Neutrophils in respiratory syncytial virus disease

Untangling the NET

Cortjens, B.

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Chapter 6

Neutrophil Extracellular Traps cause Airway Obstruction during Respiratory Syncytial Virus Disease

B. Cortjens, O.J. de Boer, R. de Jong, A.F.G. Antonis, Y.S. Sabogal Piñeros, R. Lutter, J.B.M. van Woensel, R.A. Bem

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Abstract

Human respiratory syncytial virus (RSV) is the most important cause of severe lower respiratory tract disease (LRTD) in young children worldwide. Extensive neutrophil accumulation in the lungs and occlusion of small airways by DNA-rich mucus plugs are characteristic features of severe RSV-LRTD. Activated neutrophils can release neutrophil extracellular traps (NETs), extracellular networks of DNA covered with antimicrobial proteins, as part of the first line defence against pathogens. NETs can trap and eliminate microbes, however abundant NET formation may also contribute to airway occlusion. In this study, we investigated whether NETs are induced by RSV and explored their potential anti-viral effect in vitro. Second, we studied NET formation in vivo during severe RSV-LRTD in infants and bovine RSV-LRTD in calves by examining bronchoalveolar lavage fluid and lung tissue sections respectively. NETs were visualized in lung cytology and tissue samples by DNA and immuno-staining using antibodies against citrullinated histone H3, elastase and myeloperoxidase. RSV was able to induce NET formation by human neutrophils in vitro. Furthermore, NETs were able to capture RSV, thereby precluding viral particles to bind to target cells and prevent infection. Evidence for the formation of NETs in the airways and lungs was confirmed in children with severe RSV-LRTD. Detailed histopathological examination of calves with RSV-LRTD showed extensive NET formation in dense plugs occluding the airways, either with or without captured viral antigen. Together these results suggest that although NETs trap viral particles, their exaggerated formation during severe RSV-LRTD may contribute to airway obstruction.
Introduction

The human pneumovirus respiratory syncytial virus (RSV) is the most common pathogen of lower respiratory tract disease (LRTD) in young children worldwide. The global incidence of RSV-LRTD in children under the age of 5 years has been estimated to be over 33 million per year, leading to over 3 million hospitalizations and almost 200,000 deaths. Currently, there is no licensed vaccine or effective treatment for RSV disease.

The single most important feature in histopathological studies of children with RSV-LRTD is small airway obstruction by dense plugs composed of mucus, fibrin, and debris from leukocytes and sloughed bronchial epithelial cells. Importantly, these plugs contain large amounts of DNA, which increases the viscosity of the mucus, and thus contributes to the degree of airway obstruction. The extracellular DNA in airway mucus plugs in RSV-LRTD is likely derived in part from necrotic epithelial and inflammatory cells. However, other mechanisms of intraluminal release of extracellular DNA may coexist.

Neutrophils are the predominant inflammatory cells recruited to the airways and lungs as part of the innate immune response to RSV. Neutrophils are able to take up RSV, but it is uncertain whether they support active viral replication. Neutrophils possess a broad arsenal of defensive strategies, including reactive oxygen species production, phagocytosis, release of antimicrobial granule contents and formation of neutrophil extracellular traps (NETs). NETs are web-like networks of expelled DNA covered with histones and microbicidal and cytotoxic proteins, such as elastase or myeloperoxidase. Their formation is known as NETosis and occurs upon neutrophil activation, for example by the protein kinase C activator phorbol myristate acetate (PMA), lipopolysaccharide, but also by certain chemokines, such as interleukin-8 (IL-8), which is abundantly present in the lungs during RSV-LRTD.

