have shown differences in IFN-γ and IL-10 expression between children infected with RV and RSV (37), one recent study showed that this difference dissipated when RV-bronchiolitis and RSV-bronchiolitis are accompanied by wheezing (38). This finding is in line with our results.

Interestingly, the cytokine response was not associated with the severity of illness in the RV group. However, in the RSV group, a higher expression of IFN-γ, MDC, IL-1RA, and VEGF was associated with a shorter duration of hospitalization, of which, a lower IFN-γ response has previously been shown to be

associated with a more severe clinical course (39). Of note, these cytokines are classified into a wide range of functional groups [IFN-γ, Type 1 subtype; MDC, Type 2 subtype; IL-1RA, proinflammatory activity; VEGF, regulatory T (Treg) cell activity], and these cytokines, for example, MDC (40), have overlapping properties (Type 1 and Type 2 subtypes as well as Treg).

According to a recent meta-analysis, RV-induced early wheezing has been shown to be more strongly associated with and a major risk factor for subsequent relapse or asthma when compared to RSV (6). Although data concerning the cytokine expression and long-term prognosis in young wheezing children are scarce, one study suggested that increased expression of macrophage inflammatory protein-1 alpha (MIP-1α) was associated with recurrences. However, the study did not separate viral etiologies (41). Another study on RSV-affected children showed that a decreased expression of tumor necrosis factor-alpha (TNF-α) was associated with recurrences (42). However, to our knowledge, no prior study has studied this setting in first-time RV-affected wheezing children, who are by the current knowledge at greatest risk of recurrences and development of asthma (6). In our data, increased expression of I-309 (CCL1) and TARC in the RV group was associated with fewer relapses within 2 months. Additionally, a decreased expression of IL-13 and an increased expression of I-309 (CCL1) were associated with fewer relapses within 12 months. Interestingly, a change in cytokine expression was also associated with relapse within 2 (IFN-α2) and 12 months [granulocyte colony-stimulating factor (G-CSF), fractalkine, IL-1RA, IL-1β, IL-6, MCP-1], suggesting that inadequate timing of cytokine expression might mitigate improper clearance of viral inflammation (Supplementary Table S7). However, the sample size at the 12-month follow-up for difference analysis in the no-relapse group was relatively small, and therefore, the corresponding results should be considered hypothesis-generating only.

The RV-induced wheezing illness has many asthma-like characteristics both clinically (dry cough, wheezing) and pathophysiologically (Type 2 subtype polarized immune response and pronounced atopic characteristics) (1). Although the expression of cytokines shared similarities, RV seemed to trigger more Type 2 subtype cytokine profile compared to RSV. Surprisingly, in the convalescent phase, this difference appeared to dissipate, and, ultimately, the remaining difference in the RV group was a higher expression of fractalkine, which induces chemotaxis and has antiviral properties (43). This finding is in line with the clinical phenotypes of RV- and RSV-induced bronchiolitis. The biological mechanisms of all significant cytokines are presented in Supplementary Table S5.

Although previous studies have observed differences in the cytokine expression between RV serotypes or RV genome loads (44), in our study, this difference remained concealed. This difference is possible due to differences in stimulation protocols and might be duplicable only under a similar stimulation protocol. Of note, the association of virus load with the severity of illness might be age-dependent (45). Moreover, although anti-CD3/anti-CD28 closely mimics physiological T-cell receptor (TCR)-mediated T-cell activation by antigen-presenting cells (46), different stimulation settings might present altered cytokine responses. Therefore, it is difficult to compare the results from our study with studies conducted with different stimulation protocols; our findings should be confirmed with those of similar settings. Interestingly, not all significantly different cytokines were T cell-derived. However, although stimulation of PBMCs with anti-CD3/anti-C28 activates T cells directly, it may activate other classes of lymphocytes indirectly, resulting in increased levels of non-T cell-derived cytokines (47). Our cytokine panels were broad, capable of measuring multiple inflammatory events, not just T-cell responses. However, this was partly intentional, since the study design is novel, and thus, we did not have a hypothesis of which responses and differences to anticipate.

The strengths of the current study included detailed viral diagnostics, careful characterization of the subjects, and a detailed prospective follow-up in the original trial, as well as comprehensive analyses of cytokine profiles. Our original hypothesis was to differentiate two diseases from one other, hence the absence of a "control" group. However, our study has some limitations. First, statistical power analyses were not performed, and the rather small sample size did not permit optimal analyses in the multivariable model. However, both study groups were composed of carefully characterized novel bronchiolitis subgroups. Second, a small volume of culture medium limited the ability to perform optimal dilution series, and the fluorescence of some of the cytokines exceeded the limit of quantification and therefore complicated the analyses for some cytokines. Furthermore, the number of affected cytokines was relatively low (Supplementary Table S3). Third, our results may not be generalizable to outpatients, since the majority (80%) of the subjects were enrolled from hospital wards and the sample size was too small to permit a meaningful analysis of inpatient vs. outpatient interactions. Lastly, the cytokine response from stimulated PBMCs may not reflect the response in the lower airways, and the study results may be generalizable to moderate-to-severe wheezing children only.

In summary, our current study and earlier trials support the emerging assumption that RV- and RSV-induced wheezing illnesses differentiate from each other at multiple levels—from clinical manifestation to cellular responses manifested by altered cytokine and chemokine profiles. Our findings also reveal new and early potential biomarkers for short- and medium-term prognoses in high-risk cohorts, mainly RV- or RSV-affected first-time wheezing children. However, further trials are warranted.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Turku University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

The study protocol and manuscript were written by the investigators. Data were collected by study physicians (TJ, RT, AL) and analyzed by investigators (PH and TJ), and while consulting statistician (TV). MK, MT, and PH performed literature search and reviewed previous trials. Viral analyses were supervised by the TV, participated in drafting the original study protocol, providing primers for rhinovirus detection, and writing the manuscript. BR, MA, and CA supervised the cytokine analyses. All authors were involved in writing or reviewing the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1044621/full#supplementary-material

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"Liquid biopsy" - extracellular vesicles as potential novel players towards precision medicine in asthma

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Extracellular vesicles (EVs) have emerged as vital mediators in intracellular communication in the lung microenvironment. Environmental exposure to various triggers (e.g., viruses, allergens) stimulates the EV-mediated cascade of pro-inflammatory responses that play a key role in the asthma pathomechanism. This complex EV-mediated crosstalk in the asthmatic lung microenvironment occurs between different cell types, including airway epithelial cells and immune cells. The cargo composition of EVs mirrors hereby the type and activation status of the parent cell. Therefore, EVs collected in a noninvasive way (e.g., in nasal lavage, serum) could inform on the disease status as a "liquid biopsy", which is particularly important in the pediatric population. As a heterogeneous disease, asthma with its distinct endotypes and phenotypes requires more investigation to develop novel diagnostics and personalized case management. Filling these knowledge gaps may be facilitated by further EV research. Here, we summarize the contribution of EVs in the lung microenvironment as potential novel players towards precision medicine in the development of asthma. Although rapidly evolving, the EV field is still in its infancy. However, it is expected that a better understanding of the role of EVs in the asthma pathomechanism will open up new horizons for precision medicine diagnostic and therapeutic solutions.

KEYWORDS

asthma, extracellular vesicle, exosome, precision medicine, intercellular communication, microRNA, allergy, inflammation

1 Introduction

Extracellular vesicles (EVs) are heterogenous membranous nanoparticles secreted from every cell type in our body, varying in size, morphology, and content (1). EVs carry a multitude of intracellular bioactive molecules between cells, including nucleic acids (e.g., DNA, messenger RNA (mRNA), and non-coding RNA), lipids, and proteins (2). Consequently, EVs play a vital role in intercellular communication (2, 3). It is well-established that, for instance, the composition of EV-associated RNA mirrors the type and activation status of their parent cell (4). Therefore, collecting EVs could inform on the disease status of the cells noninvasively, without removing the cells from the tissues, serving as a "liquid biopsy" (5).

EVs can be found in all human body fluids, taking part in regulation of various systemic processes such as immune function and local modulation of organ-specific reactions (6). Over the past decade, there has been a boost in EVs exploration across the scientific community, including the role of EVs in the complex lung microenvironment (7, 8).

Currently, no single optimal EVs' isolation method is recommended, since it depends on the desired balance between recovery and specificity and EV end use (e.g., basic vs. clinical research), however the most popular are ultracentrifugation and size-exclusion chromatography. According to MISEV2018, EV separation/isolation procedures should be reported in detail and multi-step characterization is needed to attribute reported function or a biomarker to EVs (1).

Rapidly accumulating evidence from *in vitro*, *in vivo*, and human studies has demonstrated that EVs have a potential role in asthma diagnostic options (9, 10). Asthma is a major chronic inflammatory lung disease affecting the quality of life of people of all ages worldwide, characterized by reversible airway obstruction causing dyspnea and cough (11, 12). Due to the heterogeneity of the disease (with various endotypes/phenotypes), there are still gaps to be filled in terms of improving patient education, implementing new diagnostics, and personalized case management (13, 14). In the asthma inflammatory microenvironment, both resident cells (e.g., epithelial, endothelial cells, fibroblasts) and inflammatory cells (e.g., eosinophils, mast cells, T cells) interact and exchange soluble mediators (15). Thus, the emerging EV field opens up new horizons to understanding asthma pathomechanism and offers new targets for precision medicine therapies.

Here, the recent updates on the role of EVs in the asthma pathomechanism in a cell type-specific manner will be discussed.

2 Types of extracellular vesicles in asthma

2.1 Epithelial cell-derived extracellular vesicles

Airway epithelial cells (AECs) play an important role in asthma development, both as a barrier and as modulators of the immune response, including innate mucosal defense (16, 17). AECs released EVs apically and basally, with side-specific functions of the miRNA cargo (Figure 1) (18, 19). Epithelial remodeling processes such as mucin hypersecretion can be significantly altered by the changed miRNA profile of AEC-EVs (16). In a recent *in vitro* study, stimulation with T2 cytokines of human AECs upregulated the release of EV proteins involved in chronic airway inflammation and decreased the expression of the antimicrobial peptide S100A7, suggesting that EVs mediate endotype-specific mechanisms related to asthma (20–22). Under T17 immune response conditions, EV-associated proteins increased neutrophil recruitment and promote neutrophilic airway inflammation (20).

Despite the current knowledge on various AECs phenotypes, we are still unable to easily detect or monitor airway epithelial cell viability/dysfunction in biosamples possessed in minimally invasive procedures, e.g., in nasal lavages, or exhaled breath condensate (EBC) (23, 24).

Some newly detected EV-related proteins were linked to asthma pathology: ezrin, contantin-1 (CNTN1) and Plexin B2 (PLXNB2). Firstly, ezrin as part of cytoskeletal elements of AEC, derives directly to EVs from AECs. EV-associated ezrin released by AECs contributed to IL-13-induced epithelial damage *via* the TNF-α-dependent pathway and was proposed as a biomarker of asthma control (25, 26). Secondly, CNTN1 promoted a Th2- and Th17-polarized immune activation through the Notch-2 pathway along with smooth muscle hyperresponsiveness (AHR) and mucus production in cell and mouse model (27). Thirdly, PLXNB2, a natural CD100 ligand, was released in AECs-EVs and augmented neutrophilic and monocytic airway inflammation in mice by activating macrophages *via* cleavage of CD100 by MMP14 (28). Furthermore, a lower expression of miR-34a, miR-92b, and miR-210 was found in EVs in nasal lavages from asthmatic children, and was associated with an obstruction of large ($FEV_1FVC_{\%pred}$) and small airways ($FEF_{25-75\%pred}$) (18).

