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ORIGINAL ARTICLE

Respiratory virome profiles reflect antiviral immune responses

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Abstract

Background: From early life, respiratory viruses are implicated in the development, exacerbation and persistence of respiratory conditions such as asthma. Complex dynamics between microbial communities and host immune responses shape immune maturation and homeostasis, influencing health outcomes. We evaluated the hypothesis that the respiratory virome is linked to systemic immune responses, using peripheral blood and nasopharyngeal swab samples from preschool-age children in the PreDicta cohort.

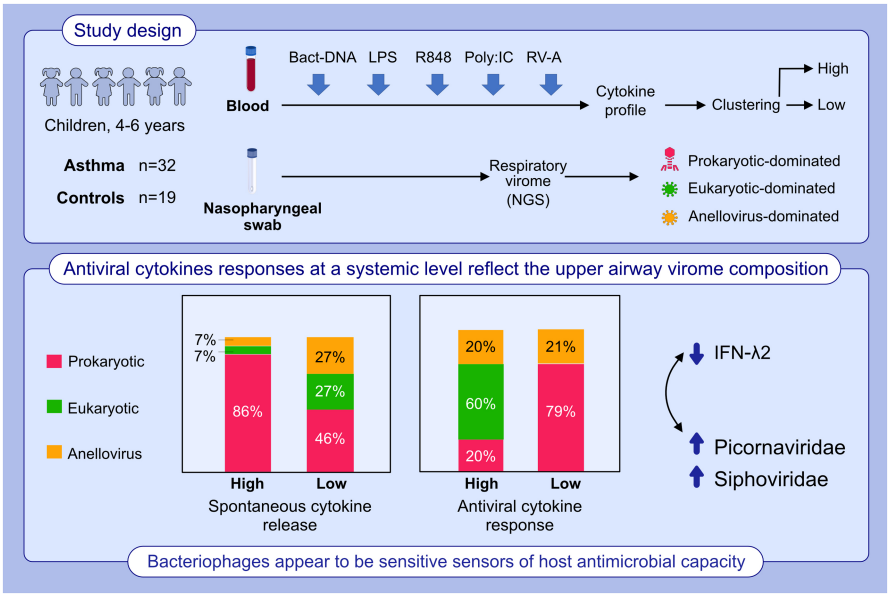
Methods: Peripheral blood mononuclear cells from 51 children (32 asthmatics and 19 healthy controls) participating in the 2-year multinational PreDicta cohort were cultured with bacterial (Bacterial-DNA, LPS) or viral (R848, Poly:IC, RV) stimuli. Supernatants were analysed by Luminex for the presence of 22 relevant cytokines. Virome composition was obtained using untargeted high throughput sequencing of nasopharyngeal samples. The metagenomic data were used for the characterization of virome profiles and the presence of key viral families (Picornaviridae, Anelloviridae, Siphoviridae). These were correlated to cytokine secretion patterns, identified through hierarchical clustering and principal component analysis.

Results: High spontaneous cytokine release was associated with increased presence of Prokaryotic virome profiles and reduced presence of Eukaryotic and Anellovirus profiles. Antibacterial responses did not correlate with specific viral families or virome profile; however, low antiviral responders had more Prokaryotic and less Eukaryotic virome profiles. Anelloviruses and Anellovirus-dominated profiles were equally distributed among immune response clusters. The presence of Picornaviridae and Siphoviridae was associated with low interferon-λ responses. Asthma or allergy did not modify these correlations.

Conclusion: Antiviral cytokine responses at a systemic level reflect the upper airway virome composition. Individuals with low innate interferon responses have higher abundance of Picornaviruses (mostly Rhinoviruses) and bacteriophages. Bacteriophages,

particularly Siphoviridae, appear to be sensitive sensors of host antimicrobial capacity, while Anelloviruses are not correlated with TLR-induced immune responses.

KEYWORDS
asthma, bacteriophages, interferon-λ, rhinoviruses, virome



GRAPHICAL ABSTRACT
Respiratory virome profiles of preschool children were associated with spontaneous cytokine release and stimulated antiviral responses from PBMCs, independent of asthma/allergy status. Prokaryotic (bacteriophage)-type viromes dominated the high spontaneous cytokine release responder cluster. High antiviral responders included more eukaryotic-dominated profiles, while low interferon lambda release correlated with increased presence of Picornaviridae and Siphoviridae.

1 | INTRODUCTION

The role of the microbiome in shaping health and disease is increasingly understood and substantiated.[1] Nevertheless, the focus on the gut microbiome has left other important niches, such as the respiratory tract, or agents, such as viruses, less well studied.[2] Particularly, little is known about the role of the respiratory virome in homeostasis across the ages, despite several studies showing that common respiratory viruses can often be found in the airways of asymptomatic individuals.[3,4]

It has been suggested that the virome may be able to mould the immune system, affecting the development of asthma and respiratory diseases in childhood.[5] Specific viruses or viral families may be able to modulate the immune responses and hence drive immune maturation, influence health and disease and be candidates for intervention strategies.[6] Clearly, infection with common cold viruses is closely linked with the development of respiratory and other allergic diseases.[7,8]

In addition, to maintain homeostasis the immune system needs to control the density and composition of the microbiome.[9] When it comes specifically to the respiratory virome, much less is known; nevertheless, there is increasing understanding regarding the development of immune surveillance to eukaryotic viruses,[10] the interplay between inert viruses such as the Anelloviruses and immune competence[11] and the non-host immune support conveyed by bacteriophages.[12]

It is therefore clear that a continuous interplay between immune surveillance, established microbial self and incoming microbes, defines a dynamic balance shaping health and disease in an interactive complexity, in which the local virome has a considerable share.

We have previously described the respiratory virome (DNA and RNA viruses) in a cohort of well-characterized preschool-age children with asthma and healthy controls across Europe, in the context of the PreDicta study.[13] Among the viral genomes identified, the most prevalent and diversified between health and asthma involved Picornaviruses, Anelloviruses and bacteriophages of the Siphoviridae family. Depending on their virome composition, individuals could be grouped into three profiles: a Eukaryotic prevailing profile (E-VPG), an Anellovirus prevailing profile (A-VPG) and a Prokaryotic prevailing profile (P-VPG).

We hypothesized that any biological interactions between the respiratory virome and the host antiviral immune responses may

be reflected in associations between viral and immune signatures. The aim of this study was to evaluate links between the presence of the prevailing viral families, as well as of the respiratory virome profile groups, with cytokine production from peripheral blood cells at baseline and following stimulation with viral- and bacterial-mimicking stimuli.