Originally, NETs have been discovered to contribute to entrapment and killing of bacteria and fungi, but over the last years their formation has also been observed in the context of viral disease, including influenza-, HIV-1, hanta- and poxvirus infection, recently reviewed. Interestingly, previous data have shown that NETs may capture HIV-1 particles in vitro, thereby preventing local spread to target cells. Similarly, a protective effect of NETs has been found in poxvirus infection in mice in vivo. On the other hand, NETs may also contribute to organ dysfunction. For example, in the airways the presence of large amounts of extracellular DNA from NETs is associated with impaired respiratory function, as has been shown in patients with cystic fibrosis and chronic obstructive pulmonary disease (COPD).
Very recently, Funchal and co-workers reported that the RSV fusion protein promotes the release of NETs from neutrophils in vitro\textsuperscript{23}, however, evidence of NET formation in \textit{vivo} during RSV infection is lacking. In the present study we hypothesized that although NETs can contribute to antiviral host defence by capturing RSV virions, exaggerated NET formation occurs in RSV-LRTD, potentially leading to areas with increased airway obstruction. To this aim, we evaluated NET formation by neutrophils in the context of RSV infection in \textit{vivo} and examined bronchoalveolar lavage fluid (BALF) samples of children with severe RSV-LRTD and lung pathology samples of calves with bovine RSV (bRSV)-LRTD.
Materials and Methods

In vitro protocol

Neutrophils were isolated from peripheral blood of healthy volunteers, using Lymphoprep (Axis-Shield PoC AS, Olso, Norway) separation and subsequent erylysis in ice-cold erylysis-buffer (155 mM NH₄ Cl, 10 mM KHCO₃, 0.3 mM EDTA). Neutrophils (2.5 x 10³ cells) were suspended in RPMI 1640 (Gibco, Grand Island, NY, USA) with 2% foetal calf serum with or without PMA (120 nM, P1585, Sigma Aldrich, St. Louis, MO, USA) for 2 hours at 37°C. After neutrophil stimulation, DNase (D-5025, Sigma Aldrich) in a final concentration of 0.15 IU/ml was added for NET degradation as negative control. Fluorescent phycoerythrin-labelled beads (HFP-0856-5, Spherotech, Lake Forest, IL, USA) were added in separate experiments to visualize nonspecific binding of particles to NETs.

For viral challenge studies, human RSV strain A2 (VR-1540, ATCC, subgroup A, obtained from E. Yasuda, AIMM Therapeutics) was labelled for 30 min. at room temperature with the far-red fluorescent, lipophilic carbocyanine DiD (D-7757, Life Technologies, Carlsbad, CA, USA) and layered on sucrose gradients. After centrifugation the interphase was collected and purified by the use of Zeba spin desalting columns (7K MWCO, Thermo scientific, Rockford, IL, USA). DiD-labelled RSV was incubated with neutrophils (MOI 0.3) for 5 min. at room temperature, in the presence of PMA and/or DNase as indicated. Recombinant green fluorescent protein expressing RSV A2 (rgRSV224) was used in RSV-GFP studies (kind gift from Dr. L. Bont, paediatric department, WKZ, Utrecht). RSV-GFP (MOI 0.04) was added to neutrophils and incubated for 30 min. at room temperature with conditions as indicated above. The culture plate with stimulated neutrophils and RSV was centrifuged for 5 min. at 800 rpm after which the supernatant was removed and added to cultured A549 cells (human lung adenocarcinoma epithelial cell line, 1.25 x 10⁴ cells/well). In DiD-RSV experiments the virus allowed to bind to A549 cells for 60 min. at 37°C, after which the supernatant was removed and cells were washed twice with PBS, trypsinized (0.5%Trypsin-EDTA) and fixed with 4% paraformaldehyde. DiD-labelled RSV attachment to A549 cells was measured using flow cytometry (APC-channel) on the Canto II (BD biosciences, Oxford, UK). In RSV-GFP studies, A549 cells were cultured for 48 hours and fluorescent pictures were taken with the Operetta (Perkin Elmer, Waltham, MA, USA) for counting of GFP positive plaques.

Live Cell Imaging

Neutrophils were isolated as described above and plated in glass-bottom 4-well plates (Cellview, Greiner Bio-One, Frickenhausen, Germany) in RPMI-2%FCS with 0.5 μM Sytox Green nucleic acid stain (S7020, Life Technologies) and immediately placed in the Leica IRBE inverted fluorescence, phase contrast microscope after stimulation with RSV as indicated.
Serial images every 5 min. were taken during three hours.

