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, 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 analyzed by Luminex for the presence of 22 relevant cytokines. Virome composition was obtained using untargeted high troughput 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 amongst immune response clusters. The presence of Picornaviridae and Siphoviridae was associated with low interferon-λ responses. Asthma or allergy did not modify these correlations. Conclusions: Antiviral cytokines 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 affected by TLR-induced 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, 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 analyzed by Luminex for the presence of 22 relevant cytokines. Virome composition was obtained using untargeted high troughput 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 amongst immune response clusters. The presence of Picornaviridae and Siphoviridae was associated with low interferon-λ responses. Asthma or allergy did not modify these correlations.

Conclusions: Antiviral cytokines 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 affected by TLR-induced immune responses.

Keywords: Asthma, bacteriophages, Interferon-λ, Rhinoviruses, virome

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 mold 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 multi-center 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, i.e. 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, 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](Supplementary Table 1). Written informed consent was obtained from the parents or by the legal guardians and the study was approved by the Regional Ethics Committee of Karolinska Institutet, Stockholm, Sweden.

Cases needed to be diagnosed with 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 Supplementary Table 1.

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, Massachusetts). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifuging at 800g for 20 min at 18-20^{o}C on Biocoll separating solution (Biochrom AG, Germany). After three washes, PBMCs were resuspended in complete medium (CM) [RPMI-1640 with HEPES 25 mM and L-Glutamine (Gibco, Life Technologies Ltd, UK), supplemented with 10ml/L Penicillin-Streptomycin USA, 50µl/L 1M β-mercaptoethanol, 20ml/L L-Glutamine plus MEM Vitamin, 20ml/L Non-essential Amino Acid, Sodium Pyruvate and 10% heat-inactivated FBS (all from Sigma-Aldrich, Germany)] at concentration 10^6 cells/ml.

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

The cell suspension was seeded in a flat-bottom 48-well tissue plate (Corning Incorporated, Costar, New York), with 5×10^5viable cells per well (500µL). 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: 4µg/ml Resiquimod (R848), 5µg/ml Endotoxin-free bacterial DNA (InvivoGen, France), 20µg/ml Polyinosinic–polycytidylic acid potassium salt (Poly I:C), 1µg/ml Lipopolysaccharides from E.Coli 0111:B4 (LPS), (Sigma-Aldrich, Germany), at 37^{o}C, 5% CO^2.

Cultures were harvested after 48 hours and, after centrifugation at 600g for 5 min, supernatants were stored at -80^{o}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, TNF-α.

2.3. Characterisation 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 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 Supplementary Data 1.

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. Pre-processing, 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 & RV1b) 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 (Supplementary table 2). 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, 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 cytokines values were compared between subjects with or without the presence of pre-specified viral families (Picornaviridae, Siphoviridae, Anelloviridae), using Wilcoxon's rank-sum 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 0.05. 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 Supplementary Data 2). 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-1b), while the second group (Cluster 2) included individuals (n=14, 27.5%) with high overall spontaneous cytokine release (Figure 1a-1b). All individual cytokines contributed to the clustering at a range of 5.5%-7.2% (Supplementary Figure 1a). 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:0.018, Chi-Square test). These findings were independent of the presence of allergy or asthma (Supplementary figure 1b-1c). 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 (Supplementary Figure 2). 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 (Supplementary Table 3). This showed that EVPG and AVPG were statistically significant and negatively associated with PVPG (EVPG p:0.034, beta: -2.92; AVPG p:0.022, -3.01) in the non-stimulated culture medium (Supplementary

Table 4), 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 & Supplementary Data 3). Hierarchical clustering identified three clusters of antibacterial immune responses (Figure 2a and Supplementary Figure 3a). 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, Cluster 3 (n=17, 33%) displayed heterogeneous and generally low cytokine responses (Figure 2b). Each cluster was characterised 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, i.e. these were independent from the antibacterial response (Figure 2c). Moreover, antibacterial responses were not associated with asthma or allergy outcomes (Supplementary Figure 3b-3c). Nevertheless, in the regression model, the geographical location (Poland p:<0.001, beta:10.95; p:<0.001, beta:-10.91; Finland p:<0.001, beta:-9.1; p:<0.001, beta:-11.37) and the presence of Siphoviridae (p:<0.001, beta:-8.67; p:<0.001, 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 (Supplementary 5), suggesting a gradient of bacteriophages in reverse correlation with responses to bacterial stimuli (i.e. high bacterial response corresponding to low levels of bacteriophages, etc).

