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Transcriptome of airway neutrophils reveals an interferon response in life-threatening respiratory syncytial virus infection

Sjanna B. Besteman[a,b], Amie Callaghan[b], Annefleur C. Langedijk[a], Marije P. Hennus[c], Linde Meyaard[b,d], Michal Mokry[e], Louis J. Bont[a,b], Jorg J.A. Calis[b,e,*]

[a] Department of Paediatrics, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
[b] Center for Translational Immunology, University Medical Centre Utrecht, Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
[c] Department of Paediatric Intensive Care, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
[d] Oncode Institute, University Medical Centre Utrecht, Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands
[e] Department of cardiology, University Medical Centre Utrecht, Utrecht, Lundlaan 6, 3584 EA Utrecht, the Netherlands

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ABSTRACT

Background: Neutrophils are the most abundant cell type infiltrating the airways during severe respiratory syncytial virus (RSV) infection. Their exact role in disease pathophysiology remains enigmatic. Therefore, we determined genome-wide RNA expression profiles of local and systemic neutrophils in RSV bronchiolitis to provide further insight into local neutrophil biology.

Methods: We performed a single-center analysis, in 16 infants, admitted to the pediatric intensive care unit with severe RSV bronchiolitis. Neutrophils were isolated from blood and tracheobronchial aspirates (sputum). After low input RNA sequencing, differential expression of genes was determined followed by gene set analysis.

Results: Paired transcriptomic analysis of airway versus blood neutrophils showed an inflammatory phenotype, characterized by NF-kB signaling and upregulated expression of IL-6 and interferon pathways. We observed distinct expression of neutrophil activation genes (TNFSF13B, FCER1G).

Discussion: Our data indicate that airway neutrophils regulate their function at the transcriptional level in response to viral infection. It also suggests that local interferon drives the neutrophil response of severe RSV bronchiolitis.

1. Introduction

Every year 3.2 million infants with respiratory syncytial virus (RSV) infection are admitted to the hospital, of which 2–12% is transferred to the pediatric intensive care unit (PICU) [1,2]. Additionally, RSV infection has a significant long-term impact on the use of healthcare-resources and coinciding costs [3]. Besides passive immunization, active immunization or effective treatment options are lacking. Although there are some vaccines on the horizon, there is still a need to gain better insight in disease pathophysiology to reveal novel treatment targets, and aid vaccine development [4].

The initial response to RSV is characterized by the production of chemokines and cytokines, by respiratory epithelial cells and lung resident macrophages [5,6]. As a result, neutrophils are recruited and comprise up to 80% of the immune cells in the airways of infected infants [7]. Despite numerous studies evaluating neutrophils in RSV disease, the molecular mechanisms behind neutrophil migration and activation during RSV are poorly understood, and their exact role in disease pathology is unknown [8–12]. Especially data on local infiltrated neutrophils are scarce, as samples from these patients are difficult to obtain, and working with neutrophils in general is difficult.

Previous transcriptomic studies in RSV patients used bulk peripheral blood mononuclear cells (PBMCs) or whole blood samples [13–17], and only few included cells from local tissue (nasal scrapings) [16,18]. All of those studies showed upregulation of neutrophil and innate immune genes, especially interferon response genes. The latter is of interest

Abbreviations: B, blood; Ctrl, control; $^{Ctrl\text{-}B}$PMNs, blood-PMNs of control patients; $^{Ctrl\text{-}S}$PMNs, sputum-PMNs of control patients; DEG, differentially expressed gene; FDR, false discovery rate; GSA, gene set analysis; IFN, interferon; ISG, interferon-stimulated gene; NET, neutrophils extracellular trap; PBMC, peripheral blood mononuclear cell; PICU, pediatric intensive care unit; PMN, polymorphonuclear cells; ROS, reactive oxygen species; RSV, respiratory syncytial virus; $^{RSV\text{-}B}$PMNs, blood-PMNs of RSV patients; $^{RSV\text{-}S}$PMNs, sputum-PMNs of RSV patients; S, sputum.

* Corresponding author at: Center for Translational Immunology/ University Medical Centre Utrecht, Room number KC02.085.2, Lundlaan 6, 3584 EA, Utrecht, the Netherlands.

E-mail address: j.j.a.calis@umcutrecht.nl (J.J.A. Calis).

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because RSV has previously been defined as a poor inducer of IFN [19]. It is unknown how well these studies represent the transcriptional response of the local tissue, i.e. the lower airways, and of individual cell types such as neutrophils. Recent studies have shown that neutrophils can modify their transcriptional profile depending on inflammatory stimuli or in response to treatment [20–24]. Therefore, we set up a study to identify paired lower airway- and blood-derived neutrophil responses of RSV patients.

To gain further insight into local neutrophils biology we characterized the transcriptomic profile of freshly sorted neutrophils from the blood and airways of infants undergoing mechanical ventilation for severe RSV infection (primary aim). Secondary, we compared this to the transcriptome of blood- and airway-derived neutrophils of patients without pulmonary infection. These unique samples allowed us to identify key biological processes involved in the function of local neutrophils during severe RSV infection in infants.

2. Methods

2.1. Study population and samples

Peripheral blood and/or trachea bronchial aspirate (this will be referred to as sputum) was obtained from 16 infants (< 24 months old) hospitalized for severe RSV bronchiolitis in the Wilhelmina Children's Hospital in the Netherlands (primary aim) [25]. Diagnosis of RSV bronchiolitis was confirmed by PCR analysis. RSV disease severity was determined as PICU admission for invasive (14 patients) or non-invasive (2 patients) mechanical ventilation (Table 1). Since we did not have access to airway samples of healthy infants, because they are not intubated, we used infants without signs of a pulmonary infection, that were undergoing surgery, as a control group (secondary aim). This control group consisted of patients (< 24 moths), without respiratory infection, undergoing surgery, such as abdominal cyst removal or correction of a heart defect. Control patients with a (suspected) pulmonary bacterial or viral infection at the time of inclusion were excluded. Pulmonary bacterial infection was defined as treatment with antibiotics for a suspected pulmonary infection diagnosed by a pediatrician, based on clinical symptoms and either CRP > 60, or positive sputum culture. All but one of the RSV patients, and all of the controls patients were sampled within 48 h after admission. The study was approved by the Medical ethical review board (NL58404.041.16).

Table 1
Characteristics of the study population.

	Respiratory syncytial virus	Controls*	*P*-value
Number of participants, *n*	16	12	–
- Blood samples for RNAseq, *n*	10	11	
- Sputum samples for RNAseq, *n*	13	7	
- Paired samples, *n*	7	6	
Female, *n* (%)	6 (37.5)	7 (58.3)	0.274
Age at recruitment, days, mean (sd)	58.6 (+69.4)	190 (+187)	**0.017**
- Neonate (< 30 days), *n* (%)	7 (43.8)	3 (25)	0.306
RSV subtype A/B$, *n*	3/10	NA	–
Day since start of symptoms, mean (sd)	7 (+2.45)	NA	
Comorbidities, *n*	6	2	–
- Prematurity, *n*	3	1	
- Dysmaturity, *n*	1	0	
- Atopy (eczema, BHR), *n*	2	1	
Pulmonary bacterial infection < 24 h within sample collection, *n* (%)†	6 (37.5)	1 (8.3)	0.078
Intubation, *n* (%)	14 (87.5)	12 (100)	0.492§
Total duration of mechanical ventilation during admission, days, mean (sd)	9 (+6.9)	1.4 (+3.7)	**0.002**

Definition of abbreviations: sd = standard deviation, BHR: bronchial hyper responsiveness.
The *P* value is derived from comparison of the two groups using a chi-square test or §Fisher exact test for the categorical data. A parametric *t*-test was used to calculate the P value for all other data. $P < 0.05$ was deemed significant (in bold).
General surgery: Abdominal cyst removal (2×), Duhamel operation, inguinal hernia. Cardiac surgery: Coarctatio aortae, atrial septal defect, Tetralogy of Fallot, mitral valve replacement, ventricular septal defect, hypoplastic left heart syndrome. Other: Button cell battery ingestion, vascular ring.
* Aetiology of diseases in control group:
$ for 3 samples subtyping was not performed.
† Pulmonary infection was defined as CRP > 60, or positive sputum culture, and requiring antibiotics.

2.2. Purification of polymorphonuclear cells (PMNs)

Whole blood samples were depleted of red blood cells. Tracheobronchial aspirates were obtained by a long suction catheter through an endotracheal tube and filtered using a 70 μm nylon filter (BD Falcon). Neutrophils were sorted by flow cytometry using monoclonal antibodies. Neutrophils were selected based on $CD66b^+/CD14^-$ staining. (Fig. 1B). During RSV there is an influx of CD16 dim (progenitor) neutrophils to the airways [26]. Therefore, we used CD66b and not CD16 in our sorting panel. Despite the use of CD66b we identified many progenitor neutrophils (characterized by myelocytes and metamyelocytes) in the sorted sputum samples (Supplementary Fig. 1B). Throughout the manuscript we will refer to the population of CD66b +/CD14- cells, including the different subsets (progenitors as well as mature neutrophils), as neutrophils. Analysis of microscopy images revealed a neutrophil purity of > 97% (Supplementary Fig. 1B). In 23 out of 24 samples cell viability was > 92% (Supplementary Fig. 1C). Moreover, neutrophils were handled with great caution during the sorting procedure by avoiding temperature alterations and sorting at a 45 degree angle into medium.

