Natural Killer Cell Functional Defects in Pediatric Patients With Severe and Recurrent Herpesvirus Infections

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Natural killer (NK) cells play a critical role in the host defense against herpesviruses. Although herpesviruses are ubiquitous in human populations, only a minority of people experience severe recurrent infections. We hypothesize that uncharacterized NK cell functional deficits predispose individuals to more significant or frequent herpesvirus infections and reactivations. To investigate this hypothesis, we broadly analyzed NK cell phenotype and functional responses in a cohort of predominantly pediatric patients with recurrent and/or severe herpesvirus infections and compared them to a healthy control population. Our results identified no global differences in cytolysis, degranulation, interferon-γ production, or surface receptor upregulation following cytokine stimulation. However, abnormal NK cell functional responses were observed in nearly one-third of patients (including 3 with hyporesponsiveness to activating signals and 1 with markedly decreased CD11b expression associated with reduced cytotoxicity and degranulation), which might contribute to those individuals’ susceptibility to herpesvirus infections.

Keywords. Herpesvirus; NK cells; CD11b; IL-2; pediatric.

Despite the high seroprevalence of herpesviruses such as herpes simplex virus 1 (HSV-1) in diverse human populations [1, 2], the majority of people with herpesvirus infections do not develop an illness necessitating significant medical attention. However, rare individuals do experience more severe and/or frequent infections requiring antiviral medications and hospitalization, including those with primary immunodeficiencies (eg, Chédiak-Higashi syndrome, Griscelli syndrome, and X-linked lymphoproliferative syndrome). A subset of people without an identified immunodeficiency also develop serious herpesvirus infections, and elucidation of the mechanisms underlying their susceptibility has the potential to significantly enhance our understanding of viral interactions with the human host.

Natural killer (NK) cells are innate lymphocytes that play a pivotal role in antiviral host defenses [3–6]. NK cell functional responses are tightly regulated by the interplay of signals from a variety of membrane-bound receptors and include the production of cytokines (eg, interferon γ [IFN-γ]) and cytolysis of infected or transformed cells via the release of preformed granules or death receptor–mediated interactions [7, 8]. Human NK cells are CD3−CD56+ lymphocytes and can be further subdivided on the basis of CD56 expression level (CD56dim or CD56bright). The CD56dim population accounts for the majority of peripheral NK cells and is more cytolytically active, whereas the CD56bright population localizes to secondary lymphoid tissue and produces large amounts of cytokines [9, 10].

Although overt deficiencies in NK cell numbers are associated with host susceptibility to herpesviruses [11, 12], abnormal NK cell function has also been reported to be a risk factor for infection with these
pathogens [13–16]. We hypothesized that previously uncharacterized defects in NK cell function may predispose patients to herpesvirus infections. To investigate this hypothesis, we used flow cytometric–based assays to characterize and compare the phenotype and functional responses of NK cells in a cohort of predominantly pediatric patients with recurrent and/or severe herpesvirus infections to those in healthy controls.

**METHODS**

**Patients and Controls**

Eighteen patients (15 pediatric patients and 3 of their parents) with a history of recurrent and/or severe herpesvirus infections were recruited through the St. Louis Children’s Hospital Outpatient Infectious Diseases and Immunology clinics. Severe infections were defined as meningoencephalitis, esophagitis, and mucocutaneous infections severe enough to warrant long-term antiviral therapy. Twenty healthy controls were recruited for comparison. The Human Research Protection Office at the Washington University School of Medicine approved the study in advance, and all subjects (or 1 parent/guardian, if the subject was a minor) gave written informed consent. Subjects with a history of immunosuppressive medication use, malignancy, or known primary or secondary immunodeficiency were excluded.

**Patient Material**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque PLUS (GE Healthcare) gradient. Cells were washed twice with Roswell Park Memorial Institute–10 (RPMI-10; RPMI 1640 medium [Sigma-Aldrich], 10% heat-inactivated fetal bovine serum [hi-FBS; Hyclone], 1% l-glutamine, and 1% penicillin/streptomycin [Invitrogen]), counted, and diluted to required concentrations.