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 and 3b & Supplementary Data 4). 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 (Supplementary Figure 4a). 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-a2, IL-25, TNF, 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 were 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 characterized as follows: Cluster 4: overall high responders, Cluster 2: RV-A responders, Cluster 1: intermediate responses, Cluster 3: low responders.

High responders had significantly higher presence of Picornaviruses (p-value: 0.036, 95%CI) in their upper airway, in comparison to low cytokine responders (Figure 3c). This was also observed regarding Siphoviridae, however with statistically marginal value (p:0.072) (Figure 3d). 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: 0.0004, 95%CI) (Figure 3c). These associations were not affected by the presence of asthma or rhinitis (Supplementary Figure 4b-4c).

In the regression model, geographical location influenced the clustering (Supplementary Table 6). The results confirmed the difference between RV-A and intermediate responders regarding to the presence of Siphoviridae and Picornaviridae (p:0.027, beta:2.83; p:<0.001, -11.63). Additionally, in low responders (Cluster 3) there were significantly more prokaryotic than eukaryotic viral group types (p:<0.001, beta:-29.67) (Supplementary Table 6).

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 and Anelloviridae 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, IL-12b were low (Figure 5). The presence of Siphoviridae was also related to low antibacterial responses (Supplementary Figure 5i), however some inflammatory cytokines (IL6, CXCL8, CCL4, TNF) were upregulated after LPS stimulation (Supplementary Figure 5ii). Regarding antiviral responses, the presence of Picornaviridae (Figure 5), as well as Siphoviridae, (Supplementary Figure 5iii) were 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-a2. 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 (Supplementary Figure 6). 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 reflect 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, IL12 and RANTES (CCL5), following innate immune stimulation [23]. Several studies have suggested that interferon deficiency is a key mechanism supporting RV replication [24,25,26] 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 [37,38].Interestingly, it has been reported that type III IFNs suppress Th2 responses in experimental asthma in mice [39], while respiratory viral pathogens have evolved mechanisms to suppress IFN-λ function or downregulate signalling, underlying their contribution to respiratory immunity at mucosal barriers [28,36].

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 [29]. 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 [30].

Bacteriophages, such as Siphoviridae, are involved in the modulation of bacterial communities and therefore potentially influencing health outcomes [13,31]. 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 (IL6, IL8, TNF) following LPS stimulation. It is probable that a robust antimicrobial capacity limits the potential of bacterial growth, consequently reducing bacteriophage proliferation [32]. 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 [13,19,31]. Nevertheless, more complex viral-bacterial interactions may contribute to these observations [13].

It is more challenging to explain the observed positive associations between the presence of anelloviruses and mostly inflammatory (IL6, MIP1b) and regulatory (IL10, IL27) cytokines following bacterial, and to a less extent also viral stimulation. Anelloviruses are apparently non-pathogenic viruses that have been associated with conditions of immune suppression [33] and are considered an integral part of the respiratory virome, particularly in asthma [5]. Anellovirus-dominated profiles as well as 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 [34, 35]. 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.

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. The number of samples was moderate, however, we used robust and state-of-the-art methodology for cytokine measurements as well as 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 was 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.

Data availability:

The datasets generated during and/or analysed during the current study are available as supplementary material.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Judit Rovira Rubió (juditroviraa@gmail.com) and Nikolaos Papadopoulos (npgallergy@gmail.com).

Author contributions:

Conceptualization: JR, SM, NP; Methodology: JR, SM, JL, NP; Software: JR, SM, JL; Visualization: JR, JL; Investigation: JR, SM, NP; Resources: MP, JL, BC, PX, CB, SF, TJ, EA, BS, CA, MA; Writing – Original

Draft: JR; Writing – Review & Editing: JR, SM, NP; Writing- Final submission: all authors; Supervision: NP.

