

Analysis of the immune response in infants hospitalized with viral bronchiolitis shows different Th1/Th2 profiles associated with respiratory syncytial virus and human rhinovirus

To the Editor,

Severe bronchiolitis is often associated with the development of wheezing and asthma during childhood, but the mechanisms behind the development of respiratory disorders after bronchiolitis remain controversial.¹ The respiratory syncytial virus (RSV) and the human rhinovirus (HRV) are the most important etiologies of bronchiolitis.^{2,3} Compared to RSV, HRV-infected infants display peculiar clinical characteristics and a correlation of HRV bronchiolitis with asthma has been suggested.⁴⁻⁷ We report here the results of a preliminary investigation aimed at the evaluation of the ongoing T-cell responses in infants with bronchiolitis of different etiology. To this purpose, we prospectively enrolled a small homogenous cohort of children hospitalized for RSV or HRV bronchiolitis during the last epidemic seasons (2016-2017) using an intracellular staining protocol based on a short-term direct stimulation of whole blood. Experimental procedures are described in this article's Supporting Information. Fifty-six children, 43 with sole RSV and 13 with sole HRV infection, were enrolled and included in this study (see Table S1). The frequencies of IFN- γ - and IL-4-producing CD4⁺ and CD8⁺ T cells in whole-blood cultures were assessed by flow cytometry. A stringent staining and gating strategy was adopted to minimize false positives (See Figure S1). The frequencies of Th1 cells (IFN- γ ⁺ of CD3⁺CD4⁺), Th2 cells (IL-4⁺ of CD3⁺CD4⁺), Tc1 cells (IFN- γ ⁺ of CD3⁺CD8⁺), and Tc2 cells (IL-4⁺ of CD3⁺CD8⁺) were calculated by subtracting background values of unstimulated samples from values of Staphylococcus enterotoxin B (SEB)-stimulated samples. SEB was chosen to amplify the ongoing acquired immune response as it induces T-cell proliferation and cytokine response similarly to physiological T-cell receptor triggering. As shown in Figure 1, HRV-infected infants displayed a Th2-oriented immune response, with significantly higher Th2 cells frequencies compared to RSV-infected subjects (Figure 1A,B). The Th2 index, calculated as the Th2/Th1 ratio, was significantly higher in HRV bronchiolitis as compared to the RSV bronchiolitis (Figure 1C). Worth of note, we did not find significant differences when comparing untreated samples from RSV and HRV infants (data not shown), thus indicating that a differential Th1/Th2 profile is evident only after amplification of the ongoing immune response. The evaluation of Tc1 and Tc2 cells frequencies induced upon whole-blood stimulation confirmed a preferential induction of Type-1 immunity in RSV bronchiolitis (see Figure S2).

To assess any correlation between the type and the magnitude of the immune response to the infectious agent and increased or

decreased pathology, T-cell frequencies were associated with the severity of the admission clinical score. In each group, clinical scores assigned to hospitalized infants ranged from 0 to 7, of a maximum of