2.3. Pre-processing and exploratory data analysis

Read counts per gene, per sample, were analyzed for global expression differences using R (version 3.5.3). TMM-normalized counts were used to assess global transcriptional profile differences of all samples by Principal Component Analysis (PCA) (10 components) and Multi Dimensional Scaling (MDS) (2 components).

2.4. Differential expression analysis

We sought to identify the transcriptome of airway neutrophils of RSV patients (primary aim), and to compare this to control patients (secondary aim). Differential expression analysis was performed with the voom-limma packages (version 3.38.3) in R (version 3.5.3). The samples were grouped as follows: Sputum-PMNs and Blood-PMNs of RSV patients ($^{RSV\text{-}S}$PMNs and $^{RSV\text{-}B}$PMNs), and Sputum-PMNs and Blood-PMNs of control patients ($^{Ctrl\text{-}S}$PMNs and $^{Ctrl\text{-}B}$PMNs). We determined differential gene expression for 5 contrasts: (1) $^{RSV\text{-}S}$PMNs versus $^{RSV\text{-}B}$PMNs; (2) $^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs; (3) $^{RSV\text{-}B}$PMNs versus $^{Ctrl\text{-}B}$PMNs; (4) $^{RSV\text{-}S}$PMNs versus $^{Ctrl\text{-}S}$PMNs; (5) ($^{RSV\text{-}S}$PMNs versus $^{RSV\text{-}B}$PMNs) versus ($^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs).

Differential expression analysis for each contrast was performed with the eBayes functionality of the limma package [27]. As a cutoff for differential expression we used a log2 Fold Change (FC) > 1, and an adjusted *p*-value (p_{adj}) < 0.05.Gene set enrichment analysis was performed for each contrast with CAMERA [28]. False Discovery Rates (FDR), to adjust for multiple testing, were determined using the Benjamini-Hochberg method. [29] FDR < 0.05 were considered significant.

A significant difference in age was observed between control and RSV patients (Table 1), and neonatal status (age < 30 days) was

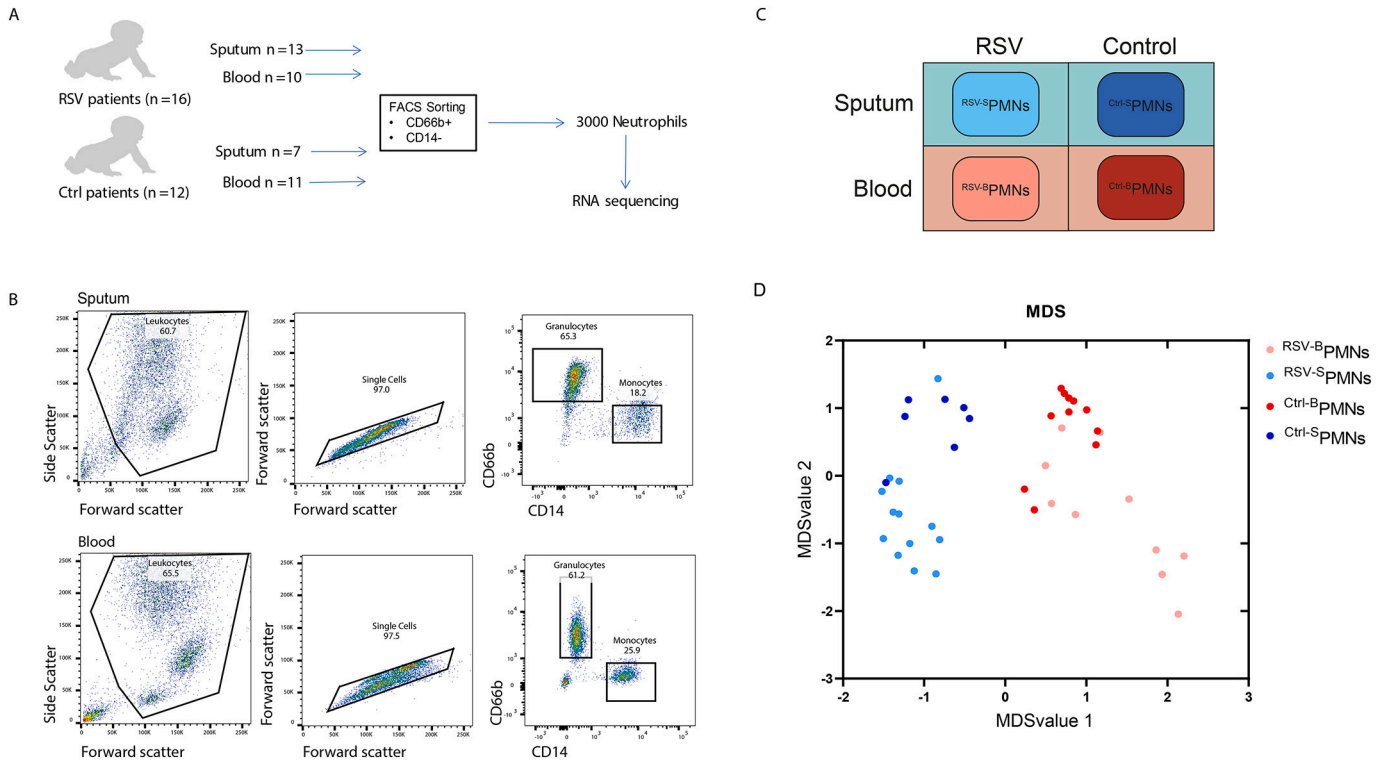

Fig. 1. Differential gene expression profiles in Sputum-PMNs and Blood-PMNs in children with and without severe RSV bronchiolitis. (A) Study design: peripheral blood and sputum were sampled from RSV (n = 16) and control (n = 12) infants admitted to the hospital. Sputum was filtered twice, and erythrocytes in whole blood samples were lysed before staining for surface markers. Cells were identified based on their characteristic forward and side scatter properties, single cells were subsequently selected and neutrophils were identified as CD66b$^+$ and CD14$^-$. Per sample, 3000 neutrophils were isolated per sample group, and used for transcriptomic analysis. (B) Flow cytometry figures showing the sorting strategy of sputum and blood samples, a representative patient is shown. (C) Schematic illustration of the four sample groups: Sputum-PMNs of RSV patients ($^{RSV\text{-}S}$PMNs), Sputum-PMNs of control patients ($^{Ctrl\text{-}S}$PMNs), Blood-PMNs of RSV patients ($^{Ctrl\text{-}B}$PMNs), and Blood-PMN of Control patients ($^{RSV\text{-}B}$PMNs). (D) Multi dimensional scaling (MDS) based on the transcriptomic profile of each PMN sample. Blue circles identify $^{Ctrl\text{-}S}$PMNs, red circles identify $^{RSV\text{-}S}$PMNs. Light blue circles identify $^{Ctrl\text{-}B}$PMNs, and pink circles identify $^{RSV\text{-}B}$PMNs. Ctrl = control patient, RSV = Respiratory Syncytial Virus patient, B = blood, S = sputum, PMN = polymorphonuclear cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified as a co-factor that explained transcriptional variation in the 4th PC (Supplementary Table 1). Therefore, transcriptomic analyses were corrected for neonatal status in gene expression modeling (main analysis). Alternative analysis were implemented to check for the robustness of presented results with respect to age: first, by including age as a linear variable; second, by including only samples from the youngest patients (age < 90 days), such that there was no significant difference in age between the control and RSV patients (37 versus 27 days old p = 0.367). Results from the main analysis are presented throughout the manuscript, and were always consistent with results from the alternative analyses.

Additional information can be found in the online supplement.

3. Results

3.1. Study population and sample characteristics

We recruited 16 infants (mean age 58.6 days) with severe RSV bronchiolitis who were mechanically ventilated for an average of 9 days. Six (38%) patients had a routine care diagnosis of bacterial infection within 24 h after sampling. We recruited 12 controls (secondary aim) without respiratory infection. Controls had a higher mean age (190 days, p = 0.017) and tended to be ventilated for only 1.4 days (p = 0.002). Blood and sputum samples were obtained from each patient, and purified neutrophils were derived consistently from each tissue sample by FACS sorting of CD66b$^+$/CD14$^-$ cells (Fig. 1A-B). Baseline characteristics are outlined in Table 1.

