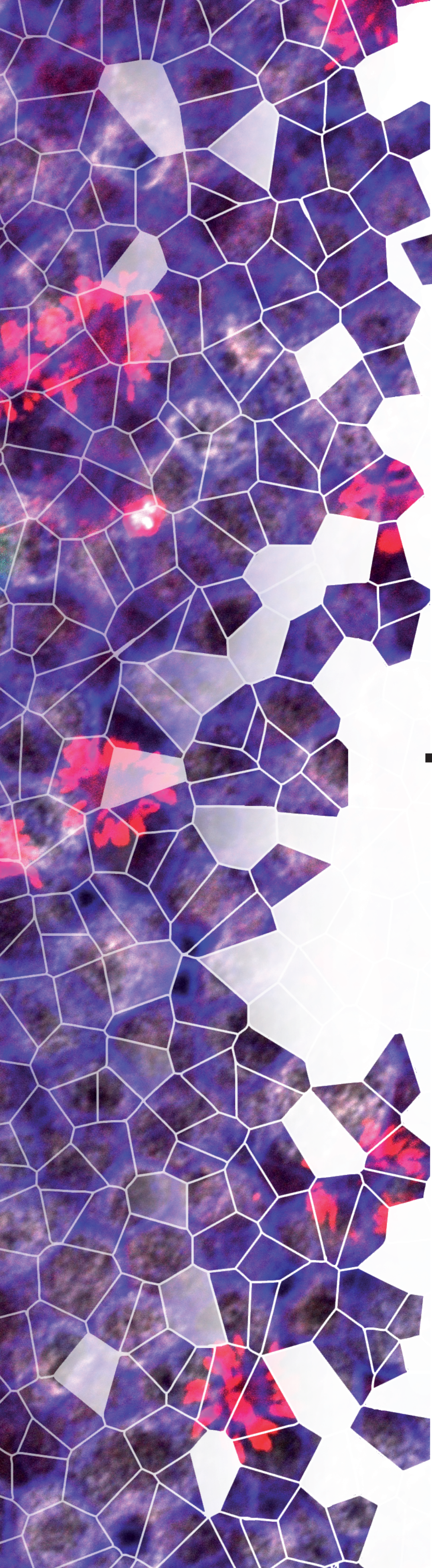

The local innate immune response during severe respiratory syncytial virus infection

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The local innate immune response during severe respiratory syncytial virus infection

Een beter begrip van de lokale aangeboren afweerreactie tegen respiratoir syncytieel virus infectie

(met een samenvatting in het Nederlands)

Proefschrift

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Table of contents

Chapter 1.	General introduction and outline of this thesis	7
Chapter 2.	Transcriptome of airway neutrophils reveals an interferon response in life-threatening respiratory syncytial virus infection *Clinical Immunology 2020*	23
Chapter 3.	Signal inhibitory receptor on leukocytes (SIRL)-1 and Leukocyte-associated immunoglobulin-like receptor (LAIR)-1 regulate neutrophil function in infants *Clinical Immunology 2020*	55
Chapter 4.	*In vitro* modeling of airway epithelium and neutrophil interaction during respiratory syncytial virus infection *Work in progress*	79
Chapter 5.	Neutrophils in respiratory syncytial virus infection: from harmful effects to therapeutic opportunities *British Journal of Pharmacology 2021*	95
Chapter 6.	Human CD14 mediates the innate immune response against respiratory syncytial virus infection *The Journal of Infectious Diseases 2022*	125
Chapter 7.	Summary and General discussion	161
Chapter 8.	Nederlandse samenvatting	177
Chapter 9.	Appendices	183
	I Fail-fast in RSV vaccine development	185
	II CD14 deficiency; translation for the general public.	189
	III Curriculum vitae	191
	IV List op publications	193
	V Dankwoord	195

1

General introduction and outline of this thesis

RESPIRATORY SYNCYTIAL VIRUS IS A MAJOR CAUSE OF CHILDHOOD PNEUMONIA WORLDWIDE

Respiratory syncytial virus (RSV) was first identified as chimpanzee coryza agent, in 1955, in chimpanzees with respiratory complaints.[1] In 1957 it was isolated from the lungs of infants with respiratory illness.[2] Nowadays, RSV is recognized as one of the most important pathogens identified in respiratory tract infections (RTI), especially in young infants, resulting in significant morbidity and mortality worldwide.[3] In children under five, RSV is estimated to lead to 3.2 million hospital admission and 125 000 deaths per year.[3, 4]

The clinical picture varies from mild upper respiratory tract symptoms, such as a runny nose and otitis, to extensive infection and inflammation of the lower airways, called bronchiolitis, leading to coughing and respiratory distress. In 2-12% of the children admitted to the hospital with RSV bronchiolitis the disease progresses to life threatening respiratory distress warranting invasive mechanical ventilation at the pediatric intensive care unit (PICU).[3, 5]

Despite over 60 years of research there is still no worldwide accessible and affordable vaccine or drug available.[6] Severe RSV disease can be prevented by palivizumab, a monoclonal antibody (mAb) against the fusion (F) protein of RSV.[7] However, this is only available for high-risk children in developed countries due to excessive costs. Ribavirin is the only registered antiviral therapeutic registered for RSV and lacks overall effectiveness.[8] Merely good hygiene and supportive measures such as supplemental oxygen supply or mechanical ventilation can aid this vulnerable group of patients. Due to a lack of resources in large parts of the world, over 90% of RSV-related childhood mortality occurs in low income countries.[4, 9] Promising preventive strategies currently being developed and tested in clinical trials include maternal immunization to protect infants, infant mAb prophylaxis with extended half-life (Nirsevimab), and the development of live-attenuated RSV vaccines.[6, 10-13] (Figure 1) Advances in understanding RSV disease pathology will aid mAb and RSV vaccine development.

Changing RSV epidemiology during the SARS-CoV-2 pandemic

Respiratory syncytial virus infection has a strong seasonality in most parts of the world. In the Netherlands, RSV seasonality is characterized by a rise during late fall and decline during early spring. However, the implementation of public health interventions aimed at preventing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission completely shifted RSV seasonality[14] The introduction of social distancing and closing day cares led to suppression of the annual RSV winter peak in the Netherlands. Once mitigations measures were released, this resulted in an unprecedentedly high infection rate during the summer of 2021.[15] Many other countries have reported a shift in RSV seasonality since the start of the SARS-CoV-2 pandemic.[14, 16] In some countries also a

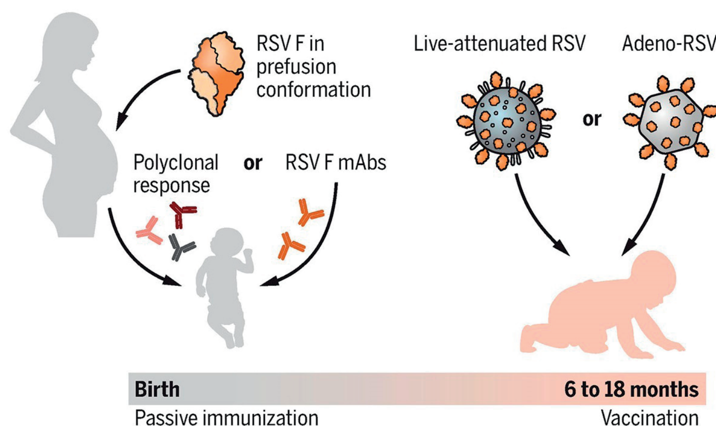

Figure 1: Passive immunization can be achieved by the administration of mAb directly to the infants or indirectly via transplacentary antibody transfer after maternal vaccination. Active immunization can be realized using live-attenuated and vector vaccines for older children. Image: Karron, R. A. (2021). "Preventing respiratory syncytial virus (RSV) disease in children." Science 372(6543): 686-687.

change in age distribution was observed, with higher infection rates than average in older children.[16] It is likely that in the upcoming years mitigation measures will be introduced on and off, and therefore RSV seasonality and epidemiology is likely changed for a longer period.[17] This poses great challenges for future RSV research but also for health care decision making.

Genetic predisposition to RSV

Risk factors associated with severe RSV infection in infants include prematurity, birth defects, chronic lung disease, and congenital heart defects. However, the majority of infants hospitalized for RSV infection are previously healthy. Genetic variation between individuals could explain why in some infants RSV disease progresses to severe bronchiolitis. A single-nucleotide variant (SNV) is a type of genetic alteration in the DNA caused by the substitution of a single nucleotide for another. SNVs include common variants, for example single nucleotide polymorphisms (SNPs), but also rare variants. Several SNPs in immunity associated genes are implicated to play a role in RSV pathogenesis, such as in genes encoding pattern recognition receptors (Toll-like receptor (TLR)3, TLR4, CD14), interferon signaling and the chemokine pathway (CCR5, RANTES).[18-23] Many of these SNPs could not be confirmed in other study populations.[reviewed in 24] Hence, the identification of RSV associated SNPs might be population- and virus strain specific. Genome wide association studies include the study of rare variants and thereby identified novel single gene-inborn errors in immunity underlying several respiratory viral infections, such as MDA5 deficiency in recurrent rhinovirus infection and IRF7 deficiency in severe influenza

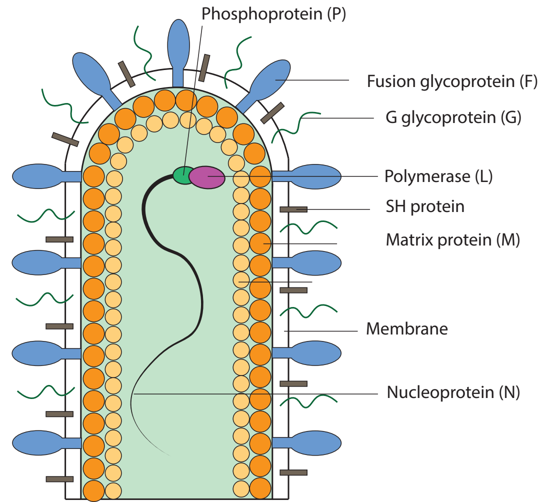

Figure 2: Respiratory syncytial virus virion. The attachment (G), and fusion (F) glycoproteins as well as the small hydrophobic (SH) protein, are embedded in the viral membrane. The viral membrane consists of a layer of matrix (M) lined with M2-1 protein, a transcriptional processivity and antitermination factor. Nucleoprotein (N) covers the viral RNA genome and associates with the large polymerase subunit (L) and the phosphoprotein polymerase cofactor (P).

influenza.[25, 26] Identifying a causal relationship between a (single) patient genetic variant and clinical phenotype can provide the next step in the understanding of diseases.[27]

The virus

RSV is an enveloped, single-stranded RNA virus and belongs to the *Orthopneumovirus* genus of the *Pneumoviridae* family. The RSV genome contains ten genes encoding 11 proteins (Figure 2). The M2 gene generates a transcription factor (M2-1), and a protein responsible for switching from transcription to replication (M2-2). Next, the non-structural proteins NS1 and NS2 are transcribed. Both proteins inhibit apoptosis and the host interferon response thereby promoting viral survival. The outer membrane of the RSV virion displays mainly two proteins: the fusion (F), and the attachment (G) glycoprotein. Less present are the small hydrophobic (SH) proteins. The virion attaches to respiratory epithelial cells of the upper respiratory tract, by attachment via the G protein, followed by fusion with the cell membrane mediated by the F protein. After attaching and fusion of the virion, RSV enters the cell by fusion, endocytosis or pacropinocytosis after which it fuses with the enodosome. It is translated and transcribed in the cytoplasm, after which new RSV virions assemble close to the plasma membrane where they bud from the cell surface and infect new cells. Recently two conformations of the F protein have been characterized: the pre-fusion and post-fusion conformation (pre-F and post-F, respectively).[28] RSV-pre-F protein is a potent inducer of neutralizing antibodies. Thereby, the

determination of the crystal structure of the RSV F glycoprotein, and stabilization of the pre-F confirmation, accelerated the development of new vaccine candidates.[6, 28-30]

In vitro, infection with RSV increases airway mucus production and induces epithelial cytotoxicity and consequently cell sloughing when studied *in vitro*. [31, 32] However, there is no clear correlation between RSV titers and disease severity.[33-40] Furthermore, antiviral therapy as a treatment for RSV bronchiolitis shows variable effectiveness even in pediatric and adult immunocompromised patients.[41, 42] These studies indicate that, in addition to virus induced pathology, disease outcome is likely determined by the host immune response.

The host

The innate immune response to RSV

The engagement of the innate immune response as an immediate response to RSV is of critical importance to reduce RSV viral burden, and determines clinical outcome. Respiratory syncytial virus primarily binds to the apical side of the ciliated and type 1 alveolar cells of the airway epithelium. Few receptors for viral entry have been described, of which the evidence for nucleolin and IGF1R is the strongest.[43, 44] Upon infection, RSV is confronted with the innate immune response by airway epithelial and lung resident cells such alveolar macrophages (AMs). In humans RSV initiates the innate immune response through several pattern recognition receptors, including TLR2, TLR4, cluster of differentiation (CD)14, and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs).[45, 46] Toll-like receptor-dependent signaling is important for activating early inflammatory responses to RSV. The production of cytokines, such as IL-6, IL-8 and type I and III interferon (IFN) by airway epithelial cells and alveolar macrophages, leads to the recruitment of innate immune cells, especially neutrophils, monocytes and dendritic cells to the lung.[45, 47]

The importance of a strong and immediate innate immune response for the eradication of RSV is highlighted by the capability of the G protein and the nonstructural proteins NS1 and NS2 of the RSV virion, to interfere with the host type I IFN response. [Reviewed in 48] This feature increases susceptibility to RSV infection and reinfection, and likely influences the induction and maintenance of adaptive immunity. Also, as mentioned above, SNPs in innate genes such as CD14, TLR4, TLR5, and IFN related genes, are associated with RSV disease susceptibility and severity.[20, 49, 50]

Adaptive immunity to RSV

This thesis will mainly focus on the innate immune response against RSV and therefore the adaptive immune response will only be discussed briefly. Upon RSV infection, alveolar macrophages recruit and activate natural killer cells to kill RSV-infected cells and to produce IFN-γ to promote Th1 responses. The adaptive immune response against RSV gives rise to high titers of virus specific antibodies and antigen specific T-cells which

will limit infection after re-infection. Despite the induction of immunological memory, symptomatic re-infection occurs throughout life, although symptoms are mostly mild.[51] Important is, that while Th1 response are important for disease resolution, Th2 driven responses are associated with RSV disease severity.[52, 53]

Inflammatory immunopathogenesis as part of RSV disease pathology

Adjacent to virus induced epithelial damage, neutrophils play a pivotal role in RSV disease pathology. Neutrophils are the predominant cell type in the lungs of RSV infected infants, representing over 80% of lung infiltrated leukocytes.[54, 55] Several studies show enhanced activation of neutrophil related genes in the blood of children with RSV bronchiolitis.[56-58] Furthermore, a common SNP upstream of the IL-8– encoding gene that is associated with increased production of IL-8, a potent neutrophil attractant, is more frequent among infants with severe RSV-induced bronchiolitis, in particular among infants without other known risk factors.[59] While it has been shown that neutrophils respond to stimulation with RSV[60-62], only one *in vitro* study indicated a beneficial role for neutrophils in RSV disease.[63] In several mice models neutrophil depletion did not impact viral load, nor contributed to disease severity after RSV infection.[64-67] Although neutropenia is associated with increased RSV mortality in patients with hematological malignancies, these studies are difficult to interpret because most of these patient are also deficient in other cell lineages and treated with immunosuppressive drugs.[42, 68] Altogether, these studies indicate that neutrophils do not limit viral replication or impact disease severity during RSV infection.

Neutrophil induced immunopathology

Neutrophils possess several features that can be detrimental to the host. The presence of neutrophils in the lung induces mucin production and airway plugging thereby contributing to disease severity.[69] Neutrophils release numerous antimicrobial and proteolytic agents, such as cathepsin G, neutrophil elastase, proteinase 3 and myeloperoxidase, which can induce bystander damage to epithelial cells.[70, 71] Co-culturing polarized airway epithelial cells together with neutrophils, showed increased epithelial barrier dysfunction that was associated with neutrophil transepithelial migration.[63, 72] RSV stimulation of human and bovine neutrophils induces the release of neutrophil extracellular traps (NETs) in a TLR4 dependent manner. [60, 61, 73] The release of NETs stimulates the local inflammatory response by airway epithelial cells, enhancing the recruitment of innate immune cells, but do not seem to induce epithelial cell damage *in vitro*.[74] *In vivo* the release of NETs does contribute to airway obstruction.[73] Additionally, neutrophils can contribute indirectly to disease severity by inducing Th17 responses. Th17 cells are highly inflammatory T-cells and their presence is associated with the severity of RSV bronchiolitis.[75-77] Finally, the presence of neutrophils in the nasal mucosa predisposes to RSV infection.[78]

Altogether, these studies suggest that in addition to viral induced epithelial damage, neutrophils contribute to RSV disease severity. Despite numerous studies evaluating neutrophils in RSV disease, their role in either controlling viral replication or, at the other hand, contributing to immunopathology remains enigmatic, and many of the molecular drivers behind this massive neutrophil activation and migration to the lung, especially in relation to disease severity, remain to be identified.

Immune balance

A balanced immune response is important to eradicate intruding pathogens, but at the same time limit immune cell mediated damage.[79] Inhibitory receptors on immune cells, also referred to as immune checkpoints, are important regulators of the immune response. [80-82] Immune checkpoints are novel targets in the treatment of cancer, auto-immune disease and infection.[83, 84] Neutrophils express several inhibitory receptors, including signal inhibitory receptor on leukocytes-1 (SIRL-1) and leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1).[85-87] Both receptors are differentially regulated during RSV infection, suggesting a role in disease pathology.[88] Previous studies have shown that neutrophil function, such as oxidative burst and neutrophil extracellular trap formation, can be inhibited through interaction via SIRL-1 and LAIR-1 *in vitro* and *in vivo*.[84, 88-91] In RSV bronchiolitis neutrophils offer a potential target for the development of novel therapeutics to reduce immune induced pathology during RSV bronchiolitis.[83, 89, 92]

In vitro models to study RSV neutrophil interaction

The airway tract is lined with a layer of pseudostratified epithelium consisting of basal cells: the primary stem cells of the lung, ciliated cells that transport debris and mucus out of the airways, and secretory cells such as goblet and club cells. Advances in single-cell RNA sequencing lead to the discovery of novel cell types and cell function such as ionocytes and tuft cells.[93] Several models can be used to study the role of RSV virus kinetics and the local immune response *in vitro* (Figure 3). Studies with non-differentiated monolayer cultures of immortalized cell lines such as Hep-2 and A549 cells cultured in 2D showed that RSV infection of epithelial cells causes cytopathology and barrier integrity dysfunction.[94, 95] However, continues cell lines are a poor representation of the complex composition of the human lung. Advances in developing cultures of morphologically and physiologically authentic, well-differentiated primary airway epithelial cells (WD-PAECs) cultured at an air liquid interface (ALI) led to a better understanding of RSV pathophysiology. These studies indicate that epithelial barrier integrity remains largely intact upon infection with RSV.[96-99] Other parameters such as viral replication, cytokine release and epithelial shedding are comparable to the studies performed in non-differentiated cultures and *in vivo* analysis of BAL fluid from RSV infected patients.[32] Finally, the recent development of 3D culture models such as nasal or bronchial epithelial

cell derived organoids offer the possibility to study RSV pathogenesis and cell –environment interaction.[100] Studies that included neutrophils or other immune cells in these (ALI or organoids) models are scarce, but remain a promising feature to provide further insight in neutrophil mediated pathology during RSV infection.[63, 72, 101, 102]

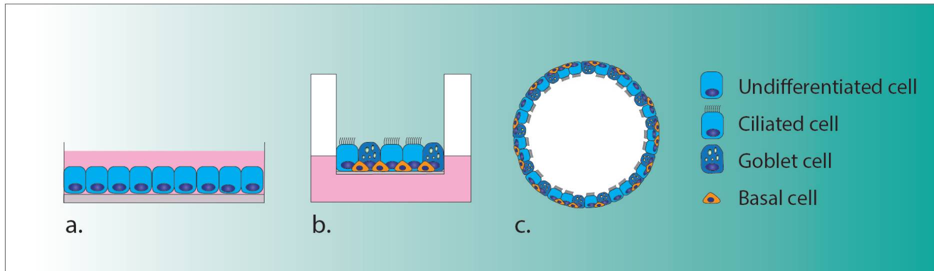

Figure 3. The figure shows a schematic cross sectional view of several culture methods that can be used to study RSV pathology. a. traditional 2D submerged epithelial culture. b. well differentiated primary airway epithelial cell cultures, cultured at an air liquid interface. c. primary airway epithelial cell derived organoid.

Gaps in knowledge and outline of this thesis

This thesis focusses on the innate immune response against RSV infection. A better understanding of disease and immune pathology offers opportunities for future research and for the development of novel treatment targets.

The first aim of this thesis was to gain insight in the role of neutrophils during severe RSV bronchiolitis in infants. Thus far, data on the role of neutrophils from the airways of severely RSV infected infants are scarce. Therefore, we set up the Neon study, which aimed to provide insight in the phenotype and function of airway neutrophils from infants with life threatening RSV. In **chapter 2** the transcriptomic profile of airway infiltrated neutrophils from infants with severe RSV bronchiolitis is described, which provides insight in molecular drivers of neutrophil migration and function during severe RSV bronchiolitis. **Chapter 3** describes how human neutrophil function can be modulated *ex vivo* by targeting inhibitory receptors during RSV bronchiolitis. Next, I aimed to identify how we can use *in vitro* well differentiated human airway epithelial cell cultures to study neutrophil – epithelial interaction during RSV infection in **chapter 4**. In **chapter 5** we present an overview of possible therapeutic targets for neutrophil modulation to improve the clinical outcome of RSV bronchiolitis.

The second aim of this thesis was to provide evidence for a causal relationship between a novel mono genetic etiology of RSV bronchiolitis and define the role of CD14 in the innate immune response against RSV. In **chapter 6** the clinical and immunological phenotype of a novel inborn error of immunity is described and identified as a possible mono-genetic cause of severe and recurrent RSV bronchiolitis.

A general discussion of the main findings of this thesis can be found in **chapter 7**.

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2

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

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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.

Keywords: Respiratory Syncytial Virus; Interferon; Neutrophil; Bronchiolitis; Transcriptome; Sputum; Immunity.

List op 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.

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 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 hours after admission. The study was approved by the Medical ethical review board (NL58404.041.16).

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 70µm nylon filter (BD Falcon). Neutrophils were sorted by flow cytometry using monoclonal antibodies. Neutrophils were selected based on CD66b+/CD14- staining. (Figure 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 Figure 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 Figure 1B). In 23 out of 24 samples cell viability was >92% (Supplementary Figure 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 pro-

file 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-SPMNs and RSV-BPMNs), and Sputum-PMNs and Blood-PMNs of control patients (Ctrl-SPMNs and Ctrl-BPMNs). We determined differential gene expression for 5 contrasts: (1) RSV-SPMNs versus RSV-BPMNs; (2) Ctrl-SPMNs versus Ctrl-BPMNs; (3) RSV-BPMNs versus Ctrl-BPMNs; (4) RSV-SPMNs versus Ctrl-SPMNs; (5) (RSV-SPMNs versus RSV-BPMNs) versus (Ctrl-SPMNs versus Ctrl-BPMNs).

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 (padj) <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 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 hours 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 (Figure 1A-B). Baseline characteristics are outlined in Table 1.

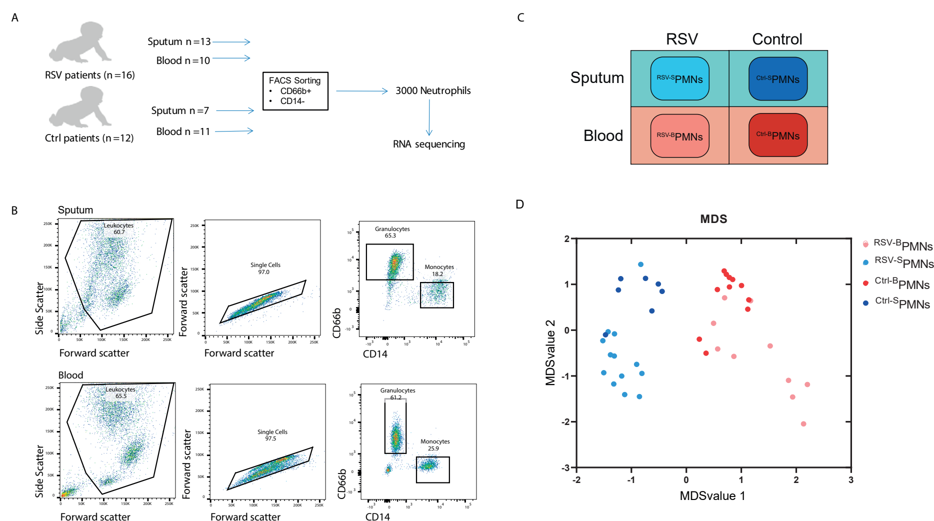

Figure 1. Differential gene expression profiles in Sputum-PMNs and Blood-PMNs in children with and without severe RSV bronchiolitis. (A) Study design: peripheral blood and sputum were sampled from RSV (n = 16) and control (n=12) infants admitted to the hospital. Sputum was filtered twice, and erythrocytes 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.

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-BPMNs, RSV-SPMNs, Ctrl-SPMNs and Ctrl-BPMNs (Figure 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) (Figure 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 (Figure 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 Figure 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-SPMNs versus RSV-BPMNs; and Ctrl-SPMNs versus Ctrl-BPMNs. In the first contrast 1476 differentially expressed genes (DEGs) were upregulated and 2110 DEGs were downregulated (Figure 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-SPMNs versus Ctrl-BPMNs) 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 (Figure 2B). The top ten overexpressed genes in Ctrl-SPMNs 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 (Figure 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 (Figure 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 (Figure 2D and 2E, 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.

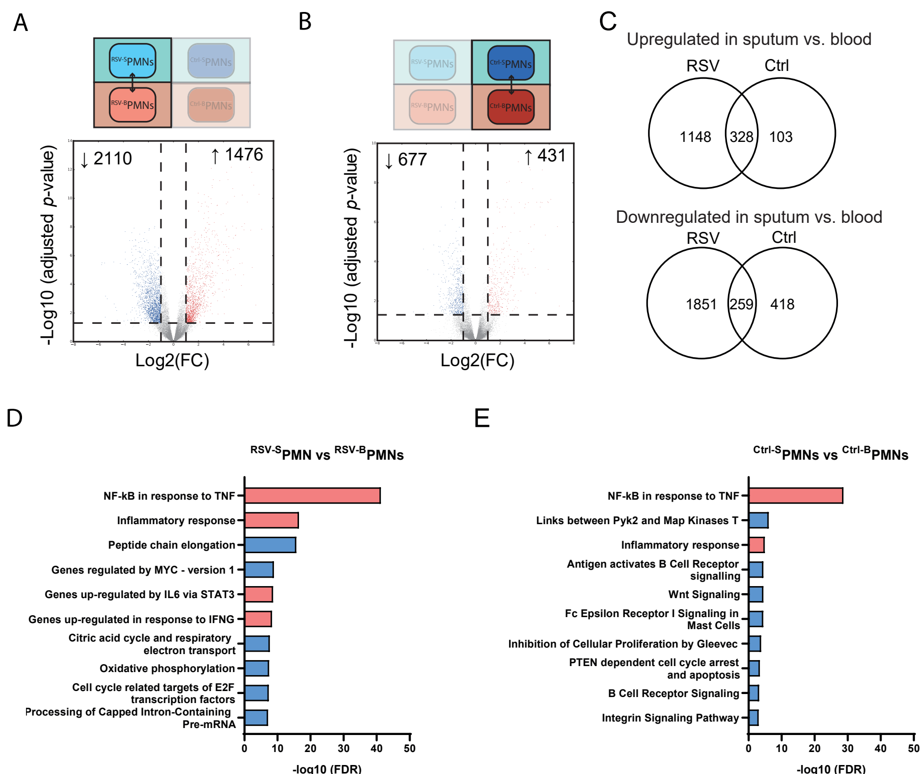

Figure 2. Distinct local neutrophil responses in the lungs of infants with and without severe RSV bronchiolitis. (A) Volcano plot showing DEGs in $^{RSV\text{-}S}$PMNs compared to $^{RSV\text{-}B}$PMNs, upregulated genes (red) and downregulated genes (blue) are marked. (B) Volcano plot showing DEGs in $^{Ctrl\text{-}S}$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.

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-BPMNs with Ctrl-BPMNs (Figure 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

(Figure 3B). Downregulated pathways, included GPCR ligand binding (padj = 0.007), and chemokine signaling (padj = 0.009, Supplementary Figure 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-BPMN samples (Supplementary Figure 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.

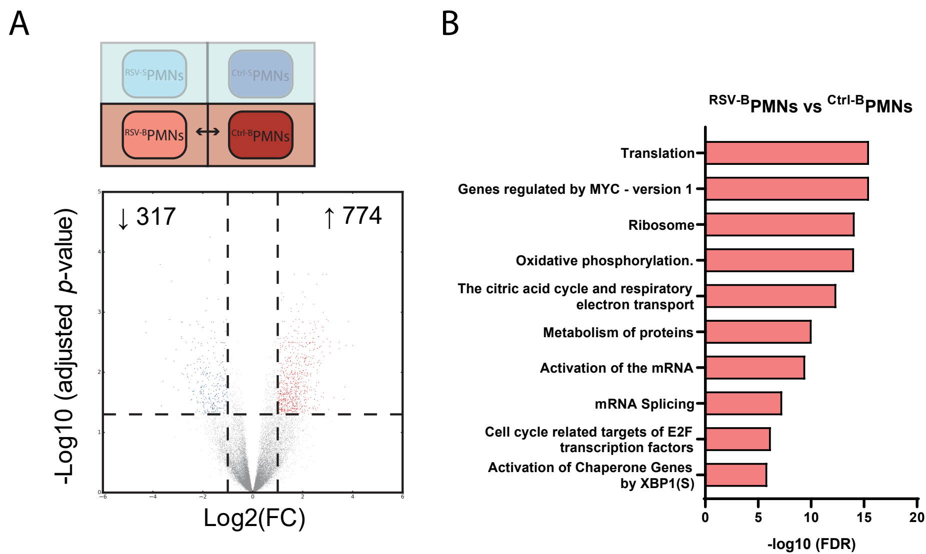

Figure 3. Distinct systemic neutrophil responses in infants with and without severe RSV bronchiolitis. Blood-PMNs from control and RSV infants were sampled. (A) Volcano plot showing DEGs in $^{RSV\text{-}B}$PMNs compared to $^{Ctrl\text{-}B}$PMNs, upregulated genes (red) and downregulated genes (blue) 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.

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-SPMNs versus Ctrl-SPMNs; second, a 2-way contrast in which the transcriptomic response to RSV in sputum neutrophils is corrected for the response in blood neutrophils: (RSV-SPMNs versus RSV-BPMNs) versus (Ctrl-SPMNs versus Ctrl-BPMNs).

The first contrast identified 399 up- and 45 downregulated DEGs (Figure 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 (padj=0.011 and padj=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-SPMNs versus RSV-BPMNs) versus (Ctrl-SPMNs versus Ctrl-BPMNs)). 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 (Figure 4C), and increased expression of most genes that are part of the type 1 IFN response gene set (Figure 4D). This response was largest observed in RSV-SPMNs samples (Figure 5). qPCR analysis on a limited number of available samples and genes, i.e. IFIT1 and CCRL2, confirmed these findings (Supplementary Figure 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 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 (padj = 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-BPMNs and RSV-SPMNs compared to controls (Figure 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 (padj 0.026, Figure 6 bottom panel). Most genes in this gene set were downregulated in RSV-SPMNs except for four genes: PTX3, TNFSF13B, IL-18 and MMP9. Altogether, we

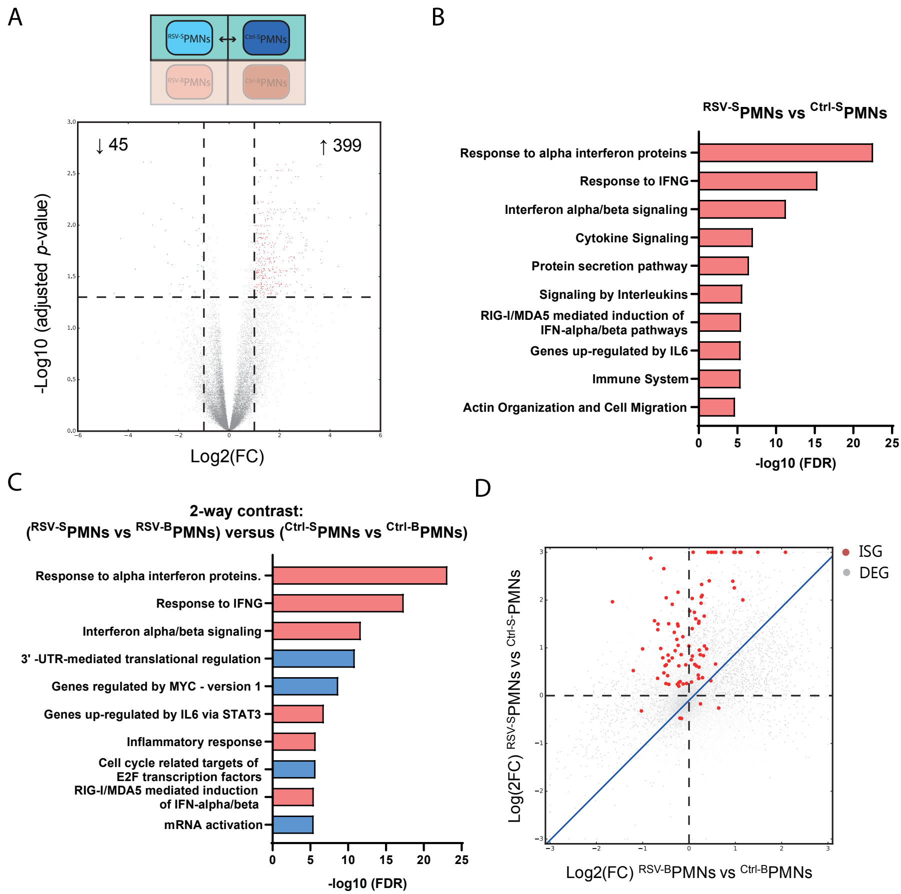

Figure 4. Transcriptomic analysis of RSV-SPMNs compared to Ctrl-SPMNs. (A) Volcano plot showing DEGs in RSV-SPMNs, compared to Ctrl-SPMNs, upregulated genes (red) and downregulated genes (blue). (B) Top 10 most significant enriched gene sets in RSV-SPMNs, compared to Ctrl-SPMNs, upregulated gene sets (red) and downregulated gene sets (blue) are marked. (C) Top 10 enriched gene sets in in RSV-SPMNs, compared to Ctrl-SPMNs, 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-SPMNs vs Ctrl-SPMNs (y-axis) and RSV-BPMNs vs Ctrl-BPMNs (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.

show that neutrophils upregulate a specific set of neutrophil activation genes, dependent on their localization during RSV infection. We confirm that gene expression profiles by blood neutrophils do not accurately reflect the transcriptomic profile of neutrophils in the airways.

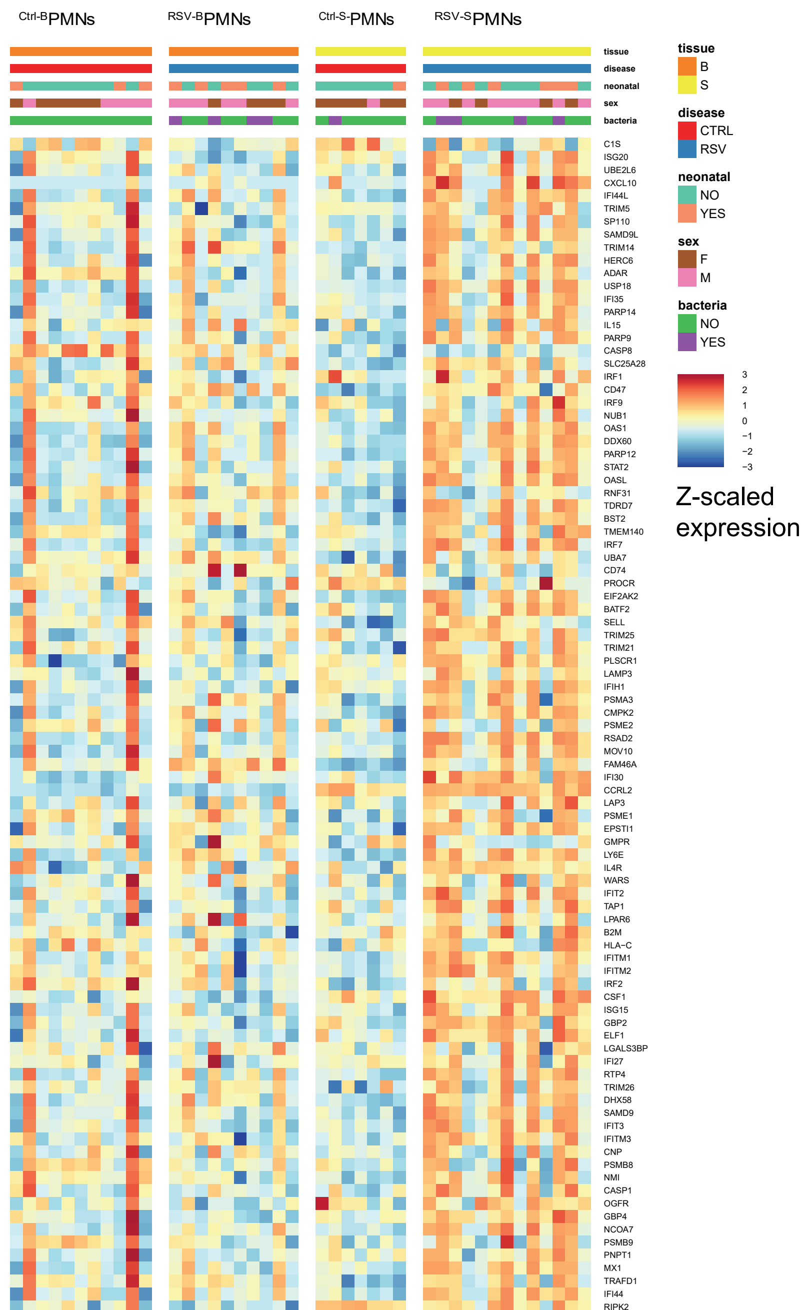

Figure 5. Heat map showing the expression of genes stimulated by IFNa (HALLMARK) in each sample. Gene expression levels are shown as row normalized Z scores with red representing higher expression and blue representing lower expression. Benjamini-Hochberg adjusted *P* values <0.05 were classified as significant. 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.

