Journal of Microbiology, Immunology and Infection 56 (2023) 282–291

Available online at www.sciencedirect.com

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Original Article

Clinical characteristics and differential cytokine expression in hospitalized Taiwanese children with respiratory syncytial virus and rhinovirus bronchiolitis

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Received 16 February 2022; received in revised form 24 August 2022; accepted 28 August 2022
Available online 9 September 2022

KEYWORDS
Bronchiolitis;
Cytokines;
Respiratory syncytial virus;
Rhinovirus

Abstract *Background:* Viral bronchiolitis presents a heterogeneous spectrum. In this study, we investigated the clinical characteristics and the cytokines/chemokines profiles among respiratory syncytial virus (RSV), rhinovirus (RV), and their dual infection in Taiwanese children with viral bronchiolitis.
Method: This study was conducted between October 2014 and June 2017. Viral etiology was identified using a Luminex respiratory virus panel and blood cytokines were evaluated using a MILLIPLEX MAP Human Cytokine/Chemokine Panel. Cytokine/Chemokine expressions were compared by clinical severity, steroid treatment, and viral entities.
Results: A total of 184 patients were evaluated; at least one respiratory virus was identified in 163 (88.6%) patients. RSV and RV were the two leading viral etiologies, with 25.5% and 17.3%, respectively. RV bronchiolitis has a comparable severity to RSV but is more common in children of an older age with a history of recurrent wheezing and blood eosinophilia. Decreased tumor necrosis factor-alpha (TNF-α) and interferon gamma (INF-γ) levels were correlated with clinical severity. Patients infected with RV exhibited higher levels of Interleukin (IL)-22, IL-23, IL-25, IL-31, and IL-33 ($p < 0.05$), whereas those with RSV had higher levels of TNF-α, INF-γ, and IL-10 ($p < 0.05$). Systemic steroid treatment was associated with higher expressions of

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https://doi.org/10.1016/j.jmii.2022.08.013

IL-4, IL-8, IL-13, and MIP-1α levels ($p < 0.05$). Cluster analysis revealed a high correlation of IL-33 and IL-31($R^2 = 0.9731$, $p < 0.0001$).
Conclusion: Different viral infections elicited the characteristic clinical presentation and immune profiles in bronchiolitis. Our findings also highlight the role of the IL-33/IL-31 axis in the immunopathogenesis of bronchiolitis.

Introduction

Typically, bronchiolitis is triggered by viral infections,[1–3] with respiratory syncytial virus (RSV) and human rhinovirus (RV) the two leading pathogens. Secondarily, in infants and young children, bronchiolitis is a major cause of hospitalization and primary care – currently without global consensus treatment.[1] Finally, recurrent bronchiolitis or wheezing in infancy is strongly linked to subsequent asthma development.[3,4]

Bronchiolitis is a heterogeneous spectrum.[5–7] Both viral and host factors play critical roles in the acute presentation of bronchiolitis and its long-term outcomes. Premature neonates, infants aged <6 months, and children with congenital cardiopathy or Down syndrome have a high risk of RSV infection.[8] Conversely, RV bronchiolitis presents with asthma-like features, affecting older children and those with a history of parental atopy or blood eosinophilia.[3,6,7] Given on the above-mentioned features, four phenotypes of bronchiolitis have been proposed in reference to two multicenter studies in the United States and Denmark.[3,5] Moreover, the proposed phenotypes have been supported through investigation of the nasal secretion immune responses in infants with RSV and RV.[9]

Studies on viral factors and immune responses have shed light on the pathogenesis of viral bronchiolitis, linking tumor necrosis factor-alpha (TNF-α), interferon gamma (INF-γ), interleukin (IL) 6, IL-8, and IL-10 to the clinical severity of RSV bronchiolitis.[10–12] Our understanding of RV-related immune responses is largely derived from experimental models and studies on human asthma.[13–15] The activation of eosinophil, associated with the expression of T helper 2 (Th2) cytokines and epithelial-derived IL-25 and IL-33, is strongly linked to RV infection–associated wheezing – though RSV often accounting for over 50% of total bronchiolitis cases may represent a bias.[1,2] Few studies have examined the difference in cytokine expressions between RSV and RV infection with a focus on viral bronchiolitis.[16]

In this study, we aimed to evaluate the association between cytokines expressions and the clinical features on bronchiolitis with various associated respiratory viral infections from the aspects of the Asian childhood population.

Materials and methods

Study participants and sample collection

This study was conducted prospectively at Chang Bing Show Chwan Memorial Hospital in Changhua, Taiwan between October 2014 and June 2017. Hospitalized children aged <24 months with acute bronchiolitis were eligible. Bronchiolitis was diagnosed in presence of rhinitis, tachypnea, wheezing, cough, or crackles; the use of accessory muscles; and/or presence of nasal flaring with or without fever. The severity of acute bronchiolitis was assessed using the Seattle Children's Hospital respiratory score. Eligible patients had to (1) be hospitalized within the previous 48 h, (2) have no previous history of physician-diagnosed asthma, and (3) have no history of malignancy, metabolic or genetic diseases, or immunodeficiency.