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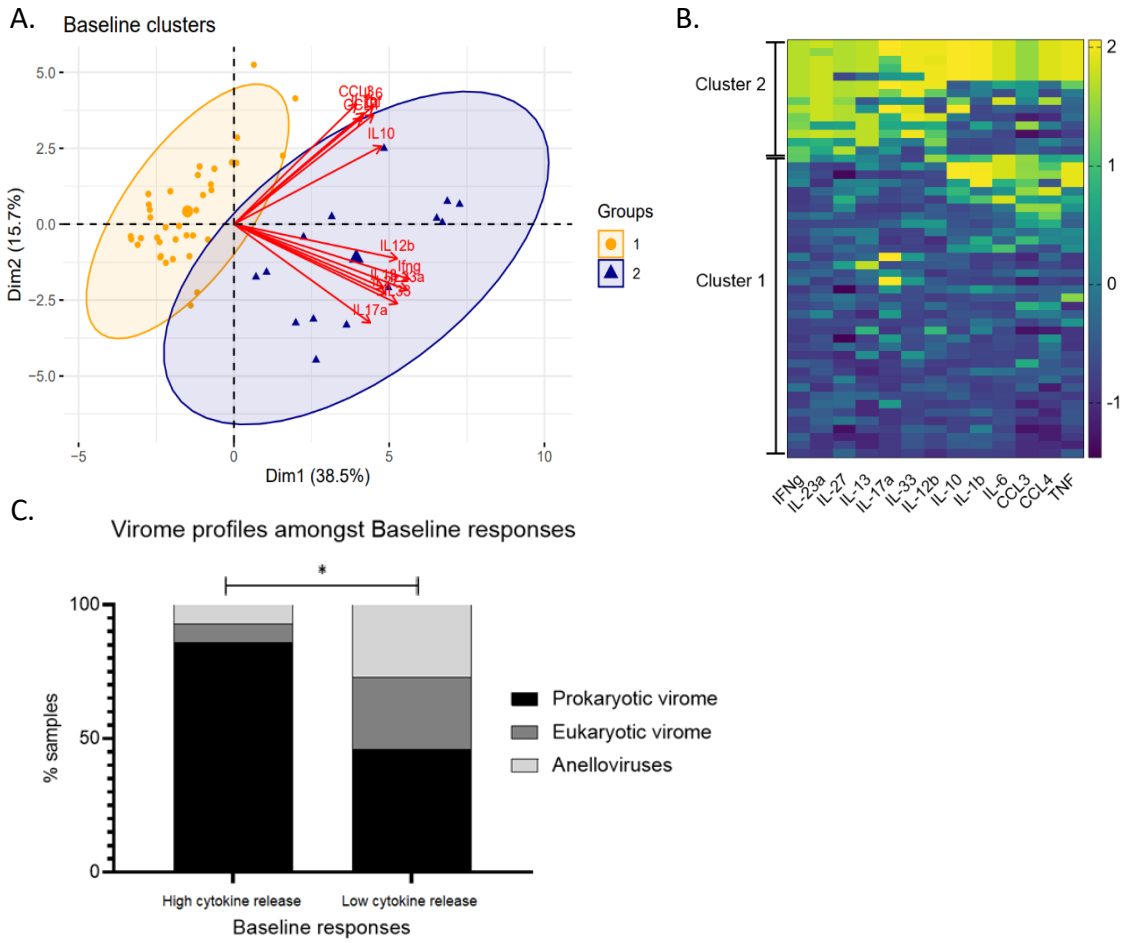

Figure 1. In preschool age children, respiratory virome profiles are associated with spontaneous cytokine release signatures from PBMCs. (A) Study subjects (n=51) were clustered in two groups based on their spontaneous cytokine release at baseline. Cytokines contributing to the clustering are represented by arrows (clockwise: CCL3, IL-6, IL-1b, CCL4, TNF, IL-10, IL-12b, IFN-ɣ, 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 color scale represents level of cytokine release. Cluster 2 subjects show higher overall values, in comparison to Cluster 1 subjects. (C) High spontaneous cytokine release from PBMC is associated with increased prokaryotic- and decreased eukaryotic- and anellovirus-dominated virome types. Cluster 1 (low spontaneous cytokine release) 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=0.018)