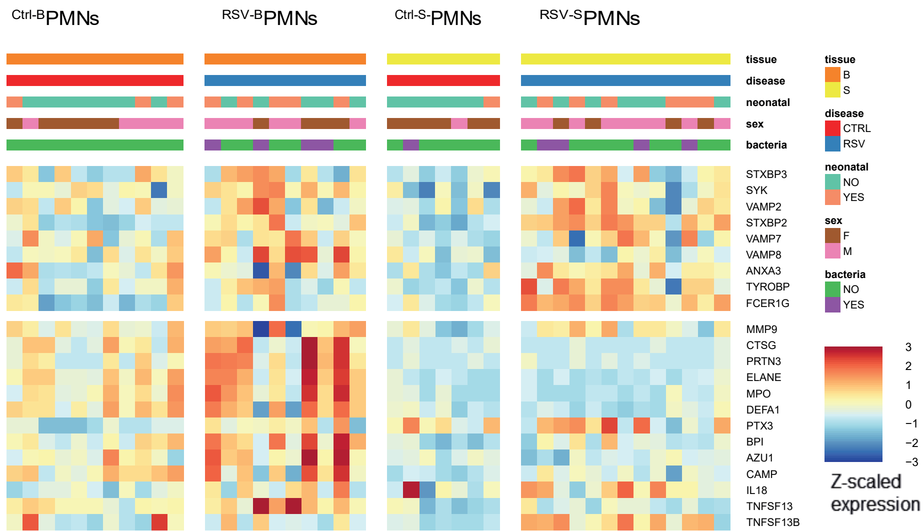

Figure 6. Transcriptomic analysis of systemic and local neutrophil activation genes in infants with and without severe RSV bronchiolitis. Heat map showing the gene expression in all samples of genes involved in neutrophil activation (GO database (top), combined with a custom list (bottom). Gene expression 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.

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 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 amongst 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 (Figure 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 Figure 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 Figure 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 Figure 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 Figure 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.

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SUPPLEMENTAL MATERIAL

1. Supplementary methods

1.1 Study population

Peripheral blood and/or sputum was obtained from 28 infants (<24months) hospitalized in the Wilhelmina Children's Hospital in the Netherlands, as part of the Neon study.[1] The inclusion period included two RSV seasons, and ranged from October 2016 to February 2018.The patients were admitted, either with severe RSV bronchiolitis (RSV group), or undergoing surgery (control group). Diagnosis of RSV bronchiolitis was based on the clinical diagnosis by a pediatrician and confirmed by PCR analysis. RSV severity was determined as being in need for admission at the PICU mechanical ventilation. Recently patients with severe RSV are getting more often treated with non-invasive mechanical ventilation (NIV) as opposed to invasive mechanical ventilation.[2] to include as much blood samples as possible we allowed for the inclusion of both invasive and non-invasive (2 patients) ventilated patients. Of these two patients we only collected blood samples and no sputum samples. Exclusion criteria: immune disease, use of immunosuppressive drugs, or (acquired) major organ dysfunction. Only in the control group we allowed for the presence of a congenital heart defect to increase the eligibility for enrolment of control patients. Additionally control patients with a suspected or confirmed pulmonary infection at the time of inclusion were excluded from the study. 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. Blood was collected in sodium heparin tubes and processed <2 hours after withdrawal. Tracheobronchial aspiration was obtained using a maximum of 2 ml of normal saline. The sputum samples of the control infants were collected through a nasal or oral tube directly after intubation. By using a long suction catheter through the tube, which extended into the lower bronchus, we avoided contamination with neutrophils derived from the upper airway. Of 12 control patients we collected 11 blood samples and 7 sputum samples of which 6 were paired. Of the 16 RSV patients we collected 10 blood samples, 13 sputum samples, of which 7 were paired. All paired samples were collected simultaneously.

Patient characteristics were obtained from all patients. The study was approved by the Medical ethical review board (review reference number NL58404.041.16), and both parents/guardians of the participants provided written informed consent.

1.2 Purification of polymorphonuclear cells (PMNs)

The whole blood sample was depleted from red blood cells, by lysing the erythrocytes by dilution with five times the initial blood volume of cold distilled water. Blood was incubated for 45 seconds with water after which 0.1x volume of 10x PBS (=1 ml) was

added to stop cell lysis. Cells were washed and remaining erythrocytes were removed by a second lysis with ammonium chloride buffer for 5 to 10 minutes, and the sample was centrifuged at 500g for 5 min at 4°C. The remaining cell pellet was washed with RPMI-1640 medium supplemented with 10% heat-inactivated Fetal Calf serum (FCS). Sputum samples were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated Fetal Calf serum (FCS) and filtered twice using a 70 µm nylon filter (BD Falcon).

Neutrophils were further sorted from blood and sputum using the FACSAria II and III fluorescence-activated cell sorter (FACS) (BD Biosciences). Cells were stained for surface markers for 20 min at 4°C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) bovine serum albumin (BSA). Cell types were identified according to their characteristic forward and side light-scatter properties, and further identified by neutrophil cell surface marker expression: $CD66b^+$/ $CD14^-$. Antibodies were obtained from BD Bioscience (CD66b) and eBioscience (CD14). Identical sorting strategies were employed to select neutrophils from the blood and sputum samples. Selected cells were lysed in Trizol LS reagent (ThermoFisher) and stored at -80°C until further processing.

1.3 Flow cytometry

When sufficient cells remained after sorting, we determined monocyte, neutrophil and eosinophils in blood and sputum, by additional flow cytometry. This was available for nine control blood samples and eight RSV blood samples, and for one control and six RSV sputum samples. Especially the control sputum samples were low in cell count and therefore often not sufficient to perform the sorting experiment as well as the additional measurement of cell types by flow cytometry. Blood and sputum samples were processed as described above: The whole blood sample was depleted from red blood cells, by lysing the erythrocytes. Sputum samples were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated Fetal Calf serum (FCS) and filtered twice using a 70 µm nylon filter (BD Falcon). Cells were stained for surface markers CD14, CD16 and CD66b for 20 min at 4°C. Cell types were identified according to characteristic forward and side light-scatter properties, using the same gating strategy as was used for sorting: granulocytes were identified by $CD66b^+$/ $CD14^-$ and monocytes were identified as $CD66b^-$/ $CD14^+$. In this staining we also included CD16 which offers the opportunity to identify eosinophils in blood. The following cell types were distinguished based on characteristic staining patterns: Whole blood derived neutrophils, $CD14^-$/ $CD66b^+$/$CD16^+$; monocytes, $CD14^+$/$CD66b^-$/$CD16^-$, eosinophils, $CD14^-$/$CD66b^+$/$CD16^-$, Sputum derived neutrophils $CD66b^+$/ $CD14^-$, and monocytes $CD66b^-$/$CD14^+$. During RSV there is an influx of CD16 dim (progenitor) neutrophils to the airways, therefore we used CD66b and not CD16 in our sorting panel, hence we could also not differentiate between eosinophils and neutrophils in sputum.[3] Positive 7-aminoactinomycin D (7-AAD) (5 µl per sample,

BD Bioscience) was used to distinguish apoptotic cells. Antibodies were obtained from BD Bioscience (CD66b), BioLegend (CD16, CD62L), and eBioscience (CD14).

1.4 Nucleic acid extraction and RSV subtyping

Nucleic acids were extracted from 250-500 µL of RSV positive nasal specimens using the MagNA Pure 96 DNA and Viral NA Large Volume kit (Roche Diagnostics, Mannheim, Germany) using the manufacturer's protocol. RSV subtyping and quantification was performed by multiplexed TaqMan RT-PCR analysis of the RSV N gene on a StepOnePlus System (Applied Biosystems), using RSV-A and RSV-B specific primers/probe mixes. RSV-A forward primer (5′ AGATCAACTTCTGTCATCCAGCAA 3′), RSV-A reverse primer (5′ TTCT-GCACATCATAATTAGGAGTATCAAT 3′), RSV-B forward primer (5′ AAGATGCAAATCATA-AATTCACAGGA 3′), RSV-B reverse primer (5′ TGATATCCAGCATCTTTAAGTATCTTTATAGTG 3′), RSV-A probe (5′ CACCATCCAACGGAGCACAGGAGAT 3′, 5′6-FAM/ZEN/3′IBFQ), and RSV-B probe (5′ TTCCCTTCCTAACCTGGACATAGCATATAACATACCT 3′, 5′ JOE NHS/ZEN/3′ IBFQ) (Integrated DNA Technologies). Three patients were diagnosed by routine molecular testing as part of clinical care, therefore there was no CT-value available for these cases.

1.5 RNA isolation and real-time quantitative PCR analysis

For transcriptomics analysis, mRNA was extracted from Trizol and used for cDNA synthesis, using iScript (Bio-Rad) and a C1000 Touch Thermal Cycler machine (Bio-Rad). For relative gene expression, cDNA transcripts were quantified by real-time quantitative PCR with IQTM SYBR Green Supermix (Bio-Rad, FR) and specific primers for RPL32 (Fw: AGGGTTCGTAGAAGATTCAAGG, Rv: GGAAACATTGTGAGCGATCTC), IFIT1 (Fw: TCATCAG-GTCAAGGATAGTCTG, Rv: GGTGTTTCACATAGGCTAGTAG), and CCRL2 (Fw: CACCAGAG-GATGAATATGATG, Rv: TTTACCAGGATAAGCACAACC. Expression levels of evaluated genes were calculated by relative quantification, using the comparative CT method: each value was corrected for the expression of one housekeeping gene: RPL32 (Quantstudio).

1.6 RNA isolation and sequencing (RNA-Seq)

Exactly 3000 neutrophils were selected from each sample for RNA extraction using Trizol. The cells were sorted and lysed in 500ul Trizol and 100 µl chloroform was added to each vial. The vials were shaken and spun down at 12000xg for 15 minutes at 4°C. The aqueous phase was transferred into a new tube and RNA was mixed with 1ul of GlycoBlue (Invitrogen) and precipitated with 250 µl isopropanolol. The low input RNA samples were prepared to obtain sequencing libraries following the Cel-Seq2 method [4] and facility-implemented (Utrecht Sequencing Facility) quality and analysis procedures. Sequencing libraries were paired-end sequenced on a NextSeq 500 at 2 x 75bp). The reads were de-multiplexed and aligned to human cDNA reference using the BWA

(0.7.13). Primary aligned reads were selected for gene expression quantification. Multiple reads mapping to the same gene with the same unique molecular identifier (UMI, 6bp long) were counted as a single read.

1.7 Pre-processing and exploratory data analysis

Read counts per gene, per sample, were analysed for global expression differences using R (version 3.5.3). Genes were selected with an expression of 1 count per million reads (CPM) in at least 8 samples, the minimum number of samples per patient-tissue type group. Read counts were TMM-normalized using the calcNormFactors function from the edgeR package (version 3.24.3), the normalized counts were used to assess global transcriptional profile differences of all samples by PCA (10 components) and MDS (2 components) analysis. For 10 Principal components (PC) from the PCA analysis, values were checked for correlation to sample characteristics (i.e. sex, neonatal status, tissue-of-origin, bacterial infection and RSV infection) by the Mann-Whitney U test implemented in the scipy package (version 0.19.0) in python (version 2.7.10). Both study groups differed in age and in the RSV group there we more patients of neonatal age than in the control group (7 vs. 3 respectively). Therefore, we used neonatal status as a characteristic to check for correlation. The characteristics of interest, i.e. tissue-of-origin and RSV infection, as well as neonatal status came out as most significantly associated ($p<0.05$) to any of the PCs (Supplementary table 1). Therefore, neonatal status was used as a co-factor in subsequent differential expression modelling. In addition we found that the modeling of age as a continuous factor or 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 $p=0.367$), did not qualitatively alter the conclusions from the differential gene set expression analysis.

1.8 Differential gene expression analysis

Differential expression analysis was performed with the voom-limma packages (version 3.38.3) in R (version 3.5.3).[5] In our study, up to two samples from the same individual are present. The correlated expression between samples from the same individual was modelled using random effects modelling, for which we needed to use limma. Voom was used to transform RNA-seq based expression counts for limma compatibility, while blocking for patient specific expression with a correlation estimate from the duplicateCorrelation function of the limma package. Gene expression was modelled to a model that included tissue-of-origin plus RSV infection as a single grouped factor, and neonatal status as a co-factor. Four groups are present in the first factor: CTRL-sputum, CTRL-blood, RSV-sputum and RSV-blood. Differential gene expression was determined for 5 contrasts between these groups: $^{\text{Ctrl-B}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{Ctrl-B}}$PMNs, $^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{RSV-S}}$PMNs, and a 2-way contrast (($^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs) versus

($^{Ctrl\text{-}S}$PMNs s $^{Ctrl\text{-}B}$PMNs)). The final contrast is a difference of differences analysis, where sputum samples are first compared to blood samples from the same patient group to negate general RSV or tissue-of-origin effects, and focus on differential expression in the specific RSV-sputum group. Differential expression statistics for each contrast were obtained through the eBayes functionality of the limma package, False Discovery Rates (FDR) were determined using the Benjamini-Hochberg method to adjust for multiple testing for all genes (see supplementary Table 2 with per gene differential expression results for each contrast).[6]

1.9 Gene set analysis

Gene set enrichment testing was performed with CAMERA[7] for the hallmark (H), canonical pathway (C2-CP) and GO term (C5) gene set collections from the Molecular Signatures Database.[8] Only neutrophil associated gene sets were selected from the GO term gene sets, based on usage of the word "neutrophil" in the gene set name. Gene sets with less than 5 genes in the set of selected genes (based on expression, see above) were excluded from the analysis. Analyses were conducted with the same linear model and contrasts as in the differential gene expression analysis (see above), and False Discovery Rates (FDR) were determined using the Benjamini-Hochberg method to adjust for multiple testing (see supplementary Table 3 with per gene set differential expression results for each contrast).[6]

SUPPLEMENTARY TABLES

	Explained variance (%)	Most significant cofactor	*P*-value
PC1	14,5	Tissue-of-origin	0,0004
PC2	10,1	Tissue-of-origin	0,0017
PC3	6,9	Tissue-of-origin	0,0084
PC4	6,6	Neonate (<30days)	0,0397
PC5	5,2	Tissue-of-origin	0,0016
PC6	4,7	RSV - status	0,0049
PC7	4,2	RSV - status	0,0208
PC8	3,6	N/A	
PC9	3,1	N/A	
PC10	2,6	N/A	

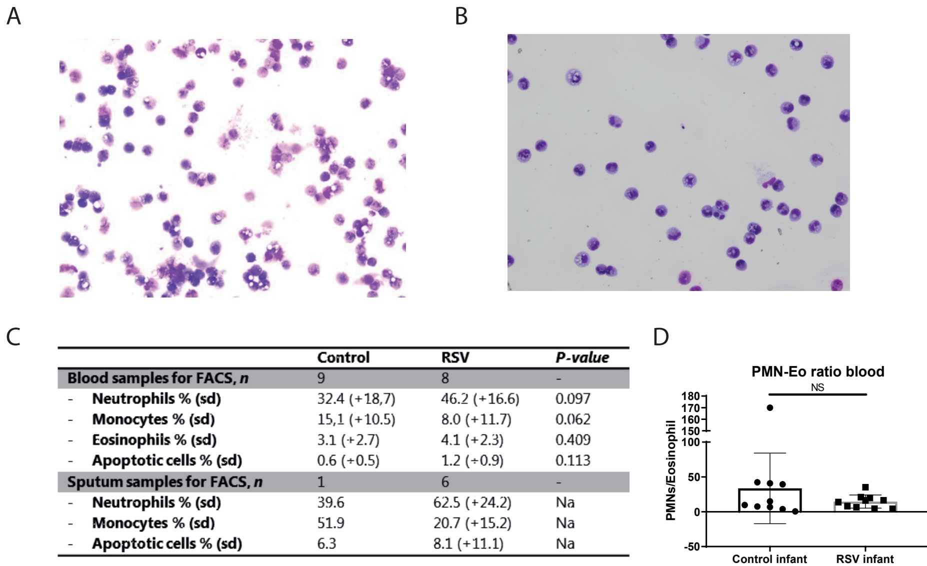
Supplementary Table 1
Explained variance and most significant cofactor associated with per principal component scores. TMM-normalized counts were used to assess global transcriptional profile differences of all samples by PCA (10 components) analysis. Values from each PC were checked for correlation to sample characteristics (i.e. sex, neonatal status, tissue-of-origin, bacterial infection and RSV infection) by the Mann-Whitney U test. Significant ($p<0.05$) co-factors are reported, in case of multiple significant cofactors, only the most significant one is reported.

Supplementary Table 2 and 3 can be found in the online supplement of the publication in Clinical Immunology. https://www.sciencedirect.com/science/article/pii/S1521661620307531?via%3Dihub

Supplementary Table 2
Result of differential gene expression analyses for these 5 contrasts: $^{\text{Ctrl-B}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{Ctrl-B}}$PMNs, $^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{RSV-S}}$PMNs, and a 2-way contrast (($^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs) versus ($^{\text{Ctrl-S}}$PMNs s $^{\text{Ctrl-B}}$PMNs)). Differential expression statistics for each contrast were obtained through the eBayes functionality of the limma package, False Discovery Rates (FDR) were determined using the Benjamini-Hochberg method to adjust for multiple testing. Results for all genes are reported here.

Supplementary Table 3
Result of differential gene set expression analyses for these 5 contrasts: $^{\text{Ctrl-B}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{Ctrl-B}}$PMNs, $^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs, $^{\text{Ctrl-S}}$PMNs vs $^{\text{RSV-S}}$PMNs, and a 2-way contrast (($^{\text{RSV-S}}$PMNs vs $^{\text{RSV-B}}$PMNs) versus ($^{\text{Ctrl-S}}$PMNs s $^{\text{Ctrl-B}}$PMNs)). Gene set enrichment testing was performed with CAMERA for the hallmark (H), canonical pathway (C2-CP) and GO term (C5) gene set collections from the Molecular Signatures Database. Only neutrophil associated gene sets were selected from the GO term gene sets, based on usage of the word "neutrophil" in the gene set name. False Discovery Rates (FDR) were determined using the Benjamini-Hochberg method to adjust for multiple testing. Results for all gene set are reported here.

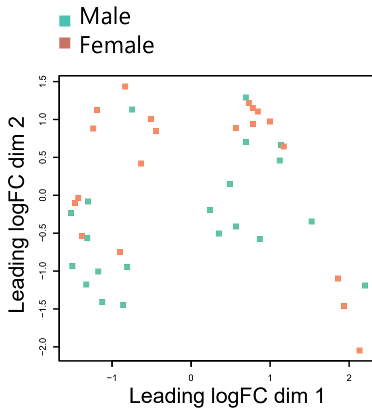
SUPPLEMENTARY FIGURES



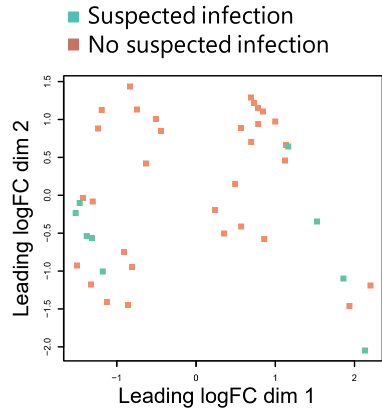
	Control	RSV	*P-value*
Blood samples for FACS, *n*	9	8	-
- **Neutrophils % (sd)**	32.4 (+18,7)	46.2 (+16.6)	0.097
- **Monocytes % (sd)**	15,1 (+10.5)	8.0 (+11.7)	0.062
- **Eosinophils % (sd)**	3.1 (+2.7)	4.1 (+2.3)	0.409
- **Apoptotic cells % (sd)**	0.6 (+0.5)	1.2 (+0.9)	0.113
Sputum samples for FACS, *n*	1	6	-
- **Neutrophils % (sd)**	39.6	62.5 (+24.2)	Na
- **Monocytes % (sd)**	51.9	20.7 (+15.2)	Na
- **Apoptotic cells % (sd)**	6.3	8.1 (+11.1)	Na

Supplementary Figure 1 (A) Cytospin and May Grunwald staining of $^{RSV-S}$PMNs before sorting (magnification 200x). (B) May Grunwald Giemsa staining of freshly sorted neutrophils from sputum of two RSV patients (augmentation 200x). (C) In cases where there was sufficient material, we performed additional flow cytometry in parallel to the sorting. Cells were stained for surface markers for 20 min at 4°C. Cell types were identified according to their characteristic forward and side light-scatter properties, using the same gating strategy as was used for sorting, followed by additional gating for CD16. Whole blood derived neutrophils: CD14$^-$/ CD66b$^+$/CD16$^+$, monocytes: CD14$^+$/CD66b$^-$/CD16$^-$, eosinophils CD14$^-$/CD66b$^+$/ CD16$^-$ Sputum derived neutrophils: CD66b$^+$/ CD14$^-$, monocytes CD66b$^-$/CD14$^+$. 7-aminoactinomycin D (7-AAD) (5µl per sample, BD Bioscience) was used to distinguish apoptotic cells. Table showing percentages of leukocyte cell populations for all available samples as identified i). (D) Neutrophil – eosinophil ratio in blood of control and RSV infants. Percentage of neutrophils was divided by percentage of eosinophils per individual patient. Columns show mean (+SD). An unpaired t-test (Mann-Whitney test) was performed to compare expression between patient groups.

A

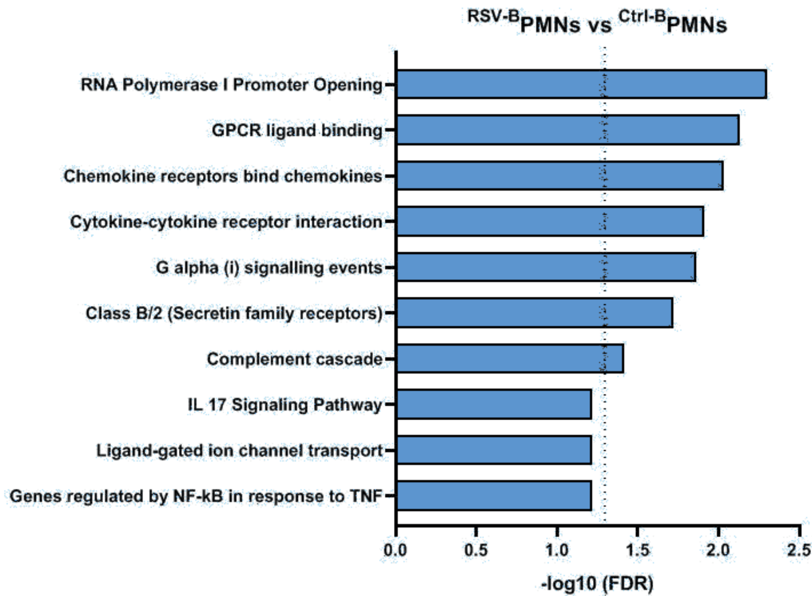


B



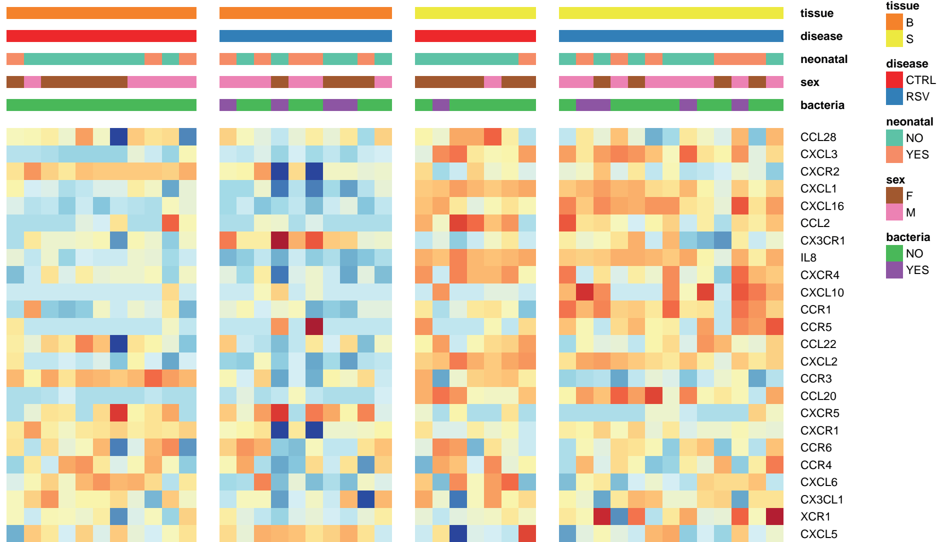
Supplementary Figure 2

MDS plot as depicted in Figure 1D of the main manuscript showing the distribution of the patient characteristics sex and bacterial infection. (A) Samples from males are indicated in green, from females in red. (B) Samples from patients that were suspected of having a pulmonary bacterial infection within 24 hours of sample collection are indicated in green, samples from patients without a bacterial pulmonary infection are depicted in red.



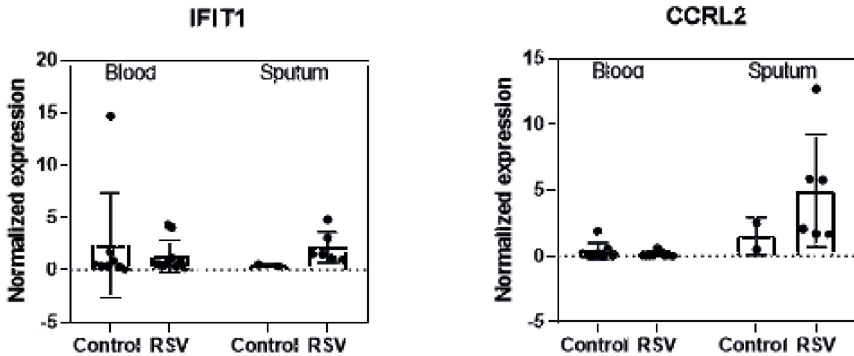
Supplementary Figure 3

Top 10 downregulated gene sets in $^{RSV-B}$PMNs compared to $^{Ctrl-B}$PMNs. FDR-values <0.05 were considered significant (indicated with the dotted vertical line).



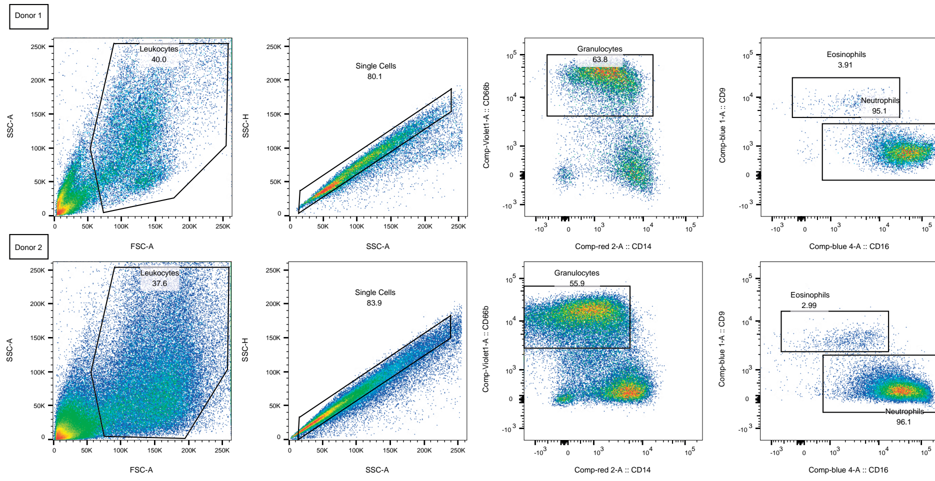
Supplementary Figure 4

Heatmap showing the gene expression in all samples of genes from the REACTOME dataset chemokine receptors bind chemokines. Gene expression levels are shown as row normalized Z scores with red representing higher expression and blue representing lower expression.



Supplementary Figure 5

Expression of Interferon stimulated genes (ISGs) relative to RPL32: IFIT1 and CCRL2, analysed by qPCR for $^{Ctrl\text{-}B}$PMNs (n=8), $^{RSV\text{-}B}$PMNs (n=10), $^{Ctrl\text{-}S}$PMNs (n=2), $^{RSV\text{-}S}$PMNs (n=6). Columns show mean (+SD). An unpaired t-test (Mann-Whitney test) was performed to compare expression between patient groups; none of the comparisons were significantly different.



Supplementary Figure 6

Flow cytometry plot of sputum cells with additional CD9 staining. Sputum was filtered twice and stained for surface markers. Cells were identified based on their characteristic forward and side scatter properties, single cells were subsequently selected and identified as follows: eosinophils as $CD9^{+}/CD66b^{+}$ and neutrophils as $CD66b^{+}/CD16^{+}$.

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3

Signal inhibitory receptor on leukocytes (SIRL)-1 and Leukocyte-associated immunoglobulin-like receptor (LAIR)-1 regulate neutrophil function in infants

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ABSTRACT

During severe respiratory syncytial virus (RSV) bronchiolitis there is a massive influx of activated neutrophils to the lungs. An exaggerated immune response contributes to lung damage and disease severity during RSV infection. We have previously shown that normal adult neutrophil function can be modulated by agonists of SIRL-1. Here we aimed to measure the potential of two immune checkpoints: SIRL-1 and LAIR-1, to regulate the function of fresh blood and sputum neutrophils from infants with and without severe RSV bronchiolitis. We show a modest inhibition of the oxidative burst through SIRL-1 and LAIR-1, in control and RSV-infected infants. In addition, SIRL-1 and LAIR-1 inhibited neutrophil extracellular traps (NET) formation by sputum neutrophils of RSV patients. Altogether our data show that inhibitory receptors LAIR-1 and SIRL-1 can be used to regulate neutrophil function.

1. INTRODUCTION

The most important viral pathogen, identified in lower respiratory tract infections in children, is respiratory syncytial virus (RSV). It causes a major global health burden, with a hospitalisation rate during infancy of around 3.2 million per year. Global mortality is estimated at 118.200–149.400 in children under five, of which 99% occurs in developing countries [1]. Despite decades of research, there is no effective treatment or globally accessible vaccine available [2].

During the acute phase of infection there is a massive influx of neutrophils to the lungs, and their effector functions have been shown to be activated by RSV ([3-8], Although neutrophils are key players in the first line of defence against foreign pathogens, their effector functions contribute to immune cell-mediated lung damage [3, 9-12]. The release of neutrophil extracellular traps (NETs), degranulation of toxic compounds, and the production of reactive oxygen species (ROS) all could contribute to their harmful potential, and induce immune pathology during pulmonary infections [13]. Hence, inhibiting neutrophil function might modulate the course of severe RSV bronchiolitis in a positive way.

One strategy to modulate the neutrophil response is ligation of inhibitory receptors, also known as immune checkpoints [13, 14]. Most inhibitory receptors contain tyrosine–based inhibition motifs (ITIMs), with few exceptions such as CD200R which signals via an NPXY motif [15]. Their main role is to keep our immune system in check, to prevent collateral damage induced by immune cells. Some pathogens exploit these receptors to evade the hosts' immune response [16, 17]. On the other hand, immune checkpoints can be used for therapeutic purposes, as has been successfully shown for cancer therapy, or to limit inflammation induced pathology [18-24].

Previous research showed that neutrophil function can be inhibited by several ITIM-bearing inhibitory receptors, such as leukocyte immunoglobulin-like receptor-(LILR) B2 and sialic acid-binding immunoglobulin-like lectins (SIGLECs) [18, 19, 25, 26]. Our group showed that neutrophil oxidative burst and NET formation by peripheral blood neutrophils can be inhibited by signal inhibitory receptor on leukocytes (SIRL)-1 [25, 27]. Additionally, *ex vivo* NET formation by neutrophils from the airways of RSV bronchiolitis patients is dampened by Leukocyte- associated immunoglobulin-like receptor (LAIR)-1 agonists [9]. *Lair1*$^{-/-}$ mice infected with RSV show enhanced airway inflammation, accompanied by increased neutrophil and lymphocyte influx, without effects on viral loads or cytokine production [28].

In this study we had unique access to fresh neutrophils from the airways of a large cohort of infants with life-threatening RSV infection and controls. We investigated whether ligation of SIRL-1 or LAIR-1 can be used to inhibit oxidative burst and NET-formation by sputum neutrophils from infants with severe RSV bronchiolitis.

2. METHODS

2.1 Patients

To study expression and function of SIRL-1, LAIR-1 and CD200R on blood and airway infiltrated neutrophils, during acute RSV infection, we included a cohort of children <2 year who were in need of invasive mechanical ventilation (Neon study). The RSV group consisted of children with PCR proven RSV bronchiolitis. As a control group we included children that were admitted and mechanically ventilated for other reasons than (respiratory) infections. Healthy adult donors were used when indicated. Exclusions criteria consisted of: severe comorbidity, such as any organ dysfunction, immune suppressed patients such as patients using immune suppressive therapy or receiving chemotherapy, any severe congenital or acquired abnormality. One year after the start of the study we only had limited numbers of control children, therefore we extended our inclusion criteria and agreed to include children with cardiac abnormalities admitted to the PICU. The modification in inclusion criteria was approved by the medical ethical review board of our hospital. A maximum of 4ml of blood was drawn from a venous or arterial line. Tracheobronchial aspiration was obtained using a maximum of 2 ml of normal saline. Patient characteristics are summarized in table 1. We defined suspected bacterial pulmonary infection based on sputum cultures with bacterial growth >100 cfu (colony-forming unit). All sputum cultures were collected because of clinical suspicion (either fever or increased ventilator settings). The Parent Advisory Board, of our RSV research team, was involved in writing the research protocol and patient information letters. The study was approved by the Medical Ethical Review Board (review reference number NL58404.041.16) and parents of the participants provided written informed consent.

2.2 Flow cytometry

Whole blood was depleted from red blood cells, by lysis with ammonium chloride buffer, and stained for surface markers for 20 min at 4°C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) bovine serum albumin (BSA). Sputum samples were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated Fetal Calf serum (FCS) and filtered twice using a 70µm nylon filter (BD Falcon). Cell types were identified according to their characteristic forward and side light-scatter properties, and by their typical cell surface markers. Whole blood derived neutrophils: CD14-/ CD16+, monocytes: CD14+CD16-, sputum derived neutrophils: CD66b+/ CD14-. Neutrophil activation was examined by surface expression of CD11b and CD62L or CD66b. 7-aminoactinomycin D (7-AAD) (5µl per sample, BD Bioscience) was used to distinguish apoptotic cells. Antibodies were obtained from BD Bioscience (CD66b, LAIR-1, mIgG1 isotype control), BioLegend (CD16, CD62L, mIgG1 isotype control), eBioscience (CD14, CD200R), or were

produced by our own laboratory as described previously (SIRL-1) [29]. Monocytes were extracted from sputum cells using a CD14-positive selection MACS kit (Miltenyi Biotec, Auburn, CA, USA).

2.3 Neutrophil isolation

Human neutrophils were isolated from venous blood by density gradient centrifugation with Ficoll (Amersham Biosciences). After centrifugation red blood cells were lysed with ammonium chloride buffer and neutrophils were re-suspended in RPMI-1640 medium supplemented with 10% heat- inactivated FCS.

2.4 Measurement of NADPH oxidase mediated ROS production

ROS production was measured in isolated primary neutrophils from whole blood, sputum cells, isolated monocytes from whole blood, or sputum cells depleted from monocytes. All experiments were performed using technical duplicates. NADPH oxidase activity was assessed as published previously [7]. In short, H2O2 formation was determined using Amplex Red (Molecular Probes, Breda, the Netherlands). Formation of H2O2 was measured directly from the start of stimulation, and continued for 60 minutes (PMNs) or 90 min (sputum cells). Fluorescence was measured at 1 min intervals (λEx/ λEm = 545/ 590 nm). We used 0.25 × 10^5 cells per well and stimulated with 4 μg/mL plate-bound anti-FcγRII (aCD32, StemCell technologies, clone IV.3) together with 5 μg/mL plate-bound anti-SIRL-1 F(Ab)2 (clone 1A5), anti-LAIR-1 F(Ab)2 (clone 8A8) or mIgG1,k F(ab)2 (Southern Biotech, clone 15H6). Incubation of cells in PBS-coated wells was used to measure background H2O2 production. We used 10μg/ml diphenyleneiodonium (DPI, Sigma) to inhibit ROS-production as a positive control. At 40 minutes almost all samples reached the maximum detection limit. The extent of inhibitory receptor-mediated inhibition of ROS production was calculated by calculating the area under the curve (AUC) until the time point of 40 minutes.

2.5 Measurement of NET formation

The protocol was based on methods that were published previously by our group [27, 30]. Sputum derived cells were washed twice with RPMI-1640 (without phenol red) supplemented with 2% FCS, 50 U/ml Penicillin-Streptomycin, and 10 mM HEPES (referred to as RPMI-pr 2% hereafter). Sputum derived cells were stimulated with 10μg/ml anti-SIRL-1, anti-LAIR-1 or an isotype control and incubated with RPMI-pr 2% containing 20 μM Hoechst 33342 for 30 min on ice. Followed by cross linking with secondary F(ab')2 goat anti-mouse IgG (20 μg/mL, Southern Biotech) for 15 min on ice. A total of 5 × 10^4 neutrophils were seeded on 0.001% poly-L-lysine (Sigma Aldrich) pre-coated wells of a clear bottom 96-wells plate (Ibidi) and incubated at 37˚C with 5% CO2 for 4 hours with 10nM of SYTOX Green (Life Technologies). After 4 hours cells were fixed using PFA 2% in

PBS. SYTOX Green fluorescence was measured with wide-field microscopy at 100-fold magnification (Pathway 855, BD). Depending on the number of available cells, all experiments were done in duplicate or triplicate.

2.6 NET quantification approach

SYTOX Green was used to stain extracellular DNA to visualize NETs. Total SYTOX Green positive area was quantified for 20 fields per condition per experiment and were analysed with ImageJ (NIH) as described in a previous study performed by our group [18]. In this study, citrullinated histone H3 was only present in Sytox Green particles with a surface above 68 µm2, confirming they were NETs. Therefore, particles smaller than 68µm2 were excluded to correct for apoptotic cells. Since in some cases there was debris skewing the SYTOX Green positive area we used the median of 20 fields per well to calculate SIRL-1 and LAIR-1 induced inhibition. The median total SYTOX Green positive area of medium control was set at 100%, and we calculated the percentage SYTOX Green positive area of anti-SIRL-1, anti-LAIR-1 and isotype control antibody, relative to medium.

2.7 Statistics

Baseline characteristics were calculated using a parametric unpaired t-test. Categorical data were compared with the Fisher's exact test. All comparisons between SIRL-1, LAIR-1 or CD200R antibodies and controls were calculated using a parametric paired t-test. Statistical significance of differences in measurements between groups of patients, or between sputum and blood, was analysed with the unpaired parametric t-test. The correlation between SIRL-1 expression and inhibition of oxidative burst was calculated using a Spearman test. Statistical analysis was performed with GraphPad Prism software version 8. P values <0.05 were considered significant.

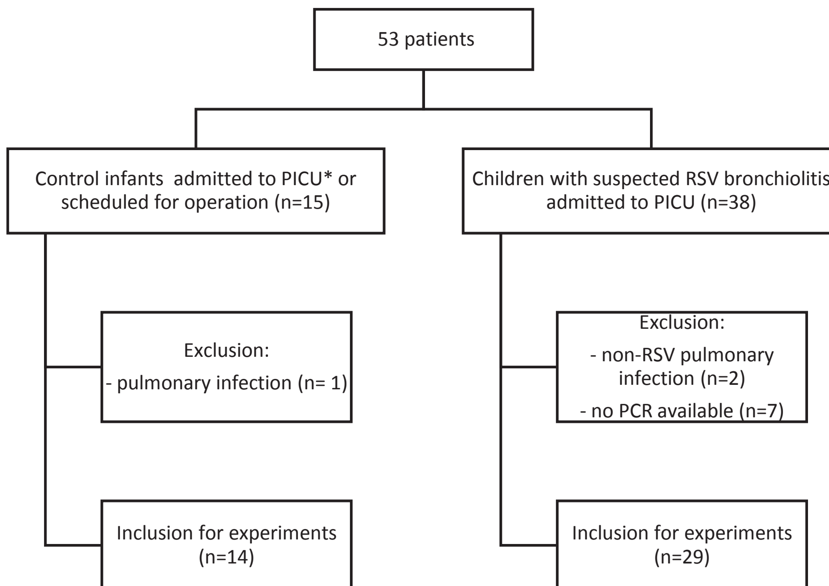
3. RESULTS

3.1 Clinical characteristics

Between November 2016 and January 2018, a total of 53 patients under two years of age, requiring intubation and mechanical ventilation, were included in our study. The study population consisted of two groups: 38 patients with severe RSV bronchiolitis and 15 control patients who were admitted for other reasons than pulmonary tract infection. Ten were excluded from analysis: in the control group there was one patient with a suspected bacterial pulmonary infection, in the RSV group there were two patients with a non-RSV pulmonary tract infection, and for seven cases there was no PCR for RSV performed. In the final analysis there were 14 control infants and 29 patients with severe RSV bronchiolitis (Fig. 1). Severity of RSV disease was defined as admission to the

paediatric intensive care unit (PICU) for invasive mechanical ventilation. All RSV patients were included within 48 hours after admission to the PICU.

