Interleukin-15 is associated with disease severity in viral bronchiolitis.

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Online Data Supplement

Supplementary methods.

Measurement of IL-15 gene expression using real time reverse transcriptase PCR

An EDTA anti-coagulated sample of blood was collected from study participants upon enrolment. PBMC were extracted from this sample by density gradient centrifugation using Lymphoprep (Axis Shield, Dundee, Scotland). The PBMC were washed, pelleted and subsequently lysed in RLT lysis buffer (Qiagen, Limburg, Netherlands) before RNA was extracted using the RNeasy micro kit (Qiagen) according to manufacturer's instructions. The quantity and purity of extracted RNA was confirmed using a spectrophotometer (Nanodrop ND-8000, Wilmington, DE, USA). RNA was stored at -70^{O}C until reverse transcription was performed as previously described. PCR reactions were performed in an AbiPrism 7900HT (Applied Biosystems) sequence detector system and all reactions were performed in triplicate. Primers and probes for IL-15 were obtained pre-customized (Applied Biosystems). Primers and probes for β-actin were made and customized as per Stordeur et al (E1). The DNA standards for β-actin and IL-15 comprised a cloned PCR product including the quantified amplicon prepared by PCR from a cDNA population including the target mRNA as per Stordeur et al [E1]. In order to quantify transcript levels, a standard curve was constructed for each PCR run for each mRNA target, using serial dilutions of the relevant standard as previously described. The results were normalized against β-actin mRNA and the values expressed as mRNA copy numbers/ 10^7 copies of β-actin mRNA.

Measurement of serum concentration of IL15 using ELISA.

Serum was obtained from whole blood clotted at room temperature and centrifuged at 900G for 10 minutes. The serum separated from the blood samples was stored at -80^{O}C until analysis. Cytokine serum concentration was measured using an electrochemiluminesce-based sandwich immunoassay kit (K151JGC-1, Meso Scale Discovery, Rockville, MD, USA)

according to the manufacturer's instructions. Each sample was measured in duplicate. The lower limit of detection was 0.46pg/mL.

Lymphocyte immunophenotyping

Lymphocyte phenotyping was performed on PBMC collected by density centrifugation. PBMC were re-suspended in approximately 50µl phosphate buffer containing 1% v/v bovine serum albumin and 0.02% sodium azide before staining. Fluorochrome-labelled monoclonal antibodies (mAb) used for phenotyping included the following; FITC-CD56, APC-CD19, PE-CD8, PE-CD4 (Immunotools, Friesoythe, Germany), PerCP-CD3 (Biolegend, San Diego, CA, USA). Cells were stained and subsequently fixed in 1% paraformaldehyde (Sigma-Aldrich) before analysis on a CyAn-ADP flow cytometer using Dako Summit software. An unstained sample was used to determine levels of autofluorescence and set voltage levels. Single colour, isotype matched controls and fluorescence-minus-one (FMO) controls were used to set compensation and gates.

Lymphocytes were identified by their characteristic forward scatter and side scatter parameters. Lymphocyte subsets were defined as follows B-cells (CD3-CD19+) and NK cells (CD3-CD56+). T cells (CD3+) were further classified as follows; helper T-cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+). Cytometry was performed using fluorochrome-conjugated monoclonal antibodies purchased from Immunotools (Friesoythe; Germany) and Biolegend (San Diego, CA, USA). Results were expressed as percentage of gated lymphocyte population or percentage of T-cells subpopulation as appropriate. The percentage of NK cells and T-cells expressing the IL2/15Rβ was measured using an APC-conjugated IL2/15Rβ antibody (Biolegend).

Intracellular Analysis of IL-15 Production.