**Patient protocol**

All patient sampling protocols were approved by the local ethical committee of the Academic Medical Centre (AMC) of Amsterdam, The Netherlands and informed consent was obtained from parents/caretakers. All procedures involving human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Five patients (mean age 46.2 ± 24.5 days) with severe RSV-LRTD admitted to the paediatric intensive care unit (PICU) of the Emma children’s hospital/AMC in Amsterdam, The Netherlands, between November 2014 and February 2015 were included. RSV infection was confirmed by direct immunofluorescence assay (Imagen, Dako, Glostrup, Denmark) and/or real time-PCR in a nasopharyngeal aspirate sample, as part of the standard hospital care. None of the RSV-LRTD patients were suspected to have a bacterial co-infection at the time of sampling. We included two age-matched control patients without RSV-LRTD undergoing elective diagnostic BALF; one patient was diagnosed with a neurological disorder, and one patient sampled for a suspected respiratory tract infection, but cultures proved to be negative. BALF was obtained on the day of intubation by two subsequent instillations of 1 ml/kg of 0.9% saline through a wedged suction catheter passed through the endotracheal tube. After each instillation, fluid was suctioned and both samples were pooled. For detection of NETs, 5 µl of the BALF sample was gently added to 50 µl PBS on glass slides. The slides were air-dried and fixed for 10 min. using 4% paraformaldehyde and stored at -80°C.

**Animal protocol**

All animal experiments were conducted in accordance with the Act on Experimental Animals of The Netherlands regulated by the local ethical review committee of the Central Veterinary Institute in Lelystad, The Netherlands. BRSV strain Odijk, subtype A (fifth in vivo passage) was originally obtained during a field outbreak in Odijk, the Netherlands, in 1991, and used for in vivo experiments with calves. Eight colostrum deprived dairy calves, aged six weeks, were infected with $10^3$-$10^4$ TCID$_{50}$ in 2ml bRSV by intranasal inoculation with an air jet nebulizer and were scored for clinical lower respiratory tract disease symptoms, including respiratory rate, dyspnoea and laboured breathing, daily as described previously. Broncho alveolar lavages were performed on day -6, 2, 5, 7 and 9 as described before. On day 9 after viral inoculation, calves were euthanized by pentobarbital overdose and exsanguination. Post mortem, lung tissue samples from a predetermined site were collected. The lung tissue sections were stored in 10% neutral buffered formalin and embedded in paraffin, after which 5 µm sections were cut for immunohistochemistry. Control animals (n=4) included age matched non-infected dairy calves. Viral loads in BALF as determined by quantitative PCR (see supplemental methods) are expressed as the 50% tissue culture infective dose (TCID$_{50}$) per mL calculated by including a
standard dilution series of virus stock with known TCID$_{50}$.