Intraexperiment controls used PBMCs from apheresis unit leukoreduction system (LRS) chambers following routine platelet donations (protocol was adapted from [17]) or fresh whole blood samples obtained from volunteers. LRS samples were diluted (ratio, 1:1) with PBS and, following PBMC isolation by Ficoll gradient, were aliquoted and frozen for later use.

**Cell Lines**

K562 cells (American Type Culture Collection [ATCC] CCL-243) and Jurkat cells (ATCC TIB-152) were grown in RPMI-10. Molt-4 cells (ATCC CRL-1593.2) and Raji cells (ATCC CCL-86) were grown in RPMI-10 supplemented with 1% sodium pyruvate [Invitrogen], 1% nonessential amino acids [Invitrogen], 1% HEPES [Invitrogen], and 4.5 g/L glucose (Sigma-Aldrich). All cells were grown to mid-log phase and frozen in aliquots. For cytotoxicity assays, K562, Jurkat, and Raji cells were labeled with 250 nM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and washed prior to aliquoting and freezing.

**Flow Cytometric Analysis and NK Cell Phenotyping**

Data were collected using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Tree Star). Lymphocytes were identified by forward and side scatter characteristics, and NK cells within the lymphocyte gate were identified with CD3 antibody (UCHT1; Becton Dickinson) and CD56 antibody (N901; Beckman Coulter). NK cells were analyzed after extracellular staining with the following antibodies: CD16 (3G8), CD25 (M-A251), DNAM-1 (DX11), KIR2DL2/3 (CH-L), CD69 (FN50), NKP44 (P44-8.1), and CD11b (ICRF44), from Becton Dickinson; interleukin 15Rα (IL-15Rα; JM7A4), NKG2D (5C6), and 2B4 (C1.7), from eBioscience; and CD94 (131412), ILT-2 (292390), interleukin 12R (69310), interleukin 18R (IL-18R; 132029), KIR3D1 (177407), LAIR-1 (342219), NKP46 (195314), CD122 (27302), KIR2DL1 (143211), NKG2A (131411), NKG2C (134591), NKP30 (210845), and NKP80 (239127), from R&D Systems. PBMCs were fixed and permeabilized using a Cytofix/Cytoperm kit (Becton Dickinson) and then stained with the following antibodies: granzyme A (CB9) and perforin (dG9), from Becton Dickinson, and granzyme B (GB12; Caltag/Invitrogen). Appropriate isotype controls were used. Results were expressed as the percentage of positively staining cells or mean fluorescence intensity (MFI) of cells in the NK cell gate. Except for phenotyping assays, which were conducted with single samples, all experiments were done in duplicate.

**NK Cell Surface Receptor Response to Stimulation With K562 Cells or Interleukin 15 (IL-15)**

A total of $2 \times 10^5$ PBMCs were incubated at 37°C for 24 hours under 3 different stimuli: (1) coculture with K562 cells at an effector-to-target (E:T) ratio of 1:1, (2) media supplemented with 10 ng/mL IL-15 (R&D Systems; IL-15 high), or (3) media supplemented with 2 ng/mL IL-15 (IL-15 low). Samples were then labeled with antibodies against CD25, CD69, IL18Rβ, IL15Rα, ILT-2, and NKP44. The MFI of these cell surface molecules on NK cells was compared to values obtained from unstimulated cells.

**NK Cell Cytotoxicity**

PBMCs were mixed with CFSE-labeled K562 or Jurkat cells ($1 \times 10^5$) at E:T ratios of 40:1, 20:1, and 10:1 and incubated at 37°C for 4 hours. Target cell death was assessed by adding 1 μL of 7-amino-actinomycin d (EMD Chemicals) 4 minutes prior to analysis. Cytolytic activity represented the percentage of target cells that were lysed, with correction for spontaneous background cell death seen in samples with target cells alone.

Raji cells are resistant to NK cell killing and were used in cytotoxicity studies of interleukin 2 (IL-2)–activated NK cells. PBMCs were incubated in RPMI-10 supplemented with IL-2 (100 U/mL; Chiron) at 37°C for 48 hours. Cytotoxicity assays at E:T ratios of 20:1 and 10:1 were performed using the
methods described above. Control cytotoxicity experiments were conducted with unstimulated PBMCs (which had been incubated concurrently in unsupplemented media) at an E:T ratio of 10:1.

