Neutrophils in respiratory syncytial virus disease
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Chapter 4

Neutrophil Subset Responses in Infants with Severe Viral Respiratory Infection

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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.
Introduction

Neutrophils are the key effector cells of the innate immune system and are vital in the host response to bacterial infection. However, increasing evidence suggest an equally important role during viral infections. 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 and they are associated with disease severity. 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 and worsen the clinical presentation.

Differentiation of immune cells into different functional phenotypes has been well described for macrophages and dendritic cells. More recently there have been several reports indicating that similar distinct functional neutrophil phenotypes exist in the circulation. 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. Besides mature neutrophils (CD16\text{high}CD62L\text{high}) they defined two other distinct neutrophil subsets: immature neutrophils (CD16\text{low}CD62L\text{high}) and suppressive neutrophils (CD16\text{high}CD62L\text{low}). 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.

Neutrophil subset responses have been poorly characterized in viral infections in general, and in local inflammatory conditions in the lungs. Nor have they been fully investigated in infants, who are known to have immature immune responses. Although CD16\text{low} and CD16\text{high} blood neutrophils have been observed during RSV infection in infants, it is unknown whether suppressive neutrophils develop and if the CD16\text{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).²²

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.

Materials and Methods

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® 368494, 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.

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 2ng/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.

**Flow cytometry**

Red blood cells were lysed in ice-cold erylisis buffer (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) immediately after collection, centrifuged for 5 min at 400g 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/CD45pos/SSChigh/CD16pos cells (Supplemental fig. 1). The sorted cells were centrifuged to a slide in the Shandon cytoospin 3 (Thermo Scientific) and stained with Diff-Quik® stain (Medi- on Diagnostics). Flow cytometry results were analyzed using FlowJo™ software (FlowJo LLC). The BAL cells were handled in the same manner described above.

**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.
Results

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, we investigated blood neutrophils after systemic LPS administration in healthy adults (Fig. 1, top row). Indeed, a prominent suppressive (CD16<sup>high</sup>CD62L<sub>low</sub>) as well as immature (CD16<sub>low</sub>CD62L<sup>high</sup>) neutrophil subset response were observed 8 h after LPS administration, confirming the results by Pillay et al. As the occurrence of neutrophil subsets based on CD16/CD62L expression has not yet been determined in infants, we investigated blood samples from several infants. First, similar to the baseline observation in healthy adults, healthy infants had only one (mature, CD16<sup>high</sup>CD62L<sup>high</sup>) neutrophil population in their peripheral blood (Supplemental fig. 2). Second, besides mature neutrophils, also immature neutrophils were observed in an infant with respiratory failure after surgery (Supplemental fig. 2). Despite being on mechanical ventilation for 3 days this infant did not develop the suppressive neutrophil subset, suggesting that mechanical ventilation did not interfere with neutrophil subset development. Third, we determined whether infants with bacterial sepsis were able to develop neutrophil subsets, similar to adults with LPS challenge. Two infants admitted to our PICU with bacterial sepsis showed presence of high numbers of suppressive neutrophils (Fig. 1, middle row), indicating that the immune system of these young children is capable of producing suppressive neutrophils.

Our next aim was to detect neutrophil subsets, and in particular suppressive neutrophils, during severe viral respiratory infections in infants. During the course of disease most blood neutrophils were mature based on CD16<sup>high</sup>CD62L<sup>high</sup> expression (Fig. 2A). We could detect immature neutrophils (CD16<sub>low</sub>CD62L<sup>high</sup>) in the blood of patients in both the virus-only and virus + bacterial co-infection groups, which peaked right after admission, but rapidly declined after day 1 (Fig. 2B). Interestingly, there were low numbers of suppressive neutrophils in the blood of patients in the virus-only group throughout the study period (Fig. 1, lower row, and fig. 2C). Only viral-infected patients with a bacterial co-infection showed marked elevation of suppressive neutrophils arising several days after admission (Fig. 2C, day 3: P = 0.03). In blood of infants with severe viral respiratory infection with and without bacterial co-infection we detected a fourth neutrophil subset, characterized by CD16<sub>low</sub> and CD62L<sub>low</sub> expression during the period of admission (Fig. 1, lower row). Interestingly, substantial expansion of this subset occurred after a prolonged disease course with maximal numbers between day 3 and 6 (Fig. 2D). Cell sorting and visual inspection of this subset revealed many myelocytes and metamyelocytes (Fig. 3), therefore this subset was termed ‘progenitor’ neutrophils. The consistent increase in the number of progenitor neutrophils during admission was statisti-
cally significant ($P < 0.001$) and was independent of the presence of a bacterial co-infection. In this relatively small patient cohort no association between disease severity (e.g. duration of mechanical ventilation) and neutrophil subset profile was observed (data not shown).

