Interleukin-15 is associated with disease severity in viral bronchiolitis

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ABSTRACT Disease severity in viral bronchiolitis in infancy is difficult to predict and has been linked to host innate immunity. The study aimed to investigate the innate cytokine interleukin-15 (IL-15) as a marker of disease severity.

A prospective single-centre observational study was conducted in a university-affiliated paediatric teaching hospital, comparing children (0–18 months) hospitalised for viral bronchiolitis, those admitted to the paediatric intensive care unit with severe disease and healthy age-matched controls. IL-15-related parameters were compared between groups. PCR and microRNA (miRNA) sequencing was undertaken on natural killer (NK) cells collected from study participants.

Samples from 88 children with viral bronchiolitis and 43 controls enrolled between 2009 and 2012 were analysed. Peripheral blood mononuclear cell (PBMC) IL-15 mRNA expression was significantly higher in those with moderate severity bronchiolitis compared with controls and those with severe disease. Serum IL-15 levels correlated with disease severity. The relative frequency of NK cells in peripheral blood was significantly reduced in participants with bronchiolitis. The NK cell miRNA transcriptome in bronchiolitis was distinct. Targets of de-regulated miRNA were differentially expressed in bronchiolitis, including *JAK3*, *STAT5A* and *NFKB1* on the IL-15 signalling pathway.

IL-15 is associated with disease severity in children hospitalised with viral bronchiolitis.

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Introduction

Viral bronchiolitis is the commonest single reason for an infant to be hospitalised in the developed world and the leading cause of lower respiratory tract infection in children worldwide [1]. It is commonest in children under 2 years and caused most frequently by respiratory syncytial virus (RSV) [2]. The disease spectrum ranges from asymptomatic infection to severe disease with respiratory failure requiring paediatric intensive care unit (PICU) admission for ventilator support [2, 3]. Some children have predisposing risk factors, but the majority of those who require PICU admission were previously healthy [4, 5]. Identifying children who are likely to develop severe disease is difficult and is further hampered by a lack of reliable laboratory predictors of disease.

Evidence from both functional and genetic associations studies suggest that disease severity in viral bronchiolitis is influenced by the host's innate immune response [6, 7]. Natural killer (NK) cells are innate lymphocytes and are "first responders" against infection [8]. This capacity to respond quickly underlines their importance in acute respiratory infection [9–11]. NK cells also shape the subsequent adaptive immune response [12, 13] and failure of this response may underlie severe disease, as suggested by autopsy studies on cases of fatal viral bronchiolitis [14].

Lymphocyte homeostasis is regulated by a family of interleukins (ILs) that share a common γ-chain receptor (γ_c) and mediate their effect by increasing expression of antiapoptotic proteins, including Bcl-2 and Bcl-x_L [15]. IL-15, in particular, regulates NK cell development and activation state [16, 17], and has previously been linked with the risk of hospitalisation for viral bronchiolitis [6, 18]. Preliminary work examining a panel of innate cytokines in viral bronchiolitis also demonstrated a relationship between IL-15 levels and disease [19].

We hypothesised that IL-15 was a marker of disease severity in viral bronchiolitis, that genetic variation might affect the host IL-15 pathway and that IL-15 influences disease severity through its effect on NK cells.

Methods

Study participants

We conducted a single-centre, prospective observational study during consecutive respiratory viral seasons (November to March) from 2009 to 2012 in the Children's University Hospital, Dublin (CUH), a tertiary level paediatric teaching hospital, and the associated neonatal outpatient department of the Rotunda Maternity Hospital, Dublin. Previously healthy Irish children of Caucasian ethnicity under the age of 18 months hospitalised for viral bronchiolitis were enrolled consecutively. Children with a history of chronic illness or immunodeficiency and children on immunosuppressants were excluded. Asymptomatic, age-matched controls, presenting for routine phlebotomy or minor day-case surgical procedures, were enrolled and were subject to similar exclusion criteria as cases. Written, informed consent was obtained from the parents/guardians of study participants. The Research Ethics Committees of the CUH and the Rotunda Hospital, Dublin approved the study.

Study procedure

All children with bronchiolitis were enrolled in CUH. Clinical and demographic information was collected by interviewing parents/guardians and examining medical records. Children were classified as having "severe" bronchiolitis if their condition was deemed by the attending paediatrician and intensivist to merit PICU admission for positive-pressure mechanical ventilator support and classified as "moderate" severity if the child was cared for on a standard inpatient ward. To further validate this classification, disease severity was also measured using a clinical score known as the Respiratory Distress Assessment Instrument (RDAI) [20]. The RDAI was calculated at the point of enrolment of each case by the same investigator (T.R.L.). Viral infection was confirmed in study participants using either direct fluorescence antibody staining of a nasopharyngeal aspirate and/or a multiplex respiratory viral real-time reverse transcription PCR of a nasopharyngeal swab [21]. Phlebotomy was performed once, shortly after enrolment, typically either on day 1 or 2 of admission. Clinical outcomes measured included length of hospital stay, the need for oxygen supplementation, PICU admission and mechanical ventilation.