Once written informed consent was provided by parents or guardians, a nasal aspirate sample was collected for detecting respiratory viral pathogens. Following the standard care of each child upon admission, residual blood samples were stored at −70 °C prior to batch analysis. The demographic data, clinical features, and laboratory results of all the enrolled patients were collected and analyzed through medical chart review.

Ethical approval

The study protocol was approved by the Institutional Review Board of Chang Bing Show Chwan Memorial Hospital (No. IRB-1030401 and 1030802).

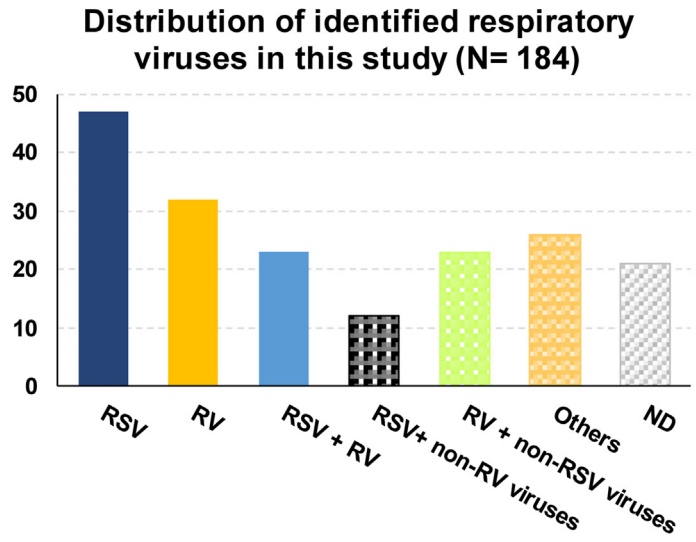
Nucleic acid extraction

Nucleic acids were extracted from the stock nasal aspirate samples using the QIAAmp Viral RNA/DNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA (or RNA) was eluted with 140 μL of elution buffer and stored at −70 °C until further analysis.

Respiratory virus detection

Viral etiology was determined using an xTAG RVP Fast Assay v2.0 (Luminex Molecular Diagnostics, Toronto, Canada) for the simultaneous detection of 19 common respiratory viral and subtype targets, including the influenza A virus, with the additional subtyping of positive specimens into H1, H3, and 2009H1N1v, influenza B virus, RSV types A and B, human coronaviruses (HCoVs, NL63, 229E, OC43, and HKU1), parainfluenza viruses types 1 to 4, human metapneumovirus, picornaviruses (RVs and enteroviruses), human bocavirus, and adenovirus. The assay was conducted using the xMAP 200 Instrument System (Luminex Molecular Diagnostics) and analyzed with TDAS RVP Fast software v2.0 (Abbott Molecular, Abbott Park, IL, USA).

A



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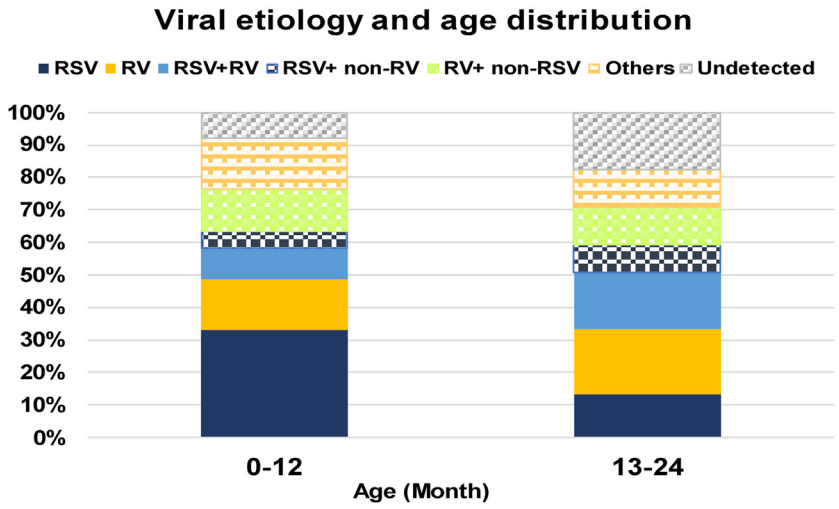

Figure 1. Distribution of the identified respiratory viruses in this study.
Viral diganostics were based on multiplex PCR analysis. The frequency of the viral causes in the sample (A), and the distribution of respiratory viruses according to the age of the hospitalized children (B).

RV detection and genotyping

Rhinovirus-specific nested polymerase chain reaction (PCR) was performed on all the RV- and enterovirus-positive samples. In brief, reverse transcription was performed using the Omniscript RT Kit (QIAGEN) and random primers. The cDNA was amplified using primers and protocols targeting the P1—P2 region.[17] Genotyping was conducted through the alignment of the RNA sequences with standard HRV sequences from the GenBank database.