**Detection of NETs**

NETs in neutrophil cell cultures and BALF samples were visualized by immunofluorescent staining using 4',6-diamidino-2-phenylindole (DAPI) staining of DNA and anti-human neutrophil elastase (NP57, Dako) and rabbit anti-human citrullinated anti-Histone H3 (citrH3, Ab5103, Abcam, Cambridge, UK). Briefly, cytology slides were blocked with Ultra V-block (TA-125-UB, Thermo Scientific) for 10 min. and incubated with the primary antibodies overnight. The next day slides were washed and incubated with goat-anti-mouse FITC and donkey-anti-rabbit Alexa647 (A-31573, Invitrogen, Carlsbad, CA, USA) for 60 min. and mounted with Prolong Gold containing DAPI (Prolong Gold, Life Technologies). The cells and NETs were visualized using the LEICA SP-8 X confocal microscope. Lung tissue sections of calves were investigated by immunohistochemistry using polyclonal rabbit anti-myeloperoxidase (A0398, Dako), rabbit anti-citrH3 (Ab5103, Abcam), mouse anti-cytokeratin Pan ab1 (MS-343-P, Thermo Scientific), goat anti-RSV (ABIN238061, Antibodies-online, Aachen, Germany). In short, paraffin sections were dewaxed and rehydrated, endogenous peroxidases were blocked in 100% Methanol/0.3%H$_2$O$_2$ for 20 min., followed by antigen retrieval using Tris EDTA (pH 9.0) for 20 min. at 98°C. After washing, the sections were subsequently incubated with appropriate dilutions of the different primary antibodies. Depending on the origin of the primary antibodies, sections were incubated with appropriate HRP conjugated anti-mouse-, anti-rabbit- or anti-goat-IgG polymer (DPVO500HRP, Immunologic, Duiven, The Netherlands). HRP activity was visualized with Novared (Vector Laboratories, Peterborough, UK). Negative controls in which the specific primary antibody had been omitted were always included. After each individual Novared staining the section was scanned using the Philips Ultra Fast Scanner 1.6RA (Philips, Eindhoven, The Netherlands), and subsequently de-stained using 60mM Tris HCl/2%SDS/0.7%-mercaptoethanol for 30 min. at 50°C, after which an additional staining was performed with a different antibody. Precise overlays of the scanned sections were created using ImageJ TrakEM2. This allowed us to perform multiple stainings in the exact same plane and depth of a single lung tissue section. Immunostaining on whole lung tissue sections were analysed using the colour deconvolution plugin followed by quantitative thresholding. CitH3-positive pixels were counted and divided by total amount of tissue pixels to calculate the percentage of positive NET staining in each specimen. We counted the total amount of NET positive intraluminal plugs with and without viral protein. In addition to the immunohistochemical detection of NETs we examined mucin expression by Alcian Blue staining.

**Statistical analysis**

Statistical analysis was performed using Graphpad Prism (V5.0, GraphPad Software, La
Jolla, CA, USA). Not normally distributed data are expressed as medians with interquartile range (IQR) or mean ± SD when distributed normally and were analysed by using the Mann-Whitney U test for differences between groups. A two-sided P value of < 0.05 was considered statistically significant.

Results

**RSV induces NET formation by human neutrophils in vitro**

Recently, it has been shown that neutrophils expel NETs upon exposure to RSV in vitro.23 First, to confirm these results we examined isolated human neutrophils exposed to PMA, RSV or both in vitro (Fig. 1). In line with the results from Funchal et al., we observed web-like networks of extracellular DNA to appear around degenerating human neutrophils challenged with RSV. These extracellular DNA structures were coated with elastase and citH3, indicating the formation of NETs (Fig. 1I-L). Intact neutrophils stained positive for citH3, representing an early stage of NETosis (Fig. 1K). NETs were present more abundantly in PMA-stimulated human neutrophils (Fig. 1E-H), as compared to RSV-challenged neutrophils (Fig. 1I-L), However, the formation of NETs was most extensive in neutrophils exposed to both RSV and PMA, suggesting a synergistic effect (Fig. 1M-P). Treatment with DNase degraded the observed extracellular structures, further confirming the formation of NETs (Fig. 1Q-T). In addition, we performed live cell imaging of neutrophils incubated with RSV or PMA, which also showed pronounced release of extracellular DNA within 30 minutes after exposure (Supplemental movie 1).

**NETs capture RSV virions and prevent viral binding and infection of epithelial cells in vitro**

NETs may have the ability to capture viral particles, as has been shown for HIV.19 We investigated whether the NETs observed in our in vitro experiments capture RSV. Incubation of PMA-stimulated neutrophils with DiD-labelled RSV indeed led to clear co-localization of RSV virions and the web-like networks of extracellular DNA (Fig. 2). Fluorescent-labelled beads also attached to NETs (Supplemental fig. 1). Together with the fact that NETs can bind a wide range of microbes14-17 this suggests non-specific binding of particles to NETs. To address a possible functional role of NETs during the antiviral response to RSV, we examined the extent of RSV binding to target lung epithelial cells in an in vitro assay. While the incubation of epithelial cells with supernatant of DiD-labelled RSV aliquots resulted in high viral binding, 5 min. pre-incubation of DiD-labelled RSV together with PMA-stimulated neutrophils already resulted in significantly reduced viral binding to epithelial cells (Fig. 3A). Importantly, the addition of DNase abrogated this effect, indicating a functional role for NETs in capturing RSV virions (Fig. 3A). Next, to address whether reduced viral binding leads to less infectivity we repeated these experiments with recombinant GFP-expressing RSV. This virus produces
NETs cause airway obstruction

Figure 1: RSV induces NET formation by human blood neutrophils in vitro.