IL-15-related analysis

Peripheral blood mononuclear cells (PBMCs) were extracted by density gradient centrifugation from an EDTA anticoagulated sample collected from each participant and quantitative real-time PCR (qPCR) for IL-15 mRNA levels was performed as previously described [22]. Serum was obtained from whole blood clotted at room temperature and IL-15 levels were measured by ELISA.

Flow cytometry

Lymphocyte phenotyping was performed on a CyAn-ADP flow cytometer instrument and analysed using Dako Summit software (Beckman Coulter, Pasadena, CA, USA). The relative frequency of lymphocyte

subsets and surface expression of IL-2/15Rβ were examined. Intracellular IL-15 production and expression of the IL-15-related antiapoptotic proteins Bcl-2 and Bcl-x_L in different lymphocyte subset populations was also measured. Absolute numbers were extrapolated from a PBMC count undertaken on each sample using a Neubauer haemocytometer.

NK cell microRNA (RNA) transcriptome and mRNA target gene analysis

To determine whether the NK cell phenotype in infants with viral bronchiolitis was related to an altered pattern of miRNA gene expression, NK cells were isolated from PBMCs of six infants with RSV-positive bronchiolitis and six age-matched controls, and the transcriptional profile of small noncoding RNA compared using gene sequencing on a SOLiD 4 next-generation sequencing (NGS) platform (Life Technologies, Carlsbad, CA, USA) [23]. Bioinformatics analysis of the miRNA sequencing data identified miRNAs differentially expressed in infants with bronchiolitis. Statistical analysis was undertaken using open-source Bioconductor software DESeq2.0 (http://bioconductor.org/packages/release/bioc/html/DESeq2.html). This uses general linear regression modelling with a negative binomial distribution to identify differentially expressed genes. Raw p-values were adjusted for multiple comparisons using the Benjamini–Hochberg method. Genes known to be targets of these miRNAs were identified by cross-referencing with miRWalk [24]. A number of these genes, known to be associated with control of the inflammatory response, were selected and their expression in NK cells compared between study groups using TaqMan (Life Technologies, Carlsbad, CA, USA) qPCR assays.

Data analysis

Descriptive analyses, means±SEM, medians (interquartile ranges (IQRs)) and frequency distributions were used to summarise the participants' baseline characteristics. As the data were, unless otherwise stated, nonparametric; study groups (controls, moderate, severe) were compared using either the Wilcoxon or Kruskal–Wallis rank sum test, with post-test comparison of moderate and severe groups using Dunn's multiple comparison test. Correlation was assessed using Spearman's rank correlation coefficient. A stepwise logistic regression model was applied to determine the independence of clinical features correlating to both IL-15 mRNA expression and serum IL-15 levels. $p<0.05$ was considered statistically significant. Statistical analysis was performed using the JMP® (SAS, Cary, NC, USA) software package.

Further details of the methodology are available in the online supplementary material.

Results

Clinical and demographic features

Over the course of the study, 88 children with bronchiolitis and 43 controls were enrolled. Enrolment occurred across three respiratory viral seasons as demonstrated in figure 1. During the respiratory viral seasons of 2009/10 and 2010/11, 66 children with viral bronchiolitis and 30 controls were successfully enrolled. Their baseline characteristics are summarised on table 1. 61 of these children had RSV-positive bronchiolitis (92%) including four children with co-infection. Virology results are summarised in online supplementary table E1. 22 of the 66 children had severe disease. A comparison of children with moderate and severe disease is presented in table 2. Samples collected from a second tranche of study participants (13 controls, 22 children with RSV-positive bronchiolitis) enrolled in 2011/12 were used for NK cell

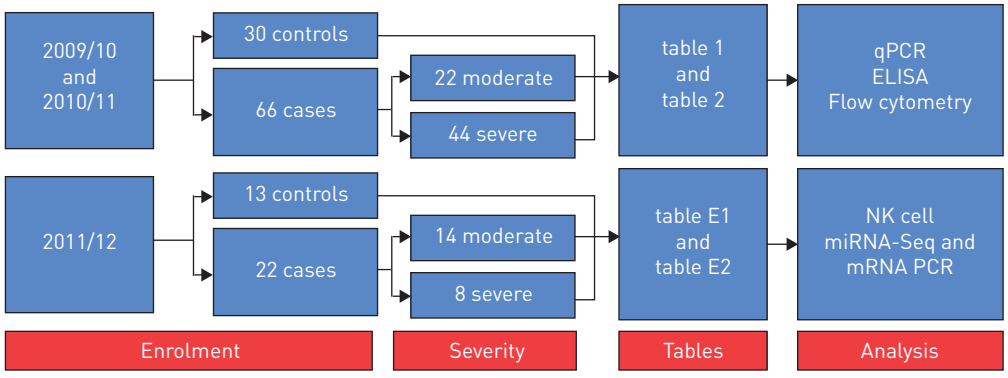

FIGURE 1 Study enrolment. Analysis was performed on samples from 88 participants with bronchiolitis and 43 controls enrolled as illustrated. qPCR: quantitative real-time PCR; NK: natural killer; miRNA-Seq: microRNA sequencing.