This helps characterize immune-microbiome interactions and biomarkers that identify health/disease gradients and unravel novel therapeutic targets.

2 | METHODS

2.1 | Description of the cohort

Our analyses were conducted using data from the PreDicta study (*Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases*), a 2-year multicentre prospective cohort study.[14] Blood and nasopharyngeal samples were collected from 233 preschool children (4–6 years) with asthma (167 subjects) and matched healthy controls (66) across 5 major European climate regions, that is Greece (Athens), Poland (Lodz), Finland (Turku), Germany (Erlangen) and Belgium (Ghent).[14] To conduct this study, cytokine measurements and metagenomics data were used from 51 subjects from the whole cohort (32 asthmatics and 19 healthy controls), based on the availability of the samples, in 3 out of the 5 centres. Our population was generally representative of the whole cohort[12] (Table S1). Written informed consent was obtained from the parents or by the legal guardians, and the study was approved by all Ethics Committees of the participating centres.

Cases needed to be diagnosed with mild/moderate asthma within the previous 2 years and have a minimum of 3 wheezing episodes within the last 12 months prior to study inclusion.[12] Exclusion criteria included severe asthma, >6 courses of oral steroids during the last 12 months, immunotherapy, chronic medication use, or the history of chronic respiratory disease other than asthma and/or allergic rhinitis. Control subjects had to demonstrate no history of wheezing/asthma.[14] Additionally, subjects needed to be away from an asthma exacerbation and/or upper respiratory tract infection for at least 4 weeks before sample collection. Subjects were balanced for sex (50.9% males, 49.1% females), of 4.95 ± 0.65 years old (range 3.26 to 6.29). Comorbidities and other characteristics are shown in Table S1.

2.2 | Treatment of blood samples and cytokine measurements

Blood samples were collected in tubes with lithium heparin (Vacutainer) and diluted with an equal volume of warm PBS (Gibco, Invitrogen). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifuging at 800 *g* for 20 min at 18–20°C on Biocoll separating solution (Biochrom AG). After three washes, PBMCs were resuspended in complete medium (CM) [RPMI-1640 with HEPES 25 mM and L-Glutamine (Gibco, Life Technologies Ltd), supplemented with 10 ml/L Penicillin–Streptomycin USA, 50 µl/L 1 M β-mercaptoethanol, 20 ml/L L-Glutamine plus MEM Vitamin, 20 ml/L Non-essential Amino Acid, Sodium Pyruvate and 10% heat-inactivated FBS (all from Sigma-Aldrich)] at concentration 10^6 cells/ml.

RV1b stock was propagated in HeLa cells and purified by centrifugation at 2500 rpm for 10 min (4°C). Suspension from HeLa lysates was used as a control. The same batch of RV1b and HeLa suspension was used throughout the study. 1 ml of cells suspension (10^6 PBMCs) was exposed to RV1b (6.7 titration) or HeLa suspension as follows: After centrifugation at 300 *g*, for 15 min, at room temperature (RT), the supernatant was carefully removed by aspiration, and cells were exposed to 0.5 ml of RV or HeLa suspension for 1 h, under rotation at RT. Subsequently, cells were washed twice with CM at 300 *g* for 15 min, RT, and resuspended in 1 ml of CM.

The cell suspension was seeded in a flat-bottom 48-well tissue plate (Corning Incorporated, Costar), with 5×10^5 viable cells per well (500 µl). PBMCs were cultured in duplicates either with complete medium alone (unstimulated control, RV1b-exposed cells and HeLa-exposed cells) or with one of the following stimulants: 4 µg/ml Resiquimod (R848), 5 µg/ml Endotoxin-free bacterial DNA (InvivoGen), 20 µg/ml Polyinosinic–polycytidylic acid potassium salt (Poly I:C) and 1 µg/ml Lipopolysaccharides from E.Coli 0111:B4 (LPS), (Sigma-Aldrich), at 37°C, 5% CO_2.

Cultures were harvested after 48 h, and, after centrifugation at 600 *g* for 5 min, supernatants were stored at −80°C until analysis. Cytokine expression levels in the culture supernatants were quantified by multiplex bead-based fluorometric immunoassay (Milliplex; Millipore) using Luminex xMAP (Luminex 200; Bio-Rad) at the Swiss Institute for Allergy and Asthma Research (SIAF) in Davos, Switzerland. The panel used contained IFNα2, IFNγ, IFNλ-2, IL-1β, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-23A, IL-25, IL-27, IL-33, CCL3, CCL4, CCL5, CXCL8, CXCL10 and TNF-α.

2.3 | Characterization of the nasopharyngeal virome

The presence of prokaryotic and eukaryotic viruses in the upper respiratory tract (nasopharynx and anterior nares) was previously investigated using metagenomic sequencing in samples obtained from 19 healthy individuals and 32 patients with mild/moderate asthma.[12] Briefly, based on the predominant viral families of these individuals, three virome profile groups (VPGs) were assigned: Prokaryotic VPG; (P-VPG, $n = 29$), contained samples with high prevalence of prokaryotic viruses and low/intermediate of eukaryotic viruses and Anelloviridae, Eukaryotic VPG (E-VPG, $n = 11$) included samples with high eukaryotic viruses' predominance and low/intermediate of Anelloviridae and Anelloviridae VPG (A-VPG, $n = 11$) contained samples with high Anelloviridae predominance. The virome characteristics of the 51 individuals are described in the Data S1.

2.4 | Statistical analysis

The data used for analysis consisted of a set of 22 cytokine concentrations in control medium and their inductions from different stimuli. Inductions representing the ratio between the stimulated values over the baseline levels were used in the downstream analysis. Preprocessing, necessary for subsequent clustering, included the following steps. First, a few missing values were imputed with the use of the random forest algorithm for imputation.[15] Second, outlier detection and correction were performed: low outliers (stimulation values lower than 1) were converted to 1, in sake of biological validity, while any high outliers were substituted with the minimum outlier value, according to the default boxplot definition. Then, all values were converted to z-scores; thus, they all possessed a mean value equal to 0 and a standard deviation equal to 1. All such variables were found to be non-parametric, with the use of the Shapiro–Wilk procedure for composite normality.

Unsupervised cluster analysis was applied to group subjects according to subsets of spontaneously released or stimulated cytokines. Stimuli were grouped according to their nature; therefore, two major conditions were generated: the antiviral (R848, Poly-IC & RVA1b) and the antibacterial (Endotoxin-free bacterial DNA & LPS) responses, alongside the baseline. After pre-processing and prior to clustering, optimal number of cluster identification took place with the use of a set of 27 appropriate criteria.[16] Then, the hierarchical agglomerative algorithm for clustering was used in order to group subjects regarding their similarity, to the pre-identified number of groups. The linkage method used was Ward's linkage. Visualization of the clustering outcome was performed with the use of principal component analysis (PCA) in the dimensions of the first two dominant principal components.