2.2 Immune cells-derived extracellular vesicles

2.2.1 EVs from mast cells

Mast cells (MCs) have been recognized as active participants in innate as well as specific immune responses (29). Their EVs were shown to play a role in positive immune regulation, including recruiting B and T cells to the lungs and facilitating the priming of naïve T cells in an *in vitro* and a mouse model (30–33). EVs from bone marrow-derived MCs, carrying high-affinity IgE receptors (FcεRI), can bind to free IgE *via* FcεRI, induce an anti-IgE effect, thus decreasing IgE levels and inhibiting the allergic cascade (34). In a mouse model of allergic asthma, these EVs modulated not only airway inflammation and AHR, but also partially the remodeling in

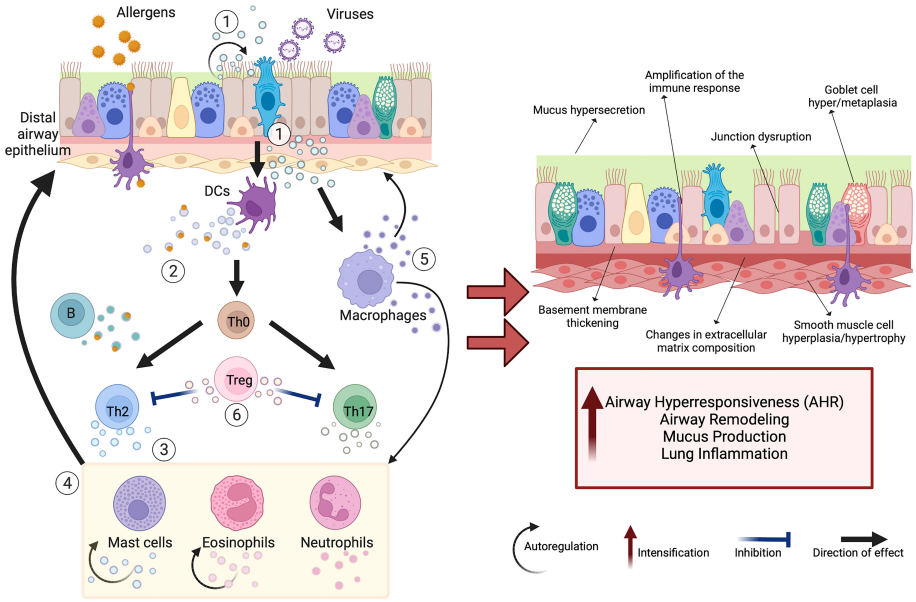

FIGURE 1
Schematic view of extracellular vesicles (EVs) as mediators of cellular interactions in the lung microenvironment. The complex EV-mediated crosstalk in the lung microenvironment occurs between many different cell types, including airway epithelial cells (AECs) and immune cells. Secreted EVs transfer specific cargo (e.g., proteins, miRNAs, mitochondria), which modulates the activity of target cells and can support tissue homeostasis or promote chronic respiratory changes. The exposure of airway epithelium to various environmental triggers (e.g., viruses, allergens) stimulates increased release of EVs with changed cargo composition that plays a critical role in asthma pathomechanism (including airway remodeling, airway hyperresponsiveness (AHR), mucus hypersecretion, increased lung inflammation). These EVs act in several ways (1): AEC-derived EVs target dendritic cells (DCs), macrophages, and themselves, promoting Th2- and Th17-polarized immune activation (2). DC-derived and B-cell-derived EVs can induce T-cell responses and serve as "antigen-presenting units" (3). T-cell-derived EVs cause Th2 skewing, eosinophil, neutrophil, and mast cell activation (4); that cells in turn produce EVs driving airway remodeling, and supply nitric oxide and reactive oxygen species, increasing the migration of other eosinophils and mast cells to the inflammation site (5). Macrophage-derived EVs also participate in airway remodeling, synthesizing leukotrienes and recruiting granulocytes to the inflammation site (6). In contrast, regulatory T cell (Treg)-derived EVs initiate anti-inflammatory activities. Created with BioRender.com.

chronic asthma (34). On the other hand, CC chemokine receptor (CCR1)-rich MCs-EVs could transfer CCR1 to other MCs *in vivo*, enhancing the co-activation of high-affinity IgE receptors (FcεRI) with CCR1 (35, 36).

Furthermore, it was proven that MCs-EVs take part in the modulation of oxidative stress. In asthmatic mice, miR-21 released in MCs-EVs promotes oxidative stress and inflammatory responses *via* the DDAH1/Wnt/β-catenin signaling axis (37). Moreover, mouse MCs-EVs exposed to oxidative stress had a different mRNA profile, transferring resistance to further oxidative damage to recipient cells; however, this mechanism is far from being elucidated (38).

2.2.2 EVs from dendritic cells

The lung-resident dendritic cells (DCs) participate in asthma development, as they have a pivotal role in establishing an allergen-specific Th2 response in the airways after stimulation with epithelial alarmins (39, 40). It has been shown that activated DCs released EVs containing various protein ligand-like OX40L that induced the proliferation of CD4+ T cells, elevated the level of IL-4, and drove Th2 differentiation *in vitro* (41). Furthermore, DC-derived EVs provided enzymes for the biosynthesis of leukotrienes (LTs), key pro-inflammatory mediators important in the pathogenesis of asthma, to smooth muscle cells. Besides, these EVs contained chemotactic eicosanoids and promoted granulocyte migration *in vitro* (42).

Majority of the studies on DCs-EVs were conducted using either bone marrow-derived or monocyte-derived DCs. Recently, it has been recognized that pulmonary DCs present different DCs subsets, including conventional types 1 and 2 DCs and plasmacytoid DCs, each playing a varying role in asthma pathogenesis (43). This evidence emphasizes the need for researchers to further specify the origin and effect of DCs-EVs in a subtype manner.

2.2.3 EVs from macrophages

The growing appreciation of macrophage plasticity and polarization in asthma pathogenesis reflects the pro-inflammatory properties of M1 polarized macrophages and the anti-inflammatory properties of M2 polarized macrophages (44–46). M2-like alveolar macrophages were reported to secrete suppressor of cytokine signaling (SOCS)-1 and SOCS-3

proteins within EVs (47, 48). Epithelial cells exposed to these EVs presented alleviated cytokine signaling *via* the JAK-STAT pathway activation. Thus, impaired delivery of SOCS proteins through EVs could serve as a significant mechanism in the dysregulated cytokine responses in asthma.

Under the stress condition, rat alveolar macrophages produced EVs carrying high levels of several microRNAs, including miR-21-5p involved in the oxidative stress. The EVs transported miR-21-5p to tracheal epithelial cells and promoted airway remodeling through the TGF-β1/Smad signaling pathway by targeting Smad7 (49). Similarly to DCs, EVs released from macrophages took part in the biosynthesis of LTs and granulocyte migration *in vitro* (42).

2.2.4 EVs from eosinophils

For decades, eosinophilia has been recognized as one of the prominent features of allergic asthma, and eosinophils are linked with the so-called T2-high asthma endotype (50). Eosinophils from asthmatic patients produced higher EV levels than those from healthy subjects, and these EVs contained molecules relevant to human asthma, such as EPO (eosinophil peroxidase), MBP (major basic protein), and eosinophil cationic protein (EPC) (51). Furthermore, these EVs induced epithelial cell apoptosis and smooth muscle cell proliferation, which are fundamental aspects of asthma pathogenesis (52). Moreover, they demonstrated properties to autoregulated and promoted eosinophil function in asthmatic inflammation by producing nitric oxide and reactive oxygen species (53). Moreover, eosinophil-derived EVs acted *in vivo* as a chemotactic factor for eosinophils due to the expression of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and integrin α2 (53). Altogether, eosinophilic EVs, acting in a feedback loop for their short-term lived parent cells, may prolong the inflammatory cellular infiltration and muscle and epithelial remodeling.

2.2.5 EVs from neutrophils

Neutrophilic airway infiltration is observed in non-allergic asthma, often in severe cases with poor response to corticosteroid treatment (54, 55). Proteomic composition of neutrophil-derived EVs, released spontaneously and upon lipopolysaccharides (LPS)-stimulation, significantly varied (56). The EVs from LPS-stimulated equine neutrophils contained higher levels of thrombospondin-1 and S100A9, and lower levels of neutrophil gelatinase–associated lipocalin and serpin peptidase inhibitor. This analysis provided evidence of neutrophils-derived EVs' contribution to tissue inflammation, apoptosis modulation, and proliferation of smooth muscle cells (56). Therefore, it supported the involvement of these EVs in the progression of asthma and the promotion of airway remodeling in severe and corticosteroid-insensitive patients with asthma. In another study, EV transfer of activated neutrophil-derived long non-coding RNA CRNDE was suggested to promote proliferation and migration of airway smooth muscle cells in asthma (57). Thereby, *in vivo* silencing of CRNDE reduced the thickness of bronchial smooth muscle in asthmatic mice.

2.2.6 EVs from T- and B-cells

T cells are of great importance in the adaptive immune responses during the asthma pathomechanism by participating in IgE antibody class switching, Th2 skewing, eosinophil and mast cell activation. A recent study, using a proteomic approach, demonstrated that EVs from activated human T cells had enhanced expression of the RAS/MAPK signaling pathway proteins, which induced ERK kinase phosphorylation in recipient immune cells *in vitro* (58). By contrast, regulatory T cells (Tregs) are a subpopulation of T cells that aim to maintain immunological tolerance, prevent autoimmunity, and limit other immune responses (59). They achieve this through various mechanisms, including Treg-derived EVs. Upon LPS stimulation, Tregs-derived EVs were found to transfer miR-150-5p and miR-142-3p to DCs, modulating DCs cytokine secretion towards an anti-inflammatory profile of increased IL-10 and decreased IL-6 (59). In another *in vitro* study, following activation of Tregs, CD73-expressing Treg-derived EVs demonstrated their suppressive activity through the production of adenosine (60).

Th2-mediated inflammation is also promoted by B cell EVs that carry allergen peptides on MHC molecules. This antigen-presenting property of B cell-derived EVs was shown *in vitro*. Birch-allergen (Bet v 1)-loaded-B-cell EVs induced T cell proliferation and secretion of IL-5 and IL-13 cytokines, key signals in driving airway inflammation and remodeling in asthma (61).

2.3 Mesenchymal-stem-cell-derived extracellular vesicles

Mesenchymal stem cells (MSCs) refer to a group of cells from bone marrow, adipose tissue and umbilical cord, that has the capacity for adherent growth (62, 63). MSCs are widely used in cell-based therapy due to their remarkable ability for proliferation, differentiation, and immune regulation. However, translating MSCs into clinic remains more difficult than expected (64). Currently, there is accumulating evidence on a promising role of MSCs-derived EVs - rather than MSCs itself, in asthma therapy. The summary of available data associating various MSCs-derived EVs with possible therapeutic options for asthma is presented in Table 1 and is discussed in more detail in chapter 3.3.

The above discussed role of EVs in asthma pathobiology, derived from major cell types within the lung microenvironment, is summarized in Figure 1.

3 Discussion and future research directions

Since the discovery of EVs decades ago, tremendous progress has been made in the deciphering how they are involved in intracellular communication, impacting various physiological and pathological events. However, this novel field is still in its infancy. To date, in asthma research, the EVs have been successfully isolated and characterized from several human biofluids, obtained *via* non-invasive methods, including saliva (75), nasal lavage fluid (18), EBC and sputum (76). Despite our growing knowledge on asthma heterogeneity, there is an unmet need for development of molecular markers guiding further the precision medicine approach, both in asthma diagnostic and therapeutic management. Notably, specially pediatricians worldwide face a great number of obstacles in the management of their asthmatic patients (77). It includes a precarious invasive collection of biological samples, lack of precise diagnostic tests, as children are often unable to perform lung function tests, and the common failure to recognize the variability of the course of asthma. This frequently leads to young asthma patients being underdiagnosed, undertreated, and inadequately controlled (78).

3.1 Role of EVs in asthma pathology

Viral respiratory infections, particularly with the rhinovirus (RV) and the respiratory syncytial virus (RSV), are major causes

TABLE 1 Mesenchymal-Stem-Cell-Derived extracellular vesicles (MSC-EVs) and their reported role in attenuating asthma pathomechanisms.

Cell type releasing EVs	EV isolation and characterization method	EV molecular signatures assessed	Key findings	Reference
Mouse ASCs	Filtration and differential ultracentrifugation; TEM, WB, NTA	CD81, CD40, calnexin	Intranasal administration of ASC-derived EVs to asthmatic mice reduced allergic airway inflammation (including the total inflammatory cells and eosinophils in the BALF), AHR, and also improved lung pathology.	Mun et al., 2021 (65)
Mouse ASCs	Filtration and differential ultracentrifugation; TEM, WB	Positive for: CD29, CD90, CD44, CD105, Negative for: CD34, vWF	Intravenous administration of mmu_circ_0001359-enriched ASC-derived EVs attenuated airway remodeling in asthmatic mouse model by targeting FoxO1 mediated M2-like macrophage polarization.	Shang et al., 2020 (66)
hBM-MSCs	Differential ultracentrifugation; TEM, WB, NTA	CD81, TSG101	Intravenous administration of hBM-MSC-derived EVs suppressed proliferation of bronchial smooth muscle cells and lung injury in asthmatic mice through the miR-188/JARID2/Wnt/β-catenin axis.	Shan et al., 2022 (67)
hBM-MSCs	Differential ultracentrifugation; TEM, WB	CD9, CD81	hBM-MSC-derived EVs promoted Tregs proliferation and immunosuppression capacity by upregulating suppressive cytokines IL-10 and TGF-β1 in PBMCs of asthmatic patient.	Du et al., 2018 (68)
hBM-MSCs	Differential ultracentrifugation; TEM, WB	CD9, CD63, CD81	hBM-MSC-derived EVs miR-1470 promoted the differentiation of CD4+CD25 +FOXP3+ Tregs isolated from peripheral blood of asthmatic patients by inducing the expression of P27KIP1.	Zhuansun et al., 2019 (69)
hBM-MSCs	Differential ultracentrifugation; TEM, NTA, flow cytometry	CD63, CD9, CD81	*In vitro* experiments showed that hBM-MSC-derived EVs modify DC function, and that delivery of miR-21-5p *via* EVs may be an important mechanistic pathway in asthma pathogenesis.	Reis et al., 2018 (70)
hUC-MSCs	Differential ultracentrifugation; WB, NTA	TSG101, HSP70, collagen-1, α-SMA, TGF-β1, HIF-1α, Gapdh, β-actin	Intravenous administration of hypoxic hUCMSC-derived EVs attenuated allergic airway inflammation and airway remodeling in chronic asthma mice more effectively than normoxic hUCMSC-derived EVs.	Dong et al., 2021 (71)
hUC-MSCs	Membrane affinity columns; TEM, NTA, flow cytometry	CD63, CD81	Intratracheal administration of hUCMSC-derived EVs ameliorated severe, steroid-resistant asthma in a mouse model by moderating inflammation, which is achieved by reshaping macrophage polarization *via* inhibition of TRAF1.	Dong et al., 2021 (72)
human iPSC-MSCs	Anion-exchange chromatography; TEM, flow cytometry	CD63, CD9, CD81, CD44, CD146, CD73, CD90, CD105	Intravenous administration of human iPSC-MSC-derived EVs reduced ILC2-dominant allergic airway inflammation at least partially through miR-146a-5p in asthmatic mouse model.	Fang et al., 2020 (73)
human iPSC-MSCs	Anion-exchange chromatography; TEM, flow cytometry	CD63, CD9, CD81, ALIX, TSG101, Calnexin	Intravenous administration of human iPSC-MSC-derived EVs ameliorated Th2-dominant allergic airway inflammation through immunoregulation on pulmonary macrophages in asthmatic mouse model.	Fang et al. (74)