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TABLE 1 Baseline characteristics of study participants

	Controls	Viral bronchiolitis	p-value
Subjects N	30	66	
Female n (%)	18 (60)	30 (45.5)	0.27#
Birthweight kg	3.4 (3.0–3.9)	3.4 (2.8–3.8)	0.70¶
Age months	5.8 (0.7–12.4)	2.2 (1.2–6.7)	0.43¶
Gestational age at birth weeks/40	39.2 (38–40.5)	40 (38–40)	0.98¶
Breastfed	18/30 (60)	21/64 (32.8)	0.015#
Number of siblings mean±SEM	1.1±0.2	1.3±0.1	0.13¶
Family history of atopy	20/29 (69.0)	35/65 (53.9)	0.18#
Parental smoking	9/29 (31.0)	36/65 (55.4)	0.04#

Data are expressed as median values (25th–75th percentile interquartile range) or n/N (%), unless otherwise stated. #: calculated using Fisher's exact test; ¶: calculated using the Wilcoxon rank sum test.

miRNA sequencing and NK cell mRNA PCR. Four of these 22 children had co-infection (three rhinovirus, two coronavirus). Their baseline characteristics are summarised in supplementary tables E2 and E3.

IL-15 mRNA expression in PBMCs and serum IL-15 levels

qPCR demonstrated a significant increase in IL-15 mRNA levels in PBMCs of children with bronchiolitis ($p<0.0001$). Participants with moderate severity bronchiolitis had statistically significantly higher expression than either controls ($p<0.0001$) or children with severe disease ($p=0.01$) (figure 2 and supplementary table E4). IL-15 protein levels as measured in the serum using ELISA (figure 2 and supplementary table E4) showed a statistically significant increment in levels from control to moderate ($p=0.0009$) and from moderate to severe bronchiolitis ($p=0.005$).

Univariate analysis including potential confounders identified in tables 1 and 2 (breastfeeding, exposure to tobacco smoke (ETS) and prematurity) was performed (supplementary table E5), suggesting an association between ETS and IL-15 mRNA expression ($p=0.02$). However, this association was not maintained in a multivariate stepwise logistic regression model, confirming the independence of the association between IL-15 mRNA expression and both the occurrence of bronchiolitis (supplementary table E6; $p=0.0003$) and its severity (supplementary table E7; $p=0.009$).

TABLE 2 Comparison of participants with moderate and severe bronchiolitis

	Moderate	Severe	p-value
Subjects N	44	22	
Demographics			
Age months	2.6 (1.2–8.5)	1.7 (1.2–2.2)	0.08#
Gestational age at birth weeks/40	40 (39–41)	37.5 (34.1–40)	0.0006#
Premature (<35/40 weeks) at birth	2/44 (4.6)	7/22 (31.8)	0.005¶
Birthweight kg	3.5 (3.1–3.9)	3.1 (2.2–3.6)	0.03#
Parental smoking	26/43 (60)	10/22 (45)	0.30¶
Breastfed	14/44 (32)	7/20 (35)	>0.99¶
Viral characteristics			
RSV infection	40/44 (90.9)	21/22 (95.5)	0.66¶
RSV qPCR C_T value	27.0 (23.8–29.4)	26.6 (24.9–32.1)	0.77#
Non-RSV bronchiolitis n	4 (9.1)	1 (4.5)	0.66¶
Co-infection (two viruses) n	4 (9.1)	1 (4.5)	0.66¶
Disease severity			
RDAI score	6 (5–8)	8.5 (7–11.25)	0.0022#
Duration of hospital stay days	3.8 (2.6–6.7)	11.1 (8.2–15.3)	<0.0001#
Duration of oxygen therapy days	2.3 (0.8–4.9)	8.3 (3.6–12.5)	<0.0001#

Data are expressed as median values (25–75th percentile interquartile range) or n/N (%), unless otherwise stated. RSV infection denotes proportion of RSV-positive bronchiolitis among proven viral bronchiolitis cohort. RSV: respiratory syncytial virus; qPCR: quantitative real-time PCR; RDAI: Respiratory Distress Assessment Instrument. #: calculated using the Wilcoxon rank sum test; ¶: calculated using Fisher's exact test.

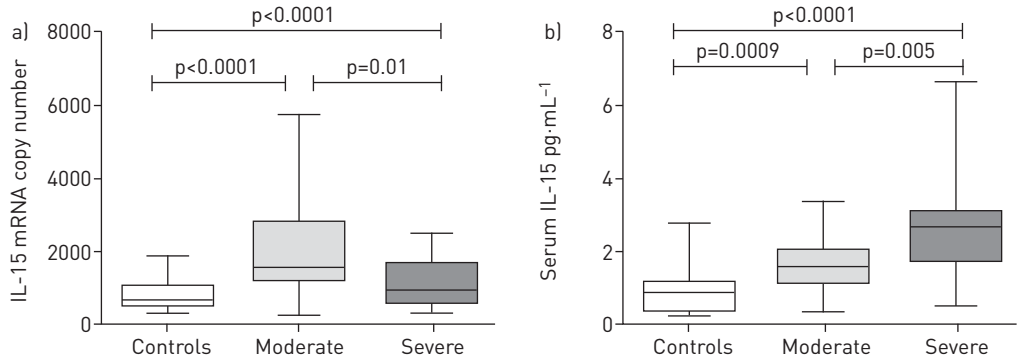

FIGURE 2 Interleukin (IL)-15 mRNA expression and serum IL-15 concentration in study participants. a) IL-15 mRNA copy number (normalised per 10 million copies of β-actin) in peripheral blood mononuclear cells of participants as measured by quantitative real-time PCR and b) serum IL-15 concentration as measured by ELISA. IL-15 mRNA copy number is highest in participants with moderate disease, whereas serum IL-15 levels are highest in those with severe disease.