Groups identified by clustering were analysed to characterize different types of response towards a stimulus or homeostasis. Comparisons between groups regarding the presence of major viral families (Siphoviridae, Picornaviridae, Anelloviridae) were performed by Pearson's chi-squared test of independence and corrected with Bonferroni's test. Furthermore, the studied categorical variables (Geography, Sex, VPGs, Rhinitis, Siphoviridae, Picornaviridae, Anelloviridae and Asthma) and age of the donors were included in a multivariate regression. To avoid multicollinearity issues in the analysis, we ran beforehand bivariate crosstab tests between the predictors (immune clusters) and all target variables, namely Pearson's chi-squared tests of independence and corrected with Bonferroni's test, to eliminate those variables that did not provide significance to the model and only retaining those that yielded a significant *p*-value to one of the predictors. In each regression, predictors' cluster 1 and target variables' 1st level were used as benchmark.

Additionally, stimulated cytokine values were compared between subjects with or without the presence of pre-specified viral families (Picornaviridae, Siphoviridae, Anelloviridae), using Wilcoxon's rank-sum test and presence of Paramyxoviridae using Kruskal–Wallis test. Since all statistical tests were non-parametric, the descriptive statistics provided were non-parametric as well (i.e. in the form of median (Q1–Q3)). All of the statistical tests were two-sided, and statistical significance was taken when p was less than .05. The statistical analysis was implemented with the usage of the R language and the RStudio interface.

3 | RESULTS

3.1 | Spontaneous cytokine release signatures in PBMCs reflect respiratory virome profiles

Based on the baseline release of 13 out of 22 measured cytokines, individuals were annotated in principal components (Figure 1 and Data S2). Unsupervised clustering identified two groups with distinct cytokine release signatures (Figure 1). The first group (Cluster 1) included individuals (n = 37, 72.5%) that were characterized by low spontaneous cytokine release (Figure 1A,B), while the second group (Cluster 2) included individuals (n = 14, 27.5%) with high overall spontaneous cytokine release (Figure 1A,B). All individual cytokines contributed to the clustering at a range of 5.5%–7.2% (Figure S1a). The majority (86%) of children in the high cytokine spontaneous release group (Cluster 2) had prokaryotic dominated viromes (PVPG) (Specifically, high Shannon diversity and richness of prokaryotic viruses), in contrast to the low cytokine spontaneous release group (cluster 1) (Figure 1C) that included evenly distributed children with all three types of virome (PVPG, EVPG and AVPG) (p: .018, chi-Square test). These findings were independent of the presence of allergy or mild/moderate asthma (Figure S1b,c). There were no significant associations between baseline cytokine clusters and the presence of specific viral families (Picornaviridae, Anelloviridae and Siphoviridae) in the upper respiratory tract virome (Figure S2). To further assess baseline responses, a logistic regression model considering geography (Greece, Poland and Finland), VPG, sex, age and presence of Picornaviridae and Anelloviridae was performed (Table S2). This showed that EVPG and AVPG were statistically significant and negatively associated with PVPG (EVPG p: .034, beta: −2.92; AVPG p: .022, −3.01) in the non-stimulated culture medium confirming that the virome groups reflected the two types of response.

3.2 | PBMC bacterial immune signatures have minimal associations with respiratory virome characteristics

Bacterially stimulated samples were annotated in a coordinate plot based on their cytokine induction profiles (n = 17) (Figure 2A and Data S3). Hierarchical clustering identified three clusters of antibacterial immune responses (Figure 2A and Figure S3a). Cluster 1 (n = 15, 30%) included children with low release of bacterial DNA-stimulated cytokines, despite high response to LPS, especially for IL-1b, IL-6, TNF, CCL3 and CCL4 (Figure 2B). In contrast, Cluster 2 (n = 19, 37%) displayed high release of TNF, CCL3, CCL4, IL-6, IL-27 and IL-1b when stimulated by bacterial DNA (Figure 2B). Finally,