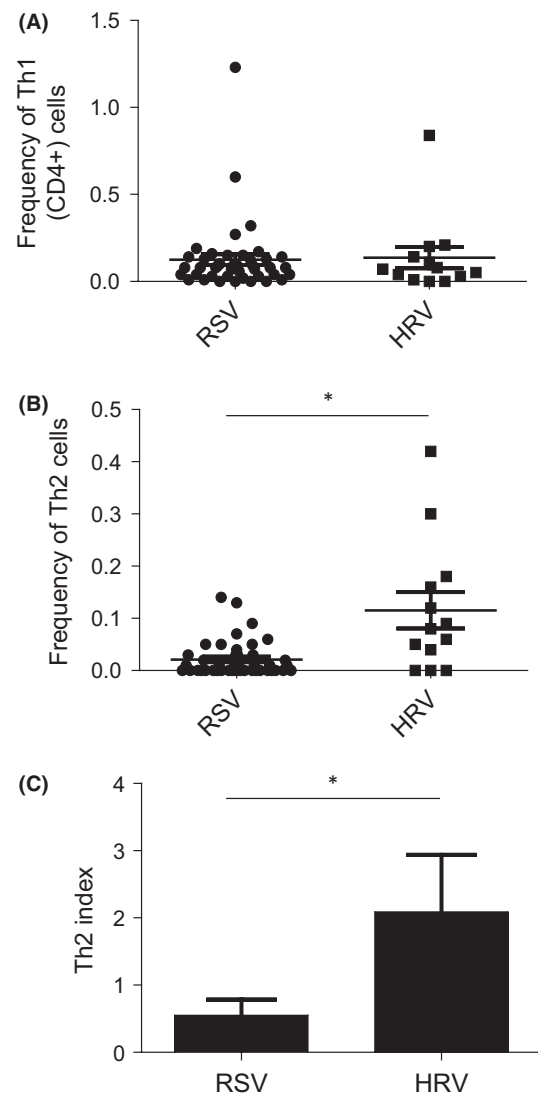


FIGURE 1 T-helper cell polarization profile in infants hospitalized with bronchiolitis. A, Frequencies of Th1 cells (IFN- γ ⁺ of CD3⁺CD4⁺) in SEB stimulated whole blood cultures of bronchiolitis infants infected with RSV and HRV. Values were obtained after subtracting background values. B, Frequencies of Th2 cells (IL-4⁺ of CD3⁺CD4⁺). C, Th2 index in infants infected with RSV and HRV, calculated as the ratio between Th2 and Th1 cells for each infant. * $P < .01$ by Mann-Whitney U test.

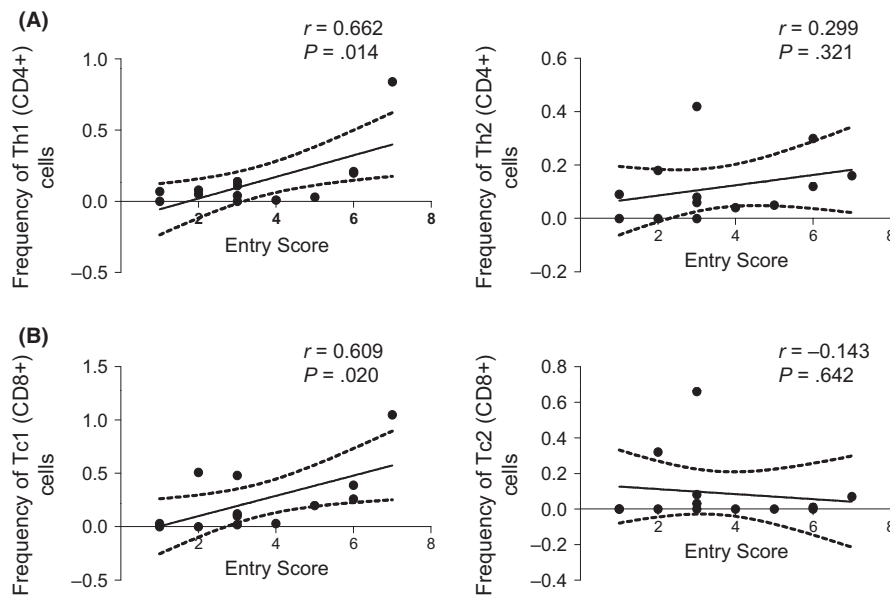


FIGURE 2 Severity of entry clinical score correlates with Type-1 immunity in HRV bronchiolitis. A, Linear regression correlating the frequency of Th1 (left) or Th2 (right) cells in HRV bronchiolitis infants with the clinical score at hospital admission; r and P values are shown. B, Linear regression correlating the frequency of Tc1 (left) or Tc2 (right) cells in diluted whole blood cultures of HRV bronchiolitis infants with the clinical score at hospital admission. Pearson's correlation coefficient (r) and P values are shown.