Premature birth (<35 weeks gestation) was found by univariate analysis to be significantly associated with reduced serum IL-15 levels (supplementary table E5; p=0.03). However the statistical significance of this association was not maintained in multivariate analysis, which confirmed the independence of the association between serum IL-15 levels, the development of bronchiolitis (supplementary table E6; p=0.0002) and severe disease (supplementary table E7; p=0.004).

Chronological age at birth was also examined as a potential confounder. Neither the correlation between chronological age at birth and IL-15 mRNA expression (Spearman rho=0.185, p>rho 0.1) nor serum IL-15 levels (Spearman rho= −0.01, p>rho 0.94) were statistically significant.

Lymphocyte phenotyping

The concentrations of total PBMCs and lymphocytes were comparable between the three study groups (supplementary table E8). There was a statistically significant reduction in the relative frequency of NK cells (p=0.008) in the peripheral blood of children with bronchiolitis as compared with the control group (supplementary figure E1). IL-2/15Rβ was expressed predominantly on NK cells and significantly more frequently than on T-cells (54% *versus* 2%; p<0.0006).

Intracellular IL-15 expression

The cellular source of IL-15 in PBMCs was explored using intracellular cytokine staining, focusing initially on monocytes ($CD3^-CD14^+$) and later by examining the dendritic cell (DC) population, gating on the $LIN1^-HLA\text{-}DR^+$ population of PBMC. The relative distributions of myeloid ($CD11c^+$), plasmacytoid ($CD123^+$) and double-negative (DN) ($CD11c^-CD123^-$) DCs are shown in supplementary table E9. Of note, children with bronchiolitis had a significantly higher percentage of DN DCs (p=0.016) and a significantly lower percentage of plasmacytoid DCs (p=0.001). The percentage of plasmacytoid DCs was significantly lower again in the severe group (p=0.017). DN DCs were the predominant source of IL-15 (figure 3 and supplementary table E9) and IL-15 expression was seen in a significantly greater proportion of DN DCs in those with bronchiolitis (p=0.008). In children with viral bronchiolitis (n=6) and controls (n=2), intracellular expression of IL-15 in monocytes was almost undetectable (median 0.7% of cells, IQR 0.5–1.4%).

Expression of IL-15-related antiapoptotic markers

Intracellular expression of Bcl-x_L was significantly greater in $CD4^+$ T-cells (p=0.007), $CD8^+$ T-cells (p=0.03) and NK cells (p=0.003) of infants with bronchiolitis (supplementary table E10 and figure 4). Furthermore, the intensity of intracellular Bcl-x_L staining correlated with the serum concentration of IL-15, particularly in NK cells (Spearman rho=0.75, p=0.0051). In children with bronchiolitis, the intracellular expression of Bcl-2 was significantly higher in $CD4^+$ T-cells (p=0.03), with a trend toward increased intracellular Bcl-2 in $CD8^+$ T-cells (p=0.08), but not in NK cells.

Impact of RSV viral load on disease severity and immunological parameters

Using the PCR cycle threshold (C_T) value as a surrogate measure of RSV viral load, we did not detect any correlation (Spearman rho=0.03, p=0.86) with disease severity (RDAI). However, there was a slight trend towards lower NK cell percentage (Spearman rho= −0.33, p=0.11), higher serum IL-15 (Spearman rho= −0.22, p=0.18) and IL-15 mRNA expression (Spearman rho= −0.21, p=0.2) with lower C_T values.