3.2. Distinct transcriptional profile in neutrophils based on tissue-of-origin and RSV bronchiolitis

To identify the molecular mechanisms involved in RSV disease pathology, we performed transcriptomic profiling in four groups of samples: $^{RSV\text{-}B}$PMNs, $^{RSV\text{-}S}$PMNs, $^{Ctrl\text{-}S}$PMNs and $^{Ctrl\text{-}B}$PMNs (Fig. 1C). Tissue-of-origin (primary aim) explained a large part of the transcriptional variation, as measured by strong associations with MDS dimensions and principal components (PC) (Fig. 1D and Supplementary Table 1). To a lesser extent, RSV status (secondary aim) also explained a substantial part of transcriptional variation. Most samples clustered with their group (Fig. 1D). Sex or the presence of a bacterial pulmonary infection did not associate with any of the PCs, and these characteristics were distributed evenly among the samples in the MDS plot (Supplementary Fig. 2). Neonatal status was identified as a co-factor that explained transcriptional variation in the 4th PC (Supplementary Table 1). Subsequent transcriptomic analyses were therefore corrected for neonatal status (see Methods). Overall, our data showed four distinct transcriptomic profiles in neutrophils, correlating independently to the tissue-of-origin (blood or sputum), and RSV infection status.

3.3. Airway neutrophils are characterized by upregulation of IL-6 and IFN signaling pathways during RSV infection

To identify how the transcriptional profile of sputum neutrophils is different from those in blood, we analyzed two contrasts: $^{RSV\text{-}S}$PMNs versus $^{RSV\text{-}B}$PMNs; and $^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs. In the first contrast 1476 differentially expressed genes (DEGs) were upregulated and 2110

DEGs were downregulated (Fig. 2A). Upregulated DEGs included genes related to transcription (CREM), neutrophil function (ICAM1, IL8, CCRL2), and TNF signaling (TNFAIP3). Downregulated genes were related to transcription (MAZ, ZNF770), tumor-suppression and cell cycle (TSPAN32, HIST1H4C) (Supplementary Table 2). The second contrast ($^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs) offers an unobstructed view on the difference between sputum and blood neutrophils, without the influence of a severe RSV infection. In this contrast fewer genes were differentially expressed: 431 upregulated and 677 downregulated DEGs were found (Fig. 2B). The top ten overexpressed genes in $^{Ctrl\text{-}S}$PMNs included genes related to neutrophil function (ICAM-1), transcription and translation activity (IVNS1ABP,CREM). The top downregulated genes were related to RNA binding (CELF2) and immune signaling (MKRN1, IL16) (Supplementary Table 2). When comparing DEGs of both contrasts we found that 76% (328 out of 431) of upregulated DEGs in control samples, were also upregulated in the RSV patient samples (Fig. 2C). Also differential gene set analysis (GSA) revealed similarity in enrichment between both contrasts, namely: inflammatory response and NF-kB signaling. However, only in RSV patient samples did we see an upregulation of IL-6 and IFN response gene sets (Fig. 2D). Gene sets related to transcription and cell cycle metabolism were downregulated in RSV airway neutrophils, while in controls we found downregulation of pathways associated with intracellular signaling and B-cell signaling (Fig. 2D and E, Supplementary Table 3). These data demonstrate an inflammatory phenotype, characterized by NF-kB signaling, in airway infiltrated neutrophils, independent of RSV status. During RSV infection IL-6 and IFN based responses are upregulated.

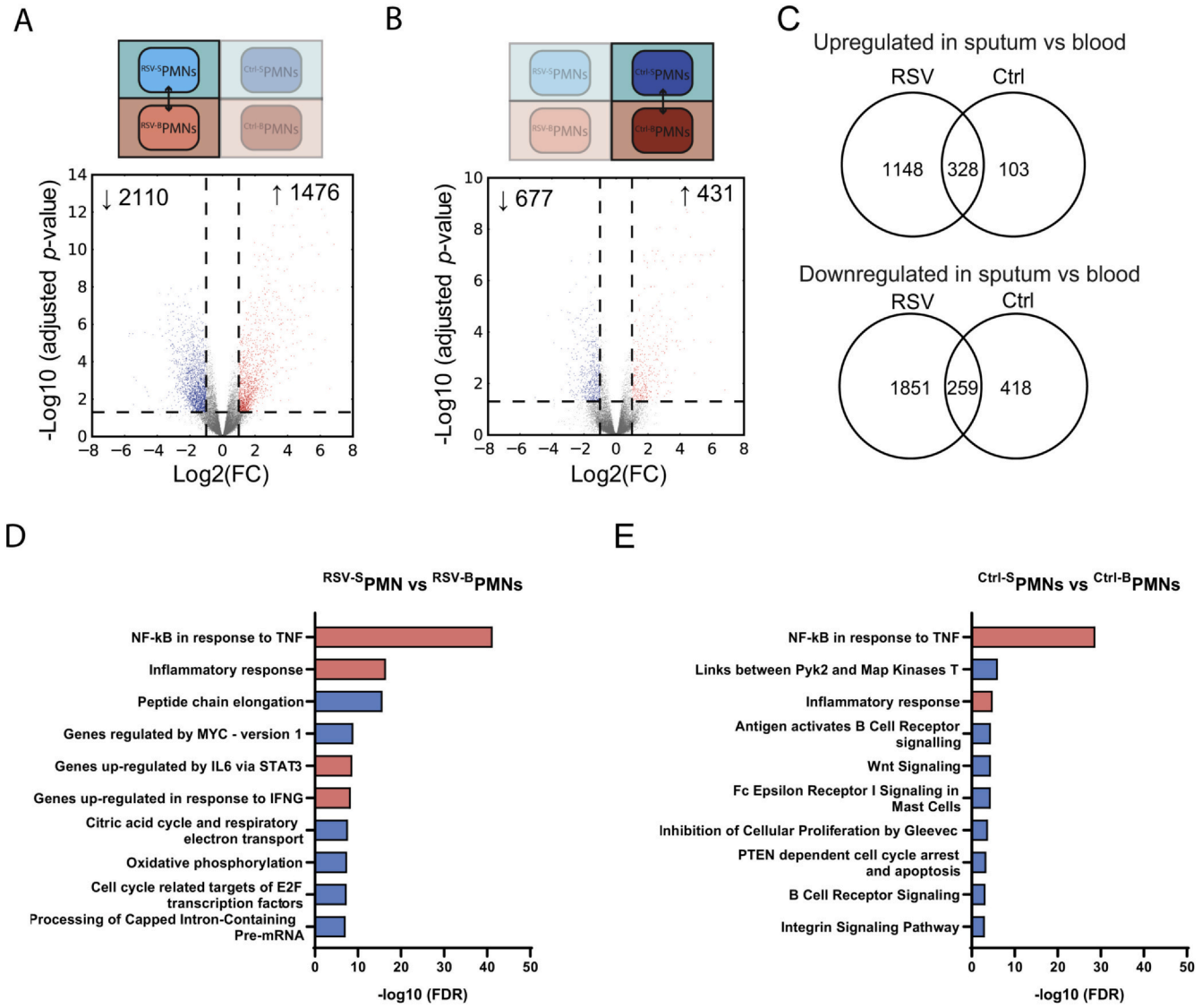

Fig. 2. Distinct local neutrophil responses in the lungs of infants with and without severe RSV bronchiolitis. (A) Volcano plot showing DEGs in $^{RSV\text{-}S}$PMNs compared to $^{RSV\text{-}B}$PMNs, upregulated genes (red) and downregulated genes (blue) are marked. (B) Volcano plot showing DEGs in $^{Ctrl\text{-}S}$PMNs compared to $^{Ctrl\text{-}B}$PMNs, upregulated genes (red) and downregulated genes (blue) are marked. (C) Venn diagram showing DEGs in $^{Ctrl\text{-}S}$PMNs vs $^{Ctrl\text{-}B}$PMNs (right circle) and $^{RSV\text{-}S}$PMNs vs $^{RSV\text{-}B}$PMNs (left circle). Top panel: upregulated genes. Bottom panel: downregulated genes. (D) Top 10 most significant gene sets enriched in $^{RSV\text{-}S}$PMNs compared to $^{RSV\text{-}B}$PMNs, upregulated gene sets (red) and downregulated gene sets (blue) are marked. (E) Top 10 most significant gene sets enriched in $^{Ctrl\text{-}S}$PMNs, upregulated gene sets (red) and downregulated gene sets (blue) are marked.
Benjamini-Hochberg adjusted FDR values < 0.05 were deemed significant. Ctrl = control patient, RSV = Respiratory Syncytial Virus patient, B = blood, S = sputum, PMN = polymorphonuclear cells, FDR = false discovery rate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Infant blood neutrophils respond to severe RSV infection by upregulation of genes related to cell adhesion, transcription, and translation

To delineate RSV specific alterations in the transcriptional response of blood neutrophils we contrasted $^{RSV\text{-}B}$PMNs with $^{Ctrl\text{-}B}$PMNs (Fig. 3A). Top ten most significantly DEGs, included upregulation of genes involved in cell adhesion (ADAM15), innate immunity (DNASE1L1), and transcription (TK2, DUS1L, ZNF770). Downregulated genes were associated with the inflammatory response (PELI1, TREM1, TLR4) (Supplementary Table 2). The GSA was in line with the DEG analysis (Supplementary Table 3). The top ten gene sets were all upregulated, and indicated increased transcription and translation activity (Fig. 3B). Downregulated pathways, included GPCR ligand binding (p_{adj} = 0.007), and chemokine signaling (p_{adj} = 0.009, Supplementary Fig. 3). Analysis and identification of the expression of genes in the chemokine signaling gene set showed that this response was only downregulated in a subset of the $^{RSV\text{-}B}$PMN samples (Supplementary Fig. 4). In conclusion, blood neutrophils of RSV-infected infants showed upregulation of genes involved in cell adhesion, metabolism, and transcription. To a smaller degree, immune related pathways were downregulated.