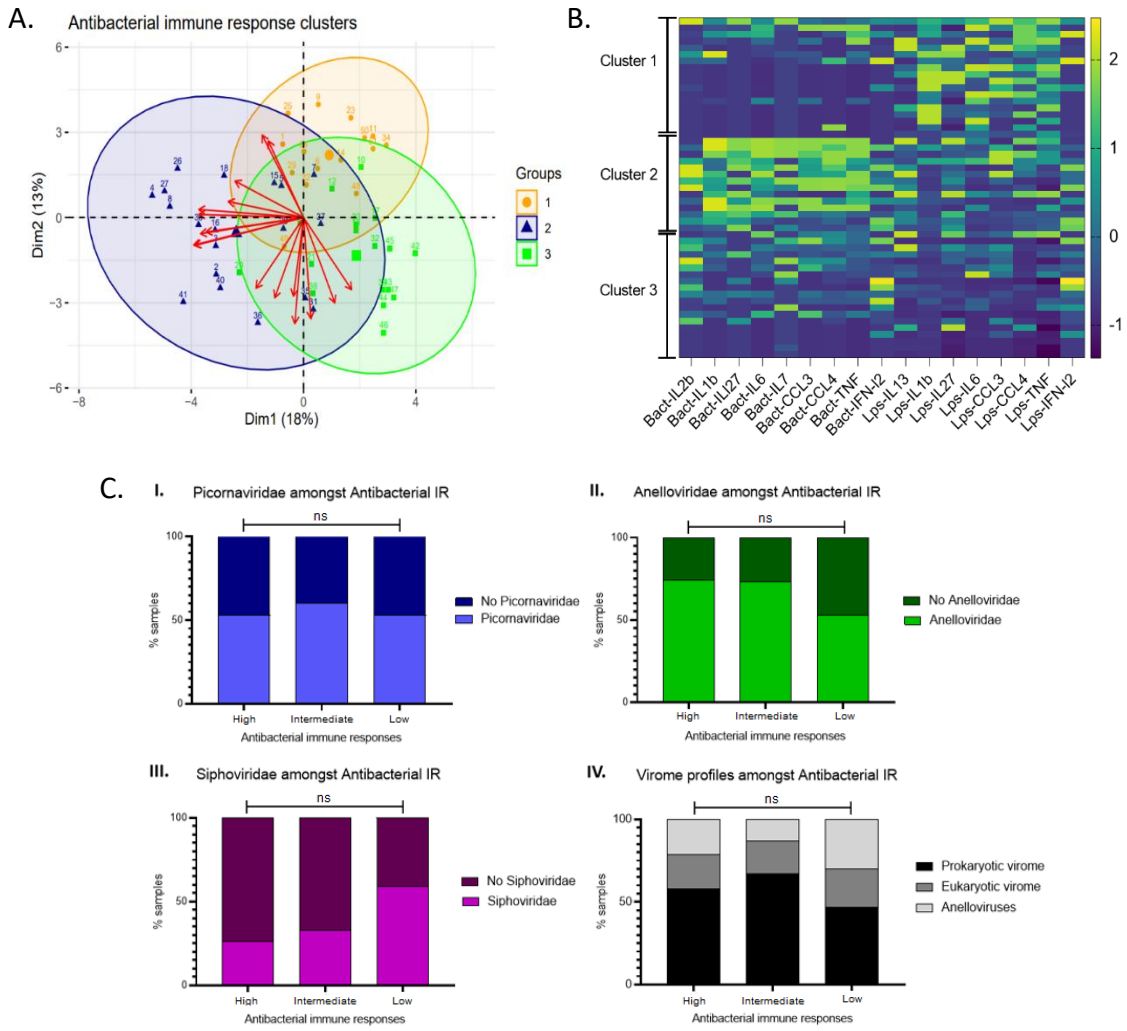

Figure 2. Bacterial immune signatures have minimal associations with respiratory viral signatures. (A) Bacterial stimuli trigger high, medium and low inflammatory responses. Subjects (n=51) were clustered based on their cytokine induction profile following stimulation with LPS and Bacterial DNA (Bact). Cytokines contributing to the clustering 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 color 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 Suppl. Table 5).