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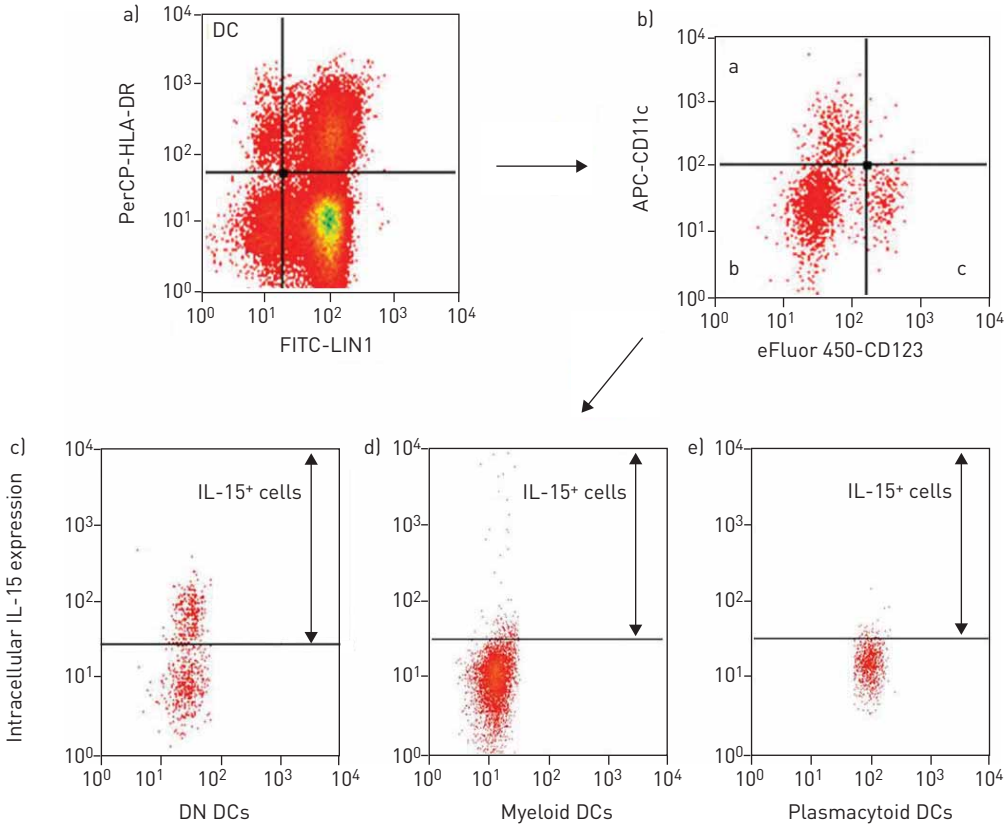

FIGURE 3 Relative intracellular expression of interleukin (IL)-15 in dendritic cell (DC) subsets. The DC population in peripheral blood mononuclear cells was identified a) as HLA-DR$^+$LIN1$^-$ (labelled DC) and subsets identified b) using cell surface staining for plasmacytoid (CD11c$^-$CD123$^+$) and myeloid (CD11c$^+$CD123$^-$) DC markers. IL-15 was found intracellularly in a significantly higher proportion of c) DN (CD11c$^-$CD123$^-$) DCs compared with d) myeloid DCs and e) plasmacytoid DCs among children with viral bronchiolitis. DN: double-negative; PerCP: peridinin chlorophyll protein; FITC: fluorescein isothiocyanate; APC: allophycocyanin.

NK cell miRNA sequencing and relative quantification of putative mRNA targets by PCR

NK cells (93% purity from extracted PBMC population) isolated from six infants with RSV bronchiolitis and six age-matched controls exhibited an array of differentially expressed miRNAs and other small noncoding RNAs as listed in table 3. Putative target genes for differentially expressed miRNAs and their relative expression in NK cells are listed in table 4.

There was a statistically significant intergroup variation in 12 of the 19 selected mRNAs targets. Seven target mRNAs, including three genes on the IL-15 pathway (*JAK3*, *STAT5A* and *NFKB1*), were differentially expressed in those with severe bronchiolitis compared with those with moderate severity bronchiolitis.

Discussion

We demonstrate, for the first time to the best of our knowledge, an association between disease severity in children hospitalised with viral bronchiolitis and 1) IL-15 mRNA expression in PBMCs and 2) IL-15 concentration in serum. We identified the source of IL-15 in PBMCs as a subpopulation of DCs that are expanded in viral bronchiolitis, and also report a correlation between IL-15 levels and intracellular expression of antiapoptotic proteins Bcl-2 and Bcl-x_L in lymphocytes of children with bronchiolitis. We identified a distinct pattern of NK cell miRNA expression in bronchiolitis and differential expression of a selection of their putative mRNA targets, among them genes for signalling molecules in the IL-15 pathway.

Children with viral bronchiolitis in our cohort demonstrated upregulation of IL-15 mRNA expression in PBMCs and increased serum levels of IL-15. This is consistent with an appropriate innate immune response to a viral infection, as it promotes activation and recruitment of NK cells to the lungs. However, while there is an increase in the serum concentration of IL-15 with increasing disease severity, in contrast children with severe bronchiolitis in our study did not demonstrate upregulation of PBMC IL-15 mRNA expression. There may be a number of explanations for the lack of correlation between serum IL-15 levels and PBMC IL-15 mRNA expression in severe bronchiolitis. First, serum IL-15 levels reflect production by

