

A Systemic Neutrophil Response Precedes Robust CD8⁺ T-Cell Activation during Natural Respiratory Syncytial Virus Infection in Infants[∇]

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Severe primary respiratory syncytial virus (RSV) infections are characterized by bronchiolitis accompanied by wheezing. Controversy exists as to whether infants suffer from virus-induced lung pathology or from excessive immune responses. Furthermore, detailed knowledge about the development of primary T-cell responses to viral infections in infants is lacking. We studied the dynamics of innate neutrophil and adaptive T-cell responses in peripheral blood in relation to the viral load and parameters of disease in infants admitted to the intensive care unit with severe RSV infection. Analysis of primary T-cell responses showed substantial CD8⁺ T-cell activation, which peaked during convalescence. A strong neutrophil response, characterized by mobilization of bone marrow-derived neutrophil precursors, preceded the peak in T-cell activation. The kinetics of this neutrophil response followed the peak of clinical symptoms and the viral load with a 2- to 3-day delay. From the sequence of events, we conclude that CD8⁺ T-cell responses, initiated during primary RSV infections, are unlikely to contribute to disease when it is most severe. The mobilization of precursor neutrophils might reflect the strong neutrophil influx into the airways, which is a characteristic feature during RSV infections and might be an integral pathogenic process in the disease.

Viral infections are characterized by a dynamic interplay between the pathogen and defensive innate and adaptive immune responses of the host (35, 38). Upon infection, virus-specific structural components are recognized by pattern recognition receptors of the host, which triggers a mechanism aimed at the suppression of virus replication and eventually virus elimination. Each virus has a characteristic signature of triggering innate immune receptors and methods to counteract immune responses of the host, which ultimately results in an immune response tailored to the particular properties of the infecting virus (6).

Most insights into the sequence of events occurring during viral infections have been obtained from animal experiments, where the immunological control of viral infections can be studied in detail. In many murine models, the crucial role of CD8⁺ T cells in complete elimination of the virus during acute infections has been well established (9, 20, 27). However, both virus-induced damage and immune pathology might contribute to the disease, depending on the type of viral infection and/or the intensity of the innate and adaptive immune responses triggered (10, 20, 37, 41, 49, 60).

Primary infections with respiratory syncytial virus (RSV) can

cause severe bronchiolitis and pneumonia in infants (24). For RSV, the mouse is not a good model to study primary disease because the virus replicates poorly in murine cells. Hence, to obtain insight into the mechanism of disease caused by RSV, infection studies in humans or nonhuman primate models are needed. We and others have shown that RSV infection causes a strong influx of neutrophils into the airways (15, 25, 48). In addition, we have recently shown that substantial virus-specific CD8⁺ T-cell responses can be elicited in infants with severe RSV infections (25). However, it is still a controversial issue whether the severe manifestations of lower respiratory tract disease are caused directly by the virus or by innate and/or adaptive immune responses triggered by RSV (8, 20, 31, 57). In our previous work, we found no relation between the severity of disease and the number of virus-specific CD8⁺ T cells in peripheral blood (25). Moreover, a direct role of the viral load or different viral strains in disease severity has not been established convincingly (11, 59).

Data on the development of primary T-cell responses in infants (<6 months old) during acute viral infections and after vaccinations are sparse. It is generally accepted that the infant immune system is immature and less effective than that of older children or adults. This has been shown by lower activation and/or Th2-polarized adaptive immune responses (1, 2, 58). For RSV-induced disease, it has been suggested that a Th2-biased immune response might be correlated with disease (39, 45, 50), but this idea has been challenged by others (4, 7, 12).

Currently, there is no RSV vaccine, and the only preventive

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treatment available is a humanized neutralizing antibody specific for the fusion protein of RSV that is administered to high-risk groups and is effective in about 60% of children (29). Immune-suppressive or antiviral treatments during severe RSV disease have marginal to no effect (3, 23, 55). Insights into the kinetics of the viral load and disease course in relation to activation of the innate and adaptive immune response will shed light on factors that are attributed to severe RSV-induced disease and will possibly provide leads for the development of curative treatment. We therefore monitored the dynamics of these parameters in infants admitted to the pediatric intensive care unit (ICU) with severe primary RSV infections. During primary RSV infection, the peak values of the viral load and disease severity were followed by the exhaustion of the peripheral blood neutrophil pool, indicating a strong innate immune response closely associated with the peak of disease. We further showed that this natural respiratory infection elicited a strong primary CD8⁺ T-cell response in the very young patients (<3 months). This T-cell response was undetectable at the moment of hospitalization, when the infants were severely ill, and peaked at convalescence. Therefore, severe primary RSV disease does not seem to be caused by inadequate or exaggerated T-cell responses but is most likely initiated by viral damage followed by intense innate immune processes.

MATERIALS AND METHODS

Study population, clinical characteristics, and sample collection. The study population consisted of 22 infants younger than 52 weeks of age admitted to the pediatric ICU of the Wilhelmina Children's Hospital during the winter seasons of 2007 to 2009. All patients required mechanical ventilation because of respiratory failure due to RSV lower respiratory tract infection (LRTI). Routine treatment consisted of supportive therapy (e.g., supplemental oxygen administration and fluid and electrolyte management) at the discretion of the attending clinicians. The patients were not treated with corticosteroids. Children with immune deficiencies were not included in the study. To measure the development of T-cell and neutrophil responses over time, 1 ml of blood was collected in EDTA tubes directly after written parental informed consent and every other day until discharge from the ICU. At the same time intervals, undiluted nasopharyngeal aspirates (NPA) and tracheal aspirates (TA) were collected for the determination of respiratory viruses. To measure the course of disease severity, the ventilation index was calculated as the product of the partial pressure of CO₂ (mm Hg) × peak airway pressure (cm H₂O) × respiratory rate (breaths per min) divided by 1,000 (5). In patients on the high-frequency oscillation ventilator (i.e., no respiratory rate available; *n* = 2) the oxygenation index was used, calculated as the inspired oxygen fraction × the mean airway pressure (cm H₂O) divided by the partial pressure of oxygen in arterial blood. The control group consisted of 7 healthy infants around 4 weeks of age without current or recent clinical symptoms of a respiratory infection. The parents of the patients and controls gave their informed written consent. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht, The Netherlands.

Respiratory virus detection and quantification. The presence of RSV and other respiratory viruses was determined by real-time (reverse transcriptase [RT]) PCR of NPA and TA. Nucleic acid was isolated as previously described (51, 52). Shortly thereafter, nucleic acids were extracted using the MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany) and eluted in 100 μl elution buffer. cDNA was synthesized by using MultiScribe RT and random hexamers (both from Applied Biosystems, Foster City, CA). Each 100-μl reaction mixture contained 40 μl of the eluted RNA. After incubation for 10 min at 25°C, reverse transcription was carried out for 30 min at 48°C, followed by RT inactivation for 5 min at 95°C.