ACSs, adipose stem cells; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; EVs , extracellular vesicles; hBM-MSCs, human bone marrowmesenchymal stem cells; hUC-MSCs ,human umbilical cord mesenchymal stem cells; iPSC-MSCs, Mesenchymal Stem Cells derived from Induced Pluripotent Stem Cells; IL, interleukin; ILC2, group 2 innate lymphoid cells; NTA, nanoparticle tracking analysis; PBMCs, peripheral blood mononuclear cells; TEM, transmission electron microscopy; TRAF1, tumor necrosis factor receptorassociated factor 1; Tregs, regulatory T cells; TSG101, tumor susceptibility 101; vWF, vonWillebrand Factor; WB, Western Blot.

of asthma development and exacerbations (79, 80). EVs offer new insight into the viral-induced chronic sequelae in the lung microenvironment. RSV-infected APCs were found to initiate EVs production that contain agents promoting inflammatory cytokine production *in vitro* in an alveolar epithelial cell line culture through IP-10, CCL2, and CXCL10 release (81). Interestingly, RSV infection of AECs was associated with significant changes in EVs RNA content (e.g., upregulated EVs-miRNAs: hsa-mir-6087, hsa-let-7e, hsa-miR-182-5p, hsa-miR-181b-5p; downregulated EVs-miRNAs: hsa-mir-223, hsa-mir-2964a, hsa-mir-205, hsa-mir-143) (81). Furthermore, EVs derived from virus- infected cells contain RSV components but do not transmit RSV infection (81). Moreover, these EVs induce pro-inflammatory mediator secretion in uninfected bystander cells, thereby impacting the additional way to modulate the immune responses during infection (81). Another comparative analysis of the airway secretory microRNAome in children under the age of three indicated that RV infection is associated with airway secretion of EVs enclosing miR-155, which *in silico* was predicted to regulate antiviral host immunity (82). Additionally, two distinct components of the inflammatory pathway regulating the immune response were revealed following RV infection and TLR3 (not TLR7) stimulation in asthmatic AECs. The first of these components highlighted a Tenascin-C protein release, including its upregulated expression in nasal lavage fluid (83). The second noted a secretion of EVs with a pro-inflammatory effect on AECs. By contrast, umbilical cord MSCs-derived EVs were shown to possess antiviral activities against other common human respiratory viruses (84).

Recent data revealed also the role of airway and even gut microbiota in asthma pathogenesis, and microbiota-derived EVs are emerging as the linking factor between microbiota and allergic reactions and asthma and are discussed in more detail elsewhere (85, 86).

3.2 EV-based asthma biomarkers

Recent evolution of high-throughput sequencing technology with advanced analytical methods enables precisely tracking changes in EVs' cargo composition and delineating complex interactions within molecular networks of asthma endotypes. For instance, circulating and/or EVs-related non-coding RNAs have been introduced as the novel, valuable biomarkers for different pathological conditions, including asthma. In particular, a promising makrer – the long non-coding RNA (lncRNA) impacts a wide range of biological processes (e.g., transcriptional activation, transcriptional interference by competitively inhibiting the effect of miRNA on downstream mRNA) (87). At this moment, the very first steps were made to explore the lncRNA-miRNA-mRNA regulatory network in T2-high asthma (57, 88). For example, PCAT19 was suggested as a lncRNA that may serve as a promising immune-related biomarker to distinguish between T2-high and T2-low asthma; however, it was not studied as a part of the EVs content (88). A novel study associated the levels of four serum EV-miRNAs (miR-21-5p, miR-126-3p, miR146a-5p, miR-215-5p) with the severity of asthmatic in adults (89). miR-21-5p and miR-126-3p, involved in Th1/Th2 differentiation, were specifically augmented in T2-high asthma. By contrast, IL-6-high patients with MSA, which were older, more obese, with higher neutrophil and basophil counts and TNF levels, manifested a decrease of miR-21-5p, miR-126-3p and miR-146a-5p. Interestingly, the researchers observed a trend towards a decreased expression of all studied miRNAs in mild asthmatics compared to healthy controls, probably due to the effect of inhaled corticosteroids (89). More research is clearly needed to clarify the role of EV-miRNAs in asthma endotyping/phenotyping and for undergoing treatment, and the current data are too limited to speculate about their possible use in clinical practice.

3.3 EV-based therapeutical strategies for asthma

Compared with other commonly used drug delivery carriers, such as liposomes, EVs have the advantages of high internal targeting ability, low immunogenicity, high modification flexibility and high biological barrier permeability, which open up an exciting avenue for modern drug delivery (63, 90). For example, experimentally engineered nanoparticles – extracellular vesicle's membrane from M2 macrophages combined with a lncRNA named methyltransferase 3A opposite strand (Dnmt3aos) smart silencer wrapped in a polylactic acid-glycolic acid (PLGA) copolymer – have been demonstrated to target M2 macrophages *in vitro* and *in vivo* and reduce airway inflammation, while not suppressing the overall immune function of the host (91). Therefore, these innovative nanoparticles can be an attractive candidate for the potential immunotherapy for asthma.

MSCs-EVs are the hot spot of current research as they have been identified to inherit the anti-inflammatory and immunomodulatory properties of stem cells, inducing the M2 polarization of macrophages, reducing inflammation, while avoiding the disadvantage of stem cells such as tumorigenicity (46). Importantly, MSCs-EVs present a specificity of the EV/macrophage axis (46). In allergic rhinitis, human MSCs-EVs could inhibit the differentiation of Th2 cells *via* the regulation of the miR-146a-5p/SERPINB2 pathway (92), which shall be extrapolated for allergic inflammation of lower airways.

Several potential therapeutic strategies for asthma were sparked by an expansion in multi-omic and EV research. Lee et al. suggested immunoregulatory effects of *Lactococcus lactis*-derived EVs by shifting the immune responses from Th2 to

Th1, mediated by DCs activation in allergic asthma (93). Studies on EV-based therapeutics for asthma treatment are highly anticipated. Preliminary findings reported an opportunity for inhalable dry powder mRNA vaccines based on EVs, which may pave the way to decreasing the risk for severe early-life respiratory infections like bronchiolitis and, consequently, de-risk further asthma development (79, 94, 95). Also, microbial EVs have recently been considered promising diagnostic and therapeutic tools for various inflammatory diseases (86).

3.4 Challenges and perspectives

Despite the magnitude of reports on EVs' advantages as diagnostic markers or therapeutic agents, there is still much to learn about their features, biological functions, and potential particle-particle interaction (96). Among the studies gathered in this review, considerable diversity in methodology could be noticed. Another key thing to remember is that most of the findings were reported by individual *in vitro* and animal studies, whereas studies on clinical samples from asthma patients are less numerous. With growing heterogeneity in EVs collection, isolation and characterization, clinical and basic researchers recognize the need for a more standardized scalable sample collection and processing methodology to obtain reproducible results. Work is underway to deliver an update in 2022 on detailed Minimal information for studies of extracellular vesicles 2018 (MISEV2018) recommendations by the International Society for Extracellular Vesicles (ISEV) (1). Moreover, from a technical point of view, long-term storage and a freeze–thaw cycle of biosamples may lead to disturbance of various structures. Evidence suggests storage of EVs isolated from bronchoalveolar lavage fluid (BALF), could destabilize the surface characteristics, morphological features, and protein content of these EVs (97). Due to the protein leakage from the EVs into the supernatant, around 50% of protein composition showed differences in abundance in BALF-EVs as a result of the storage at both +4°C and −80°C. Thus, it was proposed that airway EVs should be best analyzed immediately after isolation. This constitutes another critical challenge for the implementation of EVs into daily clinical practice.

4 Conclusions

Taken together, EVs represent a potential novel player towards precision medicine in the diagnosis and treatment of asthma. Although rapidly evolving, the EV field is still in its initial stage. In the near future, it is expected that research efforts in this area will enable further understanding of the role of EVs in the complex mechanisms underlying asthma pathogenesis; hence, providing a solid background for precision medicine diagnostic and therapeutic solutions.

Author contributions

DA prepared the first draft of the manuscript, designed the figure and the table, and made the editing following other authors' comments. AS-E, MC-K and WF reviewed the first draft of the manuscript, gave directions around the table and the figure design and worked on the editing of manuscript to help reach the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Type 2 cytokine genes as allergic asthma risk factors after viral bronchiolitis in early childhood

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Background: Genome-wide association studies of asthma have identified associations with variants in type-2 related genes. Also, specific interactions between genetic variants and viral bronchiolitis in the development of asthma has been suggested.

Objective: To conduct a gene-based analysis of genetic variants in type 2 cytokine related genes as risk factors for allergic asthma at school age, and further, to study their interaction with specific viral infections in early childhood.

Methods: A prospectively investigated cohort of children with previous bronchiolitis and controls came for follow-up at school age. The research visit, blinded to viral exposure, included detailed lung function tests, laboratory investigation, and questionnaires. Allergic asthma was defined as typical symptoms plus objective variable airway obstruction, in addition to laboratory verified atopy (elevated eosinophil count or sensitization to an allergen). Targeted and complete sequencing was performed for nine type 2 cytokine candidate genes: IL4, 5, 13, 25, 33 and 37, IL17RB, CRLF2 and TSLP.

Results: At follow-up, there were 109 children with genetic data, 91 with a history of bronchiolitis (46% respiratory syncytial virus, 24% human rhinovirus, 15% human metapneumovirus and 14% mixed viral etiology) and 18 without. The median age was 9.4 years (range 6-13) and 41 (38%) had laboratory verified atopy. Twenty-one children (19%) met the definition of allergic asthma. After adjusting for age, sex and five viral categories, IL33 achieved nominal significance ($p = 0.017$) for a positive association with allergic asthma development. In the gene-virus interaction analysis, the variant set in IL17RB

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demonstrated a nominally significant positive interaction with human metapneumovirus infection (p=0.05).

Conclusion: The results highlight the multifactorial nature of allergic asthma risk, with both viral infection and inherited genetic variants contributing to increasing risk. Results for IL33 and IL17RB were nominally significant and are potential candidate targets for designing therapeutics and early screening, but these results must be replicated in an independent study.

KEYWORDS

childhood asthma, viral respiratory infection, bronchiolitis, type 2 cytokines, genetic risk factors, gene-environment interaction (G × E)

Introduction

Asthma is the most common chronic disease among children, and is characterized by airway hyperresponsiveness, mucus hypersecretion, and an exaggerated inflammatory response usually dominated by type 2 cytokines (1). The possible role of inherited genetic risk variants related to type 2 cytokines and asthma development following viral bronchiolitis has not been considered in previous studies. There is also evidence that virus-induced bronchiolitis is associated with recurrent wheezing and asthma, particularly rhinovirus, that has been recognized as an important risk factor for asthma (2–4).

IL33 and thymic stromal lymphopoietin (TSLP) are cytokines functioning as alarmins that are released by the airway epithelium in response to viruses, allergens and other triggers and drive type 2 responses in asthma. IL33 and TSLP both have synergistic effects on type 2 cytokines production and eosinophil levels (5, 6). TSLP and IL33 may be important new targets individualized for asthma treatment, and antibody-based drugs for these pathways are being developed (7, 8). Tezepelumab is an anti-TSLP human monoclonal antibody that normalizes type 2 cytokine levels (9) and in clinical studies significantly reduced exacerbation rates vs placebo in patients with severe, uncontrolled asthma (10). Astegolimab is an anti-ST2 antibody that blocks IL33 signaling and has shown promise in reducing asthma exacerbations (11). Airway epithelial cells (AECs) from humans with asthma have increased TSLP mRNA levels (12), and over-expression of TSLP induces experimental asthma in mice (13). Rhinovirus (RV) infection in young children was found to be associated with elevated levels of TSLP (14). Further, respiratory syncytial virus (RSV) and metapneumovirus (MPV) infection induce production of TSLP in AECs *in vitro* and *in vivo*, respectively (15, 16).

IL25 also triggers expression of type 2 cytokines, activates type 2 innate lymphoid cells (ILC2 cells) and induces allergic asthma, all of which is dependent on its receptor, IL17RB (17–19). As with TSLP, overexpression of IL25 can induce asthma-like disease in mice (20, 21). Further, RV infected epithelial cells cultured from asthmatic patients have a higher capacity to produce IL25 (22). Using an established mouse model of allergic airway inflammation, infection with rhinovirus led to an increased production of IL25 as well as increased infiltration of eosinophils, neutrophils and basophils, secretion of mucus and production of type 2 cytokines. Importantly, these effects were neutralized if the animals were treated with an antibody directed against IL17RB (22).