Cytokine quantification

Nineteen cytokines and chemokines were quantified in the blood samples through use of the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel on the Luminex xMAP in accordance with the manufacturer's protocol, namely proinflammatory cytokines (IFN-γ, TNF-α, IL-6, Th1 [IL-12P70], Th2 [IL-4, IL-9, IL-10, IL-13, and IL-31], Th17 [IL-17A, IL-22, and IL-23]), epithelial-associated cytokines (IL-25 and IL-33), and chemokines (IL-8, monocyte chemotactic

Table 1 Demographical characteristics of the RSV, RV, and RSV-RV groups.

	(a) RSV	(b) RV	(c) RSV + RV	P value		
				a vs. b	a vs. c	b vs. c
Number	47	32	23			
Male (%)	74.5%	68.8%	73.9%	0.578	0.960	0.678
Age(month)	8.9 ± 6.4	11.9 ± 6.5	12.9 ± 6.4	0.047	0.016	0.558
Preterm birth (%)	18.6%	9.7%	10%	0.286	0.384	0.969
Breast milk feeding (%)	85.7%	96.8%	77.3%	0.112	0.395	0.077
Maternal atopic history (%)	21.4%	38.1%	46.2%	0.201	0.105	0.215
Eczema history (%)	26.2%	16.7%	19.0%	0.338	0.393	0.826
History of recurrent wheezing (%)	16.7%	53.8%	55%	0.001	0.002	0.938
Secondhand smoking (%)	55.6%	70.8%	72.2%	0.260	0.258	0.921
Laboratory findings						
White cell count (/ul)	9030.6 ± 3292.3	14560.3 ± 4524.4	11453.1 ± 4069.8	<0.001	0.016	0.004
Segment	2825.4 ± 1529.7	7152.0 ± 4040.6	4585.7 ± 2935.6	<0.001	0.115	0.029
Lymphocyte	5167.7 ± 2587.6	6117.5 ± 3041.1	5666.2 ± 2063.1	0.119	0.459	0.533
Eosinophil	127.6 ± 173.1	305.6 ± 209.7	235.8 ± 256.5	0.047	0.090	0.928
C-reactive protein (mg/dl)	0.7 ± 1.3	0.7 ± 0.8	1.1 ± 2.8	0.938	0.481	0.481
Clinical severity and treatment						
Presence of fever (%)	74.5%	50.0%	87.0%	0.026	0.232	0.004
Respiratory score	5.4 ± 2.0	6.5 ± 2.3	5.2 ± 1.8	0.028	0.754	0.033
Duration of hospitalization(day)	5.5 ± 1.9	5.3 ± 2.8	5.5 ± 2.3	0.670	0.957	0.759
Intensive care (%)	14.9%	29%	8.7%	0.151	0.257	0.034
Inhaled bronchodilators use (%)	85.1%	90.3%	100%	0.732	0.087	0.253
Systemic steroid use (%)	12.8%	25.8%	8.7%	0.142	1.000	0.161

Continuous data were analyzed by One-way ANOVA with least significant difference (LSD) multiple comparison; categorical data were analyzed by chi-square test.

protein-1 [MCP-1], macrophage inflammatory protein-1 alfa [MIP-1α], regulated upon activation, normal T cell expressed [RANTES], and intercellular adhesion molecule 1 [ICAM-1]). Samples with undetectable values were assigned half of the lowest detection value.

Statistical analysis

Fisher's exact test was employed for the comparison of categorical variables, and an analysis of variance (ANOVA) was applied for the comparison of continuous numeric data. We employed the Kruskal–Wallis test and Mann–Whitney U test to determine the significance of the cytokine/chemokine distribution among different viral groups and the clinical severity of the virus. Statistical analysis was performed using GraphPad Prism 9. A two-sided p-value of <0.05 was considered statistically significant, and *p* values were corrected by using Benjamini-Hochberg controlling the false discovery rate in multiple comparisons.

Results

Demographic, laboratory, and clinical features in different viral etiology groups

A total of 184 children – 126 boys (68.5%) – were enrolled during the study period, with a median age of 10.4 months. At least one respiratory virus was identified in 163 cases (88.6%). RV and enterovirus were detected in 58 samples – 53 (67.9%) verified as RV. RSV constituted the leading viral pathogen (47, 25.5%) followed by rhinovirus (32, 17.3%), a non-RSV or -RV infection (26, 14.1%), and RSV–RV coinfection (23, 12.5%; Fig. 1A). RSV (33.6%) consistently predominated in patients aged <12 months. RV replaced RSV as the prevailing viral pathogen in patients older than 1 year (Fig. 1B).