8.³ Infants were divided into 2 subgroups, with high (≥ 5) or low score (< 5). High score infants were 13 (30.2%) among RSV bronchiolitis, 4 (30.8%) among HRV infants. The frequencies of cytokine-producing CD4⁺ and CD8⁺ T-cell subsets were similar in RSV infants with low or high score. On the contrary, high score HRV infants had higher numbers of Th1, Th2 and Tc1 cells compared to low score infants, even though differences were not statistically significant (Figure S3). To investigate more in-depth this aspect, a linear regression analysis was performed on data obtained from a plot of T-cell frequency against the clinical score, for each T-cell subset. As shown in Figure 2, we found a statistically significant positive correlation between the percentage of Th1 or Tc1 lymphocytes and disease severity in infants with HRV bronchiolitis. As far as concerns RSV bronchiolitis, no significant correlation was found (data not shown).

Here, we show that in a cohort of infants with bronchiolitis, there are 2 phenotypes according to Th-1 and Th-2 response and viral etiology. It has been demonstrated that HRV infection has a major impact on the development of childhood asthma,^{3,4,8,9} suggesting that individuals prone to the development of allergic disorders are preferentially infected by HRV during the epidemic season. The main finding of this study is the identification of a predominant Th-2 polarization in HRV bronchiolitis infants as compared to RSV. The definition of bronchiolitis according to strict clinical criteria ensured the collection of homogeneous data and excluded infants with various differing clinical entities, such as wheezing bronchitis. Moreover, we choose a whole-blood short-term assay capable to identify pre-existing cytokine-producing T effector memory cells, thus mainly detecting recent or ongoing immune response.

The majority of RSV-infected infants displayed a Type-1 response, while the majority of HRV-infected infants displayed a high Th2 index. However, some RSV bronchiolitis were characterized by a Type-2 immunity and some HRV bronchiolitis by a Type-1 immunity. Noticeably, in HRV bronchiolitis the clinical score at hospital admission positively correlated with Th1- and Tc1-cell frequencies.

These observations suggest that in HRV infants the onset of a Type-1 immune response worsen the clinical picture. When a more prominent Type-2 response is ongoing, clinical symptoms are of lesser entity; nevertheless, Th-2 polarization can act as the trigger for future exacerbations.

In conclusion, this study demonstrated that infants with HRV bronchiolitis display a different acquired immunological response compared to infants with RSV bronchiolitis, yet it does not provide a clear answer to the question whether immunological data depicted in these patients are actually the result of acute viral infection (HRV vs RSV) or they are genetically determined. To this end, novel studies are being planned to investigate Th1 and Th2 immunity in bronchiolitis patients during the acute phase and at convalescence. Although preliminary, our observations highlight the importance of immunological monitoring of infants with acute bronchiolitis as a crucial aspect in the process of identifying different clinical phenotypes that can benefit from specific therapeutic and prevention strategies.

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AUTHORS' CONTRIBUTION

GF conceived the research and the experimental design, performed experiments, analyzed data, and wrote the manuscript; IS conceived the research and the experimental design, performed experiments, analyzed data, and wrote the manuscript; RN conceived the experimental design and analyzed data; AP performed experiments;

AF performed experiments; PL performed experiments; LP performed experiments; CS conceived the experimental design; FM conceived the research and the experimental design, analyzed data, and wrote the manuscript. All the authors provided comments to the manuscript.

CONFLICT OF INTEREST

All the authors declare no commercial or financial conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Analysis of the immune profile in infants hospitalized with bronchiolitis shows preferential T-helper 2 immune response induced by rhinovirus.