While genome-wide association studies of asthma have identified associations with variants in genes that encode cytokines, cytokine receptors and related proteins [e.g. IL13, IL4, IL33, IL1RL1, IL1RL2, IL18R1, TSLP; see Demanais et al. (23)], an examination of genetic variants related to excessive type 2 cytokine production and their interactions with viral infections may shed insight into specific asthma mechanisms. Here we conduct a gene-based analysis of variants in type 2 cytokine related genes (i.e. IL4, 5, 13, 25, 33 and 37, IL17RB, CRLF2 and TSLP) as risk factors for allergic asthma at school age and study their interaction with specific viral infections in early childhood (Table 1). We hypothesized that variants in type 2 cytokine related genes are associated with the susceptibility to virus-induced early wheezing and development of allergic asthma at school age, and that such associations may show interactions with specific viral infections.

Abbreviations: AEC, airway epithelial cells; BoV, bocavirus; FEV_1, forced expiratory volume in one second; ILC2, type 2 innate lymphoid cells; ISAAC, International Study of Asthma and Allergies in Childhood; MAF, minor allele frequency; MPT, metacholine provocation test; MPV, metapneumovirus; NPS, nasopharyngeal secretion; RSV, respiratory syncytial virus; RV, rhinovirus; SNP, single nucleotide polymorphism; TSLP, thymic stromal lymphopoietin.

TABLE 1 Description of the selected candidate genes and their role in type 2 responses.

Gene	Function	Main role in type 2 responses in lung
IL4	Cytokine	Activation macrophage type 2, ILC2 (24)
IL5	Cytokine	Induces eosinophilia (24)
IL13	Cytokine	Activation of macrophage type 2, fibrosis, mucus production, IgE isotype switch (24)
IL17RB	Receptor for IL-25	Activated by IL25 (17–19)
IL25	Cytokine/alarmin	Triggers type 2 cytokine expression, activates ILC2 cells, induces allergic asthma. Induces asthma in mice (17–21)
IL33	Cytokine/alarmin	Triggers type 2 cytokine expression and eosinophil levels (5, 6) and IL 33 levels corelate with asthma disease severity (25)
IL37	Regulatory cytokine (IL-1 family)	Inhibition of Th1/Th2/Th17 inflammatory mediators. Suppress allergic inflammation in asthma by suppression of innate and acquired immunity (26, 27)
CRLF2	Receptor for TSLP	Forms a complex with TSLP and stimulates type 2 inflammatory proliferation and differentiation (28)
TSLP	Cytokine/alarmin	TSLP levels correlate with asthma severity (12) and induces type 2 cytokine production and eosinophil levels, synergism with IL-33 (5, 6)

Methods

Study subjects

The study population was recruited from a prospective, regional, population-based surveillance cohort of children admitted for airway infections (The St Olav Hospital Airway Project Cohort) (29–31). Eligible participants for the present follow-up study had initially been hospitalized between 2006 and 2012 and had nasopharyngeal secretion (NPS) routinely tested with in-house polymerase chain reaction (PCR) for 17 viruses. The inclusion criteria for the follow-up study were lower respiratory tract infection before 2 years of life with referral to pediatric specialist care, or children from the original control group consisting of surgery patients and without lower viral infection exposure (see Main Effects section for details on how viral infections were classified). All journals were revised after the research visit, and two subjects were excluded because of bacterial pneumonia at exposure.

The study was approved by the Regional Committee on Medical Research Ethics (REK number 2016/540). Informed consent for the clinical follow-up at school age was collected and included consent for the genetic analyses.

Study protocol

The data from the hospitalization was prospectively collected (29–31). The follow up after 6 years of life was systematically set up for research and was not part of routine practice. Examination of the children and data collection took place at the Research Facility Ward at St Olav Hospital between March 2017 and June 2019 and was led by a pediatric asthma specialist together with a trained research nurse, both blinded for viral exposure. The research visit included lung function testing, blood sample draw after local anesthesia, a systematic medical history and clinical exam by the study pediatrician. A digital questionnaire for care givers was based on The International Study of Asthma and Allergies in Childhood Questionnaires (ISAAC) (32) related to history of asthma and atopy in childhood.

Lung function testing

Lung function was measured according to established guidelines (33, 34) using a Vyntus Pneumo APS spirometer. The results were obtained both as absolute values and in % of predicted values according to EU standard data for age, height and sex. After initial baseline flow-volume spirometry attempts all children were considered for a methacholine provocation test (MPT) to measure eventual bronchial hyper-reactivity. Contraindications for the MPT was any airway infection, ongoing antibiotic treatment or asthma exacerbation during the last two weeks, FEV_1 <70%, clinical signs of airway obstruction, technical inability to perform reproducible and repeated spirometries. The MPT test was performed with an inhalation-synchronized dosimetry nebulizer. The test procedure implicated doubling of doses of methacholine administered until a 20% fall in FEV_1 or to a maximum cumulative dose of 1,447 mg methacholine. All children concluded the lung function reversibility testing with a spirometry after inhalation of salbutamol 0.4 mg (Ventoline) through a spacer (Optichamber Diamond by Philips Respironics).

Phenotype definition

The allergic asthma diagnosis required both a clinical diagnosis of asthma based on recent guidelines and one of the

laboratory criteria (35). Clinical asthma was diagnosed if either 1) presence of one or more typical asthma symptoms in history plus variable expiratory airflow limitation at study visit or 2) recent asthma diagnosed by a pediatric specialist and ongoing treatment with inhaled corticosteroids at research visit. Asthma symptoms were defined as cough at night or prolonged cough > 14 days during viral infections, exercise induced either chest tightness, wheeze or shortness of breath, or recognition of these symptoms during methacholine testing when inducing airflow limitation. Variable airflow expiratory limitation, or reversibility, was defined as either a fall in FEV_1 of ≥ 20% during the metacholine challenge, or for those who only performed spirometry an increase of FEV_1 ≥ 12% compared to the baseline spirometry after salbutamol inhalation. The laboratory criteria were defined as either allergic sensitization (specific IgE > 0.35 kU/L to any of the tested 11 aeroallergens or 6 food allergens) or blood eosinophil count≥300 cells/μL.

Sequencing

Sequence data was generated for 111 of these subjects. Sequencing was done for nine candidate genes (i.e. IL4, 5, 13, 25, 33 and 37, IL17RB, CRLF2 and TSLP) to capture all exons, intron/exon boundaries and up- and down-stream regulatory regions. Library prep was conducted using AmpliSeq for Illumina custom DNA panel (Illumina Inc., CA), which contained 203 amplicons (in two pools) with average amplicon length of 244 bp and covered in total 28,546 base pairs (27,076 covered bases). In brief, 5 ng DNA was used as input in two target PCR amplification reactions (one of each amplicon pool) of 18 cycles (99°C for 15 sec and 60°C for 4 minutes). Secondly, FuPa reagent was used to digest primer dimers and partially digest amplicons. In the next step dual sample indexes were added. The amplicon products were then cleaned up by using Mag-Bind® TotalPure NGS (Omega Bio-tek, Inc., Georgia, GA, USA) to purify the specific amplicons away from free primers and primer dimer species. A second amplification step which amplifies the libraries to ensure sufficient quantity for sequencing on the Illumina platform were then performed, prior to a second clean up by using Mag-Bind® TotalPure NGS (Omega Bio-tek, Inc., Georgia, GA, USA). Finally, a validation of the libraries was performed using an Agilent High Sensitivity DNA Kit on a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Libraries were normalized and pooled to 8 pM and subjected to clustering on two MiSeq V3 flowcell. Finally, paired end read sequencing was performed for 2X150 cycles on a MiSeq instrument (Illumina, Inc. San Diego, CA, USA), according to the 'manufacturer's instructions. Base calling was done on the MiSeq instrument by RTA v1.18.54. FASTQ files were generated using bcl2fastq2 conversion software v2.17 (Illumina, Inc. San Diego, CA, USA).

Sequence data alignment and quality filtering

Sequencing adapters and low-quality reads were removed using Atropos (v. 1.1.18, flags -q 15 and -m 15). The processed reads were aligned against the human genome (hg38) using BWA MEM (v. 0.7.15, flags -M and -v 2). The BAM files were sorted and read group (RG) information was added, followed by cleaning, soft-clipping, and marking of duplicate reads using AddOrReplaceReadGroups, CleanSam, and MarkDuplicates (OPTICAL_DUPLICATE_PIXEL_DISTANCE = 2500, CREATE_INDEX = TRUE) from Picard tools (v. 2.18.5). Base quality scores were recalibrated using GATK's (v. 4.0.4.0) BaseRecalibrator and ApplyBQSR based on known sites of variation (dbSNP v. 151 and Mills and 1000G gold standard from GATK's resource bundle). Variants were then called for each sample separately using GATK's HaplotypeCaller, before merging all resulting gvcf files with GATK's CombineGVCFs and calling variants on the joint file using GATK's GenotypeGVCFs (flag -G StandardAnnotation). Called SNVs and indels were processed separately to filter high quality variants using 'GATK's VariantFiltration, keeping SNVs with QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, and ReadPosRankSum < -8.0, and indels with QD < 2.0, FS > 200.0, and ReadPosRankSum < -20.0.

Subject quality control

Following variant calling and initial quality control, we examined the per subject call rate to determine if there were any subjects with an indication of low-quality data across all variants. All included subjects (N=109) had a call rate across the 350 variants > 85% and therefore all were included in the analysis.

SNP quality control and annotation

Among all variants identified by sequencing, 350 were annotated to the nine genes by UCSC GTEx Gene V8 (within 20kb of the gene start and stop site). We excluded variants with call rate < 90% (N=41) and then with a Hardy–Weinberg equilibrium P-values < 10^{-4} (N=5) leaving 304 variants for analysis. The included variants were annotated by ANNOVAR (36) for functional annotation (Supplementary Table S1).

Main effects

Our main analysis examined the association with allergic asthma case/controls status. Gene-based analysis integrates

information across all included SNPs within one gene or genomic region to improve the statistical power of the association analysis. The gene-based analysis was conducted using SKAT (37). with all parameters being default values. When the sample size is less than 2000, SKAT automatically applies small sample size adjustment. Missing genotype values are imputed based on Hardy-Weinberg equilibrium for binary outcomes. Covariates include age and sex. Viral infections were classified into five categories: 1) sole RSV infection; 2) sole RV infection; 3) sole MPV infection or in combination with viruses other than RSV, RV or bocavirus (BoV); 4) mixed infection (co-infections including RSV, RV, MPV or BoV and any other virus); 5) no viral infection. Dummy coding was utilized to adjust for viral infections in the analysis. Study-wise statistical significance was determined by Bonferroni correction for the nine genes at alpha = 0.05 ($p<0.05/9 = 0.0056$) and nominal significance was considered at $p <= 0.05$.

SNP-level association tests were conducted using command "SKATBinary_Single" and p-values were computed. We ran logistic regression to calculate the direction of effect of each SNP.

Sensitivity analyses

Given the small sample size, we conducted several sensitivity analyses to test the robustness of our results to various parameters: 1) we applied the SKAT-O (38) method instead of SKAT since SKAT-O combines both the variance components and burden tests; 2) we analyzed only low-frequency variants with a minor MAF < 5% and 3) instead of dummy-coding the five indicator variables representing each viral category and adjusting for each, we merged this into one dichotomous covariate which indicates whether (viral categories 1-4) or not (viral category 5) an individual had been exposed to viral bronchiolitis.

Interaction analysis

We tested the interaction between the specific virus types, rhinovirus, RSV and MPV, and each of the nine gene-based variant sets using the R package "iSKAT" (39). We utilized the same categorization for rhinovirus, RSV, MPV described previously and adjustment for the other virus categories not contained in the interaction was done using dummy coding. In addition, age and sex were included as covariates in each model. All parameters were default values.

Results

There were 135 participants who came to the follow-up visit, 109 of these children were available for genetic analysis. Table 2 shows their clinical characteristics. The age at follow-up ranged from 6 – 13 years and there were 41 females (38%). There were 42 from the RSV group, 22 from the RV group, 14 from the MPV group, 13 from the mixed infection group and 18 with no bronchiolitis exposure. Twenty-one children (19%) met the definition for allergic asthma, all of them corresponded to mild asthma. As expected children with RV bronchiolitis exposure were more likely to have allergic asthma (29%) than not (18%), the opposite was seen for the RSV group (19% with allergic asthma vs 43% without).

Targeted sequencing of nine candidate genes was performed to identify all potential variants in each gene (N=350 variants with 304 that passed quality control [see Methods]). Using all coding variants annotated by ANNOVAR (36) to each of the nine genes, aggregate gene-based analyses were conducted for each of the variant sets. We examined the association between each of the nine gene-based aggregate of variants with allergic asthma case/controls status. After adjusting for age, sex and the five viral categories, IL33 achieved nominal significance ($P = 0.017$, Table 3). Among the samples, IL33 contained 37 coding variants, but none of the single variants achieved statistical significance after adjusting for all 304 variants but five achieved nominal significance ($p<0.05$), all of which showed an association between the minor allele and increased risk of allergic asthma (Table 4).

To test if the IL33 gene-based association was robust to different inclusion/exclusion criteria for variants, subjects and analysis method, we performed a series of sensitivity analyses (Table 5). All sensitivity analyses were nominally significant indicating that the results are robust to these inclusion/exclusion criteria. Further, restricting to low frequency variants (MAF < 5%) yielded the same p-value ($p=0.017$), indicating that low frequency variants are driving the signal in IL33 rather than common variants.