Aliquots of PBMC from both controls and participants with bronchiolitis were cryogenically frozen and later thawed for analysis of the cellular source of IL-15 in PBMC. Thawed PBMC were plated at 10^6 cells/mL in complete RPMI medium (RPMI 1640 containing Glutamax with added 25mMHEPES, 50 mg/mL streptomycin, 50 U/mL penicillin, 2 μg/mL fungizone, and 10% heat-inactivated foetal calf serum). Cells were incubated in the presence of brefeldin A (10 μg/mL, Sigma-Aldrich) to promote intracellular accumulation of cytokines. Cells were harvested and stained for cell surface expression of lymphocytes (CD3), monocytes (CD14), dendritic cell (DC) markers (CD123, CD11c), HLA-DR, and markers of non-dendritic cell PBMC (LIN1, BD bioscience, Franklin Lakes, NJ, USA) using fluorochrome-conjugated monoclonal antibodies purchased from Immunotools, BD bioscience (Oxford, UK) and eBioscience (Hatfield, UK). The DC population (LIN1 –HLA-DR+) was subclassified as myeloid (11c+,123-), plasmacytoid (123+11c-) or double negative (11c-123-) DC. Intracellular expression of IL-15 was measured using a PE-conjugated monoclonal antibody (R&D systems, Abingdon, UK). Cells were again analysed with a CyAn-ADP flow cytometer with Summit (Beckman Coulter, Pasadena, CA, USA) software. Isotype-matched, nonspecific mAbs, and fluorescence-minus-one controls were used to set compensations and gates. Dead cells were gated out using an aqua-conjugated Live-Dead stain (Invitrogen, Carlsbad, CA, USA). Results were expressed as percentage of subpopulation containing IL-15.

Analysis of expression of anti-apoptotic proteins Bcl-2 and Bcl-xL in NK cells and T cells

Intracellular expression of anti-apoptotic proteins Bcl-2 and Bcl-xL in NK cells and T cells from both controls and participants with bronchiolitis was undertaken using cell surface markers for CD3, CD56 (Biolegend), CD4, CD8 (Immunotools) and intracellular markers for Bcl-2 (BD Pharmingen) and Bcl-xL (Southern Biotech, Birmingham, AL, USA). The results

were expressed in each lymphocyte subset in each group as mean fold increase in mean fluorescence intensity (MFI).

Comparison of the miR transcriptome in NK cells of infants with bronchiolitis and age matched controls

NK cells were isolated from PBMC of infants with bronchiolitis and age-matched controls by negative selection using a magnetic NK cell isolation Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The technique was optimized to give a purity >90% of extracted PBMC. RNA extraction was performed using Ambion mirVana™ (Life technologies) and Macherey Nagel® nucleospin kits. The quantity of RNA was measured using a Qubit fluorometer (Invitrogen) and the quality assessed using an Agilent 2100 bioanalyzer system with the RNA 6000 Nano kit and the Small RNA kit (Agilent technologies). Using the SOLiD 4 NGS Platform (Life Technologies, USA), 35 base-pair barcoded short sequence reads were generated from the small RNA fraction of samples. Barcoded libraries were prepared using the Small RNA library reagents and protocol for the Applied Biosystems SOLiD™4 System (Life Technologies, USA). Libraries were quantitated using the Quantitative RT-PCR using the SOLiD™ Library TaqMan® Quantitation kit (Life Technologies, USA) then diluted to 500pM in 1X TE buffer and prepared onto templated beads using reagents and protocol from the manufacturer (Applied Biosystems SOLiD™4 System Templated Bead Preparation Guide). Multiplex fragment sequencing was performed according to the Applied Biosystems SOLiD 4 System Instrument Operation Guide and using the Applied Biosystems SOLiD 4 reagents (Life Technologies, USA) with barcode tag (BC Tag MM5) primers initially, followed by fragment library 35bp read (F3 tag MM35) primers.

SOLiD™ Sequencing quality control.

Library Size Normalisation using DESeq2.

The DESeq2 algorithm includes a normalisation step to account for differences in library size across input samples. This permits comparisons of gene counts between samples with different relative library sizes by bringing all gene count values to a common scale. In the quality control analysis (see Figures E2 and E3), there is consistent distribution of read counts in all samples regardless of the depth of sequencing. Transcripts with low average read-counts across all samples were first removed from the gene expression data to promote evidence based results. Pseudo-counts were added to any remaining transcripts with zero mapped reads to allow transformation and plotting on log scale. Gene expression data was then rlog-transformed with DESeq2 and plotted using boxplots (E2) and kernel-density estimation graphs (E3). Simple statistics show that there was no significant differences across all samples (One-Way ANOVA, n=12, p=0.99).