Detection of respiratory viruses was performed in parallel, using real-time PCR assays specific for RSV A and B; influenza viruses A and B; parainfluenza viruses 1 to 4; rhinoviruses; adenoviruses; human corona viruses OC43, NL63, and 229E; human metapneumovirus; and human bocavirus. Real-time PCR procedures were performed as previously described (51, 52). Briefly, samples were assayed in a 50-μl reaction mixture containing 20 μl of cDNA, 25 μl of 2× TaqMan universal PCR master mix (PE Applied Biosystems, Foster City, CA),

and 200 to 900 nM concentrations of forward and reverse primers and 60 to 200 nM concentrations of each of the probes. Amplification and detection were performed with an ABI Prism 7900 sequence detection system. Efficient extraction and amplification were monitored through the internal control viruses (RNA virus, murine encephalomyocarditis virus, DNA virus, and phocine herpesvirus) (53). The real-time PCR results were expressed as semiquantitative threshold cycle (*C_T*) values. For RSV, *C_T* values were converted to the number of particles/ml using standardization curves generated with electron microscopy (EM)-counted stocks. The number of infectious RSV particles present in fresh NPA was determined by an end point dilution assay. HEp-2 cells grown in 96-well plates were inoculated with serial dilutions of the NPA in culture medium. RSV quantitative standards were run in parallel with each assay. For 10 days, the cultures were observed daily for cytopathological effects. The 50% tissue culture infectious doses (TCID₅₀) were determined using the Spearman-Kärber relationship (26).

Phenotyping of immune cells. Whole blood was stained with different extracellular monoclonal antibodies (MAbs) for 30 min on ice. The following MAbs were used to phenotype CD8⁺ T-cell populations; fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (CLB-T8/4 and 4H8), anti-CD45RO (UCHL1), and anti-CCR5 (2D7); phycoerythrin (PE)-conjugated anti-CD3 (SK7), anti-CD8 (SK1), anti-CD127 (R34.34), and anti-CCR7 (3D12); peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 (SK1); and allophycocyanin (APC)-conjugated anti-CD3 (SK7) and anti-CD27 (L128), all purchased from BD Biosciences (San Jose, CA), except CD8-FITC (Sanquin, Amsterdam, The Netherlands) and CD127-PE (Immunotech, Marseille, France). For intracellular staining, cells were permeabilized and fixed using fluorescence-activated cell sorter (FACS) permeabilizing/fixation solution (Perm/Fix; BD Biosciences). Cells were stained intracellularly with FITC-conjugated anti-Ki-67 (B56), anti-perforin (δG9), Alexa 647-conjugated anti-granzyme B (GzmB) (GB11), or their isotype controls (BD Biosciences).

The following MAbs were used for the staining of neutrophils and eosinophils: PE-conjugated anti-CD49d (9F10) and Alexa 647-conjugated anti-CD16 (3G8), purchased from BD Biosciences. After cell surface staining, erythrocytes were lysed using lysis buffer containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂-EDTA for 15 min. The cells were washed twice in FACS buffer and analyzed based on selection by forward and side scatter (FSC/SSC) and marker expression patterns with a FACSCalibur flow cytometer and CellQuest software or sorted on a FACS Aria (BD Biosciences).

Statistical analysis. Data were normalized so that a value of 100% was assigned to the largest number in a patient data set and 0% to the smallest number in the same data set (GraphPad 4.0; Prism). The relationship between positive bacterial cultures (yes/no) and the neutrophil response (maximum percentage of CD16^{int} CD49d^{neg} neutrophil precursors [see below] within the total granulocyte gate) was calculated using the Mann-Whitney U test. The Pearson's correlation coefficients were calculated for (i) peak viral load (trachea), (ii) peak percentage of CD16^{int} neutrophils, and (iii) peak percentage of CCR5⁺ CCR7⁻ CD8⁺ T cells with (a) length of stay (LoS) in the ICU and (b) peak ventilation index on days 0 to 2 (SPSS 15.0 for Windows).

RESULTS

Study setup and clinical characteristics of patients. Previously, we demonstrated that during severe primary RSV infections, virus-specific T-cell responses could be detected in the peripheral blood and bronchial alveolar lavage fluids of infants with severe RSV bronchiolitis (25). We initiated the present study to elaborate on our previous work and to gain more insight into virus clearance, disease severity, and immune responses by studying the kinetics of neutrophil and developing T-cell responses in relation to the viral load and parameters of disease severity.

Twenty-two patients <52 weeks of age were included in the study. They all had severe RSV bronchiolitis and required mechanical ventilation due to respiratory failure (Table 1 lists the demographics). During the study, five patients were excluded because they developed signs and symptoms of a superimposed bacterial infection during their stay at the ICU and they had a repeat bacterial culture taken >2 days after admis-

TABLE 1. Clinical characteristics of patients and healthy controls

Characteristic	Value	
	RSV patients (n = 17)	Healthy controls (n = 7)
Male/female (no.)	11/6	3/4
Age (wks) [mean (SEM)]	7.2 (1.2)	5.6 (0.4)
Premature birth at <36 wks [no. (%)]	3 (18)	0 (0)
Symptom onset ^c (days) [mean (SEM)]	5.4 (0.7)	NA ^e
Viral culture positive for RSV ^d [no. (%)]	11 (65)	NA
Viral coinfection [no. (%)]	5 (31) ^a	NA
Bacterial culture positive at ≤2 days [no. (%)]	12 (71) ^b	NA
Duration of ventilation (days) [mean (SEM)]	10.4 (0.6)	NA
Length of stay (days) [mean (SEM)]	11.3 (0.6)	NA

^a Rhinovirus (n = 5) and coronavirus (n = 1).

^b *Haemophilus influenzae* (n = 7), *Staphylococcus aureus* (n = 5), and *Moraxella catarrhalis* (n = 4) were most commonly found.

^c Before admittance to the ICU.

^d All patients were positive by RSV PCR.

^e NA, not applicable.

sion that was positive. Of the 17 patients who were included in the final analysis, 16 were <3 months of age, and 1 child was 5 months old. Peripheral blood samples, NPA, and TA were taken at the same time intervals. Upon study inclusion, the onset of symptoms (rhinitis, cough, fever, wheezing, apnea, dyspnea, vomiting, and diarrhea) reported by parents, gestational age at birth, and birth weight were recorded. On average, the RSV patients were symptomatic for 5.4 (standard error of the mean [SEM], 0.7) days before admission to the ICU. The severity of illness was assessed using (i) LoS in the ICU (days) and (ii) the parameters of mechanical ventilation (Fig. 1). Since the exact moment of infection was unknown, the day of first clinical symptoms of a respiratory infection as reported by the parents was regarded as day zero. The disease was most severe in the first 2 to 3 days on the ICU in all patients.

Kinetics of the RSV load. Viral RNA loads were measured in NPA and TA by real-time PCR and quantitative viral culture. The viral-RNA load was maximal in the first samples in both NPA and TA (Fig. 2A and B) and decreased during the

hospital stay but could still be detected in most patients in the NPA at the time of discharge (around day 17 after onset of symptoms) (Fig. 2A). Interestingly, RSV RNA was cleared more rapidly in the lower than in the upper airways (Fig. 2B versus A). In only 65% of the patients we were able to detect infectious virus particles by viral culture, and virus could never be cultured beyond day 10 after the onset of symptoms. This was well before discharge from the ICU in the majority of patients (data not shown).