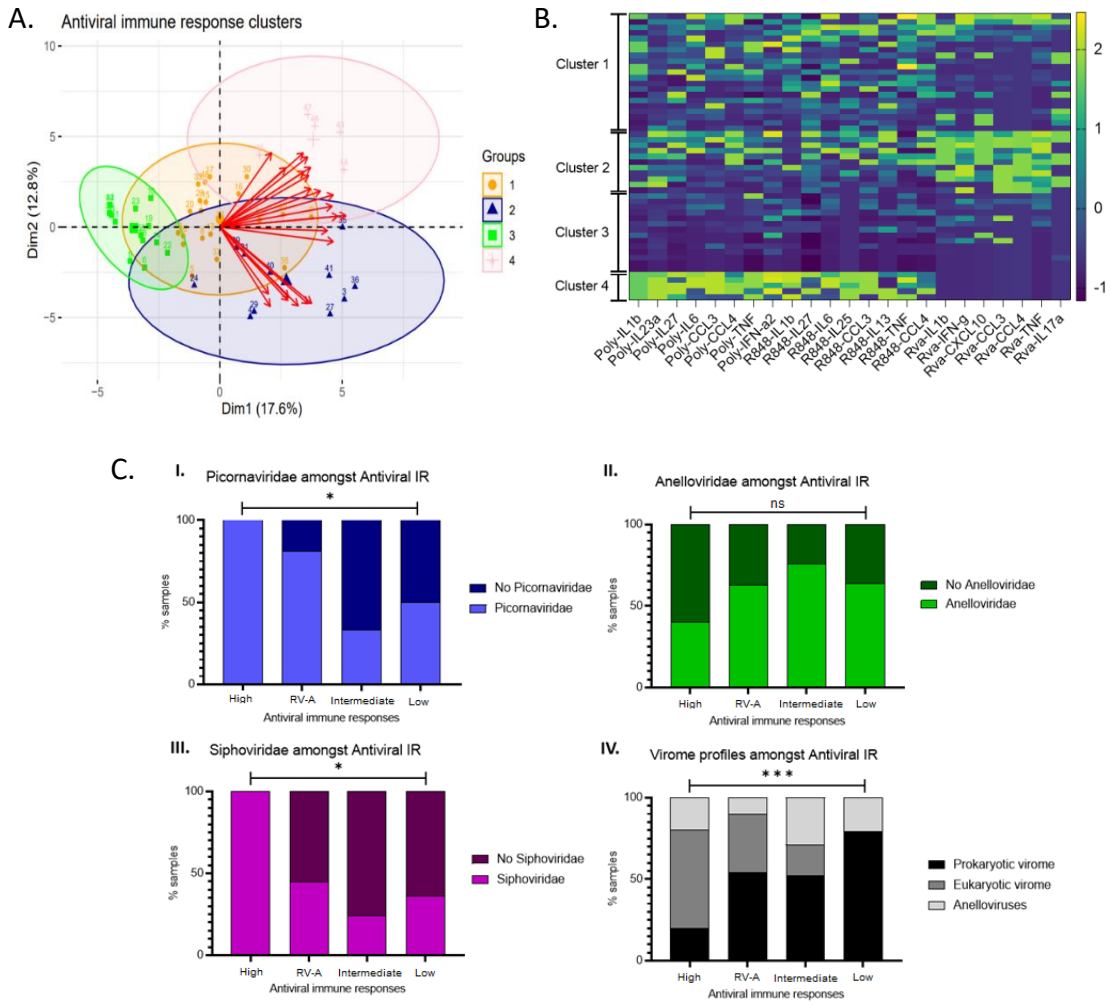

Figure 3. Viral immune signatures correlate to the presence of picornaviruses and bacteriophages in the respiratory virome. (A) Viral stimuli trigger four distinct types of responses. Subjects (n=51) were clustered based on their cytokine induction profile following stimulation with R848, polyIC and RV-A. Cytokines contributing to the clustering are represented by arrows (clockwise: R848-IL1b, R848-TNF, Poly-TNF, Poly-IL1b, 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-ɣ, 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 color scale represents level of induction. Cluster 3 subjects show low induction, in contrast to Clusters 2 and 4 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), as well as 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).