The mean age was 199 days for the infants in the control group, and 53 days for the infants in the RSV group. In the RSV group 55% of patient had a suspected bacterial superinfection defined as a positive sputum culture. Flow cytometric analysis of sputum cells showed comparable numbers of neutrophils in the control and RSV group (Table 1) Cytospin and May-Grünwald staining of a representative sputum sample indicated the same distribution of cells (Fig. 2A). Baseline characteristics are depicted in table 1.



*PICU: pediatric intensive care unit.

Fig. 1: Study flowchart showing the included and excluded participants.

3.2 Distinct inhibitory receptor expression by airway infiltrated neutrophils.

First, we characterised surface expression of the ITIM-bearing inhibitory receptors LAIR-1, SIRL-1 and the non-ITIM-bearing CD200R on blood neutrophils of control and RSV infants (Fig. 2B-C). Both SIRL-1 and LAIR-1 showed higher expression on circulating neutrophils derived from patients with RSV bronchiolitis compared to controls (Fig. 2D-E). In contrast, CD200R expression was decreased in RSV patients compared to controls (Fig. 2F).

In infants with severe RSV bronchiolitis, we compared surface expression of LAIR-1, SIRL-1 and CD200R between blood and sputum derived neutrophils. SIRL-1, LAIR-1 and CD200R were expressed by circulating neutrophils (Fig. 2). As was reported previ-

Table 1: Characteristics of participants

	Control group (n=14)*	Severe RSV bronchiolitis (n=29)	p-value
Clinical information			
Sex, male (%)	6 (40%)	19 (65%)	ns
No. preterm infants	0	1	ns
Age on admission, mean days (SD)	198.6 (+176.1)	53.1 (+53.1)	P<0.005
CRP at inclusion	25 (+4)	71.8 (+54.2)	ns
No. suspected bacterial pulmonary infection (SD)	0	16 (55%)	P<0.005
Sputum characteristics			
No. of samples	2	21	
% neutrophils (SD)	65.8 (+4.5)	61.8 (+19.3)	ns
% monocytes (SD)	16.7 (+5.8)	13.2 (+8.8)	ns

Note: SD: standard deviation. * Aetiology of diseases in control group: general surgery (Abdominal cyst removal (2x), inguinal hernia (2x)), cardiac surgery (Nikaidoh procedure, correction of coarctatio aortae, atrial septal defect closure, Tetralogy of Fallot correction, mitral valve replacement, ventricular septal defect closure, correction of interrupted aortic arch type A.), other (button cell battery ingestion, supraglottic stenosis, vascular ring).

ously [27], LAIR-1 surface expression on sputum neutrophils was increased compared to circulating neutrophils. Likewise, surface expression of CD200R was upregulated on sputum neutrophils. Only SIRL-1 was down-regulated by almost 70% (Fig 2D-F). Sputum neutrophils displayed high expression of CD66b and low expression of CD62L, indicative of cellular activation (Fig. 2G-H). In control infants there were only two sputum samples available for flow cytometry (data not shown). Therefore, we did not compare expression between the two groups, or between blood and sputum in control infants. The second most present leukocyte population in the lung consisted of monocytes. The surface expression profile of inhibitory receptors on monocytes was more heterogeneous compared to neutrophils. In RSV patients, sputum monocytes showed no significant difference in LAIR-1 expression between blood and sputum monocytes. There was a slight decrease in SIRL-1 expression and an increase in CD200R expression compared to circulating monocytes (Suppl. Fig. 1). In conclusion, we show distinct inhibitory receptor expression on circulating and airway leukocytes during severe RSV bronchiolitis.

3.3 SIRL-1 inhibits ROS production by normal infant neutrophils

Previously we have shown SIRL-1 dependent ROS inhibition in adults. Here we tested SIRL-1 mediated inhibition of oxidative burst in control infants [20]. Simultaneous SIRL-1 and FcγR stimulation inhibited ROS production compared to FcγR stimulation in the presence of isotype control antibody, by polymorphonuclear neutrophils (PMNs), derived from healthy adult donors and control infants. LAIR-1 stimulation did not inhibit oxidative burst in adult donors, it did modestly in control infants (Fig. 3A-D).

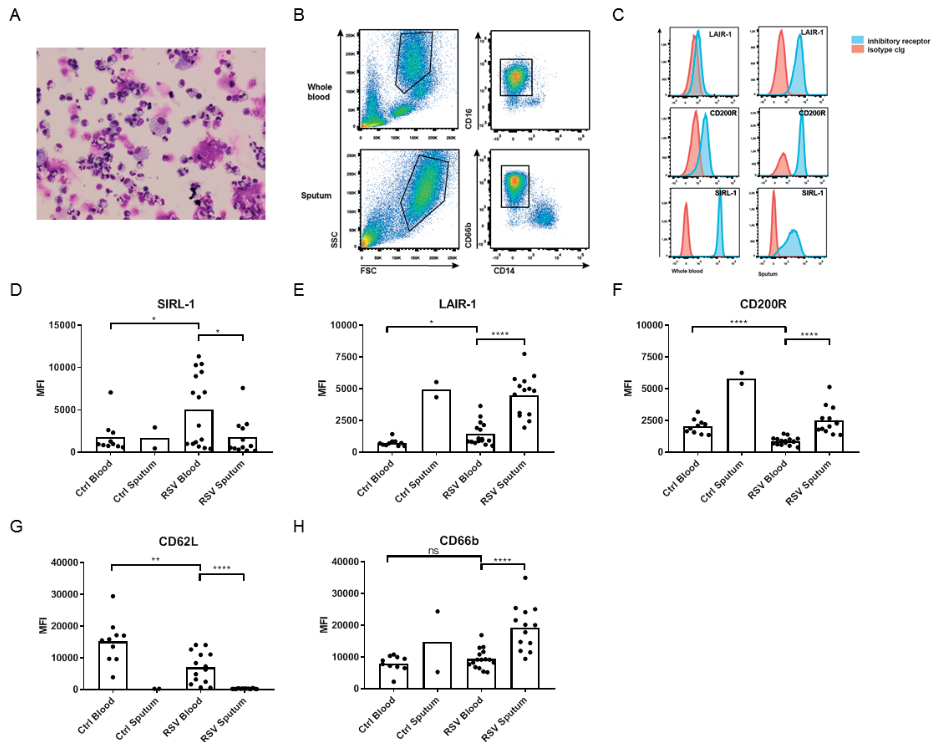


Fig. 2: Circulating and airway infiltrated neutrophils from RSV patients express SIRL-1, LAIR-1 and CD200R. Cytospin of sputum sample of an RSV patient shows mainly neutrophils (magnification 200x) (A). Cells were stained for cell lineage and activation markers, and a panel of inhibitory receptors. Neutrophils were gated based on their forward and side scatter and further characterised as CD14-/ CD16+ staining for circulating neutrophils, and CD66b+/CD14- for sputum neutrophils. Flow cytometry plots of a representative donor of blood and sputum cells from a patient with severe RSV bronchiolitis is shown (B). Blood and sputum neutrophils are compared for expression of SIRL-1, LAIR-1, and CD200R to isotype control-Ig (cIg), a representative RSV patient is shown (C). Primary neutrophils from blood and sputum are compared for expression of inhibitory receptors per patient group; control infants and RSV bronchiolitis patients. The plot shows mean MFI of SIRL-1, LAIR-1, and CD200R, each dot represents an individual patient (D-F). Blood and sputum neutrophils were stained for activation markers CD62L and CD66b, expression was compared between both groups (G-H). Ctrl blood n = 8, ctrl sputum n= 2, RSV blood n = 16, RSV sputum n = 13. A paired t-test was performed to compare expression between samples. An unpaired t-test was performed to compare expression between patient groups and between blood and sputum samples * $p = 0.05$, ** $p = <0.01$, *** $p < 0.001$ **** $p <0.0001$.

3.4 SIRL-1 and LAIR-1 inhibit ROS production by blood and airway infiltrated neutrophils from infants with severe RSV.

Blood and sputum from children admitted with severe RSV bronchiolitis were collected and oxidative burst was measured. Blood and sputum derived neutrophils from infants with RSV bronchiolitis showed higher background ROS release (Fig. 4A, B). Stimulation with intact SIRL-1 and LAIR-1 antibodies inhibited ROS production in non-stimulated and FcR-stimulated circulating neutrophils and sputum cells. (Suppl. Fig. 2). To exclude that this was due to Fc-mediated stimulation by intact antibodies of the isotype control,

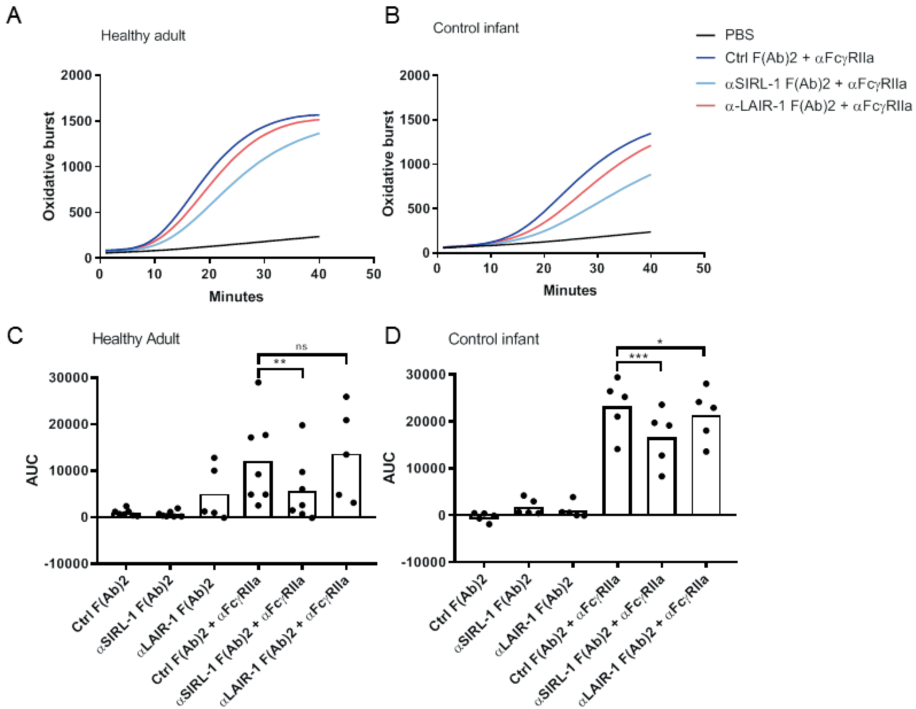


Fig. 3 SIRL-1 reduces anti-Fc gamma RII induced ROS production by circulating neutrophils in adults and children. PMNs from seven healthy adult donors and five infant controls were stimulated with anti-Fc gamma RII and anti-SIRL-1 F(Ab)2, anti-LAIR-1 F(Ab)2 or control F(Ab)2. The plots are a representative of one donor per group (A-B). After stimulation with anti-Fc gamma RII and anti-SIRL-1 F(Ab)2, anti-LAIR-1 F(Ab)2 or control F(Ab)2, background of unstimulated samples was subtracted and area under the curve was subsequently calculated for all samples. A paired-sample t-test was used to compare ROS production after stimulating FcγRIIa with anti-SIRL-1, anti-LAIR-1 F(Ab)2 or F(Ab)2 control. Each dot represents an individual patient, horizontal bar indicates mean. (C-D). * =p <0.05 ** =p <0.01 *** = p < 0.001

we manufactured F(Ab')2 for further experiments. Also with F(Ab')2 fragments we were able to inhibit FcR-induced oxidative burst by SIRL-1 in both sputum and blood derived neutrophils (mean 25% and 38.2% respectively), (Fig. 4C,D). Since sputum cells have increased surface expression of LAIR-1 and CD200R, we investigated whether we could inhibit ROS production by sputum cells through these receptors. Concomitant stimulation with aLAIR-1 F(Ab)2 did indeed inhibit ROS production by 12.3 and 14,7% in sputum and blood respectively. In contrast, CD200R did not inhibit FcR induced oxidative burst (Fig 4E-F).

Sputum derived cells also release ROS without additional stimulation with an FcR-ligand. Therefore, we used non-stimulated sputum cells, together with aSIRL-1-F(Ab)2 or aLAIR-1-F(Ab)2. Ligation of SIRL-1 or LAIR-1 with F(Ab)2 did not decrease ROS-production compared to isotype control in non-stimulated sputum cells (data not shown). Likewise, when using an antibody that targets a different epitope of LAIR-1 (clone DX26)

or combining both clones did not inhibit ROS production (data not shown). To confirm whether ROS production could be reduced in non-stimulated sputum cells we used diphenyleneiodonium (DPI), a known inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) as a positive control which abrogated ROS production completely (Fig. 4G).

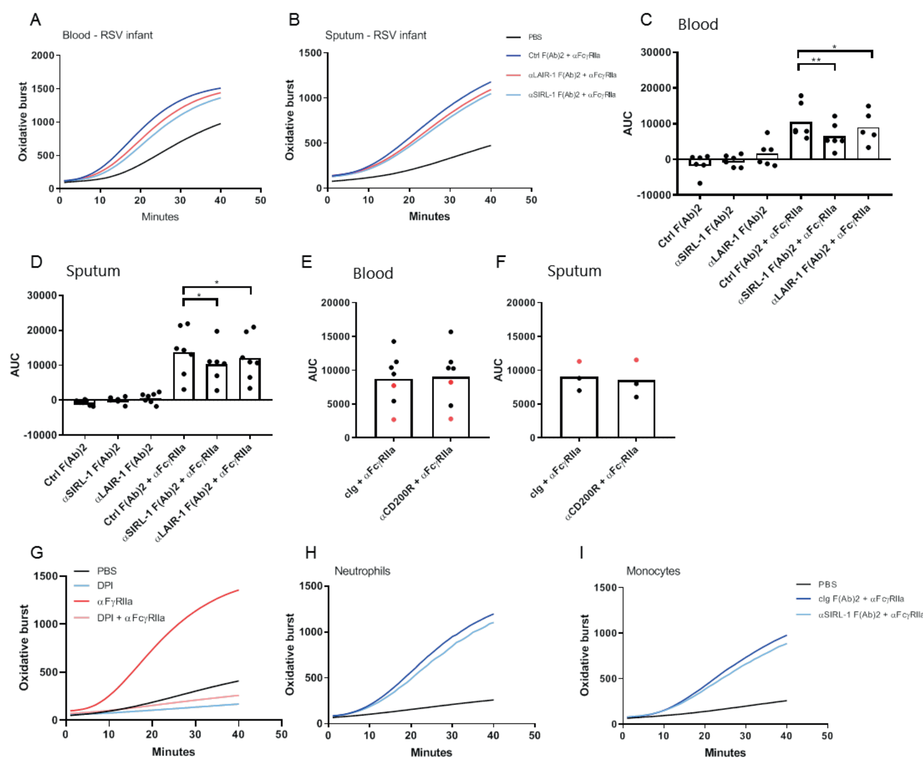


Fig. 4: SIRL-1 and LAIR-1, but not CD200R, reduce anti-Fc gamma RII induced ROS production in circulating and sputum neutrophils derived from children with severe RSV bronchiolitis. Primary blood derived neutrophils from six infants with severe RSV were stimulated with anti-Fc gamma RII and anti-SIRL-1 F(Ab)2, anti-LAIR-1 F(Ab)2 or isotype-matched control F(Ab)2. One donor is shown (A). Sputum cells obtained from seven infants with severe RSV bronchiolitis were stimulated with and without anti-Fc gamma RII and anti-SIRL-1 F(Ab)2, anti-LAIR-1 F(Ab)2 or control F(Ab)2. The plot is a representative of one donor (B). After stimulation with anti-Fc gamma RII and anti-SIRL-1 or F(Ab)2, anti-LAIR-1 F(Ab)2 or isotype-matched control F(Ab)2, background of unstimulated samples was subtracted and area under the curve was subsequently calculated for all samples for blood neutrophils (C) and sputum samples(D). Circulating (E) and sputum (F) neutrophils from three and seven infants respectively with severe RSV were stimulated with anti-Fc gamma RII and anti-CD200R Ab or isotype-matched control Ab. After stimulation, background of unstimulated samples was subtracted and area under the curve was subsequently calculated for all samples (E-F). Due to limited number of participants we also included the suspected RSV bronchiolitis patients (n= 1 in the PMN group and n=2 in the sputum group (in red). Sputum cells were stimulated with and without DPI. A representative of four donors is shown (G). Sputum cells were deprived from monocytes by MACS sorting. CD14 negative (H) and CD14 positive fraction (I) was stimulated with anti-Fc gamma RII and aSIRL-1 F(Ab)2 or control F(Ab)2. One representative donor of 2 experiments is shown (n=2). A paired samples t-test was used to compare ROS production between samples. * = $p<0.05$ ** = $p<0.01$

Given that monocytes are the next largest population, in sputum from RSV patients, we wondered whether this could influence oxidative burst measured in sputum cells. Therefore, we performed a ROS assays on sputum cells depleted from monocytes with CD14-beads, in two donors. CD14-negative and CD14-positive cell fractions showed a similar effect of SIRL-1 mediated inhibition (Fig. 4H-I). There was no significant correlation between SIRL-1 surface expression and inhibition of ROS production in RSV patients and control infants (Suppl. Fig. 3). Together, this data demonstrates that SIRL-1 and LAIR-1 inhibit FcR-mediated ROS production in polymorphonuclear cells derived from both blood and sputum of children with severe RSV bronchiolitis.

3.5 SIRL-1 and LAIR-1 signalling control NET formation by sputum cells in RSV bronchiolitis patients

To confirm previous data by our group we investigated the regulation of NETosis by SIRL-1 and LAIR-1 using a recently published method [30]. We confirm that airway infiltrated neutrophils from severe RSV patients release NETs without additional *ex vivo* stimulation when incubated for 90 minutes *in vitro* (movie 1 and 2). SIRL-1 and LAIR-1 agonistic antibodies modestly inhibited this spontaneous NET-formation by airway neutrophils of children with RSV bronchiolitis (Fig. 5 A-B).

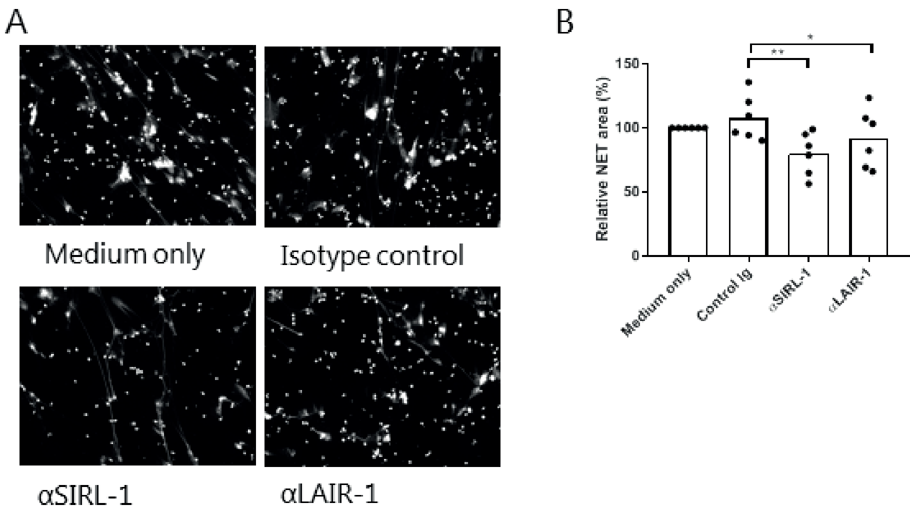


Fig. 5: Stimulation of SIRL-1 and LAIR-1 inhibits NET formation by sputum derived neutrophils in patient with severe RSV bronchiolitis.

Primary immune cells were obtained from sputum of six RSV bronchiolitis patients and incubated in vitro with anti-SIRL-1, anti-LAIR-1, an isotype-matched control antibody, or medium only. LAIR-1, SIRL-1, and isotype-matched control antibody were cross-linked with secondary goat anti-mouse Ig F(ab')2 fragments. SYTOX Green was used to stain extracellular DNA to visualize NETs. Samples were fixed using PFA 2% after 4 hours' incubation. Images (100x) of a representative patient is shown (A). Total SYTOX Green positive area, was quantified for 20 fields per condition per experiment and normalized to medium control for six patients. Each dot represents an individual patient, horizontal bar indicates mean (B). A paired samples t-test was used to compare NET production between samples. *= p <0.05 ** = p <0.01

4. DISCUSSION

RSV is a major global health burden, especially in infants [1, 31]. To date there is no effective treatment available. In this study we focussed on neutrophils because during RSV bronchiolitis there is a massive neutrophil influx to the lungs. Accumulated evidence supports the idea that this excessive neutrophil influx and neutrophil response, in the form of ROS production, the release of NETs and microbicidal compounds, leads to immune cell mediated pathology during infectious and inflammatory processes [9, 32-36]. Previous studies proposed that inhibitory immune receptors could function as regulators of innate immune responses during infections [14, 24, 37, 38]. Modulating the neutrophil immune response, by targeting inhibitory receptors, offers an attractive treatment strategy to reduce immune mediated damage during RSV bronchiolitis.

In this study we focused on the role of inhibitory receptors SIRL-1, LAIR-1 and CD200R expressed by circulating and airway infiltrated neutrophils in infants. There was a minimal increase in surface expression of SIRL-1 on circulating PMNs of RSV patients, compared to control infants. This is surprising because we previously showed a decrease in SIRL-1 expression after *in vitro* stimulation with TNF-alpha [20]. Furthermore, LAIR-1 surface expression is increased on circulating neutrophils from RSV patients compared to controls. LAIR-1 is stored in cytoplasmatic granules of neutrophils, and expression on the cell surface is low or absent. Upon inflammatory stimuli these granules are released, and LAIR-1 is expressed on the cell surface. [27]. Apparently, systemic inflammation in RSV patients initiates cell surface expression of LAIR-1 on neutrophils before neutrophils enter the lung. In line with previous studies we see a further increase in LAIR-1 expression on sputum neutrophils, while SIRL-1 expression is downregulated on sputum neutrophils. The decrease in SIRL-1 expression could be the result of SIRL-1 shedding from the cell membrane or a downregulation of transcription of the *vstm1*-gene. Indeed, when comparing *vstm*-1 mRNA expression in sputum and blood, a two-fold decrease in *vstm*-1 mRNA expression in sputum compared to blood in patients with severe RSV bronchiolitis can be seen (data unpublished).

In this study, we prove that cross-linking SIRL-1 inhibits Fc induced ROS production by circulating neutrophils in both healthy adults and control infants. We previously found similar expression levels of most inhibitory receptors for both neonates and adults. Only LAIR-1 surface expression is known to be increased on circulating neutrophils from neonates opposed to adults [39]. This might explain the modest LAIR-1 mediated inhibition of ROS production in control infants and not in adults. Moreover, control infants were not healthy, in contrast to the control adults, therefor a difference in inflammatory state, due to the underlying condition, might influences differences we found between adults and infants.

The percentage of oxidative burst inhibited by SIRL-1 and LAIR-1 in RSV patients is lower compared to control infants. This might be caused by several factors. Firstly, downregulation of SIRL-1 on sputum neutrophils reduces the capability of inhibition by this receptor. In our study SIRL-1 expression did not show a significant correlation to percentage inhibition of oxidative burst (Suppl. Fig. 3). However, the number of studied subjects is small and therefor this conclusion has to be taken with caution. Secondly, background ROS production is much higher in RSV infants than in control infants in both blood and sputum. This is most likely the result of an increased activated status of circulating and airway infiltrated neutrophils from RSV patients [40]. This is supported by our flow cytometry data, which showed increased CD66b and decreased CD62L surface expression on RSV neutrophils compared to circulating neutrophils from controls (Fig. 2G-H). Both markers are indicative of cellular activation [40]. Neutrophil migration into the airways causes phenotypical changes, such as altered receptor expression, which contributes to an altered response to stimuli *in vivo* and *in vitro* [41-43]. Lastly, neutrophil function can be altered dependent on inflammatory and infectious conditions. For instance, neutrophils show decreased respiratory burst and killing activity *in vitro*, after they transmigrated in the presence of a bacterial co-infection [44]. Overall, SIRL-1 was more potent than LAIR-1 in regulating neutrophil oxidative burst. Even when SIRL-1 was down regulated on sputum neutrophils in RSV patients, the expression was still similar to the expression on blood neutrophils from control infants. Possibly even limited SIRL-1 expression on neutrophils is sufficient to regulate neutrophil oxidative burst. As opposed to LAIR-1 and SIRL-1, there was no inhibitory effect on oxidative burst by ligation of CD200R. This could be due to the fact that CD200R uses different signalling pathways compared to the ITIM bearing receptors LAIR-1 and SIRL-1 [15]. CD200R may have an indirect effect on neutrophilic inflammation in pulmonary infections, as has been described for influenza and bacteria, and in this way play a role in maintaining immune homeostasis in the lung [45-48].

RSV virions are known to induce NETs in healthy donor neutrophils by triggering the classical ROS-dependent NETosis pathway [4]. Airway infiltrated neutrophils from severe RSV patients show spontaneous NET-release and a previous study by our group showed a significant decrease in NETosis using LAIR-1 antibodies [27]. In this study, we show modest SIRL-1 and LAIR-1 mediated inhibition of NETosis in sputum cells of RSV bronchiolitis patients. Surprisingly the effect of LAIR-1 on the control of NET-release was much lower than in our previous study. There are a few possible explanations for this difference in results. Firstly, the RSV population might be different from previous years; for instance, a different number of bacterial co-infections or a different type of genotype causing RSV. Several studies have shown that RSV genotypes might be related to disease severity [49, 50], and that bacterial co-infection influences neutrophil function [44]. Unfortunately, we do not have access to information on viral load or bacterial co-infection

of the previous study. Secondly, in the former study we used neutrophils collected via bronchial alveolar lavage (BAL). Neutrophils that are collected by BAL are mainly derived from the lower airways. These neutrophils might be functionally different from the neutrophils we collected in sputum, which consist mostly of neutrophils from the bronchi.

This study has potential limitations. Firstly, *in vitro* study of neutrophils may not reflect patterns of response *in vivo*. Secondly, there were additional pulmonary bacterial infections in the RSV group that were not present in the control group. This is a common finding in children admitted with RSV bronchiolitis in the PICU [51]. We analysed differences in surface expression of SIRL-1, LAIR-1 and CD200R between RSV patients with and without a suspected bacterial pulmonary infection. There was no significant difference between the groups (Suppl Fig. 4A-C). Considering the oxidative burst and ROS assay, a large proportion of patients was suspected of a bacterial superinfection but all showed inhibition of ROS-production and NET-formation (Suppl. Fig. 4D-F). While considering the limited numbers of patients in this study, our results indicate a potential role for SIRL-1 and LAIR-1 to inhibit neutrophil function in the presence of bacteria. Thirdly, due to practical limitations infants in the RSV group were younger than the control infants. Lastly, the control group consisted of eight patients with a cardiac disease of which three were known to have an oxygen saturation below 92% which might have influenced neutrophil function. Children with congenital heart disease who undergo cardiac surgery with cardiopulmonary bypass have a risk of developing systemic inflammatory response syndrome (SIRS) [52]. Also adults with congenital heart disease have increased inflammatory markers [53]. If anything, the neutrophils of these patients will have an inflammatory phenotype, and the differences we found between RSV patients and controls might be underestimated.

Overall, our results demonstrate that SIRL-1 and LAIR-1 inhibit ROS-production by sputum cells and blood derived neutrophils in infants with and without RSV bronchiolitis. Furthermore, SIRL-1 and LAIR-1 inhibit ROS-production in sputum cells from infants with severe RSV bronchiolitis. Finally, we show that SIRL-1 and LAIR-1 modestly inhibit NETosis by sputum cells from infants with severe RSV bronchiolitis. Future research should define the potential of synergy by combining antibodies to different inhibitory receptors, such as LAIR-1 and SIRL-1. We conclude that immune checkpoints on neutrophils are promising therapeutic targets to improve the outcome of one of the most important infections during infancy.

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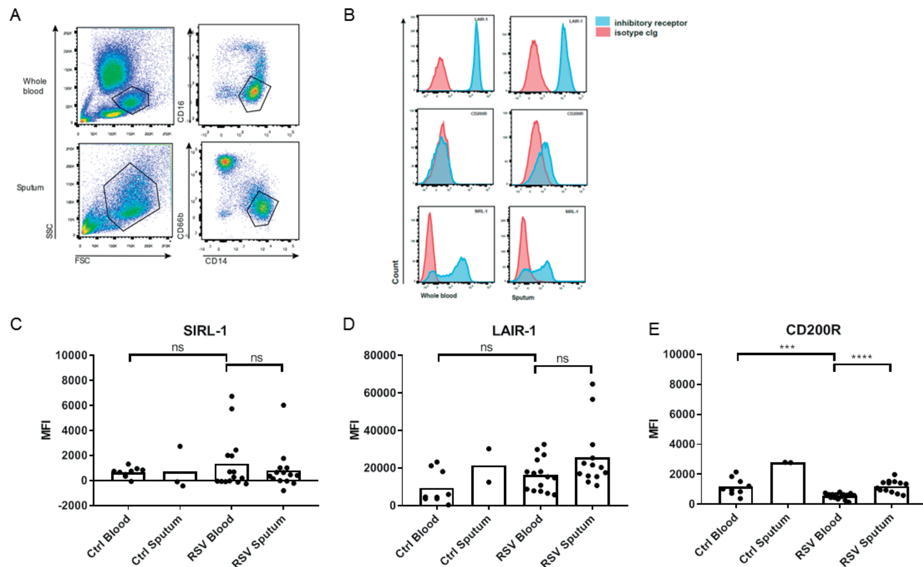
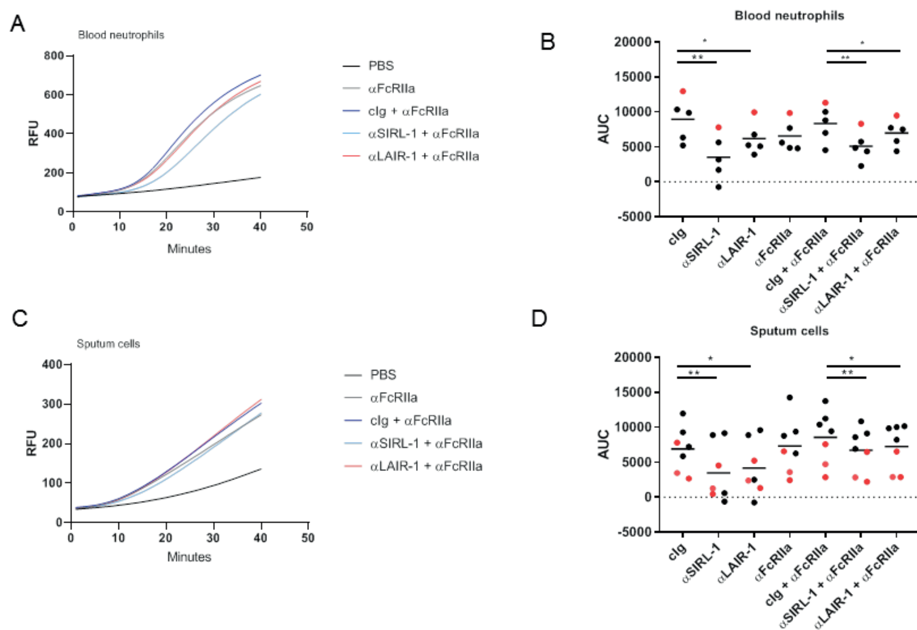
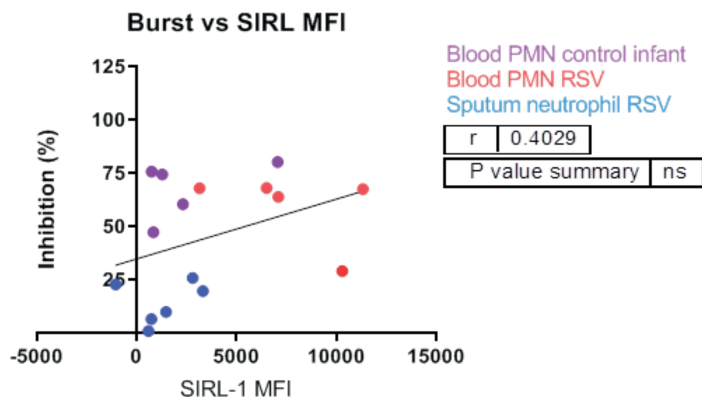
SUPPLEMENTARY FIGURES



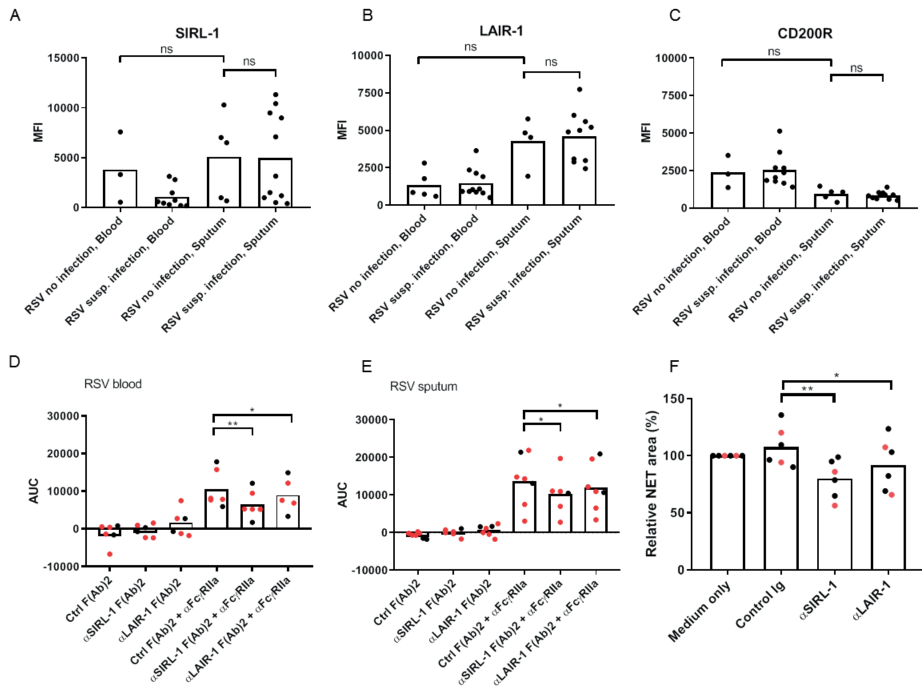
Suppl. Fig. 1: Sputum derived airway monocytes express LAIR-1, SIRL-1 and CD200R inhibitory receptors. Expression of inhibitory receptors on monocytes isolated from whole blood or sputum cells obtained from RSV patients. Monocytes were gated based on their forward- and sidelight scatter and further characterised as CD16-/CD14+ for peripheral blood monocytes or CD66b-/CD14+ for sputum monocytes (A). Cells were stained for a panel of inhibitory receptors. Flow cytometry plots of a representative donor of blood and sputum cells from a patient with severe RSV bronchiolitis is shown (B). Blood and sputum monocytes of control infants and RSV patients are compared for expression of LAIR-1, SIRL-1 and CD200R. Ctrl blood n = 8, ctrl sputum n= 2, RSV blood n = 16, RSV sputum n = 13 (C-E).
An unpaired t-test was performed to compare expression between patient groups and between blood and sputum samples * $p<0.05$, ** $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Note: compensation settings were set up using neutrophils. SRIL-1 surface expression on monocytes is lower than on neutrophils, therefor SIRL-1 MFI is negative for some of the samples.



Suppl. Fig. 2: SIRL-1 and LAIR-1 inhibit ROS production in blood and sputum cells from patients with severe RSV bronchiolitis. Circulating neutrophils and sputum cells obtained from infants with RSV bronchiolitis were stimulated with and without aFc gamma RII and anti-SIRL-1, anti-LAIR-1 or isotype-matched control antibody. The plots are a representative of both cell populations: circulating neutrophils (A) and sputum cells (C). After stimulation with anti-Fc gamma RII and anti-SIRL-1, anti-LAIR-1 or isotype matched antibody, background was subtracted, AUC was calculated for all samples (B, D). Due to limited number of participants we also included the suspected RSV bronchiolitis patients (red dots). A paired samples t-test was used to compare H2O2 production between samples * <0.05 ** = <0.01



Suppl. Fig. 3: SIRL-1 surface expression compared to percentage inhibition of ROS. Percentage ROS inhibition relative to isotype was calculated. SIRL-1 surface expression level (mean MFI) was compared to percentage inhibition of ROS for three groups: control PMNs (purple), RSV PMNs (red) and RSV sputum cells (blue). Spearman correlation was calculated. Ns = not significant.



Suppl. Fig. 4: SIRL-1 and LAIR-1 regulate neutrophil function in blood and sputum cells from patients with severe RSV bronchiolitis with, and without suspected pulmonary bacterial infection. Circulating neutrophils and sputum cells obtained from infants with RSV bronchiolitis were stained for inhibitory receptors, for gating strategy we refer to figure 1. Surface expression on both cell populations are shown for patients with and without suspected bacterial pulmonary infection (A-C). An unpaired samples t-test was used to compare samples * <0.05 ** = <0.01 Circulating neutrophils and sputum cells obtained from infants with RSV bronchiolitis were stimulated with and without aFc gamma RII and anti-SIRL-1, anti-LAIR-1 or isotype-matched control antibody. Oxidative burst was measured, and background was subtracted, AUC was calculated for all samples. The plots are a representative of both cell populations: circulating neutrophils (D) and sputum cells (E). A paired samples t-test was used to compare H2O2 production between all samples * <0.05 ** = <0.01. For NET analysis, primary immune cells were obtained from sputum of six RSV bronchiolitis patients and incubated in vitro with anti-SIRL-1, anti-LAIR-1, an isotype-matched control antibody, or medium only. LAIR-1, SIRL-1, and isotype-matched control antibody were cross-linked with secondary goat anti-mouse Ig F(ab')2 fragments. SYTOX Green was used to stain extracellular DNA to visualize NETs. Total SYTOX Green positive area, was quantified for 20 fields per condition per experiment and normalized to medium control for six patients. Each dot represents an individual patient, horizontal bar indicates mean (F). Patients with a suspected pulmonary bacterial infection are indicated in red. A paired samples t-test was used to compare conditions (all samples were included) * <0.05 ** = <0.01.