Recruitment of neutrophil precursors into the blood during RSV infection. RSV infection is characterized by a massive influx of mainly neutrophils into the airways compared to control patients undergoing surgery for non-respiratory tract-related disease (15, 25, 48). Neutrophils were phenotyped in order to monitor the systemic effects involved in neutrophil recruitment into the airways of RSV patients. Neutrophils were identified based on FSC/SSC patterns and staining with anti-FcγRIII (CD16) and VLA-4 (CD49d) (CD16^{hi} CD49d^{neg}) and could easily be separated from eosinophils (CD16^{neg} CD49d^{hi}) (Fig. 3A). Interestingly, during the course of RSV infection, we observed the appearance of a population of neutrophils with intermediate CD16 expression (Fig. 3B). Banded neutrophils or their precursors recruited from the bone marrow into the blood by granulocyte colony-stimulating factor (G-CSF) or inflammatory conditions in general have lower expression of CD16 (28, 36). To confirm that this was also the case during RSV infections and was not due to prolonged stimulation, we sorted granulocytes into three different populations based on negative (CD16^{neg}), intermediate (CD16^{int}), and high (CD16^{hi}) CD16 expression (56). Cytospins of these sorted cell populations were performed, and the presence of eosinophils (CD16^{neg}) and mature/activated neutrophils (CD16^{hi}) was identified by May-Günwald Giemsa staining (Fig. 3C). The population of cells characterized by CD16^{int} was a heterogeneous population of neutrophil precursors consisting of myelocytes, metamyelocytes, and banded neutrophils, which are neutrophil precursors normally residing in the bone marrow (Fig. 3C) (13, 14). The same cell types were also observed in direct blood films from RSV patients (data not shown).

We quantified the percentage of CD16^{int} CD49d^{neg} neutrophil precursors within the total granulocyte gate (neutrophils and eosinophils) over time during the stay at the ICU. We found very low numbers of neutrophil precursors in the blood of RSV patients at admission to the ICU, but they appeared a

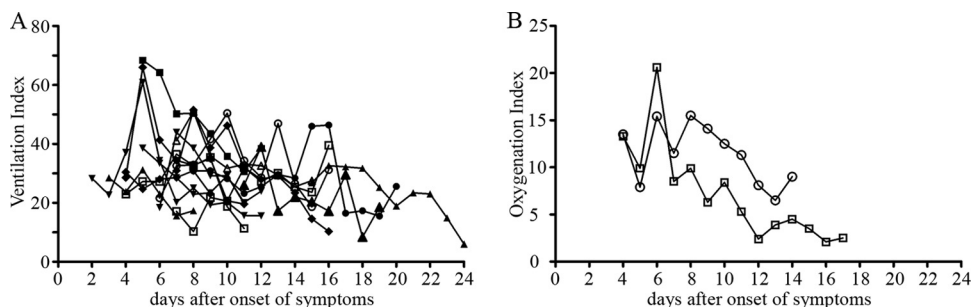


FIG. 1. Kinetics of the parameters of mechanical ventilation. Day 0 denotes the first reported symptomatic day. Shown is the disease severity of RSV patients, based on the ventilation index (VI) (A) or the oxygenation index (OI) (B) determined during the stay at the ICU. Each line and symbol (open or closed) represents the VI (A) or OI (B) in time in a single patient.

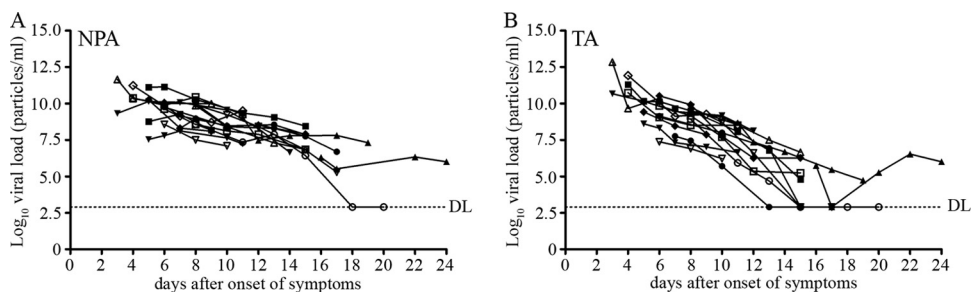


FIG. 2. Decrease in the viral load. The RSV load was determined by PCR in NPA (A) or TA (B) during the stay in the ICU and plotted as \log_{10} viral particles/ml. Each line (and symbol) represents the decreasing viral load over time in a single patient. The lower limit of detection of the PCR was 2.9 \log_{10} viral particles/ml RSV.

few days after admission to the ICU (Fig. 4A). The main increase in neutrophil precursors for all of the RSV patients was between days 7 and 9 after the onset of symptoms (Fig. 4B) and subsided a few days later.

To determine whether nasopharyngeal-carrier status was related to the neutrophil response, the percentages of $CD16^{int} CD49d^{neg}$ neutrophil precursors within the total granulocyte gate were compared for patients with a positive bacterial culture and patients with a negative bacterial culture. The neutrophil responses were found to be similar in the two groups (mean percentage, 35% in both groups; $P =$ not significant).

Identification of activated $CD8^+$ T cells in peripheral blood. Because in infants younger than 6 months of age the majority of T cells are still naïve, the presence of an RSV-specific T-cell response could be readily identified by a large set of phenotypic markers that are known to be expressed on activated and/or memory $CD8^+$ T cells (25). To monitor $CD8^+$ T-cell kinetics, we stained whole-blood samples with different combinations of activation markers. First, we used the combination of antibodies recognizing the chemokine receptors CCR5 and CCR7. CCR7 is expressed on naïve and central memory T cells, but not on effector T cells. CCR7 is the receptor for the

ligands CCL19 and CCL21, which are mainly expressed in lymphoid organs (16). The downregulation of CCR7 allows T cells to leave lymphoid organs, while upregulation of CCR5 directs the migration to inflamed sites, where the ligand RANTES (CCL5) is expressed, as in the RSV-infected lung (18, 46). Figure 5A shows an example of the presence of activated $CCR7^- CCR5^+$ effector $CD8^+$ T cells (35.1% of $CD8^+$ T cells) in a patient during severe RSV bronchiolitis compared with a typical example of a healthy age-matched control child (0.9% $CD8^+$ T cells).

A second way to identify effector $CD8^+$ T cells in this young age group is the combination of CD127 and CD45RO staining. CD127 is the receptor for interleukin 7 (IL-7) that is expressed on naïve and memory T cells and is required for homeostatic proliferation (33). The IL-7 receptor is downregulated on effector T cells that are also identified by upregulated expression of CD45RO. Figure 5A shows that the combination of these markers identifies a similar fraction of effector T cells in the RSV patient that is enhanced compared to the control (29.4% versus 4.5%, respectively, of $CD8^+$ T cells). The combination staining of GzMB and perforin identifies $CD8^+$ T cells with lytic potential, while Ki-67 stains proliferating or recently di-

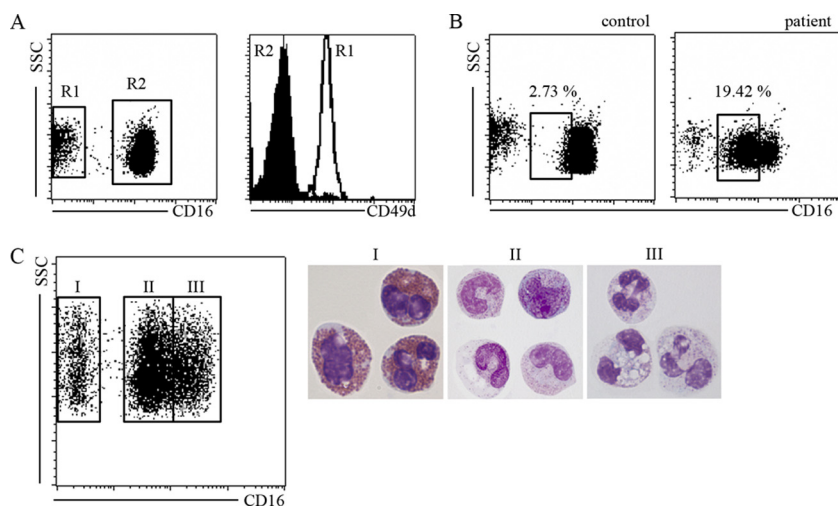


FIG. 3. Identification of blood neutrophils. (A) Whole blood of a healthy control was stained with anti-CD16 and anti-CD49d. Granulocytes were identified based on FSC/SSC patterns and CD16 staining (left), and neutrophils were identified based on low expression of CD49d, while eosinophils had high expression of CD49d (right). (B) Expression pattern of CD16 on granulocytes from a healthy control (left) or an RSV patient (right). (C) (Left) Gating strategies (FACS plot) for sorting of eosinophils and neutrophils of an RSV patient; (right) cytopsin with May-Günwald Giemsa staining of sorted eosinophils, neutrophil precursors, and mature neutrophils.