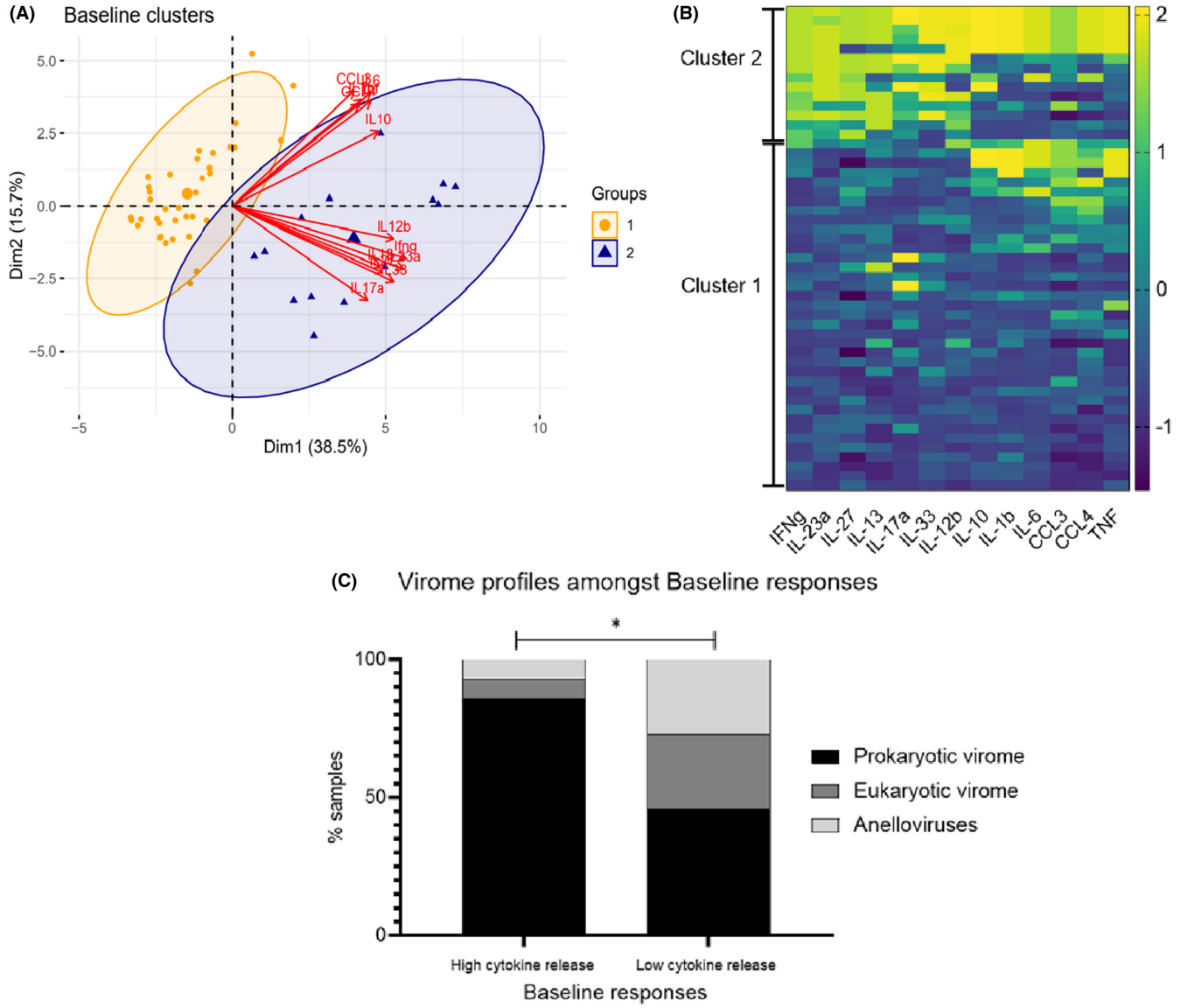


FIGURE 1 In preschool age children, respiratory virome profiles are associated with spontaneous cytokine release signatures from PBMCs. (A) Study subjects ($n = 51$) were clustered in two groups based on their spontaneous cytokine release at baseline. Cytokines contributing the most for the characterization of both clusters were used for analysis and are represented by arrows (clockwise: CCL3, IL-6, IL-1b, CCL4, TNF, IL-10, IL-12b, IFN-γ, IL-23a, IL-13, IL-27, IL-33 and IL-17a). (B) A quantitative response pattern differentiates baseline spontaneous release clusters. Each line represents a subject and each column a cytokine. The colour scale represents level of cytokine release. (C) High spontaneous cytokine release (cluster 2) from PBMC is associated with increased prokaryotic- and decreased eukaryotic- and anellovirus-dominated virome types. Low spontaneous cytokine release (cluster 1) subjects ($n = 37$) were divided between the virus profile groups (prokaryotic (PVPG) 46%, eukaryotic (EVPG) 27%, anellovirus (AVPG) 27%). In contrast, subjects with high spontaneous cytokine release ($n = 14$) included mostly PVPG (86%) and only 7% of EVPG and AVPG. ($p = .018$). Clusters in the *x*-axis were ordered by biological meaning (from 'high' to 'low').

Cluster 3 ($n = 17$, 33%) displayed heterogeneous and generally low cytokine responses (Figure 2B). Each cluster was characterized according to its features: Cluster 3 children were identified as low responders, Cluster 1 as intermediate responders, and Cluster 2 as high responders. We then explored possible correlations with the presence of specific viral families (Picornaviridae [$n = 28$, 55%], Anelloviridae [$n = 34$, 66%], Siphoviridae [$n = 20$, 39%]) and the virome profiles groups (VPGs) (Figure 2C) to each type of responder. Neither the presence of different virus families, nor the type of virome significantly differ across the three cytokine clusters, that is these were independent from the antibacterial response (Figure 2C). Moreover, antibacterial responses were not associated with mild/moderate asthma or allergy outcomes (Figure S3b,c). Nevertheless, in the regression model, the geographical location (Poland p: <.001, beta:10.95; p: <.001, beta: −10.91; Finland p: <.001, beta: −9.1; p: <.001, beta: −11.37) and the presence of Siphoviridae (p: <.001, beta: −8.67; p: <.001, beta: 13.81) had significant inference in high and low responders, respectively (Cluster 2 and 3) over intermediate responders (cluster 1) regarding their antibacterial responses (Data S5), suggesting a gradient of bacteriophages in reverse correlation with responses to bacterial stimuli (i.e. high bacterial response corresponding to low levels of bacteriophages).