To minimize the potential confounding influences from multiple viral coinfections, we compared three groups: RSV, RV, and RSV-RV coinfection (Table 1). Overall, the RSV group was characterized by patients of a younger age ($p < 0.05$) and a higher proportion of those with a preterm birth history compared with the other groups ($p < 0.05$). Conversely, a lower frequency of fever upon admission and higher rate of maternal atopic history, second-hand smoking exposure, and recurrent wheezing history were more common in the RV group – only the history of recurrent wheezing reached statistical significance (RV versus RSV; $p = 0.001$). For the laboratory findings, leukocytosis (>10000/μL) and eosinophilia were significant in the RV groups compared with those in the RSV group ($p < 0.001$ and $p = 0.047$, respectively). The C-reactive protein level was comparable across the three groups ($p = 0.86$).

Regarding clinical severity, the RV group had the highest mean respiratory score (RS; RS = 6.5 ± 2.3, $p = 0.0743$, Fig. 2A) and intensive care rate (29%, $p = 0.23$) (see Table 1). No difference was observed in

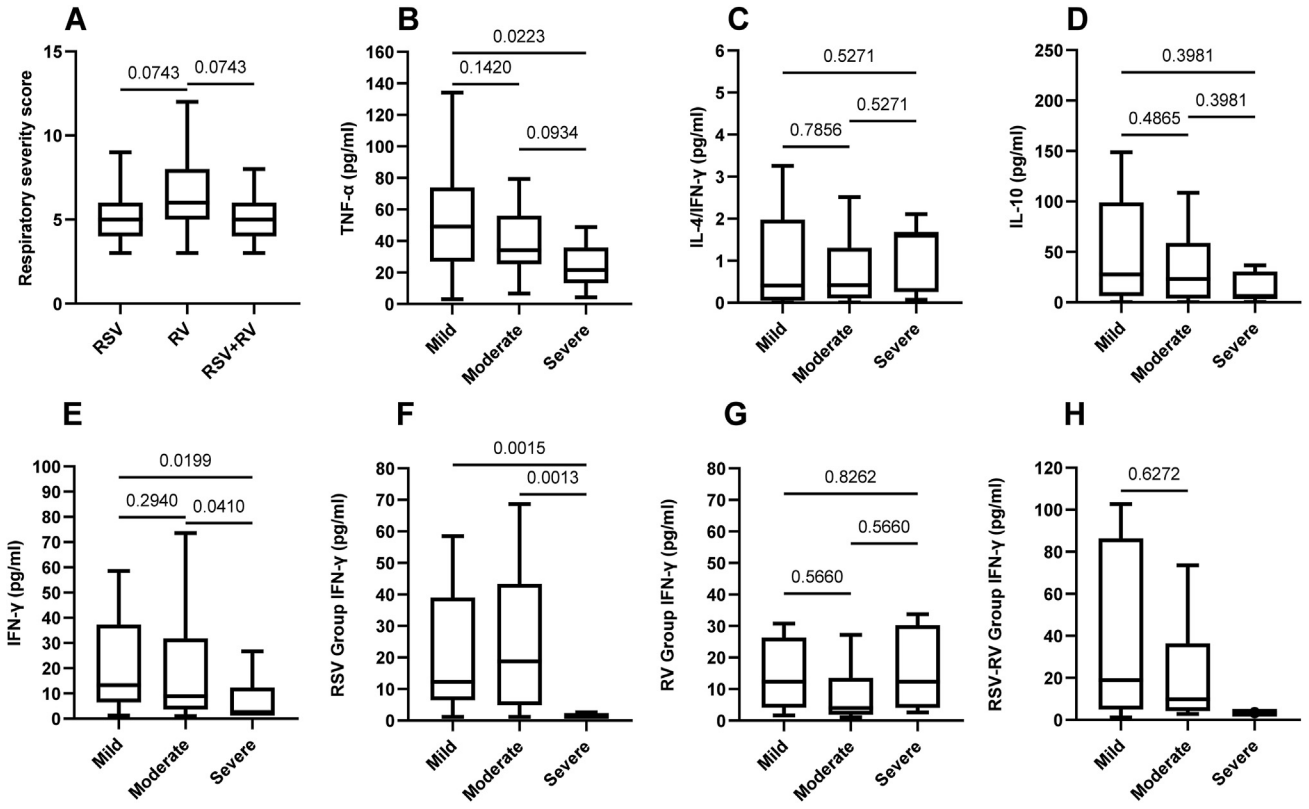

Figure 2. Comparison of the expression of cytokines and chemokines in relation to different clinical severity levels. Comparison of the clinical severity level in the different viral groups (A). Comparison of the serum TNF-α (B), IL-4/INF-γ (C), IL-10 (D) and INF-γ (E) levels among the severity groups. The median value and interquartile ranges are presented. Comparison of the serum INF-γ according to infectious virus and clinical severity (F—H). Statistical analysis was performed using the Kruskal—Wallis test or Mann—Whitney U test as appropriate. Significance was set at $p < 0.05$ and adjusted by false discovery rate method.

hospitalization duration or the use of aerosol bronchodilator therapy among the three groups.