SUPPORTING INFORMATION

Materials and Methods

Study population

Fifty-six infants hospitalized for viral-bronchiolitis at the Paediatric Emergency Department of Policlinico Umberto I Hospital, “Sapienza” University of Rome from November 2016 to April 2017 were enrolled in the study. These infants belong to the Brome (Bronchiolitis in Rome) cohort of unrelated, full-term previously healthy infants, aged less than 1 year and non-low birth weight. Bronchiolitis was defined as the first episode of acute lower airway infection, characterized by a history of upper respiratory tract infection followed by acute onset of respiratory distress with cough, tachypnea, retractions and diffuse crackles on auscultation (2). Nasopharyngeal washing was collected from each infant and RSV, HRV and other respiratory viruses were detected using a panel of reverse transcription-polymerase chain reaction (PCR) or nested PCR assays, as reported (30). Patients with virus-negative bronchiolitis or co-infections were excluded from the study. At admission to the hospital and during hospitalization a clinical severity score, ranging from 0 to 8, was assigned to each infant according to respiratory rate, arterial oxygen saturation on room air, presence of retractions, and ability to tolerate oral feeding, as previously described (2). The Ethics Committee of Policlinico Umberto I Hospital approved the study and informed consent was obtained from parents before enrolling the children.

Whole blood stimulation and permeabilization

Children’s peripheral whole blood was collected directly into Na-Heparin-containing vacutainers (BD Biosciences, San Jose, USA) and processed within 1 hour. Whole blood (300 µl) was diluted 1:1 with RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), then was either left unstimulated or stimulated with Staphylococcal enterotoxin B (SEB), at 1 µg/mL (Sigma-

Aldrich, St. Louis, MO, USA). Diluted blood was incubated in polypropylene tubes at 37 °C for 24 h, and Brefeldin-A (Sigma-Aldrich) was added during the last 18 h of incubation (10 µg/mL) to inhibit cellular secretion. Diluted whole blood cultures were then harvested and red blood cells lysed with FACS lysing solution (BD Biosciences). The remaining white cells were extensively washed with FACS buffer (PBS containing 0.5% BSA and 0.09% NaN₃) and stained with Live/Dead Fixable Violet Dead Cell Stain Kit (Thermo Fisher Scientific). Cells were fixed by 1% PFA for 15 minutes at room temperature and permeabilized using FACS buffer and 5% Saponin (Sigma-Aldrich).

Intracellular cytokine staining and flow cytometry

Cells were incubated with a panel of fluorochrome-conjugated monoclonal antibodies (mAbs) for 30 min at 4 °C. The composition of panel was: anti-CD3-FITC, anti-CD4-APC-H7, anti-CD8-Alexa Fluor 700, anti-IL-4 APC (all from BD Biosciences), anti-IFN-γ-PerCPCy5.5 (Biolegend, San Diego, CA, USA), anti-CD14-eFluor450 and anti-CD19-eFluor450 (both from eBioscience, Thermo Fisher Scientific). Saponin (5%) was added to the antibody mix. Cells were then suspended in 250 µL 1% paraformaldehyde and acquired on a Gallios Flow Cytometer (Beckman Coulter, Miami, FL, USA) with a standard stopping gate set at 50,000 CD3⁺ T-cells. Compensation was performed using VersaComp Antibody Capture Beads (Beckman Coulter) stained with each individual mAb and compensation matrices were calculated with Kaluza 1.3 software (Beckman Coulter). Data were analyzed using Kaluza software. Gating strategy and an example of raw flow cytometry data is shown in Supplementary Fig.1. Frequencies of cytokine-expressing CD4⁺ and CD8⁺ T-cells stimulated by SEB were determined after subtracting percentages of cytokine-expressing cells detected in unstimulated controls.

Statistical analysis

We used chi-square test and Mann–Whitney U test to evaluate differences in percentages and median values, respectively. Pearson's correlation was calculated to analyze the correlation between

continuous variables. A p-value below 0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism software, version 5.0 (San Diego, CA).