3.5. Airway infiltrated neutrophils are characterized by an interferon signature

We assumed that the transcriptome of airway neutrophil of RSV patients was defined by RSV status and tissue localization. Therefore, we evaluated the transcriptional response to RSV infection by local neutrophils using two contrasts: first a contrast of $^{RSV\text{-}S}$PMNs versus $^{Ctrl\text{-}S}$PMNs; second, a 2-way contrast in which the transcriptomic response to RSV in sputum neutrophils is corrected for the response in blood neutrophils: ($^{RSV\text{-}S}$PMNs versus $^{RSV\text{-}B}$PMNs) versus ($^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs).

The first contrast identified 399 up- and 45 downregulated DEGs (Fig. 4A). The top ten overexpressed genes were related to neutrophil degranulation (STXBP2, GRN, DNASE1L1). The top ten downregulated genes were related to cell-cell interaction (EMR3), the complement pathway (C1QB) and translation (FAM86B1) (Supplementary Table 2). Differential GSA revealed an increase in pathways associated with the IFN signaling response, protein secretion, cytokine signaling and migration. Pathways related to complement and IL-5 signaling, although outside the top 10 of DEG sets, were significantly downregulated (p_{adj} = 0.011 and p_{adj} = 0.045, Supplementary Table 3). To identify genes that are only differentially expressed in local neutrophils upon RSV infection, we analyzed differential expression using the second contrast (($^{RSV\text{-}S}$PMNs versus $^{RSV\text{-}B}$PMNs) versus ($^{Ctrl\text{-}S}$PMNs versus $^{Ctrl\text{-}B}$PMNs)). Even though the power in this analysis was lower, we found six upregulated and seven DEGs downregulated. Upregulated genes were associated with cell metabolism (SLC5A9), cell adhesion (cytohesin 1), and immune response (TLR4) (Supplementary Table 2). GSA along the second contrast confirmed a distinct immune response to RSV in local neutrophils. We observed enrichment in pathways associated with the response to interferon, IL-6 and NF-kB signaling (Fig. 4C), and increased expression of most genes that are part of the type 1 IFN response gene set (Fig. 4D). This response was largest observed in $^{RSV\text{-}S}$PMNs samples (Fig. 5). qPCR analysis on a limited number of available samples and genes, i.e. IFIT1 and CCRL2, confirmed these findings (Supplementary Fig. 5). Altogether, we show a strong age-independent IFN signature by airway infiltrated neutrophils of life-threatening RSV infected patients.

3.6. Neutrophils show enrichment of genes associated with activation in blood and sputum of RSV patients

Interferon is known to induce and amplify the formation of neutrophils extracellular traps (NETs) and production of reactive oxygen

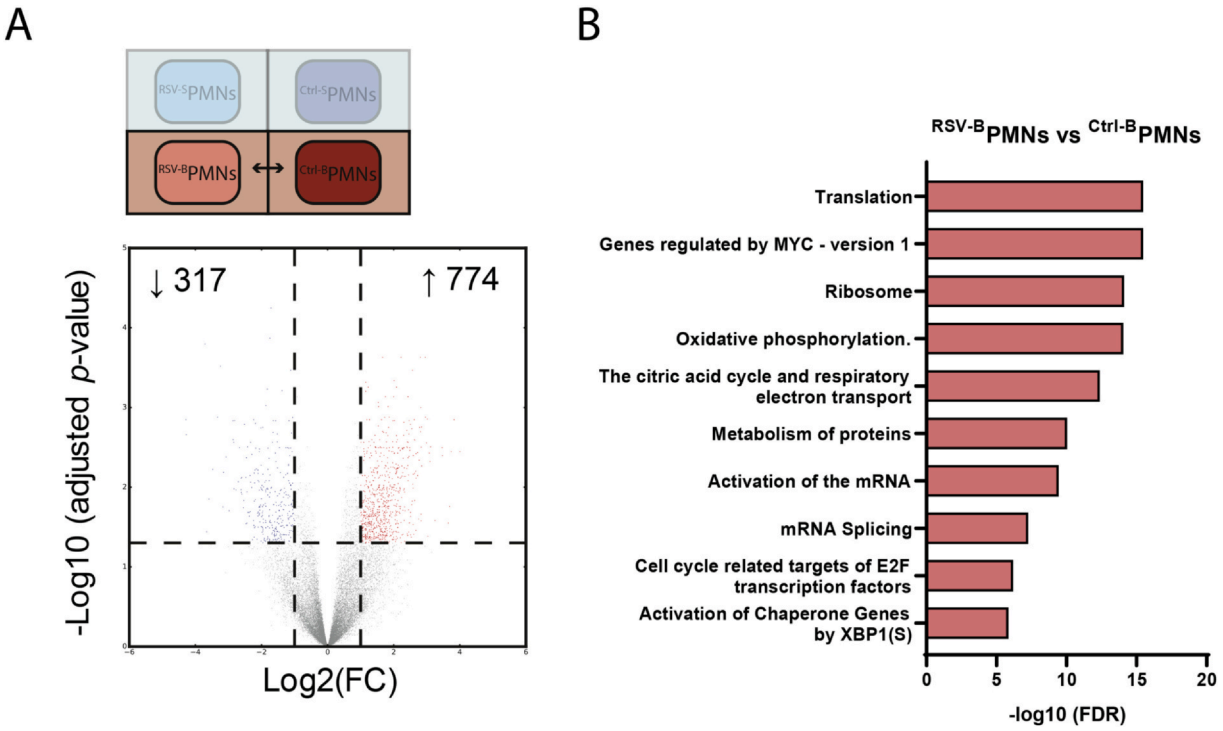

Fig. 3. Distinct systemic neutrophil responses in infants with and without severe RSV bronchiolitis. Blood-PMNs from control and RSV infants were sampled. (A) Volcano plot showing DEGs in $^{RSV\text{-}B}$PMNs compared to $^{Ctrl\text{-}B}$PMNs, upregulated genes (red) and downregulated genes (blue) are marked. (B) Top 10 most significant enriched gene sets (all upregulated) in $^{RSV\text{-}B}$PMNs compared to $^{Ctrl\text{-}B}$PMNs. Benjamini-Hochberg adjusted *P* values < 0.05 were classified as significant.
Ctrl = control patient, RSV = Respiratory Syncytial Virus patient, B = blood, S = sputum, PMN = polymorphonuclear cells, FDR = false discovery rate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