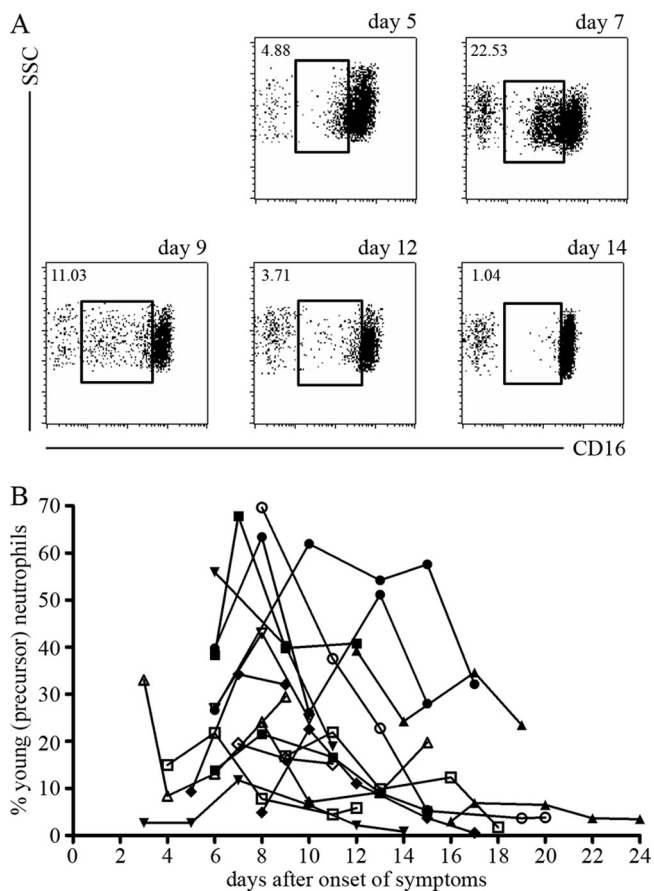


FIG. 4. Influx of neutrophil precursors into peripheral blood. (A) Whole blood of an RSV patient was stained with anti-CD16, and the percentage of precursor neutrophils within the granulocyte gate was enumerated on the indicated days after the onset of symptoms. (B) Kinetics of neutrophil precursor (CD16^{int} expression) recruitment into the blood, where day 0 denotes the first reported symptomatic day. Each line and symbol (open or closed) represents the dynamic influx of precursor neutrophils in peripheral blood over time in one individual RSV patient.

vided cells (19, 34, 43). Figure 5A shows that in the same patient, these combinations of markers also identified a similar fraction of CD8⁺ T cells, GzmB-perforin (31.6% versus 0.2% in the control), or GzmB/Ki67 (31.2% versus 0.9% in the control). In previous work, we showed that these markers were all coexpressed on recently activated cells that were in large part also virus specific (reference 25 and unpublished results). Figure 5B and C show examples of the developing CD8⁺ T-cell response over time in a second RSV patient using the combination staining for CCR7 and CCR5 (Fig. 5B) and GzmB-perforin (Fig. 5C). These data illustrate that the kinetics of the CD8⁺ T-cell response can be visualized by both combinations of markers.

The identification of CCR5⁺ CCR7⁻ and GzmB⁺ perforin⁺ effector CD8⁺ T cells in peripheral blood allowed us to track the kinetics of the CD8⁺ T-cell response in patients during the hospital stay. In Fig. 5D, the kinetics of the CD8⁺ T-cell response are summarized for the patient group, based on expression of CCR5⁺ CCR7⁻. The data represent the percentages of effector CD8⁺ T cells found on the depicted days,

where day zero was the day of the first clinical symptoms. While the peak levels of the T-cell response varied significantly between patients (from 5% to 35%), the kinetics of the response were similar in most patients, peaking between days 11 and 15 after the onset of symptoms (Fig. 5D). In one patient, the peak of the T-cell response was found 22 days after the onset of symptoms. This child had symptoms of the common cold for 2 weeks before admission to the hospital. Although we did not detect a different virus in this patient, it remains possible that a different viral infection preceded the RSV infection.

On admission, when the viral load and disease severity peaked, the percentage of effector CD8⁺ T cells was extremely low and comparable to the percentages observed in the seven healthy control children (1.5%; SEM, 0.2). After extensive expansion, the number of effector CD8⁺ T cells in the blood of RSV patients dropped from peak levels by the time of discharge. The kinetics and peak values in the effector CD8⁺ T-cell response as measured by chemokine markers (CCR5⁺ CCR7⁻ CD8⁺ T cells) were very similar to observations made with GzmB⁺ perforin⁺ stained CD8⁺ T cells (data not shown).

Combined kinetics of viral and immune parameters during RSV infection. The kinetics of the neutrophil precursors and activation of CD8⁺ T cells combined with the quantitative data on the viral load and disease severity were normalized and grouped for every 2 days to eliminate variations in the heights of responses and the time point of sampling between the patients. The period between the first signs of infection, disease manifestation, and subsequent admission to the ICU was variable between patients due to so far unknown parameters (range, 2 to 14 days; average, 5.4 days). The viral load was shown to be maximal at or before admission to the ICU, and disease severity was highest around days 2 and 3 after admission to the ICU (Fig. 6A and B). While the disease severity and viral load were declining, there was an influx of neutrophil precursors in the blood, followed by the activation of CD8⁺ T cells (Fig. 6C and D). Upon discharge from the ICU, RSV RNA was still detectable in the nose, the percentage of neutrophil precursors dropped to levels seen in healthy controls, and the level of CD8⁺ T-cell activation also declined but remained higher than in healthy controls. Together, these data showed a sequence of events whereby (i) the viral load peaked before or upon hospital admission, when the disease was most severe, and (ii) neutrophil precursors peaked in the blood around days 7 to 9 after the onset of symptoms, while (iii) CD8⁺ T-cell responses peaked in the peripheral blood toward the end of the ICU stay, around days 11 to 15 after the onset of symptoms.

We did not observe a correlation between the immune response activation (neutrophil precursors and effector T cells), the viral load, and the ventilation index. However, we observed a weak correlation between the height of the T-cell peak and the length of stay. This observation should be confirmed in a larger group of patients in the future.