**NK Cell Degranulation**
A total of $2 \times 10^5$ PBMCs were incubated with K562, Jurkat, or Molt-4 cells (ratio, 1:1) at 37°C for 4 hours. In addition, PBMCs from 7 subjects were preincubated in RPMI-10 supplemented with IL-2 (100 U/mL) for 24 hours at 37°C prior to incubation with K562 cells (1:1). After 1 hour, monensin (GolgiStop; Becton Dickinson) was added per the manufacturer’s instructions. Following extracellular staining with CD3 and CD56, cells were fixed, permeabilized, and stained with IFN-γ (4S.B3; eBiosciences) antibody. Values were corrected for background staining observed in PBMC samples incubated without target cells.

**Statistical Analysis**
All statistical calculations were conducted with Prism 5 software (GraphPad Software). On the basis of the data distribution, a nonparametric, 2-tailed Mann–Whitney $U$ test or an unpaired, 2-tailed test was used to assess for significant differences between groups. A paired $t$ test was used to compare MFIs between individual patients for the receptors studied in the K562 and IL-15 stimulation assays. Statistically significant differences were defined as those with $P$ value of $< .05$.

**RESULTS**

**Demographic and Clinical Characteristics of Patients**
Eighteen patients with recurrent and/or severe herpesvirus infections and 20 healthy controls (Table 1) were recruited and had their NK cell phenotypes and functional responses analyzed. The mean age ($\pm$SD) of the patient cohort was $17.1 \pm 13.4$ years (median age, 13.2 years). Controls were not significantly older (mean age $\pm$SD, $23.1 \pm 8.9$ years; $P = .11$). The majority of patients and controls were white, and the groups were relatively similar in terms of sex. The majority of patients (78%) had HSV infections with orolabial or facial recurrences. Two patients had recurrent HSV encephalitis, 1 had HSV esophagitis, and 1 had severe human herpesvirus 6 encephalitis. Most patients (94%) were receiving antiviral suppressive therapy with either acyclovir or valacyclovir. Additional immunologic testing performed during the clinical evaluation of each of the patients is noted in Supplementary Table 1.

**Phenotypic Profile of NK Cells From Patients With Recurrent/Severe Herpesvirus Infections**
Although several patients had NK cell percentages that were below the 10th percentile of age-specific normal values (Supplementary Table 2), no significant differences were seen in the mean percentage ($\pm$SD) of NK cells in the peripheral blood of the patient and control groups (4.4% $\pm$ 2.4% and 6.2% $\pm$ 4.3%, respectively; $P = .28$; Figure 1A and 1B). Furthermore, the percentages of CD56$^{bright}$ NK cells in peripheral blood samples from patient and control groups were similar (9.0% $\pm$ 6.7% and 6.5% $\pm$ 3.5%, respectively; $P = .33$; Figure 1C). Extensive extracellular staining for activating and inhibitory receptors, cytokine receptors, and activation markers and intracellular staining for cytolytic components was conducted to characterize the phenotype of NK cells in patients and controls. Most of the 26 molecules analyzed showed no significant difference between groups (Table 2). However, minor but statistically significant differences in expression of 2 receptors (NKG2D and NKp44) and 2 cytotoxic granule constituents (granzyme A and perforin) were observed between patient and control groups. Specifically, NKp44 expression was modestly higher on NK cells in the patient group, while expression of NKG2D, granzyme A, and perforin were each slightly lower.