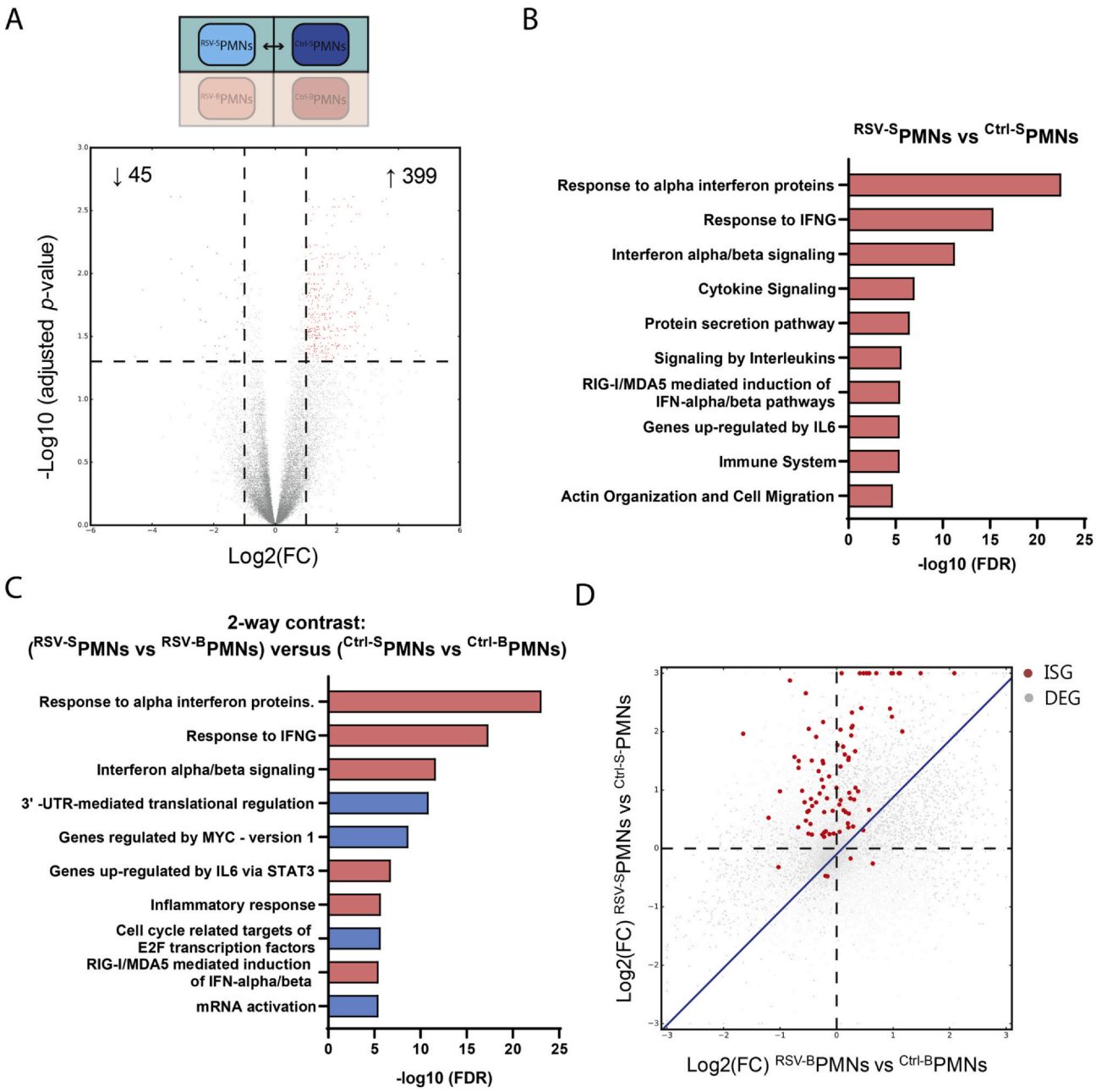

Fig. 4. Transcriptomic analysis of $^{RSV\text{-}S}$PMNs compared to $^{Ctrl\text{-}S}$PMNs. (A) Volcano plot showing DEGs in $^{RSV\text{-}S}$PMNs, compared to $^{Ctrl\text{-}S}$PMNs, upregulated genes (red) and downregulated genes (blue). (B) Top 10 most significant enriched gene sets in $^{RSV\text{-}S}$PMNs, compared to $^{Ctrl\text{-}S}$PMNs, upregulated gene sets (red) and downregulated gene sets (blue) are marked. (C) Top 10 enriched gene sets in in $^{RSV\text{-}S}$PMNs, compared to $^{Ctrl\text{-}S}$PMNs, after correcting for expression of DEGs in blood (2-way contrast), upregulated gene sets (red) and downregulated gene sets (blue) are marked. (D) Plot showing all DEGs (grey dots) and their Log2(FC) for $^{RSV\text{-}S}$PMNs vs $^{Ctrl\text{-}S}$PMNs (y-axis) and $^{RSV\text{-}B}$PMNs vs $^{Ctrl\text{-}B}$PMNs (x-axis). Genes annotated in the gene set for IFNa (HALLMARK) are depicted in red.
Ctrl = control patient, RSV = Respiratory Syncytial Virus patient, B = blood, S = sputum, PMN = polymorphonuclear cells, FDR = false discovery rate, ISG = interferon-stimulated gene. DEG = differentially expressed gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species (ROS) by neutrophils [21,30,31]. We and others have previously shown enhanced migration, NETosis and ROS production by sputum-derived neutrophils of RSV bronchiolitis patients [10,11,32–34]. We analyzed the expression of a group of genes that are associated with neutrophil activation (GO: 0042119). The expression of this gene set was significantly upregulated in the airways of RSV infants compared to controls (p_{adj} = 0.0002). Within this gene set we identified an increased intensity of neutrophil activation genes, mainly related to degranulation (STXBP2, STXBP3 and VAMP2), in both $^{RSV\text{-}B}$PMNs and $^{RSV\text{-}S}$PMNs compared to controls (Fig. 6 top panel). Next, we selected a panel of genes associated with viral protection such as IL-18, TNFSF13B (also known as B-cell activating factor) and MMP9, or neutrophil induced lung injury, including myeloperoxidase (MPO), neutrophil elastase (ELANE), and Azurocidin-1 (AZU1) [8,35]. During severe RSV infection this module was not significantly differentially expressed in airway neutrophils, while it was in blood neutrophils (p_{adj} 0.026, Fig. 6 bottom panel). Most genes in this gene set were downregulated in $^{RSV\text{-}S}$PMNs except for four genes: PTX3, TNFSF13B, IL-18 and MMP9. Altogether, we show that neutrophils upregulate a specific set of neutrophil activation genes, dependent on their localization

Fig. 5. Heat map showing the expression of genes stimulated by IFNa (HALLMARK) in each sample. Gene expression levels are shown as row normalized Z scores with red representing higher expression and blue representing lower expression. Benjamini-Hochberg adjusted P values < 0.05 were classified as significant. See Supplementary Fig. 7 for a larger version of the heat map.
Ctrl = control patient, RSV = Respiratory Syncytial Virus patient, B = blood, S = sputum, PMN = polymorphonuclear cells, FDR = false discovery rate, ISG = interferon-stimulated gene. DEG = differentially expressed gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during RSV infection. We confirm that gene expression profiles by blood neutrophils do not accurately reflect the transcriptomic profile of neutrophils in the airways.

4. Discussion

An incomplete understanding of the host immune response to RSV hinders the development of future therapies and vaccines. Neutrophils are implicated in disease pathology, but their exact role remains enigmatic [8]. Here, we aimed to identify key-biological processes that control airway infiltrated neutrophil function in severe RSV bronchiolitis, by transcriptomic analysis of freshly isolated neutrophils from blood and lungs.

Our study builds on recent studies that showed upregulation of neutrophil response genes in blood of RSV infected infants [15–18,36,37]. Therefore, we have examined the neutrophil response directly, identified their biological response mechanisms, and extended this analysis to the lungs. Paired transcriptomic analysis of airway and blood neutrophils from RSV patients showed that pathways associated with IL-6 and IFN gamma were upregulated. Comparison of airway

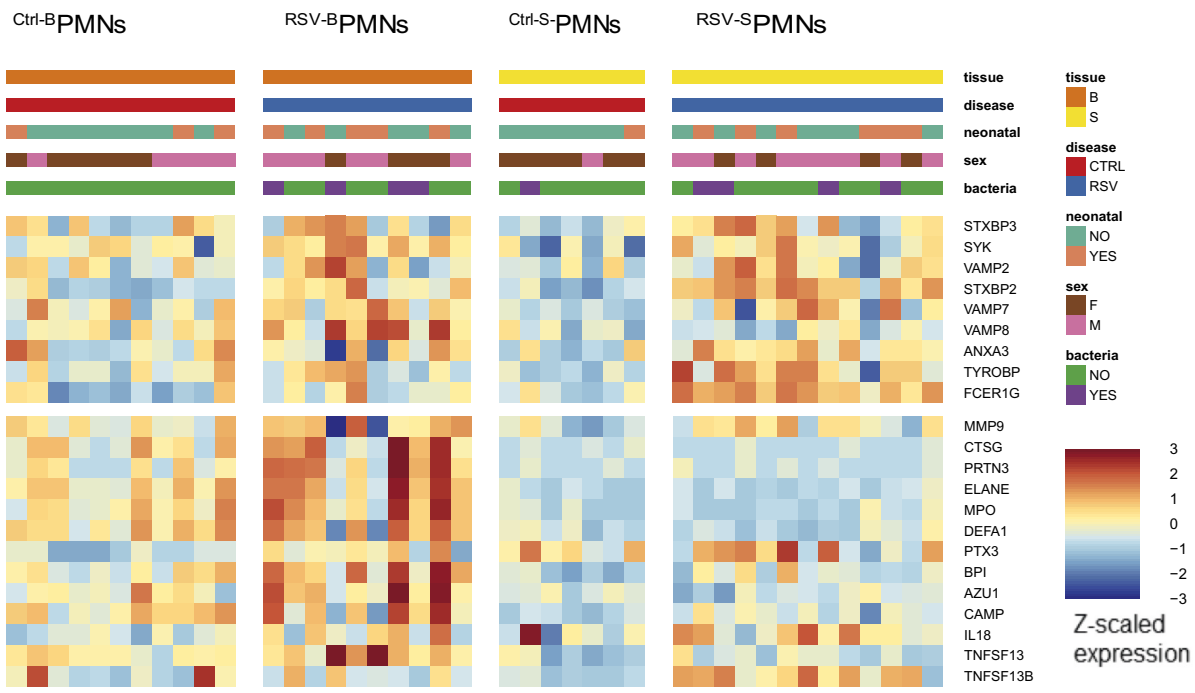

Fig. 6. Transcriptomic analysis of systemic and local neutrophil activation genes in infants with and without severe RSV bronchiolitis. Heat map showing the gene expression in all samples of genes involved in neutrophil activation (GO database (top), combined with a custom list (bottom). Gene expression levels are shown as row normalized Z scores with red representing higher expression and blue representing lower expression.
Ctrl = control patient, RSV = Respiratory Syncytial virus patient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neutrophils from RSV patients to those from control patients, revealed a strong, age independent, IFN response indicating that airway neutrophils of infants with severe RSV alter their gene expression profile in response to virus-induced IFNs.

