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Neutrophil subset responses in infants with severe viral respiratory infection

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ABSTRACT

Neutrophils are the predominant inflammatory cells recruited to the respiratory tract as part of the innate immune response to viral infections. Recent reports indicate the existence of distinct functional neutrophil subsets in the circulatory compartment of adults, following severe inflammatory conditions. Here, we evaluated the occurrence of neutrophil subsets in blood and broncho-alveolar lavage fluid during severe viral respiratory infection in infants based on CD16/CD62L expression. We show that during the course of severe respiratory infection infants may develop four heterogeneous neutrophil subsets in blood (mature, immature, progenitor, and suppressive neutrophils), each with distinct activation states. However, while isolated viral respiratory infection was characterized by a relative absence of suppressive neutrophils in both blood and lungs, only patients with bacterial co-infection were shown to produce suppressive neutrophils. These data suggest the occurrence of distinct and unique neutrophil subset responses during severe viral and (secondary) bacterial respiratory infection in infants.

1. Introduction

Neutrophils are the key effector cells of the innate immune system and are vital in the hosts response to bacterial infection [1]. However, increasing evidence suggest an equally important role during viral infections [2]. For example, neutrophils are the predominant inflammatory cells recruited to the respiratory tract as part of the innate immune response to viral respiratory infections. They are commonly found in airway samples taken from infants with respiratory syncytial virus (RSV) infections [3–7] and they are associated with disease severity [8]. Neutrophils possess a broad arsenal of defensive strategies, such as: reactive oxygen species production, phagocytosis, release of antimicrobial granule contents and the formation of neutrophil extracellular traps (NETs). These functions serve to protect against invading pathogens, but may also cause collateral host tissue injury [9,10] and worsen the clinical presentation. Their role during viral respiratory infections, including RSV, remains elusive and important questions remain unanswered.

Differentiation of immune cells into different functional phenotypes has been well described for macrophages and dendritic cells [11]. More recently there have been several reports indicating that similar distinct functional neutrophil phenotypes exist in the circulation [12–15]. Pillay and co-workers have characterized neutrophil subsets in experimental studies with healthy adults challenged with systemic LPS and in patients with severe inflammation originating from bacterial sepsis and trauma based on CD16 (FcγRIII receptor) and CD62L (L-selectin) expression [12]. Besides mature neutrophils ($CD16^{high}CD62L^{high}$) they defined two other distinct neutrophil subsets: immature neutrophils ($CD16^{low}CD62L^{high}$) and suppressive neutrophils ($CD16^{high}CD62L^{low}$) [12]. Immature neutrophils may arise after depletion of mature neutrophils from the bone marrow and are deemed incompetent in anti-microbial immune functions. The suppressive neutrophils show a hypersegmented nucleus which implies increased maturation compared to mature neutrophils. Interestingly, the suppressive neutrophils subset was described to be capable of T cell suppression and could play an important role in the dampening of severe inflammatory responses. However, they could also cause immune paralysis, limiting the effectivity of the immune system against invading pathogens [16].

Neutrophil subset responses have been poorly characterized in viral infections in general, and in local inflammatory conditions in the lungs.

Abbreviations: CRP, C-reactive protein; EDTA, ethylenediaminetetraacetic acid; G-MDSC, granulocytic myeloid derived suppressor cell; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; ROS, reactive oxygen species; RSV, respiratory syncytial virus.

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Nor have they been fully investigated in infants, who are known to have immature immune responses [17]. Although $CD16^{low}$ and $CD16^{high}$ blood neutrophils have been observed during RSV infection in infants [13], it is unknown whether suppressive neutrophils develop and if the $CD16^{low}$ neutrophils can be further defined using other cellular expression markers (e.g. CD62L). Finally, it is unclear whether the suppressive neutrophil subset sustains or develops during longer periods of time after infection. Previous studies with LPS administration only detected suppressive subsets after relative short periods of time (hours) [12].

Further characterization of the dynamics of the neutrophil response could improve our understanding in key immune regulators involved in severe viral (e.g. RSV)-induced respiratory disease in infants. To this aim, we evaluated the occurrence of neutrophil subsets in blood and broncho-alveolar lavage fluid (BAL) during severe viral respiratory infection in infants with and without bacterial co-infection.