Comparison of mRNA expression of selected targets of differentially expressed miRs in controls and participants with bronchiolitis

Bioinformatics analysis of the miR-Seq data identified miRNAs differentially expressed in infants with bronchiolitis. Genes known to be targets of these miRNAs were identified by cross-referencing with miRWalk [E2], an online database of both experimentally validated and computationally predicted miRNA-target interactions (MTIs). A number of these genes known to be associated with control of the inflammatory response were selected and their expression compared between controls and study participants with bronchiolitis. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to convert total RNA to single stranded cDNA using 15ng RNA per sample. Predesigned RT-PCR TaqMan® assays for AKT1, AKT2, BAX, CXCR4, FAS, FLT3, FOXO3, IRAK1, JAK3, MAPK1, MAPK8, NFATC2, NFKB1, STAT1, STAT5A, TGFB1, TGFB2, TNFRSF10A were performed in 20µl using 2µl cDNA template per reaction with CDKN1B as an endogenous control. All samples were assayed in triplicate on a 7500HT Fast RT-PCR instrument (Applied Biosystems, USA) using a standard ramp rate.

References

E1. Stordeur P, Poulin LF, Craciun L, et al. Cytokine mRNA quantification by real-time PCR. J Immunol Methods 2002:259(1-2):55-64.

E2. Dweep H, Sticht C, Pandey P, et al. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011:44(5):839-847.

Figure Legends

Figure E1. Peripheral blood NK cell relative frequency in study groups. Lymphocyte subset analysis demonstrated a significant reduction in the relative percentage of NK cells in the peripheral blood of children with viral bronchiolitis.

Figure E2. **Boxplot of rlog-transformed expression data** Transcript counts were rlog-transformed across the input samples. This plot show that there are no significant changes in the overall distribution of gene expression values across all samples.

Figure E3. Kernel density estimation (KDE) of rlog transformed expression data Kernel-density estimation (KDE) was applied to show an even distribution of read counts across all samples. For each input sample, KDE plots the rlog transformed expression data (x-axis) against the probability of finding a transcript in that sample with a given expression value (y-axis). All input samples are shown individually, coloured by group. This plot illustrates a consistent distribution of transcript counts across the input samples.

Table E1. Respiratory viruses detected in participants with viral bronchiolitis enrolled, 2009-11	
Severe Group (N=22)	**Moderate Severity group (N=44)**
RSV only - 21	**RSV only – 36**
	RSV + coinfection – 4
	hMPV-2
	Coronavirus-1
	Influenza A-1
Non-RSV infections	**Non-RSV infections**
Rhinovirus and Coronavirus - 1	Rhinovirus and Coronavirus -1
	Bocavirus-1
	Rhinovirus - 1
	Coronavirus-1

Table E2. Baseline characteristics of participants in NK cell miR sequencing			
	Controls (N=6)	**Viral bronchiolitis (N=6)**	***P***
Gender; No.(%) female	4/6 (67)	2/6 (33)	0.56†
Birthweight (Kg)	3.62 (2.6- 3.8)	3.24 (2.1- 3.7)	0.34*
Age (months)	3.7 (1.1-6.8)	3.7 (1.4 – 7.2)	0.81*
Gestational age at birth (weeks/40)	40 (39-40)	39.5 (36.3 – 40.1)	0.74*
Breastfed; n/N (%)	1/6 (17)	3/6 (50)	0.55†
# Siblings; mean, SEM	0.83 (0.33)	2.0 (0.33)	0.03*
Family history of atopy; n/N(%)	3/6 (50)	4/6 (67)	>0.99†
Parental smoking; n/N(%)	2/6 (33)	3/3 (50)	>0.99†

Participants are a subset of cohort described in Table E3. Data expressed as median values (25^{th}-75^{th} IQR) unless otherwise stated. *P* calculated using Wilcoxon* or Fisher's exact test† as appropriate.