Supll. Movie 1 and 2: NET release by primary immune cells derived from sputum from an RSV patient show spontaneous NETosis. To access these movies scan the QR code:
https://www.sciencedirect.com/science/article/pii/S1521661619304383?via%3Dihub#ec0005

Primary immune cells were obtained from sputum of an RSV bronchiolitis patient. SYTOX Green was used to stain extracellular DNA to visualize NET's, Hoechst was used as a nuclear dye to indicate neutrophils (red). Cells were imaged using BD pathway and pictures were taken every 10 minutes during 4 hours. Movies of two representative donors are shown (n=3).

4

In vitro modeling of airway epithelium and neutrophil interaction during respiratory syncytial virus infection

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Work in progress

ABSTRACT

Introduction: Respiratory syncytial virus (RSV) is a major cause of severe viral airway disease in young children. Interaction between infected airway epithelial cells and neutrophils contribute to disease pathology. It is unclear what the exact mechanism is behind this interaction. To study the epithelial response to RSV, air-liquid interface cultured well-differentiated human bronchial epithelial cells (ALI-HBECs) are frequently used.

Methods: To increase our understanding of local neutrophil biology during RSV infection, we developed an *in vitro* co-culture model of RSV infected ALI-HBECs and freshly isolated polymorphonuclear cells (PMNs). RSV infected ALI-HBECs, in the presence and absence of PMNs were used to study viral replication, epithelial barrier integrity and epithelial immune responses.

Results: Our cultures showed a normal ALI-HBEC phenotype with typical airway cell differentiation. Infection with RSV-GFP showed productive infection in ciliated cells and induced IL-8 production. Infection did not reduce epithelial barrier integrity. Moreover, the presence of neutrophils during RSV infection did not induce epithelial damage.

Implications: Our study provides a RSV/ALI-HBEC/PMN co-culture model. This model offers the opportunity to further examine the local host response during RSV respiratory tract infection in a controlled setting.

Abbreviations:

ALI: air-liquid interface; FITC-D: 4-kDa fluorescein-conjugated dextran; dpi: day post infection; PMN: polymorphonuclear cell; RSV: Respiratory syncytial virus; TEER: Trans epithelial electrical resistance; HBEC: human bronchial epithelial cells.

INTRODUCTION

Respiratory syncytial virus (RSV) bronchiolitis is characterized by the development of extensive epithelial injury in the lungs of previous healthy infants.[1] RSV mainly infects the airway epithelium, specifically ciliated cells, which results in epithelial damage. This has been studied using *in vitro* cultures of non-differentiated airway epithelial cell lines, which showed that RSV infection causes cytopathology and barrier integrity dysfunction.[2, 3] However, continues cell lines are a poor representation of the complex composition of the human airway epithelium, which is composed of differentiated ciliated and secretory epithelial cells. Therefore, infection studies using more physiologically authentic airway epithelial cells cultures may increase our understanding of RSV bronchiolitis pathophysiology.[4-7] Air-liquid interface cultures of well-differentiated bronchial epithelial cells (ALI-HBEC) are more representative of the human airway epithelium *in vivo*. In this model, primary airway epithelial cells differentiate into pseudostratified epithelial cells including ciliated and secretory cells.[7] This model has been used by others to examine RSV infections, showing that ALI-HBEC maintained intact barrier integrity upon RSV infection.[4, 7-11] This suggest a lack of virus-induced epithelial damage, which is in line with observational studies in patients showing no correlation between RSV disease severity and viral titers.[12-14] Apparently, virus induced damage to the airway epithelium is limited, suggesting that other factors determine disease outcome. In addition to a direct effect of RSV on infected airway epithelial cells, it has been proposed that neutrophils may contribute to airway epithelial injury.[15-19] During the acute phase of RSV bronchiolitis there is an extensive infiltration of neutrophils to the lungs, mainly driven by IL-8 secretion by infected airway epithelial cells.[20-22] In *ex vivo* studies, using airway immune cells from severely RSV infected infants, it has been shown that airway infiltrated neutrophils display features of activation and degranulation upon interactions with RSV-infected airway epithelial cells.[23-25] We recently reviewed how this mechanism may induce epithelial injury due to the cytotoxicity of degranulated antimicrobial armaments. [26, 27] Based on this, we hypothesized that RSV induced activation of neutrophils causes damage to airway epithelial cells. To test this hypothesis, we aimed to set up an *in vitro* model of RSV infected ALI-HBEC co-cultured with neutrophils. This model was subsequently used to examine the contribution of neutrophils to the loss of airway epithelial barrier integrity during RSV infection.

METHODS

Culturing of air liquid interface differentiated primary human bronchial epithelial cells (ALI-HBEC)

Human bronchial epithelial cells (HBECs) were derived from three different adults lung donors. Basal cells were isolated and expanded in 5% collagen IV coated 6-wells culture plates with expansion medium. Culture medium was refreshed three times a week, until the cell cultures were confluent and transferred to ALI. Cells were seeded on 1% collagen coated 6.5 mm Transwell® with 0.4 µm Pore Polyester Membrane Inserts (Corning) in a density of 0,2x10^6 cells/insert in 200 µl apical, 800 µl basal differentiation medium. When confluent, typically after two days, the cells were air exposed by removing the apical culture medium. ALI HBECs were cultured for three to four additional weeks to obtain a complete differentiated epithelium. Culture medium was refreshed twice a week (Monday and Thursday or Tuesday and Friday), and the apical side of the cultures was washed with PBS once a week.

RSV infection of ALI-HBEC

ALI-HBEC cultures were washed with PBS and infected at the apical surface with RSV-GFP (A2 strain) at an ~MOI 1.0. Number of epithelial cells per filter was estimated at 300.000 cells to calculate MOI. Infections were conducted in duplicates and medium was used as mock control. Infected cells were incubated at 37 °C, 5% CO_2 for two hours. After incubation the apical surface was washed carefully with warm PBS. Cultures were kept in the incubator for the designated time points and medium in the basal compartment was changed three times a week. At the designated time points the apical surface washings were collected by rinsing the cells with medium for 30 minutes. Washings were subsequently stored at -80 until further use.

Purification of polymorphonuclear cells (PMNs)

Blood was obtained through the "blood donor service for scientific research" (*Mini donordienst*) of our hospital. Neutrophils were isolated from venous blood by density gradient centrifugation with Ficoll (Amersham Biosciences). After centrifugation red blood cells were lysed with ammonium chloride buffer (15 minutes on ice) and neutrophils were re-suspended in RPMI-1640 medium supplemented with 10% heat- inactivated FCS. Before adding neutrophils to the filters they were counted, washed and reconstituted to a concentration of 1x10E6/ml in optiMEM, 100µl was added per filter.

Determination of RSV infection

RSV replication in the apical surface washings was determined by measuring changes in Ct values by real time PCR at the National Institute for Public Health and the Environment (RIVM).

Airway epithelial barrier integrity assays

Airway epithelial cell damage was quantified by measuring the trans-epithelial electrical resistance (TEER) using an EVOM Voltcom meter. In addition to TEER measurements, cell permeability was determined of fluorescein isothiocyanate dextran (FITC-D) (Sigma 46944-100), with an average molecular weight of 4000. Permeability was measured by addition of 0.1 ml FITC-dextran in PBS (1 mg/ml) to the apical surface of the ALI-HBEC cultures. After 60 minutes of incubation, the medium in the bottom chamber was transferred to a black 96 wells plate. Each sample was run in triplicate and fluorescence was measured w at 485/535 nm with the Fluoroskan Ascent. Finally, we also measured epithelial sloughing. This was determined by counting the number of cells that detached from the membrane using a 4',6-diamidino-2-fenylindool (DAPI) stained cytospin of apical washes, counted using an EVOS microscope.

ELISA

Concentrations of IL-8 in apical and basal medium were measured conform manufacturers' protocol (Ready-Set-Go! (V2) (Ebioscience)).

Immunocytochemistry and fluorescence microscopy

ALI-HBEC was fixed with 200µl 4% paraformaldehyde (PFA), at room temperature (RT) for 15 minutes. For whole mount staining, ALI-HBEC was permeabilized with 200µl permeabilization buffer (PBS + 0,2% Triton) at RT for 30 minutes. Next, the cells were blocked with 200µl blocking buffer (PBS + 0,2% Triton + 0,1% BSA) at RT for one hour. After blocking, the cells were stained with 100µl staining buffer (blocking buffer + diluted primary antibodies) at RT for two hours. After incubation, the cells were washed and incubated with 100µl secondary staining buffer (blocking buffer + secondary antibodies) at RT for 30 minutes. Cells were washed two times with 150µl PBS. Primary antibodies: mouse anti-β-tubulin (IgG1), rabbit anti-MUC5AC (IgG), Mouse anti-cc10 (IgG1) (all 1:500). Secondary antibodies: Goat-aMouse AF488 and AF647 (IgG1), and goat-aRabbit AF 488 (IgG) (all Invitrogen). Images were taken with a Zeiss or Leica confocal laser microscope.

Statistics

Differences between Mock and RSV infected group were analyzed by unpaired t-test. P –value <0.05 was considered significant.

RESULTS

Characterization of ALI-HBEC cultures and RSV infections

First, we set up the ALI-HBEC cultures and RSV infection model (Fig. 1). To compare our ALI-HBEC to previously published models, we assessed the expression of airway epithelial cell differentiation markers and epithelial barrier integrity. Confocal microscopy showed the presence of ciliated, secretory club cells, while goblet cells were lacking, in line with a distal airway phenotype (Fig. 1B). The airway epithelial barrier integrity, as measured by TEER, was within the normal range (Fig. 1C).

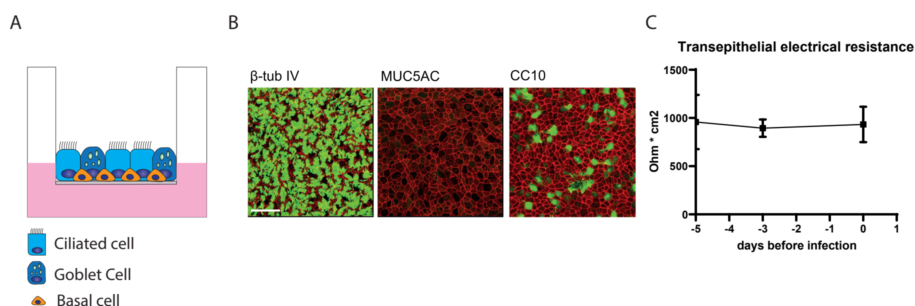

Figure 1. Well differentiated human bronchial epithelial cells cultured at an air liquid interface.
(A) Schematic model of well differentiated human bronchial epithelial cells cultured at air liquid interface (ALI-HBEC). (B) En face confocal images of ALI-HBEC stained for the cytoskeleton (red), and β-tubulin (green, left panel), $MUC5AC^{+}$ goblet cells (green, middle panel) and club cells (green, right panel). (C) Trans epithelial electrical resistance (TEER) was measured nine days after air exposure of ALI-HBEC. TEER was measured with a volt-ohm meter at 5, 3 and 0 days before RSV infection was initiated (= day 0). (n=1 experiment, 3 donors, 12 filters per donor were measured).

Next, ALI-HBEC cultures were infected with RSV-GFP A2 strain, ~MOI 1 (Fig. 2A). Infection was restricted to a subset of ciliated cells (Fig. 2B). RSV infection peaked at day seven and persisted until termination of the experiment at day 14 (Fig. 2C-D). In RSV infected cultures IL-8 secretion was increased by 6.8-fold on day five (p=0.06), and by 2.7-fold on day seven post infection (p=0.01) (Fig. 2E), this is in line with previous *in vitro* and *in vivo* studies showing epithelial innate immune responses upon RSV infection.[7, 18] During daily monitoring of infected ALI-HBEC we observed no leakage of medium to the upper chamber, suggesting no major cytopathic effects or culture deterioration over 14 days compared with uninfected controls. In line with this, the barrier integrity measured by TEER and FITC-D permeability was similar between mock and RSV infected cultures (Fig. 2F-G). Apical epithelial cell sloughing is a common finding during RSV infection, and previous studies with ALI-HBEC used this as a sign of cytopathic effects.[5, 7, 25] However, there was no significant difference in epithelial shedding between RSV infected and control cultures (Fig. 2H). Altogether, our data confirm previous studies showing that RSV can infect well-differentiated ALI-HBEC cultures, induces IL-8 release, but does not induce a loss of epithelial barrier integrity.

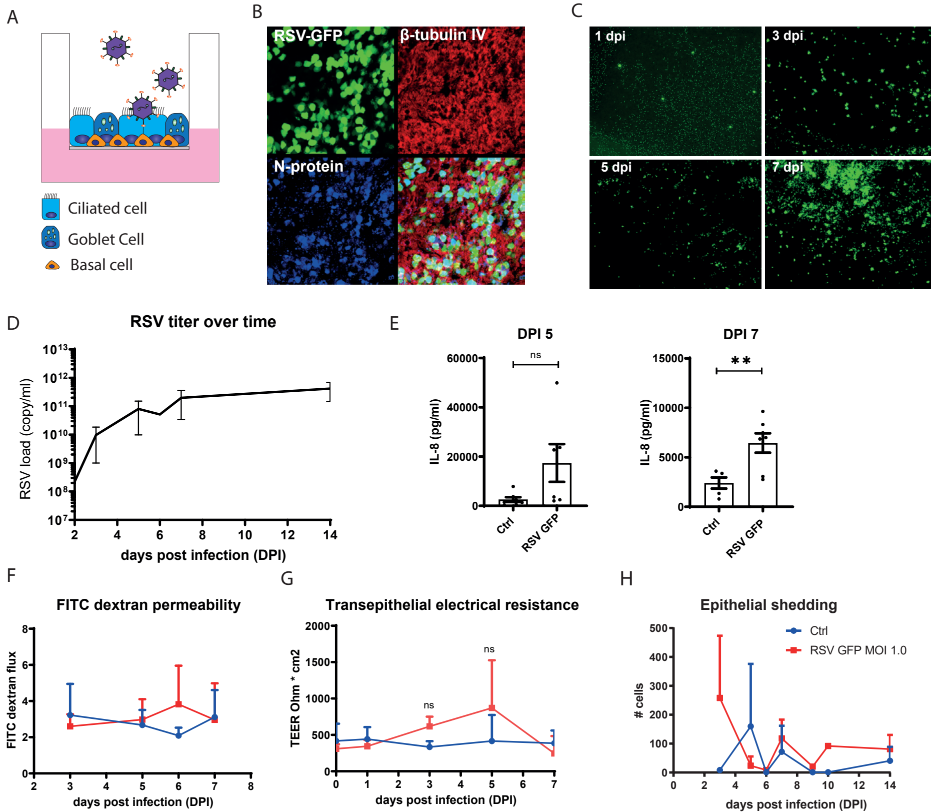

Figure 2. RSV infection does not disrupt the barrier function of bronchial epithelial cells.
(A) Schematic model of RSV infection of ALI-HBEC. (B) En face confocal image of RSV-infected ALI-HBEC, stained for nuclei (blue), RSV N protein (green) and β-tubulin (red). (C) Cultures were infected with RSV-A2 (GFP version) at an MOI of 1.0. Microscope images of RSV infected ALI-HBEC culture at 1, 3, 5 and 7 dpi. RSV-infected cells are GFP positive (green). One representative culture is shown (Magnification: 40×). (D) Virus growth kinetics determined by RSV titer (copy/ml) in apical washes at 24-h intervals following infection. Data are presented as mean ±SD. (N>3 individual experiments). (E) IL-8 protein expression was determined in assay supernatant at day five and day seven post infection (n=5 individual experiments). (F) Permeability assays were performed with 4-kDa fluorescein-conjugated dextran (FITC-D) up to seven dpi. The data are representative of 1-4 independent experiments depending on the time point. (G) TEER was measured with a volt-ohm meter at up to 14 days post-RSV infection (n=3 individual experiments). (H) Epithelial shedding after infection. Cells in apical washes were stained with DAPI and counted (n = 3).

Neutrophils do not induce airway epithelial damage during RSV infection

Next, we co-cultured RSV-infected ALI-HBEC with blood-derived neutrophils. First, neutrophils, unstimulated or pre-activated with immune complexes, were added in different cell concentrations to the apical surface of uninfected ALI-HBEC cultures for 4 hours ALI-HBEC (Fig. 3A). This did not alter the barrier integrity based on no differences in TEER measurements compared to control cultures (Fig. 3B). Due to inter assay variation in the TEER measurement, we decided to use FITC-D permeability in the following experiments, to determine epithelial barrier integrity during RSV infection.

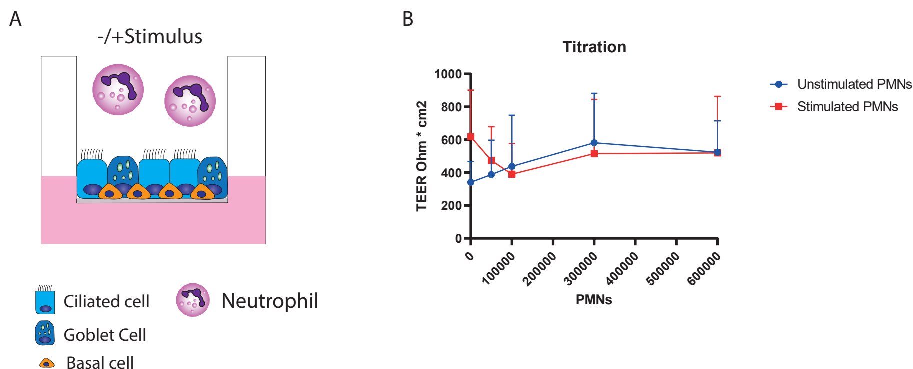

Figure 3. Activated neutrophils do not induce a loss of barrier integrity *in vitro*
(A) Schematic model of ALI-HBEC co-culture with neutrophils (± activation with immune complexes). (B) Trans epithelial electrical resistance (TEER) was measured after four hours of incubation with different concentrations of activated and non-activated neutrophils. (n=2 individual experiments)

ALI-HBECs were infected for five or seven days, afterwards neutrophils (100.000/ well) were added for four hours (Fig. 4A). Confocal microscopy showed adherence of some neutrophils to the epithelial surface (Fig. 4B). In the presence of neutrophils, the permeability of FITC dextran of RSV infected ALI-HBEC was increased 1.7-fold at five dpi (p=0.07), however not at 7 dpi (Fig. 4C-D). To investigate whether the duration of the co-culture with neutrophils determined the effect on FITC-D permeability we performed a neutrophil co-culture for up to 24 hours at five dpi. In contrast to the initial experiment we observed no significant differences in FITC-D permeability between cultures with or without neutrophils (Fig. 4E). Finally, the presence of neutrophils in the cultures had no effect on viral replication when compared to RSV infected cultures without neutrophils (Fig. 4F). In conclusion, these results suggest that blood derived neutrophils co-cultured with RSV infected ALI-HBEC do not induce a consistent loss of airway epithelial barrier integrity and do not affect viral replication.

DISCUSSION

In this study we provide an *in vitro* model which can be used to gain further inside into the role of neutrophil-epithelial interactions during RSV bronchiolitis. We attempted to examine whether neutrophils contribute to airway epithelial damage upon RSV infection. Consistent with previous studies, we first showed that RSV infected and replicated in ciliated airway epithelial cells, and induced IL-8 production.[5, 7, 28] Moreover, our data showed no effect of RSV infection on the epithelial barrier integrity as measured by FITC-D flux or TEER, in ALI-HBEC cultures, in line with others.[4, 7-11] This suggests that repairing mechanisms of airway epithelial cells compensates for the cytopathic effects

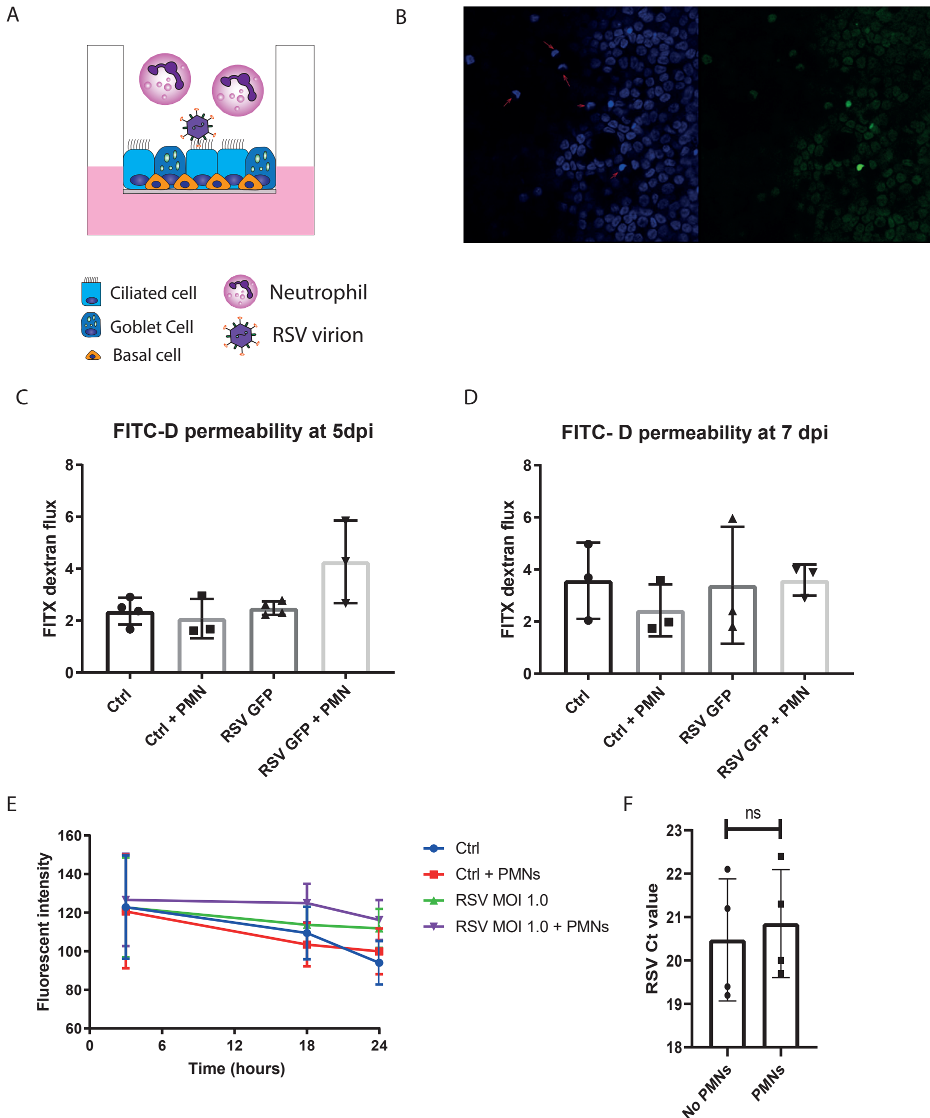

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Figure 4. Peripheral blood neutrophils do not induce a loss of barrier integrity during RSV infection *in vitro*
(A) Schematic model of RSV infection of ALI-HBEC co-culture with neutrophils. ALI-HBEC cultures were infected with RSV-A2 (GFP version) at an MOI of 1.0 at day five or seven dpi, 100.000 neutrophils were added per well. (B) En face confocal image of RSV infected ALI-HBEC co-cultured with neutrophils (green), and stained for nuclei (blue). Red arrows indicate neutrophils (650x). (C-D) Permeability assays were performed with 4-kDa fluorescein-conjugated dextran (FITC-D) after 4 hours incubation of with neutrophils on day five (C) and seven (D) dpi. (n$\geq$3 individual experiments) (E) Permeability assays were performed with FITC-D after 4, 18 and 24 hours incubation of with neutrophils (n= 2 individual experiments). (F) Virus growth kinetics determined by RSV titer (copy/ml) in apical washes at 24-h intervals following infection. Data are presented as mean $\pm$SD (n>3 individual experiments).

induced by RSV, or that RSV in itself induces little to no injury to bystander cells. Lastly, in preliminary studies we observed that co-culturing with peripheral blood neutrophils did not increase epithelial permeability.

Although already proposed by Villenave and colleagues in 2013 [29], we identified only two studies that investigated the effect of neutrophils on well-differentiated airway epithelial cell cultures during RSV infection [24, 25], emphasizing the difficulty of such co-culture systems. Herbert et al. used an ALI model with differentiated nasal airway epithelial cell cultures on inverted plates which allowed basolateral to apical neutrophil migration. This study showed that neutrophil migration through the epithelial barrier induced epithelial injury, while the epithelial barrier measured by red dextran flux and TEER remained intact.[24] In our study we also observed no effect on FITC-D permeability and TEER in co-cultures after 4 and 24 hours. In another study by Herbert et al., a reduced expression of the tight junction protein ZO-1 was observed in RSV infected nasal epithelial cell cultures after 1h co-culture with apical addition of neutrophils.[23] In contrast to both studies by Herbert et al., we did not identify that the presence of neutrophils induced a reduction in RSV viral load.[22, 24] Our observation is in line with a study showing no effect on RSV viral load in neutrophil depleted mice.[15] The discrepancy with the studies of Herbert et al. may be explained by the differences in epithelial cell types (nasal vs. bronchial cells) that were used, the number of neutrophils used, or the duration and timing of the experiment.

Blood and airway neutrophils of RSV patients are functionally and phenotypically different. Upon trafficking from the circulation to the RSV infected airway tissue, neutrophils display an altered gene expression profile and express different type of receptors, giving rise to distinct neutrophils subsets and activation level in the blood and airways.[19, 21, 30-32] Thus far no studies have been performed using airway-derived neutrophils, while previous studies by our group have shown that it is possible to collect them and perform functional assays.[21, 30] For future studies we recommend using neutrophils isolated from the airways of RSV infected and non-infected children (e.g. from children undergoing intubation for surgery).

While setting up our model we identified several challenges. First, we observed substantial variation between experiments, likely caused by the use of primary airway cells. Factors that could determine donor variation include variations in airway composition (e.g. percentage of ciliated cells), and baseline differences in host immune response.[18] In future studies the amount of cell cultures from independent donors should be increased to correct for donor variations. Moreover, further optimized culture conditions may lead to uniform airway cell differentiation between different donors. Secondly, in our study neutrophils were added to the apical surface of the culture, and therefore did not migrate through the epithelial layer. Trans epithelial migration has been shown to induce epithelial damage during RSV infection.[25] Third, TEER is a frequently used

method to assess barrier integrity but the stability of this assay is limited. Difficulties in TEER measurement have previously been described and factors such as temperature, medium composition, timing of medium change, and cell passage number all influence TEER measurement.[33] Indeed, we found that even in uninfected ALI cultures TEER varied significantly over time. Also, the use of TEER or FITC-D permeability as the only outcomes for assessing epithelial injury is likely not sufficient. Two recent publications showed that RSV-infected epithelial cell co-cultured with neutrophils displayed a reduced expression of tight junctions and LDH release as measurement of cytotoxicity, whereas no increased epithelial permeability was observed in.[23, 25] In a study by Herbert et al. neutrophils accumulated in epithelial gaps and therefore FITC-D permeability or TEER remained normal, while other variables indicative of epithelial damage such as LDH release and the loss of epithelial cells changed.[25] This suggests that FITC-D permeability and TEER measurement are not the most optimal outcome measurements for epithelial injury in co-culture systems. Finally, we only investigated the effects of neutrophils during a limited time frame. It is more likely that as a result of changes in the local inflammatory milieu during infection, the number, subsets and functionality of neutrophils change time. [23, 25, 31]

In order to overcome these limitations in future studies we propose to use inverted plates which enables the study of epithelial trans-migration[25, 34], use freshly isolated neutrophils from the airways of RSV infected infants. In addition, we propose to use a combination of methods to determine epithelial damage and cytotoxicity, including paracellular flux and assessment of tight junction expression. Finally, advanced techniques such as secretome analysis, RNA sequencing and flow cytometry on neutrophil and epithelial cells may be used to study the expression of protein biomarkers, neutrophil activation markers, immune checkpoints and epithelial viral entry receptors.[17, 18, 31, 35-38] Table 1 provides an overview of the optimal set up for the future study of neutrophil-epithelial interaction in RSV infected airway epithelial cell ALI. This model can also be used to study the pathophysiology of other respiratory viruses and to test therapeutic targets.[39-42] Moreover, in addition to ALI-cultures the use of airway and lung derived organoids will offer the potential to study neutrophil-epithelial interaction in an even more complex environment.[37, 43-45]

In summary, our co-cultures of ALI-HBEC and neutrophils are a useful starting point towards a model to study neutrophil-epithelial interaction during RSV infections, but also other respiratory viruses. It offers the possibility to study local neutrophil biology, immune cell induced epithelial injury, virus kinetics, and therapeutic targets thereby improving the understanding of the local immune response of current and emerging respiratory infections.

Table 1: Recommendations for the optimal study of neutrophil –epithelial interaction in RSV infected airway epithelial cell culture cultured at an air-liquid interface

	Experiment	References*
Cell type	Nasal epithelial cells	[23]
	Bronchial epithelial cells	[7, 36]
Set up	Inverted plates with blood derived neutrophils	[24, 25, 34]
	Regular plates with airway derived neutrophils	none
	Outcomes	
Barrier integrity	Paracellular flux	[10, 23-25]
	Tight junction expression	[10, 23]
Cytotoxicity	LDH release	[25]
	Epithelial shedding	[23, 25, 46]
	Ciliary beat frequency	[23, 25]
Virus kinetics	RSV titer	[7, 23-25, 36]
	RSV-GFP expression	[7, 23-25, 36]
Neutrophil function	Functional assays: NET-formation, ROS production, phagocytosis	[21, 30, 47]
	Viability	[23]
Novel techniques	RNA sequencing	[38]
	Flow cytometry	[23, 31, 35, 36]
	Secretome analysis	[17]

*in the context of human RSV studies

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5

Neutrophils in respiratory syncytial virus infection: from harmful effects to therapeutic opportunities

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ABSTRACT

Respiratory syncytial virus (RSV) is an important infectious agent in infants and young children. In most cases, RSV infection only causes mild disease, but in some, it requires invasive ventilation. Although antiviral drugs are obvious candidates to treat viral illness, and some have shown antiviral effects in humans, antivirals such as GS-5806,, ALX-0171, ALS-8176 did not yet meet their expectations. Since the inappropriate or dysregulated immune response against RSV leads to harmful immune pathology, a robust immune cascade is probably underway by the time patients reach the hospital. RSV infection is associated with a strong neutrophil influx into the airway. It is unclear how these cells contribute to antiviral defense or whether they contribute to lung pathology. This article discusses the protective and harmful roles of neutrophils during RSV infection and provides an overview of mechanisms by which neutrophil function could be targeted to prevent tissue injury and preserve homeostasis.

Keywords: RSV, neutrophil, inflammation, immunopathology, immunomodulators

ABBREVIATIONS

Respiratory syncytial virus, RSV; Acute lower respiratory tract infection, ALRI; Pattern recognition receptors, PRRs; Nuclear factor-kB, NF-kB; interferon regulatory factor, IRF; Interleukin, IL; Tumor necrosis factor-α, TNF-α; Chemokine (C-C motif) ligand, CCL; Dendritic cells, DCs; Natural killer, NK; Interferon, IFN; Toll-like receptors, TLRs; Antigen presenting cells, APCs; T helper, Th; Neutrophil extracellular traps, NETs; eosinophil cationic protein, ECP; Human neutrophil peptides, HNPs; Pentraxin, PTX; Myeloperoxidase, MPO; Reactive oxygen species, ROS; DC natural killer lectin group receptor-1, DNGR-1; Src homology region 2 domain-containing phosphatase 1, SHP-1; Macrophage inflammatory protein 2, MIP-2; Cannabinoid receptor 2, CB2; Junctional adhesion molecule, JAM; Neutrophil inhibitory factor, NIF; targeted neutrophil inhibitory-hirulog, TNHH; Cystic fibrosis, CF; Chronic obstructive pulmonary disease, COPD; Gastrin-releasing peptide receptor, GRPR; Mitogen-activated protein kinase, MAPK; Leukocyte immunoglobulin-like receptor subfamily B member 2, LILRB2; Leukocyte-associated Immunoglobulin-like receptor-1, LAIR-1; Signal inhibitory receptor on leukocytes-1, SIRL-1; Sialic acid binding Ig-like lectin E, Siglec-E; Mitochondrial antiviral-signaling protein, MAVS, soluble NSF attachment protein, SNAP; Phosphoinositide 3-kinase, PI3K; N-formyl-methionyl-leucyl-phenylalanine, fMLP; Phosphodiesterase type 4, PDE4, Cyclic adenosine 3',5'-monophosphate, cAMP; Exchange protein directly activated by cAMP-1, Epac-1; Granulocyte colony stimulating factor, G-CSF; neutrophil elastase, NE; Peptidyl arginine deaminase 4, PAD4; Acetylsalicylic acid, ASA; neonatal NET inhibitory factor, nNIF; Cysteine protease dipeptidyl peptidase 1, DPP1; Anti-citrullinated protein antibody, tACPA; doxorubicin, DOX;

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory tract infections (ALRI) in infants and young children worldwide (Stein et al., 2017). The virus is associated with about 28% of all childhood ALRI episodes and 13-22% of all ALRI mortality, with 99% of the deaths occurring in developing countries (Shi et al., 2017). The clinical and economic burden of RSV-induced ALRI in childhood is considerable, at least in part, due to the lack of licensed vaccines or effective drugs against the virus (Mazur et al., 2018). RSV infection in infancy is also associated with long-term respiratory morbidity, especially recurrent wheezing and asthma (Fauroux et al., 2017).

The immune response to RSV infection is characterized by an exacerbated inflammatory response in the lung. The inflammatory response to RSV infection acts as a "double-edged sword" (Fig.1). On the one hand, it contains a wide range of antiviral

properties, limiting viral replication, and spread (Currie et al., 2016). On the other hand, inappropriate or dysregulated responses can be pathogenic, causing disease-enhancing inflammation that contributes to short- and long-term effects (Openshaw et al., 2017). The importance of inflammation in the pathogenesis of RSV infection (known as immune pathology) is highlighted in human and animal studies (Peebles & Graham, 2005; Rosenberg & Domachowske, 2012).

An intense neutrophil influx characterizes inflammation in RSV-induced ALRI to the airways and the consequent release of antimicrobial products, which likely drive immunopathology (Geerdink et al., 2015; Habibi et al., 2020; McNamara et al., 2003). Neutrophil-mediated inflammation is a common trait of RSV infection, but it is not clear which parts are beneficial and detrimental to disease outcome (Geerdink et al., 2014). However, increased neutrophil responses correlate to clinical severity (Lukens et al., 2010). Furthermore, studies considering the correlation between RSV titers and disease severity are contradictory, although evidence is accumulating that disease severity does not correlate to viral titers (Thwaites et al., 2018; Uusitupa et al., 2020). It seems that the severity of infection mostly depends on the magnitude and timing of the innate immune and inflammatory response in the airways (Tahamtan et al., 2020).

Here, we discuss neutrophils' broad action as a critical factor in the pathogenesis of RSV infection and the mechanisms by which neutrophils are regulated to prevent tissue injury and preserve homeostasis. Progress in the knowledge of protective and harmful immunity to RSV infection will lead to new prevention and therapy approaches.

IMMUNE RESPONSE TO RSV INFECTION

RSV infects the upper airway's superficial ciliated cells, the small bronchioles' epithelium, and type-I alveolar pneumocytes. Upon infection, the airway epithelial cells and lung resident immune cells, such as alveolar macrophages, detect the virus through pattern recognition receptors (PRRs) (Lambert et al., 2014). Subsequently, this results in the activation of nuclear factor-kB (NF-kB) and the interferon regulatory factor (IRF) family members, which enhance the expression of cytokines, chemokines, and anti-viral factors (Lambert et al., 2014). Many inflammatory mediators such as interleukin (IL)-6, IL-8, tumor necrosis factor-α (TNF-α), chemokine (C-C motif) ligand (CCL)3, and CCL5 are present in the airways at high concentrations and attract innate immune cells to the infected airways (Lambert et al., 2014; McNamara et al., 2005; McNamara et al., 2004). Neutrophils are recruited leukocytes in the early stages of bronchiolitis (Lambert et al., 2014). However, the protective or harmful role of neutrophilia during RSV infection is not yet fully understood. Furthermore, other immune cells, such as dendritic cells (DCs) and natural killer (NK) cells, are crucial for the immune response's progression. In this case, NK cells

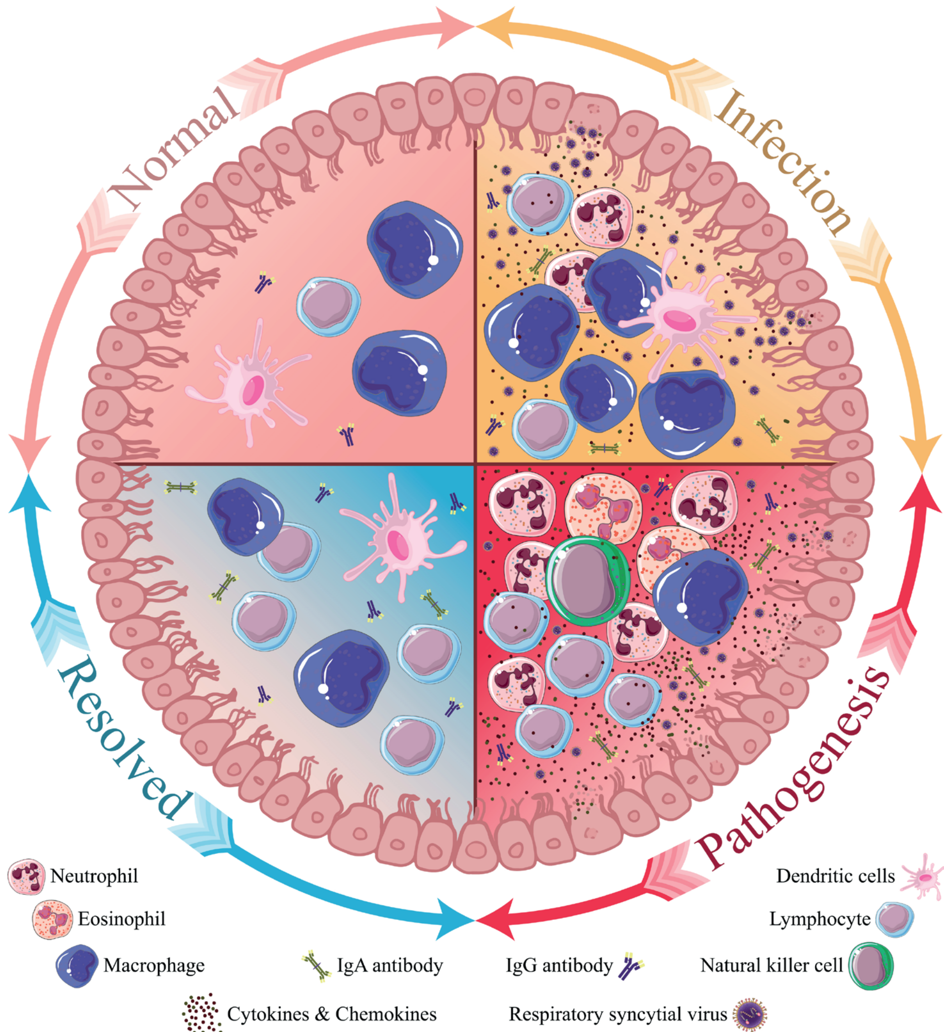

Figure 1. Immune factors affecting the outcome of RSV infection. Several immune cell types, chemokines, and cytokines are involved in the host immune response to RSV infection and the establishment of an inflammatory milieu. Innate and adaptive immunity is vital for viral clearance and disease resolution but may be associated with inappropriately polarized responses and immunopathology.

provide interferon (IFN) -γ to help DCs activation and prime the T cell responses(Culley, 2009; Kaiko et al., 2010). In the neonatal immune response to RSV infection, reduced levels of interferons and toll-like receptors (TLRs) signaling, altered functions of antigen presenting cells (APCs), low IL-12, enhanced production of IL-6 and IL-23, and reduced activation of regulatory T-cells skew the adaptive immune responses towards a T helper (Th) 2 and Th17 response, and away from protective Th1 and cytotoxic T cell (Kollmann et al., 2009; Lambert et al., 2014). Additionally, impaired follicular helper T cell activation

combined with little or no B cell memory results in low antibody titers and low antibody affinity (Lambert et al., 2014; Mastelic et al., 2012). A detailed discussion of the immune response against RSV can be found in Supplementary File 1.