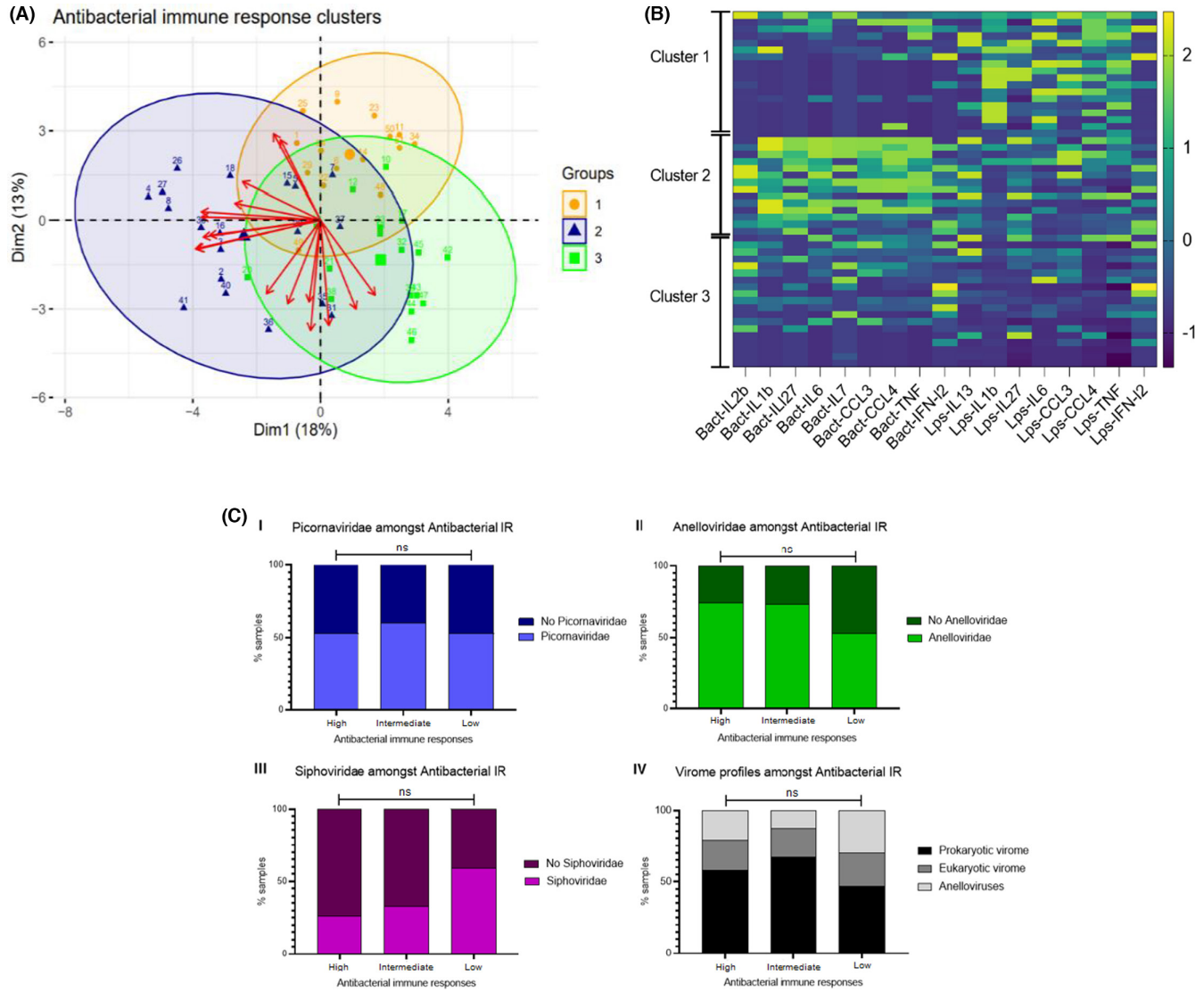


FIGURE 2 Antibacterial immune signatures have minimal associations with respiratory viral signatures. (A) Bacterial stimuli trigger high, medium and low inflammatory responses. Subjects (n = 51) were clustered based on their cytokine induction profile following stimulation with LPS and bacterial DNA (Bact). Cytokines contributing the most for the characterization of the different clusters were used for analysis and are represented by arrows (counterclockwise: LPS-IFN-λ2, Bact-IFN-λ2, Bact-IL12b, Bact-IL7, Bact-CCL3, Bact-CCL4, Bact-IL27, Bact-IL1β, Bact-IL6, Bact-TNF, LPS-IL13, LPS-IL27, LPS-CCL3, LPS-CCL4, LPS-TNF, LPS-IL6, LPS-IL1β). (B) Antibacterial immune response clusters. Each line represents a subject and each column a cytokine (induced by either LPS or Bacterial DNA (Bact)). The colour scale represents level of induction. Cluster 2 subjects show higher overall induction values, while Cluster 1 includes low responders. Cluster 3 represents an intermediate response. (C) Innate immune responses against bacterial stimuli are mostly independent from the virome composition. The presence of Picornaviridae (I) and Anelloviridae (II) is equally distributed among cytokine response clusters. A gradient in regard to the presence of Siphoviridae (III) did not reach statistical significance in this analysis, but was significant in multivariate regression (see Table S3). Clusters in the x-axis were ordered by biological meaning (from 'high' to 'low').

3.3 | PBMC antiviral immune signatures correlate with the presence of Siphoviridae and Picornaviridae in the upper respiratory virome

We identified 4 clusters describing PBMC responses to viral stimuli (Figure 3A,B and Data S4). IL-1b, CCL3, CCL4 and TNF in all virus-like stimulants had a major influence in clustering the samples, among a total of 23 significant conditions (Figure S4a). Cluster 4 children (n = 5, 10%) had a high and homogeneous response to TLR3 (Poly:IC) and TLR7/8 (R848) stimulated cytokines, namely IL-1b, IL-23a, IL-27, IL-6, CCL3, CCL4, TNF, IFN-α2, IL-25, TNF and IL-13, but not to rhinovirus A (RV-A) (Figure 3B). In contrast, the release of IL-1b, IFN-γ, CXCL10, CCL3, CCL4, TNF and IL-17a in response to RV-A was high in Cluster 2 children (n = 11, 21.5%) (Figure 3B). Children with the lowest overall cytokine responses were grouped on Cluster 3 (n = 14, n = 27.5%), while the largest group of subjects displayed a heterogenous pattern in their responses and were assigned to Cluster 1 (n = 21, 41%) (Figure 3B). Consequently, each group was