Table S1. Clinical and demographic parameters in infants with viral bronchiolitis^a

	RSV n (=43)	HRV n (=13)	P- value^b
Demographic Characteristics			
Gender [n, (%)]			
Male	21/43 (48.8%)	7/13 (53.8%)	ns
Female	22/43 (51.2%)	6/13 (46.1%)	
Age at admission (days)	67.00 (50 - 96)	51.00 (42- 82.50)	ns
Gestational age (weeks)	38.00 (38 - 40)	39.00 (37 - 40)	ns
Birth weight (Kg)	3.080 (2.700 - 3.450)	3.00 (2.775 - 3.450)	ns
Disease Severity			
Hospital length-of-stay (days)	5.50 (4 - 7)	3.00 (3 - 7)	0.036
Admission score	4.00 (3 - 5)	3.00 (2 - 5.5)	ns
Hospitalization score	5.00 (3 - 6)	3.00 (2 - 4)	0.024

^aData are expressed as medians, 25%-75% interquartile range (IQR).
^bPercentages and continuous variables were analyzed using chi-square and Mann-Whitney test, respectively.

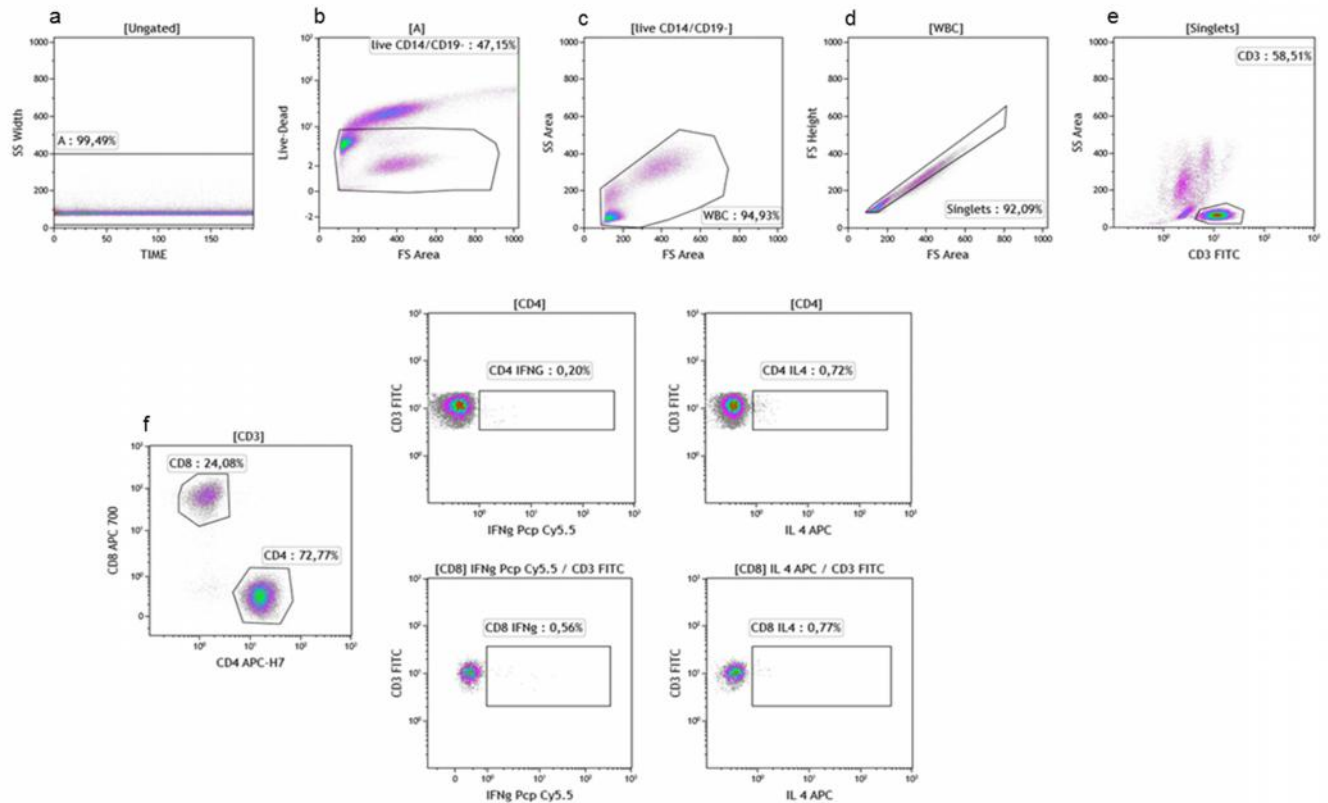


Figure S1

Gating strategy used to identify cytokine producing T-cell subsets.

