Immune response to herpesvirus infections in immunocompromised children
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Citation for published version (APA):
Chapter 6

Absence of circulating NK cells and primed CD8$^+$ T cells in life-threatening varicella

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The Journal of Infectious Diseases, in press
Abstract

Five pediatric patients with no history of immunodeficiency experienced a life-threatening course of varicella. Strikingly, NK cells were absent from the circulation in all patients and in spite of active viral infection, up to 98% of the CD8+ T-cells were naive. Primary immunodeficiencies were excluded since NK cells and primed CD8+ T-cells re-appeared in the circulation, granzymes were detectable in plasma early in infection, and no abnormalities could be detected in the function of the IL15-receptor. Our data indicate that varicella-zoster virus (VZV) has a unique capability to seclude primed CD8+ T-cells and NK cells from the circulating lymphocyte pool. This seclusion may be the consequence of an overwhelming immune response to VZV, influenced by factors such as infectious dose, age, and presence of maternal antibodies during infancy. Since both homozygous twin-sisters included in this study developed a severe course of varicella, particular genetic factors may contribute to the development of severe varicella.

Introduction

Primary infection with varicella-zoster virus (VZV), an α-herpesvirus, results in varicella (chickenpox), a highly contagious disease which is characterized by fever, malaise and a generalized vesicular rash (exanthem). After primary infection, the virus remains latent in dorsal root ganglia. In countries of temperate climates where VZV vaccination is not implemented in routine childhood vaccinations, such as The Netherlands, more than 95% of children have been infected before the age of 5 years. Although varicella will lead to discomfort, it is usually a benign and self-limiting illness. Nonetheless, complications of varicella may be observed in otherwise healthy children, such as bacterial superinfection of the cutaneous lesions with S. aureus or S. pyogenes, VZV pneumonia, cerebellar ataxia and viral encephalitis [1]. In immunocompromised individuals, the risk of these complications during primary VZV infection is highly increased [1]. Prior to the appearance of the vesicular exanthem, abundant VZV replication is initially suppressed by the innate immune system. However, the innate immune system by itself is incapable of containing the virus completely. The adaptive immune response is subsequently activated by clonal expansion of VZV-specific T and B cells that interfere with viral replication and spread of the virus. VZV-specific CD4+ T cells secrete cytokines such as interleukin (IL)-2 and interferons (IFN), and may exhibit MHC class II-restricted cytotoxicity [2-4]. So far, VZV-specific CD8+ T cells could not be detected, due to paucity of experimental tools. Nevertheless, CD8+ T cells are thought to constitute one of the main effector arms in the immune response, being responsible for viral clearance and protection on re-encounter. Studies on other persistent viruses have shown that during primary infection, CD8+ T cells expand and differentiate. CD8+ T cells can be subdivided into functional subsets by markers such as CD45RA, the co-stimulatory molecule CD27 [5], and CCR7 [6], a chemokine receptor involved in lymphocyte migration to the lymph nodes. During acute infection, virus-specific CD8+ T cells show a CD45RA CD27-CCR7- phenotype and abundantly express perforin and proteases such as granzymes [7]. Beside CD4+ and CD8+ T cells, natural killer (NK) cells, which are part of the innate immune system, are also believed to be important in herpesvirus infections [8,9]. Biron et al. described a female patient with a severe course of VZV infection accompanied by a complete
Absence of NK cells and primed CD8+ T cells in severe VZV

Absence of NK cells, which persisted after resolution of the infection [10]. NK cells normally constitute up to 15% of circulating human lymphocytes and are defined by the expression of CD56, and lack of CD3 surface antigens. Development of NK cells is dependent on cell-cell contact between their progenitors and bone marrow stromal cells, as well as on cytokines such as IL-15, and growth factors like Flt3 ligand (FL) and stem cell factor (SCF) [11]. Although the precise mechanisms of activation of NK cells are as yet unresolved, it appears that the regulation of NK cell activity is the result of a balance between signals from activating and inhibitory receptors on the cell, the latter being dominant in steady-state [12,13].