Cytokine and chemokine expression with clinical severity and steroid use

Clinical severity was categorized using the following RS scale: mild (0—4), moderate (5—8), and severe (9—12). Of all tested cytokines and chemokines, only TNF-α and INF-γ were inversely correlated with clinical severity (Fig. 2B and E) in RSV infected group, but not in the RV or RSV-RV infected group (Fig. 2 F—H). We cannot compute significance because of the limited number of severe cases of each viral entity. No significant association was found between IL-4/INF-γ ratio and IL-10 levels with RSV bronchiolitis severity (Fig. 2C and D). For systemic steroid therapy, it was more frequently used in the RV group (28.6%), but the difference was nonsignificant (RSV:13.6% and RSV-RV group: 9.5%, $p > 0.05$). Overall, IL-4, IL-8, IL-13, and MIP-1α levels correlated with the steroid-use group (Fig. 3A—D, each $p = 0.030$, 0.048, 0.042, and 0.045). While looking further into steroid use and infecting virus, IL-4, IL-6, IL-13 and MIP-1α levels were significantly higher in steroid use group of RSV infected patients(Fig. 3E—H, each $p = 0.018$, 0.049, 0.041, and 0.035), but only IL-8 was higher in steroid use group of RV infected patients (Fig. 3I, $p = 0.049$).

Cytokine and chemokine expressions in different viral entities

The proinflammatory cytokine and chemokine levels in the RSV, RV, and RSV-RV coinfection groups, namely IL-6, IL-8, INF-γ, MIP-1α, MCP-1, RANTES, and ICAM-1 levels, are listed in Table 2, and only the IL-6 level in the three groups differed significantly and was lowest in the RV group ($p = 0.036$). In the RSV group, TNF-α (Fig. 4A) and IL-10 (Fig. 4B) levels were higher than RV groups ($p = 0.0110$ and < 0.0001, respectively), but no difference in IL-4 among three groups (Fig. 4C). By contrast, IL-22, IL-23, IL-25, IL-31, and IL-33 expressions were significantly higher in the RV and RSV—RV groups (Fig. 4D—H). In term of sole RV group, 29 out of 32 samples were successfully genotyped: 8 of HRV-A, 1 of HRV-B, and 20 of HRV-C. There were no differences in clinical features and cytokine expression profiles, at least between HRV-A and C (Table S1). A heatmap generated by using R Graphics package to visualize the infectious viruses and immune responses (Fig. 4I). The cytokines/chemokines were categorized into 7 clusters and showed that expressions of TNF-α, IL-10, IL-22, IL-23, IL-25,