Successive gates were applied to: a) exclude anomalies in the acquisition, visualized when events are displayed as a function of the time parameter; b) exclude dead cells, CD14+ monocytes and CD19+ B-cells; c) identify white blood cells; d) identify singlet cells; e) identify CD3+ T-cells; f) identify CD4+ and CD8+ T-cell subsets. The percentages of IFN- γ and IL-4 producing cells were then assessed within the CD4+ or the CD8+ T-cell subsets. Representative dot plots are shown.

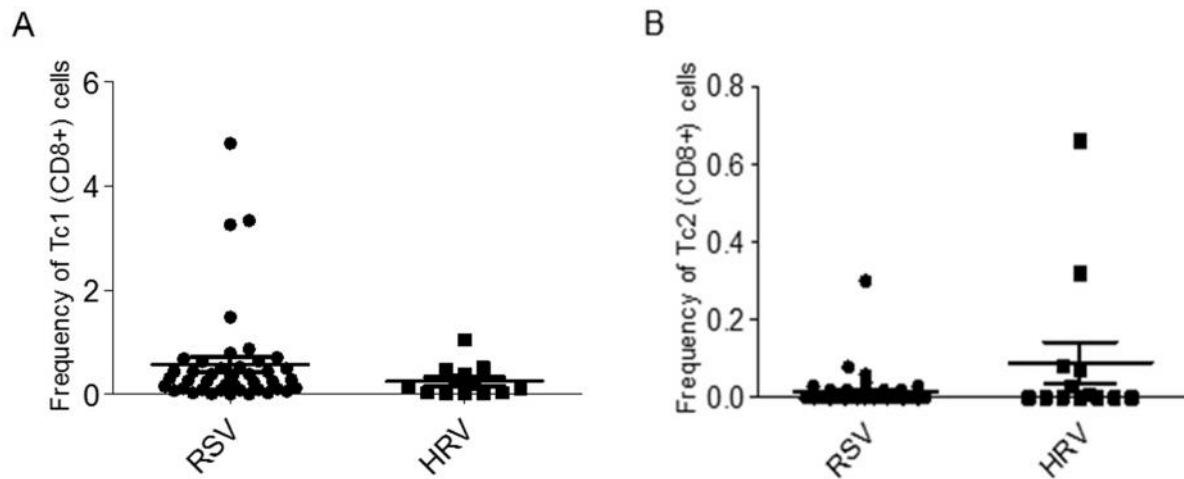


Figure S2

Cytotoxic T cell polarization profile in infants hospitalized with bronchiolitis.

(A) Frequencies of Tc1 cells (IFN- γ ⁺ of CD3⁺CD4⁺) in SEB stimulated diluted whole blood cultures of bronchiolitis infants infected with RSV and HRV. Values were obtained after subtracting background values. (B) Frequencies of Tc2 cells (IL-4⁺ of CD3⁺CD8⁺).