DISCUSSION

We explored the dynamics of human neutrophil and CD8⁺ T-cell responses during acute severe RSV-induced bronchiolitis in infants younger than 6 months of age. To our knowledge,

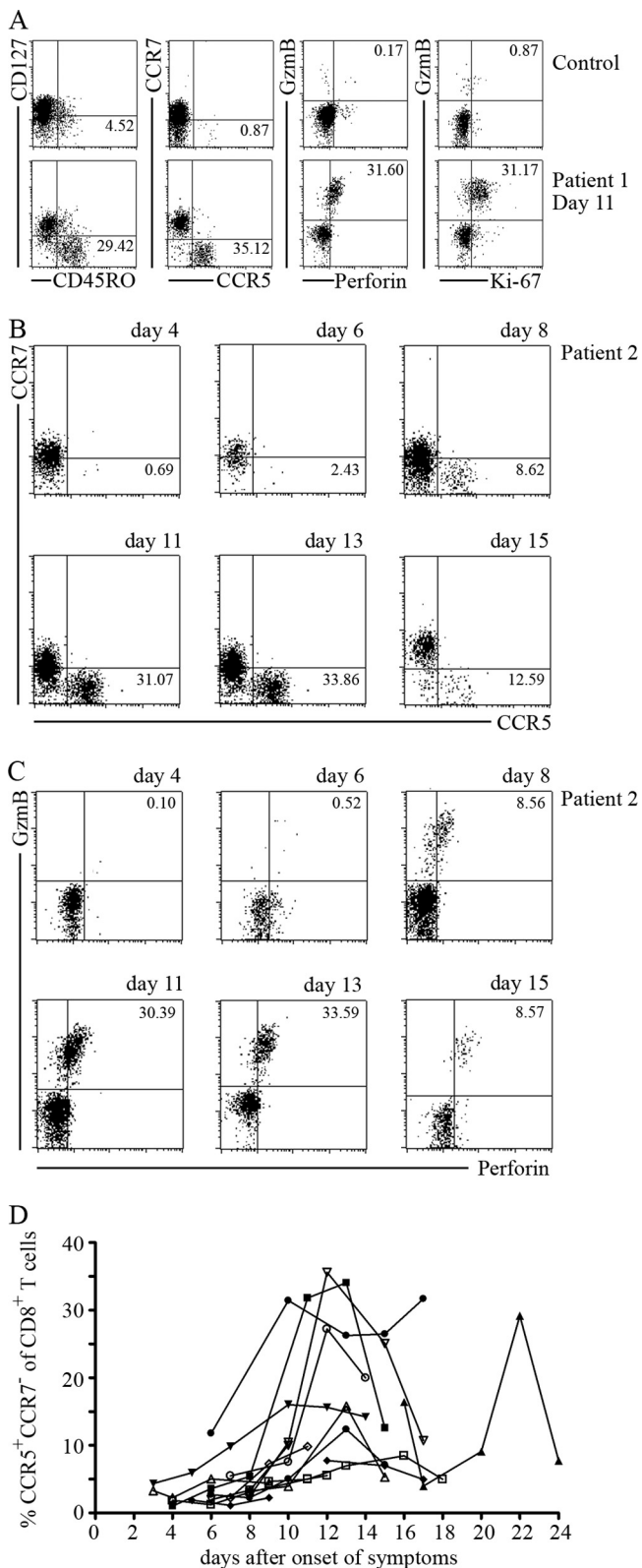


FIG. 5. Identification of activated CD8⁺ T cells in blood. (A) Whole blood of a healthy control infant (top) and a 12-week-old RSV patient (bottom) drawn on day 11 after the onset of symptoms was surface stained with anti-CD3, -CD8, -CD45RO, -CD127, -CCR5, or -CCR7 or stained intracellularly with anti-GzmB, -perforin, and -Ki-67. CD8⁺ T cells were identified based on FSC/SSC lymphocyte

this is the first longitudinal analysis correlating the parameters of innate and adaptive immune responses with the viral load and the parameters of disease severity during a natural primary viral infection in this age group. After admission to the ICU, both the viral load (as determined by real-time PCR) and, 2 to 3 days later, disease severity declined, indicative of the fact that these children were admitted to the ICU with peak values of viral load and disease severity. The decline in the viral load in children hospitalized with RSV disease has been described before by other groups (22, 54). However, these studies used quantitative viral culture or evaluated the viral load for a short period after admission (<4 days). We found infectious RSV by viral culture in only 65% of the patients, whereas all patients were RSV positive by real-time PCR. With real-time PCR we could still demonstrate RSV RNA in the nasal area in nearly all patients at discharge, although in smaller quantities than at admission (Fig. 2B and C). The observed presence of non-infectious virus might indicate that the virus was neutralized by antibodies. Interestingly, although serum antibodies were low in peripheral blood after RSV infection in the youngest infants (<6 months), antibody responses in nasal secretions were fast and were similar in magnitude and quality in infants and older children (30). It has recently been shown that during RSV LRTI, B lymphocytes and plasma cells are recruited to the lungs of infants at the peak of RSV illness (44). This mucosal B-cell activation most likely occurs in a T-cell-independent way, because CD4⁺ T cells were not found in the lung tissue at the time B cells were abundantly present (44), consistent with our observation that CD4⁺ T-cell responses were hardly detectable in our patients and appeared late, i.e., at the time of recovery (data not shown). Interestingly, the RSV RNA load declined more rapidly in the lower airways than in the nasal areas. Because serum IgG antibodies are somewhat more efficient in accessing the lower respiratory tract, it is possible that more efficient clearance in the lower airways might indicate a role for IgG (47).

Primary RSV infection is characterized by a strong influx of neutrophils into the airways (15, 25, 48). Furthermore, increased numbers of neutrophil precursors in peripheral blood are a characteristic feature of severe inflammatory conditions (28, 42). Therefore, the recruitment of bone marrow-derived

gating and then on the expression of CD3⁺ CD8⁺. (B and C) Example of developing CD8⁺ T-cell response over time in a second (2-week-old) RSV patient. Whole blood was drawn at admission and every other day until discharge. The blood was surface stained with anti-CD3, -CD8, -CCR5, or -CCR7 (B) or stained with anti-CD3 or -CD8 and intracellularly with anti-GzmB and -perforin (C). The percentages of activated CD8⁺ T cells are given in the respective quadrants. The experiments shown are representative examples of 13 individual RSV patients and 7 healthy controls. In all patients and controls, similar patterns of activation markers and chemokine receptor expression were observed; however, the levels of activation differed between patients. (D) Summary of the kinetics of CD8⁺ T-cell activation measured in whole blood of 13 patients during the stay at the ICU based on the expression patterns of CCR5 and CCR7 on CD3⁺ CD8⁺ lymphocytes. Due to technical reasons, we could measure T-cell activation in only 13 out of the 17 patients. Each line and symbol (open or closed) represents the CD8⁺ T-cell kinetics in the blood of one individual RSV patient.