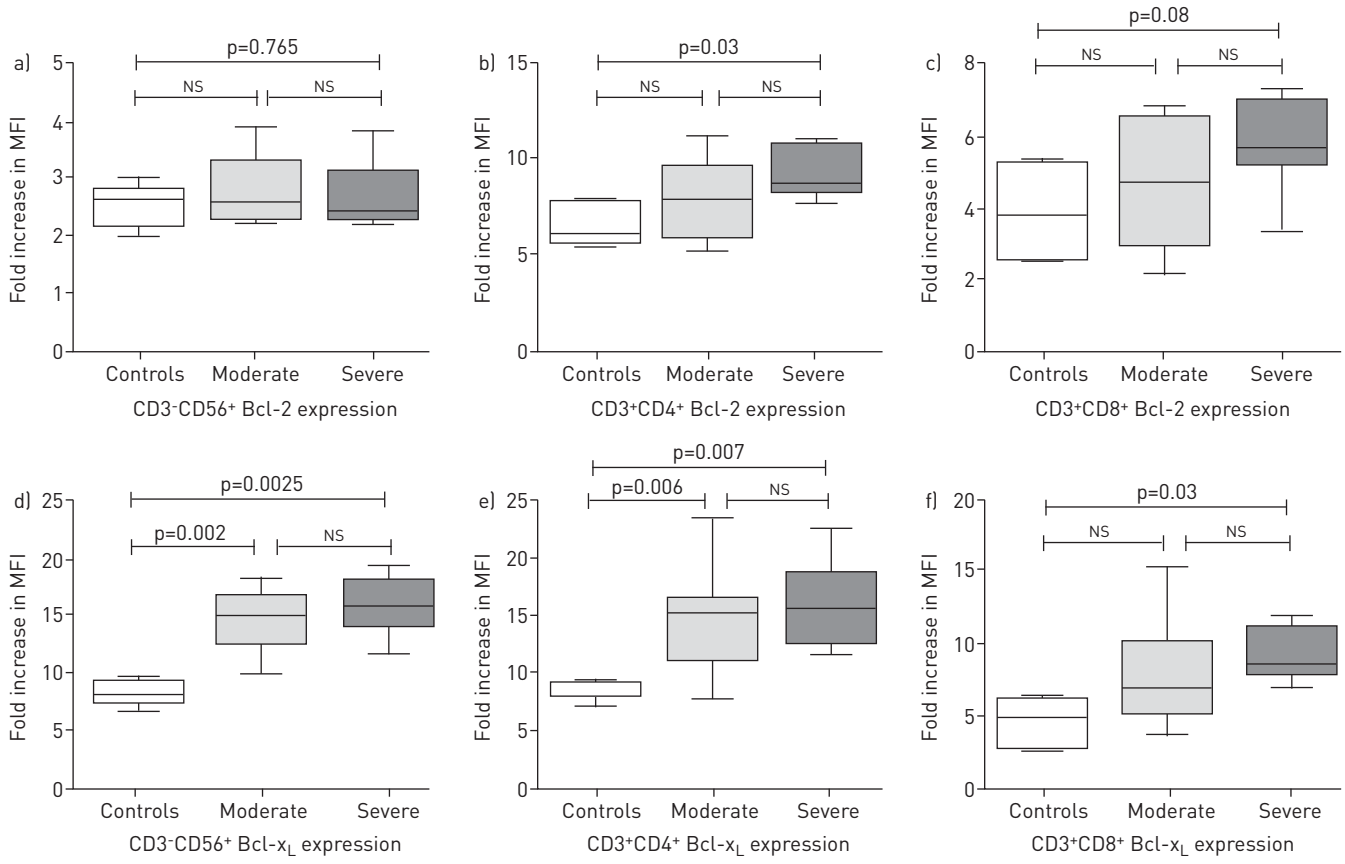

FIGURE 4 Intracellular expression of antiapoptotic proteins by lymphocyte subset in study participants. Bcl-2 expression in a) NK cells did not differ between controls and children with bronchiolitis, unlike b) $CD4^+$ T-cells and c) $CD8^+$ T-cells. Bcl-x_L expression was increased in participants with bronchiolitis in d) NK cells, e) $CD4^+$ T-cells and f) $CD8^+$ T-cells. MFI: mean fluorescence intensity; NK: natural killer.

all cells, whereas IL-15 mRNA expression, as measured in our experiment, reflects only mRNA expression in the extracted PBMC. The difference may also reflect a homeostatic measure. IL-15 expression is tightly regulated at the mRNA level, as unrestrained NK cell activation may have harmful effects and has been shown to lead to pulmonary fibrosis in the mouse model [25]. In addition, IL-15 produced by antigen-presenting cells (APCs) such as DCs and mononuclear phagocytes within the PBMC compartment is trans-presented to the target cell together with IL-15Rα, allowing the target cells to respond at lower physiological levels of IL-15 [26]. Therefore, PBMC IL-15 mRNA expression may be more carefully regulated than in nonimmune cells.

Alternatively, failure to upregulate IL-15 mRNA expression may represent evidence of an "immunoparesis" among those children with severe bronchiolitis, analogous to that demonstrated by our research group in sepsis and pneumonia in other patient cohorts [27]. Immunoparesis in this context may be genetically mediated and may, at least in part, account for the inherited susceptibility to severe bronchiolitis seen among some children [28]. Support for this hypothesis comes from genetic association studies undertaken and published by our colleagues in Utrecht, The Netherlands [6]. In one of the biggest studies of its kind, Janssen *et al.* [6] showed an overall association of innate immunity genes with genetic susceptibility to RSV infection in a candidate gene study of 480 hospitalised children and >1000 controls. Among other associated loci, they showed a nominal association with alleles of the *IL15* gene variant rs2254514 (OR 1.22, 95% CI 1.02–1.46; p=0.026) and hospitalisation for RSV. Interestingly, rs2254514 is a proxy for another *IL15* SNP rs6837991 (linkage disequilibrium; r^2=0.87), which has a RegulomeDB (http://www.regulomedb.org/) score of 1f (*i.e.* it is likely to affect *IL15* gene expression). Thus, while the association is weak, due to the limitations of power of the original study, there is inferential evidence of a genetic association with RSV susceptibility and *IL15* expression [29]. Finally, absence of PBMC IL-15 upregulation in severe bronchiolitis may reflect a viral pathogenic effect. RSV, for example, has been shown to infect human monocyte-derived DCs *in vitro*, and impair their ability to subsequently activate and proliferate T-cells [30]. A similar phenomenon might explain the findings in this study.