NEUTROPHILS AND RSV INFECTION

Following RSV infection, neutrophils infiltrate the airways in high frequencies: approximately 80% of infiltrated cells are neutrophils (McNamara et al., 2003). Post-mortem lung tissues from suspected fatal cases of RSV-induced ALRI reveal extensive neutrophilia, highlighting a potential central role of neutrophils in RSV disease pathology (Welliver et al., 2007). Additionally, neutrophils change their phenotype and behavior depending on the tissue and environment (Ballesteros et al., 2020).Four distinct neutrophil subsets have been identified in the blood and lung during acute RSV-bronchiolitis. Based on differential expression of CD16 and CD62L, mature, immature, suppressive, and progenitor neutrophils can be identified, each with distinct activation states. Interestingly, severe viral respiratory infection is characterized by a relative absence of suppressive neutrophils in the blood, except in the case of bacterial co-infection (Cortjens et al., 2017; Sebina & Phipps, 2020). The interaction of different neutrophil subsets with other cells in the immune system and their exact role in the course of RSV infection is not clear (Cheung et al., 2018). It has been shown that neutrophils have a protective role in particular viral infections, such as influenza and HSV, but are also associated with tissue damage (Geerdink et al., 2015). Distinct neutrophil functions during RSV infection are shown in Fig.2.

The association of neutrophils with asthma severity and lung function is also remarkable. In RSV infection, severe wheezing may result from excessive infiltration of neutrophils to the lung. Several studies have shown a relation between neutrophil inflammation and asthma (Li et al., 2006; Wood et al., 2012). There is an association between sputum neutrophil count and objectively recorded cough frequency and impaired lung function in asthma. Therefore, neutrophilic inflammation could be a biomarker of severe asthma (Ray & Kolls, 2017). Next, the release of dsDNA from neutrophil extracellular traps (NETs) in the airway may induce asthma exacerbations.

Interestingly, neutrophil cytoplasts (enucleated cell bodies) can also trigger $CD4^+$ T cell helper responses, particularly Th17 immune responses. A mouse model of allergic asthma showed increased asthma-like pathology in the lung that resulted from augmented DC-mediated Th17 responses in the lymph nodes due to the neutrophil cytoplasts (Krishnamoorthy et al., 2018; Sebina & Phipps, 2020). Moreover, in a rhinovirus-infected mouse model of asthma, it is shown that NETosis and the released dsDNA from the recruited neutrophils to the airways induce the release of type-2 cytokines from

Th2 cells. In turn, this increases the inflammation by eosinophil recruitment, resulting in the exacerbation of asthma-like symptoms in the mice (Busse, 2017). While many studies indicate an association between RSV infection and asthma development later in life, a definite link has not been established (Scheltema et al., 2018). Whether there is an association between neutrophil infiltration during RSV infection and the development of post-viral wheezing or asthma is unknown. RSV disease severity is correlated to the neutrophil influx and the risk of developing childhood asthma. Conceivably, neutrophilic airway inflammation in RSV infection leads to excessive mucus production, epithelial damage, and airway remodeling, contributing to impaired lung function and lung morbidity later in life. Therefore, targeting neutrophil during RSV infection might improve long term pulmonary outcomes in children.

Protective role of neutrophils during RSV infection

Neutrophils can detect and be activated by different viruses through PRRs, especially TLR-7 and 8 (Thomas & Schroder, 2013). However, it seems that in RSV infection, neutrophils may not directly recognize intact RSV, and they are activated at sites of infection as a result of the cytokines/chemokines and inflammatory mediators released by infected cells (Bataki et al., 2005). Lung-resident cells such as epithelial cells, DCs, and alveolar macrophages, as the initiators of the innate immune response to the infection, commence with the secretion of cytokines and chemokines (Nuriev & Johansson, 2019). In this case, IL-8 produced by epithelial cells leads to the influx of neutrophils, contributing to the inflammation by releasing chemokines and granular enzymes (Jaovisidha et al., 1999). RSV induces the release of CC chemokines CCL3 and CCL4 by neutrophils (Jaovisidha et al., 1999). CCL3 stimulates eosinophil and basophil chemotaxis and degranulation,, which leads to the recruitment of these cells and the subsequent release of eosinophil cationic protein (ECP) and histamine in the airways (Jaovisidha et al., 1999). CCL4 may enhance the proliferation of Th2 lymphocytes in the airways (Jaovisidha et al., 1999). Besides, the type 2 cytokine IL-9, produced by airway neutrophils during RSV infection, may skew the response to a harmful type 2 immune response (McNamara et al., 2004).

Neutrophils mediate direct antimicrobial effects through phagocytosis, degranulation, and NETs formation (Amulic et al., 2012). Antiviral properties of neutrophil are shown in Table 1. They can eliminate virus-infected cells by phagocytosis and engulf virions, thus preventing further viral replication (Hashimoto et al., 2007; Watanabe et al., 2005). In response to RSV infection, neutrophils release hundreds of proteins with broad antimicrobial action, such as α-defensins 1-4 or human neutrophil peptides (HNPs) 1-4, cathelicidins (i.e., LL-37), pentraxin (PTX) 3, and Myeloperoxidase (MPO) into the microenvironment (Hazrati et al., 2006; Jaovisidha et al., 1999; Wang et al., 2004). HNPs are small, cationic amphipathic peptides that inhibit viral entry into the cell and promote

virion aggregation and phagocytosis (Demirkhanyan et al., 2012; Tecle et al., 2007). It has been demonstrated that the cationic antimicrobial peptide LL-37 exerts direct antiviral activity against RSV virions, protects epithelial cells from infection and cell death, and inhibits virion production (Currie et al., 2013). This is consistent with a report showing that low serum levels of LL-37 precursors are associated with severe RSV bronchiolitis (Mansbach et al., 2012). PTXs are soluble pattern recognition molecules that mediate innate humoral immunity, activate complement, and act as opsonins, which reduce virion infectivity (Bottazzi et al., 2010; Han et al., 2012; Reading et al., 2008). MPO catalyzes the production of hypochlorous acid, a potent antimicrobial reactive oxygen species (ROS), because of its ability to chlorinate and oxidize a wide variety of biomolecules (Klebanoff & Coombs, 1992; Wilson et al., 2013). The recruitment and degranulation of airway neutrophils following RSV infection are also associated with increased expression of bactericidal proteins, which contributes to the regulation of commensal bacteria in the upper respiratory tract (Sande et al., 2019). RSV induces classical ROS-dependent NETs formation known as NETosis in human neutrophils (Muraro et al., 2018). NETs are cytotoxic structures comprised of decondensed strands of nuclear DNA released into the extracellular environment. These chromatin webs carry a strong negative charge. They are studded with both nuclear proteins, such as histones and proteins derived from neutrophil granules, including defensins, elastase, cathepsins, lactoferrin, and MPO (Brinkmann et al., 2004). Studies *in vitro* and *in vivo* have demonstrated that NETs can bind and sequester virions, including RSV, preventing them from reaching their target cells (Cortjens et al., 2016; Jenne et al., 2013; Scapini & Cassatella, 2014).

In addition to the direct antimicrobial effects against RSV infection described above, neutrophils can regulate adaptive immune responses in multiple ways (Scapini & Cassatella, 2014). There is some evidence that neutrophils can present viral antigens to $CD8^+$ T-cells and stimulate proliferation, IFN-γ production, and cytolytic activity (Beauvillain et al., 2007; Duffy et al., 2012; Hufford et al., 2012). Neutrophils release chemoattractants, such as cathepsin G and azurocidin, involved in the recruitment of other immune cells, particularly monocytes/macrophages (Kumar et al., 2018). Neutrophils recruit, activate, and polarize DCs but can also suppress their activity (Charmoy et al., 2010). Neutrophil products such as cathelicidins, lactoferrin, α-defensins, and chemokines such as CCL3, CCL4, CCL5, and CCL20 are responsible for DC mobilization to sites of infection (Mayadas et al., 2014; Schuster et al., 2013). Neutrophil life-death status, including mechanism of cell death (apoptosis, necrosis, NETosis), determines its impact on DC activation (Schuster et al., 2013). Also, activated, not resting, neutrophils can induce maturation of DCs that enables these cells to trigger T cell proliferation and Th1 polarization. This neutrophil-DC interaction is driven by the binding of the DC-specific, C-type lectin DC-SIGN to the β_2-integrin Mac-1 (van Gisbergen et al., 2005). Furthermore, TNF-α produced by activated neutrophils is essential for inducing DC maturation (van Gisbergen

et al., 2005). Charmoy et al. have shown that neutrophil-secreted CCL3 is essential in chemotaxis of immature DCs in an experimental model of *Leishmania major* infection (Charmoy et al., 2010). In contrast, phagocytosis of apoptotic neutrophils may inhibit DC function, and neutrophil-derived ectosomes can inhibit DC maturation and lead to immune tolerance (Schuster et al., 2013). Also, neutrophils maintain NK cell homeostasis by collaboration with DCs (Costantini et al., 2011; Jaeger et al., 2012). Splenic neutrophils produce cytokines that induce immunoglobulin class-switch recombination, somatic hypermutation, and antibody production in the marginal zone of the spleen (Puga et al., 2011). Significantly, the T-cell activity can be suppressed by neutrophils through direct interactions with T-cells and by limiting stimulatory interactions between T-cells and DCs (Odobasic et al., 2013; Pillay et al., 2012). Furthermore, phagocytosis of apoptotic neutrophils by macrophages induces a regulatory phenotype in macrophages, which might contribute to the resolution of inflammation (Filardy et al., 2010; Shirey et al., 2010). Despite *in vitro* and theoretic data indicate that neutrophils are important in the antiviral response against RSV, *in vivo* evidence is limited. Moreover, a recent study using neutropenic mice showed no effect on disease severity or viral load (Kirsebom et al., 2020). On the other hand, neutrophils could contribute to a protective antiviral response supported by clinical reports indicating high mortality following RSV infection among patients with neutropenia (Vakil et al., 2018).

Table 1 Potential targets to modulate neutrophil functions and migration during RSV infection

Mechanism	Effect	Virus/model	References
Phagocytosis	Eliminates virus-infected cells and virions	HSV, CMV and Influenza virus/*in vitro and in vivo*	Hashimoto et al, Watanabe et al.
HNP-1	Inhibits viral entry into cell	HIV/*in vitro*	Hazrati et al.
HNP-1 and HNP-2	Promotes virion aggregation and phagocytosis	Influenza/*in vitro*	Wang et al.
LL-37	Inactivates virions, protects epithelial cells from infection and cell death, inhibits virion production	RSV/*in vitro* and clinical association	Currie et al, Mansbach et al.
PTX3	Reduces virion infectivity	Influenza virus and MHV-1/ *in vitro* and *in vivo*	Reading et al, Han et al.
MPO	Inactivates virions through HClO production	HIV-1/*in vitro*	Klebanoff et al.
NETosis	Reduces viral infectivity and spread, inactivates virions	Influenza virus and myxoma poxvirus/*in vitro* and *in vivo*	Jenne et al.
ROS	Limits viral dissemination	HIV-1/*in vitro*	Wilson et al.
Defensins	Binds and inactivates virions	HIV-1, HSV, CMV and VSV	Hazrati et al, Wang et al.
Unknown	Reduces viral load	RSV/*in vitro*	Muraro et al.

*Adapted and updated from Geerdink et al JACI 2015

Neutrophil induced immune pathology

It is believed that the influx of neutrophils in the airways and their subsequent action play a substantial role in the pathology of RSV disease (Geerdink et al., 2015). Due to neutrophils' promiscuous role, unwanted neutrophilic inflammation induces immune injury during viral respiratory infection (Geerdink, 2014). In this regard, the degree of neutrophilic inflammation is associated with RSV-induced disease severity (Yasui et al., 2005). Genes related to neutrophil function, such as those encoding α-defensin-1 and elastase, are overexpressed in patients with severe disease, and expression levels positively correlate with severity (Mejias et al., 2013). A common single nucleotide polymorphism in the IL-8 encoding gene that is associated with increased production of this potent neutrophil chemoattractant is more frequent in cases with severe RSV bronchiolitis (Hull et al., 2000). Neutrophils can induce lung pathology following RSV infection through different mechanisms (Fig.2). *First,* neutrophils induce mucus production limiting access of viral particles to the epithelium but contributes to airway obstruction (Stokes et al., 2013). *Second,* the infiltrating neutrophils block the small airways of young infants, leading to impaired gas exchange and manifesting clinically in reduced blood oxygen saturation and hypoxia (Sande et al., 2019). *Third,* a significant enrichment of the neutrophil degranulation pathway, characterized by increased expression of neutrophil effector proteins, is found in RSV-infected children (Mejias et al., 2013). Degranulation of neutrophils releases antimicrobial mediators that are cytotoxic to host cells. For example, elastase released following neutrophil recruitment to the lungs disrupts the lung extracellular matrix and adds to the inflammatory response (Cavarra et al., 1996). *Fourth,* increased expression of neutrophil granule proteins in the airway is also associated with reduced oxygen saturation (Sande et al., 2019). *Fifth,* neutrophil-derived ROS is potentially antimicrobial but also damages host cellular structures (Hosakote et al., 2011). *Sixth,* the formation of NETs by neutrophils can capture and inactivate viral particles but also damage healthy bystander cells (Jenne & Kubes, 2015). Many of the NET components, including elastase and histones, are cytotoxic, leading to endothelial damage, exposure of the sub-endothelium, coagulation, and exacerbated inflammation (Fuchs et al., 2010; Xu et al., 2009). Finally, RSV infection increases neutrophil trans-epithelial migration that induces epithelial disruption and lung damage (Herbert et al., 2020). Notably, severe neutrophilic lung inflammation during infancy with effects on pulmonary architecture and immune homeostasis could tenably predispose children to subsequent wheezing and onset of asthma (Geerdink et al., 2015).

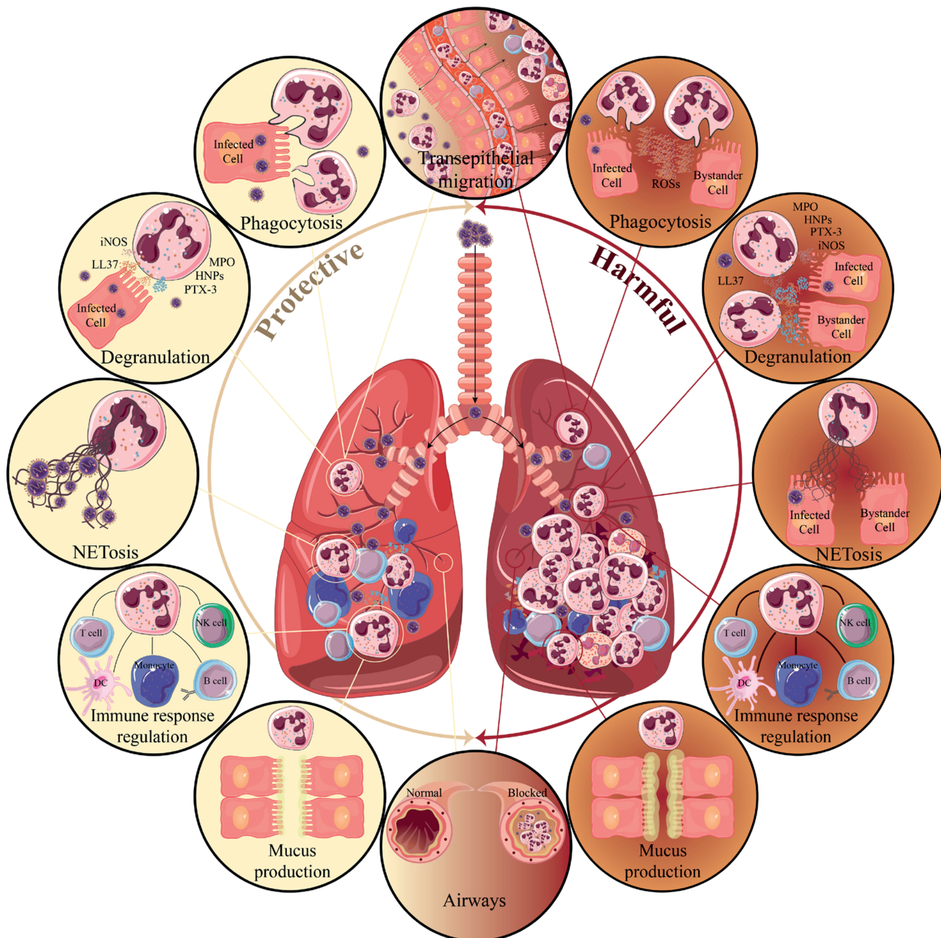

Figure 2. Broad roles of neutrophils during RSV infection. Neutrophils mediate direct antimicrobial effects through phagocytosis, degranulation, and NETs formation. Neutrophils can also regulate immune responses such as T-cells proliferation, DCs activity, NK cell homeostasis, immunoglobulin class-switch recombination, somatic hypermutation, and antibody production in the marginal zone of the spleen. The influx of neutrophils in the airway and their actions play a substantial role in the pathology of RSV disease. RSV infection increases neutrophil trans-epithelial migration that induces epithelial disruption and lung damage. Neutrophils induce mucus production that limits access of viral particles to the epithelium but contributes to airway obstruction. The infiltrating neutrophils block the small airways of young infants, leading to impaired gas exchange and manifesting clinically in reduced blood oxygen saturation and hypoxia.

TARGETING NEUTROPHILS DURING RSV INFECTION

The outcome of RSV infection is determined by the balance between efficient neutrophil-mediated antimicrobial activity and lung immune pathology. In patients with severe RSV infection, the balance between neutrophil activity and tissue damage shifted towards a pathologic response. Modulation of neutrophil recruitment and

function represents a promising therapeutic strategy for the treatment of RSV disease. Several potential therapeutic methods can be applied to dampen neutrophil-mediated lung damage (Supplementary table 1): 1) using neutrophil-modulating molecules; 2) blocking neutrophil chemokine receptors; 3) targeting neutrophil-expressed inhibitory receptors; 4) modifying signaling pathways; 5) modulating cytokine signaling; 6) inhibiting neutrophil-derived products; and 7) the usage of advanced technologies such as nanoparticles or miRNA.

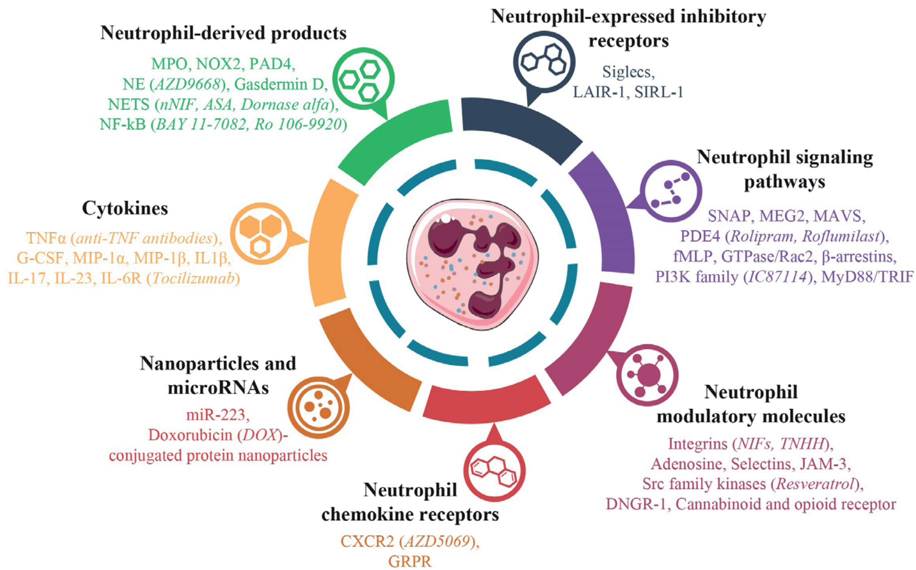

Figure 3. Potential targets to modulate neutrophil function and migration during RSV infection. There are several potential therapeutic methods that can be applied to dampen neutrophil-mediated lung damage: (1) using neutrophil-modulating molecules; (2) blocking neutrophil chemokine receptors; (3) targeting neutrophil-expressed inhibitory receptors; (4) modifying signalling pathways; (5) modulating cytokine signalling; (6) inhibiting neutrophil-derived products; and (7) the use of advanced technologies such as nanoparticles or miRNAs. Siglec, Sialic acid binding Ig-like lectin; LAIR-1, Leukocyte-associated immunoglobulin-like receptor-1; SIRL-1, Signal inhibitory receptor on leukocytes-1; SNAP, Soluble NSF attachment protein; PDE4, Phosphodiesterase4; fMLP, N-Formylmethionine-leucyl-phenylalanine; PI3K, Phosphoinositide 3-kinases; NIF, Neutrophil inhibitory factor; TNHH, Targeted neutrophil inhibitory-hirulog; JAM-3, Junctional adhesion molecule; DNGR-1, DC natural killer lectin group receptor-1; GRPR, Gastrin-releasing peptide receptor; MPO, Myeloperoxidase; NOX2, NADPH oxidase 2; PAD4, Peptidylarginine deiminases; NE, Neutrophil elastase.

Neutrophil modulatory molecules

Different types of molecules determine neutrophil function and migration.. Agonizing/antagonizing neutrophil-modulatory molecules may help limit immunopathology during RSV infection. It has been shown that engagement of DC natural killer lectin group receptor-1 (DNGR-1) limits tissue damage by dampening neutrophil recruitment via the activation of Src homology region 2 domain-containing phosphatase 1 (SHP-1) and inhibition of macrophage inflammatory protein 2 (MIP-2) expression in classical DC1s

(Del Fresno et al., 2018). Cannabinoid and opioid receptors on neutrophils can modulate their activity and migration during inflammation (Kurihara et al., 2006; Souza & Rosas, 2018; Tahamtan et al., 2016; Tahamtan et al., 2016). Kapellos et al. demonstrated that cannabinoid receptor 2 (CB2) plays an important role during acute neutrophil mobilization to sites of inflammation in which CB2 deficiency resulted in enhanced neutrophil in the dorsal air pouch model of inflammation in mice (Kapellos et al., 2019). Modulation of neutrophil function by targeting adenosine signaling has been relevant in various disease models such as ischemia-reperfusion injury, sepsis, and non-infectious acute lung injury (Inoue et al., 2008; Mirakaj et al., 2010; Thiagarajan et al., 1997). Adenosine is an endogenously released purine nucleoside with a short half-life, which is a product of ATP breakdown. Neutrophils can release adenosine and also express adenosine receptors. The effect of adenosine on neutrophil function is dependent on its concentration; low concentrations have a proinflammatory effect, while high concentrations inhibit neutrophil migration and effector functions (Barletta et al., 2012). The third member of the endothelial expressed family of junctional adhesion molecules (JAMs), JAM-3, mediates neutrophil transmigration in a Mac1-dependent manner. Inhibition of JAM-3 with soluble mouse JAM-3 resulted in a 50% reduction of neutrophil migration in a mouse model of acute peritonitis. Hence, JAM-3 could be targeted to modulate neutrophil transmigration to inflamed sites (Chavakis et al., 2004). An additional strategy to modulate the neutrophil function and trafficking is by interfering with integrin binding by a neutrophil inhibitory factor (NIF). These factors block the adhesion of activated human neutrophils to vascular endothelial cells and the release of H2O2 from activated neutrophils (Anbu & Joshi, 2008). NIF derived from canine hookworm (Ancylostoma caninum) inhibits the integrin CD11b/CD18-dependent neutrophil function *in vitro* (Moyle et al., 1994; Schnyder-Candrian et al., 2012). In an acid-induced lung injury model in rabbits, NIF and anti-CD18 antibodies showed lung injury attenuation, but with a high number of accumulated neutrophils in the air space. This indicates that NIF rather blocks neutrophil cytotoxicity than interfering with their recruitment (Folkesson & Matthay, 1997). Targeted neutrophil inhibitory-hirulog (TNHH) is another NIF which is described as a potential drug candidate for acute ischemic stroke. This drug is a recombinant glycoprotein, which binds selectively to thrombin and CD11b/CD18 integrin on neutrophils. The TNHH can be combined with Mac-1 molecules on the membrane of white blood cells to inhibit the adhesion, migration, invasion, and other activities of neutrophils (Gou et al., 2019). Although integrin and selectin blockers showed promising effects in animal studies, several human studies' results are disappointing (Németh et al., 2020).

Neutrophil chemokine receptors

Targeting chemokine receptors on neutrophils is another way to modulate neutrophil trafficking. The most critical neutrophil chemokine receptor is CXCR2, and antagonists against CXCR2 are under investigation in clinical studies for the treatment of cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and neutrophilic asthma (Chapman et al., 2009; Németh et al., 2020). For instance, CXCR2-blocking AZD5069 has been investigated in patients with asthma in a phase II study, which resulted in a decreased number of neutrophils in the sputum of these patients (O'Byrne et al., 2016). Moreover, there are some other chemokine receptor antagonists such as repertaxin, navarixin, and danirixin, which have the potential as treatment of inflammatory diseases such as COPD, CF, and asthma (Mårdh et al., 2017). In a study by Czepielewski et al., a gastrin-releasing peptide receptor (GRPR) antagonist inhibited CXCL2-induced migration *in vivo* and decreased neutrophil activation through CD11b and CD62L modulation. Moreover, antagonizing GRPR decreased IL8-driven neutrophil chemotaxis *in vitro* independently of CXCR2 internalization and induced activation of Mitogen-activated protein kinases (MAPKs) (p38 and ERK1/2) and downregulation of neutrophil adhesion molecules CD11b and CD66b (Czepielewski et al., 2017). GRPR is expressed in the developing lung and increases in response to hypoxia or lung radiation therapy (Tighe et al., 2019). The effect of blocking GRPR in the lung concerning lung neutrophilia and neutrophil induced lung injury remains a question for future studies.

Neutrophil-expressed inhibitory receptors

Various inhibitory receptors are expressed on the surface of neutrophils that act as feedback mechanisms to prevent unwanted tissue damage and preserve homeostasis. Some of these receptors, such as Leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2) and leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1), are stored in intracellular granules and rapidly move to the cell surface upon neutrophil stimulation (Geerdink et al., 2018). The collagen receptor LAIR-1 and signal inhibitory receptor on leukocytes (SIRL)-1, which are expressed on the majority of human peripheral blood mononuclear leukocytes, have been recently identified as inhibitory receptors on activated airway neutrophils. They negatively regulate neutrophil recruitment during murine RSV infection and limit NET formation and ROS production during severe RSV infection in infants (Besteman et al., 2020; Geerdink et al., 2018; Kumawat et al., 2019). Kumawat et al. found that LAIR-1-deficient (*Lair1*$^{-/-}$) mice infected with RSV have enhanced airway inflammation characterized by increased neutrophil and lymphocyte recruitment to the airways (Kumawat et al., 2019). Neutrophils express a range of other inhibitory receptors, including Sialic acid binding Ig-like lectin E (Siglec-E), which limited neutrophil recruitment to the lung in a mice model of acute lung inflammation (McMillan et al., 2013; Spence et al., 2015). Due to their ability to attenuate neutrophils' immune

response, targeting inhibitory receptors are attractive targets for controlling neutrophil induced immunopathology.

Neutrophil signaling pathways

Recent insights in molecular mechanisms that drive neutrophil trafficking and function offer opportunities for selective targeted therapies to interfere with RSV-driven neutrophil signaling pathways. It has been recently identified that neutrophil recruitment to the lung following RSV infection is dependent on MyD88/TRIF signaling. In contrast, neutrophil activation is dependent on type I IFN-driven, pro-inflammatory lung environment induced by Mitochondrial antiviral-signaling (MAVS) (Kirsebom et al., 2019). Thus, interfering with the MyD88/TRIF signaling pathway could alter neutrophil airway migration, while inhibiting the type I IFN response could limit neutrophil activation. Moreover, recent observations indicated that granule release from neutrophils depends on the activation of intracellular signaling pathways, including β-arrestins, the Rho guanosine triphosphatase (GTPase) Rac2, soluble NSF attachment protein (SNAP) receptors, the *Src* family of tyrosine kinases, and the tyrosine phosphatase MEG2 (Lacy, 2006). Additionally, Phosphoinositide 3-kinase (PI3K) family signaling affects different cells and pathologic processes, including those in which neutrophils play a dominant role (Hawkins & Stephens, 2015). Resveratrol, a drug that has been shown to have beneficial effects on inflammatory diseases, cancer, neurodegenerative diseases, and cardiovascular disorders, also has the potential to be used for the treatment of inflammatory lung diseases (Tsai et al., 2019). Resveratrol is a natural stilbenoid and can be found in various foods, such as grapes, red wine, and berries (Dvorakova & Landa, 2017). Studies demonstrated that resveratrol reduces respiratory burst, degranulation, integrin expression, and cell adhesion of activated human neutrophils via inhibition of Src family kinases (Nosáľ et al., 2014; Tsai et al., 2019). Some of the PI3K isoforms are ubiquitously expressed, and others are almost restricted to specific cells. PI3Kγ is an essential regulator of neutrophil effector responses expressed highly in myeloid cells (Mårdh et al., 2017). Another isoform, PI3Kδ, is mostly restricted to myeloid and lymphoid cells (Okkenhaug, 2013). Using IC87114, a PI3Kδ-selective inhibitor, demonstrated blockade of both N-formyl-methionyl-leucyl-phenylalanine- (fMLP-) and TNF-α-induced neutrophil superoxide generation and elastase exocytosis in a mouse model of inflammation (Sadhu et al., 2003). Other drugs targeting PI3K such as IPI-145, RV1729, and RV6153 are under evaluations in phase 2 clinical trials in asthma (Mårdh et al., 2017). The Phosphodiesterase type 4 (PDE4) inhibitors, roflumilast-N-oxide, and rolipram, significantly reduce the numbers of neutrophils and eosinophils in the airways and inhibit the migration of neutrophils in response to CXCL1 and LTB4 in a concentration-dependent manner (Dunne et al., 2019; Hohlfeld et al., 2008). Roflumilast is an approved compound which suppresses sputum neutrophilia in COPD patients by inhibiting neutrophil chemotaxis. It inhibits neutrophil

chemotaxis via a cyclic adenosine 3',5'-monophosphate (cAMP)-mediated mechanism requiring activation of exchange protein that is directly activated by cAMP-1 (Epac-1) (Dunne et al., 2019; Giembycz & Field, 2010). As a therapeutic window, these pathways offer ample opportunity to study new interventions to alter neutrophil activation and migration.

Cytokines

Interfering with cytokine signaling can alter neutrophil dynamics and behavior. Several studies suggested granulocyte colony-stimulating factor (G-CSF) as a potential therapeutic target, affecting neutrophil activation through increased CXCR2 expression (Campbell et al., 2016; Cornish et al., 2009; Lee et al., 2017). Targeting the IL-23-IL-17 axis as a regulator of G-CSF production limits neutrophil migration and has been developed for many inflammatory diseases such as psoriasis and arthritis (Gaffen et al., 2014). IL-8, a potent neutrophil chemoattractant, could also be targeted to limit neutrophil recruitment to the airways. Additionally, targeting cytokine production or cytokine receptors on neutrophils by using IL-6 receptor antibodies or TNFa antibodies could dampen neutrophilic inflammation (Wright et al., 2010).

Neutrophil-derived products

The formation of NETs and the release of granular enzymes, such as neutrophil elastase (NE), proteinase 3, and MPO are important hallmarks of neutrophilic inflammation. As NET formation is reliant on MPO, NOX2, NE, peptidyl arginine deiminase 4 (PAD4), and gasdermin D, these molecules could serve as targets to dampen NET-mediated pathology (Chen et al., 2018; Porto & Stein, 2016; Sollberger et al., 2018). It has been shown that acetylsalicylic acid (ASA) treatment and NF-kB blockade, as NET suppressing strategies, could be useful to avoid undesired effects of persistent neutrophil activation (Lapponi et al., 2013). In addition, BAY 11-7082 [(E)-3- [4-methylphenylsulfonyl]-2-propenenitrile] and Ro 106-9920 [6 (phenyl sulfonyl) tetrazolo [1,5-b] pyridazine], two structurally unrelated NF-kB inhibitors, markedly impair NET formation in the peritoneum of infected mice (Lapponi et al., 2013). Furthermore, it is reported that the removal of NETs by treatment with DNase 1 (dornase alpha) limited NET formation in the airways of RSV infected calves (Cortjens et al., 2018). Treatment with DNase 1 also reduced pulmonary NET formation and improved mucus plugging and symptoms in CF patients (Papayannopoulos et al., 2011). Novel NET inhibitors are being studied.. Recently, neonatal NET inhibitory factor (nNIF) has been isolated from umbilical cord plasm and shown to inhibit NET formation by human neutrophils (Yost et al., 2016). NE inhibitors, which will inhibit NE mediated NET formation as well as the cytotoxic effects of NE itself, are also under investigation in clinical studies. The NE inhibitor AZD9668 has been studied in patients with bronchiectasis and demonstrated improved lung function and reduced sputum

inflammatory biomarkers in these patients in phases I and II clinical trials (Stockley et al., 2013). The cysteine protease dipeptidyl peptidase 1 (DPP1, also known as cathepsin C) activates another kind of granule-associated enzymes, neutrophil serine proteases (NSPs), via cleavage of a dipeptide. Targeting the neutrophil weaponry by DPP1 inhibitors, AZD7986 and GSK2793660 to treat COPD and bronchiectasis are in phase 1 of the clinical trial (Doyle et al., 2016; Mårdh et al., 2017).

It is demonstrated that the antidiabetic drug metformin significantly reduced the concentrations of NET components elastase, proteinase-3, histones, and double-strand DNA in the blood of patients with pre-diabetes (Menegazzo et al., 2018). Gasdermin D was only recently identified as a driver of NET formation by providing a feed-forward mechanism; gasdermin D inhibitors could, therefore, attenuate the process of NETosis (Sollberger et al., 2018). Also, a new therapeutic approach using the anti-citrullinated protein antibody (tACPA) showed a broad therapeutic potential. It is shown that NET-mediated pathological symptoms in diseases such as inflammatory arthritis, pulmonary fibrosis, inflammatory bowel disease, and sepsis were prevented via treatment with tACPA in various mouse models (Chirivi et al., 2020). Finally, targeting NET inducing factors, such as macrophage-derived IL1beta, could dampen inflammation (Meher et al., 2018).

Nanoparticles and microRNAs

Controlled drug release facilitated by nano-delivery systems may advance the option for selective modulation of neutrophil activation, migration, and function (Bartneck & Wang, 2019). As an example, doxorubicin (DOX)-conjugated protein nanoparticles can selectively target inflammatory neutrophils for intracellular delivery of DOX that induces neutrophil apoptosis. It is shown that DOX release triggers by acidic environments in neutrophils and subsequently inhibits neutrophil transmigration and inflammatory responses (Zhang et al., 2019). Finally, as microRNAs are known to regulate most protein-coding genes, it offers a potential strategy for several targets in neutrophils (Tahamtan et al., 2020).

FUTURE PERSPECTIVES

Antiviral therapy as a treatment for RSV bronchiolitis shows variable effectiveness even in pediatric and adult immunocompromised patients.. Currently, Ribavirin is the only antiviral therapeutic registered for RSV (Chemaly et al., 2014; Hoover et al., 2018). The low effectiveness of antiviral therapy might be due to the relatively late use during disease (Griffiths et al., 2017). When a patient reaches the hospital, RSV disease already progressed to a phase that cannot be limited by antivirals. In severe cases, an appropri-

ate immune response shifts towards a harmful one, characterized by the neutrophil influx and activation, as is shown by RNA sequencing and immune phenotyping of immune cells from the blood of RSV patients (Jones et al., 2019; Mejias et al., 2013). To what extent the blood's findings can be translated to neutrophilia in the lung is unknown since no data is available on lung neutrophils in children with mild and moderate disease. From a patient with severe RSV infection who enter the intensive care unit, we know that neutrophil influx is already prominent from the day of admission, which is typically at day seven since the start of symptoms (Besteman et al., 2020; Geerdink et al., 2018). Future studies using new technologies such as RNA sequencing, proteomics, and extensive immune phenotyping are needed to identify clinical or diagnostic parameters representing neutrophil activation to determine the optimal timing of the administration of neutrophil targeted therapy.

Additionally, studies identifying risk factors and prognostic markers could identify patients with increased risk of exaggerated neutrophil responses. Repeated sampling within large patient cohorts would provide additional insight into neutrophil biology during different disease phases. Finally, the type of therapy is essential in determining the time of intervention. For instance, ROS inhibition and NET degrading strategies would be beneficial when a patient shows signs of enhanced activation, which is the case in intubated and mechanically ventilated patients in ICU (Besteman et al., 2020; Cortjens et al., 2016; Cortjens et al., 2018; Geerdink et al., 2018). For strategies that target molecular pathways, it could be beneficial to be administer them earlier in the course of the disease. Since we cannot rule out a possible antiviral effect of neutrophils, we propose to target neutrophil function under an umbrella of antivirals as a promising combination strategy to treat one of the most important diseases during infancy.

CONCLUSION

Understanding the mechanisms and factors that govern protective and deleterious neutrophil responses against RSV is crucial to develop effective therapies. While neutrophils might be involved in the antiviral response against RSV disease, their recruitment and activation must be tightly regulated to minimize lung immune pathology. New treatment options for severe RSV infection are required to ameliorate disease and reduce long-lasting sequelae. One treatment strategy might be combining neutrophil-regulating therapeutics under the protection of an antiviral umbrella. Our review serves as a stepping stone to guide future studies that establish neutrophils' role in RSV disease and open a new therapeutic window.