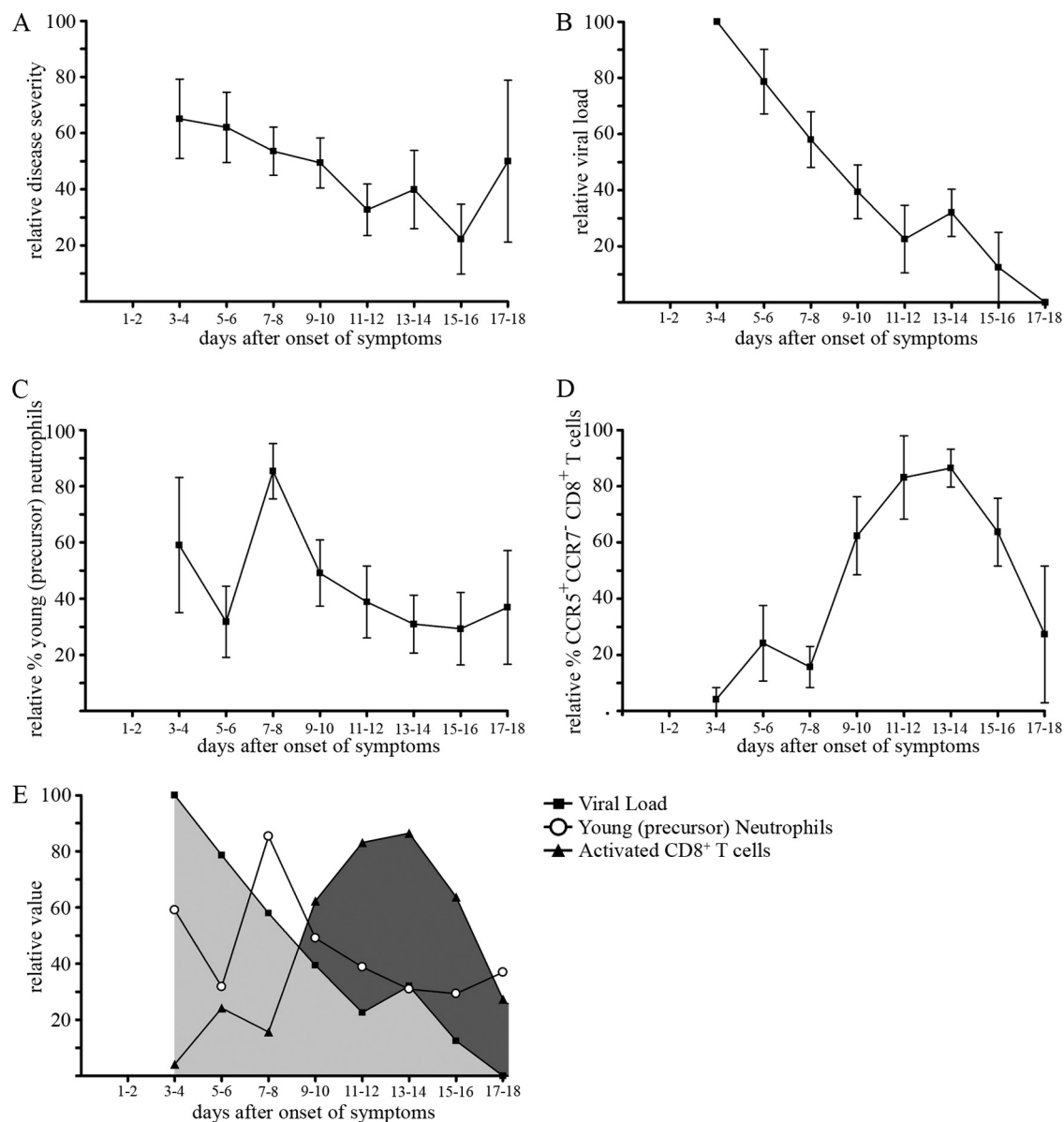


FIG. 6. Dynamics of disease parameters during primary RSV infection in infants. (A and B) Kinetics of normalized and grouped data of disease severity (A) and viral load (B) in TA during the stay at the ICU. (C and D) Normalized values of precursor neutrophil percentages (C) and activated CCR5⁺ CCR7⁻ CD8⁺ T-cell recruitment into peripheral blood (D). The error bars represent the SEM. (E) Combined dynamics of viral load, neutrophil precursor recruitment, and CD8⁺ T-cell activation.

neutrophil progenitors indicated a dramatic involvement of neutrophils during RSV disease, and the RSV infection presumably exhausted the banded-neutrophil pool in the peripheral blood (Fig. 4B). Mechanical ventilation can induce a local and systemic inflammatory response and enhanced neutrophil activation and recruitment to the lungs (17). However, in non-ventilated children with severe RSV disease a strong neutrophil response has also been observed, demonstrating that marked involvement of neutrophils is a characteristic feature of RSV infection (57). Nevertheless, it is possible that during the first days of respiratory support ventilation-induced effects might enhance neutrophil responses and even delay recovery.

T-cell activation in peripheral blood was quantified based on

a set of antibodies directed against well-known activation-associated surface markers and cytotoxic proteins: CD27, CD45RA, CD45RO, CD127, CCR5, CCR7, perforin, Ki-67, and GzmB. Based on these parameters, we showed that, despite the young age of the patients, a robust CD8⁺ T-cell response developed during primary RSV infection. In all patients, CD8⁺ T-cell responses peaked between 11 and 15 days after the onset of the first symptoms. The phenotype of the CD8⁺ T cells showed that they were actively dividing cells (Ki67 expression) (19). They acquired the ability to migrate to a peripheral site of inflammation, because we observed the reciprocal downregulation of CCR7, a lymph node homing receptor, and the upregulation of CCR5, a chemokine receptor

involved in the migration of T cells to inflamed tissues. Moreover, the presence of components of the lytic machinery, GzmB and perforin, showed their functional potential (43).

Recently, Miller et al. described the longitudinal analysis of CD8⁺ effector and memory T-cell responses to live smallpox (Dryvax) and yellow fever virus (YFV-17D) vaccines in adult volunteers (40). These vaccines are attenuated live-virus vaccines that cause acute infections. Based on phenotypic analysis (CD38⁺ HLA-DR⁺ Bcl-2^{low}, and Ki-67⁺) and HLA tetramer staining, peak CD8⁺ T-cell effector responses were found at day 15 after vaccination and declined sharply by day 30. YFV was detected in serum by PCR, and the viral load peaked on day 7 after vaccination and became undetectable by day 11. Peak CD8⁺ T-cell responses based on phenotypic analysis ranged from 2 to 13% for YFV and 10 to 40% for Dryvax. The CD8⁺ T-cell responses we detected in infants during natural infection with RSV were very similar to the T-cell responses in adults described by Miller after Dryvax and YFV vaccination. First, we found the peak of the CD8⁺ T-cell response between days 11 and 15 after the first symptoms, which occurred 3 to 5 days later than virus inoculation (32). In addition to the similarities in kinetics, we observed a range of responding CD8⁺ T cells (between 1 and 40% of CD8⁺ T cells) in our patient population comparable to the responses in healthy adults. The striking similarity between the kinetics and the height of the response in healthy adults and the predominantly <3-month-old infants during the different viral infections is remarkable. The infant immune system is generally considered to be immature and Th2 biased and is thought to be weaker than that of adults (1). However, our findings indicate that, despite their young age, infants can develop robust CD8⁺ T-cell responses against a respiratory viral infection. In contrast to the complete virus eradication in serum observed after YFV vaccination, we still detected RSV by PCR in the airways of children at discharge from the ICU, which was around day 17 after the onset of symptoms. Hence, in this location, virus eradication seemed less efficient in RSV patients than systemic viremia in YFV-vaccinated individuals. In earlier work, we found that one-third of the effector CD8⁺ T cells of RSV patients, identified by phenotypic analysis, responded with gamma interferon (IFN- γ) production upon RSV stimulation (25). Also, this observation was similar to the observations made by Miller et al. in adults, where one-third of the CD8⁺ T cells also responded by production of IFN- γ after antigenic stimulation. It is currently unclear why only one-third of the effector CD8⁺ T cells produce IFN- γ upon stimulation with virus antigen. In the infants in our study, the T-cell compartment consisted mostly of naive cells, which makes substantial bystander activation unlikely. Also, after live-vaccine-induced CD8⁺ T-cell responses in adults, no evidence of bystander T-cell activation was observed (40).