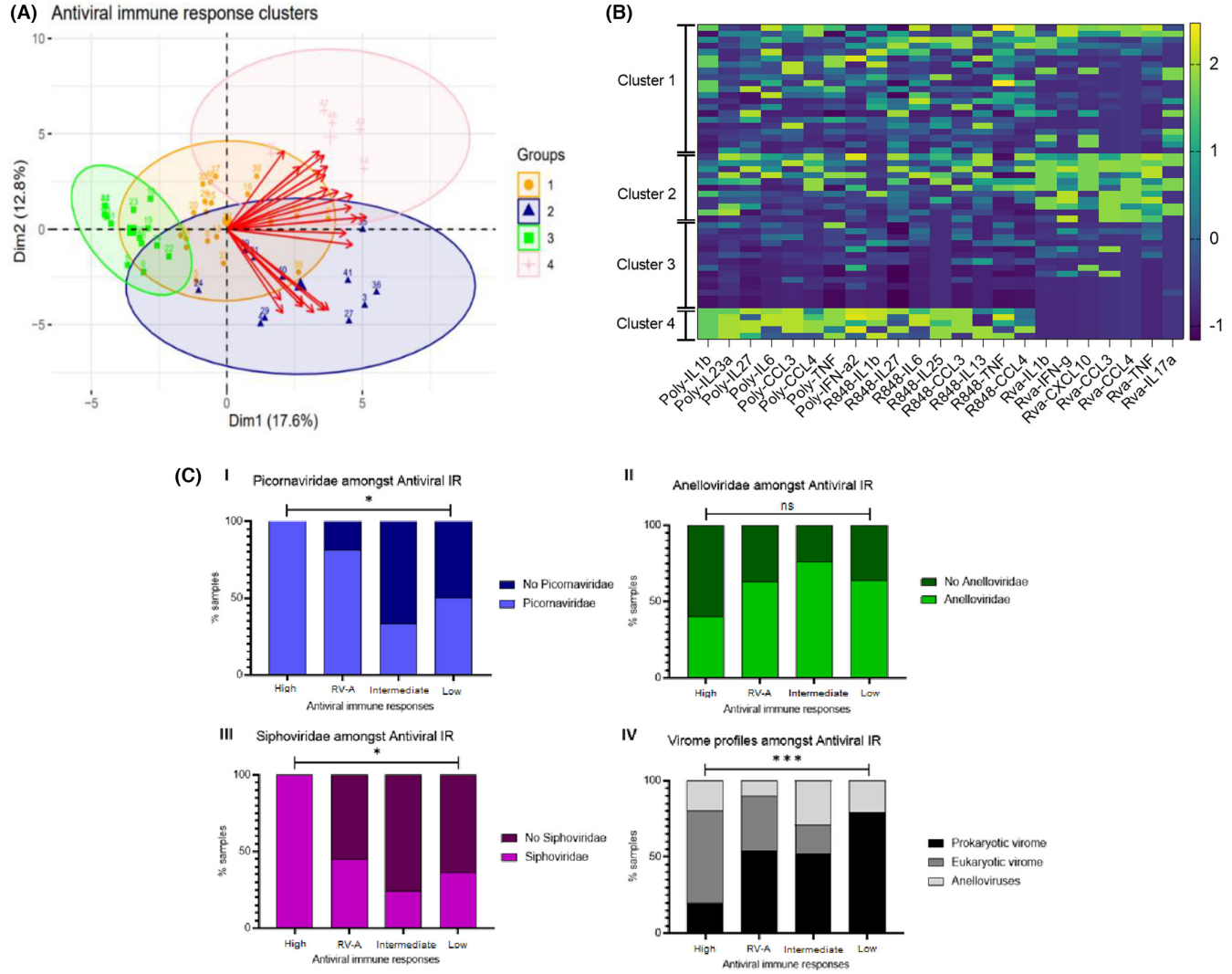


FIGURE 3 Antiviral immune signatures correlate to the presence of picornaviruses and bacteriophages in the respiratory virome. (A) Viral stimuli trigger four distinct types of responses. Subjects ($n = 51$) were clustered based on their cytokine induction profile following stimulation with R848, polyIC and RV-A. Cytokines contributing the most for the characterization of the different clusters were used for analysis and are represented by arrows (clockwise: R848-IL1b, R848-TNF, Poly-TNF, Poly-IL-1b, Poly-IL6, R848-IL6, Poly-CCL3, Poly-IFN-α2, R848-CCL3, R848-IL25, Poly-IL23a, Poly-IL27, Poly-CCL4, R848-CCL4, R848-IL27, RVA-TNF, RVA-CCL4, RVA-IFN-γ, RVA-IL6, RVA-IL13, RVA-CXCL10, RVA-IL17a, RVA-CCL3). (B) Antiviral immune response clusters. Each line represents a subject and each column a cytokine (induced by either R848, polyIC, or RV-A). The colour scale represents level of induction. Cluster 3 subjects show low induction, in contrast to Clusters 2 (RV-A) and 4 (high responders) which include high responders to RV-A or polyIC/R848, respectively. Cluster 1 represents an intermediate response. (C) Correlation of antiviral immune responses with virus families and virome patterns. While Anelloviridae (II) are equally distributed among antiviral response groups, Picornaviridae (I) and Siphoviridae (III) are more prevalent in subjects with strong antiviral responses. Subjects with low antiviral responses have significantly more prokaryotic and less eukaryotic viromes (IV). Clusters in the *x*-axis were ordered by biological meaning (from 'high' to 'low').

characterized as follows: Cluster 4: overall high responders, Cluster 2: RV-A responders, Cluster 1: intermediate responses and Cluster 3: low responders.

High responders had significantly higher presence of Picornaviruses (*p*-value: .036, 95%CI) in their upper airway, in comparison with low cytokine responders (Figure 3C). This was also observed regarding Siphoviridae, however, with statistically marginal value (*p*: .072) (Figure 3C). No differences were observed considering the presence of Anelloviridae. The comparison of the virome profiles confirmed a significantly biased virome composition between children in the different cytokine clusters (*p*-value: .0004, 95%CI) (Figure 3C). These associations were not affected by the presence of mild/moderate asthma or rhinitis (Figure S4b,c).

In the regression model, geographical location influenced the clustering (Table S4). The results confirmed the difference between RV-A and intermediate responders regarding to the presence of Siphoviridae and Picornaviridae (*p*: .027, beta: 2.83; *p*: <.001, −11.63). Additionally, in low responders (Cluster 3), there were significantly more prokaryotic than eukaryotic viral group types (*p*: <.001, beta: −29.67) (Table S4).

3.4 | Presence of viral families in the airway and its association with antibacterial and antiviral cytokine induction in PBMCs

To further describe potential associations between antibacterial and antiviral PBMC responses with viral presence in the nasopharynx, we investigated cytokine induction levels in the presence of the Picornaviridae, Siphoviridae, Anelloviridae and additionally Paramyxoviridae viral families (Figure 4). Among stimulants, bacterial DNA and LPS were considered for the antibacterial responses and Poly:IC, R848 and RV-A for the antiviral. When Picornaviridae were present in the nasopharynx, bacterially stimulated production of IFN-λ-2, CCL5 and IL-12b were low (Figure 5). The presence of Siphoviridae was also related to low antibacterial responses (Figure S5a); however, some inflammatory cytokines (IL6, CXCL8, CCL4, TNF) were upregulated after LPS stimulation (Figure S5b). Regarding antiviral responses, the presence of Picornaviridae (Figure 5), and Siphoviridae (Figure S5c) was associated with low levels of IFN-λ-2 responses; Siphoviridae were also associated with reduced IL-7, IL-23a and IL-12b, but increased IFN-α2. Paramyxoviridae showed similar results to Picornaviridae (Figure 4), in addition to downregulation of IL-12b and IL-23a after bacterial and R848 stimuli and upregulation of LPS-stimulated IL-6, CXCL-10 and CCL4. In contrast, presence of Anelloviridae coincided with increased production of CCL4, IL-6, IL-27 and IL-10 against bacterial stimuli and TNF and IL-7 against viral stimuli (Figure S6). In all, Siphoviridae displayed the broadest association with PBMC derived cytokines following either bacterial or viral stimulation (Figure 4).

4 | DISCUSSION

This is the first study showing that antiviral immune responses at a systemic level are associated with the upper airway virome composition during free acute infections periods. Although most attention is currently given to the mechanisms by which the microbiota and/or their components shape the immune responses,[17] we are also well aware that host immune responses can regulate microbial expansion and therefore control microbiota.[18] Analysing the immune status in combination with the microbiome is thought to be necessary for understanding the mechanisms involved in microbial influence of clinical outcome.[17] Another relevant finding of the study is the higher abundance of particular viral families, such as Picornaviridae and Siphoviridae, in individuals with low innate interferon responses.