Immunofluorescent cytochemistry for detection of NETs by DAPI (DNA), elastase and citH3-stainings with the right column showing merged images. (A-D) Unstimulated neutrophils do not form NETs. In contrast, stimulation of neutrophils with PMA (E-H), RSV (I-L) or both (M-P) induces NET formation. (Q-T) Addition of DNase degrades the NETs, but early NETosis as indicated by citH3-positive neutrophils can still be seen. Representative images are shown; neutrophils from all three donors produced NETs after PMA or RSV stimulation (N = 3).
Figure 2: NETs capture RSV in vitro.

Immunofluorescent cytochemistry detection of extracellular DNA (DAPI, blue) and DiD-labelled RSV (red). (A-C) Unstimulated neutrophils without virus. (D-F) PMA stimulation of neutrophils leads to extracellular DNA accumulation due to NET formation. Viral capture (co-localization) by the extracellular DNA networks is observed upon addition of RSV to PMA stimulated (G-I) and non-PMA stimulated neutrophils (J-L). Representative images are shown; neutrophils from all three donors produced NETs after RSV stimulation with co-localization of RSV within the NETs (N = 3).

GFP after translation, which is indicative of productive infection of target cells. Incubation of GFP-RSV with PMA-stimulated neutrophils reduced infection by 36.3% compared to incubation of GFP-RSV with unstimulated neutrophils (60.5% ± 9.4% versus 96.8% ± 13.9%, P = 0.008, fig. 3B). Addition of DNase abolished this effect (P = 0.03, Fig. 3B-C). Together, these data are indicative of reduced spread and subsequent decreased productive infection of RSV in lung epithelial cells due to the formation of NETs by stimulated neutrophils. Importantly, both the viral binding (Fig. 3A) and viral replication assay (Fig. 3B) demonstrated no difference between the conditions with virus alone and virus with unstimulated neutrophils, suggesting there was no relevant uptake by neutrophils of the DiD-RSV and RSV-GFP during the short period of incubation.
NETs cause airway obstruction

Figure 3: NETs functionally capture RSV virions by preventing viral attachment to target lung epithelial cells in vitro.

(A) Mean fluorescent intensity (MFI) as measured by flow cytometry to detect viral attachment of DiD-labelled RSV to cultured A549 lung epithelial cells under the indicated conditions. Addition of RSV to A549 cells causes a high fluorescent signal as evidence for viral attachment to target cells. Prior incubation of DiD-labelled RSV with PMA stimulated neutrophils reduces RSV virion binding (** P = 0.002). This reduction in RSV binding to target cells can be abrogated by addition of DNase to PMA stimulated neutrophils prior to viral incubation (* P = 0.04), suggesting functional capture of RSV virions by NETs. Data are shown as bars depicting the mean ± SD from two separate experiments performed in triplicate. (B) NETs prevent productive infection of RSV-GFP in target lung epithelial cells in vitro. Data is expressed as mean ± SD of the percentage of GFP-positive plaques compared to viral infection of A549 cells alone (= 100% infection). Prior incubation of RSV-GFP with PMA-stimulated neutrophils reduces active replication at 48 hours, which is abrogated by addition of DNase (** P = 0.008, * P = 0.03). Each condition was tested in quadruplicate with neutrophils from 5 donors. (C) Representative images of RSV-GFP infected A549 cells in different conditions; viral incubation with unstimulated neutrophils (Neu), stimulated neutrophils (Neu + PMA) and stimulated neutrophils with degradation of NETs by DNase (Neu + PMA + DNase). Neu; Neutrophils, PMA; Phorbol 12-myristate 13-acetate
NETs cause airway obstruction