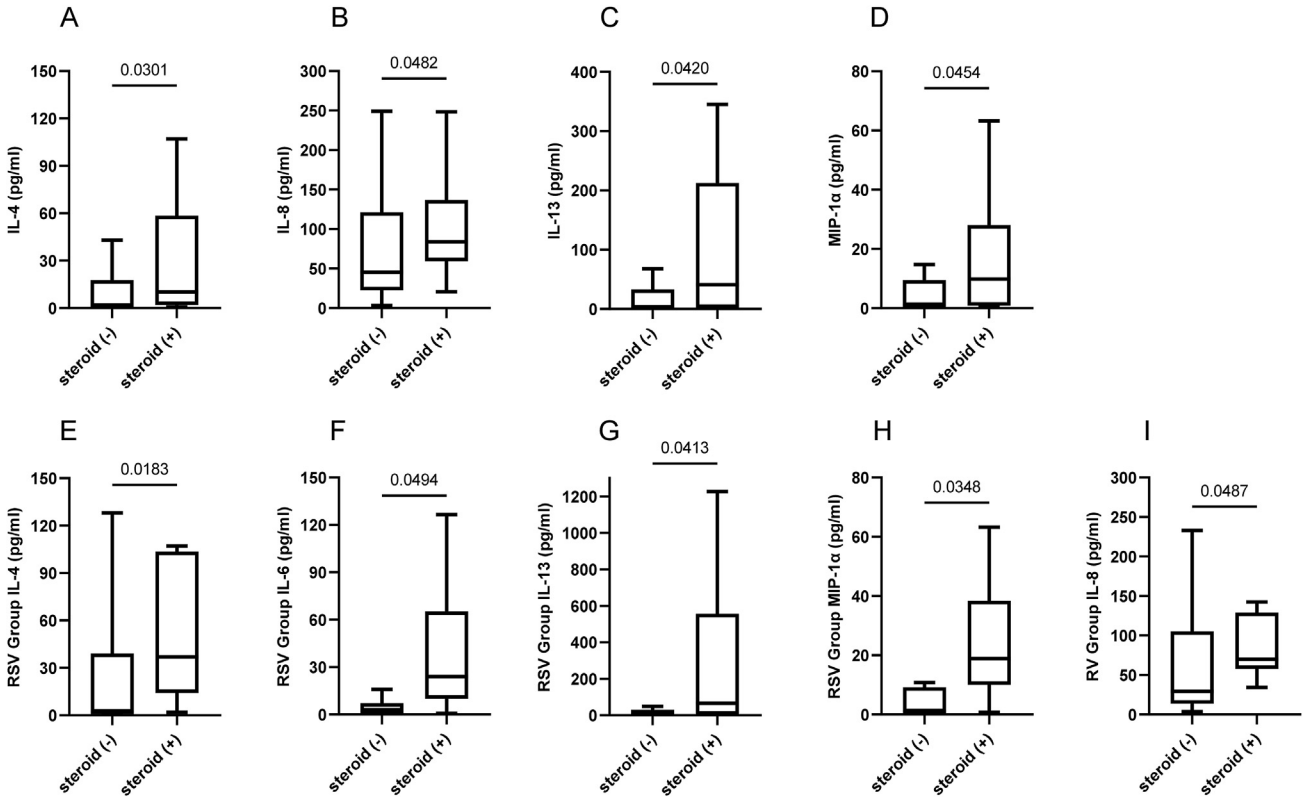

Figure 3. Comparison of the serum cytokine/chemokine expressions by the use of systemic steroid therapy.
Overall, the serum IL-4 (A), IL-8 (B), IL-13(C), and MIP-1α(D) were associated with the use of systemic steroid. For RSV infected patients, IL-4 (E), IL-6 (F), IL-13(G) and MIP-1α(H) levels were associated with systemic steroid use. Instead, only IL-8 (I) level was linked to the use of systemic steroid in RV infected patients. All results were analyzed using ANOVA test, and significance was set at $p < 0.05$.

IL-31, and IL-33 differed by infecting virus. Of note, IL-31 and IL-33 were grouped to the same cluster with a high correlation ($R^2 = 0.9731$, $p < 0.0001$).

Discussion

In this study, RSV and RV were the two leading viruses causing bronchiolitis in Taiwanese children. RSV, though with less than 50% rate, was still the leading viral pathogen. Comparatively, older age, a positive personal history of atopy, recurrent wheezing, eosinophilia, and higher rate of systemic steroid use were distinct features of RV bronchiolitis.[6,7] Our findings were consistent with other reports and support the heterogenous nature of bronchiolitis. Regarding clinical severity, Van Leeuwen JC et al.[18] and our study showed no preferential tendency was observed between RSV and RV bronchiolitis. Respiratory viral infections can trigger not only local epithelial immune reactions but also elicit systemic inflammatory responses. Although nasal aspirates become popularly used for microbiological and immunological investigation in recent years, blood cytokine profiling is still valuable for infectious diseases. Taking COVID-19 infection for example, blood cytokine profiles were shown to be highly correlated with clinical severity and outcome,[19,20] therefore, serum IL-6 level is widely used clinically to assess COVID 19 severity and outcome prediction. Previous studies show several blood cytokines and chemokines proposed as potential biomarkers for RSV bronchiolitis severity[8,10,12,21,22]: A high expression of IL-8, MIP-1α, and INF-γ are positively associated with RSV bronchiolitis severity and the need for ventilation or supplemental oxygen, alternatively, low serum or nasal TNF-α, INF-γ, and IL-8 level has been associated with severe disease, prolonged hospitalization, and the need for intensive care in relation to RSV bronchiolitis.[16,23—25] Furthermore, several in vitro studies have reported that high levels of TNF-α and INF-γ have protective functions against severe RSV infection.[26,27] In line with the aforementioned results, our study demonstrated that clinical severity was independent of viral etiology but associated inversely with TNF-α and INF-γ levels, implying that impaired innate immunity in the infant to toddler population is an important factor influencing viral bronchiolitis severity.

Steroid therapy is generally not recommended for acute bronchiolitis in most guidelines.[1] However, growing evidence inferred bronchiolitis is not a single illness but can have different "endotypes" and "phenotypes," based on age, personal or family history of atopy, etiology, and pathophysiological mechanism.[3,5] Our data showed who likely received systemic steroid therapy were RV infected patients and those with elevated Th2 responses. Short term prednisolone therapy has been evaluated in rhinovirus induced wheezing patients, and demonstrated its efficacy