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SUPPLEMENTAL TABLE 1

Potential targets to modulate neutrophil functions and migration during RSV infection

Methods	Target	Effect
Neutrophil modulatory molecules	SHP-1 & MIP-2 (*DNGR-1*)	Limiting tissue damage by dampening neutrophil recruitment
	Cannabinoid and opioid receptors	Modulating neutrophil activity and migration during inflammation
	Adenosine signaling	Inhibiting neutrophil effector functions and migration in high concentrations
	JAM-3	Reducing neutrophil transmigration
	NIFs (*TNHH*)	Blocking the adhesion of neutrophils and H2O2 release
	Selectin & integrin	Inhibiting the adhesion, migration and invasion of neutrophils
Neutrophil chemokine receptors	CXCR2 *(AZD5069, Danirixin)*	Decreasing neutrophil activation and neutrophil chemotaxis
	CXCR1/2 (*Repertaxin, Navarixin)*	Decreasing neutrophil activation and neutrophil chemotaxis
	GRPR	Decreasing neutrophil activation and neutrophil chemotaxis
Neutrophil-expressed inhibitory receptors	LILRB2	Decreasing neutrophil recruitment, limiting NET formation, and ROS production
	LAIR-1	Decreasing neutrophil recruitment, limiting NET formation, and ROS production
	SIRL-1	Decreasing neutrophil recruitment, limiting NET formation, and ROS production
Neutrophil signaling pathways	MyD88/TRIF signaling	Altering neutrophil airway migration
	MAVS signaling	Limiting neutrophil activation
	β-arrestins	Interfering with granule release from neutrophils
	GTPase/Rac2	Interfering with granule release from neutrophils
	SNAP	Interfering with granule release from neutrophils
	Src kinase family *(Resveratrol)*	Reducing respiratory burst, degranulation, integrin expression, and cell adhesion of activated human neutrophils
	MEG2	Interfering with granule release from neutrophils
	PI3K family signaling (*IC87114, RV1729, IPI-145, RV6153*)	Blocking neutrophil superoxide generation and elastase exocytosis
	fMLP	Blocking neutrophil superoxide generation and elastase exocytosis
	PDE4 (*Roflumilast, Rolipram*)	Inhibiting the migration and reducing the numbers of neutrophils
Cytokines	G-CSF	Inhibiting neutrophil production and activation
	IL-17	limiting neutrophil migration
	IL-23	limiting neutrophil migration

6

Human CD14 mediates the innate immune response against respiratory syncytial virus infection

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ABSTRACT

Background: Recurrent severe respiratory syncytial virus (RSV) infection is a rare event and the underlying mechanism is unknown. We aimed to determine the role of CD14-mediated immunity in the pathogenesis of recurrent severe RSV infection.

Methods: We performed extensive genotyping and longitudinal immunophenotyping of the first patient with a genetic CD14 deficiency who developed recurrent severe RSV infection. We analyzed gene expression profiles and IL-6 production in response to RSV pre- and post-fusion (F) protein. We generated CD14 deficient air-liquid interface airway cultures of patient-derived airway cells, and after CRISPR-based gene editing of control airway cells. We analyzed viral replication upon RSV infection.

Results: Sanger sequencing revealed a homozygous single-nucleotide deletion in *CD14*, resulting in absence of the CD14 protein in the index patient. *In vitro,* viral replication was similar in WT and *CD14*$^{-/-}$ HNEC-ALI. Loss of immune cell CD14 led to impaired cytokine and chemokine responses to RSV pre- and post-F protein, characterized by absence of IL-6 production.

Conclusion: We report an association of recurrent severe RSV bronchiolitis with a loss of CD14 function in immune cells. Lack of CD14 function led to defective immune responses to RSV pre- and post-F protein without a change in viral replication.

Abbreviations:

CD14 (Cluster of differentiation 14), HC (healthy control), HNEC-ALI (human nasal epithelial cells cultured at air-liquid interface), Ig (immunoglobulin), LBP (lipopolysaccharide -binding protein), LPS (lipopolysaccharide), MD-2 (myeloid differentiation factor 2), post-F (post-fusion), pre-F (pre-fusion), RSV (respiratory syncytial virus), RTI (respiratory tract infection), TLR (Toll-like receptor), WT (wild type).

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most important viral pathogens identified in respiratory tract infections (RTI) in children. It causes a major global health burden, with a hospitalization rate of ~3.2 million and an estimated yearly mortality rate of 125,000 in children under five.[1, 2] Most infants who develop severe infection and require admission to the intensive care unit, are below the age of three months.[3] Recurrent RSV hospitalization is rare and without a known genetic etiology. The recent discovery and stabilization of the pre-fusion (pre-F) conformation of the RSV surface F glycoprotein accelerated vaccine development.[4] Still, designing an effective RSV vaccine or therapy remains challenging.[5] Defining immunological determinants of RSV susceptibility and disease severity may aid the advancement of new therapeutics and vaccine development.[5, 6]

RSV F is required for infection of respiratory epithelial cells. After entering the lungs, RSV is confronted with the innate antiviral responses mediated by the airway epithelium and lung resident immune cells such as alveolar macrophages.[6] These first molecular events upon infection define the size and direction of the inflammatory response. A dysregulated inflammatory response can lead to increased viral burden, as well as enhanced pulmonary inflammation.[6] Hence, robust innate defenses that partially control virus replication will reduce the level of adaptive immune responses needed to clear infection, reducing immunopathology.[7] Toll-like receptors (TLRs) such as TLR2 and TLR4, and the co-receptor CD14, have been shown to modulate the immune response to RSV and accelerate viral clearance.[8-10] The CD14 receptor exists in two forms; membrane bound by a GPI-anchor to immune and epithelial cells (mCD14), and soluble (sCD14), circulating in the blood and at mucosal sites.[11] CD14 functions primarily as a lipopolysaccharide (LPS) transferase, greatly accelerating the binding of LPS to a complex consisting of TLR4 and myeloid differentiation factor 2 (MD2).[12, 13] The association of CD14 function with RSV disease outcome remains controversial, and the clinical relevance of CD14 as part of the putative RSV receptor complex has not been shown.[14-17] It has even been proposed that RSV evolved the ability to stimulate the CD14-TLR4-MD2 axis for its own benefit.[14] Over the years the role of CD14 has been extensively studied in cell lines and animal models, but a human model has yet to be described. Here, we aimed to determine the role of local and systemic CD14-mediated immunity in the pathogenesis of RSV infection. Additionally, we describe the first child with a homozygous frameshift mutation in *CD14*, leading to autosomal recessive CD14 deficiency, as a novel genetic etiology associated with recurrent severe RSV bronchiolitis.

MATERIAL AND METHODS

Study Participants

The patient and infant controls were initially enrolled through a study previously performed by our group (Neon Study: IRB review reference number NL58404.041.16).[18] All participants gave written informed consent. The Parent Advisory Board of our RSV research team was involved in writing a patient information letter specifically for the index patient.

Immunophenotyping

We performed flow cytometry to identify CD14 surface expression on patient and control immune cells. Whole blood was depleted from red blood cells. Cell were stained for surface markers for 20 min at 4°C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) bovine serum albumin (BSA). Cell types were identified according to their characteristic forward and side light-scatter properties, and by their typical cell surface markers: neutrophils: $CD14^{-}/CD16^{+}$, monocytes: $CD14^{+}/CD16^{-}$. 7-aminoactinomycin D (7-AAD) was used to distinguish apoptotic cells. Generation of RSV-pre and post-F probes and identification of RSV-pre and post-F binding B-cell was performed as described previously.[4]

Monocyte isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood using Ficoll. Monocytes were isolated from the PBMCs by Percoll gradient. Standard Isotone Percoll (SIP) was prepared by adding 10:1 10x PBS. PBMCs were taken up in 60% SIP, followed by gentle addition of 47.5% and 34% SIP, centrifuged for 45 minutes, 1750 RCF, at room temperature. Monocytes were collected from the upper ring and washed twice with PBS. After isolation, PBMCs or monocytes were stimulated with a panel of Toll-like receptor (TLR) agonists: LPS (S. typhosa (Sigma Aldrich) or E. coli Ultra pure) 1, 10 and 100 ng/mL, PAM3CSK 30 and 100 ng/mL, ODN 0.3 and 1 mM, R848 1 or 3 µg/mL, Poly I:C 0.1 and 1 µg/mL, flagellin 1 and 5 µg/mL, FSL-1 1 and 10 µ/mL, Lipoteichoic acid 0.1 and 1µg/mL (Sigma Aldrich). All TLR stimuli were obtained from Invivogen, unless stated otherwise. Where indicated monocytes were pre-incubated for 30 minutes at 4°C with 10µg/mL anti-hCD14-IgG (Invivogen) or mouse anti-human HLA-ABC (BD Pharmingen) as control. Polymyxin B 10µg/mL (Invivogen) was added to the PBMCs 10 minutes prior to the addition of LPS or RSV F. For heat inactivation, LPS and RSV F were heated during 10 min at 99°C before adding this to the PBMCs. All assays with cells derived from the index patient were performed within the age range of 10 months to 3 years.

Collection and differentiation of nasal epithelial cells

Primary human nasal epithelial cells (HNECs) were obtained from healthy children, and the index patient by nasal brushes. All participants or their caregivers provided written informed consent. The study was approved by a specific ethical board for the use of biobanked materials TcBIO (Toetsingscommissie Biobanks), an institutional Medical Research Ethics Committee of the University Medical Center Utrecht (protocol ID: 19/678). Nasal airway cells were collected, isolated, expanded and differentiated as previously described.[47] The basal cells were differentiated on Transwell® inserts (Corning) in an air-liquid interface (ALI). They were cultured for ~18 days to obtain a complete differentiated epithelium. Culture medium was refreshed twice a week, including an apical wash of 120 µl PBS (5 minutes incubation).

Generation of CD14 knockouts in airway epithelial basal cells using CRISPR-Cas9

CD14 knock-out cell lines were made with CRISPR-Cas9 technologies as described previously.[48, 49] Before electroporation, sgRNA (30 µM, Synthego) and Cas9 protein (20 µM, Synthego) were mixed with optiMEM supplemented with Y27632 (10 µM) and incubated for 10 minutes at room temperature to generate RNP complexes. One million trypsinized basal cells per sample were dissolved in 75 µL of optiMEM supplemented with Y27632 (10 µM) and added to the RNP complex mixture. The NEPA21 electroporator was used for transfection according to previously published settings [50]. After electroporation the basal cells were expanded. 0.2 million cells were used for DNA isolation and the other cells were seeded for ALI differentiation. DNA was isolated according to the manual of the Quick-DNA Microprep Kit (Zymo research). Regions of interest were amplified in a PCR reaction with GoTaq G2 Flexi DNA polymerase with primers, and PCR-amplified samples were run on 1,2% TBE-agarose gel for size separation. DNA fragments were excised from the gel, purified according to the gel extraction kit (Qiagen), and sent for Sanger sequencing with sequencing primers (supplementary figure S5).

Viral infection of HNEC-ALI

One day before infection, culture medium of the HNEC-ALI was refreshed. RSV-A2-GFP was diluted in serum-free Opti-MEM (Gibco) to obtain a multiplicity of infection (MOI) of 1. HNEC-ALI were infected with 100 µl RSV-A2-GFP or serum-free Opti-MEM only (mock), and incubated at 37°C, 5% CO_2 for two hours. After incubation, apical medium was aspirated carefully and the apical surface was washed twice with 125µl of warm PBS. After infection the cultures were incubated for up to 72 hours. Each day, basal medium was refreshed and stored, without washing the apical compartment. Pictures were taken each day with an EVOS microscope using a 40x and 100x objective. At the indicated day post infection (dpi), 200µl of serum-free Opti-MEM was added to the inserts and

incubated for 30 minutes at 37°C, 5% CO2. Supernatant from both the apical and basal compartment were stored for ELISA. Subsequently, cultures were lysed for gene expression by real-time quantitative PCR. From the apical wash, viral titers were measured by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

RNA isolation and real-time quantitative PCR analysis of ALI

For RNA isolation, cells were lysed using RLT + 1% β-mercaptoethanol. Biological duplicate lysates were pooled to obtain a final cDNA concentration of 500ng/μl. Extracted mRNA (RNeasy Mini Kit QIAGEN) was used for cDNA synthesis, using iScript (Bio-Rad) and a T100 Thermal Cycler machine (Bio-Rad).

For relative gene expression, cDNA transcripts were quantified by real-time quantitative PCR (CFX Connect Real time PCR, CFX-96 Real time PCR Bio-Rad) with IQTM SYBR Green Supermix (Bio-Rad, FR) and specific primers for RPL13A, B-ACTIN, P63, MUC5AC, FOXJ1, and CD14. Expression levels of evaluated genes were calculated by relative quantification, using the standard curve method. Additionally, each value was corrected for the expression of the following housekeeping genes: B-ACTIN or RPL13A.

ELISA and quantification of RSV F protein-binding antibody

Standard ELISA kits were used according to the manufacturers' protocols to measure: IL-6 and IL-8 (Ready-Set-Go! (V2) Ebioscience), CD14 and LBP (both RnD systems). Serum binding assays were performed as described previously.[4]

RSV IgG concentrations and avidity assays

Analysis of IgG in serum was performed using a RSV multiplex immunoassay as described earlier. The avidity of the RSV-specific antibodies was determined using the same RSV multiplex immunoassay with some adaptations. After incubation of the sera with the RSV proteins, all samples were additionally incubated for 10 minutes, at room temperature, in threefold on the same plate in the presence of 1.5 M Ammonium thiocyanate (NH_4SCN), 3.0 M NH_4SCN or PBS (pH 7.4). The avidity index (AI) was expressed as the percentage of residual MFI IgG signal in comparison to the undenatured (PBS) signal which was set at 100%.

RSV neutralization assays

Neutralization by sera from the index patient, 15 adult controls of a previous study [21], and the BEI NR-4020 control sera were measured by a fluorescence plate reader neutralization assay described previously.[4]

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 8.3.0. *P* values <0.05 were considered significant.

Additional information on the applied methodologies can be found in this article's online supplement.

RESULTS

Clinical phenotype of CD14 deficiency

The index patient, a boy, had life-threatening RSV type B bronchiolitis at the age of nine months (figure 1a). He required intubation and invasive mechanical ventilation at the pediatric intensive care unit (PICU) for a period of 11 days. He had higher levels of RSV (RSV Ct-value: 19.18), compared to control infants admitted to the PICU with severe RSV (mean RSV Ct-value: 24.51) (figure 1b), measured in broncho tracheal aspirates. He had three additional episodes of RSV bronchiolitis at the age of 14 and 22 months and 4 years, requiring hospital admission for observation of the respiratory status, and supplemental oxygen supply. The patient tested negative for RSV between episodes. At the time of the first RSV episode we discovered the patient's CD14 deficiency studying the patient's leukocytes via flow cytometry (supplementary figure S1).[18] This was confirmed by Sanger sequencing which showed a single-nucleotide deletion in the *CD14* gene: a homozygous variant (NM_000591.4:c.196del), resulting in a frameshift and truncation in the CD14 protein (NP_000582.1:p.Leu66*) (figure 1c and supplementary figure S2). This rare variant is present in the European (Non-Finnish) population with an allele frequency of 4.54e-05 and is absent in the homozygous state (Gnomad). Both parents were heterozygous for the mutation, and the sister had a normal genotype. Both parents had normal CD14 cell surface expression (supplementary figure S3). No other de novo or recessive variants were identified that score as variant of uncertain significance or higher according to the 2015 ACMG-AMP guidelines.[19] The child was admitted three times with non-RSV respiratory tract infections, including human rhinovirus (HRV)/enterovirus infection. Figure 1a, shows an overview of the events in the patient's medical history, and an extended case report is presented in an online repository. Altogether, the clinical phenotype of CD14 deficiency is characterized by respiratory infections, in particular recurrent and severe RSV bronchiolitis.

Immunological phenotype of human CD14 deficiency

Evaluation of patient's leukocytes by flow cytometry showed no CD14 cell surface expression (truncated or intact), and there was no intact sCD14 detected in the plasma

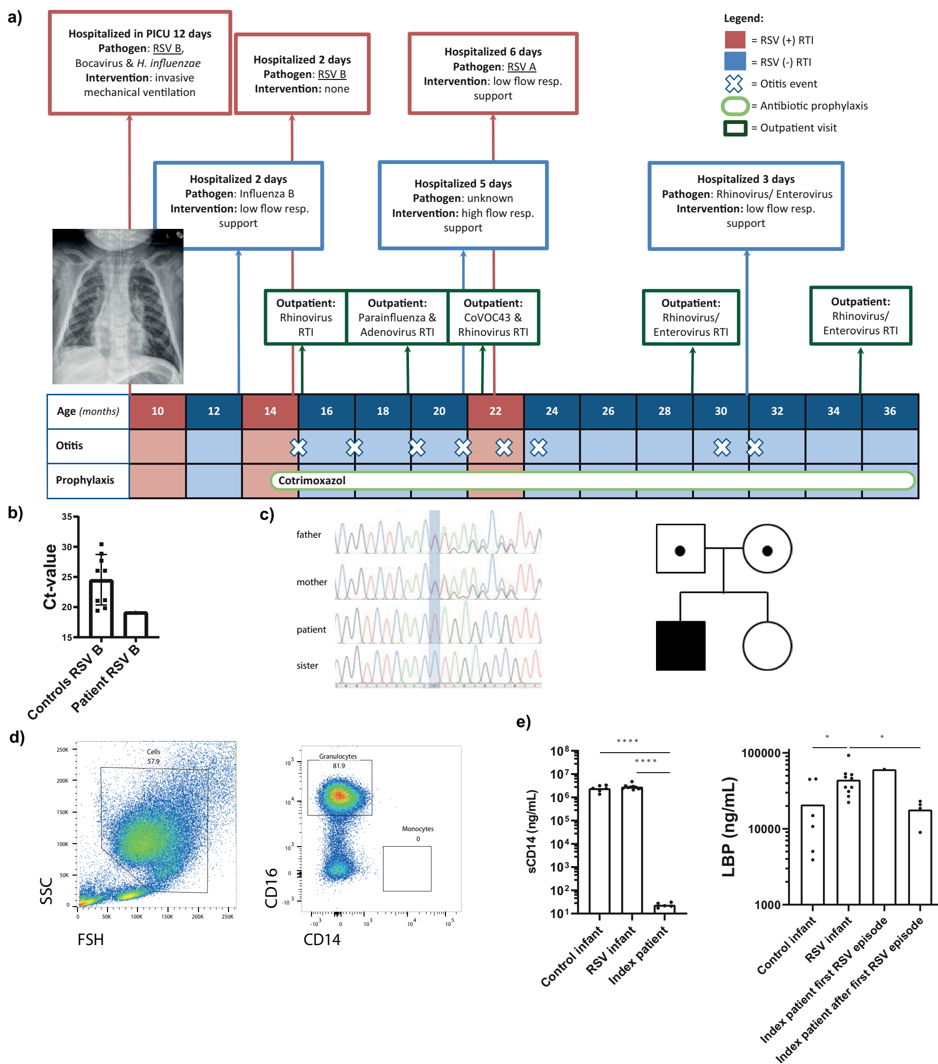


Figure 1. Phenotypic and genetic analysis of CD14 deficiency

(a) Timeline of critical events in the patient's medical history, according to his age in months. (b) RSV Ct-value of the index patient during the first RSV infection and of nine controls admitted with RSV at the PICU in the same season. (c) Genomic DNA sequence of *CD14* comparing the patient (*CD14*$^{-/-}$) with his parents (both *CD14*$^{+/-}$) and sister (wild type). The blue band indicates the deletion of C in the index patient. In the family pedigree circles and squares denote female and male family members, black dots represent a heterozygous mutation in *CD14*, the black square represents the index patient. (d) Flow cytometry plot of the index patient. Cells were gated based on their forward and side scatter properties, single, non-apoptotic cells were selected. Monocytes were identified a CD14^{+}/CD16^{-}. (e) Plasma levels of sCD14 in the index patient compared to control infants. Levels of LBP during and after the first RSV episode in the index patient, compared to infant controls. An unpaired t-test was performed to compare expression between patient groups. Only significant values are shown. * p = <0.05, p = <0.0001.

RTI = respiratory tract infection, resp. = respiratory, ($^{-/-}$) = genetic knock out, CRP = c-reactive protein, CoVOC43 = Human coronavirus OC43.

(figure 1d-e). In case a truncated form of CD14 is present in the circulation, we predicted this would not be functional since it lacks the pocket rim needed for LPS binding (supplementary figure S2b).[20] We measured LPS-binding protein (LBP) levels in patient plasma during his first RSV episode and at several time points thereafter. Our index patient, and other infants hospitalized with severe RSV had mildly increased LBP) levels compared to control infants. After the first RSV episode, the index patient had normal LBP levels (figure 1e).

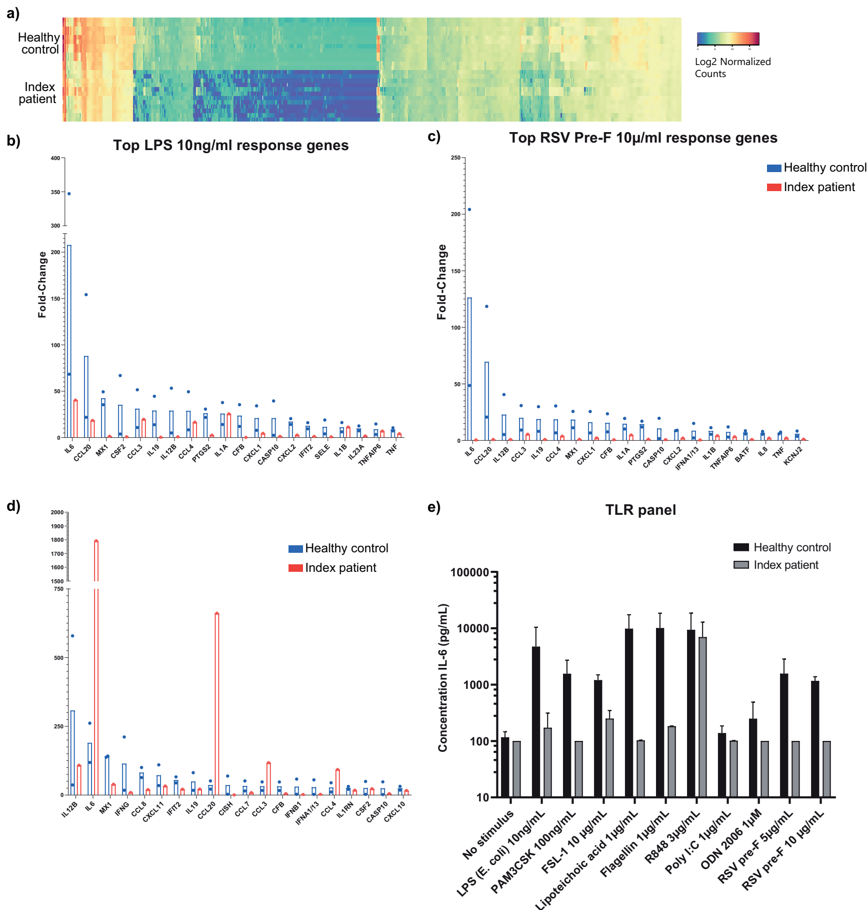


Figure 2. Impaired innate immune response to TLR 1/2, 2/6, 4 and 5 stimulation in CD14 deficiency
(a) Gene expression by PBMCs after 4 hours of stimulation with a panel of TLR ligands, quantified using NanoString Technology^{TM}. Log2 normalized counts are represented for the index patient (top), and a healthy control (bottom). Upregulated genes are depicted in red, downregulated genes in blue. Each row represents a stimulus. Each column represents one gene. (b-d) Expression of the top 20 response genes measured in A, after stimulation with LPS (E. coli) (10 ng/mL), R848 (1 µg/mL) and RSV-pre-F (10 µg/mL). Fold change compared to unstimulated is shown for a healthy control (blue bars) and the index patient (red bars). (e) IL-6 protein expression by patient and control PBMCs after TLR-stimulation. Values show the mean of two independent experiments.
HC = healthy control, IP = index patient.

CD14 deficiency leads to impaired Toll-like receptor signaling

Next, we studied the functionality of CD14-dependent signaling pathways by stimulating PBMCs of the patient, and healthy adult controls with TLR agonists and measured innate immune gene expression by NanoString™, a methodology for measuring mRNA levels in the absence of amplification. The innate immune response to TLR 1/2, 2/6, 4 and 5, but not TLR 7/8 stimulation was impaired in the patient's PBMCs compared to healthy controls (figure 2a-c). This included an absent *IL-6* mRNA response to RSV-pre-F in the index patient (figure 2d). Additionally, mRNA expression of several chemokines (*CCL2, CCL20*) and interferon-stimulated genes (*MX1, IFNa 1/13*) were compromised (figure 2d). Subsequent protein measurement of IL-6 in supernatant confirmed the *IL-6* mRNA-data (figure 2e). In line with these results, prolonged stimulation of patient-derived PBMCs (supplementary figure S4) with LPS, but not the TLR7/8 ligand R848, with varying concentrations, showed impaired IL-6 protein production. We conclude that this patient, with a homozygous autosomal recessive CD14 deficiency, demonstrated a loss of known CD14 functions.

Airway epithelial derived CD14 does not inhibit RSV infection

Next we studied whether the recurrent severe RSV infections in our patient were caused by the absence of CD14 in airway epithelial cells. First, we compared RSV infections in healthy control (HC) and CD14 deficient patient derived ALI-HNEC (figure 3a). As expected, soluble CD14 protein levels could be detected in HC ALI-HNEC culture supernatants, but not in the supernatants of the CD14 deficient patient (figure 3b). However, we did not observe differences in RSV replication between HC and CD14 deficient patient-derived cells in time (figure 3c). We further confirmed the lack of effect of CD14 deficiency on RSV infection in airway epithelial cells by using $CD14^{-/-}$ cells developed with CRISPR-Cas9 based gene editing (supplementary figure S5). Corresponding with CD14 deficient patient derived cells, $CD14^{-/-}$ cells displayed a lack of CD14 expression compared with wild type (WT) control cells (figure 3d, 3e). Furthermore, RSV replication in $CD14^{-/-}$ ALI-HNEC was comparable with WT controls (figure 3f). Based on these findings, we conclude that in the absence of immune cells, epithelial derived CD14 does not inhibit RSV infection in the airway epithelium.

CD14 expression on immune cells mediates the innate immune response against RSV fusion protein

To investigate the role of systemic CD14 in the immune response to RSV F, patient immune cells were stimulated for up to 24 hours with pre-F or post-F protein. This resulted in impaired IL-6 response to both proteins in the patient (figure 4). In line, surface blockade of CD14 with a monoclonal antibody in healthy donor monocytes inhibited the IL-6 protein response to pre-F and post-F (figure 5), which confirmed previous observations.

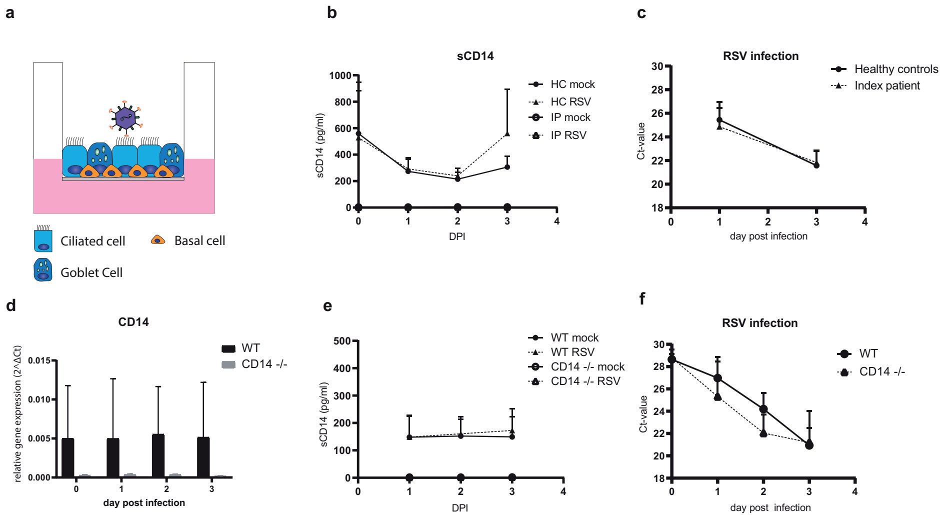


Figure 3. Airway epithelial derived CD14 does not inhibit RSV infection

(a) Schematic view of RSV infection of HNEC-ALI. HNEC-ALI was derived from healthy control children (n=2), the index patient, WT (n=4) and CD14 (n=4). (b) Cytokine production of sCD14 at day 1, 2 and 3 post infection in cultures of the index patient and controls. (c) Cultures were infected with RSV-A2-GFP, no significant difference in Ct-value was observed between the index patient and controls. (d) Plot shows mRNA expression of CD14 in WT and CD14$^{-/-}$ cultures. We confirmed the absence of CD14 mRNA expression in CD14$^{-/-}$ cultures. (e) Cytokine production of sCD14 at day 1, 2 and 3 post infection in WT and CD14$^{-/-}$ cultures. (f) Cultures were infected with RSV-A2-GFP, no significant differences in Ct-value were observed between WT and CD14$^{-/-}$ cultures.

HC = healthy control child, IP = index patient, WT = wild type, CD14$^{-/-}$ = CD14 knock out, DPI = day post infection, HNEC-ALI = human nasal epithelial cells cultured at air liquid interface.

[9] To rule out that this response was caused by LPS contamination of RSV F stocks, we measured IL-6 after heat inactivation, and in the presence of LPS-neutralizing peptide polymyxin B. After stimulation with heat inactivated RSV F IL-6 was not detectable in culture supernatant, while it was present after addition of polymyxin B, ruling out LPS contamination (supplementary figure S6). Additionally, we investigated adaptive immune responses to RSV. The patient had normal seroconversion and concentrations of RSV specific antibody titers, with normal avidity (figure 6a-b). Furthermore, the binding and neutralizing activity of total pre-F and pre-F exclusive RSV antibodies in the serum was normal (figure 6c). Finally, B cell phenotyping revealed that memory IgG+ B cells binding to pre-F, post-F, or both probes fell within the expected range (figure 6d), demonstrating an intact memory B-cell response.[21] In conclusion, we show that systemic CD14 mediates the innate immune response against RSV infection.

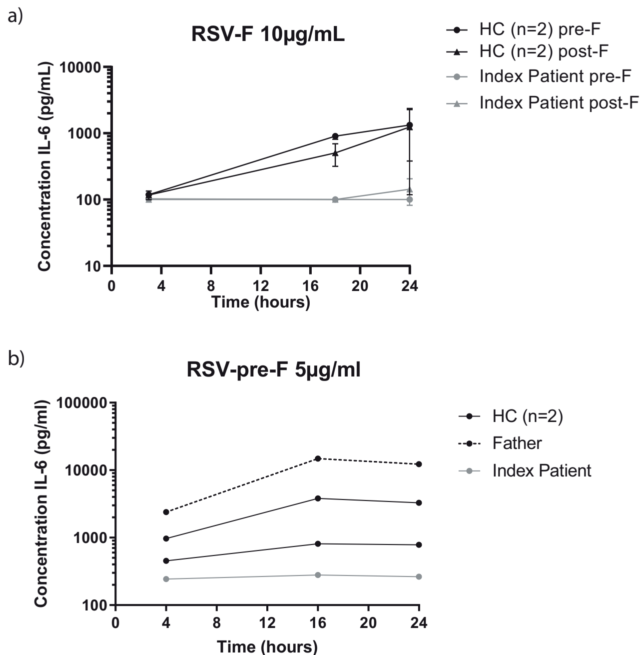


Figure 4. CD14 expression on monocytes mediates the IL-6 response to RSV-F

(a) IL-6 protein response after 4, 18 and 24 hours of stimulation by RSV-pre-F and post-F (10µg/mL), by PBMCs of two healthy controls and the index patient. One experiment, performed in duplicates, lines indicate mean per individual (±SD). (b) IL-6 protein response during 24 hours of stimulation by RSV-pre-F (5µg/mL) by Percoll-isolated monocytes of two healthy controls, the index patient and his father. One experiment, lines indicate mean per individual.
HC = healthy control, IP = index patient.

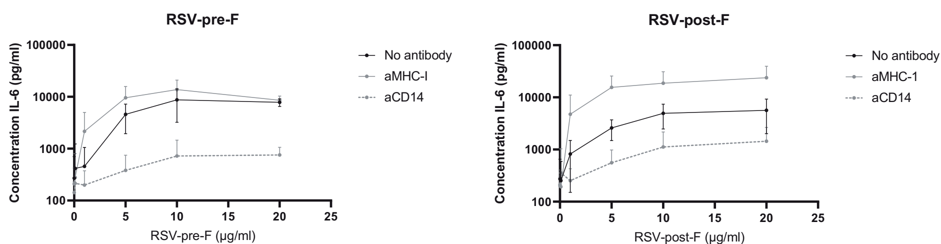


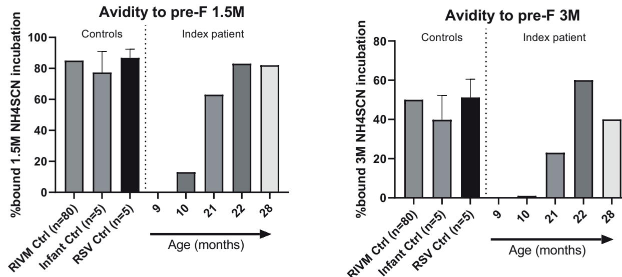
Figure 5. Impaired IL-6 protein response to RSV-F after blocking CD14 in healthy adult donor monocytes.

The figure shows IL-6 protein production after stimulation of healthy donor monocytes with RSV pre-F (left) or post-F (right), by healthy donor monocytes, after Percoll isolation, in the presence of CD14 blocking antibodies (aCD14), control antibody (aMHC-1) or no antibody. The lines indicate the mean (±SD) of three independent experiments with a total of seven donors.

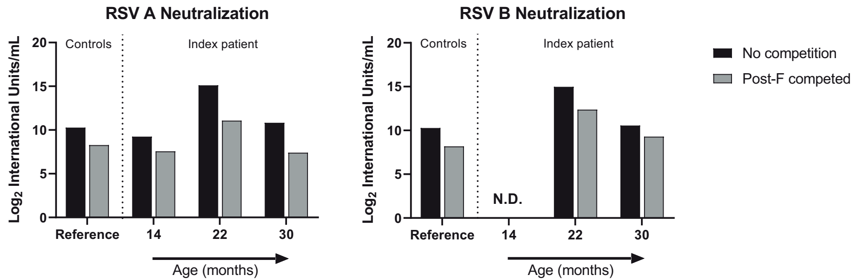
a)

Age (mo)	RSV status	Ga	Gb	Post F	N	Pre f
9	First RSV episode	0	0	1.0	0.6	1.8
10	-	7.9	15.2	947.5	1765.5	320.9
14	Second RSV episode	No sample available				
21	-	0	2.3	224.3	646	109.6
22	Third RSV episode	14.2	28.5	5973.8	11210.4	7770.4
28	-	110.7	7.2	1629.9	2100.2	1386.5

b) Normal RSV antibody maturation



c) Normal RSV A and B neutralization



d) Normal B-cell response

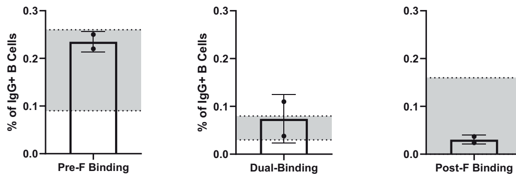


Figure 6. Normal pre-F-specific antibody response in CD14 deficiency

(a) Seroconversion of RSV specific antibodies after the first RSV infection in the index patient. Depicted are the concentrations IgG against Ga, Gb, Post-F, N and Pre-F in arbitrary units/mL at different time points during and in between RSV infections. (b) Percentage pre-F specific IgG bound after incubation with 1.5M NH4SCN (left graph) and 3M NH4SCN (right graph), at the time points indicated in a. (c) Conformation-specific RSV A and B neutralizing activity in sera obtained at 14, 22, and 30 months, in the absence and presence of excess post-F. Bars represent mean neutralizing activity normalized to International Units of Neutralization. (d) B-cell specific binding to RSV-pre- and post-F,8 months after the 2nd RSV infection and 6 months after 3rd RSV infection. Bars show assembled percentage of total IgG+ B cells that bind pre-F, post-F, or both probes (dual binding) in the index patient. In grey the interquartile range of healthy controls is depicted.
RIVM = Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment), N. D. = not determined due to too little available serum, Q1 = first quartile, Q3 = third quartile.

DISCUSSION

Life-threatening RSV infection at age 9 months, followed by recurrent RSV-related hospitalizations, is rare [3], and a possible genetic predisposition is unknown. Although single nucleotide polymorphisms (SNPs) in the *CD14* and *TLR4* genes have been associated with severe RSV bronchiolitis, a genetic link to recurrent severe disease has not been described.[22, 23] We identified the first CD14 deficient patient with an impaired innate immune response against RSV. We show that airway epithelium derived CD14 did not affect RSV viral infection and that CD14 on immune cells impaired the immune response against RSV. Our data suggest a beneficial role for CD14 in RSV immune signaling, and we extend these findings to a clinical phenotype for CD14 deficiency associated with recurrent respiratory tract infections.

Casanova showed that susceptibility to common severe infections can be linked to single-gene inborn errors of innate immunity.[24] Previous TLR signaling deficiencies linked to disease in humans include: TLR3 deficiency as a cause of herpes simplex encephalitis, MyD88-IRAK4 deficiency in pyogenic infections, MDA5 deficiency underlying severe HRV infection, inherited IRF7 and IRF9 deficiencies associated with severe influenza pneumonitis, and TIRAP deficiency as a cause of staphylococcal disease.[25-31] We demonstrated that a deficiency of CD14 was characterized by, but not limited to, recurrent severe RSV infections. The patient was also hospitalized with RTIs caused by other viruses, which might also be part of the CD14 deficiency phenotype, but a link between CD14 and these viruses has not been reported in literature. *In vitro,* the patient's immune cells showed an impaired innate immune response to the RSV F protein. Lung resident cells, including airway epithelial cells, alveolar macrophages and dendritic cells, together with infiltrating monocytes and neutrophils initiate the innate immune response against RSV.[6, 32] Impaired innate immune responses by monocytes, as in our patient, are likely to affect the host's susceptibility to RSV. This is supported by our previous observation that SNPs in innate immune genes are related to predisposition to RSV infection.[33] Our data show that without CD14-mediated engagement of innate immune responses, adaptive immunity to RSV may be inadequate to control viral replication and protect from recurrent severe RSV disease.

In humans, CD14 has been shown to interact with TLR2 and TLR4 [34], while in mice CD14 also associates with TLR3, TLR7 and TLR9.[35, 36] We identified an impaired immune response to agonists against TLR1/2, 2/6, 4 and 5, but not TLR 7/8 in the index patient. We previously found that TLR2/6 and TLR4 can interact with RSV F to promote innate immune responses.[8, 9] It is uncertain which TLR or co-molecules are required for CD14- RSV F signaling in our patient, but direct interaction between CD14 and RSV F protein, facilitated by the presence of MD2, has been reported.[9, 10, 37] Dysregulation of TLR signaling impacts various functions within the immune system, including

negative regulation of dendritic cell maturation, and changing B-cell proliferation.[38, 39] Defective TLR signaling has been associated with enhanced RSV disease caused by poor antibody affinity maturation.[40] In our patient, antibody affinity, avidity and B- cell memory were normal, suggesting intact antibody maturation in CD14 deficiency.

A strength of our study is that we were able to perform extensive clinical, genetic, epithelial and immunological phenotyping. We were able to address the longstanding question about the clinical relevance of CD14 in RSV disease. Furthermore, the broad clinical phenotype of recurrent respiratory infections, and our *in vitro* TLR data, indicated a central role for CD14 in innate immune signaling that extends beyond TLR4. Soluble CD14 is studied as a marker for severe disease during viral infections, including in individuals with HIV infection.[41] Therapeutics targeting CD14, have been studied and are currently being developed, such as the use of a monoclonal anti-CD14 antibody (IC14) for the treatment of severe COVID-19.[34, 42] Our study exposes potential adverse effects of such therapies.