Formation of NETs in children with severe RSV-LRTD

Next, we investigated the presence of NETs in the lower respiratory tract of children with severe hRSV-LRTD in vivo. We performed immunofluorescent staining of BALF cytology samples from five patients with RSV-LRTD with respiratory failure admitted to the PICU. In all of these children there was marked positive intracellular staining for citH3 of lung neutrophils, indicative of early NETosis, as compared to uninfected control patients (Fig. 4E-H). Likewise, surrounding the (degenerating) neutrophils there were extracellular networks of DNA coated with elastase and citH3, indicating the presence of NETs (Fig. 4I-L).

Figure 4: NETs are formed in the lungs during severe RSV-LRTD in children.

Representative images of immunofluorescent cytiochemistry of BALF samples for detection of NETs by DAPI (DNA), elastase- and citH3-stainings with the right column showing merged images. (A-D) Non-infected control patients show no evidence of extracellular DNA structures or positive citH3-staining in neutrophils (elastase-positive cells). In contrast, neutrophils in BALF from children with RSV-LRTD show intracellular citH3-positive staining, indicating early NETosis (Patient 1, E-H) and formation of NETs (Patient 2, I-L). NETs were detected in all RSV patients (N = 5), but in none of the control patients (N = 2).
**NETs cause airway obstruction**

**NETs are formed in plugs obstructing the airways in bRSV-LRTD in calves**

Our in vitro experiments suggested that NETs may play a role in the antiviral response to RSV. However, extensive formation of NETs in the airways, as we observed in BALF from children with RSV-LRTD, has also been implicated in local obstruction in patients with non-infectious conditions, like cystic fibrosis and COPD.\(^{21,22}\) We therefore examined lung tissue sections of calves with bRSV-LRTD, a cognate host-pneumovirus model, which mirrors many aspects of RSV disease in children including strong neutrophil recruitment and histopathological lung alterations.\(^{20}\) LRTD symptoms in experimentally infected calves started to appear 5 days after inoculation, peaking on day 8 around the peak in viral loads in BALF (Fig. 5H) with substantial LRTD symptoms persisting on the day of analysis (day 9, Fig. 5F). BALF analysis showed pronounced neutrophil influx on day 7 to 9 (Fig. 5G). The lungs of calves with bRSV-LRTD revealed widespread NETosis and deposition of NETs as indicated by positive intracellular and extracellular citH3 and MPO staining (Fig. 5B-C). In addition, we found marked mucin staining within the NET-containing airway plugs (Fig. 5D). The fold increase of NET-positive pixels, normalized for total tissue pixels in bRSV-infected calves, compared to non-infected control calves, was 16.02; IQR 8.43-22.59 vs.1.06; IQR 0.27-1.67 (\(P = 0.004\), Fig. 5E). We found two patterns of NET deposition: intra-luminal in the (small) airways and interstitial at the airway and alveolar level (Fig. 5B-C). More detailed histopathological examination revealed that intra-luminal airway NET formation was most prominent during bRSV-LRTD. These NETs co-localized in the dense cellular plugs containing shed epithelial cells and large amounts of neutrophils, which obstructed the smaller airways and larger bronchi (Fig. 6). Interestingly, 63.25% of the NET-plugs did not contain virus (Fig. 6L) opposed to 36.75% that did contain virus (Fig. 6H; plugs without and with virus per lung tissue section: 13.25 ± 17.66 versus 7.7 ± 11.3).
Figure 5: Widespread formation of NETs in the lungs of calves with bRSV-LRTD.