We conducted a gene-virus analysis to identify potential interactions with specific viral types that may be interacting with the gene-based variant sets to alter the risk for asthma. The variant set in IL17RB demonstrated a nominally significant interaction with MPV infection ($p=0.050$; Table 6).

Discussion

Using children from a well described bronchiolitis cohort and their controls that collected detailed phenotype and clinical data at school-age, we were able to detect a nominal association with asthma for the gene IL33. Further, we were able to detect a nominally significant interaction with MPV infection and variants in IL17RB despite a small sample size. These are promising findings that will need to be confirmed in a larger study sample and contribute to the growing literature of gene-virus interactions contributing to asthma development.

TABLE 2 Clinical characteristics study subjects.

Characteristics	Allergic asthma at follow-up (%[1])	Controls (%[1])
Total	21	88
Age [years, IQ-range]	9.1 (8.0 -11.1)	9.5 (8.5 – 10.8)
Female	11 (52)	31 (35)
Parental asthma or allergy	18 (86)	57 (65)
Sensitization or eosinophilia	21 (100)	20 (23)
Virus etiology[2]		
RSV[3]	4 (19)	38 (43)
RV[4]	6 (29)	16 (18)
MPV[5]	4 (19)	10 (11)
Mixed infection[6]	4 (19)	9 (10)
no respiratory tract infection exposure[7]	3 (14)	15 (17)

[1]Unless otherwise noted.
[2]For the four virus positive categories, these subjects were hospitalized with respiratory tract infection with viral etiology based on PCR on nasopharyngeal samples (31).
[3]RSV: sole RSV infection.
[4]RV: sole RV infection.
[5]MPV: sole MPV infection or in combination with viruses other than RSV, RV or BoV.
[6]Mixed infection: co-infections including RSV, RV, MPV or BoV and any other virus.
[7]no viral infection.

Type 2 cytokine hyperresponsiveness is a hallmark of both asthma and bronchiolitis following rhinovirus (40), RSV (41) and MPV (42, 43) infections. Therefore, an examination of variants within the genes that code for cytokines and their receptors is warranted to identify their potential role in interactions with viral infection and asthma risk. Previous studies identified an association between asthma susceptibility and a variant in IL17RB (44) as well as IL33 which was also associated across diverse ancestries (45, 46).

TABLE 3 Gene-based association results using SKAT.

Gene[1]	P-value	Variants (N)	MAC[2]	m[3]
IL37	0.850	18	362	109
IL17RB	0.229	23	414	98
TSLP	0.425	47	686	102
IL5	0.293	8	24	18
IL13	0.457	12	184	65
IL4	0.158	6	120	34
IL33	**0.017**	**37**	**674**	**102**
IL25	0.898	19	263	71
CRLF2	0.094	26	121	78

[1]Genes with a nominally significant (p<0.05) p-value are indicated in bold.
[2]MAC: total minor allele count.
[3]m: number of subjects with minor allele(s).

IL33 induces expression of a number of Th2 cytokines and thus increases eosinophilic inflammation. A rare loss of function variant in IL33 has been shown to be protective against asthma (8). While our analysis did not include any loss of function variants, this does further demonstrate the potential importance of low frequency and rare variants in the etiology of asthma. Variants included in our analysis of IL33 both increased and decreased risk for asthma development (Table 4), however, we do not yet know if the minor alleles increase or decrease expression of IL33 in relevant tissues following viral exposure.

IL17RB encodes the cytokine receptor interleukin-17 receptor B, a receptor specific for IL17B and IL25 (IL17E). IL17 knock-out mice appear to have a lower inflammatory response following MPV infection (42). Further, there is evidence of an intronic IL17RB variant (+5561G>A) in which the minor allele is protective against asthma and associated with lower IL17RB expression (44). Taken together, these observations suggest that genetic variations that increase IL17RB expression may increase the risk for asthma, particularly in those exposed to respiratory infections.

Unlike previous studies of gene-virus interactions for asthma, we chose a sequence-based approach to identify all variants in the coding regions of our selected candidate genes. Our primarily analyses aggregated variants across all those annotated to each gene rather than examining them individually. Our results for IL33 demonstrate that while several single variants within this gene were nominally associated with asthma, no single variant achieved the same

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TABLE 4 Single variant results for IL33.

Variant[1]	Chr[2]	Pos[3]	Gene[4]	P-value[5]	MAF[6]	Direction[7]	Annotation[8]
rs555998964	9	6215178	IL33	0.229	0.005	+	UTR5
rs746808236	9	6215231	IL33	0.608	0.005	–	intronic
rs1431798023	9	6215727	IL33	0.125	0.005	+	intronic
rs17498168	9	6237186	IL33	0.211	0.028	–	intronic
NA	9	6250936	IL33	0.496	0.037	–	intronic
rs1317230	9	6251012	IL33	0.205	0.317	–	intronic
rs73398552	**9**	**6252689**	**IL33**	**0.039**	**0.041**	**+**	**intronic**
rs149045797	9	6252690	IL33	0.535	0.005	–	intronic
rs1397714619	9	6252710	IL33	0.142	0.005	+	intronic
NA	9	6252720	IL33	0.142	0.005	+	intronic
rs10975519	9	6253571	IL33	0.861	0.367	+	exonic (synonymous)
rs562550122	9	6253690	IL33	0.068	0.005	+	intronic
rs10975520	9	6253710	IL33	0.660	0.358	+	intronic
NA	9	6255774	IL33	0.626	0.005	–	intronic
NA	9	6255781	IL33	0.626	0.005	–	intronic
rs12336076	9	6255789	IL33	0.602	0.321	–	intronic
NA	9	6255807	IL33	0.600	0.239	–	intronic
NA	9	6255808	IL33	0.785	0.028	–	intronic
NA	9	6255814	IL33	0.784	0.028	–	.
rs1013300156	9	6255821	IL33	0.819	0.133	+	intronic
NA	9	6255826	IL33	0.870	0.101	–	intronic
rs1332290	9	6255881	IL33	0.403	0.417	+	intronic
rs146597587	9	6255967	IL33	0.535	0.005	–	splicing
rs1240440599	9	6256010	IL33	0.732	0.005	–	exonic (synonymous)
NA	9	6256013	IL33	0.732	0.005	–	exonic (synonymous)
rs35375147	9	6256078	IL33	0.344	0.014	–	exonic (synonymous)
rs1048274	9	6256292	IL33	0.835	0.372	–	UTR3
NA	9	6256471	IL33	0.542	0.050	–	UTR3
NA	9	6256476	IL33	0.789	0.023	+	UTR3
rs55726619	**9**	**6256678**	**IL33**	**0.039**	**0.041**	**+**	**UTR3**
NA	**9**	**6256741**	**IL33**	**0.035**	**0.005**	**+**	**UTR3**
rs12000491	**9**	**6257367**	**IL33**	**0.039**	**0.041**	**+**	**UTR3**
rs189961633	9	6257571	IL33	0.787	0.009	+	UTR3
NA	9	6257597	IL33	0.099	0.014	+	UTR3
rs553100713	9	6257606	IL33	0.068	0.009	+	UTR3

(Continued)

TABLE 4 Continued

Variant[1]	Chr[2]	Pos[3]	Gene[4]	P-value[5]	MAF[6]	Direction[7]	Annotation[8]
NA	9	6257718	IL33	0.607	0.005	–	UTR3
rs73398574	**9**	**6257724**	**IL33**	**0.039**	**0.041**	+	**UTR3**

[1]Variant identification number (rsID), if assigned. Nominally significant associations with allergic asthma are presented in bold.
[2]Chromosome.
[3]Base pair position on the chromosome, in hg38 coordinates.
[4]Gene in which the variant was identified.
[5]P-value from the SKAT test.
[6]Minor Allele Frequency.
[7]Direction of effect estimated using logistic regression, where "+" represents a variant with a minor allele that has a higher frequency among asthmatic children and "-" represents a variant with a minor allele that has a lower frequency among asthmatic children.
[8]Functional annotation according to refGene; UTR3 = 3' untranslated region; UTR5 = 5' untranslated region.

TABLE 5 Sensitivity analysis results (P-values).

Gene[1]	SKAT	SKAT-O	MAF<5% (M = 210)	Virus/no-virus covariate coding
IL37	0.850	0.504	0.882	0.901
IL17RB	0.229	0.376	0.231	0.129
TSLP	0.425	0.638	0.547	0.422
IL5	0.293	0.428	0.293	0.238
IL13	0.457	0.520	0.563	0.682
IL4	0.158	0.205	0.120	0.144
IL33	**0.017**	**0.020**	**0.017**	**0.042**
IL25	0.898	0.104	0.952	0.949
CRLF2	0.094	0.172	0.084	0.171

[1]Genes with a nominally significant interaction p-value are indicated in bold along with the p-value for the respective tests.

TABLE 6 Gene x virus interaction analysis results (P-values).

Gene[1]	RV	RSV	MPV
IL37	0.694	0.065	0.450
IL17RB	**0.510**	**0.846**	**0.050**
TSLP	0.631	0.884	0.201
IL5	0.612	NA[2]	0.241
IL13	0.146	0.822	0.506
IL4	0.622	0.186	0.814
IL33	0.490	0.672	0.370
IL25	0.076	0.097	0.766
CRLF2	0.545	0.510	0.258

[1]A gene(s) with a nominally significant interaction p-value are indicated in bold along with the p-value for the respective test(s).
[2]NA: Due to the fact that no subject with a rare variant in IL5 was also positive for an RSV infection the interaction matrix was 0 across all subjects and thus the interaction analysis was unable to be performed.

level of statistical significance as they did in aggregate. By using a variance-components based method to analyze our aggregated variants, we increased our power to detect associations since the directions of effect of the variants in the IL33 gene were relatively evenly split between risk and protective. Further, our sensitivity results show that the gene-based association between IL33 and asthma is driven by low frequency variants (MAF <5%) which we had no power to detect associations with individually.

Unfortunately, we do not detect any association signals between allergic asthma and the remaining eight candidate genes, despite all of them being strong candidate genes for this phenotype. This could be due to out sequencing strategy of focusing primarily on coding and regulatory regions which may miss many of the tagging genetic variants that were identified in previous genetic studies. Further, we are likely underpowered to detect associations in many of these genes.

These results highlight the multifactorial nature of asthma risk, with both viral infection and inherited genetic variants contributing to increasing asthma risk. While the results presented for both IL33 and IL17RB are statistically significant and are potential targets for designing therapeutics and early

screening, these results must be replicated in an independent study. Further work must also be done to determine if the genetic effects on asthma risk are dependent on specific treatment responses, a question requiring a much larger sample size.

Data availability statement

The dataset presented in this article is not readily available because Norwegian law limits the sharing of sensitive data which includes genetic sequence data. Requests to access the dataset should be directed to Drs. Kari Risnes and Andrew DeWan.

Ethics statement

The study was approved by the Regional Committee on Medical Research Ethics (REK number 2016/540). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

KR, IJ and AD conceived of the overall study design. IJ, AM, HD and KR led the clinical follow-up and data collection. AM performed the clinical data analysis and phenotype generation. ZD performed the genetic analyses. KR, IJ and AD supervised the work. ZD, AM, IJ, TJ, HD, KR and AD interpreted the results. ZD, AM, KR and AD drafted the initial manuscript. All authors contributed to and approved the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1054119/full#supplementary-material

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Integrative genetics-metabolomics analysis of infant bronchiolitis-childhood asthma link: A multicenter prospective study

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Background: Infants with bronchiolitis are at high risk for developing childhood asthma. While genome-wide association studies suggest common genetic susceptibilities between these conditions, the mechanisms underlying the link remain unclear.

Objective: Through integrated genetics-metabolomics analysis in this high-risk population, we sought to identify genetically driven metabolites associated with asthma development and genetic loci associated with both these metabolites and asthma susceptibility.

Methods: In a multicenter prospective cohort study of infants hospitalized for bronchiolitis, we profiled the nasopharyngeal metabolome and genotyped the whole genome at hospitalization. We identified asthma-related metabolites from 283 measured compounds and conducted metabolite quantitative trait loci (mtQTL) analyses. We further examined the mtQTL associations by testing shared genetic loci for metabolites and asthma using colocalization analysis and the concordance between the loci and known asthma-susceptibility genes.

Results: In 744 infants hospitalized with bronchiolitis, 28 metabolites (e.g., docosapentaenoate [DPA], 1,2-dioleoyl-sn-glycero-3-phosphoglycerol, sphingomyelin) were associated with asthma risk. A total of 349 loci were associated with these metabolites—161 for non-Hispanic white, 120 for non-Hispanic black, and 68 for Hispanics. Of these, there was evidence for 30 shared loci between 16 metabolites and asthma risk (colocalization posterior probability

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≥0.5). The significant SNPs within loci were aligned with known asthma-susceptibility genes (e.g., *ADORA1*, *MUC16*).

Conclusion: The integrated genetics-metabolomics analysis identified genetically driven metabolites during infancy that are associated with asthma development and genetic loci associated with both these metabolites and asthma susceptibility. Identifying these metabolites and genetic loci should advance research into the functional mechanisms of the infant bronchiolitis-childhood asthma link.