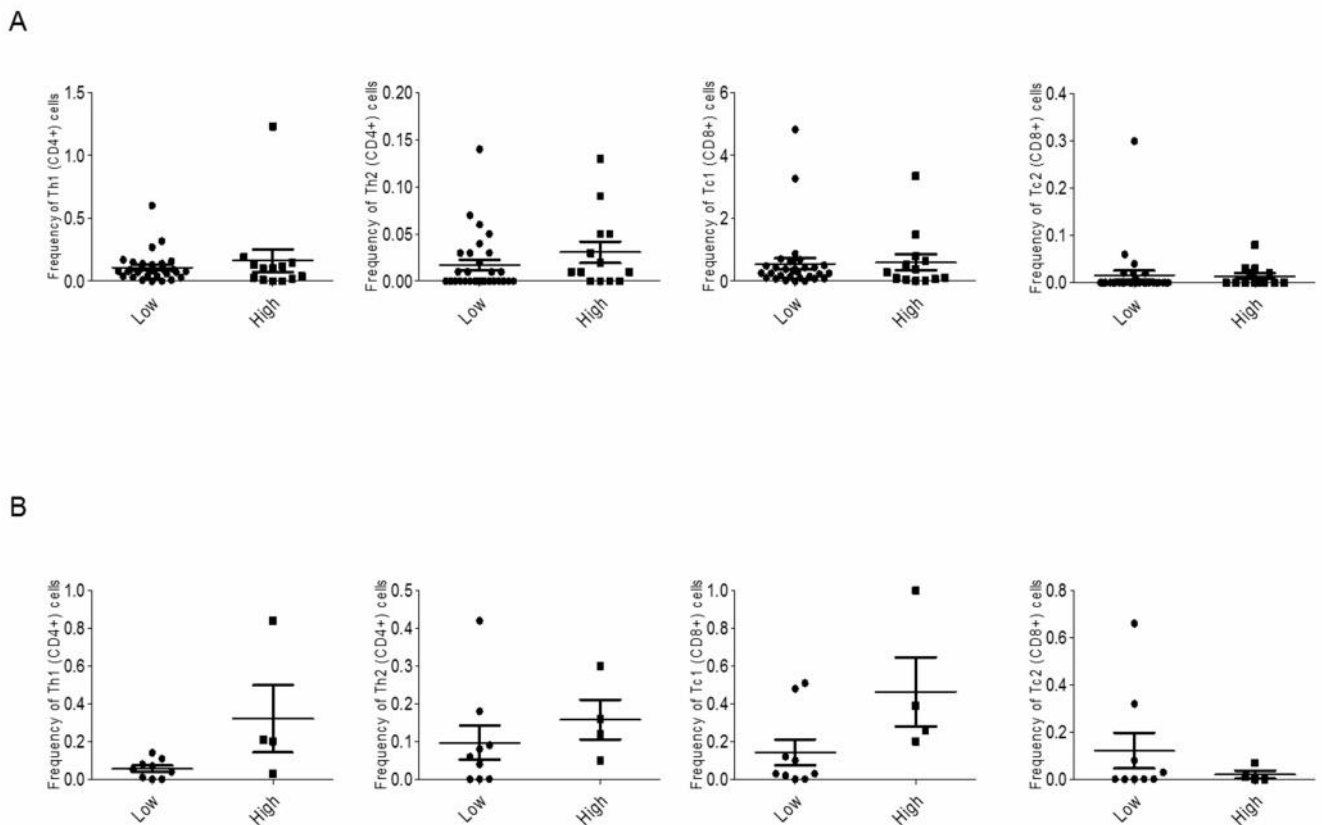


Figure S3

T-cell polarization profile in infants with low or high entry clinical score.

(A) Frequencies of Th1, Th2, Tc1 and Tc2 cells in RSV-infected bronchiolitis infants with low (<5) or high (≥ 5) clinical score at hospital admission. (B) Frequencies of Th1, Th2, Tc1 and Tc2 cells in HRV-infected bronchiolitis infants with low (<5) or high (≥ 5) clinical score at hospital admission.