The transcriptomic response by neutrophils to IFN in the lungs of infants with severe RSV disease prompts the question to what extent this is a protective or immunopathological response. Determining the type of IFN response (eg. type 1 or 2) based on RNA sequencing data is hard, as the same IFN stimulated genes (ISGs) are upregulated in response to both IFN 1 and 2. Type 1 IFNs are produced by among others dendritic cells and epithelial cells, and modulate several immune functions, including neutrophil recruitment and activation, and play a crucial role in anti-viral immunity [21,35,38]. Additionally, numerous studies indicated pro-inflammatory features of the type 1 IFN response in pulmonary infections, such as Influenza, tuberculosis and *S. pneumoniae* infection [16,39–43]. Data on the role of type 1 IFN in the pathogenesis of RSV are conflicting. In order to be protective the timing of this response is important. For example, to have an antiviral affect, ISG15 has to accumulate in the cell prior to RSV infection [44], and in mice, IFNa administration decreased viral burden when administered before RSV infection [45]. In children with RSV, intramuscular administration of IFN-a-2a shortly after the start of symptoms, slightly decreased respiratory complaints, while it did not have an effect on viral shedding [46]. Additionally, IFN-alpha/beta receptor$^{-/-}$ mice showed a reduction of pro-inflammatory cytokines, but reduced disease severity after RSV infection, without an effect on viral replication [47,48]. The type 2 IFN, IFN-gamma, is produced by T cells and natural killer cells, and has been shown to be increased in the nose during moderate RSV cases compared to severe RSV cases, indicating a protective role [49]. Apparently, the timing, duration and type of IFN production is of importance in order to limit viral replication, while maintaining a balanced immune response, and preventing immunopathology. These studies and our data suggest that in the case of severe bronchiolitis, the local IFN response might not only be protective, but at least coincides with immunopathology.

Our data showed upregulation of specific genes associated with neutrophil transcription, translation and activation during severe RSV, with some interesting differences between tissues of origin. In particular, genes related to degranulation were upregulated in airway-derived neutrophils, while expression of genes encoding granular proteins was increased in blood (Fig. 6). This is interesting because gene expression patterns change during neutrophil differentiation and maturation [31]. Moreover, many neutrophil effector proteins are stored in intracellular granules that develop during maturation, the release of these proteins is not directly dependent on their transcription [35]. Several genes encoding NET localized proteins, such as elastase, MPO and Azurocidin 1, were enriched in blood and downregulated in sputum, which could be a result of NET-formation, and subsequent apoptosis, by airway infiltrated neutrophils. In line with this we found upregulation of apoptosis related gene sets in the sputum but not in the blood neutrophil samples of RSV patients (Supplementary Table 3). Thus, our data suggests that neutrophil activation commences in the blood, after which matured neutrophils migrate to the lungs where, in response to IFNs, degranulation and the process of NET-formation is activated.

In contrast with previous studies by Mejias et al. and Jones et al., we did not find an IFN response in the blood of patients with RSV bronchiolitis [16,17,50]. This might be related to the moment of sample collection, or differences in age or disease severity, which are both associated with suppression of IFN in blood [19,49,51]. Finally, we focused on the neutrophil specific response which might be distinct from the transcriptomic analysis of whole blood.

Our study has strengths. It is the first study identifying DEGs in freshly isolated sputum neutrophils from the airways of infants with RSV bronchiolitis, which comprises the primary site of infection. In addition, it is the first study comparing sputum neutrophils from children without pulmonary infections, to blood neutrophils, which reveals molecular mechanisms underlying the function and the presence of

neutrophils in the absence of pulmonary infection. Despite a modest patient cohort, we were able to identify clear gene expression differences between RSV patients and controls, by studying paired samples from both tissues.

Also limitations require discussion. First, our control patients are older than the RSV patients; as they were selected on requiring intubation as part of their treatment. For logistic reasons it was impossible to obtain airway material from healthy children, because they are not intubated. Even though control patients with an infection were excluded, underlying disease or age might have influenced the results. Nevertheless, modeling of age as a continuous factor or analysis including only samples from the youngest patients (age < 90 days), did not qualitatively alter the conclusions from the differential gene set expression analysis, indicating that our results are age independent. Second, as expected, a trend for RSV patients to more often show signs of bacterial pulmonary infections compared to controls was observed in our study [52]. Previous studies suggested that RSV is the dominant pathogen in determining the transcriptomic response during viral or bacterial co-infection [14,18]. While we were not able to confirm these findings for viral co-infection, because we did not attempt to test for viral co-infections, we could confirm that bacterial co-infection did not explain transcriptional variation in the first 10 PCs (Supplementary Table 1). Third, the sorting strategy used for selecting neutrophils did not exclude eosinophils (Supplementary Fig. 1). Eosinophils in sputum of RSV patients are reported to be less than 1% of total cells [7], compared to over 80% for neutrophils [7]. We identified that $< 1.5\%$ of total sputum cells considered eosinophils (Supplementary Fig. 6). A study using a similar sorting strategy on sputum samples with CD66b magnetic beads, reported a neutrophil purity of 99% [22]. This is in line with our analysis of microscopy images showing a neutrophil purity of $> 97\%$ (Supplementary Fig. 1). Also, in our dataset, we did not observe significant differential expression patterns for the eosinophil associated genes Siglec-8 and IL5RA, (Supplementary Table 2). Based on the literature, RNA sequencing data and small fraction of eosinophils in the granulocyte population, we referred to our CD66b selected cells as neutrophils. Fourth, migration to the lung, as well as RSV infection, influences the phenotype of neutrophils, giving rise to dynamic and heterogeneous neutrophil subsets in blood and lung [12,26,53]. In our study, sputum neutrophils of RSV patients showed diverse neutrophil subsets, including many progenitor neutrophils (Supplementary Fig. 1). In future studies, it would be interesting to address the transcriptomic response in different neutrophil subsets. Finally, because (therapeutic) interventions and repeated sampling in children are difficult to organize, we have to base our analysis on gene sets that are derived from previous studies and collected in the molecular signatures database (MSigDB). A limitation of this approach is that a possibly unknown pathway involved in RSV pathophysiology and neutrophil biology that would not be in the MSigDB, will also not be part of our analysis. Our data offer a novel insight into local neutrophil biology during RSV infection in infants. A caveat of this transcriptomics study is that differences in RNA expression are not necessarily translated to distinct protein profiles or neutrophil function. Future studies are needed to validate the functional relevance of our findings.

In summary, we studied neutrophil-specific gene expression profiles in the airways of RSV bronchiolitis patients. This study demonstrates that during severe RSV bronchiolitis, airway neutrophils displayed a distinct IFN signature, and showed overexpression of activation and degranulation genes. These results confirm that infants are capable of mounting an IFN response to RSV infection and that during severe RSV bronchiolitis this response results in a profound IFN signature in airway neutrophils. To develop therapeutic targets, we now need to address the harmful and protective aspects of the airway IFN response during RSV bronchiolitis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2020.108593.

Authors' contribution

SB, LB, MM, MH and JC designed the study. SB, AC, MH and AL included the patients. SB and AC performed the experiments. JC, SB, LM and LB analyzed the data and interpreted the results. All authors were involved in writing the paper and gave final approval of the submitted and published versions.

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References

[1] T. Shi, D.A. McAllister, K.L. O'Brien, E.A.F. Simoes, S.A. Madhi, B.D. Gessner, F.P. Polack, E. Balsells, S. Acacio, C. Aguayo, I. Alassani, A. Ali, M. Antonio, S. Awasthi, J.O. Awori, E. Azziz-Baumgartner, H.C. Baggett, V.L. Baillie, A. Balmaseda, A. Barahona, S. Basnet, Q. Bassat, W. Basualdo, G. Bigogo, L. Bont, R.F. Breiman, W.A. Brooks, S. Broor, N. Bruce, D. Bruden, P. Buchy, S. Campbell, P. Carosone-Link, M. Chadha, J. Chipeta, M. Chou, W. Clara, C. Cohen, E. de Cuellar, D.-A. Dang, B. Dash-yandag, M. Deloria-Knoll, M. Dherani, T. Eap, B.E. Ebruke, M. Echavarria, C.C. de Freitas Lázaro Emediato, R.A. Fasce, D.R. Feikin, L. Feng, A. Gentile, A. Gordon, D. Goswami, S. Goyet, M. Groome, N. Halasa, S. Hirve, N. Homaira, S.R.C. Howie, J. Jara, I. Jroundi, C.B. Kartasasmita, N. Khuri-Bulos, K.L. Kotloff, A. Krishnan, R. Libster, O. Lopez, M.G. Lucero, F. Lucion, S.P. Lupisan, D.N. Marcone, J.P. McCracken, M. Mejia, J.C. Moisi, J.M. Montgomery, D.P. Moore, C. Moraleda, J. Moyes, P. Munywoki, K. Mutyara, M.P. Nicol, D.J. Nokes, P. Nymadawa, M.T. da Costa Oliveira, H. Oshitani, N. Pandey, G. Paranhos-Baccalà, L.N. Phillips, V.S. Picot, M. Rahman, M. Rakoto-Andrianarivelo, Z.A. Rasmussen, B.A. Rath, A. Robinson, C. Romero, G. Russomando, V. Salimi, P. Sawatwong, N. Scheltema, B. Schweiger, J.A.G. Scott, P. Seidenberg, K. Shen, R. Singleton, V. Sotomayor, T.A. Strand, A. Sutanto, M. Sylla, M.D. Tapia, S. Thamthitiwat, E.D. Thomas, R. Tokarz, C. Turner, M. Venter, S. Waicharoen, J. Wang, W. Watthanaworawit, L.-M. Yoshida, H. Yu, H.J. Zar, H. Campbell, H. Nair, Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study, Lancet 390 (2017) 946–958.