KEYWORDS

asthma, bronchiolitis, childhood asthma, genetics, integrated-omics, metabolomics, phosphatidylglycerol, sphingolipids

Introduction

Bronchiolitis is the leading cause of infant hospitalization in the U.S., accounting for 110,000 hospitalizations each year (1). Its chronic morbidity is also substantial. Among infants hospitalized with bronchiolitis (i.e., severe bronchiolitis), ~30% subsequently develop childhood asthma (2–6). Yet, the mechanisms underlying the bronchiolitis-asthma link remain unclear. Our limited understanding has hindered the development of asthma prevention strategies.

Asthma is a complex syndrome that is influenced by both genetic and environmental factors (e.g., early-life virus infection) (7). Metabolomics systematically profiles small molecules in a biological system, which represent the downstream functional products of these genetic and environmental interactions. Studies have suggested metabolites involved in asthma pathobiology—e.g., sphingolipids (e.g., sphingomyelins) (8, 9), phospholipids (e.g., phosphatidylglycerol [PG]) (10), and fatty acids (e.g., docosapentaenoate [DPA]) (11). In addition to metabolomics, genome-wide association studies (GWASs) have identified genetic loci for childhood asthma susceptibility (12–17). For example, *ORMDL3* located at chromosome 17q21—a major regulator of sphingolipid metabolism—plays an important role in asthma development (12, 15). Metabolomics and genetics studies have *independently* attempted to elucidate the mechanisms underlying asthma pathobiology. However, no study has yet examined the integrated relationship of genetics, airway metabolome, and asthma development in children—let alone in infants at high risk for asthma development.

To address this knowledge gap, we applied an integrative genetics-metabolomics approach to data from a multicenter prospective cohort study of infants with severe bronchiolitis. We sought to identify the genetically driven metabolites and the genetic loci regulating those metabolites associated with the development of childhood asthma.

Abbreviations: ADORA1, adenosine receptor A1; CAAPA, Consortium on Asthma among African-ancestry Populations in the Americas; cAMP, cyclic adenosine monophosphate; DPA, docosapentaenoate; ER, endoplasmic reticulum; FDR, false discovery rate; GWAS, genome-wide association studies; GPE, glycerophosphorylethanolamine; GPG, glycero-3-phosphoglycerol; IL, interleukin; IRF7, interferon regulatory factor 7; MARC, multicenter airway research collaboration; mtQTL, metabolite quantitative trait loci; NF-κB, nuclear factor-κB; PG, phosphatidylglycerol; PP.H4, posterior probability of hypothesis 4; RSV, respiratory syncytial virus; SNP, single nucleotide polymorphisms; TRAF6, tumor necrosis factor receptor associated factor 6.

Materials and methods

Study design, setting, and participants

We analyzed data from the 35th Multicenter Airway Research Collaboration (MARC-35) study—a multicenter prospective cohort study (18). Details of the study design, setting, participants, data collection, testing, and statistical analysis may be found in the Supplementary Methods. Briefly, investigators enrolled 1,016 infants (age <1 year) hospitalized with attending physician-diagnosis of bronchiolitis at 17 sites across 14 U.S. states (Table S1) in 2011–2014. The diagnosis of bronchiolitis was made according to the American Academy of Pediatrics bronchiolitis guidelines (19), defined as an acute respiratory illness with a combination of rhinitis, cough, tachypnea, wheezing, crackles, or chest retractions. We excluded infants with a known heart-lung disease, immunodeficiency, immunosuppression, or gestational age of <32 weeks. All patients were treated at the discretion of the treating physicians.

Of 1,016 infants enrolled in the MARC-35 cohort, the current analysis investigated 744 infants who underwent both genotyping and nasopharyngeal metabolome profiling (Table S2). The institutional review board at each participating hospital approved the study with written informed consent obtained from the parent or guardian.

Data collection

Clinical data (patients' demographic characteristics, medical, environmental, and family history, and details of the acute illness) were collected *via* structured interviews and chart reviews using a standardized protocol (8, 9). After the index hospitalization for bronchiolitis, trained interviewers began interviewing parents/legal guardians by telephone at 6-month intervals in addition to medical record review by physicians. All data were reviewed at the Emergency

Medicine Network Coordinating Center at Massachusetts General Hospital (Boston, Massachusetts, USA) (18). By using a standardized protocol (8), investigators collected peripheral blood specimens (for genotyping) and nasopharyngeal specimens (for metabolome profiling) within 24 hours of hospitalization. We described the details of the data collection and measurement methods in the Supplementary Methods.

Genotyping

We used the Illumina Multi-Ethnic Genotyping Array (San Diego, California) for genotyping. For genotype imputation, we used the TOPMed reference panel on the TOPMed Imputation Server (20) and removed variants with an imputation score of <0.6 from the imputed dataset. We also removed rare variants with a minor allele frequency of <0.01 from the dataset. Subsequently, we included a total of 10,852,874 autosomal variants for the downstream association study. We described the details of genotype imputation and quality control in the Supplementary Methods.

Nasopharyngeal airway metabolome profiling

We profiled the nasopharyngeal metabolome using liquid chromatography with tandem mass spectrometry (LC-MS/MS) at Metabolon (Morrisville, North Carolina). The laboratory processed the blinded specimens in random order. Instrument variability was 4%, as determined by calculating the median relative standard deviation for the internal standards. The metabolome profiling identified 283 known metabolites from 76 sub-pathways within 7 super-pathways. We described the details of metabolome profiling in a previous study (21) and Supplementary Methods.

Clinical outcome measure

The clinical outcome of interest is the development of asthma by age 6 years. Asthma was defined using a commonly used epidemiologic definition: physician-diagnosis of asthma, with either asthma medication use (e.g., albuterol, inhaled corticosteroids) or asthma-related symptoms (e.g., wheezing, nocturnal cough) in the preceding year (22).

Statistical analysis

The objectives of the present study are (i) to identify genetically driven metabolites that are associated with the risk of developing asthma and (ii) to examine the genetic loci that are associated with both these metabolites and asthma susceptibility. The analytic workflow is summarized in Figure 1. The details of the statistical analysis may be found in the Supplementary Methods.

Briefly, we first constructed logistic regression models with Lasso regularization that examines the association of the nasopharyngeal metabolome with asthma development to identify candidate metabolites for the subsequent metabolite quantitative trait loci (mtQTL) analysis. Second, we performed the mtQTL analysis with an additive linear regression genetic association analysis adjusting for age, sex, and the first two ancestry principal components to examine the association between the genotypes and candidate metabolites in

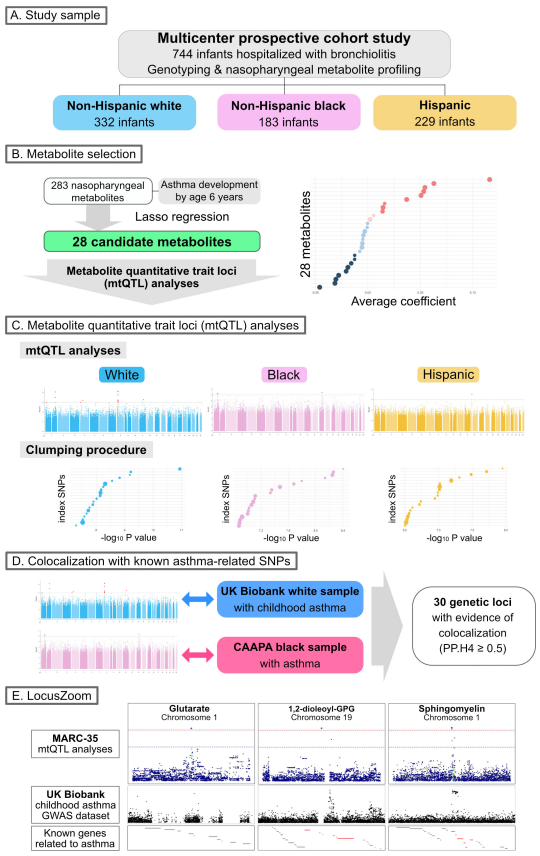

FIGURE 1
Analytic workflow of the study **(A)**. Of 744 infants (age <1 year) hospitalized with bronchiolitis, the current analysis investigated 744 infants—332 (45%) were non-Hispanic white, 183 (25%) were non-Hispanic black, and 229 (31%) were Hispanics—who underwent both genotyping and nasopharyngeal metabolome profiling. **(B)**. The association of 283 nasopharyngeal metabolites of the infants with asthma development by age 6 years was examined—by constructing Lasso regression models—to identify candidate metabolites for the downstream metabolite quantitative trait loci (mtQTL) analysis. **(C)**. The mtQTL analysis was performed to examine the association between 28 candidate metabolites and genotypes in each of the three major racial/ethnic subgroups of infants with bronchiolitis. The clumping procedure was also conducted to determine mtQTLs that are independent of each other. **(D)**. Bayesian colocalization analysis using summary statistics of the mtQTLs of MARC-35, UK Biobank (white sample with childhood asthma), and CAAPA (African-admixed sample with asthma) was conducted to identify shared genetic loci between the mtQTLs and the independent GWAS summary statistics for asthma. **(E)**. The association peaks of the 30 loci with colocalization evidence were compared between the GWAS summary statistics of the mtQTL analysis of MARC-35 and the UK Biobank. The association peaks of the three loci were aligned with those in the same genetic regions of UK Biobank summary statistics for childhood asthma, and there were concordances between the peaks of the two loci and known genes related to asthma. Abbreviations: CAAPA, Consortium on Asthma among African-ancestry Populations in the Americas; GPG, glycero-3-phosphoglycerol; GWAS, genome-wide association studies; MARC, multicenter airway research collaboration; PP.H4, posterior probability of hypothesis 4; SNP, single nucleotide polymorphism.

each of the three major racial/ethnic samples (non-Hispanic white, non-Hispanic black, and Hispanics) in the MARC-35 cohort. The significance threshold for these analyses was set to $P<1\times10^{-6}$. Third, we conducted clumping procedures to determine mtQTLs that are independent of each other. Fourth, we conducted pathway analyses to examine the biological significance of the genetic loci identified in the mtQTL analysis by using all genes within the clumped region of those loci. Fifth, we conducted Bayesian colocalization analyses to examine whether genetic loci for candidate metabolites are shared with those for asthma by using the GWAS summary statistics of the mtQTL analysis from MARC-35 and those of two independent datasets—the UK Biobank (white sample with childhood asthma (23–25)) and the Consortium on Asthma among African-ancestry Populations in the Americas (CAAPA; African-admixed sample with asthma (26)). We selected variants within 500 kb of the index SNP—the SNP with the smallest (i.e., most significant) P-value in each clumped region—at each of the shared loci in the non-Hispanic white and non-Hispanic black samples, and estimated the posterior probability that the two traits (i.e., each metabolite and asthma risk) share one common causal variant (a posterior probability of hypothesis 4 [PP.H4]). We considered loci with a posterior probability of ≥0.5 to colocalize. Lastly, we visualized the index SNPs colocalized with childhood asthma-risk loci from the UK Biobank GWAS summary statistics by using LocusZoom (27).

Results

Of 1,016 infants enrolled in the MARC-35 cohort, the current study focused on 744 infants with severe bronchiolitis who underwent both genotyping and nasopharyngeal metabolome profiling. The analytic (n=744) and non-analytic (n=272) cohorts did not differ in the patient characteristics (P≥0.05; Table S2), except for the proportion of racial/ethnicity and rhinovirus infection. Of the infants in the analytic cohort, the median age was 3 (interquartile range [IQR], 2-6) months and 40% were female; 45% were non-Hispanic white, 25% were non-Hispanic black, and 31% were Hispanics (Table 1). Overall, 26% subsequently developed asthma by age 6 years (Table S3).

mtQTL analysis reveals nasopharyngeal airway metabolites at infant bronchiolitis that are genetically driven

Of 283 nasopharyngeal metabolites identified in infants with bronchiolitis, 28 candidate metabolites were associated with the risk of developing asthma based on the Lasso regression models (Figure 2). Of these 28 candidate metabolites, 13 were lipids (e.g., DPA, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol [GPG], glutarate, sphingomyelin [d17:1/16:0, d18:1/15:0, d16:1/17:0]), 5 were amino acids (e.g., N-acetylarginine), 4 were carbohydrates (e.g., arabitol), and 6 were other classes of metabolites.

Based on the mtQTL analysis for each of the 28 candidate metabolites, 900 SNPs were at a suggestive significance level (28) ($P<1\times10^{-6}$)—524 in non-Hispanic white, 259 in non-Hispanic black, and 117 in Hispanics (Figure S1 and Table S4). The clumping procedure for these SNPs showed that 349 loci were independently associated with the candidate metabolites—161 loci associated with 26 metabolites in non-Hispanic white, 120 loci with 28 metabolites in non-Hispanic black, and 68 loci with 25 metabolites in Hispanics (Figure 3, Tables S5, S6). The pathway analysis showed the biological importance of these loci with significant pathways (FDR<0.05; Figure S2), which are relevant to both bronchiolitis and asthma development—e.g., interferon-α/β (29), tumor necrosis factor receptor-associated factor 6 (TRAF6) mediated interferon regulatory factor 7 (IRF7) activation pathways (30).