Representative images of immunohistochemistry staining for citH3 with haematoxylin counterstain in lung tissue sections from calves. (A) Normal lung architecture without positive citH3-staining in a non-infected control calf (magnification 5×). (B) Marked positive citH3-staining in the inflamed lungs of a calf with bRSV-LRTD (magnification 5×), with evidence for both intracellular (arrowheads) and extracellular (arrows) citH3-staining (C, magnification 80×). (D) Mucin staining within the NET-containing airway plug, as shown by Alcian blue stain (magnification 80×). (E) The fold increase in NET-positive pixels (normalized for total tissue pixels) in bRSV-infected calves (N = 8) as compared to non-infected control calves (N = 4). Data are shown as median + IQR, P = 0.004. Clinical bRSV-LRTD scores (F), neutrophil concentration per mL BALF (G) and lung viral loads determined by PCR (H) in bRSV-infected calves (N = 8) during the course of LRTD.
Figure 6: Formation of NETs in plugs obstructing the airways of calves with bRSV-LRTD.

Representative images of immunohistochemistry staining for Pan-cytokeratin, myeloperoxidase (MPO), citH3 and bRSV antigen in lung tissue sections from calves. (A-D) Normal airway architecture with intact epithelium without positive staining for MPO and citH3 in a non-infected control calf (magnification 40×). (E-H) Large airway obstruction by plugs consisting of epithelial cells (cytokeratin), neutrophils (MPO) and evidence for NETosis/NETs (positive intracellular and extracellular staining for citH3 and MPO), with captured viral particles (magnification 20×). (I-L) Small airway obstruction by plugs with positive citH3-staining indicating NETosis and formation of NETs, but without the presence of viral antigens (magnification 40×).
NETs cause airway obstruction

Discussion

The goal of the present study was to explore the formation of NETs by neutrophils during RSV infection. We found that RSV is able to induce NETosis and that NETs can functionally capture RSV particles in vitro thereby preventing infection of target epithelial cells. Moreover, marked formation of NETs is observed during RSV-LRTD in vivo, including accumulation of NETs in dense plugs obstructing the airways without captured viral antigen, suggesting an exaggerated adverse response.

Our study contributes to the understanding of neutrophil biology in RSV infection. Neutrophils are the most abundant inflammatory cells present in the lungs and airways of children with severe RSV-LRTD. Prominent neutrophil attracting chemokines such as IL-8, MIP1α and IL-17A can be found in the airways during infection and recruit neutrophils towards RSV infected areas. Neutrophils become highly activated in this pro-inflammatory microenvironment, but appear relatively inert to activation by RSV itself. So far, the precise role of neutrophils in either anti-RSV host defence or immunopathology is largely unknown. Potentially neutrophils may, like in other viral infections contribute to RSV clearance and limit viral spread. For example in influenza virus models neutrophil depletion results in more severe disease and higher viral replication during influenza infection with virulent strains, but less so after infection with avirulent strains. As part of their anti-microbial defence system, stimulated neutrophils are able to form NETs, which can capture and neutralize a diverse spectrum of pathogens. Besides bacteria and fungi, some viruses, including HIV-1 have been found to induce NET formation by neutrophils, which can trap and neutralize viral particles. This viral-induced NETosis appears to be a TLR-dependent mechanism. Interestingly, in a recent study by Funchal et al. it has been reported that the fusion protein of RSV induces the formation of NETs in vitro, a process that appears dependent on TLR-4. Our study confirms these findings of RSV-induced NETosis in human neutrophils in vitro, but further reveals evidence for the release of NETs in children with RSV-LRTD in vivo. Additionally we show that NETs can capture RSV virions, which prevents viral binding to target epithelial cells. As such, the formation of NETs by neutrophils may be an important mechanism of the local anti-RSV response to limit viral spread.