Figure 1: Blood neutrophil subsets develop after LPS, bacterial infection and viral infection.

Flow cytometry plots of CD16 and CD62L expression on blood neutrophils defining mature neutrophils (CD16$^{\text{high}}$CD62L$^{\text{high}}$), the immature subset (CD16$^{\text{low}}$CD62L$^{\text{high}}$, light grey box), the suppressive subset (CD16$^{\text{high}}$CD62L$^{\text{low}}$, dark grey box) and the progenitor subset (CD16$^{\text{low}}$CD62L$^{\text{low}}$, black box) in adults before and after LPS administration (top row panels), and in infants with bacterial sepsis (middle row panels) or severe viral respiratory infection (virus-only group, low row panels). All image plots on one row are obtained from a single patient and found representative for the group.
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Figure 2: Neutrophil subset formation in blood.

Mean (± SEM) percentage of the mature (A), immature (B), suppressive (C) and progenitor (D) neutrophils that are released during severe viral respiratory infection without bacterial co-infection (white circles) and with bacterial co-infection (black squares) in infants up to 6 days after admission. The number of patients on each time point is depicted below the graph. Mann-Whitney U test *: P = 0.03.

Figure 3: Morphological characterization of progenitor neutrophils.

Representative Diff-Quik® stained cytospin images of flow cytometry assisted cell sorted progenitor neutrophils (CD16<sub>low</sub>CD62L<sub>low</sub>, red squares) and mature neutrophils (CD16<sup>high</sup>CD62L<sup>high</sup>, green squares). The progenitor population consists of myelocytes and metamyelocytes whereas the mature population consists of lobulated mature neutrophils. The cytospin neutrophils are digitally copy-pasted to fit the square.
**Blood neutrophil subsets are differentially activated.**

In order to determine the activation state of the heterogeneous neutrophil subsets in blood of infants with severe viral respiratory infection we evaluated the expression of several activation and degranulation markers (Fig. 4A,B). There were no differences in expression between the subsets from the virus-only group and the virus with bacterial co-infection group, indicating that these subsets show a similar phenotype regardless of their trigger (Fig. 4A,B). Compared to mature neutrophils the expression of the activation marker CD11b was elevated in suppressive neutrophils (Fig. 4B, $P = 0.005$). In addition, this subset showed the highest expression of CD63 on their surface, indicative of active degranulation (Fig. 4B, $P = 0.005$). The progenitor neutrophils exhibited an activated phenotype with high expression of CD63 and CD66b (Fig. 4A,B). Yet, compared to mature cells we noticed a lower expression of markers involved in neutrophil migration and extravasation. By subset definition they present with lower expression of CD62L (Fig. 3), but CD54 was also decreased on the surface of these cells (Fig. 4A,B, $P = 0.02$ for both groups).

![Figure 4: Neutrophil subset activation.](image)

The relative expression of four markers (CD11b, CD54, CD63, CD66b) on the surface of blood neutrophil subsets as compared to the expression of mature neutrophils (expression = 1) in infants with severe viral respiratory infection (A, $N = 8$) or severe viral respiratory infection with bacterial co-infection (B, $N = 11$) the first time point of each patient was taken. Suppressive neutrophils show an activated phenotype with elevated expression of CD11b and CD63. Progenitor neutrophils express high levels over CD63 and CD66b, and low levels of CD11b and CD54. Data are displayed as mean ± SEM, Wilcoxon signed-rank test *: $P < 0.05$, **: $P < 0.01$.

**Neutrophil subsets can be detected in the lungs of infants with severe viral respiratory infection.**

Thus far, heterogeneous subsets have only been detected in neutrophils in the circulation. It is unknown whether neutrophils subsets also occur in relevant target organs, for example in the lung during viral respiratory infections. In order to determine the presence of neutrophils subsets in the lungs we were able to collect BAL from a subgroup of the patients ($N = 8$) and analyzed these samples using flow cytometry. Importantly, gating the neutrophil subsets in BAL was found to be hampered by the overt loss of CD62L in BAL neutrophils, which is likely caused by partial shedding of CD62L during the process of extravasation and migration into...
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Indeed, we observed a much lower mean fluorescent intensity CD62L expression in BAL neutrophils as compared to blood neutrophils (Fig. 5A, BAL mean ± SD: 1259 ± 943, versus blood 9848 ± 4224, \( P < 0.001 \)). Despite this drawback, by far the largest part of neutrophils in BAL appeared to be mature neutrophils, which was true throughout admission up to day 6 (Fig. 5D). In addition, smaller populations of immature and occasionally suppressive neutrophils could be detected (Fig. 5B,C). Suppressive neutrophils appeared during admission, and peaked on day 6 (Fig. 5D, showing the combined data from both groups). However, similar to the data from the blood samples only patients in the virus + bacterial co-infection group had a suppressive neutrophil subset of > 5% (representative example shown in fig. 5C). In contrast to the findings in neutrophils in the circulation, none of the BAL samples contained detectable progenitor neutrophils (Fig. 5B-D), which is in line with our observation of the relatively low expression of extravasation markers on blood progenitor neutrophils.