We here describe five children with a severe course of varicella with symptoms including generalized varicella, skin infection, pneumonitis, hepatitis, or encephalitis. None of these children had a history of recurrent infection or known immunodeficiency. Due to the severity of their disease, four of these children were admitted to the Pediatric Intensive Care Unit (PICU). After more than four to twelve weeks of intensive treatment because of their clinical condition, four of the children recovered, whereas the fifth child died of multi-organ failure (pt. 3). We studied functions and phenotypes of T and NK cells to investigate the incapability of these children to control the virus adequately.

Materials & Methods

Table 1 Characteristics of the patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at onset of varicella</th>
<th>Admission</th>
<th>Symptoms</th>
<th>Severity score</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>M</td>
<td>8 months</td>
<td>4</td>
<td>vesicular rash, pneumonitis, hepatitis, encephalitis</td>
<td>30</td>
<td>acyclovir, IVIG, clindamycin</td>
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<td>10 months</td>
<td>7</td>
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<td>25</td>
<td>acyclovir, IVIG, clindamycin</td>
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<tr>
<td>Pt. 3</td>
<td>F</td>
<td>5 years, † wk 6</td>
<td>5</td>
<td>vesicular rash, encephalitis, multi-organ failure</td>
<td>30</td>
<td>acyclovir, IVIG, clindamycin</td>
</tr>
<tr>
<td>Pt. 4c</td>
<td>F</td>
<td>11 months</td>
<td>2</td>
<td>vesicular rash, pneumonitis, hepatitis</td>
<td>24</td>
<td>acyclovir, clindamycin</td>
</tr>
<tr>
<td>Pt. 5c</td>
<td>F</td>
<td>11 months</td>
<td>1</td>
<td>vesicular rash, pneumonitis</td>
<td>16</td>
<td>acyclovir</td>
</tr>
</tbody>
</table>

a days after onset of rash; number of lesions remained <500 in all cases
b according to Vazquez et al. [14]: mild disease when ≤7 points, moderately severe when 8-15 points, severe when ≥16 points on a scale to assess severity of varicella.
c homozygous twins
IVIG = intravenous immunoglobulins

Patients

Five patients with a severe course of varicella were admitted to the Emma Children's Hospital at the Academic Medical Center (AMC). Four of these patients (pt. 1-4) were admitted to the PICU because of respiratory and circulatory failure. The cohort included one female homozygous pair of twins, of which one child was admitted to the PICU (pt. 4), whereas the other child was admitted experiencing a severe, yet not life-threatening course of varicella (pt. 5). The patients differed in age and sex (Table 1). According to the clinical scoring system of Vazquez et al. [14], the variables rash (number of lesions), character of the lesions (macular, vesicular, hemorrhagic), height of fever, systemic signs (abdominal pain, interstitial pneumonia, encephalitis) and a subjective assessment of the patient's
appearance (not, moderately or severely ill), assess the severity of varicella. The total score of points in each of these patients allowed categorization as "severe disease" caused by VZV: i.e. >16 points (Table 1). Treatment of the patients consisted of intravenous acyclovir or without intravenous immunoglobulins, beside the medication for respiratory and circulatory support. Peripheral blood samples were drawn from the patients at "early" (within 10 days) and "late" (4-6 weeks) time points after appearance of the exanthem. These patients are referred to as index patients.

As controls for our index patients, we included three patients experiencing varicella who were admitted to the PICU because of respiratory and circulatory failure due to complications (bacterial superinfections; n=3). One of these three children had <50 vesicles and suffered from epiglottitis (age 4 months). Bacterial cultures were positive for *Haemophilus influenzae* type b (HiB); she was vaccinated once for HiB in the past. The second child (age 11 months) had bilateral lobar pneumonia; blood cultures were positive for *Streptococcus pneumoniae*. The third child (age 2 years) was admitted with a blood culture-positive septic shock due to group A β-hemolytic Streptococci spreading from impetiginized vesicles. These three patients had a rash of <200 vesicles and looked severely ill. All three patients had a severity score of 14 and had "moderately severe disease" [14]. A second group of controls (diagnosed and followed at the outpatient care unit) consisted of children experiencing mild varicella with a severity score of 6.2 (n=8; mean age: 3.8 years) [14]. Blood samples of these controls were collected and stored when allowed by parental consent.