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TABLE 3 Differentially expressed small noncoding RNA in enriched natural killer cells from infants with respiratory syncytial virus-positive bronchiolitis compared with healthy age-matched controls

Gene	Transcript type	Base mean	Log_2 fold increase	Adjusted p-value
Upregulated				
SNORD82	snoRNA	117.497	1.87	0.022
MIR370	miRNA	132.383	3.785	0.041
SCARNA6	snoRNA	134.66	1.589	0.022
MIR3074	miRNA	160.91	1.083	0.009
MIR379	miRNA	177.244	3.233	0.022
SNORD85	snoRNA	247.918	1.332	0.014
SCARNA18	snoRNA	289.76	1.644	0.022
MIR222	miRNA	485.265	2.288	0.014
AC078899.5	miRNA	691.042	1.41	0.01
SCARNA5	snoRNA	1265.42	1.473	0.014
MIR101-1	miRNA	1378.251	1.335	0.022
MIR30D	miRNA	1427.109	1.041	0.027
U8	snoRNA	1445.441	1.349	0.004
snoU2-30	snoRNA	1538.635	1.735	0.017
SNORD73A	snoRNA	3053.163	1.384	0.022
MIR873	miRNA	6541.255	1.113	0.049
SNORD51	snoRNA	7180.6	1.328	0.029
SNORD74	snoRNA	7471.941	1.786	0.017
RNY4P10	misc_RNA	13 473.821	2.792	0.022
MIR103A2	miRNA	63 043.74	1.264	0.013
MIR221	miRNA	156 248.869	2.233	0.045
Downregulated				
RN7SL610P	misc_RNA	340.915	−1.865	0.014
RPPH1	misc_RNA	794.029	−2.07	0.022
Y_RNA	misc_RNA	2615.626	−1.219	0.025
RN7SL364P	misc_RNA	101.251	−1.716	0.025
LA16c-360H6.3	Antisense RNA	7897.37	−1.635	0.027
RP11-1129I3.1	Sense overlapping	150.593	−2.302	0.049
MIR199B	miRNA	1706.133	−2.165	0.049

snoRNA: small noncoding RNA; miRNA: microRNA; misc_RNA: miscellaneous RNA.

IL-15 is particularly important for NK cell homeostasis and activation [31]. We hypothesised that children with severe bronchiolitis had a defect in the APC–IL-15–NK cell axis that prevented the development of a competent innate response to respiratory viral infection. This might account for the paucity of NK cells in lung tissue seen at autopsy studies of children who died from severe viral bronchiolitis [32]. Lymphocyte phenotyping by flow cytometry revealed a significant drop in the relative percentage of NK cells seen in the peripheral blood among children with bronchiolitis. While noteworthy, a similar finding has been reported previously [33]. Furthermore, Larranaga *et al.* [34] reported changes in NK cell subsets, in particular unregulated $CD94^+$ activated NK cells, correlating with disease severity in RSV bronchiolitis. These changes in NK cell and subset distribution may represent sequestration of activated NK cells to the site of infection rather than apoptosis. In support of this conjecture, we found increased expression of the IL-15-related antiapoptotic marker Bcl-x_L in lymphocytes (including NK cells) in children with bronchiolitis. In contrast, Bcl-2 expression was not upregulated in NK cells of children with bronchiolitis. The significance of this finding needs to be explored further in a larger cohort.

We identified a subpopulation of DCs as the source of IL-15 in PBMCs of infants and found this population to be expanded among children with bronchiolitis. This argues against cellular depletion as an explanation for decreased IL-15 mRNA in severe bronchiolitis. DCs are known to be a cellular source of IL-15, but the change in DC subset distribution and IL-15 production demonstrated in children with bronchiolitis has not been reported previously [35].

Results from qPCR, ELISA and flow cytometry experiments led us to screen for differential expression of miRNA in NK cells of infants with RSV bronchiolitis, miRNA now being increasingly recognised as central to NK cell function [36]. We observed that NK cells of infants with RSV exhibited a distinct pattern of miRNA expression. Even with our limited sample size (n=6 in each group), statistically significant differences were detectable using current analytic methods [37].