Figure 5: Neutrophil subsets in the lungs.

(A) Mean fluorescent intensity (MFI) of CD62L expression on mature neutrophils in peripheral blood (black bar) and BAL (white bar), showing substantial reduction in CD62L expression on BAL mature neutrophils (Mann-Whitney U test: \( P < 0.0001 \)). (B,C) Representative flow cytometry plots of CD16 and CD62L expression on BAL neutrophils defining mature neutrophils (CD16\text{high}CD62L\text{high}), the immature subset (CD16\text{low}CD62L\text{high}, light grey box), the suppressive subset (CD16\text{low}CD62L\text{low}, dark grey box) and the progenitor subset (CD16\text{low}CD62L\text{low}, black box) in an infant with severe viral respiratory infection (left panel, virus-only, day 0) or severe viral infection with bacterial co-infection (right panel, day 6) during admission. The progenitor subset is absent in all patients. The gates are set based on the mature neutrophil subset in individual patients. (D) Mean (± SEM) percentage of the neutrophil subsets that are released during severe respiratory infection in infants (\( N = 8 \)) up to 6 days after admission. The number of patients on each time point is depicted below the graph.
Discussion

The goal of this study was to identify neutrophil subset responses in infants with severe viral respiratory infection, with and without bacterial co-infection. We found that both the circulating and local neutrophil response during severe lung inflammation in these patients is characterized by the occurrence of heterogeneous subsets with distinct activation profiles. Interestingly, there appears to be a relative paucity in the development of a suppressive neutrophil subset response in severe viral respiratory infection in infants, when compared to findings obtained from sepsis patients and patients with a bacterial co-infection. In addition, an activated progenitor neutrophil subset residing in the blood compartment was identified.

In this study, infants with severe viral respiratory infection developed relatively low numbers of suppressive blood neutrophils, as defined by CD16<sup>high</sup>CD62L<sup>low</sup> according to Pillay et al..<sup>12</sup> Similarly, scarce suppressive neutrophils were found in the lungs. The suppressive neutrophil subset has previously been found to play an immune modulatory role by suppressing T cell activity by release of hydrogen peroxide into the immunological synapse.<sup>12</sup> Interestingly, severe viral respiratory infection in infants, such as RSV infection, is characterized by low numbers of recruited lymphocytes to the lungs, suggesting immunosuppression.<sup>20</sup> Apparently, immunosuppression by suppressive neutrophils preceding the lymphocyte response is not responsible for this relatively paucity of lymphocytes. Whether suppressive neutrophils are important in dampening the local inflammatory response to avoid host tissue damage is currently unknown. Suppressive neutrophils have also been connected to the development of immune paralysis, rendering patients more susceptible to bacterial (co-)infection.<sup>16</sup> This may cause important problems in critically ill patients with severe inflammation due to bacterial sepsis.<sup>21</sup> In this light, the finding that infants in our study with the highest suppressive neutrophils numbers had a bacterial co-infection is interesting as these patients may be prone to immune paralysis.

The release of young neutrophils from the bone marrow either as a result of enduring inflammation or in response to a specific stimulus has been suggested to contribute to immune dysfunction.<sup>16</sup> Besides the immature neutrophil subset (CD16<sup>low</sup>CD62L<sup>high</sup>) we observed a fourth distinct subset of progenitor neutrophils in blood, which arose a few days after admission. These cells were identified by CD16<sup>low</sup>CD62L<sup>low</sup>. The occurrence of young neutrophils in the blood of children with severe viral respiratory infection has also been reported by Lukens et al..<sup>13</sup> They observed a CD16<sup>low</sup> subset in blood neutrophils of RSV-patients 2-5 days after hospital admission. We confirmed these findings in our cohort and showed that these young neutrophils can be divided into immature neutrophils (CD16<sup>low</sup>CD62L<sup>high</sup>) and progenitor neutrophils (CD16<sup>low</sup>CD62L<sup>low</sup>). The gradual increase in the numbers of progenitor neutrophils after admission was consistent in infants with severe viral respiratory infection, but
was also detected in patients with bacterial sepsis. Non-specific or iatrogenic inflammatory triggers in critically ill patients may contribute to the release of this subset. However, we included one post-operative patient on mechanical ventilation who did not develop progenitor or suppressive neutrophil subsets after 1 and 3 days of mechanical ventilation, suggesting that mechanical ventilation alone is not sufficient to initiate subset formation.