**Determination of VZV-specific CD4⁺ T cells by intracellular cytokine staining**

PBMCs were isolated using standard density gradient centrifugation techniques by use of Lymphoprep (Nycome, Pharma, Oslo, Norway). PBMCs were cryopreserved until use and thawed in accordance with standard procedures. VZV-specific CD4⁺ T-cell frequencies were determined as described previously [4]. In brief, PBMCs were stimulated for 6 hours with VZV-antigen (20 μl/ml; Microbix Biosystems, Toronto, Canada), the final 5 hours in presence of brefeldin-A (10 μg/ml; Sigma Chemical, St. Louis, MO). PBMCs were costimulated with anti-CD28 (2 μg/ml; CLB 15E8; Sanquin, Amsterdam, The Netherlands) and anti-CD49d (1 μg/ml; BD Biosciences, San Jose, CA). Next, the cells were incubated with FACS lysing solution and thereafter with FACS permeabilisation solution (BD Biosciences). The cells were stained for CD4-PerCPCy5.5, IFN-γ-FITC (both BD Biosciences), and CD69-APC (Caltag Laboratories, Burlingame, CA). The CD4⁺CD69⁺IFN-γ⁺ T cells were designated antigen-specific CD4⁺ T cells. Negative controls consisted of stimulation with medium, and positive controls of stimulation with *Staphylococcus aureus* enterotoxin B (SEB; Sigma, St Louis, MO). Percentages of VZV-specific CD4⁺ T cells were corrected for background staining.

![Figure 1 VZV load in blood during the course of VZV infection. High VZV loads were detected in blood from pt. 3 during the early phase of infection (within 10 days after appearance of the vesicular rash), which remained high throughout the infection. Normal VZV loads were observed in blood from pt. 1, 4, and 5, as compared with controls consisting of pediatric patients with a mild course of varicella. No data were available for pt. 2. Early = within 10 days after the appearance of the rash. Late = 4-6 weeks after the appearance of the rash. Differences in VZV loads between the patient groups were not statistically significant.](image-url)
Absence of NK cells and primed CD8\(^+\) T cells in severe VZV

VZV Polymerase Chain Reaction (PCR)
Quantitative PCR was performed in blood samples as described elsewhere [15]. For comparison, VZV DNA loads were also determined in blood samples from pediatric controls experiencing mild to moderate varicella. The electrochemiluminescence (ECL) signal was measured by an M-8 analyzer (iGEN, Oxford, UK).

Immunofluorescent staining and flow cytometry
Thawed PBMCs were resuspended in RPMI, containing 10% fetal calf serum (FCS) and antibiotics. Cells were washed in phosphate-buffered saline (PBS) containing 0.01% (w/v) Na\(_2\) and 0.5% (w/v) bovine serum albumin. A total of 200,000 PBMCs were incubated with fluorescent-labeled conjugated monoclonal antibodies (mAbs; concentrations in accordance with manufacturer's instructions) for 30 minutes at 4°C. Different combinations of mAbs were used to analyze lymphocyte subsets: CD3-FITC, CD4-PerCP, CD8-PerCP, CD8-APC, CD16-PE, CD27-PE, CD45RA-FITC, and CD56-APC (all BD Biosciences). Analysis of cells was performed using a FACSCalibur flowcytometer and CellQuest software (BD Biosciences).

Cytokine production
Thawed PBMCs were stimulated with PMA (2 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) for 4.5 hours, of which 3.5 hours in presence of the protein-secretion inhibitor Brefeldin A (10 μg/ml). After stimulation, cells were washed and incubated with FACS lysis solution and thereafter with FACS permeabilisation solution (BD Biosciences). Cells were stained with IFN-γ-FITC, IL-2-PE, IL-4-PE, CD4-PerCP, and CD8-APC (all BD Biosciences). Intracellular detection of the cytokines was analyzed using a FACSCalibur flow cytomter and CellQuest software (BD Biosciences).

CFSE labeling and cell culture
Thawed PBMCs were resuspended in PBS at a final concentration of 5-10x10^6 cells/ml. PBMCs were labeled with 5-(and-6)-CarboxyFluorescein diacetate Succinimidyl Ester (CFSE, 2.5 μM; Molecular Probes, Europe BV, Leiden, The Netherlands) in PBS for 8 minutes while shaking at 37°C. Labeling was stopped by addition of IMDM supplemented with 10% FCS. Cells were washed and subsequently resuspended in IMDM supplemented with 10% human pool serum (HPS), antibiotics and 3.57 x 10^4% (v/v) β-mercapto ethanol (Merck) (culture medium). CFSE labeled cells were cultured for five days in 24 wells plates at a concentration of 0.5-1x10^6 cells/ml in culture medium in presence or absence of IL-15 (10 ng/ml; R&D systems, Abingdon, UK) for 5 days. Analysis of cells was performed using a FACSCalibur flowcytometer and CellQuest software (BD Biosciences).