Table E3. Baseline characteristics of participants in NK cell mRNA PCR experiment			
	Controls (N=13)	**Viral bronchiolitis (N=22)**	***P***
Gender; n/N (%) female	9/13 (69%)	10/22 (45%)	0.29†
Birthweight (Kg)	3.47 (2.64-3.75)	3.16 (2.18-3.6)	0.32*
Age (months)	3.8 (0.9-7.3)	1.6 (0.8-4.1)	0.40*
Gestational age at birth (weeks/40)	40 (38.5-40)	38.8 (35-40)	0.11*
Breastfed; n/N (%)	10/13 (77%)	11/22 (50)	0.16†
# Siblings; mean, SEM	0.61 (0.24)	1.5 (0.18)	0.006*
Family history of atopy; n/N(%)	5/13 (38.4)	11/22 (50)	0.73†
Parental smoking; n/N(%)	7/13 (54)	15/22 (68.2)	0.48†

Data expressed as median values (25^{th}-75^{th} IQR) unless otherwise stated. *P* calculated using Wilcoxon* or Fisher's exact test† as appropriate

Table E4. Interleukin-15 cytokine gene expression and serum concentration by study participant category

	Control	N	Moderate bronchiolitis	N	Severe bronchiolitis	N	P^1	P^2
IL-15 mRNA expression*	823 (663 -983)	29	2118 (1632-2605)	36	1128 (816-1439)	17	<0.0001	0.01
Serum IL-15 level†	0.9 (0.6-1.2)	19	1.6 (1.4-1.9)	33	2.5 (1.95-3.1)	21	<0.0001	0.005

Results expressed as mean (95% CI of mean) no of copies of cytokine mRNA per 10 million β-actin mRNA copy numbers* and as picograms/mL†. *P* calculated using Kruskal Wallis Rank sum test (P^1), and Dunn's multiple comparison test (P^2) to compare moderate vs severe bronchiolitis.

Table E5. Univariate analysis of IL15 mRNA expression and serum IL15 level by participant characteristics

		IL15 mRNA expression* (median, IQR)	*P*	Serum IL15 concentration† (median, IQR)	*P*
Ever Breastfed	No	1446 (585-1907)	0.38	1.7 (0.9-2.7)	0.08
	Yes	1050 (639-1534)		1.2 (0.8-1.8)	
Premature (<35 weeks gestational age at birth)	No	1000 (596-1848)	0.47	2.7 (1.5-3.0)	0.03
	Yes	1195 (411-1688)		1.5 (0.9-2.1)	
Environmental (parental) tobacco smoke	No	875 (531-1385)	0.02	1.6 (0.9-2.1)	0.31
	Yes	1587 (724-2020)		1.6 (1.0-2.7)	

Results expressed as mean no of copies of cytokine mRNA per 10 million β-actin mRNA copy numbers* and as picograms/mL†.
P calculated using Wilcoxon non-parametric Rank Sum Test

Table E6. Multivariate analysis of IL15 mRNA expression and serum IL15 level by participant characteristics

Variable	IL15 mRNA expression		Serum IL15 concentration	
	F-ratio	*P*	F-ratio	*P*
Environmental (parental) tobacco smoke	0.91	0.34	0.562	0.46
Premature (<35 weeks gestational age at birth)	0.18	0.67	4.2	0.05
Viral Bronchiolitis (cases vs controls)	14.3	0.0003	15.836	0.0002

Stepwise logistic regression model. P represents Probability>F ratio

Table E7. Multivariate analysis of IL15 mRNA expression and serum IL15 level in participants with viral bronchiolitis				
Variable	**IL15 mRNA expression**		**Serum IL15 concentration**	
	F-ratio	*P*	F-ratio	*P*
Environmental (parental) tobacco smoke	0.075	0.79	0.22	0.64
Premature (<35 weeks gestational age at birth)	0.657	0.42	2.856	0.1
Disease severity (moderate vs severe)	7.389	0.009	9.104	0.004
Stepwise logistic regression model. P represents Probability>F ratio				