On the other hand, powerful neutrophil immune responses may contribute to pathology during RSV-LRTD. Tissue injury and organ dysfunction by exaggerated or prolonged activation of neutrophils is implicated in a number of diseases, including the development of acute lung conditions. Similarly, a number of studies have proposed adverse effects of neutrophil responses in RSV-LRTD. For example, Stokes et al. found that neutrophil depletion results in less airway inflammation and mucin expression in RSV-infected mice, suggesting that neutrophils can contribute to airway obstruction during RSV-LRTD. One
of the potential additional mechanisms of neutrophil-mediated immunopathology is the formation of NETs. For example, in patients with cystic fibrosis and COPD extensive NET formation is seen during exacerbations.\textsuperscript{23,43} This is interesting as (small) airway occlusion by dense mucus- and cell-rich plugs is a characteristic feature during RSV-LRTD, and is of important clinical relevance in the development of respiratory failure and atelectasis.\textsuperscript{42} Our results from the bRSV calf-model provide morphological evidence for the strong presence of NETs in these plugs during RSV-LRTD. We observed NET-rich plugs containing RSV antigen, underlining the results of viral capture by NETs in vitro. Yet most areas of airway occlusion with NETs did not contain viral antigen, which suggests disproportional formation of NETs. Although speculative, the relatively high local secretion of IL-8, a known powerful trigger of NETosis\textsuperscript{14}, in patients with RSV-LRTD\textsuperscript{12,44,45} may contribute to this imbalance in neutrophil response. As such, the present study reveals a potential novel pathological mechanism of airway obstruction in RSV-LRTD.

The NETs observed in the lower respiratory tract of children with RSV-LRTD contribute to the amount of local extracellular DNA. Addition of DNA to mucus increases the viscosity in vitro.\textsuperscript{8} Interestingly in this context, some studies have assessed the effects of nebulized recombinant DNase in children with RSV-LRTD to reduce mucus viscosity and decrease airway obstruction.\textsuperscript{10,46-48} One clinical trial showed strong radiological improvement\textsuperscript{10}, and one case-series in mechanically ventilated infants with severe RSV-LRTD showed clinical and radiological improvement.\textsuperscript{46} Unfortunately, in two more recent randomized placebo-controlled trials DNase treatment did not reduce the duration of admission or oxygen need of hospitalized infants with RSV-LRTD.\textsuperscript{47,48} However, it is possible that this negative result is related to the relative mild RSV disease in the populations studied or to the difficulty of the agent to reach the more distal and heterogeneous distributed airway plugs. Whether direct degradation of NETs as end products or rather targeting of early events leading to NETosis is beneficial in the course of RSV-LRTD remains to be investigated.

In conclusion, RSV is able to induce NET formation in vitro and NETs can capture RSV virions, indicating an anti-viral role. However, exaggerated NET formation is observed in the lungs and cell-rich plugs, with and without captured viral antigen, occluding the small airways during severe RSV-LRTD. This suggests NETs contribute to airway obstruction and immunopathology in RSV disease.
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Statement of contribution

BC, JW, RJ, AA and RB designed the study. BC, RJ and AA conceived and carried out the animals experiments. BC, YS and OB carried out the immunohistochemistry and analysed the data. BC, YS and RL carried out the in vitro experiments and analysed the data. BC, JW and RB interpreted the results. All authors were involved in writing the paper and had final approval of the submitted and published versions.
References


RSV induces extracellular DNA deposition. Isolated human neutrophils were cultured in glass-bottom 4-well plates with addition of the DNA marker Sytox Green in RPMI-2%FCS, in control conditions neutrophils die after 1-3 hours and become green fluorescent, the DNA remains inside the cell. The RSV treated group developed rapid expanding extracellular DNA structures.

Supplemental figure 1. Fluorescent phycoerythrin (PE)-labelled beads binding to NETs in vitro.

Beads (0.77µm) were added to PMA-stimulated neutrophils to visualize co-localisation on NETs. (A-C) Fluorescent beads (yellow) are trapped within the DNA structures (blue staining of extracellular DNA by DAPI). (D-F) Higher magnification of A-C.

**Supplemental methods**

**BRSV PCR**

BRSV RNA from 200µL BALF was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (03038505001, Roche, Almere, The Netherlands). The conserved N-gene was detected with a primer/taqman probe mix by realtime PCR using the QuantiFast Multiplex RT-PCR Kit (204954, QiaGen, Venlo, The Netherlands) according to the manufacturer’s instructions. All assays included reverse transcription of RNA into cDNA and were run on the Applied Biosystems 7500 under optimized cycling conditions (N = 40). Threshold cycle (Ct) values of the samples were compared with Ct values of a standard curve from a virus stock with a known TCID₅₀/mL determined by virus titration on Embryonic Bovine Trachea cells.