Granzyme ELISA
Cell-free plasma samples were collected from heparin-anticoagulated blood from the patients. ELISA's to measure the levels of granzyme A and B were performed as described elsewhere [16].

NK cell cytotoxicity assay
Target cell line K562, a human leukemic cell line that is highly sensitive to killing by NK cells, was prepared by labeling these cells with \(^{51}\)Cr (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h at 37°C, 5% CO\(_2\). \(^{51}\)Cr-labeled target cells were then incubated in triplicates with NK cells at various effector:target ratios for 4 h at 37°C, 5% CO\(_2\). Spontaneous release was determined by incubation of labeled target cells with medium. Maximal release was determined by culturing labeled target cells with NP40 solution. Supernatants were harvested and counted in a γ-radiation detector. Percentage specific lysis was calculated from the formula "percentage specific lysis = ((experimental release – spontaneous release) / (maximal release – spontaneous release)) x 100%".

Statistics
Where applicable, differences in means between groups were determined by a Student's t test. P<0.05 was considered statistically significant. All analyses were done using SPSS version 11.5 (SPSS, Chicago).
**Results**

**A**  
\(CD3^-\) lymphocytes:

<table>
<thead>
<tr>
<th></th>
<th>Pt. 1</th>
<th>Pt. 2</th>
<th>Pt. 3</th>
<th>Pt. 4</th>
<th>Pt. 5</th>
<th>control</th>
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<tr>
<td>Early</td>
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<td>Late</td>
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</table>

**B**  
\(CD8^+\) lymphocytes:

<table>
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<tr>
<th></th>
<th>Pt. 1</th>
<th>Pt. 2</th>
<th>Pt. 3</th>
<th>Pt. 4</th>
<th>Pt. 5</th>
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<tr>
<td>Late</td>
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</table>

**C**  
\(CD4^+\) lymphocytes:

<table>
<thead>
<tr>
<th></th>
<th>Pt. 1</th>
<th>Pt. 2</th>
<th>Pt. 3</th>
<th>Pt. 4</th>
<th>Pt. 5</th>
<th>control</th>
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<tr>
<td>Late</td>
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</table>

**Figure 2** Disturbed pattern of NK cells and primed CD8\(^+\) T cells in peripheral blood from the patients. During the early phase of infection ("early"), NK cells (A, gated on CD3^- lymphocytes; numbers indicate % of CD3^-CD56^- cells within the total lymphocyte pool) and primed CD8\(^+\) T cells (B, gated on CD8\(^+\) T cells) were absent from peripheral blood from the patients, despite acute viral infection, whereas these cells appeared in peripheral blood during the late phase of infection ("late"). NK cells and primed (i.e. CD45RA^-CD27^, CD45RA^-CD27^, or CD45RA^CD27^-) CD8\(^+\) T cells were detectable in peripheral blood at all time points after VZV infection in one of the representative pediatric controls, who experienced a mild course of varicella. (C) By contrast, CD4\(^+\) T cells were normally distributed throughout the infection. For patients "early" refers to within 10 days, and "late" to 4-6 weeks after the appearance of the vesicular exanthem. Since controls experiencing mild varicella resolved the infection within one week, "early" refers to 0-3 days and "late" to 7-10 days after appearance of the rash in these children.
Absence of NK cells and primed CD8+ T cells in severe VZV

Viral load in blood samples
Quantitative PCR analysis to determine VZV DNA loads in the blood from the patients showed that VZV loads of patients 1, 4 and 5 during the early phase of infection were within the range of loads observed in pediatric patients experiencing a mild to moderate course of varicella (Fig. 1). The loads declined during the course of infection to background levels similar to those observed in controls after resolution of the infection (Fig. 1). By contrast, the VZV load of pt. 3 was high during the early phase of infection and although the load decreased, it remained high throughout the infection. VZV loads were not determined in serial blood samples of pt. 2. Taken together, the differences between the index patients and the control children suffering from mild to moderate VZV infection did not reach statistical significance.