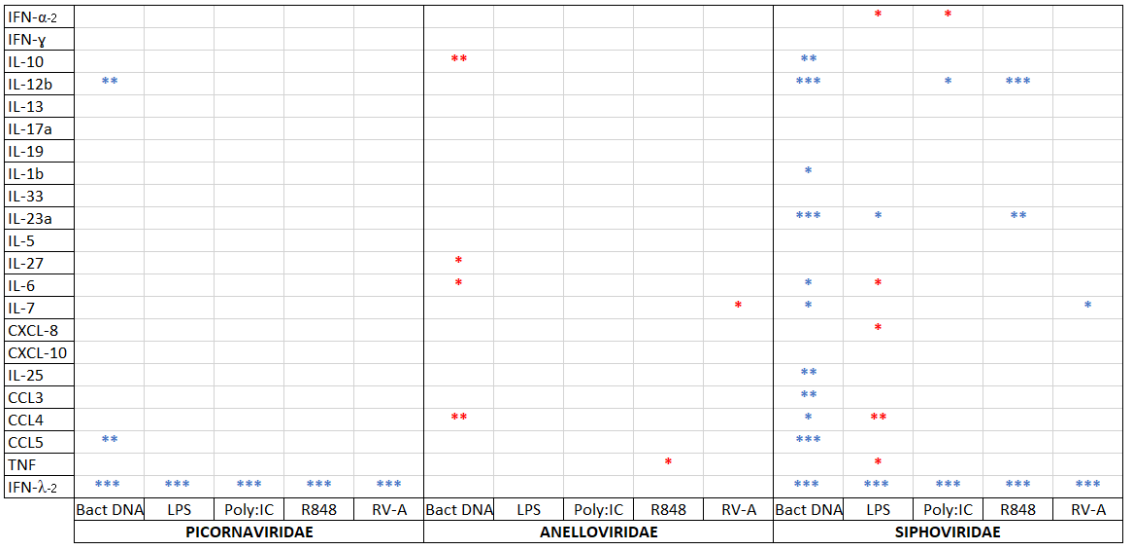

Figure 4. Cytokine changes in the presence versus absence of Picornaviridae, Anelloviridae and Siphoviridae following bacterial and viral stimuli. The p-value is represented by asterisks: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. Red asterisks show a significant increase of the corresponding cytokine in presence of the viral family, blue asterisks a decrease. Antibacterial responses: bacterial DNA, LPS; antiviral responses: Poly:IC, R848, RV-A

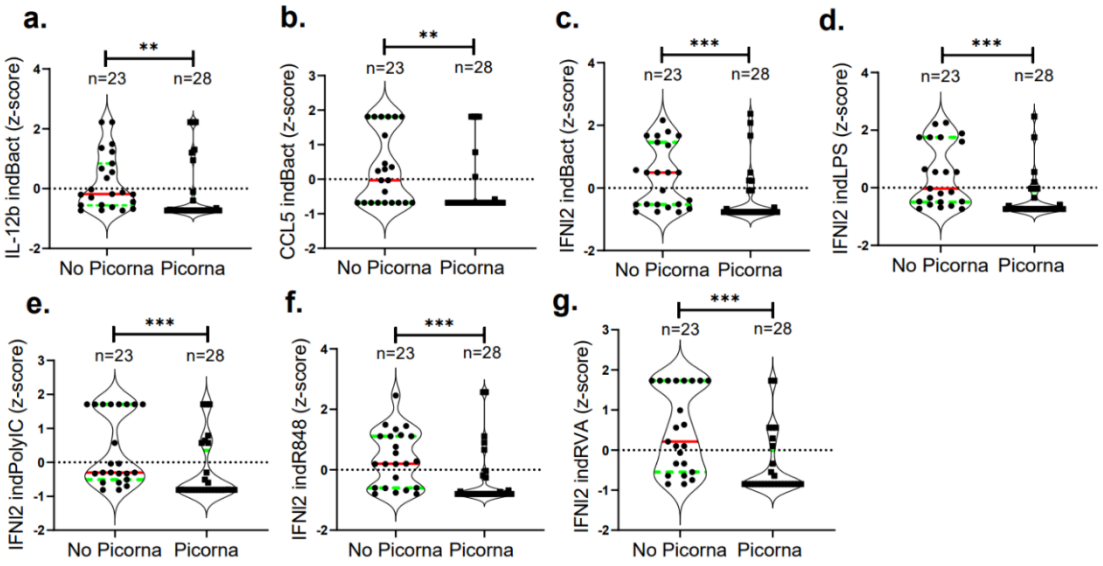

Figure 5. In children with Picornaviridae in their nasopharynx, reduced IFN responses, to both viral and bacterial stimuli are observed. (a) Bacterially-induced IL-12b (p:0.004), (b) Bacterially-induced CCL-5 (p:0.019), (c) Bacterially-induced IFN-λ-2 (p:0.001), (d) LPS-induced IFN-λ-2 (p:0), (e) PolyIC-induced IFN-λ-2 (p:0.001), (f) R848-induced IFN-λ-2 (p:0), (g) RVA-induced IFN-λ-2 (p:0).