**NK Cell Activation Following Cytokine or Target Cell Stimulation**
Cytokine stimulation results in NK cell activation, characterized by enhanced cytolytic ability, proliferation, and upregulation of CD25, CD69, and NKp44 [18–23]. Therefore, we evaluated target cell and cytokine-stimulated upregulation of several cell surface receptors and activation markers in patients and controls. PBMCs were incubated for 24 hours with either K562 target cells or IL-15. Upregulation of CD25, CD69, NKp44 (an activating NK cell receptor), ILT-2 (an inhibitory NK cell receptor), IL15Rα, and IL18Rβ was assessed by comparison of the MFIs of these molecules on stimulated and unstimulated NK cells. NK cells from patients and controls showed significant increases in CD25 and CD69 following stimulation (Figure 2A). Both groups also had significant upregulation of ILT-2 and NKp44 following IL-15 stimulation (Figure 2B). However, no statistically significant differences were identified between the cohorts.
NK Cell Functional Responses

To evaluate the cytolytic function of NK cells, PBMCs from patients and controls were incubated with CFSE-labeled target cells over a series of E:T ratios. Experiments were conducted with freshly isolated PBMCs mixed with K562 or Jurkat cells, as well as with PBMCs that were preactivated with IL-2 prior to coculture with NK-resistant Raji cells (Figure 2C and D). With this panel of targets and conditions, no significant differences were found between cohorts.

Cytolytic granule exocytosis was assessed in our cohort using a CD107a degranulation assay. PBMCs were incubated with K562, Jurkat, or Molt-4 cells, and the accumulation of CD107a on the cell surface was analyzed by flow cytometry (Figure 2E). Modest degranulation was observed with all target cell lines, with the most robust degranulation stimulated by K562 cells (mean value [±SD], 12.5% ± 8.4% and 12.0% ± 5.6% in patients and controls, respectively). No significant differences were found between the cohorts. Four patients and 3 controls also had degranulation assays done using PBMCs that had been activated with IL-2 prior to incubation with K562 cells (Figure 2E). CD107a surface accumulation was significantly higher in all subjects when compared to non–cytokine-stimulated NK cells. However, no significant difference was seen between the patients and controls.

IFN-γ is a prominent cytokine produced by NK cells [24] and is critical for controlling viral infections [25]. Analysis of intracellular IFN-γ production following incubation with K562 cells revealed no significant difference between cohorts.

### Table 1. Clinical and Demographic Details of Study Subjects and Controls

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site and Species of Infecting Herpesvirus</th>
<th>Age at Enrollment, y</th>
<th>Race</th>
<th>Sex</th>
<th>Age at First Infection</th>
<th>HSV Type</th>
<th>Suppressive Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Orofacial HSV</td>
<td>12.6</td>
<td>AA</td>
<td>M</td>
<td>7 y</td>
<td>1</td>
<td>V</td>
</tr>
<tr>
<td>2</td>
<td>Genital/facial HSV</td>
<td>5.7</td>
<td>W</td>
<td>M</td>
<td>Neonate</td>
<td>U</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>Orofacial HSV</td>
<td>9.7</td>
<td>W</td>
<td>F</td>
<td>6 mo</td>
<td>U</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>Pericocular HSV</td>
<td>4.3</td>
<td>W</td>
<td>M</td>
<td>2 y</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>Facial/conjunctival HSV</td>
<td>13.0</td>
<td>W</td>
<td>M</td>
<td>7 y</td>
<td>U</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>Oral HSV, recurrent HSV encephalitis</td>
<td>1.0</td>
<td>W</td>
<td>F</td>
<td>7 mo</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>Orolabial HSV</td>
<td>11.6</td>
<td>W</td>
<td>M</td>
<td>8 y</td>
<td>U</td>
<td>A/V</td>
</tr>
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<td>8</td>
<td>HSV esophagitis, EBV mono</td>
<td>19.1</td>
<td>W</td>
<td>M</td>
<td>18 y</td>
<td>1</td>
<td>V</td>
</tr>
<tr>
<td>9</td>
<td>HSV encephalitis</td>
<td>4.0</td>
<td>W</td>
<td>F</td>
<td>2 y</td>
<td>1</td>
<td>A</td>
</tr>
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<td>10</td>
<td>Recurrent herpetic whitlow, orolabial HSV</td>
<td>8.4</td>
<td>W</td>
<td>M</td>
<td>11 mo</td>
<td>U</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>Orolabial HSV, HSV esophagitis, VZV, EBV mono</td>
<td>20.8</td>
<td>W</td>
<td>F</td>
<td>18 y</td>
<td>1</td>
<td>V</td>
</tr>
<tr>
<td>12</td>
<td>Orolabial HSV</td>
<td>9.6</td>
<td>W</td>
<td>F</td>
<td>9 y</td>
<td>1</td>
<td>A</td>
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<tr>
<td>13</td>
<td>HHV-6 encephalitis</td>
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<td>W</td>
<td>M</td>
<td>10 mo</td>
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<td>None</td>
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<tr>
<td>14</td>
<td>Orolabial HSV</td>
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<td>W</td>
<td>F</td>
<td>9 y</td>
<td>1</td>
<td>V</td>
</tr>
<tr>
<td>15</td>
<td>Genital/facial HSV</td>
<td>13.3</td>
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<td>F</td>
<td>5 yrs</td>
<td>U</td>
<td>V</td>
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<tr>
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<td>M</td>
<td>U</td>
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<td>V</td>
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<tr>
<td>17</td>
<td>Orolabial HSV, VZV (x2), EBV mono</td>
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<td>W</td>
<td>F</td>
<td>6 mo</td>
<td>U</td>
<td>V</td>
</tr>
<tr>
<td>18</td>
<td>Orolabial HSV</td>
<td>45.5</td>
<td>W</td>
<td>F</td>
<td>U</td>
<td>U</td>
<td>V</td>
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**Subject summary**