Degranulation markers CD63 and CD66b were high on progenitor neutrophils compared to immature and mature neutrophils in blood. Yet, the expression of extravasation markers CD62L (L-selectin) and CD54 (ICAM-1) was low on these cells. CD62L is an essential surface-receptor facilitating neutrophil migration and lack of CD62L leads to reduced neutrophil migration in mice.\textsuperscript{22,23} As progenitor cells lack high levels of CD62L is it possible that they are confined to the systemic compartment, at least until further maturation. Indeed, we could not detect progenitor cells in the BAL of our patients. Despite having higher CD63 expression, indicating increased degranulation, we could not determine whether progenitor cells are ‘reactive’ cells, causing damage within the bloodstream. Possibly, these young neutrophils are part of the heterogeneous group of granulocyte myeloid-derived suppressor cells (G-MDSCs). G-MDSCs consist of a broad group of granulocytic cells with immune inhibitory properties.\textsuperscript{24} It is tempting to speculate that these progenitor cells could function as suppressive cells in the systemic compartment preventing systemic immune damage and/or aiding in tissue repair, as they appear in the bloodstream days after the peak of the infection.

Although the detection of distinct neutrophil subset profiles in viral-infected patients with and without bacterial co-infection appears very interesting from a diagnostic perspective, a limitation of this study is the lack of functional tests to further address the roles of the different neutrophil phenotypes in innate immunity. In addition, this study was not designed to detect an association between subset profile and clinical outcome. While the suppressive functions of the CD16\textsuperscript{high}CD62L\textsuperscript{low} neutrophil subset have clearly been described before,\textsuperscript{12} future studies must address the functional properties of the newly described progenitor subset. A second limitation of this study is the use of CD62L as a subset defining marker. While blood neutrophils are easily separated with the use of this marker, BAL neutrophils shed CD62L during migration and activation, which may cause less well defined separation between mature and suppressive neutrophils. However, we found that the percentages and dynamics of the BAL suppressive neutrophils closely mimic the blood suppressive cells, suggesting our marker identification strategy was also usable for detecting BAL neutrophil subsets. Further study and identification of other steady markers is essential for in-depth analysis of BAL neutrophil subsets.
In conclusion, our results show that severe viral respiratory infection in infants is associated with the occurrence of four heterogeneous neutrophil subsets in blood characterized by different expression of CD16 and CD62L. These subsets consist of mature, immature, suppressive and progenitor neutrophils, each with distinct activation states. These neutrophil subsets, except the progenitor neutrophils, can also be detected locally in the lungs. While severe viral respiratory infection is characterized by the relatively absence of suppressive neutrophils in blood, our data suggests that only in patients with bacterial co-infection suppressive neutrophils are found in the circulation. This indicates distinct and unique neutrophil subset responses during severe viral respiratory infections with and without bacterial co-infection. Further studies are needed to determine the functional roles of these different neutrophil subsets, as well as their potency as diagnostic markers during (severe) viral respiratory infection in infants.

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Statement of contribution

BC, JW, AV and RB designed the study. BC and SI carried out the flow cytometry and analyzed the data. BC, LK, AV, JC, JW and RB interpreted the results. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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References

Supplemental figures

(A) Neutrophils were gated based on CD45<sup>pos</sup>SSC<sup>high</sup> (Top left panel), doublets were removed (second panel, FSC-H/FSC-W) and viable CD45<sup>pos</sup> cells were selected (Bottom left panel). Eosinophils were excluded based on CD16<sup>neg</sup> expression (Bottom second panel). (B) Broncho-alveolar lavage was analyzed in a similar way but eosinophils were absent.

Supplemental figure 2: Neutrophil subsets in healthy infants.

(A) Representative flow cytometry plots of CD16 and CD62L expression on blood neutrophils defining mature neutrophils (CD16<sup>high</sup>CD62L<sup>high</sup>), the immature subset (CD16<sup>low</sup>CD62L<sup>high</sup>, light grey box) in one post-operative patient on mechanical ventilation on day 1 and day 3, showing the formation of immature neutrophils and presence of mature neutrophils but absence of suppressive or progenitor neutrophils. (B) Two healthy infants (left and right panel) visiting the outpatient clinic for routine follow-up of non-infectious/respiratory disorders only show mature neutrophils in their peripheral blood.