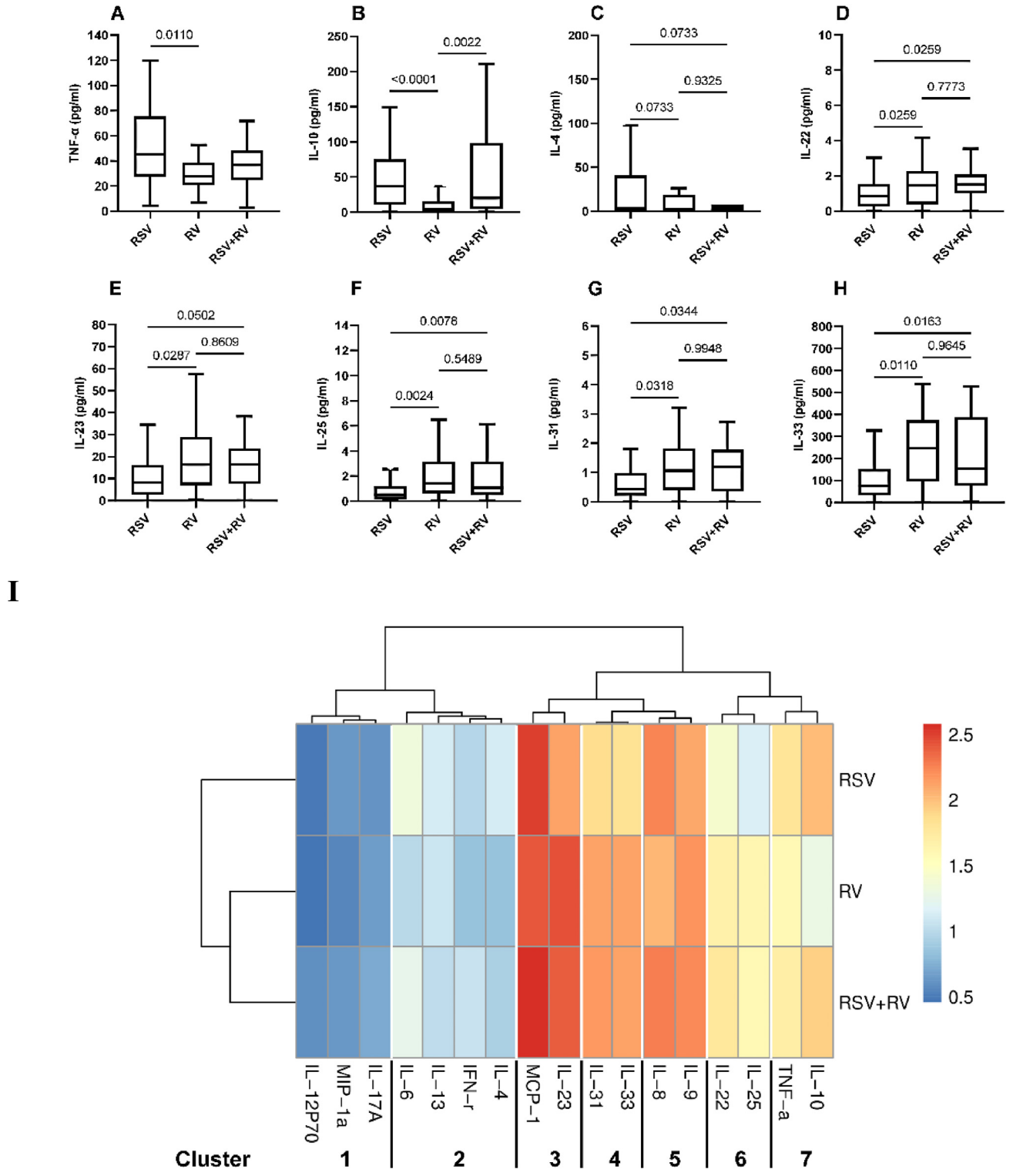

Figure 4. Cytokines/chemokines expression association with RSV, RV, and RSV-RV co-infection.
The boxplots of serum TNF-α (A), IL-10 (B), IL-4 (C), IL-22 (D), IL-23 (E), IL-25 (F), IL-31(G), and IL-33 (H) levels were presented by the viral etiologies. Values for the cytokines are described in picogram/mililiter, except IL-25 (F), which is presented as nanogram/mililiter. The results were analyzed using ANOVA test, and significance was set at $p < 0.05$ and adjusted by false discovery rate method. The heatmap and cluster analysis of cytokine profiles for 3 viral categories (I). Log10-transformed cytokine expression is presented as a difference from the baseline: red denoting high expression and blue low expression. Hierarchal clustering was used to generate the cluster dendrogram and cytokine response groups were assigned based on the branching of the dendrogram. Cytokine names are shown at the bottom of the chart.

Table 2 Comparison of proinflammatory cytokines and chemokines in different viral groups.