Disturbed distribution of CD8+ T cells and NK cells in peripheral blood during the early phase of VZV infection
Concerning humoral immunity, Ig spectra were normal. Moreover, the patients had developed normal antibody responses to childhood vaccinations (DTP-Polio-Hib at 2, 3, 4, and 11 months of age (all patients), or Mumps–Measles–Rubella (MMR) at 14 months of age (pt. 3). Regarding varicella, IgM and IgG antibodies to VZV could be detected in the blood from the patients at admission (prior to treatment with immunoglobulins) and 3–4 months after convalescence of varicella (data not shown).

Since T cells and NK cells play a key role in the control of VZV infection, the presence and differentiation of these cells was studied. Significantly lower levels of NK cells were found in the five index patients as compared with the three patients admitted to the PICU because of circulatory and respiratory failure due to bacterial super-infection during the episode of acute varicella (Table 2; P=0.001 (absolute numbers); P=0.02 (percentages)). Furthermore, NK cells can be detected in children experiencing a mild course of varicella (Fig. 2A). The levels of NK cells in the index patients normalized during convalescence (Table 2).

Table 2 NK cells in VZV-induced life-threatening varicella compared with varicella complicated by bacterial super- or co-infection

<table>
<thead>
<tr>
<th>Severity score</th>
<th>Severe VZV disease (n=5)</th>
<th>Complicated VZV disease (n=3)</th>
<th>Age controls (&lt;1 year, n=34)</th>
<th>Age controls (1-4 years, n=18)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cells (per µL)</td>
<td>20 ± 20</td>
<td>510 ± 100</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>NK cells (% lympho)</td>
<td>0.9 ± 0.6</td>
<td>10.2 ± 3.4</td>
<td></td>
<td></td>
<td>0.020</td>
</tr>
<tr>
<td>Reconvalescent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cells (per µL)</td>
<td>230 ± 90</td>
<td>520 ± 320</td>
<td>480 ± 290</td>
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<td>0.06</td>
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<tr>
<td>NK cells (% lympho)</td>
<td>6.5 ± 3.6</td>
<td>10.1 ± 8.1</td>
<td>10.8 ± 3.6</td>
<td></td>
<td>0.35</td>
</tr>
</tbody>
</table>

a patients (n=3) suffering from epiglottitis, pneumococcal pneumonia and GABHS septicemia, respectively
b the controls <12 months and of 1-4 years of age were of mixed racial background
c severity score according to Vazquez et al. [14]: mild disease ≤7, moderate 8-15, and severe ≥16

Although the index patients experienced an acute viral infection, the majority of CD8+ T cells in the early phase of infection were of the naïve subset (i.e. CD45RA+CD27-). Significantly
lower levels of primed CD8+ T cells were detectable in the index patients, compared with the control PICU patients (Table 3). Furthermore, in control patients experiencing a mild course of varicella, CD8+ T cells showed a primed phenotype (i.e. CD45RA CD27+, CD45RA·CD27-, or CD45RA·CD27-) and were already detectable in peripheral blood within 5 days after onset of the vesicular rash (Fig. 2B) [5]. In the index patients a naïve phenotype in the CD4+ T cells was present during the early phase of infection and seemed to persist, although this distribution of naïve and primed CD4+ T cells was variable (Fig. 2C & Table 3).

During the late phase of infection, NK cells appeared in peripheral blood from the index patients (Fig. 2A & Table 2), and primed CD8+ T cells with memory and effector phenotypes were found in the circulation (Fig. 2B & Table 3) [5]. Primed and naïve CD4+ T cells and B cell numbers remained stable (Table 3 & data not shown). At all time points studied, circulating CD4+ and CD8+ T cells were able to secrete IFN-γ, IL-2 and IL-4 upon PMA/iono stimulation (data not shown).