All these similarities suggest that the neonatal response to an acute viral infection is comparable to primary T-cell responses induced in adults with respect to kinetics, magnitude, and function. The sequence of events in these children, with high viral loads and disease parameters upon admission to the ICU while T-cell responses were absent, suggests that these T cells play a minor role in RSV immune pathology. In contrast, massive neutrophil influx in the airways and subsequent recruitment of neutrophil precursors a few days after the peak

viral load and disease severity indicated that these cells might be important contributors to RSV disease (15, 25, 48). We cannot formally exclude the possibility that lung CD8⁺ T-cell numbers peak before CD8⁺ T-cell numbers in the peripheral blood. However, in mouse models of localized influenza virus infections, peak virus-specific CD8⁺ T-cell numbers in the lung, peripheral blood, and spleen coincided (21). Moreover, in a study by Welliver et al., who described lung tissue analysis in autopsy materials from RSV- and influenza virus-infected children (57), lymphocytes were hardly detected in the lungs of the children who died from severe RSV LRTI. In contrast, strong neutrophil influxes were found in the autopsy material of these children (57). These findings also suggested that at the peak of disease T cells do not play a significant role, because responses were low or had not fully developed at the time the children were severely ill.

It has been shown that neutralizing antibodies and antivirals have no protective ability when given during established disease (55). This observation suggests that lowering the viral load during disease has no beneficial effect. Because T-cell responses peak at convalescence and therefore do not appear to contribute to pathology, manipulating innate immune parameters might be the only option for disease treatment, while replicating vaccines administered early might induce protective T-cell immunity.

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REFERENCES

- Adkins, B., C. Leclerc, and S. Marshall-Clarke. 2004. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* 4:553.
- Barrios, C., P. Brawand, M. Berney, C. Brandt, P. H. Lambert, and C. A. Siegrist. 1996. Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *Eur. J. Immunol.* 26:1489.
- Blom, D., M. Ermers, L. Bont, W. M. Van Aalderen, and J. B. van Woensel. 2007. Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing. *Cochrane. Database. Syst. Rev.* CD004881.
- Bont, L., C. J. Heijnen, A. Kavelaars, W. M. Van Aalderen, F. Brus, J. T. Draaisma, S. M. Geelen, H. J. van Vught, and J. L. Kimpen. 1999. Peripheral blood cytokine responses and disease severity in respiratory syncytial virus bronchiolitis. *Eur. Respir. J.* 14:144.
- Bont, L., A. Kavelaars, C. J. Heijnen, A. J. van Vught, and J. L. Kimpen. 2000. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. *J. Infect. Dis.* 181:1772.
- Bowie, A. G., and L. Unterholzner. 2008. Viral evasion and subversion of pattern-recognition receptor signalling. *Nat. Rev. Immunol.* 8:911.
- Brandenburg, A. H., A. Kleinjan, L. B. van Het, H. A. Moll, H. H. Timmerman, R. L. de Swart, H. J. Neijens, W. Fokkens, and A. D. Osterhaus. 2000. Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. *J. Med. Virol.* 62:267.
- Cannon, M. J., P. J. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J. Exp. Med.* 168:1163.
- Claassen, E. A., G. M. van Bleek, Z. S. Rychnavska, R. J. de Groot, E. J. Hensen, E. J. Tijhaar, W. van Eden, and R. G. van der Most. 2007. Identification of a CD4 T cell epitope in the pneumonia virus of mice glycoprotein and characterization of its role in protective immunity. *Virology* 368:17.
- de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. V. Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. V. Cam, D. Q. Ha, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar.