TABLE 4 Relative quantification of mRNA targets of microRNA genes with altered expression by study group

	Control	Moderate	Severe	p-value[#]	p-value[¶]
Subjects n	13	14	8		
AKT1	1.1 (1.0–1.2)	1.3 (1.2–1.5)	1.3 (1.0–1.8)	0.01	NS
AKT2	1.7 (1.4–1.9)	1.9 (1.7–2.1)	1.7 (1.5–2.5)	0.18	NS
BAX	1.2 (0.9–1.4)	1.7 (1.4–1.9)	1.7 (1.5–2.0)	0.0029	NS
CXCR4	1.2 (0.5–2.4)	1.6 (1.0–2.0)	1.4 (0.7–2.9)	0.78	NS
FAS	0.2 (0.2–0.3)	0.5 (0.4–1.0)	0.7 (0.6–1.3)	0.0001	0.0478
FLT3	1.1 (0.5–1.5)	1.3 (0.5–1.9)	3.4 (2.2–4.2)	0.0066	0.0041
FOXO3	0.5 (0.4–1.2)	0.7 (0.6–0.9)	0.6 (0.5–2.1)	0.86	NS
GADPH	1.0 (0.9–1.1)	1.3 (1.0–1.6)	3.3 (1.8–5.0)	0.0008	0.0041
IRAK1	1.1 (0.9–1.1)	1.4 (1.2–1.6)	1.8 (1.4–2.2)	0.0013	NS
JAK3	0.6 (0.5–0.8)	0.9 (0.8–1.0)	1.9 (1.3–2.9)	<0.0001	0.0021
MAPK1	0.9 (0.9–1.0)	1.1 (1.0–1.3)	1.3 (1.1–1.7)	0.0037	NS
MAPK8	1.2 (1.2–1.3)	1.2 (1.1–1.6)	1.3 (1.0–1.9)	0.75	NS
NFATC2	1.4 (1.3–1.5)	1.7 (1.3–2.0)	1.3 (0.4–2.0)	0.14	NS
NFKB1	0.7 (0.6–0.8)	1.0 (0.9–1.1)	1.3 (1.0–1.7)	<0.0001	0.0203
STAT1	0.4 (0.4–0.7)	0.8 (0.6–1.6)	1.3 (0.6–3.1)	0.007	NS
STAT5A	1.0 (0.7–1.2)	1.2 (1.0–1.5)	1.9 (1.3–2.6)	0.0048	0.0290
TGFB1	1.3 (1.1–1.4)	1.3 (1.2–1.6)	1.3 (1.1–1.7)	0.63	NS
TGFB2	1.0 (0.6–2.9)	1.1 (0.7–2.1)	4.9 (1.3–8.2)	0.17	NS
TNFRSF10A	1.8 (1.2–2.0)	1.9 (1.1–2.6)	3.9 (2.3–5.4)	0.0155	0.0203

Relative quantity values are expressed as median (interquartile range), unless otherwise stated. NS: nonsignificant. [#]: calculated using the Kruskal–Wallis rank sum test to compare all three groups; [¶]: calculated using Dunn's multiple comparison test to compare moderate *versus* severe bronchiolitis.

Bioinformatics analysis identified putative target genes for these deregulated miRNAs. Differential expression of a selection of these target genes was confirmed in NK cells of infants with moderate and severe bronchiolitis. These differentially expressed target genes included genes regulating cell activation and expansion; notably *STAT5A*, *JAK3* and *NFKB1*, which code for proteins on the IL-15/γ_c signalling pathway, reinforcing the hypothesis that IL-15 is linked to disease severity in RSV bronchiolitis [38].

The patterns of miRNA expression that characterised the NK phenotype of infants with bronchiolitis are distinct from those reported in prior *in vitro* studies where NK cells were activated by γ_c cytokines IL-2 and IL-15 [23, 39]. However, *in vitro* experiments of NK cell activation may not reflect the complexity of a clinical disease process. In this regard, our study adds to the available NK cell literature, providing a novel insight into factors that regulate NK cell activation in infants with viral infection.

Our study has several limitations. Being a single-centre study, our capacity to recruit large numbers was limited, restricting the power of our study. We attempted to mitigate this risk by collaborating with colleagues in The Netherlands, who having access to a much larger cohort of patients, report a link between IL-15 single nucleotide polymorphisms (SNPs) and disease severity. There are limitations to this approach, as patient cohorts are distinct and recruited by separate investigators. However, we consider that the large size of the Dutch cohort adds considerable strength to the conclusions from our smaller study of IL-15 gene expression. Some potential confounders, notably gestational age at birth, breastfeeding rate and ETS, distinguish those in the moderate and severe groups. However, the link between disease and IL-15 was maintained when these confounders were accounted for in multivariate analysis. Small volumes of blood were obtained from those patients recruited, which meant that not all tests could be performed on each patient sample. The technical challenges associated with extracting sufficient miRNA from small volumes of blood containing relatively few NK cells introduced an element of bias into the sequencing data, as those with the most severe disease had the fewest NK cells, precluding their analysis. This precluded an exploration of the relationship between NK cell miR, disease progression and resolution. The results of miRNA transcriptomal screening involving multiple comparisons and performed in relatively small numbers of patients are inferential in nature. To validate these inferential transcriptomal findings, we confirmed differential expression of miRNA target genes in NK cells. As with all biomarker studies, external validation of these findings in cohorts from different patient populations using different assays and platforms is needed.

Despite these limitations, data garnered from SNP analysis, qPCR, ELISA, flow cytometry and NGS all suggest an association between IL-15 and disease severity in children hospitalised with viral bronchiolitis. This may serve to prompt research into novel RSV vaccination and treatment strategies. Further research is

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needed to examine the relative impact of the other γ_c cytokines on disease severity in RSV bronchiolitis, as well as the specific contribution of other innate cells to the clinical phenotype.

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