### Table 3 T cell subsets in VZV-induced life-threatening varicella compared with varicella complicated by bacterial super- or co-infection

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Acute severe VZV disease</th>
<th>Reconvalescent VZV disease</th>
<th>Complicated VZV disease</th>
<th>Age controls (≤1 year)</th>
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<tbody>
<tr>
<td><strong>CD4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve (CD45RA+CD27+)</td>
<td>89.6 ± 3.3</td>
<td>88.4 ± 6.4</td>
<td>78.0 ± 3.5 (P=0.005)</td>
<td>80.8 ± 5.0 (P=0.003)</td>
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<tr>
<td>Mem (CD45RA-CD27+)</td>
<td>9.5 ± 3.5</td>
<td>10.6 ± 7.2</td>
<td>18.7 ± 4.0 (P=0.01)</td>
<td>16.6 ± 4.7 (P=0.01)</td>
<td></td>
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<tr>
<td>Eff/mem (CD27+)</td>
<td>1.2 ± 0.5</td>
<td>1.4 ± 1.1</td>
<td>3.3 ± 1.5 (P=0.02)</td>
<td>2.4 ± 1.4 (P=0.12)</td>
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<tr>
<td><strong>CD8</strong></td>
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<tr>
<td>Naïve (CD45RA+CD27+)</td>
<td>92.6 ± 3.4</td>
<td>75.0 ± 11.0</td>
<td>70.0 ± 5.6 (P=0.0003)</td>
<td>74.8 ± 14.9 (P=0.009)</td>
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<tr>
<td>Mem (CD45RA-CD27+)</td>
<td>5.0 ± 1.6</td>
<td>17.6 ± 12.6</td>
<td>23.0 ± 6.6 (P=0.0008)</td>
<td>13.9 ± 9.9 (P=0.01)</td>
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<tr>
<td>Eff/mem (CD27+)</td>
<td>1.8 ± 1.6</td>
<td>4.8 ± 3.6</td>
<td>6.9 ± 3.2 (P=0.0001)</td>
<td>12.6 ± 8.3 (P=0.09)</td>
<td></td>
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</tbody>
</table>

*index patients (n=5)  
*b patients (n=3) suffering from epiglottitis, pneumococcal pneumonia and GABHS septicemia, respectively; P-values are derived from Student's t-test on acute severe VZV disease vs. complicated VZV disease  
*c age controls <12 months, n=16; mixed racial background; P-values are derived from Student's t-test on acute severe VZV disease vs. age controls (<1 year)

### High levels of granzymes in plasma

Granzymes A and B are produced by cytotoxic T cells and NK cells and are important mediators of cytolysis of virus-infected cells. The level of GrA in plasma was relatively high during the early phase of infection in pt. 1, whereas the level of GrB was relatively high in pt. 1 and 4. The levels of these granzymes detected in the other patients were within the range of pediatric controls experiencing mild to moderate varicella (Fig. 3). The granzyme levels normalized during the course of infection to levels as observed in controls after resolution of VZV infection (data not shown). Since granzymes A and B were detectable both during the early and late phase of infection, we believe that primed CD8+ T cells and NK cells were
Absence of NK cells and primed CD8\(^+\) T cells in severe VZV present throughout the infection and were functional *in vivo*, even though they could not be detected in peripheral blood in the early phase of infection. During early and late infection, the values of granzymes between the two patient groups did not show any statistically significant difference on comparison.

![Graph](https://example.com/graph.png)

**Figure 3 Secretion of granzymes A and B in plasma.** During the early phase of VZV infection, all patients secreted granzymes A and B in plasma. High levels of these cytolytic proteins were detected in plasma of pt. 1, as compared with the pediatric controls experiencing mild varicella. The levels of granzyme secretion normalized in these patients during the course of infection. Early = within 10 days after the appearance of the vesicular exanthem. Late = 4-6 weeks after the appearance of the rash. Dotted horizontal line = detection limit of ELISA. At both the early and late time point the differences in granzyme concentrations between the patient groups were not statistically significant.

**Delayed appearance of VZV-specific CD4\(^+\) T cells in peripheral blood**

The VZV-specific immune response of these patients was studied with a functional assay in which VZV-specific CD4\(^+\) T cells were detected by upregulation of CD69 and production of IFN-\(\gamma\) after 6 hours of stimulation with a VZV lysate (VZV antigen) [4]. Only very low frequencies of VZV-specific CD4\(^+\) T cells could be detected in peripheral blood from three of the patients during the early phase of infection by this assay, whereas these cells were absent from the circulation in the other two patients (Table 4). Increased frequencies of virus-specific CD4\(^+\) T cells were detected during the late phase of infection, after treatment with antiviral therapy (Table 4). By contrast, these cells were already detectable in the early phase of VZV infection in peripheral blood from pediatric patients experiencing a mild course of varicella.