Colocalization analysis demonstrates 30 genetic loci associated with both metabolites and asthma-susceptibility

To test for genetic loci that are in common between the candidate metabolites and asthma risk, colocalization analyses were performed for the 281 loci identified in the clumping procedure—161 in non-Hispanic white and 120 in non-Hispanic black—by comparing the GWAS summary statistics of the mtQTL analysis in MARC-35 to two independent datasets—the UK Biobank and CAAPA (Table S5 and Figure S3). There was evidence for 30 shared genetic loci between 16 metabolites and asthma risk (PP.H4 ≥0.5; Table 2)—27 loci in UK Biobank (e.g., chromosome 6q26 with DPA, chromosome 1q21 with glutarate, chromosome 1q32 with sphingomyelin [d17:1/16:0, d18:1/15:0, d16:1/17:0]), 1 locus in CAAPA (chromosome 6q12 with N-acetylarginine), and 2 loci with both datasets (e.g., chromosome 14q31 with 1,2-dioleoyl-GPG).

Genetic loci are concordant to known asthma-susceptibility genes

The association peaks for the 30 loci with colocalization evidence were compared between the mtQTL analysis and UK Biobank (Figure S4). For example, the association peaks of glutarate on chromosome 1q21 (e.g., rs2232187), 1,2-dioleoyl-GPG on chromosome 19p13 (e.g., rs113043905), and sphingomyelin [d17:1/16:0, d18:1/15:0, d16:1/17:0] on chromosome 1q32 (e.g., rs12752641) were aligned with the association peak in the same genetic region from the UK Biobank statistics of childhood asthma. Furthermore, there were apparent concordances between the association peaks for two loci (i.e., chromosome 19p13 with 1,2-dioleoyl-GPG, chromosome 1q32 with sphingomyelin [d17:1/16:0, d18:1/15:0, d16:1/17:0]) and genes that are known to be related to asthma (e.g., *MUC16*, *ADORA1*; Figure 4).

Discussion

By applying an integrated genetics-metabolomics approach to multicenter prospective cohort data of 744 infants with severe bronchiolitis, we identified 28 metabolites associated with asthma development and 349 independent genetic loci associated with these metabolites. Additionally, of these loci, colocalization analysis (with

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TABLE 1 Baseline characteristics and clinical course of 744 infants hospitalized with bronchiolitis, according to race/ethnicity.

Characteristics	Overall (n=744; 100%)	Non-Hispanic white (n=332; 45%)	Non-Hispanic black (n=183; 25%)	Hispanic (n=229; 31%)	P value
Demographics					
Age (month), median (IQR)	3 (2-6)	3 (2-6)	3 (2-6)	4 (2-6)	0.67
Female sex	300 (40)	134 (40)	80 (44)	86 (38)	0.45
Prematurity (32.0-36.9 weeks)	136 (18)	55 (17)	43 (24)	38 (17)	0.11
C-section delivery	250 (34)	118 (36)	60 (34)	72 (32)	0.57
Previous breathing problems (count)					0.76
0	587 (79)	268 (81)	142 (78)	177 (77)	
1	122 (16)	51 (15)	30 (16)	41 (18)	
2	35 (5)	13 (4)	11 (6)	11 (5)	
Previous ICU admission	12 (2)	4 (1)	2 (1)	6 (3)	0.35
History of eczema	111 (15)	45 (14)	39 (21)	27 (12)	0.02
Ever attended daycare	170 (23)	78 (24)	57 (31)	35 (15)	0.001
Parental history of asthma	246 (33)	107 (32)	73 (40)	66 (29)	0.04
Parental history of eczema	143 (19)	64 (19)	55 (30)	24 (11)	<0.001
Clinical presentation					
Weight (kg), median (IQR)	6 (5-8)	6 (5-8)	6 (5-8)	6 (5-8)	0.39
Respiratory rate (per minute), median (IQR)	48 (40-60)	48 (40-60)	52 (40-61)	48 (40-60)	0.08
Oxygen saturation					0.01
<90%	62 (9)	27 (8)	9 (5)	26 (12)	
90-93%	113 (16)	60 (19)	17 (9)	36 (16)	
≥94%	553 (76)	233 (73)	156 (86)	164 (73)	
Blood eosinophilia (≥4%)	70 (11)	32 (11)	17 (11)	21 (11)	0.99
IgE sensitization	155 (21)	52 (16)	54 (30)	49 (21)	0.001
Clinical course					
Positive pressure ventilation use*	39 (5)	20 (6)	5 (3)	14 (6)	0.21
Intensive treatment use†	114 (15)	50 (15)	23 (13)	41 (18)	0.32
Length-of-day (day), median (IQR)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)	0.03
Respiratory virus					
RSV only	421 (57)	210 (63)	93 (51)	118 (52)	0.004
RV only	52 (7)	17 (5)	17 (9)	18 (8)	0.17
RSV/RV coinfection	81 (11)	34 (10)	24 (13)	23 (10)	0.54
Other pathogen‡	179 (24)	72 (22)	45 (25)	62 (27)	0.33

ICU, intensive care unit; IgE, immunoglobulin E; IQR, interquartile range; RSV, respiratory syncytial virus; RV, rhinovirus.
Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100 because of rounding and missingness.
*Infants with bronchiolitis who underwent continuous positive airway ventilation and/or mechanical ventilation.
†Infants with bronchiolitis who were admitted to ICU and/or who underwent positive pressure ventilation.
‡Adenovirus, bocavirus, Bordetella pertussis, enterovirus, human coronavirus NL63, OC43, 229E, or HKU1, human metapneumovirus, influenza A or B virus, Mycoplasma pneumoniae, and parainfluenza virus 1-3.

independent GWAS datasets for asthma) revealed 30 shared loci between 16 metabolites and asthma risk. Furthermore, the significant SNPs within two loci were aligned with known asthma-susceptibility genes (e.g., *ADORA1*, *MUC16*). To the best of our knowledge, this is the first study that has investigated infant bronchiolitis-childhood asthma link with an integrative genetics-metabolomics approach and demonstrated genetically driven metabolites and related genetic loci associated with asthma risk.

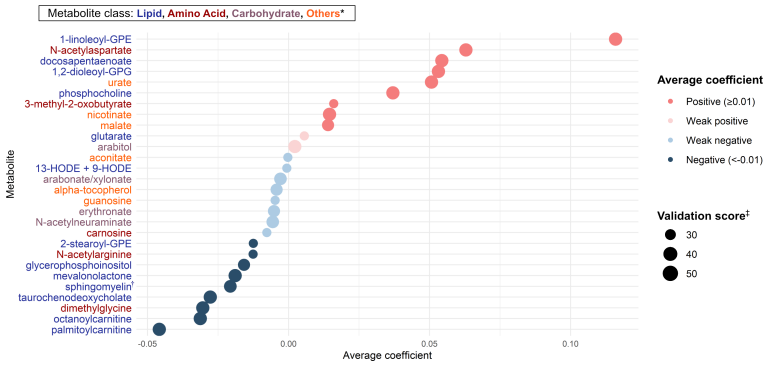

FIGURE 2
Metabolites selection for the mtQTL analyses By applying the Lasso regression models on nasopharyngeal metabolome (283 metabolites) data and the asthma outcome data of 744 infants hospitalized for bronchiolitis, we identified 28 candidate metabolites for the downstream mtQTL analyses. The average coefficient indicates the magnitude of the metabolite-outcome association averaged over times selected as a feature in 100 Lasso regression model executions. * Of 28 metabolites, 13 were lipids, 5 were amino acids, 4 were carbohydrates, and 6 were other classes (e.g., vitamins, nucleic acids) of metabolites. † Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0) ‡ The number of times selected as a feature of the Lasso regression model in 100 model executions. Abbreviations: GPE, glycerophosphorylethanolamine; GPG, glycero-3-phosphoglycerol; HODE, hydroxyoctadecadienoic acid.

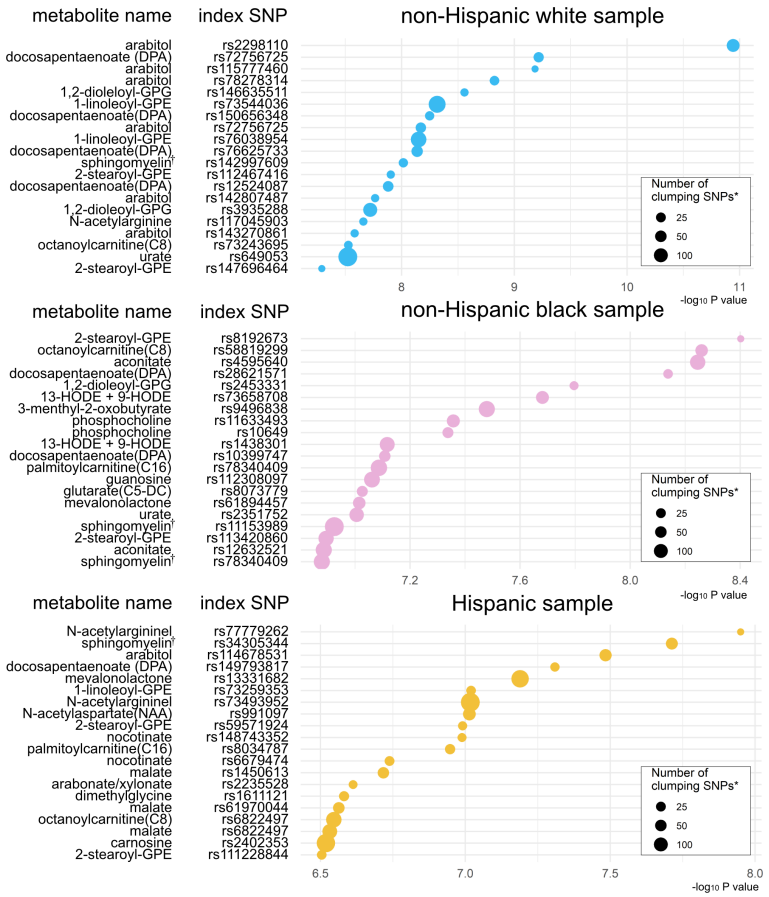

FIGURE 3
Significant index SNPs identified in metabolite quantitative trait loci (mtQTL) analyses The 20 index SNPs with the lowest P-values associated with the candidate metabolites were identified in the mtQTL analysis of each racial/ethnic sample after the clumping procedure. * Variants with a P value of $<1 \times 10^{-3}$, R^2 of ≥ 0.2, and <500 kb away from the index SNP were assigned to the clump. † Sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0) Abbreviations: GPE, glycerophosphorylethanolamine; GPG, glycero-3-phosphoglycerol; HODE, hydroxyoctadecadienoic acid.

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TABLE 2 Summary of the 30 genetic loci associated with both the candidate metabolites and asthma risk.

Metabolite		Locus									
Metabolite class	Metabolite name	Index SNP*	N†	Chr	Index SNP* position	Alt allele	P value		PP.H4‡		Known asthma genes within index SNP* ± 500KB
							Non-Hispanic white	Non-Hispanic black	UKB white	CAAPA black	
Significant loci in the non-Hispanic white sample											
Lipid	1,2-dioleoyl-GPG (18:1/18:1)	rs116459436	1	1	7227920	T	5.0×10^{-07}	0.009	0.513	0.267	
		rs1637750	16	7	2188176	G	9.3×10^{-07}	0.740	0.656	0.259	*AMZ1*
		rs146635511	2	10	124772492	T	2.8×10^{-09}	NA	0.651	0.235	*LHPP*
		rs142277549	3	14	88122695	T	2.1×10^{-07}	0.109	0.691	0.695	
		rs113043905	1	19	8717141	G	6.0×10^{-07}	0.453	0.580	0.251	*ADAMTS10, ACTL9, MUC16, OR1M1*
Lipid	docosapentaenoate (DPA; 22:5n3)	rs78388829	5	2	15120216	G	5.1×10^{-07}	0.784	0.695	0.278	*FAM84A, DDX1*
		rs12524087	12	6	163619816	G	1.3×10^{-08}	0.574	0.841	0.287	*QKI*
Lipid	glutarate (C5-DC: glutarylcarnitine)	rs2232187	3	1	147759651	A	6.5×10^{-07}	0.656	0.768	0.277	
Lipid	mevalonolactone	rs4752744	11	11	1697036	G	2.7×10^{-07}	0.869	0.967	0.237	*MUC5B*
Amino acid	N-acetylarginine	rs9363451	29	6	65822620	A	2.9×10^{-07}	0.001	0.145	0.556	
		rs117045903	2	15	66627247	A	2.2×10^{-08}	0.294	0.698	0.289	*SMAD3, SMAD6, LINC01169*
Carbohydrate	arabitol	rs2298110	34	1	19906558	G	1.1×10^{-11}	0.077	0.936	0.398	
		rs113812800	1	2	3901708	C	2.4×10^{-07}	0.053	0.738	0.383	*ALLC*
		rs143270861	2	4	182064534	A	2.6×10^{-08}	0.707	0.698	0.258	*MGC45800*
		rs185153229	7	6	34748997	C	7.0×10^{-08}	0.767	0.625	0.203	*TCP11, SCUBE3*
		rs115777460	1	13	52988125	A	6.6×10^{-10}	0.817	0.728	0.381	
		rs77244206	7	15	31337658	G	7.6×10^{-07}	0.610	0.957	0.377	
Carbohydrate	N-acetylneuraminate	rs10101380	119	8	5147537	C	9.1×10^{-07}	0.760	0.586	0.187	*CSMD1*
Energy	aconitate	rs148027659	4	10	74609570	G	5.8×10^{-07}	0.633	0.514	0.214	
Significant loci in the non-Hispanic black sample											
Lipid	2-stearoyl-GPE (18:0)	rs12494581	36	3	55257122	C	0.524	7.3×10^{-07}	0.812	0.506	
Lipid	palmitoylcarnitine (C16)	rs79141561	4	2	105989161	G	NA	8.1×10^{-07}	0.550	0.472	
Lipid	sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)	rs12752641	8	1	203053298	C	0.475	5.0×10^{-07}	0.688	0.226	*PPFIA4, MYOG, ADORA1, MYBPH, CHI3L1, CHIT1*
		rs78340409	29	1	234553708	G	0.852	1.3×10^{-07}	0.659	0.164	