Table E8. Lymphocyte immunophenotyping by study participant group

	Control	N	Moderate	N	Severe	N	P^1	P^2
Total PBMC (x 10^6/ml)	6.1 (4.6-8.2)	24	6.7 (5.4-8.8)	16	7.1 (4.0-11.3)	14	0.57	NS
Total lymphocyte (x 10^6/ml)	5.3 (3.8-7.1)	24	4.9 (4.2-7.3)	16	4.6 (3.3-9.5)	14	0.84	NS
B-cell (% of lymphocyte)	13.8 (7.1-17.1)	24	13.0 (8.6-15.9)	18	13.9 (7.1-17.0)	15	0.95	NS
NK cell (% of lymphocyte)	6.9 (5.0-9.5)	24	4.2 (3.8-5.3)	18	4.7 (2.9-5.6)	13	0.008	NS
T-cell (% of lymphocyte)	68.7 (64.7-76.7)	24	73.3 (67.2-81.2)	18	65.3 (61.2-71.5)	15	0.21	NS
CD4+ T-cell(% of T cells)	75.9 (70.5-78.6)	24	79.2 (72.5-84.6)	18	77.1 (67.9-84.5)	15	0.27	NS
CD8+ T-cell(% of T cells)	21.3 (18.5-27.3)	24	17.8 (13.2-23.2)	18	19.6 (13.1-25.4)	15	0.22	NS
Dendritic cell (% of PBMC)	2.3 (1.5-3.1)	8	1.2 (1.0-1.4)	8	1.6 (1.0-2.1)	7	0.02	NS

P calculated using Kruskal Wallis Rank sum test (P^1), and Dunn's multiple comparison test (P^2) to compare moderate vs severe bronchiolitis. NS = not significant (P>0.05)

Table E9. Distribution of Dendritic Cell subsets by study participant category					
	Control (N=8)	**Moderate (N=12)**	**Severe (N=6)**	P^1	P^2
Myeloid DCs	40.6 (28.4-58.9)	43.1 (34.7-63.3)	54.5 (35.9-61.0)	0.88	NS
Plasmacytoid DCs	40.6 (19.0-48.2)	13.1 (6.8-22.0)	5.6 (3.4-8.4)	0.001	0.017
"double negative" DCs	12.3 (6.7-19.2)	36.2 (21.3-55.1)	40.7 (33.8-56.2)	0.016	NS
Intracellular Interleukin-15 expression among DC subsets (% of cells)					
Myeloid DCs	1.0 (0.2-7.9)	6.0 (1.8-43.0)	2.8 (1.1-9.0)	0.21	NS
Plasmacytoid DCs	0.3 (0.0-2.2)	2.5 (0.2-17.2)	3.7 (0.0-14.4)	0.3	NS
"double negative" DCs	5.0 (2.4-6.1)	16.6 (7.8-46.9)	30.1 (8.2-39.4)	0.008	NS
Values expressed as median (interquartile range) percentage of dendritic cell population. Groups compared using Kruskal-Wallis Rank Sum test (P^1). Post-test comparison of moderate and severe bronchiolitis groups using Dunn's Multiple Comparison test (P^2). IQR= interquartile range, NS = non-significant (P>0.05).					

Table E10. Intracellular expression of anti-apoptotic markers by study participant group

	Lymphocyte subset	Control (N=5)	Moderate (N=13)	Severe(N=7)	P^1	P^2
Bcl-2	**CD4+ T-cells**	6.1 (5.5-7.8)	7.9 (5.9-9.6)	8.8 (8.2-10.8)	0.03	NS
	CD8+ T-cells	3.8 (2.6-5.4)	4.8 (2.9-6.6)	5.7 (5.2-7.2)	0.08	NS
	NK cells	2.6 (2.1-2.8)	2.6 (2.3-3.3)	2.4 (2.2-3.1)	0.77	NS
Bcl-xL	**CD4+ T-cells**	9.1 (7.8-9.2)	15.1 (11.1-16.6)	15.7 (12.3-18.8)	0.007	NS
	CD8+ T-cells	4.9 (2.7-6.4)	6.7 (5.2-10.3)	8.5 (7.7-10.8)	0.03	NS
	NK cells	8.4 (7.5-9.5)	15.0 (12.4-16.8)	15.9 (14.0-18.1)	0.003	NS

Values expressed as median (interquartile range) of mean fold increase in Mean Fluorescence intensity. Groups compared using Kruskal-Wallis Rank Sum test (P^1). Post-test comparison of moderate and severe bronchiolitis groups using Dunn's Multiple Comparison test (P^2). IQR= interquartile range, NS = non-significant ($P > 0.05$).