<table>
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<tr>
<th>Age at Enrollment, y</th>
<th>Race</th>
<th>Sex</th>
<th>Age at First Infection</th>
<th>HSV Type</th>
<th>Suppressive Therapy</th>
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<tr>
<td>17.1</td>
<td>AA</td>
<td>M</td>
<td>6%</td>
<td>C 94%</td>
<td>...</td>
</tr>
<tr>
<td>17.1</td>
<td>C 94%</td>
<td>M</td>
<td>50%</td>
<td>F 50%</td>
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**Control summary**

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<th>Age at Enrollment, y</th>
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<th>Sex</th>
<th>HSV Type</th>
<th>Suppressive Therapy</th>
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<tr>
<td>23.1</td>
<td>AA</td>
<td>M</td>
<td>5%</td>
<td>C 75%</td>
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<tr>
<td>23.1</td>
<td>F 65%</td>
<td>M</td>
<td>35%</td>
<td>Other 20%</td>
</tr>
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</table>

Abbreviations: A, acyclovir; AA, African American; EBV, Epstein–Barr virus; F, female; HHV-6, human herpesvirus 6; HSV, herpes simplex virus; M, male; NA, not applicable; U, unknown; V, valacyclovir; VZV, varicella zoster virus; W, white.

a Patients 16, 17, and 18 were parents of patients 2, 4, and 7, respectively.

b “Other” included Asian (2 subjects), Hispanic (1 subject), and biracial (1 subject was African-Asian).

c HSV type was obtained from serum antibody testing or real-time polymerase chain reaction annealing temperature curves available from the patient’s medical records.

d Data are for 20 controls.
Furthermore, no significant differences were seen between the cohorts in experiments in which IL-2 was added during the 8-hour incubation with target cells (Figure 2G).

Individual Patient Abnormalities

Although our analysis revealed no significant global differences between the cohorts, a number of individual patients had novel NK cell abnormalities that might contribute to their susceptibility to severe and/or recurrent herpesvirus infections. These observed functional defects are outlined below.

NK cells from patient 1 expressed CD11b at levels 7 SD below the mean level for the control cohort (Figure 3A). CD11b, αM integrin, and CD18 form a heterodimer, Mac-1, which is 1 of 4 β2-integrin complexes (LFA-1, Mac-1, αxβ2, and αDβ2). CD11b serves as a marker of NK cell maturation [26] and is important for NK cell adhesion, chemotaxis, and cytotoxic effects [27]. This patient also had poor degranulation against K562 cells (Figure 3B) and killing of K562 and Jurkat cells (Figure 3C and D). The remainder of his NK cell functional testing, including degranulation against Jurkat and Molt-4 cells and cytokine-stimulated Raji killing, was normal.