[2] L. Bont, P.A. Checchia, B. Fauroux, J. Figueras-Aloy, P. Manzoni, B. Paes, E.A. Simoes, X. Carbonell-Estrany, Defining the epidemiology and burden of severe respiratory syncytial virus infection among infants and children in Western countries, Infect. Dis. Ther. 5 (2016) 271–298.

[3] E.A.F. Simoes, V. Chirikov, M. Botteman, Y. Kwon, A. Kuznik, Long-term assessment of healthcare utilization five years after respiratory syncytial virus infection in US infants, J. Infect. Dis. 221 (8) (2019) 1256–1270.

[4] N.I. Mazur, D. Higgins, M.C. Nunes, J.A. Melero, A.C. Langedijk, N. Horsley, U.J. Buchholz, P.J. Openshaw, J.S. McLellan, J.A. Englund, A. Mejias, R.A. Karron, E.A. Simoes, I. Knezevic, O. Ramilo, P.A. Piedra, H.Y. Chu, A.R. Falsey, H. Nair, L. Kragten-Tabatabaie, A. Greenough, E. Baraldi, N.G. Papadopoulos, J. Vekemans, F.P. Polack, M. Powell, A. Satav, E.E. Walsh, R.T. Stein, B.S. Graham, L.J. Bont, F. Respiratory Syncytial Virus Network, The respiratory syncytial virus vaccine landscape: lessons from the graveyard and promising candidates, Lancet Infect. Dis. 18 (2018) e295-e311.

[5] T.S. Hallstrand, T.L. Hackett, W.A. Altemeier, G. Matute-Bello, P.M. Hansbro, D.A. Knight, Airway epithelial regulation of pulmonary immune homeostasis and inflammation, Clin. Immunol. 151 (2014) 1–15.

[6] P.J.M. Openshaw, C. Chiu, F.J. Culley, C. Johansson, Protective and harmful immunity to RSV infection, Annu. Rev. Immunol. 35 (2017).

[7] P.S. McNamara, P. Ritson, A. Selby, C.A. Hart, R.L. Smyth, Bronchoalveolar lavage cellularity in infants with severe respiratory syncytial virus bronchiolitis, Arch. Dis. Child. 88 (2003) 922–926.

[8] R.J. Geerdink, J. Pillay, L. Meyaard, L. Bont, Neutrophils in respiratory syncytial virus infection: a target for asthma prevention, J. Allergy Clin. Immunol. 136