When spontaneous cytokine release was evaluated, high producers were dominated by prokaryotic virome profiles. There are two, non-mutually exclusive, possible explanations: one, a high cytokine secretion status may result in reduction of Anellovirus and Picornavirus presence and diversity, or, high baseline responses might be the result of concurrent bacterial expansion, which in turn favours the proliferation of bacteriophages.[19] However, the latter explanation is less likely, considering that the extent PBMCs get activated by confronting bacteria during homeostasis, is minor.[20]

Regarding antibacterial responses, we observed a differential response against LPS versus bacterial-DNA. Although both stimuli activate antibacterial responses in the cell, they initiate TLR signalling from distinct locations: LPS does not require internalization to activate the signal cascade, while bacterial DNA does.[21] This distinct immune activation pattern has been previously reported.[22]

In line with the established understanding of rhinovirus (RV) biology, we have observed a correlation between the presence of picornaviruses (mostly RVs) and low levels of IFN-λ2, IL-12 and RANTES (CCL5), following innate immune stimulation.[23] Very similar results were obtained in regard to Paramyxoviridae, who are similarly pathogenic upper respiratory RNA viruses. Several studies have suggested that interferon deficiency is a key mechanism

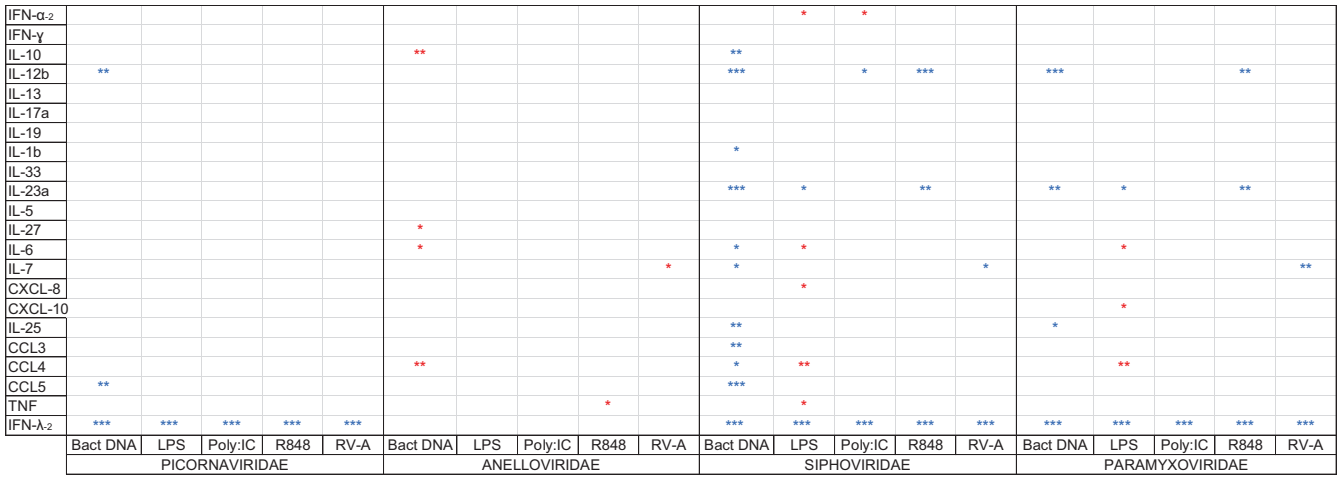


FIGURE 4 Cytokine changes in the presence versus absence of Picornaviridae, Anelloviridae, Siphoviridae and Paramyxoviridae following bacterial and viral stimuli. The *p*-value is represented by asterisks: $p < .05^{*}$, $p < .01^{**}$, $p < .001^{***}$. Red asterisks show a significant increase of the corresponding cytokine in presence of the viral family, blue asterisks a decrease. Antibacterial responses: bacterial DNA, LPS; antiviral responses: Poly:IC, R848, RV-A.

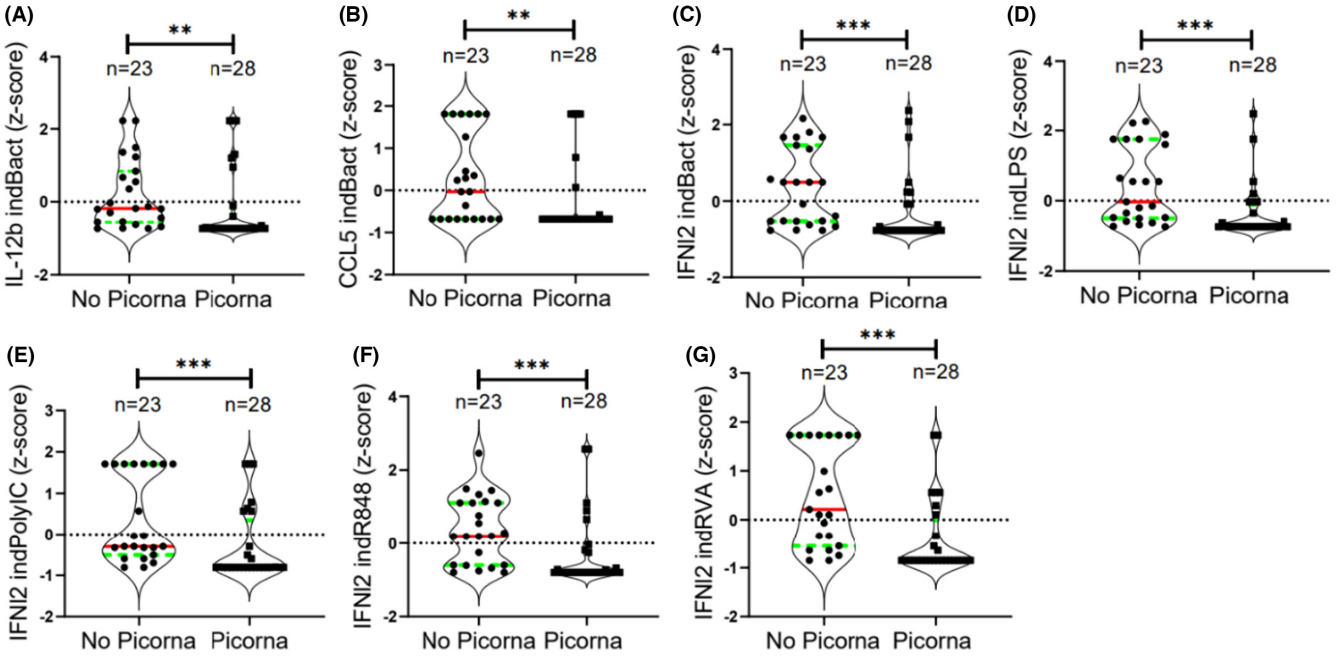


FIGURE 5 In children with Picornaviridae in their nasopharynx, reduced IFN responses, to both viral and bacterial stimuli are observed. (A) Bacterially induced IL-12b (*p*: .004), (B) Bacterially induced CCL-5 (*p*: .019), (C) Bacterially induced IFN-λ-2 (*p*: .001), (D) LPS-induced IFN-λ-2 (*p*: 0), (E) PolyIC-induced IFN-λ-2 (*p*: .001), (F) R848-induced IFN-λ-2 (*p*: 0), (G) RVA-induced IFN-λ-2 (*p*: 0).