(Continued)

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TABLE 2 Continued

Metabolite		Locus									
Metabolite class	Metabolite name	Index SNP*	N†	Chr	Index SNP* position	Alt allele	P value		PP.H4‡		Known asthma genes within index SNP* ± 500KB
							Non-Hispanic white	Non-Hispanic black	UKB white	CAAPA black	
Lipid	taurochenodeoxycholate	rs72816230	7	17	1219933	G	0.023	6.2×10^{-07}	0.626	0.249	
Amino acid	dimethylglycine	rs8052562	1	16	3491830	T	0.436	7.2×10^{-07}	0.686	0.268	*TRAP1*
Amino acid	N-acetylaspartate	rs11714340	64	3	27610676	C	0.211	5.0×10^{-07}	0.581	0.220	
Carbohydrate	N-acetylneuraminate	rs76840346	84	13	53885705	C	0.063	3.5×10^{-07}	0.511	0.272	
Cofactors and vitamins	alpha-tocopherol	rs12942941	13	17	80129214	G	0.476	9.0×10^{-07}	0.652	0.239	
Energy	aconitate	rs12632521	29	3	32682166	A	0.920	1.3×10^{-07}	0.667	0.219	*GLB1, TRIM71, TMPPE, CRTAP, SUSD5, CCR4*
Nucleotide	guanosine	rs112308097	27	2	230821995	T	NA	8.7×10^{-08}	0.576	0.301	

*The SNP with the smallest (i.e., most significant) P value in each clumped region in the mtQTL analysis.
†The number of SNPs with a P value of $< 1 \times 10^{-3}$ in each clumped region in the mtQTL analysis.
‡We considered genetic loci with a posterior probability of H4 (PP.H4) of ≥0.5 to colocalize.
Alt, alternative; Chr, chromosome; CAAPA, consortium on asthma among African-ancestry populations in the Americas; DPA, docosapentaenoate; KB, kilobyte; GPE, glycerophosphorylethanolamine; GPG, glycerophosphoglycerol; PP.H4, posterior probability of H4; SNP, single nucleotide polymorphism; UKB, UK Biobank.

Childhood asthma is a heterogeneous syndrome that results from complex interactions between genetic and environmental factors in early childhood (7). Recent research has studied the mechanisms by applying high-throughput approaches to survey the metabolome—the downstream functional products of a child's genetic make-up and environmental exposures (e.g., virus respiratory infection) (21, 31–40). Consistent with our findings, for example, recent studies of infants with acute respiratory infection have reported associations of

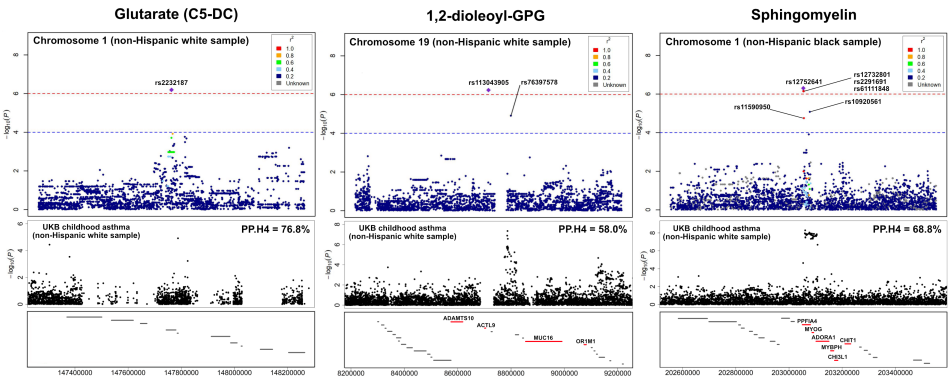

FIGURE 4
LocusZoom for the genetic loci with the UK Biobank summary statistics of childhood asthma To compare the association peaks of the loci between the mtQTL analysis in infants with bronchiolitis and the UK Biobank summary statistics for childhood asthma, we applied LocusZoom to glutarate (C5-DC: glutarylcarnitine) on chromosome 1q21, 1,2-dioleoyl-GPG on chromosome 19p13, and sphingomyelin [d17:1/16:0, d18:1/15:0, d16:1/17:0] on chromosome 1q32. The association peaks of these three loci were aligned with those in the same genetic regions of UK Biobank summary statistics for childhood asthma. The red lines at the bottom boxes represent the genetic location of the genes that are known to be related to asthma. There were concordances between the association peaks of two loci (i.e., chromosome 19p13 with 1,2-dioleoyl-GPG, chromosome 1q32 with sphingomyelin) and known asthma genes (e.g., *ADORA1, MUC16*). Abbreviations: GPG, glycero-3-phosphoglycerol; PP.H4, posterior probability of hypothesis 4; UKB, UK Biobank.

the upper airway (21, 34–38), serum (36, 38), and urine (39) metabolome signature (e.g., altered sphingolipid, phospholipid, and fatty acid metabolism)—with the subsequent development of asthma. Independent from these metabolomics investigations, GWASs have identified genetic regions associated with respiratory syncytial virus (RSV) infection (41–43), severe bronchiolitis (41, 42, 44), and asthma risk (12–17, 43, 45–49). For example, multiple studies have identified *ORMDL3* as an asthma susceptibility gene (12, 15, 46, 48) with asthma risk. ORMDL3 is a major regulator of serine palmitoyltransferase—the rate-limiting enzyme of sphingolipid biosynthesis (50). While most research independently have applied genetics and metabolomics, few studies have examined the integrated relationships of genetic variants and altered metabolism with prevalent asthma in adults (51, 52). For example, Johnson et al. recently conducted an mtQTL analysis of 348 adults (59 with prevalent asthma) from Tangier Island and found several serum metabolites (e.g., linoleoyl ethanolamide) associated with asthma risk (51). Our multicenter prospective study—integrating the genome and nasopharyngeal airway metabolome data from high-risk infants—corroborates these earlier reports, and extends them by demonstrating genetic loci and genetically driven metabolites associated with the risk of developing asthma.

The exact mechanisms underlying the observed relationship of genetic loci and metabolites—e.g., genes within chromosome 1q32 (e.g., *ADORA1*, *PPFIA4*) and sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)—with asthma risk warrant further clarification. Studies have shown that genes on chromosome 1q32, including *ADORA1* and *PPFIA4* are associated with asthma risk (13, 53, 54), and *ADORA1* may interact with sphingolipids to enhance airway inflammation (55–59). For example, adenosine receptor A1 (ADORA1) encoded by the *ADORA1* gene contributes to bronchoconstriction, mucus secretion, and inflammation in bronchial epithelial cells through the sphingolipid signaling pathway (55, 56). Experimental studies have also shown that ADORA1 regulated cyclic adenosine monophosphate (cAMP) and sphingomyelin-derived lipids to mobilize intracellular calcium stores in bronchial smooth muscle cells, leading to the contraction of the bronchial smooth muscles and airway remodeling (57–59). Sphingolipids are not only integrated components of the human cell membrane (60) but also have molecular signaling functions with roles in the immune response to infections, inflammation, and cell proliferation, thereby contributing to asthma pathobiology (61). Accordingly, studies have suggested ADORA1 (62) and sphingolipids (63) as therapeutic targets for asthma.

In addition to sphingomyelins, we also observed a relationship between genes on chromosome 19p13 (e.g., *MUC16*), 1,2-dioleoyl-GPG, and asthma risk. 1,2-dioleoyl-GPG is one of the phosphatidylglycerols (PGs)—a main component of pulmonary surfactant (64). An experimental study has suggested that PG inhibits proinflammatory protein expression in alveolar macrophages through downregulation of NF-κB activation (65, 66). In contrast, the depletion of pulmonary surfactant PG leads to asthma-associated surfactant dysfunction (10). Another study has also reported that PG inhibits RSV infection by blocking viral attachment to epithelial cells (67). Besides, Mucin-16—also known as CA125 and encoded regulated by the *MUC16* gene—is the largest membrane-associated mucin synthesized in the endoplasmic reticulum (ER) of the bronchial epithelial cells (68–70). Research has demonstrated that the expression of mucin-16 was promoted by NF-κB activation and protects against ER stress (71). ER stress regulates proinflammatory signaling in epithelial cells through pulmonary surfactant dysfunction that is also induced by PG depletion (72). Accordingly, *MUC16* gene, mucin-16, and GPG jointly play roles in ER stress (73). Notwithstanding the complexity of these potential mechanisms, the identification of genetically driven metabolites associated with the development of childhood asthma is an important finding. Our data—in conjunction with the literature—should advance further research into the pathobiological mechanisms underlying the bronchiolitis-asthma link.

The current study has several potential limitations. First, the study did not have "healthy controls." Yet, the objective of the study was not to identify the genetic loci and metabolites related to incident bronchiolitis (i.e., bronchiolitis yes vs. no) but to investigate the functional consequences of genetic risk factors for asthma (i.e., genetically driven metabolites) in this high-risk population. Second, compared to the non-analytic cohort, the analytic cohort had an overrepresentation of solo rhinovirus infection (3% vs. 7%), which might have led to selection bias. Third, it is possible that asthma diagnosis is misclassified and that some children are going to develop asthma at a later age. To address these potential limitations, the cohort is currently being followed up to age 9 years. In addition, in the current study, children who had asthma-related symptoms but did not receive asthma medication might have been over-diagnosed with asthma. Fourth, the sample size of the current analysis was relatively small, partially because of the stratified analysis across the different racial/ethnic subgroups. To accommodate the limited statistical power, we performed the mtQTL analyses with the significance threshold ($P < 1\times10^{-6}$) proposed in a previous study (28). The statistical power of the analyses calculated using the GAS Power Calculator (74) was 0.72 in non-Hispanic white, 0.71 in non-Hispanic black, and 0.71 in Hispanics. Fifth, the CAAPA dataset provided summary statistics for an African-admixed population with asthma (26). Therefore, the sample from the other racial/ethnic groups might have affected the colocalization estimates of our non-Hispanic black sample due to population stratification (75). Sixth, the lack of publicly available asthma GWAS data in the Hispanic sample precluded us from conducting colocalization analysis for our Hispanic sample. Lastly, our inferences may not be generalizable to infants without severe bronchiolitis (i.e., infants with mild-to-moderate bronchiolitis). Nonetheless, our observations remain directly relevant to the 110,000 infants hospitalized annually in the U.S (1).—a large population with a substantial morbidity burden.

Conclusions

By integrating the genetics and nasopharyngeal airway metabolomics data from a multicenter, prospective cohort study of infants hospitalized for bronchiolitis, we identified genetically driven metabolites (e.g., 1,2-dioleoyl-GPG, sphingomyelin) associated with asthma development and genetic loci associated with both these metabolites and asthma susceptibility genes (e.g., *ADORA1*, *MUC16*). These associations were also confirmed by colocalization analyses with independent GWAS datasets for asthma. Identifying

these metabolites and genetic loci should advance research into the functional consequences of genetic risk factors for the development of asthma. Furthermore, these findings will, in turn, accelerate the understanding of the bronchiolitis-asthma link and the development of prevention strategies for childhood asthma.

Data availability statement

The data presented in the study are deposited in the ImmPort repository, accession number SDY2157.

Ethics statement

The studies involving human participants were reviewed and approved by Alfred I. duPont Hospital for Children, Wilmington, DE; Arnold Palmer Hospital for Children, Orlando, FL; Boston Children's Hospital, Boston, MA; Children's Hospital of Los Angeles, Los Angeles, CA; Children's Hospital of Philadelphia, Philadelphia, PA; Children's Hospital of Pittsburgh, Pittsburgh, PA; The Children's Hospital at St. Francis, Tulsa, OK; The Children's Mercy Hospital & Clinics, Kansas City, MO; Children's National Medical Center, Washington, D.C.; Cincinnati Children's Hospital and Medical Center, Cincinnati, OH; Connecticut Children's Medical Center, Hartford, CT; Dell Children's Medical Center of Central Texas, Austin, TX; Norton Children's Hospital, Louisville, KY; Massachusetts General Hospital, Boston, MA; Phoenix Children's Hospital, Phoenix, AZ; Seattle Children's Hospital, Seattle, WA; Texas Children's Hospital, Houston, TX. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

TO carried out the main statistical analysis, drafted the initial manuscript, and approved the final manuscript as submitted. ZZ carried out the data processing, developed the methodology, analyzed and interpreted data, reviewed and revised the initial manuscript, and approved the final manuscript as submitted. LL, JC, BH, AH, ER, and RF collected the study data, reviewed and revised the initial manuscript, and approved the final manuscript as submitted. CC and KH conceptualized the study, obtained funding, supervised the statistical analysis, reviewed and revised the initial manuscript, and approved the final manuscript as submitted. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JC received research materials from Merck Rahway, NJ in order to provide medications free of cost to participants in an NIH-funded study, unrelated to the current work.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1111723/full#supplementary-material

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