	(a) RSV	(b) RV	(c) RSV + RV	P value		
				a vs. b	a vs. c	b vs. c
IL6 (pg/ml)	48.9 ± 151.9	4.3 ± 5.6	39.6 ± 88.7	0.100	0.754	0.270
IL8 (pg/ml)	574.5 ± 1829.7	82.8 ± 92.8	662.9 ± 2645.4	0.249	0.849	0.252
INF-γ(pg/ml)	24.9 ± 29.7	55.7 ± 187.4	35.6 ± 64.1	0.239	0.697	0.512
MCP-1 (pg/ml)	803.3 ± 668.4	608.2 ± 394.8	1022.2 ± 1096.6	0.263	0.251	0.047
MIP-1α(pg/ml)	11.9 ± 20.2	7.5 ± 11.7	9.4 ± 12.5	0.264	0.561	0.683
RANTES (pg/ml)	105221.3 ± 92536.3	125467.1 ± 115148.4	107500.3 ± 122941.8	0.510	0.943	0.616
ICAM-1 (pg/ml)	56520.7 ± 39438.8	58158.9 ± 30772.1	62320.0 ± 22837.3	0.884	0.666	0.778

Data were analyzed by One-way ANOVA with least significant difference (LSD) multiple comparison.

by shortening clinical symptoms, and reducing respiratory medication and risk of recurrent wheezing.[28—30] Even corticosteroid dose not be routinely used in treating bronchiolitis, we can expect the coming change in bronchiolitis prevention and management in personalized medicine era.

IL-10 is a counter-inflammatory cytokine that balances Th1 and Th2 responses. Studies have revealed the association between IL-10 level — variants included — and post-bronchiolitis wheezing and asthma development.[11,31] However, the association between IL-10 and RSV bronchiolitis severity was variable. Leahy et al.[21] revealed an inverse association between high IL-10 levels and severe disease, but Mella et al.,[32] in line with our findings, reported that serum IL-10 levels played no significant role in disease severity. Studies comparing the IL-10 expression of RSV and RV bronchiolitis are scarce. In one small-sample study, no difference was observed in the IL-10 level in nasal wash between RSV and RV bronchiolitis groups.[16] Our study revealed lower serum IL-10 levels in the RV group compared with other groups and a positive correlation between IL-10 and INF-γ, in consistency with a Finnish observational study.[22]

Th17 cells also play a role in the pathogenesis of RSV and RV infection[33,34]; the elevation of plasma IL-17 cytokine was associated with the severity of RSV bronchiolitis. In our study, both IL-22 and IL-23 plasma levels in RV infection were higher than in RSV. Because IL-23 is essential for the differentiation of Th17 subsets and promotion of the secretion of IL-22, plasma IL-17 cytokine levels were likely elevated in both infections. In RSV infection, Th17 cells are involved in diminishing viral clearance activity, enriching a Th2 environment, and promoting mucus production[33]; thus, Th17 cells may be associated with RV-related airway hyperresponsiveness.[14]

Respiratory epithelia act as the first line of defense against microorganisms and allergens and are linked to many immune responses. Notably, IL-25 and IL-33 play crucial roles in airway inflammation and asthma exacerbation.[14,15] Both RSV and RV infection induce the expression of these two cytokines.[13,15,35] In neonatal mice, RSV induced significantly higher IL-33 levels than in adult mice, proportionally to the severity of lung inflammation. However, RV infection highly increases airway IL-33 levels and induces Th2 responses in patients with asthma.[13,15] Limited data were available to compare the two cytokine levels present in RSV and RV infection. In this study, we demonstrated that RV infection was associated with higher IL-25 and IL-33 plasma levels than RSV infection. Furthermore, the RSV-RV coinfection group exhibited higher levels of IL-25 and IL-33 than the RSV group and significant positive correlation between IL-33 and IL-31. IL-31 is a novel cytokine produced by T cells that is highly involved in atopic dermatitis.[36] Regarding the role of IL-31 in asthma and allergic airway diseases, our study, consistently with Vocca et al., showed that IL-33 was involved in Th2/IL-31 responses and in positive correlation with allergic rhinitis and asthma.[37] The current evidence emphasizes the critical roles and biomarker potential of IL-25, IL-33, and IL-31 for phenotypic classification in viral bronchiolitis.

This study has several limitations: First, the sample size of this study was too small to enroll enough severe bronchiolitis cases and to draw conclusions on the cytokine levels and clinical severity. Second, only immune responses at the single time point upon admission was assessed. Thus, this work cannot present the dynamic changes of the investigated cytokines throughout the whole disease course nor evaluate the longitudinal impact. Third, the small sample size cannot avoid potential confounding factors and cause the statistical errors in multiple comparison, even we tried using a clinical scoring system and adapting Benjamini-Hochberg controlling the false discovery rate in this study. More large-scale studies are required to elucidate the dynamics of cytokines and chemokines and their influence on the disease course and outcome.

In summary, this investigation demonstrated comparable clinical presentations and related immune profiles of viral bronchiolitis between Asian ethnic children and previous reports from Western countries. The clinical severity was independent of viral etiology but associated inversely with TNF-α and INF-γ levels. Respiratory viruses elicited robust IL-33 and IL-25 expressions, which orchestrated Th17 and IL-31 responses.

Acknowledgments

We fully appreciated all participants, their parents' kindness and help to this study, and the comments from Professor Larry J. Anderson, Department of Pediatrics, Emory University, Georgia, USA.

This study was funded by Chang Bing Show Chwan Memorial Hospital (RD103032 and 103037).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.08.013.