(2015) 838–847.
[9] K. Kumawat, R.J. Geerdink, M.P. Hennus, M.A. Roda, I. van Ark, T. Leusink-Muis, G. Folkerts, A. van Oort-Jansen, A. Mazharian, S.P. Watson, F.E. Coenjaerts, L. Bont, L. Meyaard, LAIR-1 limits neutrophilic airway inflammation, Front. Immunol. 10 (2019) 842.
[10] F.C.M. Kirsebom, F. Kausar, R. Nuriev, S. Makris, C. Johansson, Neutrophil recruitment and activation are differentially dependent on MyD88/TRIF and MAVS signaling during RSV infection, Mucosal Immunol. 12 (2019) 1244–1255.
[11] S.P. Muraro, G.F. De Souza, S.W. Gallo, B.K. Da Silva, S.D. De Oliveira, M.A.R. Vinolo, E.M. Saraiva, B.N. Porto, Respiratory syncytial virus induces the classical ROS-dependent NETosis through PAD-4 and necroptosis pathways activation, Sci. Rep. 8 (2018) 14166.
[12] M.V. Lukens, A.C. van de Pol, F.E. Coenjaerts, N.J. Jansen, V.M. Kamp, J.L. Kimpen, J.W. Rossen, L.H. Ulfman, C.E. Tacke, M.C. Viveen, L. Koenderman, T.F. Wolfs, G.M. van Bleek, A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants, J. Virol. 84 (2010) 2374–2383.
[13] V.L. Jong, I.M. Ahout, H.J. van den Ham, J. Jans, F. Zaaraoui-Boutahar, A. Zomer, E. Simonetti, M.A. Bijl, H.K. Brand, I.W.F. van, M.I. de Jonge, P.L. Fraaij, R. de Groot, A.D. Osterhaus, M.J. Eijkemans, G. Ferwerda, A.C. Andeweg, Transcriptome assists prognosis of disease severity in respiratory syncytial virus infected infants, Sci. Rep. 6 (2016) 36603.
[14] W.A. de Steenhuijsen Piters, S. Heinonen, R. Hasrat, E. Bunsow, B. Smith, M.C. Suarez-Arrabal, D. Chaussabel, D.M. Cohen, E.A. Sanders, O. Ramilo, D. Bogaert, A. Mejias, Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection, Am. J. Respir. Crit. Care Med. 194 (2016) 1104–1115.
[15] A. Mejias, B. Dimo, N.M. Suarez, C. Garcia, M.C. Suarez-Arrabal, T. Jartti, D. Blankenship, A. Jordan-Villegas, M.I. Ardura, Z. Xu, J. Banchereau, D. Chaussabel, O. Ramilo, Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection, PLoS Med. 10 (2013) e1001549.
[16] A.C. Jones, D. Anderson, S. Galbraith, E. Fantino, D. Gutierrez Cardenas, J.F. Read, M. Serralha, B.J. Holt, D.H. Strickland, P.D. Sly, A. Bosco, P.G. Holt, Personalized transcriptomics reveals heterogeneous Immunophenotypes in children with viral bronchiolitis, Am. J. Respir. Crit. Care Med. 199 (2019) 1537–1549.
[17] S.E. Vieira, S.Y. Bando, M. de Paulis, D.B.L. Oliveira, L.M. Thomazelli, E.L. Durigon, M.B. Martinez, C.A. Moreira-Filho, Distinct transcriptional modules in the peripheral blood mononuclear cells response to human respiratory syncytial virus or to human rhinovirus in hospitalized infants with bronchiolitis, PLoS One 14 (2019) e0213501.
[18] L.A.H. Do, J. Pellet, H.R. van Doorn, A.T. Tran, B.H. Nguyen, T.T.L. Tran, Q.H. Tran, Q.B. Vo, N.A. Tran Dac, H.N. Trinh, T.T.H. Nguyen, B.T. Le Binh, H.M.K. Nguyen, M.T. Nguyen, Q.T. Thai, T.V. Vo, N.Q.M. Ngo, T.K.H. Dang, N.H. Cao, T.V. Tran, L.V. Ho, B. De Meulder, C. Auffray, J.J. Hofstra, J. Farrar, J.E. Bryant, M. de Jong, M.L. Hibberd, Host Transcription Profile in Nasal Epithelium and Whole Blood of Hospitalized Children Under 2 Years of Age With Respiratory Syncytial Virus Infection, J. Infect. Dis. 217 (2017) 134–146.
[19] D.R. Hijano, L.D. Vu, L.M. Kauvar, R.A. Tripp, F.P. Polack, S.A. Cormier, Role of type I interferon (IFN) in the respiratory syncytial virus (RSV) immune response and disease severity, Front. Immunol. 10 (2019) 566.
[20] F.S. Lakschevitz, M.B. Visser, C. Sun, M. Glogauer, Neutrophil transcriptional profile changes during transit from bone marrow to sites of inflammation, Cell. Mol. Immunol. 12 (2015) 53–65.
[21] B.C. Rocha, P.E. Marques, F.M.S. Leoratti, C. Junqueira, D.B. Pereira, L. Antonelli, G.B. Menezes, D.T. Golenbock, R.T. Gazzinelli, Type I interferon transcriptional signature in neutrophils and low-density granulocytes are associated with tissue damage in malaria, Cell Rep. 13 (2015) 2829–2841.
[22] M. Conese, S. Castellani, S. Lepore, O. Palumbo, A. Manca, T. Santostasi, A.M. Polizzi, M. Copetti, S. Di Gioia, V. Casavola, L. Guerra, A. Diana, P. Montemurro, M.A. Mariggio, C. Gallo, A.B. Maffione, M. Carella, Evaluation of genome-wide expression profiles of blood and sputum neutrophils in cystic fibrosis patients before and after antibiotic therapy, PLoS One 9 (2014) e104080.
[23] Y.V. Subrahmanyam, S. Yamaga, Y. Prashar, H.H. Lee, N.P. Hoe, Y. Kluger, M. Gerstein, J.D. Goguen, P.E. Newburger, S.M. Weissman, RNA expression patterns change dramatically in human neutrophils exposed to bacteria, Blood 97 (2001) 2457–2468.
[24] M.A. Khan, N. Palaniyar, Transcriptional firing helps to drive NETosis, Sci. Rep. 7 (2017) 41749.
[25] S.B. Besteman, A. Callaghan, M.P. Hennus, G.H.A. Westerlaken, L. Meyaard, L.L. Bont, Signal inhibitory receptor on leukocytes (SIRL)-1 and leukocyte- associated immunoglobulin-like receptor (LAIR)-1 regulate neutrophil function in infants, Clin. Immunol. 211 (2020) 108324.
[26] B. Cortjens, S.A. Ingelse, J.C. Calis, A.P. Vlaar, L. Koenderman, R.A. Bem, J.B. van Woensel, Neutrophil subset responses in infants with severe viral respiratory infection, Clin. Immunol. 176 (2017) 100–106.
[27] C.W. Law, Y. Chen, W. Shi, G.K. Smyth, voom: precision weights unlock linear model analysis tools for RNA-seq read counts, Genome Biol. 15 (2014) R29.
[28] D. Wu, G.K. Smyth, Camera: a competitive gene set test accounting for inter-gene correlation, Nucleic Acids Res. 40 (2012) e133.
[29] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Stat. Soc. Ser. B Methodol. 57 (1995) 289–300.
[30] G.S. Garcia-Romo, S. Caielli, B. Vega, J. Connolly, F. Allantaz, Z. Xu, M. Punaro, J. Baisch, C. Guiducci, R.L. Coffman, F.J. Barrat, J. Banchereau, V. Pascual, Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus, Sci. Transl. Med. 3 (2011) 73ra20.
[31] S. Martinelli, M. Urosevic, A. Daryadel, P.A. Oberholzer, C. Baumann, M.F. Fey, R. Dummer, H.U. Simon, S. Yousefi, Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation, J. Biol. Chem. 279 (2004) 44123–44132.
[32] R.J. Geerdink, M.P. Hennus, G.H.A. Westerlaken, A.C. Abrahams, K.I. Albers, J. Walk, E. Wesselink, R. Janssen, L. Bont, L. Meyaard, LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis, J. Allergy Clin. Immunol. 141 (2018) 811–814.
[33] B. Cortjens, O.J. de Boer, R. de Jong, A.F. Antonis, Y.S. Sabogal Pineros, R. Lutter, J.B. van Woensel, R.A. Bem, Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease, J. Pathol. 238 (2016) 401–411.
[34] G.A. Funchal, N. Jaeger, R.S. Czepielewski, M.S. Machado, S.P. Muraro, R.T. Stein, C.B. Bonorino, B.N. Porto, Respiratory syncytial virus fusion protein promotes TLR-4-dependent neutrophil extracellular trap formation by human neutrophils, PLoS One 10 (2015) e0124082.
[35] A. Mantovani, M.A. Cassatella, C. Costantini, S. Jaillon, Neutrophils in the activation and regulation of innate and adaptive immunity, Nat. Rev. Immunol. 11 (2011) 519–531.
[36] K.L. Bucasas, A.I. Mian, G.J. Demmler-Harrison, A.C. Caviness, P.A. Piedra, L.M. Franco, C.A. Shaw, Y. Zhai, X. Wang, M.S. Bray, R.B. Couch, J.W. Belmont, Global gene expression profiling in infants with acute respiratory syncytial virus broncholitis demonstrates systemic activation of interferon signaling networks, Pediatr. Infect. Dis. J. 32 (2013) e68–e76.
[37] H.O. Fjaerli, G. Bukholm, A. Krog, C. Skjaeret, M. Holden, B. Nakstad, Whole blood gene expression in infants with respiratory syncytial virus bronchiolitis, BMC Infect. Dis. 6 (2006) 175.
[38] E. Pylaeva, S. Bordbari, I. Spyra, A.S. Decker, S. Haussler, V. Vybornov, S. Lang, J. Jablonska, Detrimental effect of type I IFNs during acute lung infection with *Pseudomonas aeruginosa* is mediated through the stimulation of neutrophil NETosis, Front. Immunol. 10 (2019) 2190.
[39] M. Comerlato Scotta, D. Greff Machado, S. Goecks Oliveira, A. de Moura, G. Rhoden Estorgato, A.P.D. de Souza, B. Nery Porto, P.D. de Araujo, E.E. Sarria, P.M. Pitrez, M.H. Jones, L. Araujo Pinto, R. Tetelbom Stein, F.P. Polack, R. Mattiello, Evaluation of nasal levels of interferon and clinical severity of influenza in children, J. Clin. Virol. 114 (2019) 37–42.
[40] J.C. Gomez, M. Yamada, J.R. Martin, H. Dang, W.J. Brickey, W. Bergmeier, M.C. Dinauer, C.M. Doerschuk, Mechanisms of interferon-gamma production by neutrophils and its function during Streptococcus pneumoniae pneumonia, Am. J. Respir. Cell Mol. Biol. 52 (2015) 349–364.
[41] L. Moreira-Teixeira, K. Mayer-Barber, A. Sher, A. O'Garra, Type I interferons in tuberculosis: foe and occasionally friend, J. Exp. Med. 215 (2018) 1273–1285.
[42] J. Dunning, S. Blankley, L.T. Hoang, M. Cox, C.M. Graham, P.L. James, C.I. Bloom, D. Chaussabel, J. Banchereau, S.J. Brett, M.F. Moffatt, A. O'Garra, P.J.M. Openshaw, M. Investigators, Progression of whole-blood transcriptional signatures from interferon-induced to neutrophil-associated patterns in severe influenza, Nat. Immunol. 19 (2018) 625–635.
[43] D. Damjanovic, C.L. Small, M. Jeyanathan, S. McCormick, Z. Xing, Immunopathology in influenza virus infection: uncoupling the friend from foe, Clin. Immunol. 144 (2012) 57–69.
[44] R. Gonzalez-Sanz, M. Mata, J. Bermejo-Martin, A. Alvarez, J. Cortijo, J.A. Melero, I. Martinez, ISG15 is upregulated in respiratory syncytial virus infection and reduces virus growth through protein ISGylation, J. Virol. 90 (2016) 3428–3438.
[45] S.A. Cormier, B. Shrestha, J. Saravia, G.I. Lee, L. Shen, J.P. DeVincenzo, Y.I. Kim, D. You, Limited type I interferons and plasmacytoid dendritic cells during neonatal respiratory syncytial virus infection permit immunopathogenesis upon reinfection, J. Virol. 88 (2014) 9350–9360.
[46] R.Y. Sung, J. Yin, S.J. Oppenheimer, J.S. Tam, J. Lau, Treatment of respiratory syncytial virus infection with recombinant interferon alfa-2a, Arch. Dis. Child. 69 (1993) 440–442.
[47] M. Goritzka, L.R. Durant, C. Pereira, S. Salek-Ardakani, P.J. Openshaw, C. Johansson, Alpha/beta interferon receptor signaling amplifies early proinflammatory cytokine production in the lung during respiratory syncytial virus infection, J. Virol. 88 (2014) 6128–6136.
[48] M. Ansar, N. Komaravelli, T. Ivanciuc, A. Casola, R.P. Garofalo, Detrimental role of type I interferon signaling in respiratory syncytial virus infection, 200 (2018) (Issue 1 supplement 60.6).
[49] R.S. Thwaites, M. Coates, K. Ito, M. Ghazaly, C. Feather, F. Abdulla, T. Tunstall, P. Jain, L. Cass, G. Rapeport, T.T. Hansel, S. Nadel, P. Openshaw, Reduced nasal viral load and IFN responses in infants with respiratory syncytial virus bronchiolitis and respiratory failure, Am. J. Respir. Crit. Care Med. 198 (2018) 1074–1084.
[50] A. Mejias, Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection, PLoS Med. 10 (2013).
[51] H.L. Chung, H.J. Park, S.Y. Kim, S.G. Kim, Age-related difference in immune responses to respiratory syncytial virus infection in young children, Pediatr. Allergy Immunol. 18 (2007) 94–99.
[52] K. Thorburn, S. Harigopal, V. Reddy, N. Taylor, H.K. van Saene, High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis, Thorax 61 (2006) 611–615.
[53] E. Fortunati, K.M. Kazemier, J.C. Grutters, L. Koenderman, J. Van den Boschv, Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease, Clin. Exp. Immunol. 155 (2009) 559–566.