supporting RV replication[24–26] and induction of exacerbations in patients with asthma.[27] Interestingly, although IFN-λ2 showed decreased levels in presence of picornaviruses, IFN-α2 protein levels remained unchanged. IFN-λ2 (IL-28A) belongs to IFN III-type and INF-α2 to IFN I-type. In contrast to type I IFNs, type III IFNs are not ubiquitously expressed and are mainly found at barrier epithelial surfaces such as the respiratory tract where they exhibit unique not-redundant antiviral functions.[28,29] Interestingly, it has been reported that type III IFNs suppress Th2 responses in experimental asthma in mice,[30] while respiratory viral pathogens have evolved mechanisms to suppress IFN-λ function or downregulate signalling, underlying their contribution to respiratory immunity at mucosal barriers.[31,32] Low IFN release leads to an increased RV replication, which in turn may result in extended inflammation and therefore a high antiviral response.[27,33]

TLR3 is believed to mediate the antiviral response. However, poly:IC is not always a good model for studying the biology of live virus infection. A published report claimed that blocking TLR3 during a RV infection increased cellular proinflammatory responses in bronchial epithelial cells. They also hypothesized that there is a balance of responses induced by TLR3 activation in response to RV that is not apparent for poly:IC.[34] The increased inflammatory cytokine release in response to RV stimuli and not to TLR3-agonist in cluster 2 (RV responders) might be explained by similar mechanisms.

Our findings indicate that this is a wider mechanism that controls the extent of RV presence in the upper airway mucosa, in which RV is a frequent, but transient visitor.[35] Picornaviruses were also present in all samples from subjects with high antiviral responses, but low responses to RV (cluster 4). This can be due to RV-specific defects, as the ones that have been described on a genetic basis.[36]

Bacteriophages, such as Siphoviridae, are involved in the modulation of bacterial communities and therefore potentially influencing health outcomes.[13,37] In our cohort, the presence of Siphoviridae was extensively negatively correlated with both antibacterial DNA and antiviral cytokine immune responses, while there was a positive correlation of inflammation-related cytokines (IL-6, IL-8, TNF) following LPS stimulation. It is probable that a robust antimicrobial capacity limits the potential of bacterial growth, consequently reducing bacteriophage proliferation.[38] This finding may have important implications, as it suggests a potential role of bacteriophages as sensitive sensors of host immunity. Although data are scarce, the effect of bacteriophages on the immune system appears to be mostly indirect, through their impact on their target bacteria.[13,19,37] Nevertheless, more complex viral-bacterial interactions may contribute to these observations. Viral-bacteria and particularly phage-bacterial dynamics are complex and unexplored. Reduced phage presence might influence bacterial colonization through the absence of appropriate ecological interactions. In addition, increased co-occurrence of eukaryotic viruses may trigger a transition to increased susceptibility for viral and bacterial infections, consequently compromising the immune system's stability and resilience.[13] In our study, we found evidence that reduced eukaryotic and anelloviruses virome profiles characterize subjects with a high cytokine release state, reinforcing the concept that microbial dysbiosis might reflect a lack of the system's robustness.

It is more challenging to explain the observed positive associations between the presence of anelloviruses and mostly inflammatory (IL-6, MIP1b) and regulatory (IL-10, IL-27) cytokines following bacterial, and to a less extent also viral stimulation. Even though we found no association between its presence and an increased IFN response, previous studies have stated that anelloviruses can induce innate cells to produce and secrete IFN-γ and stimulate inflammatory responses.[39] Anelloviruses are apparently non-pathogenic viruses that have been associated with conditions of immune suppression[40] and are considered an integral part of the respiratory virome, particularly in asthma.[5] Anellovirus-dominated profiles and anellovirus presence were equally distributed among both antibacterial and antiviral immune response clusters, suggesting that anellovirus presence may be controlled by mechanisms other than TLR-stimulation. It is possible that inflammatory instead of antiviral responses may facilitate anellovirus proliferation.

When studying the microbiome, it is challenging to differentiate the causal host-microbiome associations from secondary microbial changes. In many immune-related conditions, abnormal viral-bacterial interactions can be considered as either a cause or a marker of the disease state.[41,42] Individual variations in the quality of T-cell responses involving RV-specific PBMC proliferation and IFN-γ secretion might be a key determinant regarding the degree of viral replication.[43] Our results highlight important correlations between the respiratory virome and immune signatures; however, we cannot establish causality. A noteworthy observation is that even though the cohort was comprised of healthy and atopic asthmatic individuals, disease was not a modifier of the correlations, suggesting a fundamental mechanism of immune-microbial interaction; or the result of insufficient statistical power to identify such patterns. The power of the study may be limited in its ability to find an association between virome profiles and asthma or allergy development; thus, it does not imply there is none, also considering severe asthmatics were excluded from the study.

One limitation of the study is the measurement of one post-induction time point, so we could only describe cross-sectional associations instead of a complete response curve, due to practical limitations. Despite all the benefits of the PBMC model, it is composed by a mixed population of isolated mononuclear immune cells whose composition is dependent on the donor's physiological status; hence, it increases variability and excludes granulocytes. Also, the number of samples was moderate; however, we used robust and state-of-the-art methodology for cytokine measurements and for the characterization of the virome. Subjects from a wide geographic representation were included. All participating centres followed a validated and synchronized approach for PBMC cultures with common training and reagents. The processing and assessment for nasopharyngeal samples and immune responses were independent.

In conclusion, there are tight parallels between the upper airway virome and the host immune status and potential innate immune responses. Viral stimulation has the capacity of directing immune responses, while immune responses themselves may control microbial composition. The unravelling of such interactions offers new opportunities for intervention towards disease prevention.

AUTHOR CONTRIBUTIONS

JR, SM and NP involved in conceptualization. JR, SM, JL and NP involved in methodology. JR, SM and JL involved in software. JR and JL involved in visualization. JR, SM and NP involved in investigation. MP, JL, BC, PX, CB, SF, TJ, EA, BS, CA and MA involved in resources. JR involved in writing—original draft. JR, SM and NP involved in writing—review and editing. All authors involved in writing—final submission. NP involved in supervision.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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