**Table 4 VZV-specific CD4\(^+\) T cells**

<table>
<thead>
<tr>
<th>Index patients</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>0.02%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>0.03%</td>
<td>0.06%</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>0.00%</td>
<td>0.05%</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>0.02%</td>
<td>0.06%</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>0.00%</td>
<td>0.02%</td>
</tr>
<tr>
<td>Mild VZV patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.23%</td>
<td>0.05%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.13%</td>
<td>0.03%</td>
</tr>
</tbody>
</table>

*Numbers indicate the percentages of specific IFN-\(\gamma\)/CD69\(^+\) T cells within the CD4\(^+\) T cell population. From two controls serial samples were available at identical time points as tested in the index patients.*
Chapter 6

Proper function of the IL-15 receptor

Signaling of IL-15 through the IL-15 receptor is important in the maturation and differentiation of NK cells and CD8\(^+\) T cells. We therefore tested the functionality of this receptor on our patients. CD8\(^+\) T cells were able to proliferate upon stimulation with IL-15 as demonstrated by dilution of CFSE on day 5 of culture, whereas no proliferation was detected when stimulated with medium (Fig. 4A). CD8\(^+\) T cells collected at the late phase of infection exhibited increased proliferative capacity upon IL-15 stimulation than CD8\(^+\) T cells collected during the early phase of infection. Furthermore, NK cells (only detectable during the late phase of infection) proliferated upon stimulation with IL-15 (Fig. 4B).

**Figure 4** Proliferation of CD8\(^+\) T cells and NK cells upon IL-15 stimulation.

(A) Stimulation with IL-15 resulted in proliferation of CD8\(^+\) T cells on day 5 of culture (thick line), whereas CD8\(^+\) T cells cultured in medium only (thin line) did not proliferate. CD8\(^+\) T cells in peripheral blood during the late phase of infection ("late") exhibited increased proliferative potential as compared with CD8\(^+\) T cells during the early phase of infection ("early"). (B) NK cells that appeared in peripheral blood from the patients during the late phase of infection proliferated upon stimulation with IL-15. Data from one representative patient (pt. 3) are shown.

NK cell cytotoxicity

Cytotoxicity of the NK cells appearing in peripheral blood from the patients during the late phase of infection was determined by lysis of target cell line K562. As shown in Fig. 5, NK cells of all patients were capable of lysing target cell line K562, at levels comparable to NK cells derived from pediatric controls (data from one representative control are shown).

Discussion

We here describe the immunological response of five patients experiencing a life-threatening, and in one case fatal course of chickenpox. Most strikingly, NK cells and primed CD8\(^+\) T cells were nearly absent from the circulation during the early phase of primary VZV infection, whereas these cells could be detected in control patients experiencing varicella, who were admitted to the PICU because of bacterial super- or co-infection. The absence of these cell populations, which are believed to be crucial in the antiviral defense [7,8,17,18], might be the result of a primary defect in the development or differentiation of these cells. Several observations excluded a severe primary defect in the generation and maintenance of these cell populations in our patients. First, CD8\(^+\) T cells as well as NK cells of the patients proliferated upon stimulation with IL-15, a key factor in the differentiation and homeostasis of these cells [19-24], excluding defects in the IL-15Ra, IL-2R\(\beta\), the common \(\gamma\)-chain (CD132), or signaling molecules coupled to this receptor [25]. Second, plasma granzymes A and B were detectable in the patients during the early phase of infection, which suggested that
Absence of NK cells and primed CD8\(^+\) T cells in severe VZV