Limitations also require discussion. First, we described a single CD14 deficient patient, and no other patients could be identified because of the low allele frequency. However, there are several other loss of function mutations identified in the CD14 gene (combined allele frequency of 2,5x10e-4), although no other CD14 deficient patient has been described thus far. Second, the amount of blood limited the number of tests we could do. For instance, we did not investigate phagocytosis or monocyte responses to viruses other than RSV. Third, for practical reasons adult controls were used for stimulation experiments with immune cells. We found impaired TLR 1/2, 2/6, 4, and 5 responses in the index patient up to age three, when TLR responses are at adult level in normal children.[43-46] For the epithelial studies we were able to use healthy pediatric controls.

We described a novel single-gene immunodeficiency resulting in a phenotype characterized by, but not limited to, recurrent severe RSV infections. This patient establishes an important role for CD14 in RSV pathogenesis. Furthermore, CD14-mediated innate immune responses appear particularly critical to protect against pathogens in the respiratory tract. We predict that other loss of function mutations in the CD14 signaling pathway may be similarly associated with recurrent severe RSV infections.

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ONLINE SUPPLEMENT

CASE DESCRIPTION

The index patient was the second child of two non-consanguineous parents. He was born at term with a birthweight of 4470 grams. He exhibited normal growth and reached developmental milestones at the appropriate age. At the age of six months, he presented at the outpatient clinic with mild viral wheeze and started with salbutamol inhalations for occasional use. There were no signs of eczema or allergy. He was regularly immunized according to the national immunization program. The family history was negative for atopy, immuno-deficiencies or gastrointestinal diseases. Although both parents had the same ethnic background, their ancestors came from different regions. Therefore, we cannot confirm consanguinity.

At the age of nine months, the patient presented in the emergency department with vomiting, diarrhea and minimal fecal blood loss. He was diagnosed with mild viral gastro-enteritis, without signs of dehydration, and sent home. There was no pathogen identified. One week later, he presented again in the emergency department. The enteral complaints subsided, but he presented with a fever of 39°C, dyspnea and wheezing. He was admitted to the pediatric ward for monitoring and supplemental oxygen support through a high flow nasal cannula. Due to progressive dyspnea, he required intubation for invasive mechanical ventilation in the pediatric intensive care unit (PICU). At the PICU, he was diagnosed with RSV bronchiolitis with a bacterial superinfection of beta-lactamase positive *Haemophilus influenzae*. He was prescribed a seven day course of amoxicillin and clavulanic acid. However, two days after the discontinuation of the antibiotic therapy, he became increasingly ill with fever of 40.1 °C, a rise in CRP levels to a maximum of 205 mg/L and deteriorating pulmonary status leading to the need of high ventilator pressure. Sputum cultures showed a resurgence of *H. influenzae* growth, and urine cultures revealed the growth of *E. coli* and *Klebsiella pneumoniae*. Blood cultures were negative. The patient was put on ceftazidime intravenously for seven days. Because of anemia with a hemoglobin level 4.4mmol/L, he received packed cells. During admission, he developed mild edema, which was treated with furosemide and spironolactone. Sedation medication consisted of intravenous midazolam and morphine. He was mechanically ventilated for 11 days and discharged the next day to the pediatric ward. In the ward, he required supplemental oxygen through a low flow nasal cannula for 14 days. Furthermore, the patient developed a delirium, due to withdrawal of sedatives, which was treated with risperidone and methadone. After 15 days in the pediatric ward, he was discharged home.

During the admission to the PICU, the patient was enrolled in a study regarding neutrophil function during RSV bronchiolitis.[1] In this study, we found absence of the CD14 receptor on the patient's monocytes by flow cytometry (supplementary figure S1). Neutrophil function - oxidative burst by sputum neutrophils and neutrophil extracellular trap-formation by blood neutrophils - were comparable to other infants with severe RSV. The leukocyte differential count was normal, with average monocyte counts. We excluded paroxysmal nocturnal hemoglobinuria as a cause of absent CD14, since other GPI-anchored receptors were present (FLAER, CD157 and CD59). Finally, Sanger sequencing of the CD14 gene revealed a single-nucleotide deletion in the CD14 gene: a homozygous variant (NM_000591.4:c.196del), resulting in a frameshift and truncation in the CD14 protein (NP_000582.1:p.Leu66*) (figure 1, C and supplementary figure S2). Both parents were heterozygous for the same mutation, with normal CD14 expression on monocytes (supplementary figure S3). This confirmed the diagnosis of an autosomal recessive (AR) CD14 deficiency.

In the subsequent months, the index patient developed frequent episodes of otitis media with otorrhea, treated with antibiotic auricular drops (containing dexamethasone, framycetin and gramicidin), and recurrent cervical lymphadenitis. At the age of 13 months, he was prescribed antibiotic prophylaxis with trimethoprim-sulfamethoxazole. He continued to use salbutamol inhalations on occasion, and at the age of 2.5 years he was prescribed corticosteroid inhalation therapy. His weight and height gain were normal.

At the age of 14 and 22 months and 4 years, he was again admitted to the pediatric ward with respiratory distress due to RSV bronchiolitis. At age 14 months, he received no supplemental oxygen and was released after one day. At 22 months and age 4 years, he required a low flow nasal cannula for respiratory support over a period of four and two days respectively. Additionally, he was admitted three times with acute respiratory tract infection (ARTI) and wheezing due to other viral pathogens in the first four years of life. At the age of 21 months we explored microbial flora in the airways of the patient, this showed normal commensal flora. Table S2 and S3 show an overview of all virology and bacteriology results.

To evaluate the possibility of permanent lung damage as a result of recurrent RTI's and CD14 deficiency, we performed a CT scan of the lungs at the age of 21 months. The CT scan showed mild ground glass phenomena surrounding the apex and in the right and left lower lobe (supplementary figure S7). IgE was modestly elevated (176 kU/L) and showed no specific IgE against respiratory allergens. Additional immunological investigation did not reveal abnormalities in other immune cell functions (normal B and T cell subsets, IgA, IgM and IgG, and a normal response to 10-valent pneumococcal vaccination) (table S4 and S5). Broad viral serology was negative for EBV, influenza A and B, and positive for CMV, and VZV. Vaccination responses to polio, measles, diphtheria and

tetanus were normal. Mumps vaccination failed to elicit a protective response after the first immunization at age 14 months (table S6). A SNP-array showed no abnormalities. Whole exome sequencing showed, besides the CD14 mutation, a heterozygous mutation in the ABCB4 gene. This is rarely associated with progressive familial intrahepatic cholestasis (#OMIM 602347), but liver enzymes tested normal in our patient. No other de novo or recessive variants were identified that score as variant of uncertain significance or higher according to the 2015 ACMG-AMP guidelines.*[2]*

SUPPLEMENTARY METHODS

Patient and samples

The patient and infant controls were initially enrolled through a study previously performed by our group and published elsewhere (Neon Study: IRB review reference number NL58404.041.16).[1] All participants, or the legal guardian in the case of a minor, gave written informed consent. Healthy adult donors were used when indicated. For the index patient, all procedures were based on standard of care and established clinical guidelines were followed. The Parent Advisory Board of our RSV research team was involved in writing a patient information letter specifically for the index patient.

Collection of blood and plasma samples of the control children were within 48 hours after admission to the PICU. Nasopharyngeal swabs for determination of the RSV subtype and Ct value were taken within 48 hours after admission to the PICU. Severe RSV was defined as being in the need for mechanical ventilation at the PICU.

Sanger sequencing

Sanger sequencing confirmed CD14 deficiency. Specifically, CD14 coding exons were amplified and sequenced with gene specific primers (primer sequences and cycling conditions available upon request).

PBMC and monocyte isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood using Ficoll. First, the blood was diluted 1:1 with PBS, added on top of Ficoll and centrifuged for 30 minutes, 1000 RCF, acceleration 2, break 2, at room temperature. PBMCs were collected and washed twice. Monocytes were isolated from the PBMCs by Percoll gradient. Standard Isotone Percoll (SIP) was prepared by adding 10:1 10x PBS. SIP was then diluted to a concentration of 34%, 47.5% and 60% with IMDM containing 1% FCS. PBMCs were taken up in 60% SIP, followed by gentle addition of 47.5% and 34% SIP. This was centrifuged for 45 minutes, 1750 RCF, acceleration 2, break 2, at room temperature.

Monocytes were collected from the upper ring and washed twice with PBS. A sample was taken to determine monocyte purity by flow cytometry.

After isolation, PBMCs or monocytes were counted and transferred to a plate: 100.000 cells per well in a 96 well plate, 500.000 cells per well in a 24 well plate or 1.000.000 cells per well in a 12 well plate. They were stimulated with a panel of Toll Like receptor (TLR) agonists: LPS (S. typhosa (Sigma Aldrich) or E. coli Ultra pure) 1, 10 and 100 ng/mL, PAM3CSK 30 and 100 ng/mL, ODN 0.3 and 1 mM, R848 1 or 3 µg/mL, Poly I:C 0.1 and 1 µg/mL, flagellin 1 and 5 µg/mL, FSL-1 1 and 10 µ/mL, Lipoteichoic acid 0.1 and 1µg/mL (Sigma Aldrich). All TLR stimuli were obtained from Invivogen, unless stated otherwise. Where indicated monocytes were pre-incubated for 30 minutes at 4°C with 10µg/mL anti-hCD14-IgG (Invivogen) or mouse anti-human HLA-ABC (BD Pharmingen) as control. Polymyxin B 10µg/mL (Invivogen) was added to the PBMCs 10 minutes prior to the addition of LPS or RSV F. For heat inactivation, LPS and RSV F were heated during 10 min at 99°C before adding this to the PBMCs. Supernatant was collected at the designated time points, and cells were lysed using RLT plus (Qiagen). Samples were stored at -80°C degrees until further processing. All assays with cells derived from the index patient were performed within the age range of 10 months to 3 years.

Flow Cytometry

We performed flow cytometry to identify CD14 surface expression on patient and control immune cells. The whole blood sample was depleted from red blood cells, by lysing the erythrocytes by incubation of 45 second with 5x the initial blood volume of cold distilled water, after which 0.1x volume of 10x PBS (=1 mL) was added to halt cell lysis. Cells were washed and remaining erythrocytes were removed by a second lysis with ammonium chloride buffer for 5 - 10 minutes, and centrifuged at 500g for 5 min at 4°C. The remaining cell pellet was washed with RPMI-1640 medium supplemented with 10% heat-inactivated Fetal Calf serum (FCS). Cell were stained for surface markers for 20 min at 4°C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) bovine serum albumin (BSA). Flow cytometric analysis was performed using a CANTO II (BD) and analyzed with FlowJo 10.2 software (FlowJo LLC). Cell types were identified according to their characteristic forward and side light-scatter properties, and by their typical cell surface markers: neutrophils: $CD14^{-}/CD16^{+}$, monocytes: $CD14^{+}/CD16^{-}$. 7-aminoactinomycin D (7-AAD) was used to distinguish apoptotic cells. Absence of CD14 surface expression in patient samples was demonstrated by flow cytometry using two different clones (aCD14 clones RMO52 and 61D3). Clone 61D3 has been shown to compete for binding to CD14 with aCD14 clone MEM18[3], which has been mapped to amino acids 51 to 63 of CD14 protein[4]. Both clones (61D3 and MEM18) act also as functional blockers of CD14: macrophages pre-incubated with either clone fail to produce TNF in response to

LPS.[4] We are therefore confident that we are using an anti-CD14 clone that is capable of detecting also the truncated mutant version of CD14.

To evaluate monocyte purity after Percoll, monocyte subtypes were identified as follows: lymphocytes were selected based on their forward and side scatter properties, single cells were selected, leukocytes were identified by CD45 surface expression. Monocyte subtypes were identified based on CD16 and CD14 surface expression. Non-classical monocytes: CD16$^+$/CD14$^-$, Intermediate monocytes: CD16$^+$/CD14$^+$, Classical monocytes: CD16$^-$/CD14$^-$. The mean monocyte purity after Percoll isolation was 65%.

The generation of RSV-pre and post-F probes and identification of RSV-pre and post-F binding B-cell was performed as described previously.[5] Briefly, frozen PBMCs were thawed and stained for viability with the Fixable Blue Dead Cell Stain Kit (Thermofisher Scientific), and then surface stained with the B cell antibody panel in table S1. Samples were collected on a LSR-X50 cytometer (BD) and data were analyzed with FlowJo 10.2 software. Table S1 shows a list of reagents and dilutions used in flow cytometry studies. The gating strategy is depicted in supplementary figure S8.

mRNA expression

Extracted mRNA (RNeasy Mini Kit Qiagen) was used for cDNA synthesis, using iScript. NanoString analysis was performed using the nCounter SPRINT profiler, using the immunology_v2_C2328 Codeset, which simultaneously measures mRNA expression levels of 579 inflammation-related genes, according to the manufacturer's instructions (NanoString Technologies^{TM}).

ELISA and quantification of RSV F protein-binding antibody

Standard ELISA kits were used according to the manufacturers' protocols to measure: IL-6 (Ready-Set-Go! (V2) (Ebioscience)), CD14 and LBP (both RnD systems).

RSV IgG concentrations and avidity assays

Analysis of IgG in serum was performed using a RSV multiplex immunoassay as described earlier.[6] The IgG concentrations against five RSV proteins (Ga, Gb, prefusion F, postfusion F and nucleoprotein) were quantified by interpolation from a five-parameter logistic standard curve, converting mean fluorescence intensities (MFI) to arbitrary units per ml (AU/mL).

The avidity of the RSV-specific antibodies was determined using the same RSV multiplex immunoassay with some adaptations. After incubation of the sera with the RSV proteins, all samples were additionally incubated for 10 minutes, at room temperature, in threefold on the same plate in the presence of 1.5 M Ammonium thiocyanate (NH_4SCN), 3.0 M NH_4SCN or PBS (pH 7.4). The avidity index (AI) was expressed as the

percentage of residual MFI IgG signal in comparison to the undenatured (PBS) signal which was set at 100%.

RSV neutralization assays

Neutralization by sera from the index patient, 15 adult controls of a previous study (reference standard)[7], and the BEI NR-4020 control sera were measured by a fluorescence plate reader neutralization assay described previously.[5] In brief, diluted sera were mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from subtype A (strain A2) or subtype B (strain 18537), and incubated at 37°C for 1 hour. Next, the mixture was added to H28 cells that had been seeded in a 384-well black optical bottom plate. After 24 hours of incubation plates were analyzed on a spectrophotometer (588/635 nm). Neutralization assays used to address the contribution of pre-F -exclusive antibodies were performed by dilution of sera in post-F protein from RSV A2 (20 µg/mL) prior to the addition of the virus.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 8.3.0. *P* values <0.05 were considered significant.

SUPPLEMENTARY TABLES

Table S1. Overview of antibodies used for flow cytometry

Panel used in figure 1a				
Target	**Label**	**Clone**	**Company**	**Dilution**
CD16	PeCy7	3G8	BioLegend	100x
CD14	APC-eF780	61D3	eBioscience	200x
7-aminoactinomycin D (7-AAD)			BD Bioscience	5 µl/sample
Panel used to determine monocyte purity				
Target	**Label**	**Clone**	**Company**	**Dilution**
CD14	V450	G10F5	BD Bioscience	50x
CD16	PeCy7	3G8	BioLegend	100x
CD45	APC-eF780	61D3	eBioscience	200x
Panel used to determine monocyte subsets				
Target	**Label**	**Clone**	**Company**	**Dilution**
CD3	PerCP-Cy5.5	UCHT1	Biolegend	100x
CD19	PerCP-Cy5.5	HIB19	eBioscience	100x
CD56	PerCP-Cy5.5	B159	BD	100x
CD66b	PerCP-Cy5.5	G10F5	Biolegend	100x
CD14	APC-eF780	61D3	eBioscience	200x
CD16	V500	3G8	BD	50x
CD86	FITC	2331 (FUN-1)	BD	50x
CD33	PE	WM53	Biorad	20x
CD64	APC	10.1	Biolegend	20x
CCR2	BV421	K036C2	Biolegend	150x
HLA-DR	PE-Cy7	L243	Biolegend	100x
Panel used to quantify RSV F-specific B-cells				
Target	**Label**	**Clone**	**Company**	**Volume used per 100 µl test**
IgA	FITC	S11-8E10	Miltenyi	2.5 µl
IgM	PerCpCy55	G20-127	BD	5 µl
CD8	BV510	RPA-T8	Biolegend	0.5 µl
CD3	BV510	OKT3	Biolegend	0.625 µl
CD56	BV510	HCD56	Biolegend	0.625 µl
CD14	BV510	M5E2	Biolegend	0.31 µl
CD27	BV605	O323	Biolegend	2 µl
CD11c	BV650	3.9	Biolegend	0.25 µl
CD72	BV711	J4-117	BD Bioscience	0.15 µl
CD19	ECD	J3-119	Beckman	2.5 µl
CD21	PeCy5	B-ly4	BD Bioscience	2.5 µl
CD71	PeCy7	CY1G4	Biolegend	0.078 µl

IgD	BUV395	IA6-2	BD Bioscience	1 µl
UV Blue			Thermofisher	0.125 µl
CD38	BUV661	HIT2	BD Bioscience	1.25 µl
IgG	A700	G18-145	BD Bioscience	2.5 µl
CD20	APCCy7	2H7	Biolegend	0.5 µl
Pre-F Probe	APC		In-House Conjugate	0.24 µg
Post-F Probe	BV421		In-House Conjugate	0.24 µg

Table S2. Overview of virology test results

Age (months)	Material	Method	Result
9	NP swab	PCR	Boca virus & RSV B positive
11	NP swab	PCR	Boca virus & RSV negative
14	NP swab	FARP	RSV B positive
15	NP swab	FARP	Rhino virus positive, RSV negative
18	NP swab	PCR	Parainfluenza virus, type 1 & 3, & Adenovirus positive
21	NP swab	ePlex	rhinovirus/enterovirus & coronavirus positive
22	NP swab	ePlex	RSV A positive, rhinovirus/enterovirus & coronavirus negative
22	NP swab	PCR	RSV negative
25	NP swab	ePlex	rhinovirus/enterovirus positive
28	NP swab	ePlex	rhinovirus/enterovirus negative
29	NP swab	ePlex	rhinovirus/enterovirus positive
29	NP swab	ePlex	rhinovirus/enterovirus positive
31	feces	PCR	adenovirus positive
32	feces	PCR	adenovirus negative
33	NP swab	ePlex	rhinovirus/enterovirus positive

NP = nasopharyngeal, PCR = polymerase chain reaction, FARP = FilmArray respiratory panel, Eplex = multiplex molecular based assay.

Table S3. Overview of bacterial culture results

Age (months)	Culture	Microorganism
9	urine (from indwelling catheter)	negative
	blood culture	negative
	bronchotracheal aspirate	*H. influenzae*
10	bronchotracheal aspirate	*H. influenzae*
	catheter urine	*Klebsiella pneumoniae*
		E. coli
	blood culture	negative
13	ear swab	*H. influenzae*
20	ear swab (right ear)	*Streptococcus pyogenes*
	ear swab (left ear)	*Streptococcus pyogenes*
21	ear swab	negative
21	bronchotracheal aspirate*	*Streptococcus mitis* group
		Rothia mucilaginosa
		Neisseria subflava
		Prevotella spp,

* To explore the microbial flora in the airways of the patient, sputum bronchotracheal aspirate was obtained at the age of 21 months and cultured using routine media and additional media (anaerobic culture and yeast/mold culture). All visually discernible microorganisms were identified by MALDI-TOF-MS. The identified microorganisms fit with normal commensal flora from the oropharynx and the upper airways. No yeasts or molds were isolated. All other cultures were performed in relation to upper respiratory tract complaints with routine procedures only.

Table S4. Normal immune phenotyping and immunoglobulin levels

Immune Phenotype			
Test	**Result**	**Reference**	**Unit**
Relative T-cell	53	53-74	% in Ly
Absolute T-cell	2,630	1800-5900	per mm^3
Relative CD4	25.40	33.0-55.0	% in Ly
Absolute CD4	1,267	1902-2977	per mm^3
Relative CD8	24	14.0-26.0	% in Ly
Absolute CD8	1,197	667-1473	per mm^3
CD4/CD8 ratio	1.10	1.4-3.7	
Active T cell (CD4)	1.70	0.3-1.3	% in CD4
Naive T cell (CD4)	72.80	82.3-95.1	% in CD4
Memory T cell (CD4)	27.20	4.6-17.3	% in CD4
Active T cell (CD8)	2.90	0.4-4.4	% in CD8
Naive T cell (CD8)	30.40	77.5-98.5	% in CD8
Memory T cell (CD8)	69.60	1.0-11.1	% in CD8
Relative B cell	31.10	16.3-26.8	% in Ly
Absolute B cell	1,534	871-1553	per mm^3
Naive B cells	62	62-69	% in B
Immature peripheral B cell	20.10	9.7-17.9	% in B
IgM B cell	4	4.6-15.0	% in B
IgG B cell	1.1	1.5-4.2	% in B
IgA B cell	1.4	0.8-1.7	% in B
NK cell Relative	15	3-14	% in Ly
Absolute NK cell	717	100-1100	per mm^3
Eosinophils	0.49	0.10-0.70	x10^9/L
Basophils	0.07	0-0.20	x10^9/L
Neutrophils	13.8	1.5-8.50	x10^9/L
Monocyte	2.05	0.00-0.80	x10^9/L
Lymphocyte	5.48	2-8	x10^9/L
Leukocytes	21.9	5.5-15.5	x10^9/L
IgM	1.3	0.1-0.87	g/L
IgA	1.2	0.19-1.1	g/L
IgG	12.7	2.6-13.9	g/L
IgE	176	0-32	kU/L

Table S5. Normal response to routine 10-valent pneumococcal vaccination

Polysaccharide antibodies*	Result	Unit
anti-Pneumococcus type 6B	N/A	μg/mL
anti-Pneumococcus type 8	0.21	μg/mL
anti-Pneumococcus type 9V	1.9	μg/mL
anti-Pneumococcus type 14	2.3	μg/mL
anti-Pneumococcus type 15B	<0.040	μg/mL
anti-Pneumococcus type 19F	>40	μg/mL
anti-Pneumococcus type 20	0.13	μg/mL
anti-Pneumococcus type 23F	0.27	μg/mL
anti-Pneumococcus type 33F	<0.040	μg/mL

*10 months after the third and final, routine vaccination with 10-valent pneumococcal vaccine (Synflorix), as part of the regular Dutch vaccination schedule.

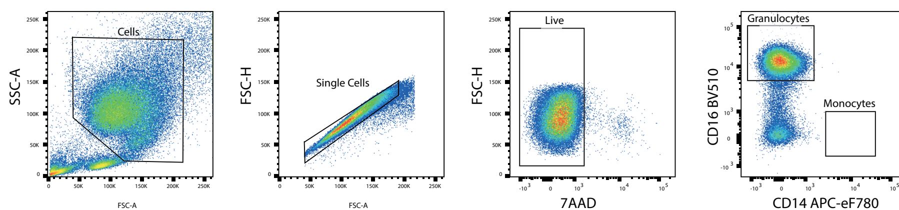
Table S6. Serology results of the index patient at age 3 years

Virus	Result	Unit
Cytomegalovirus IgG	17.1/Pos	ratio
Diphtheria antibodies	**0.55/Protective**	IU/mL
Epstein-Barr virus VCA IgG	0.360/Neg	ratio
Epstein-Barr virus NA IgG	0.363/Neg	ratio
Hepatitis B virus anti-HBs	**>1000.00/Protective**	IU/L
Influenza B virus antibodies	<1:10	titer
Influenza A virus antibodies	<1:10	titer
Measles virus IgG	**7366/Protective**	mIU/mL
Mumps virus IgG	**0.49/Neg**	ratio
Poliovirus type 1 antibodies	**1:128/Protective**	titer
Poliovirus type 3 antibodies	**1:384/Protective**	titer
Rubella virus IgG	**51.8/Protective**	IU/mL
SARS-CoV-2	0.04/Neg	ratio
Tetanus toxin antibodies	**1.34/Protective**	IU/mL
Varicella Zoster virus IgG	>2338/Pos	mIU/mL

Pos = positive, Neg = negative
Vaccination induced responses are depicted in bold

SUPPLEMENTARY FIGURES

Index patient



Healthy control

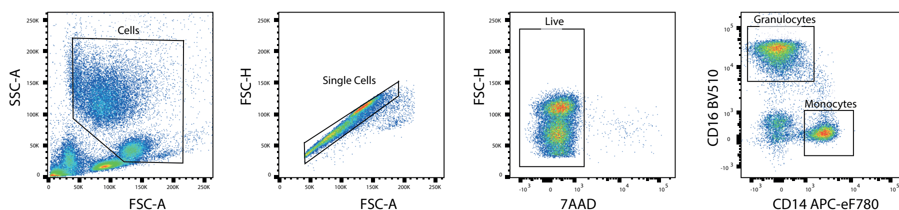


Figure S1. Gating strategy for granulocytes and monocytes in controls and index patient.
Flow cytometry plot of whole blood of the index patient (top row) and an infant control patient (bottom row). Cells were gated based on their characteristic forward and side scatter properties, subsequently single and live cells (7-AAD$^-$) were selected. Monocytes were identified as CD14$^+$/CD16$^-$; granulocytes were identified as CD14$^-$/CD16$^+$.

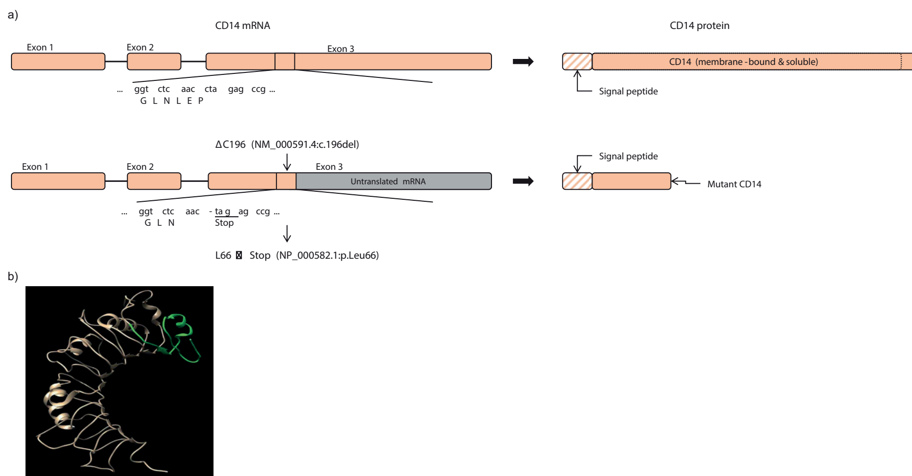


Figure S2. Schematic view of CD14 mutation.
a) Sanger sequencing of the CD14 gene revealed a single-nucleotide deletion in the CD14 gene (NM_000591.4:c.196del), for which the patient was homozygous. The wild type (top) and ΔC196 (bottom) alleles of the CD14 gene (left), and the proteins they encode (right) are schematically represented. The full-length CD14 protein consists of 375 amino acids (aa). The ΔC196 deletion is a frameshift mutation that changes the codon encoding leucine at position 66 into a stop codon (NP_000582.1:p.Leu66). (b) Characterization of the CD14 molecule with in green a prediction of the mutant form of CD14 in our patient, lacking the pocket rim for LPS binding.[4]

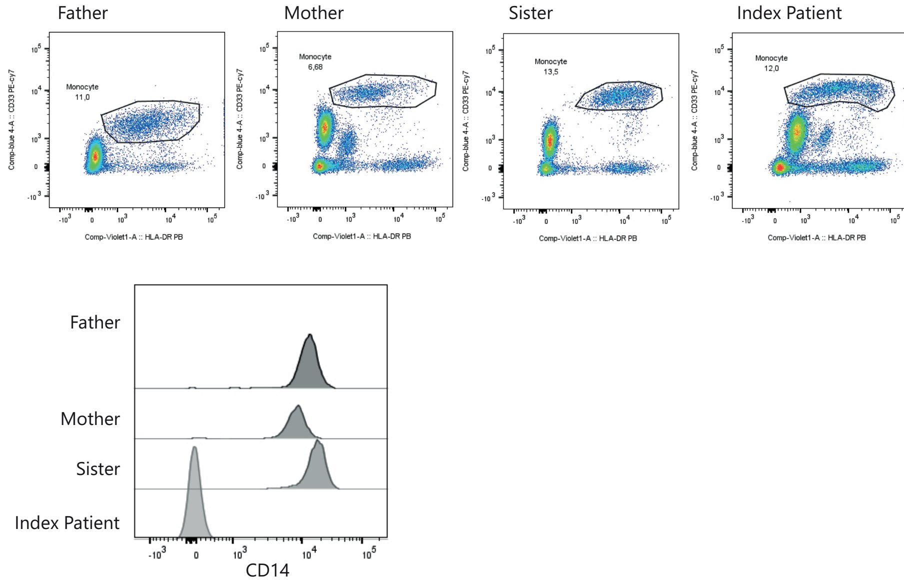


Figure S3. Normal CD14 surface expression on immune cells of parents and sibling of the index patient.
Flow cytometry plot of whole blood of both parents, sister and the index patient. Cells were gated based on their characteristic forward and side scatter properties, subsequently monocytes were identified based on CD33$^+$/HLA-DR$^+$ surface staining. On the bottom a histogram shows monocyte surface expression of CD14 in all family members, while this is absent in the patient.

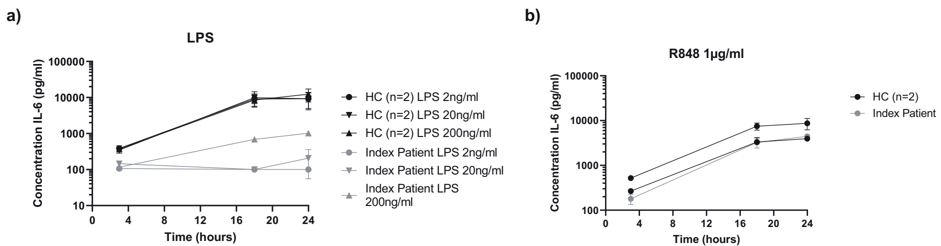
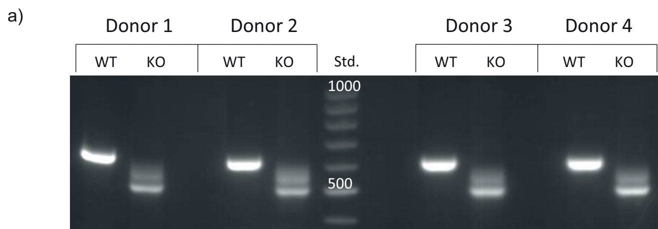


Figure S4. Impaired IL-6 production after prolonged stimulation of index patient PBMCs by TLR agonists LPS, but not R848.
(a) IL-6 protein response to different concentration of LPS (E. coli) after 4, 18 and 24 hours of stimulation, by PBMCs of two healthy controls and the index patient. (b) IL-6 protein response to R848 1 µg/mL after 4, 18 and 24 hours of stimulation, by PBMCs of two healthy controls and the index patient.

a)



b)

Primer	Sequence
CD14_Fw	GCCTACCAGTAGCTGAGCAG
CD14_Rv	AGAGAGGTGGGGAGGTGATC
CD14_seq	GCATCGACGCGCTTTAGAAA
sgRNA1	CCUCUACUGCAGACACACAC
sgRNA2	CGGAGAAGUUGCAGACGCAG
sgRNA3	GUCGCAGAGACGUGCACCAG

Figure S5. Confirmation of the CD14 knock out cell line.
(a) Agarose gel images with standard (std; in base pairs) from wild-type (WT) and knockout (KO) CD14. (b) Primer sequences used for PCR and Sanger sequencing.

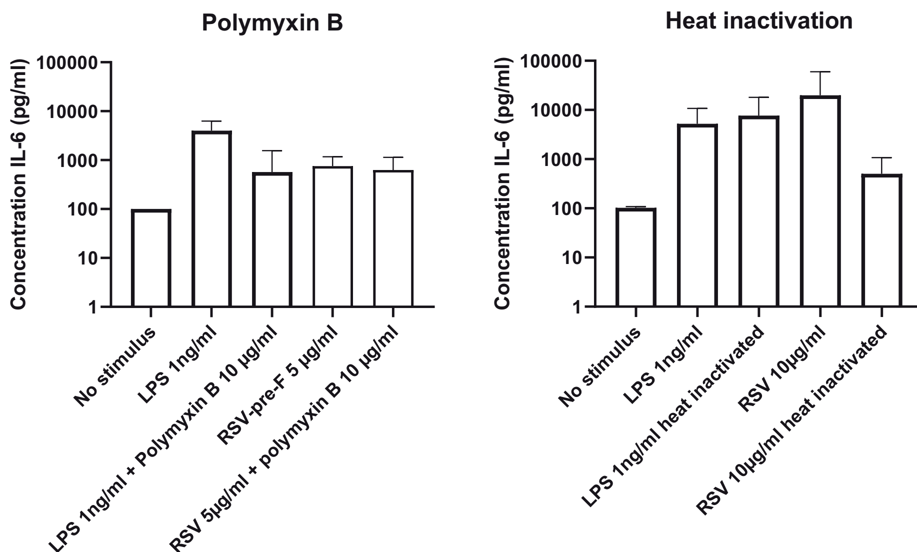


Figure S6. No LPS contamination of RSV-pre-F.
(a) IL-6 production after 16 hours of stimulation with LPS (1 ng/mL) or RSV pre-F (5 µg/mL) in the presence or absence of polymyxin B (10 µg/mL). N = 3 experiments with a total of 5 donors. (b) Shows IL-6 production after 16 hours of stimulation with LPS (1 ng/mL) or RSV pre-F (5 µg/mL) after heat inactivation. N = 3 experiments with a total of 8 donors.

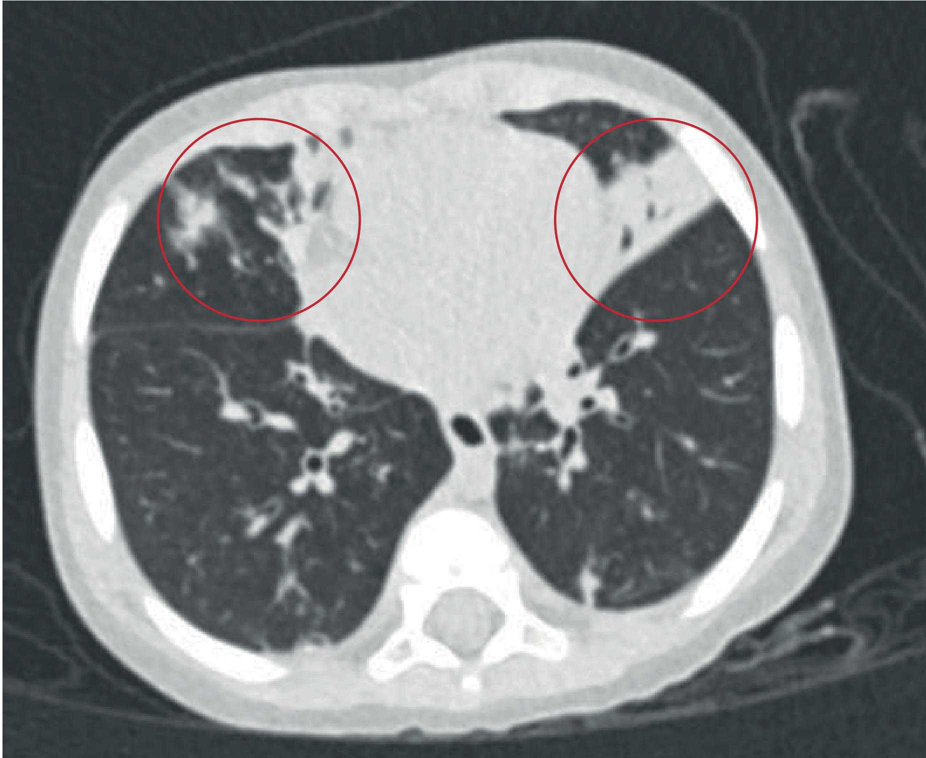


Figure S7. Pulmonary computed tomography (CT) of index patient.
CT-image of a cross-section of the lungs of the index patient at the age of 21 months. Red circles indicate areas suspected of ground-glass opacification in the left and right lung.

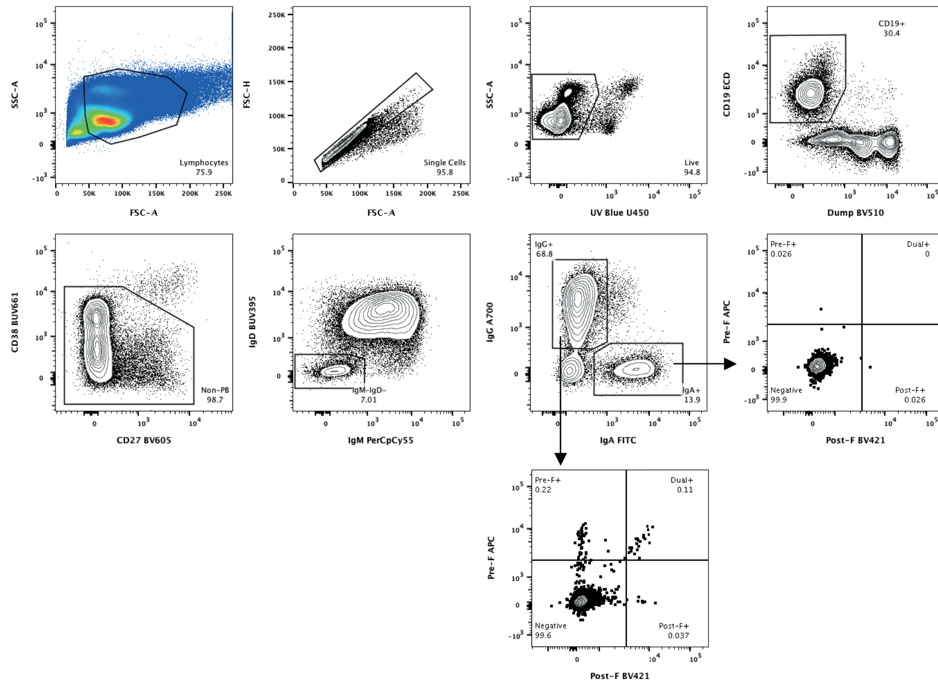


Figure S8. Gating strategy for IgG and IgA positive memory B-cells.

Gating strategy used to identify pre-F and post-F probe-binding IgG+ and IgA+ memory B-cells in human PBMCs. Lymphocytes were selected based on their forward and side scatter properties, live cells and surface expression of CD19^{+}. Plasma blasts (CD38^{++}CD27^{++}) were excluded and populations were further gated on isotype-switched cells (IgM^{-}IgD^{-}) to identify probe-binding IgG^{+} and IgA^{+} memory B cells.