Patient 6 presented at 7 months of age with seizures and bilateral frontal lobe and thalamic subacute infarcts. Polymerase chain reaction (PCR) of cerebrospinal fluid (CSF) was positive for HSV-1. Three months after completing an appropriate course of treatment, she had a second episode of PCR-positive HSV-1 encephalitis, followed by a possible third episode 1.5 years later (results of CSF PCR were negative), shortly after discontinuing prophylactic antiviral medication. Results of extensive immune evaluations were negative, including testing for UNC-93B, TLR3, and TRIF deficiencies [28–31]. Interestingly, her NK cell cytolytic activity against K562 cells was severely depressed 3 weeks after her second episode of encephalitis but had returned to normal when it was reevaluated 7 months later (Figure 3E). Despite negligible K562 cytosis, no other functional deficiencies were noted, although a Jurkat cytolytic assay was not conducted because of sample limitations. However, an inverse correlation was observed between NK cell cytotoxic activity and the percentage of ILT-2+ NK cells. Following an episode of encephalitis, patient 6 had poor NK cell cytolytic activity and a high percentage of ILT-2+ NK cells, while 7 months later, during an asymptomatic period, she was found to have a very low percentage of ILT-2+ NK cells and normal NK cell cytolytic activity (Figure 3F). Unfortunately, NK cells from the second patient in our cohort with HSV encephalitis (patient 9) were analyzed only once, 2 years after her HSV encephalitis episode. At that time, she had normal NK cell cytolytic activity and a low percentage of ILT-2+ NK cells, similar to the follow-up studies involving patient 6 (data not shown).

The NK cells from 3 patients with recurrent orolabial HSV (patients 7, 11, and 18) displayed poor responses to activating stimuli. In particular, exposure of their NK cells to IL-2, a cytokine involved in NK cell activation [9, 32], did not
enhance IFN-γ production (Figure 4A) or Raji cell cytolysis (Figure 4B) to the same extent as observed in controls and other patients. In addition, analysis of NK cells following K562 cell or IL-15 stimulation showed no upregulation of key activation markers (ie, CD25 and CD69) in patients 7 and 18 (Figure 4C), suggesting a more general impairment in NK cell activation in these 2 patients. NK cells from patient 11 responded essentially normally to these non-IL-2-activating stimuli (data not shown). However, in contrast to findings for the other patients and controls, NK cells from patient 11 showed minimal augmentation of degranulation against K562 cells following IL-2 preincubation (Figure 4D; similar degranulation studies were not performed for patients 7 and 18). Also, while not clearly linked to IL-2, impairment in Jurkat cell cytolysis was seen in all 3 patients, although less robustly for patient 7 (Figure 4E). Interestingly, patient 7 is the child of patient 18 and displayed clinical and NK cell functional phenotypes similar to those of his mother. Patient 11 had a more significant herpesvirus history than the 2 related subjects, including severe HSV esophagitis and gingivostomatitis, and her NK cells displayed a more profound defect in IFN-γ production and in cytolysis of Raji cells.

**DISCUSSION**

This study quantitatively evaluated the phenotypic and functional characteristics of NK cells from a cohort of patients with recurrent and/or severe herpesvirus infections and represents one of the largest studies to perform such evaluations in pediatric patients. The majority of patients had HSV-1 infections; however, several had a history of infection with multiple herpesviruses, and 1 had recurrent human herpesvirus 6 encephalitis.

NK cell defects in herpesvirus infections are characterized by diminished IFN-γ production and cytolytic activity. NK cell dysfunction can be assessed using a variety of methods, including flow cytometry, enzyme-linked immunospot (ELISPOT) assays, and spontaneous and induced degranulation assays. Our findings support the hypothesis that persistent exposure to herpesviruses can lead to a state of chronic inflammation, which may affect NK cell function. This is consistent with previous studies showing that chronic activation of NK cells can result in functional impairment due to receptor suppression or cytokine exhaustion. In addition, our results indicate that NK cell dysfunction may be associated with a more generalized impairment in immune function, as evidenced by the decreased expression of activation markers in patients 7 and 18.