2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* **12**:1203.
11. DeVincenzo, J. P., C. M. El Saleeby, and A. J. Bush. 2005. Respiratory syncytial virus load predicts disease severity in previously healthy infants. *J. Infect. Dis.* **191**:1861.
 12. de Waal, L., L. P. Koopman, I. J. van Benten, A. H. Brandenburg, P. G. Mulder, R. L. de Swart, W. J. Fokkens, H. J. Neijens, and A. D. Osterhaus. 2003. Moderate local and systemic respiratory syncytial virus-specific T-cell responses upon mild or subclinical RSV infection. *J. Med. Virol.* **70**:309.
 13. Elghetany, M. T. 2002. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol. Dis.* **28**:260.
 14. Elghetany, M. T., Y. Ge, J. Patel, J. Martinez, and H. Uhrova. 2004. Flow cytometric study of neutrophilic granulopoiesis in normal bone marrow using an expanded panel of antibodies: correlation with morphologic assessments. *J. Clin. Lab. Anal.* **18**:36.
 15. Everard, M. L., A. Swarbrick, M. Wright, J. McIntyre, C. Dunkley, P. D. James, H. F. Sewell, and A. D. Milner. 1994. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. *Arch. Dis. Child.* **71**:428.
 16. Forster, R., A. C. Valos-Misslitz, and A. Rot. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* **8**:362.
 17. Frank, J. A., and M. A. Matthay. 2003. Science review: mechanisms of ventilator-induced injury. *Crit. Care.* **7**:233.
 18. Galkina, E., J. Thattai, V. Dabak, M. B. Williams, K. Ley, and T. J. Braciale. 2005. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. *J. Clin. Invest.* **115**:3473.
 19. Gerdes, J., H. Lemke, H. Baisch, H. H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* **133**:1710.
 20. Graham, B. S., L. A. Bunton, P. F. Wright, and D. T. Karzon. 1991. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J. Clin. Invest.* **88**:1026.
 21. Haanen, J. B., M. Toebes, T. A. Cordaro, M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher. 1999. Systemic T cell expansion during localized viral infection. *Eur. J. Immunol.* **29**:1168.
 22. Hall, C. B., R. G. Douglas, Jr., and J. M. Geiman. 1975. Quantitative shedding patterns of respiratory syncytial virus in infants. *J. Infect. Dis.* **132**:151.
 23. Hall, C. B., E. E. Walsh, J. F. Hruska, R. F. Betts, and W. J. Hall. 1983. Ribavirin treatment of experimental respiratory syncytial viral infection. A controlled double-blind study in young adults. *JAMA* **249**:2666.
 24. Hall, C. B., G. A. Weinberg, M. K. Iwane, A. K. Blumkin, K. M. Edwards, M. A. Staat, P. Auinger, M. R. Griffin, K. A. Poehling, D. Erdman, C. G. Grijalva, Y. Zhu, and P. Szilagyi. 2009. The burden of respiratory syncytial virus infection in young children. *N. Engl. J. Med.* **360**:588.
 25. Heidema, J., M. V. Lukens, W. W. van Maren, M. E. van Dijk, H. G. Otten, A. J. van Vught, D. B. van der Werf, S. J. van Gestel, M. G. Semple, R. L. Smyth, J. L. Kimpen, and G. M. van Bleek. 2007. CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. *J. Immunol.* **179**:8410.
 26. Hierholzer, J. C., and R. A. Killington. 1996. Virus isolation and quantitation, p. 25. *In* B. W. Mahy and H. O. Krago (ed.), *Virology methods manual*. Academic Press Ltd., London, United Kingdom.
 27. Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz. 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J. Immunol.* **149**:1319.
 28. Hubl, W., S. Andert, G. Thum, S. Ortner, and P. M. Bayer. 1997. Value of neutrophil CD16 expression for detection of left shift and acute-phase response. *Am. J. Clin. Pathol.* **107**:187.
 29. IMpact-RSV Study Group. 1998. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. *Pediatrics* **102**:531.
 30. Jensen, I. P., E. Thisted, G. Glikmann, N. Obel, P. E. Kofoed, M. Sambo, N. H. Valerius, and C. H. Mordhorst. 1997. Secretory IgM and IgA antibodies to respiratory syncytial virus in nasopharyngeal aspirates: a diagnostic supplement to antigen detection. *Clin. Diagn. Virol.* **8**:219.
 31. Johnson, J. E., R. A. Gonzales, S. J. Olson, P. F. Wright, and B. S. Graham. 2007. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod. Pathol.* **20**:108.
 32. Johnson, K. M., R. M. Chanock, D. Rifkind, H. M. Kravetz, and V. Knight. 1961. Respiratory syncytial virus. IV. Correlation of virus shedding, serologic response, and illness in adult volunteers. *JAMA* **176**:663.
 33. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* **4**:1191.
 34. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**:31.
 35. Kawai, T., and S. Akira. 2006. Innate immune recognition of viral infection. *Nat. Immunol.* **7**:131.
 36. Kerst, J. M., M. de Haas, C. E. van der Schoot, I. C. Slaper-Cortenbach, M. Kleijer, A. E. von dem Borne, and R. H. van Oers. 1993. Recombinant granulocyte colony-stimulating factor administration to healthy volunteers: induction of immunophenotypically and functionally altered neutrophils via an effect on myeloid progenitor cells. *Blood* **82**:3265.
 37. Kobasa, D., S. M. Jones, K. Shinya, J. C. Kash, J. Copps, H. Ebihara, Y. Hatta, J. H. Kim, P. Halfmann, M. Hatta, F. Feldmann, J. B. Alimonti, L. Fernando, Y. Li, M. G. Katze, H. Feldmann, and Y. Kawaoka. 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* **445**:319.
 38. Kohlmeier, J. E., and D. L. Woodland. 2009. Immunity to respiratory viruses. *Annu. Rev. Immunol.* **27**:61.
 39. Legg, J. P., I. R. Hussain, J. A. Warner, S. L. Johnston, and J. O. Warner. 2003. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *Am. J. Respir. Crit. Care Med.* **168**:633.
 40. Miller, J. D., R. G. van der Most, R. S. Akondy, J. T. Glidewell, S. Albott, D. Masopust, K. Murali-Krishna, P. L. Mahar, S. Edupuganti, S. Lalor, S. Germon, R. C. Del, M. J. Mulligan, S. I. Staprans, J. D. Altman, M. B. Feinberg, and R. Ahmed. 2008. Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* **28**:710.
 41. Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund, and T. D. Randall. 2004. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat. Med.* **10**:927.
 42. Orr, Y., J. M. Taylor, P. G. Bannon, C. Geczy, and L. Kritharides. 2005. Circulating CD10/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. *Br. J. Haematol.* **131**:508.
 43. Peters, P. J., J. Borst, V. Oorschot, M. Fukuda, O. Krahenbuhl, J. Tschopp, J. W. Slot, and H. J. Geuze. 1991. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**:1099.
 44. Reed, J. L., T. P. Welliver, G. P. Sims, L. McKinney, L. Velozo, L. Avendano, K. Hintz, J. Luma, A. J. Coyle, and R. C. Welliver, Sr. 2009. Innate immune signals modulate antiviral and polyreactive antibody responses during severe respiratory syncytial virus infection. *J. Infect. Dis.* **199**:1128.
 45. Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz. 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. *Am. J. Respir. Crit. Care Med.* **156**:190.
 46. Sheeran, P., H. Jafri, C. Carubelli, J. Saavedra, C. Johnson, K. Krisher, P. J. Sanchez, and O. Ramilo. 1999. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. *Pediatr. Infect. Dis. J.* **18**:115.
 47. Siber, G. R., D. Leombruno, J. Leszczynski, J. McIver, D. Bodkin, R. Gonin, C. M. Thompson, E. E. Walsh, P. A. Piedra, and V. G. Hemming. 1994. Comparison of antibody concentrations and protective activity of respiratory syncytial virus immune globulin and conventional immune globulin. *J. Infect. Dis.* **169**:1368.
 48. Smith, P. K., S. Z. Wang, K. D. Dowling, and K. D. Forsyth. 2001. Leucocyte populations in respiratory syncytial virus-induced bronchiolitis. *J. Paediatr. Child Health* **37**:146.
 49. Sun, J., R. Madan, C. L. Karp, and T. J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat. Med.* **15**:277.
 50. Sung, R. Y., S. H. Hui, C. K. Wong, C. W. Lam, and J. Yin. 2001. A comparison of cytokine responses in respiratory syncytial virus and influenza A infections in infants. *Eur. J. Pediatr.* **160**:117.
 51. van de Pol, A. C., A. M. van Loon, T. F. Wolfs, N. J. Jansen, M. Nijhuis, E. K. Breteleur, R. Schuurman, and J. W. Rossen. 2007. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. *J. Clin. Microbiol.* **45**:2260.
 52. van de Pol, A. C., T. F. Wolfs, N. J. Jansen, A. M. van Loon, and J. W. Rossen. 2006. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. *Crit. Care* **10**:R61.
 53. van Doornum, G. J., J. Guldemeester, A. D. Osterhaus, and H. G. Niesters. 2003. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J. Clin. Microbiol.* **41**:576.
 54. van Woensel, J. B., R. Lutter, M. H. Biezeveld, T. Dekker, M. Nijhuis, W. M. Van Aalderen, and T. W. Kuijpers. 2003. Effect of dexamethasone on tracheal viral load and interleukin-8 tracheal concentration in children with respiratory syncytial virus infection. *Pediatr. Infect. Dis. J.* **22**:721.
 55. Ventre, K., and A. G. Randolph. 2007. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. *Cochrane Database Syst. Rev.* **CD000181**.
 56. Wagner, C., R. Deppisch, B. Denefleh, F. Hug, K. Andrassy, and G. M. Hansch. 2003. Expression patterns of the lipopolysaccharide receptor CD14, and the Fcγ receptors CD16 and CD64 on polymorphonuclear neu-

- trophils: data from patients with severe bacterial infections and lipopolysaccharide-exposed cells. *Shock* **19**:5.
57. **Welliver, T. P., R. P. Garofalo, Y. Hosakote, K. H. Hintz, L. Avendano, K. Sanchez, L. Velozo, H. Jafri, S. Chavez-Bueno, P. L. Ogra, L. McKinney, J. L. Reed, and R. C. Welliver, Sr.** 2007. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. *J. Infect. Dis.* **195**:1126.
58. **Winkler, S., M. Willheim, K. Baier, D. Schmid, A. Aichelburg, W. Graninger, and P. G. Kremsner.** 1999. Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum* malaria. *J. Infect. Dis.* **179**:209.
59. **Wright, P. F., W. C. Gruber, M. Peters, G. Reed, Y. Zhu, F. Robinson, S. Coleman-Dockery, and B. S. Graham.** 2002. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *J. Infect. Dis.* **185**:1011.
60. **Zampieri, C. A., N. J. Sullivan, and G. J. Nabel.** 2007. Immunopathology of highly virulent pathogens: insights from Ebola virus. *Nat. Immunol.* **8**:1159.