primed CD8\(^+\) T cells and NK cells were present and were functional. Third, primed CD8\(^+\) T cells and NK cells re-appeared in the circulation during reconvalescence. Alternatively, the absence of these cytotoxic cells from the circulation could be the consequence of their redistribution to target sites. A major caveat of studies of viral infections in humans is the limitation of compartments that can be studied, being largely restricted to the circulating pool of immune cells. The majority of NK cells and primed T cells express chemokine receptors such as CCR5 and CXCR3, whereas they do not express the secondary lymphoid homing receptor CCR7 \[6,7,26\]. This pattern of chemokine receptor expression enables them to migrate from the circulation to inflamed tissue to eliminate virus-infected cells \[27-30\]. Due to this migration, the distribution of virus-specific cells in peripheral blood may differ from their distribution in target sites \[31,32\]. Indeed, several studies suggested that primed cells may be secluded from the circulation during viral infection. Virus-specific T cells were present at higher frequencies in non-lymphoid tissue than in lymphoid tissue during acute infection in murine models \[31,33\]. In addition, a few studies on hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in humans, showed that the percentages of virus-specific T cells are higher in the liver, being the major target site for these viruses, than in the circulation \[34-36\].

As our study showed, significantly less primed CD4\(^+\) T cells were observed in the early phase of infection in the index patients. A low number of VZV-specific CD4\(^+\) T cells appeared in the circulation after intensive antiviral treatment. The appearance of virus-specific CD4\(^+\) T cells was delayed compared with patients experiencing mild varicella. These data are consistent with observations from human cytomegalovirus (CMV) infection and human immunodeficiency virus (HIV) infection, which showed that during the acute phase of HIV and symptomatic CMV infection, virus-specific CD4\(^+\) T cells are kept out of the circulation, and only appear upon control of the virus by antiviral therapy \[37,38\]. The absence of primed CD8\(^+\) T cells and NK cells from the circulation during life-threatening varicella in our patients, seems at present unique for VZV, since in CMV- and HIV-infection primed CD8\(^+\) T cells are detectable in the circulation, regardless of therapeutic intervention \[37,39\]. Whereas the targeting of CD4\(^+\) T cells by CMV, HIV and VZV from the circulation probably only involves virus-specific CD4\(^+\) T cells, all primed CD8\(^+\) T cells were absent from the circulation in the early phase of these severe courses of varicella, but not during mild courses of varicella. We therefore suggest that in some cases, VZV might be able to seclude primed CD8\(^+\) T cells and NK cells from the circulation.

The patients described in this study probably suffered from an overwhelming immune response to VZV. The ongoing infection may induce high levels of chemokines at the inflamed sites, leading to targeting of cytotoxic cells at these sites. Since chemokine receptors that enable migration to target sites, are not only present on VZV-specific CD8\(^+\) T cells but on the majority of primed CD8\(^+\) T cells, cytotoxic cells of broad antigenic specificities may be secluded from the circulation. Only upon normalization of the chemokine levels due to spontaneous recovery or intervention with antiviral therapy, these cells might be enabled to return to the circulation. In addition, the ongoing infection may lead to the exhaustion of primed CD8\(^+\) T cells and NK cells, due to a disturbed balance between the generation of these cells and apoptosis \[40-42\]. NK cells were not only absent from the circulation during the early phase of infection, but also from bone marrow (<100 per 10\(^5\) cells (n=3) compared with 2480 ± 960 per 10\(^5\) cells in bone marrow from pediatric controls (n=7)), whereas they could be cultured from CD34\(^+\) selected hematopoietic stem cells in vitro for the patients from
whom sufficient bone marrow was obtained (pt. 1 and 3; H. Spits, unpublished observation). Although never described, the infection of NK cells by VZV may induce apoptosis of these cells. However, granzymes were secreted, indicating the presence of functional NK cells. Furthermore, the restriction of this phenomenon to NK cells seems implausible.

Severe courses of varicella are rare in otherwise healthy children. Primary VZV infection at infancy has been defined as one of the risk factors for developing complicated chickenpox [43], which may partly explain the severity of the disease in four of our patients. In addition, infectious viral dose, duration of antigen exposure, and passive protection by maternal antibodies in infancy may influence the induction of the antiviral immune response and thereby the severity of the course of varicella. Furthermore, genetic factors may contribute to the outcome of VZV infection, since both homozygous twin-sisters included in this study developed a complicated course of varicella.

Figure 5 NK cell cytolysis. NK cells that re-appeared in peripheral blood from the patients during recovery from the infection were capable of lysing the NK-cell sensitive target K562. nd = not done

Acknowledgements

The authors would like to thank Prof. Dr. H. Spits for the experiments on NK cell development in vitro.

References

Chapter 6