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7

Summary and general discussion

SUMMARY

The aim of this thesis was to provide further insight into the role of the innate immune response against RSV. First, I studied the transcriptomic profile of local and systemic neutrophils during severe bronchiolitis in children admitted to the pediatric intensive care unit (**Chapter 2**). Using low input RNA sequencing on freshly isolated neutrophils from the blood and airways of 16 infants with RSV, I identified an interferon response in airway neutrophils during severe RSV infection. These data indicate that neutrophils alter their transcription in response to virus induced interferons. The fact that this interferon response is present late during infection in severe disease might indicate that this response is insufficient in protecting the host from severe infection, and at least coincides with immune-induced pathology. Second, in **Chapter 3**, in order to therapeutically modulate the neutrophil immune response, the potential of two inhibitory receptors: SIRL-1 and LAIR-1 as immune modulators, was evaluated. Targeting these receptors with specific agonistic antibodies moderately inhibited oxidative burst and neutrophil extracellular trap formation by airway neutrophils from RSV patients *ex vivo*. Third, in **Chapter 4** I aimed to develop a model to study epithelial and neutrophil interaction during RSV infection *in vitro*. Culturing and differentiating bronchial epithelial cells at an air liquid interface mimicked *in vivo* features of the airway epithelium. Infection with RSV in co-culture with neutrophils offered the potential to study neutrophil induced epithelial injury. When this model will be further optimized this will provide an additional and highly warranted tool to better understand the harmful versus protective role of neutrophils during RSV infection. Fourth, we presented an overview of the mechanisms by which neutrophil function could be targeted to prevent tissue injury and preserve homeostasis (**Chapter 5**). This review can be used as a guideline for future studies regarding neutrophil modulation. Finally, I identified a novel monogenetic disease as a possible cause of recurrent severe RSV bronchiolitis (**Chapter 6**). The chapter contains a description of the first patient with inherited CD14 deficiency and I identified a link with recurrent RSV disease. My work shows that CD14 mediates the immune response against RSV, and that the clinical phenotype of CD14 deficiency is characterized by, but not limited to, recurrent severe RSV infection.

Implications of the main findings of this thesis will be further discussed in the general discussion.

GENERAL DISCUSSION

In this section of the thesis I will discuss how a balanced and timely innate immune response is crucial to protect from infection with respiratory syncytial virus (RSV), while

dysregulation of this response facilitates the induction of inflammatory immunopathology.

Homeostasis in the lung

Processes in our body are aimed at maintaining homeostasis and preventing unwanted inflammatory responses from occurring. Balance is maintained by a circuit of sensors, effectors and mediators, which often involves a feedback loop (Figure 1). [1, 2] Under normal conditions homeostasis in the lung is preserved by the presence of an intact airway epithelium and the presence of lung resident immune cells such as alveolar macrophages.[3, 4] Barrier integrity of the epithelium is maintained by preservation of the epithelial architecture and the regenerative capacity of basal cells that underline the epithelium. Although cell turnover in the lung is low compared to other barrier organs such as the skin or the gut, in response to injury, the lung has a prompt capacity and flexibility to repair or replace damaged cells.[3, 5] Being the first line of defense against intruding pathogens, epithelial cells are sentinels that recognize a spectrum of pathogenic and nonpathogenic molecular patterns via the expression of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) 1-9.[6, 7] After TLR-stimulation epithelial cells produce a range of chemokines and cytokines that amplify the immune response. To prevent the airway to elicit unwanted responses to nonpathogenic stimuli, these responses are kept in check by inhibitory molecules and cytokines such as programmed death-ligand 1 (PD-L1) and CD200.[8-10, reviewed in 11] Studies using RNA sequencing of airway cells also showed expression of inhibitory receptor LAIR-1, but the exact role in the airway epithelium is unknown.[8, 9] The next players in airway homeostasis are alveolar macrophages (AMs). The AMs are long lived cells that are mainly replenished by self-renewal, although recent studies show that AMs can generate from circulating monocytes.[12] Alveolar macrophages are the sentinels of the lungs and are designed to sense danger and pathogen associated molecular patterns (DAMPs and PAMPs) via a range of receptors including TLRs. They aim to inhibit pro-inflammatory responses to tissue debris or to innocent (self) antigens, while effective immune responses to pathogenic microorganisms remain intact.[Reviewed in 4] A recent study implicated that AMs also have the potential to prevent RSV infection.[13] Collectively, epithelial cells and alveolar macrophages maintain a complex network of interaction through intra-cellular crosstalk via cell surface-expressed receptors and secreted mediators.

The role of neutrophils in the upper respiratory tract during homeostatic conditions remains largely unknown. A recent RSV human challenge study by Habibi et al. showed that neutrophilic mucosal inflammation in the upper airway, prior to RSV virus infection, predisposes to RSV disease development.[14] This suggests that presence of neutrophils could have a disruptive effect on local homeostasis thereby predisposing to RSV infection.

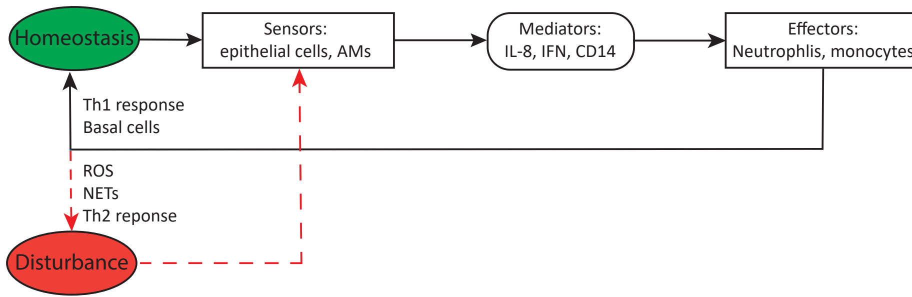

Figure 1: circuit of sensors, effectors and mediators. *Depicted are the most important cells and molecules involved in airway homeostasis during RSV infection (in green). Mediators thar are involved in the disturbance of airway homeostasis are depicted in red. (AMs = alveolar macrophages, IFN = interferon, ROS = reactive oxygen species, NET = neutrophil extracellular trap). Adapted from Medzhitov*[2]

Respiratory syncytial virus infection

Homeostatic conditions in the lung can be disrupted by many agents, including smoke, toxins, and bacterial or viral pathogens. A few receptors have been identified as RSV entry receptors of which insulin growth factor 1-receptor (IGF1R), nucleolin, and TLR4 are the best characterized.[15-18] Blocking or inhibiting these receptors limits viral infection.[16] It is unknown whether the host itself can interfere with RSV-receptor binding and thereby limit viral infection and spread. For dengue it has been shown that pre-treatment of cells with LPS, the known ligand for TLR4, limits viral infection in a CD14 dependent manner.[19] In other words, LPS-CD14 binding was necessary for the LPS induced inhibitory effect on viral replication. Therefore, I speculate that this mechanism (LPS-CD14 binding) could also inhibit RSV infection. Hence, a lack of CD14 would result in increased viral replication and spread. However, my data using RSV-infected patient derived and CD14 knockout airway cell cultures did not show a difference in RSV replication. Future studies including pretreatment of cultures with LPS are needed to further evaluate my hypothesis.

Sensors of respiratory syncytial virus infection

The initial immune response by the airway epithelium, AMs and dendritic cells occurs within the first three days post-infection. Respiratory syncytial virus motifs are recognized by TLR2, 3, 4 and 7 on AMs and airway epithelial cells which initiates a pro-inflammatory response.[15, 17, 20-22] Upon infection the airway epithelium produces anti-viral IFN III and releases several pro-inflammatory mediators such as CD14, triggering receptor expressed on myeloid cells 1 (TREM1) and (high mobility group box 1 (HMGB1), which are all involved in TLR signaling.[23-29] The importance of AMs in the host response against RSV is illustrated by several mouse studies showing that AMs limit RSV infection by the production of IFN I and II thereby attracting antiviral monocytes. Additionally, they limit airway occlusion by removing cell debris.[30-33]

First responders to respiratory syncytial virus infection

A few days (2-4 days) after the initial infection, effector cells such as neutrophils and monocytes migrate from the blood to the airways. Neutrophils are the first to arrive and comprise up to 80% of the leukocyte population in the lung.[34, 35] They possess several effector functions that are believed to induce epithelial damage and enhance RSV disease severity, although direct evidence is very limited.[36-39] Sequencing studies using blood from RSV patients showed upregulation of neutrophil genes (neutrophil activation, degranulation).[40-42] Blood and airway neutrophils differ in effector function during RSV infection; airway neutrophils have an activated phenotype and show increased NET-formation and ROS production compared to blood neutrophils.[35, 43, 44] I investigated isolated neutrophils from blood and airways of RSV-infected infants and found distinct gene expression patterns. Blood derived neutrophils are characterized by upregulation of genes related to migration and degranulation such as genes encoding the attachment molecule intercellular adhesion molecule 1 (ICAM1) and TREM1, and degranulation molecules progranulin and syntaxin binding proteins 2 and 3. Airway neutrophils showed upregulation of apoptotic and IFN induced genes.[45, 46] Thus, despite the fact that RSV infection is restricted to the airway epithelium, there are systemic changes in the neutrophil transcriptome during RSV infection. Additionally, we showed that inhibiting NET formation and ROS production only had a modest effect in airway neutrophils, likely caused by the high level of activation of these cells once they have migrated towards the inflamed lungs. This is in line with the RNA sequencing data showing upregulation of neutrophil activation genes in blood. If it holds true that transcriptomic alterations in blood neutrophils result in an irreversible activation level of airway migrated neutrophils, interfering with neutrophil effector functions at the local level (i.e. in the airway) might be too late.. Thus, it could be more beneficial to prevent neutrophil activation or migration to the lung with systemic therapy than to try to inhibit an over-activated neutrophil using local airway therapy. Possible therapeutic targets to modulate neutrophil function are highlighted in chapter 5 of this thesis. Future studies that investigate modification of systemic versus local neutrophil responses will help understanding disease pathogenesis and the design of targeted therapy.

Monocytes: a neglected player in respiratory syncytial virus infection

Due to the large number in the lungs of RSV-infected infants, neutrophils are a major topic of RSV research. Monocytes and lung resident macrophages are the second largest leukocyte population in the lung (up to 40% in some cases). In the context of RSV they are much less studied than and their exact contribution to RSV pathology is unclear.[45] Airway monocytes and monocyte derived cells, such as AMs, are shown to play a role in several respiratory tract infections, including rhinovirus and influenza infection.[47, 48] During influenza infection macrophages and monocytes induce epithelial damage and

thereby contribute to disease severity.[48-50] The role of local monocytes and macrophages during RSV infection and how they interact with neutrophils, especially in humans, is not fully understood and studies regarding the role of this particular cell type are lacking.[51] In mice it has been shown that antiviral monocytes are recruited by AMs and that these cells are crucial in limiting RSV disease.[31] TLR4 and CD14 are expressed on AMs and airway epithelial cells. [52-55] Our data showing impaired immune responses by CD14 deficient monocytes in response to RSV-F protein stimulation could implicate that CD14 deficiency also results in defective TLR4/CD14 signaling in other CD14 expressing cells, such as AMs and airway epithelial cells.

Resolution of infection versus progression to severe disease

Which immunological factors determine whether a child is able to clear RSV infection without progressing towards severe disease? This has been the main research question of many studies. At day five post infection symptoms usually peak and either reside, or continue as a syndrome characterized by a prolonged and exaggerated immune response including local neutrophil influx and activation, and enhanced Th2 responses.[45, 56-62] When an appropriate innate and adaptive response is induced, NK cells and TCs kill virus infected cells with the help of DCs, macrophages clean apoptotic cells and debris, and the injured airway epithelium will be repaired and replaced by new cells. Finally, the lung will return to its homeostatic state. It is unknown what happens at, or even before, this point and which factors determine whether the balance shifts to resolution of disease or enhanced inflammation. Based on the paragraphs above we can identify some conditions that favor a good outcome of disease: an optimal functioning epithelium with the ability of self-renewal, well-functioning sentinels, effector cells that are kept in check, correct timing, and appropriate integral cross-talk with the adaptive immune response. Disorders in either of these conditions can affect the immune response in a negative way by either delaying the immune response, inducing an immune response that is too weak resulting in increased viral induced pathology, or too strong (i.e. exaggerated) and contributing to immunopathology (Figure 2).

1. Epithelial response: dysfunction of the airway epithelium, for instance under developed lungs in premature infants or chronic inflammation as a result of asthma in young children pre-dispose to RSV infection.[63-66] Dysfunctional responses in mucus production, the production of antimicrobial compounds or immune modulatory molecules increase the risk of a complicated course of RSV infection.[5] The expression of inhibitory molecules by epithelial cells, could be a future target to modify the epithelial immune response.
2. Defective sensing: an immediate and adequate innate immune response to RSV infection is a critical determinant of disease outcome and depends on adequate sensing of intruding pathogens by the epithelium and phagocytic system. [reviewed in]

7

[5, 51] SNPs in several innate genes including, *TLR4*, *CD14*, and *IFN* related genes, such as *CCR5*, have been shown to be associated with RSV disease severity.[67-70] We additionally found that human CD14 deficiency resulted in a defective innate immune response, characterized by an almost absent IL-6 response, to RSV-F, leading to recurrent RSV infection.

3. Bad timing: that timing of the immune response is important is underlined by a mouse study which showed that early IL-6 production is warranted to limit RSV disease, while late or prolonged IL-6 production leads to more severe disease.[71] Furthermore, a SNP in the IL-6 promotor increases the susceptibility to RSV, while increased IL-6 levels during RSV infection are associated with enhanced disease severity.[72, 73] The same holds true for other innate cytokines including the timing of the IFN response.
4. Derailed effector cells: is the harmful role of neutrophils determined by enhanced neutrophil activation or a lack of inhibitory signals? Neutrophils express a range of inhibitory receptors, and cell surface expression and function depends on the inflammatory state. Any modification in the cell surface composition and expression of these receptors or presence of ligands might influence the activation state of neutrophils.[74] Soluble forms of these inhibitory receptors, such as sSIRL-1, might have an additional effect on neutrophil function.
5. Integral cross-talk with the adaptive immune response: is there a failure in the link between innate and adaptive immunity? Neutrophils are not operating individually, but instruct and induce adaptive immune responses by the production of cytokines, NETS or cell-to-cell contact. [Reviewed in 75] A dysfunctional neutrophil response can therefore impair the adaptive response necessary for RSV recovery. Neutrophils have been shown to promote and accelerate antibody production and maturation by B-cells via B-cell activating factor (BAFF), APRIL and IL-21. In line with these data we showed that airway neutrophils upregulate BAFF during RSV, while APRIL and IL-21R were not significantly differentially expressed, which could indicate poor B-cell cross talk.[45] Furthermore, neutrophils induce Th17 responses by promoting the production of IL-17 by T-cells, thereby skewing the response to a deleterious type 2 immune response.[76] Finally, CD14 has the potential to skew the immune response from a harmful Th2 response towards a protective Th1 response.[77] If this holds true, sCD14 or agonistic CD14 antibodies could offer a potential treatment target during severe RSV disease.

Concluding remarks and future work

Severe RSV bronchiolitis is caused by a delayed, derailed or prolonged innate immune response. Defects in innate immunity can contribute to these processes in several ways. A deficit in detectors (sensors) will result in impaired recognition of RSV virions with an

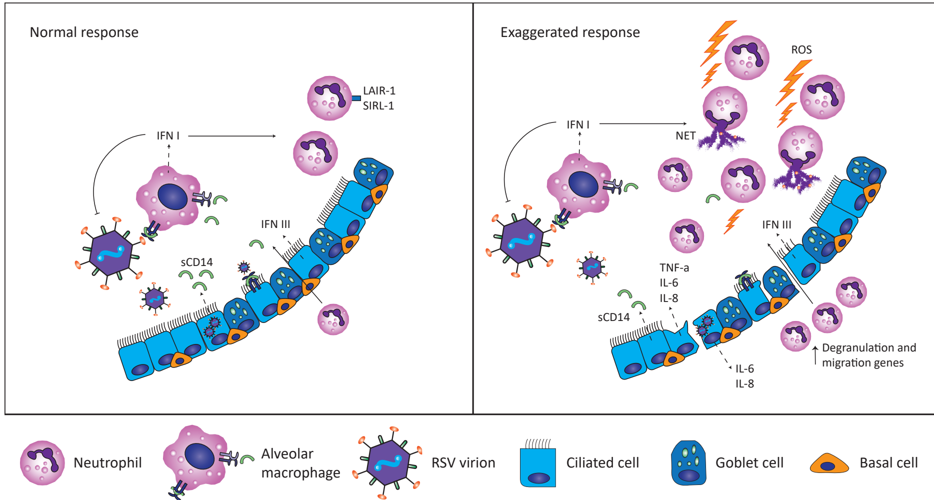

Figure 2: summary of different mechanisms that contribute to severe RSV disease. *Left panel depicts an appropriate and balanced response. The F-protein of the RSV virion is recognized by TLR2/4 and CD14 by alveolar macrophages and epithelial cells, which induces an IFN response that limits viral infection. Neutrophils are present in low numbers and are kept in check by inhibitory molecules. Right panel depicts an exaggerated neutrophil response. The epithelial release of IL-8, IL-6 and TNF-a attracts leads to neutrophil migration from the blood and neutrophil activation. Neutrophil degranulation. the release of NETs and production of ROS induce epithelial injury.*

sCD14 = soluble CD14, ROS = reactive oxygen species, NET= neutrophil extracellular trap, IFN = interferon, LAIR-1= leukocyte-associated immunoglobulin-like receptor 1, SIRL-1 = signal inhibitory receptor on leukocytes-1.

impaired or delayed immune response as a result, as is the case in human CD14 deficiency. It is unknown whether this leads to excessive viral replication and virus induced pathology, or that the defect in itself dysregulates the immune response towards a harmful response. Dysfunctional effector cells, such as early or prolonged activation of neutrophils, can determine disease outcome by inducing immune pathology. Ultimately both of these defects can lead to impaired resolution of disease by a dysfunctional cross talk with other immune cells, such as M2 macrophages and T cells.

Future work

The exact drivers of immune-induced pathology during severe RSV bronchiolitis and the contribution to disease pathophysiology remain to be clarified. Key questions include: what is the contribution of the immune response by the airway epithelium to neutrophil activation; what is the role of direct viral sensing by neutrophils, such as has been described for TLR-4 mediated NET formation,[78] in contrast to cytokine mediated neutrophil activation; what is the contribution of alveolar macrophages and other monocyte-derived cells in RSV control; and what is the exact role of epithelial (s)CD14 in viral sensing and entry?

To address these questions, future studies should focus on the use of sophisticated culture methods with human primary cells such as HNEC-ALI and 3-dimensional airway organoids. Improving these models as a co-culture system enables the study of interaction between neutrophils and monocytes with the RSV-infected airway epithelium in further detail. These models can also be used to identify the role of (s)CD14 in local RSV pathophysiology.

Paired analysis of single-cell RNA sequencing of local (in the upper and lower airway) and systemic (blood) immune cells during severe RSV infection, including alveolar macrophages and monocytes, is crucial to unravel new cell types and functions in airway immune biology. This should be accompanied by immune cell phenotyping of patients over time in RSV-infected patients with variable disease severity. Preferably, this would be done in a prospective manner, e.g. before and after the development of severe disease or by use of the human challenge model. As I have argued elsewhere, to force advances in the RSV research field we should adopt a fail-fast culture (appendix I). The human challenge model offers an opportunity to efficiently analyze immune responses at different time points during infection, and can be used to speed up vaccine development, thereby obviating the need for costly and complex trials. Only once we master a better understanding of the dynamics of the immune response can we begin to overcome the gaps in knowledge that currently prevent us from developing an effective vaccine or treatment option for RSV bronchiolitis.

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8

Nederlandse samenvatting

Respiratoir syncytieel virus (RSV of RS-virus) is een van de belangrijkste ziekteverwekkers van luchtweginfecties bij kinderen. Iedereen wordt in zijn leven meerdere keren besmet met dit virus. De klachten van RSV infectie variëren van een simpele verkoudheid (ontsteking van de bovenste luchtweg) tot bronchiolitis (een ontsteking van de lagere luchtwegen). Bij jonge kinderen kan dit ernstige benauwdheid tot gevolg hebben waarvoor een ziekenhuisopname of zelfs opname op de kinder intensive care nodig is. Bij kinderen onder de vijf jaar leidt RSV wereldwijd naar schatting tot 3,2 miljoen ziekenhuisopnames en 125.000 sterfgevallen per jaar. Na Malaria is het de belangrijkste doodsoorzaak op de kinderleeftijd. Meer dan 90% van deze sterfgevallen vindt plaats in ontwikkelingslanden. Dit maakt het bestrijden en voorkomen van het RS-virus belangrijk voor de gezondheid van kinderen wereldwijd.

De afweerreactie tegen RSV komt tot stand door activatie van cellen van het aangeboren en verworven afweersysteem (immuunsysteem). Belangrijke cellen binnen het aangeboren immuunsysteem zijn neutrofielen en monocyten. Deze cellen kunnen bacteriën en virussen herkennen en direct onschadelijk maken. Het verworven immuunsysteem bevat o.a. B-cellen en T-cellen. Deze cellen zijn vooral belangrijk bij de afweer die ontstaat na vaccinatie of het doormaken van een infectie. Daarnaast speelt in de verdediging tegen luchtwegvirussen ook de afweerreactie van cellen in de luchtweg zelf een rol. De luchtweg is bekleed met een laag epitheelcellen die trilharen hebben of slijm produceren zodat virussen en bacteriën uit de longen worden verdreven.

Om het virus tegen te gaan is een gebalanceerde afweerreactie van het lichaam nodig zodat het virus wordt bestreden maar er zo min mogelijk schade aan gezond weefsel wordt toegebracht. Ondanks een goede afweerreactie worden sommige kinderen toch ernstig ziek van RSV. We begrijpen nog niet goed waarom dat gebeurd. Mogelijk is het zo dat niet alleen het virus de longen ziek maakt, maar ook overactiviteit van bepaalde cellen van het afweersysteem, dit noemen we immuun gemedieerde pathologie.

Het doel van mijn onderzoek was om meer inzicht te krijgen in zowel de beschermende als de schadelijke rol van de aangeboren immuunrespons tijdens ernstige RSV infectie. Tijdens RSV infectie dringen afweercellen, met name neutrofielen, de luchtwegen binnen. Uit eerder onderzoek is gebleken dat deze neutrofielen geactiveerd zijn en stoffen uitscheiden die schadelijk kunnen zijn voor de longen. Stoffen die worden uitgescheiden door neutrofielen zijn bijvoorbeeld zuurstofradicalen (reactieve oxygen species of ROS) en NETs (neutrophil extracellular traps). Om te onderzoeken door welke mediatoren neutrofielen van RSV patiënten geactiveerd raken hebben we bloed afgenomen en sputum (slijm dat kan worden opgezogen uit de beademingsbuis van beademende kinderen) opgevangen van ernstig zieke RSV patiënten en vergeleken met controle patiënten. Uit het bloed en sputum hebben we neutrofielen geïsoleerd en daarvan het transcriptoom onderzocht (**Hoofdstuk 2**). Met behulp van RNA-sequencing toonden we aan dat de luchtwegneutrofielen activatie lieten zien van de *NfKb* en *IL-6*

pathway. Daarnaast was er een interferonrespons in luchtwegneutrofielen tijdens ernstige RSV-infectie. Op het moment van onderzoek waren de kinderen nog erg ziek; het lijkt er dus op dat deze interferonrespons en de reactie van de neutrofielen het kind niet voldoende tegen een ernstige infectie beschermen. Mogelijk draagt deze reactie bij aan immuun gemedieerde pathologie.

Afweercellen hebben verschillende receptoren op hun celoppervlak. Deze receptoren kunnen bijvoorbeeld bacteriën of virussen herkennen. Na herkenning van een virus of bacterie zal de cel geactiveerd worden en een signaal uitzenden om de afweerreactie op te wekken. Daarnaast hebben afweercellen receptoren op het oppervlak die er juist voor zorgen dat cellen niet overmatig geactiveerd wordt, zogenaamde remmende receptoren (immune check points of check point inhibitors). Om te kijken of het mogelijk was geactiveerde neutrofielen uit de luchtwegen van RSV patiënten te remmen heb ik het potentieel van twee remmende receptoren: SIRL-1 en LAIR-1 geëvalueerd (**Hoofdstuk 3**). Behandeling van de neutrofielen uit de luchtwegen van RSV-patiënten met specifieke antilichamen tegen SIRL-1 en LAIR-1 remde de neutrofielfunctie (ROS en NETs). Dit toont aan dat remmende receptoren, zoals SIRL-1 en LAIR-1, gebruikt kunnen worden om de neutrofielfunctie te beïnvloeden. Inmiddels is ook in muizenstudies aangetoond dat antilichamen tegen LAIR-1 de toestroom van neutrofielen naar de longen verminderen en zo inflammatie remmen. Dit betekent dat dit soort antilichamen in de toekomst mogelijk gebruikt kunnen worden als behandeling bij ziekten waar neutrofielen een rol spelen in de pathologie, zoals tijdens RSV bronchiolitis.

Om beter te begrijpen hoe geactiveerde neutrofielen schade aanrichten aan met RSV-geïnfecteerd luchtwegepitheel hebben we een model ontwikkelt om dit te bestuderen (**Hoofdstuk 4)**. Om de luchtweg na te bootsen werden luchtwegcellen van gezonde mensen gekweekt en gedifferentieerd op een filter dat is blootgesteld aan de lucht aan de ene kant en aan vloeistof met voedingsstoffen (medium) aan de andere kant. Deze speciale luchtwegkweken bevatten de verschillende cellen uit de luchtweg waaronder cellen met trilharen en cellen die slijm produceren. Wanneer deze kweken met RSV geïnfecteerd worden ontstond er geen duidelijke schade aan de epitheelcellen. Daarnaast werd door de RSV geïnfecteerde epitheelcellen IL-8 geproduceerd. De afweerstof IL-8 is een afweer stof dat neutrofielen aantrekt. Vervolgens heb ik in een pilotstudie het effect van neutrofielen op de RSV geïnfecteerde kweken onderzocht. In de huidige studieopzet kon niet worden aangetoond dat neutrofielen het luchtwegepitheel tijdens RSV beschadigen. Dit model kan gebruikt worden om het effect van infectie met RSV of andere virussen op luchtwegepitheel te bestuderen. Daarnaast kan je bestuderen wat het effect is van neutrofielactivatie en -migratie op geïnfecteerd epitheel. In **hoofdstuk 5** bespreek ik verschillende receptoren en moleculen die gebruikt kunnen worden om neutrofielfunctie te moduleren en zodoende immuungemedieerde weefselbeschadiging te voorkomen. Veelbelovende methoden omvatten o.a. het blok-

keren van stoffen die neutrofielen aantrekken (bijvoorbeeld IL-8), het stimuleren van de op neutrofielen tot expressie gebrachte remmende receptoren en het remmen van stoffen die neutrofielen uitscheiden en die potentieel schadelijk zijn zoals NETs.

In **hoofdstuk 6** bespreek ik de ontdekking van een patiënt met een nieuwe monogenetische ziekte als oorzaak van recidiverende ernstige RSV-bronchiolitis. De patiënt had in zijn eerste 4 levensjaren terugkerende, soms ernstige RSV-ziekte. Herhaaldelijke RSV infectie waarvoor ziekenhuisopname nodig is komt vrijwel niet voor bij gezonde kinderen. Het betrof de eerste patiënt met een mutatie in het *CD14* gen wat resulteerde in een CD14-deficiëntie. CD14 is een receptor op de afweercellen van het aangeboren immuunsysteem. Deze receptor herkent bacteriën en virussen en zorgt ervoor dat deze bacteriën en virussen worden vernietigd. In de afwezigheid van CD14 was er geen goede afweer reactie tegen het RSV-F eiwit, wat werd gekenmerkt door een vrijwel afwezige IL-6 respons. De afweerstof IL-6 is bij gezonde mensen betrokken bij de afweer tegen bacteriën en virussen. Mijn studie toont aan dat CD14 de immuunrespons tegen RSV medieert (Appendix II).

Conclusie en toekomst

Ernstige RSV-bronchiolitis wordt veroorzaakt door een vertraagde, ontspoorde of langdurige activatie van de aangeboren immuunrespons. Defecten in de aangeboren afweer kunnen daar op verschillende manieren aan bijdragen. Een voorbeeld hiervan is de verstoorde herkenning van het virus door afweercellen in de long (zoals in het geval van CD14 deficiëntie), of door overactiviteit van neutrofielen (zoals bij ernstig zieke kinderen op de kinder intensive care). Ondanks de vooruitgang die de afgelopen decennia is geboekt op het gebied van RSV onderzoek, ontbreekt het ons nog steeds aan een wereldwijd beschikbaar veilig en effectief RSV-vaccin. Daarnaast is er ook geen behandeling die ziekte-ernst vermindert of de ziekteduur verkort. Beter begrip van de afweerreactie door epitheelcellen en door het aangeboren en verworven immuunsysteem vergroot de kans op het vinden van een behandeling en het versnellen van vaccinontwikkeling. Door toekomstig onderzoek te richten op de dynamiek van de immuunrespons, dat wil zeggen door onderzoek bij patiënten gedurende de gehele ziekteperiode, kunnen we de hiaten in de kennis overbruggen die ons momenteel beletten effectieve vaccins en behandelingen voor RSV-bronchiolitis te ontwikkelen.

9

Appendices

I Fail-fast in RSV vaccine development

II CD14 deficiency; translation for the general public.

III Curriculum vitae

IV List op publications

V Dankwoord

FAIL-FAST IN RSV VACCINE DEVELOPMENT

Sjanna B. Besteman, Louis J. Bont

American Journal of Respiratory and Critical Care Medicine 2019

Despite of progress made for the past decades in the field of RSV, we are still lacking a safe and effective RSV vaccine. In the absence of a correlate of protection to RSV, vaccine developers are working in the dark, and therefore often find out that their product is insufficiently effective[1]. For example, Novavax developed a nanoparticle-based RSV vaccine for older adults, went through a large phase 2b trial, only to find out it was not effective in a large phase 3 trial [2]. Novavax got back on its feet and is currently unblinding their next phase 3 trial, now in pregnant women [3]. In order to accelerate RSV vaccine development, developers adopted the fail-fast approach.

Fail-fast systems were designed to immediate report failure in order to stop product development, rather than continue developing a product which likely will never be good enough. The fail-fast approach has been adopted by Pharmaceutical Industry, and became the mantra of many start-up companies. Not only to prevent wasting efforts, but also to create a healthy society in which entrepreneurs can fail, learn and improve [4]. However, the fail-fast strategy has its own challenges, which is illustrated by dr. Stephanie Ascough and colleagues in the current issue of the Journal. In their paper they describe the results of a phase 1 study of a novel needle-free RSV vaccine: SynGEM. The vaccine is based on a stable pre-fusion F antigen of the virus, and uses a bacterial-like-particle (BLP) as an immune-enhancing carrier. The vaccine had already been shown to protect against infection in mice and cotton rats upon RSV challenge [5]. Now, healthy adult volunteers were vaccinated using a prime-boost approach. The authors used conventional methods to measure induced antibodies, including nasal IgA levels, serum neutralization (PRNT) and palivizumab competing assays (PCA). The vaccine was safe and induced a 2-fold rise in specific antibodies, also when participants were seropositive at the start of the trial. RSV-specific mucosal IgA concentrations against RSV were variable, with the strongest increase in individuals with low pre-existent levels of mucosal antibodies. Furthermore, the vaccine induced an increase in concentration of circulating RSV-specific B-cells determined by ELISpot. However, despite the use of a subunit vaccine based on the stabilized pre-F protein, there was no induction of neutralizing antibodies. Unfortunately, these results were not convincing enough to grant funding for a human challenge trial.

This study is important and well performed, given the challenges of doing this with limited resources of an early phase clinical trial. The study sets an example that collaboration between a relative small Biotech Company, such as Mucosis, and the highly experienced RSV research team at Imperial College, has the potential of developing

vaccines with worldwide impact. Unfortunately, the study didn't reach the endpoint threshold and SynGEM was withheld from proceeding to next phase trials. Was this a rushed decision?

In order to answer this question we should think about RSV vaccine development in general. The development of an RSV vaccine is hampered by several problems. First, there is no optimal animal model. Mice can be infected with human RSV, but lack many of the clinical characteristics of RSV bronchiolitis in children. Cotton rats are often used to develop therapeutics, but do not reliably predict efficacy of antivirals or vaccines against RSV. Additionally, the formalin-inactivated RSV vaccine, which caused vaccine-augmented disease, led to great caution taking an RSV vaccine into seronegative infants[6]. Most importantly, we lack serological markers of protection to RSV. There is evidence that suggests mucosal IgA is protective against RSV, and neutralizing antibodies against RSV-pre-F show high neutralizing activity[7-8]. Still, this does not guarantee that the vaccine protects against RSV infection. Until we have established correlates of protection to RSV, we are left with trial-and-error approaches, like for SynGEM in this paper. Considering the risk that vaccines might still fail during late-stage clinical trials, manufacturers are vexed on how to move forward with clinical development. This uncertainty may well have contributed to the premature ending of potentially safe and effective RSV vaccines, which would be detrimental to the development of a working vaccine. A possible way to negate these adverse effects – and a more secure option in the fail-fast approach – would be to use the human challenge model (HCM).

In the case of SynGEM, the decision to discontinue vaccine development resulted in discontinuation of funding and the bankruptcy of Mucosis. Could this public-private partnership have used a human challenge study to add value to the decision whether or not to continue the clinical development of SynGEM? The human challenge model has limitations, including the use of a single viral strain, absence of the target population (infants) and relatively mild disease severity. Nevertheless, human challenge studies can quickly provide proof-of-concept of efficacy of novel RSV therapeutics as is also acknowledged by the world health organization and regulators [9-12]. The authors are part of the publicly funded prestigious HIC-Vac network which is hosted at Imperial College, London, which can perform a human challenge with SynGEM in healthy volunteers. As Virtuvax has now taken over Mucosis' Vaccine Technology, this may still be a viable option to get the answer to the critical question whether or not immunity by SynGEM could be protective.

In conclusion, the authors have provided compelling evidence that BLP-based vaccines may have a future, in particular for RSV infection. However, the conclusions of this study leave us with a dilemma. On the one hand, the vaccine may deserve a second chance in a human challenge study to define clinical protection. On the other, following the fail-fast culture, we should be bold enough to terminate a program without hesita-

tion, as this allows us to move on and develop an even better program to fight one of the most deadly diseases during infancy.

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CD14 DEFICIENCY; TRANSLATION FOR THE GENERAL PUBLIC.

The graphics in figures 1 and 2 were made by Tessa van Steenbergen, Norely Wijnholds and Ymke Wilgenburg from Het Streek Lyceum, Ede (the Netherlands). They were created as part of their master assignment in their final year.

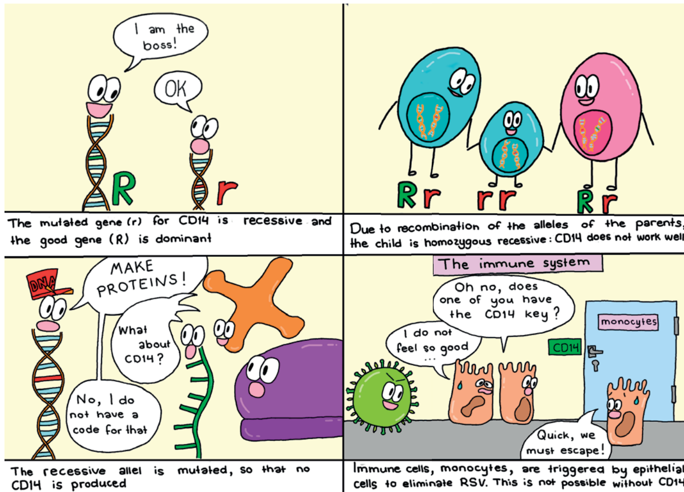

Figure 1: monogenetic recessive CD14 deficiency. *Top left: the mutated recessive gene and intact dominant gene for CD14. Top right: inheritance of the mutation from heterozygous parents. Bottom left: mutation in CD14 gene leads to absence of CD14 protein. Bottom right: due to absence of CD14 the immune response to RSV is impaired.*

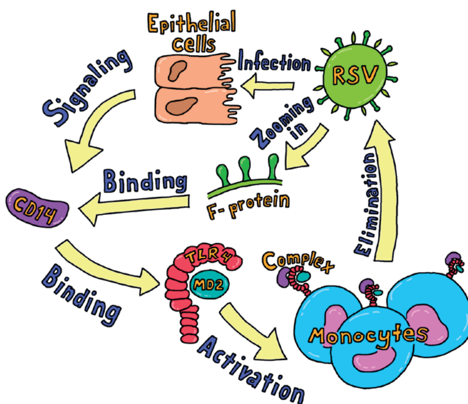

Figure 2: the hypothetical sequence of events after RSV infection and involvement of CD14. *RSV infects epithelial cells in the airway; the RSV-F protein is recognized by the TLR-MD2-CD14 complex on monocytes; monocytes are acivated and facilitate elimniation of RSV.*

CURRICULUM VITAE

Sjanna Bo Besteman was born on the 10th of September 1985 in Heemskerk, the Netherlands. She Received her secondary education at St. Michael College in Zaandam. In 2004 she started medical school in the Academical Medical Center at the University of Amsterdam. During her study she completed the extracurricular minor International Development studies and an extra-curricular course in medical ethics. Also, she was active as a volunteer with the non-governmental organization Medicine du Monde. In the fourth year of her medical study she did a scientific internship on the management of hypertension in Aracaju, Brazil.

After receiving her medical degree in 2011 she started working as a resident not in training at the pediatric department of St. Antonius Ziekenhuis, Nieuwegein. After a voluntary two month pediatric cardiology internship with the non-profit organization Safe a Child's Heart, at Wolfson Medical Center in Holon Israel, she started her residency in pediatrics in 2014 which takes part in the Wilhelmina Children's Hospital in Utrecht. The PhD project described in this thesis started in 2016 in the Center for Translational Immunology at the University Medical Center Utrecht, under the supervision of Prof. L. Bont and Prof. L Meyaard. In 2019 she obtained her University Teaching Qualification (BKO). She will complete her residency at the beginning of 2023.

She lives in Amsterdam with Elon Heymans and their two sons, Aviv (born in 2018) and Ami (born in 2021).

LIST OP PUBLICATIONS

This thesis

1. **Sjanna B. Besteman**, Emily Phung*, Henriette H.M. Raeven*, Gimano D. Amatngalim, Matevž Rumpret, Juliet Crabtree, Rutger M. Schepp, Lisa W. Rodenburg, Susanna G. Siemonsma, Nile Verleur, Rianne van Slooten, Karen Duran, Gijs van Haaften, Jeffrey M. Beekman, Lauren A. Chang, Linde Meyaard, Tjomme van der Bruggen, Guy A.M. Berbers, Nicole Derksen, Stefan Nierkens, Kaitlyn M. Morabito, Tracy J. Ruckwardt, Evelyn A. Kurt-Jones, Douglas Golenbock, Barney S. Graham, Louis J. Bont. Recurrent respiratory syncytial virus infection in a CD14 deficient patient. Sjanna B. Besteman et al. *The Journal of infectious disease. 2022.* *These authors contributed equally.
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3. **Sjanna B. Besteman,** Amie Callaghan, Annefleur C. Langedijk, Marije P. Hennus, Linde Meyaard, Michal Mokry, Louis L. Bont, Jorg J.A. Calis. Transcriptome of airway neutrophils reveals an interferon response in life-threatening respiratory syncytial virus infection. *Clinical Immunology*. 2020.
4. **Sjanna B. Besteman,** Amie Callaghan, Marije P. Hennus, Geertje H.A. Westerlaken, Linde Meyaard, Louis J. Bont. Signal inhibitory receptor on leukocytes (SIRL)-1 and Leukocyte- associated immunoglobulin-like receptor (LAIR)-1 regulate neutrophil function in infants. *Clinical Immunology.* 2020.
5. **S. Besteman, L. Bont**. Fail-Fast in RSV Vaccine Development. Am. J. Respir. Crit. Care Med. Feb. 2019.

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