2. Materials and methods

2.1. 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 Fortaleza (2013). Between January 2015 and March 2016, 19 patients (median [IQR] age 1.5 [0.6–7.1] months) with respiratory failure admitted to the pediatric intensive care unit (PICU) of the AMC/Emma Children's Hospital in Amsterdam, The Netherlands, were included (see Table 1 for patient characteristics). Blood was collected from an arterial catheter in lithium-heparin tubes (Vacutainer® 368,494, BD) on the day of admission (day 0) and days 1, 3, and 6, as long as there was an arterial catheter in situ. In a subgroup of patients who were mechanically ventilated ($N = 8$) BAL was obtained on the same days 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, the fluid was aspirated and both samples were pooled. The patients were divided in two groups: viral infection only (virus-only, $N = 8$) or viral infection with evidence of bacterial co-infection (virus + bacterial co-infection, $N = 11$). Viral infection was confirmed by real time-PCR in nasopharyngeal aspirate samples, as part of the standard hospital care. Patients were considered to have a bacterial co-infection if there was a positive blood culture *or* positive endotracheal sputum culture *and* a clinical suspicion of bacterial co-infection based on occurrence of fever, elevated CRP levels and/or chest X-ray abnormalities indicative of bacterial pneumonia. Clinical management was similar in both patient groups with the exception of antibiotic treatment in the bacterial co-infection group. The use of corticosteroids for the patient population under study is not standard practice in The Netherlands and were not administered in our patients.

Table 1
Patient characteristics.

	Virus ($N = 8$)	Virus + bacterial co-infection ($N = 11$)
Age (mths)	2.8 [1.1–6.2]	1.1 [0.5–9.1]
Male (%)	5/8 (62%)	6/11 (55%)
Ventilation duration (days)	8.5 ± 2.6	9.9 ± 6.0
Detected viral pathogens[a]		
- RSV	5	9
- Boca	1	2
- Corona	0	2
- Rhino	2	0
- Metapneumo	1	0
Detected bacterial pathogens[a]		
- *H. Influenzae*	–	7
- *S. aureus*	–	3
- *M. Catarhalis*	–	2
- *S. Pneumoniae*	–	1
- *P. aeruginosa*	–	1

[a] Multiple pathogens per patient could be detected.

We performed an extensive series of additional sampling and analysis for positive and negative controls. These included blood samples from: 1. healthy adults participating in an experimental challenge study using systemic LPS, described before [18]. In short, healthy volunteers ($N = 18$) were challenged with intravenous LPS *E. coli* 2 ng/Kg. Blood samples were taken prior LPS exposure and up to 8 h after exposure; 2. infants with acute respiratory failure due to bacterial sepsis ($N = 2$, age 0.8 and 39.6 months, both with samples on day 1 and 3 after admission); 3. infant with (post-operative) respiratory failure without respiratory disease or infection ($N = 1$, age 3 months, samples on day 1 and 3 after admission); and 4. healthy infants who visited the outpatient clinic for routine follow up for non-infectious/respiratory disorders ($N = 2$, age 12 days and 6 months). All samples were processed and analyzed by flow cytometry using the same protocol.

2.2. Flow cytometry

Red blood cells were lysed in ice-cold erylysis buffer (168 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) immediately after collection, centrifuged for 5 min at 400*g* and resuspended in PBK (5 mMol K-EDTA, 1%BSA, PBS). The cells were stained for 30 min at room temperature with the following antibodies: CD16 APC (Clone 3G8, Immunotools), CD62L PE (DREG-56, BD Pharmingen), CD45 AlexaFluor 700 (HI30, BD Pharmingen), Viability Cf594 (Invitrogen), CD11b APC-Cy7 (ICRF44, BD Pharmingen), CD54 FITC (84H10, Beckman Coulter), CD63 PE-Cy7 (H5C6, BD Pharmingen), and CD66b PerCP-Cy5.5 (G10F5, BD Pharmingen). After antibody staining the cells were washed in PBK and measured on the BD FACS Canto II or BD FACS Verse or sorted with the Sony SH800 Cell Sorter. Neutrophils were gated according to viable/$CD45^{pos}$/SSC^{high}/$CD16^{pos}$ cells (Supplemental Fig. 1). The sorted cells were centrifuged to a slide in the Shandon cytospin 3 (Thermo Scientific) and stained with Diff-Quik® stain (Medion Diagnostics). Flow cytometry results were analyzed using FlowJo™ software (FlowJo LLC). The BAL cells were handled in the same manner described above.

2.3. Statistical analysis

Statistical analysis was performed using Graphpad Prism (V5.0, GraphPad Software). Data are expressed as mean with standard error (SE). Neutrophil subset percentages between groups were compared using the Mann-Whitney *U* test. For the comparison of expression markers between subsets we used the Wilcoxon signed-rank test. The increase of neutrophil subset populations over time was analyzed using a linear mixed model, which corrected for the occurrence of bacterial co-infection. A two-sided *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Blood neutrophil subsets occur in the peripheral blood during severe viral respiratory infection in infants

In a first series of control experiments to identify previously reported heterogeneous neutrophil subsets based on CD16 and CD62L expression during acute inflammatory conditions [12], we investigated blood neutrophils after systemic LPS administration in healthy adults (Fig. 1, top row). Indeed, a prominent suppressive ($CD16^{high}CD62L^{low}$) as well as immature ($CD16^{low}CD62L^{high}$) neutrophil subset response were observed 8 h after LPS administration, confirming the results by Pillay et al. [12]. As the occurrence of neutrophil subsets based on CD16/