**Table 2. Extracellular Receptor and Cytotoxic Granule Enzyme Levels of Natural Killer Cells in Patients With Recurrent and/or Severe Herpesvirus Infections and Healthy Controls**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD11b</th>
<th>CD16</th>
<th>CD25</th>
<th>CD69</th>
<th>CD94</th>
<th>CD122</th>
<th>KIR2DL1</th>
<th>KIR2DL2/3</th>
<th>KIR3DL1</th>
</tr>
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<tbody>
<tr>
<td>Patients</td>
<td>93.4 (7.3)</td>
<td>86.2 (8.1)</td>
<td>4.4 (3.2)</td>
<td>32.6 (21.1)</td>
<td>71.0 (12.9)</td>
<td>66.6 (11.5)</td>
<td>11.4 (8.9)</td>
<td>28.6 (11.1)</td>
<td>29.2 (16.1)</td>
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<tr>
<td>Controls</td>
<td>95.3 (3.7)</td>
<td>90.1 (7.5)</td>
<td>3.2 (2.7)</td>
<td>41.8 (20.0)</td>
<td>75.2 (9.5)</td>
<td>67.2 (14.4)</td>
<td>12.5 (7.7)</td>
<td>29.9 (7.3)</td>
<td>31.6 (14.0)</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td>Patients</td>
<td>47.7 (17.5)</td>
<td>48.4 (18.6)</td>
<td>71.4 (11.7)</td>
<td>68.6 (21.5)</td>
<td>15.5 (6.2)</td>
<td>81.3 (5.0)</td>
<td>82.7 (7.2)</td>
<td>95.4 (4.2)</td>
<td>83.3 (9.5)</td>
</tr>
<tr>
<td>Controls</td>
<td>56.2 (19.4)</td>
<td>55.0 (19.3)</td>
<td>78.7 (11.3)</td>
<td>67.9 (23.8)</td>
<td>11.3 (5.3)</td>
<td>73.8 (17.2)</td>
<td>85.9 (4.6)</td>
<td>96.7 (3.7)</td>
<td>85.0 (8.4)</td>
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<tr>
<td>P</td>
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<td>.045</td>
<td>.033</td>
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</tr>
<tr>
<td>Patients</td>
<td>11.9 (10.5)</td>
<td>94.3 (3.9)</td>
<td>31.6 (11.1)</td>
<td>13.7 (15.1)</td>
<td>17.0 (20.0)</td>
<td>78.2 (9.4)</td>
<td>94.6 (4.3)</td>
<td>87.0 (19.8)</td>
<td></td>
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<tr>
<td>Controls</td>
<td>16.6 (11.5)</td>
<td>94.2 (4.4)</td>
<td>33.3 (13.7)</td>
<td>13.0 (13.6)</td>
<td>22.7 (25.7)</td>
<td>83.7 (11.8)</td>
<td>96.1 (3.0)</td>
<td>94.8 (4.0)</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>.048</td>
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</table>

Data are mean (SD) and represent the average percentage of NK cells staining positive for each noted molecule in the patient cohort (n = 18) and the control cohort (n = 20). Comparison of patient and control populations was calculated using a 2-tailed Mann-Whitney U test. When significant (P < .05), P values are provided.

Abbreviations: GZMA, granzyme A; GZMB, granzyme B; IL-12R, interleukin 12R; IL-15Rα, interleukin 15Rα; IL-18R, interleukin 18R; NS, not significant.

* Peripheral blood mononuclear cells were fixed and permeabilized for intracellular staining of granzymes and perforin in the patient cohort (n = 17) and the control cohort (n = 20).
hantavirus [38], and chikungunya virus [39] infections. Of note, we did not observe a differential expansion of NKG2C+ NK cells between our patient cohort and controls; however, cytomegalovirus serologic status was not assessed in our subjects.

Despite the absence of global defects in NK cell function in our cohort, 5 patients had specific NK cell abnormalities that might contribute to their herpesvirus susceptibility. We identified an individual with low NK cell expression of CD11b associated with diminished NK cell cytotoxicity, borderline degranulation, and severe orolabial and facial HSV-1 reactivations. Complete $\beta_2$-integrin complex deficiency results in leukocyte adhesion deficiency I. Interestingly, impaired NK cell cytotoxicity of HSV-infected cells has been reported in this...
In contrast to the total loss of β₂-integrin complexes in leukocyte adhesion deficiency I [41], nearly 70% of our patient's NK cells were positive for CD11b. Decreased CD11b expression has been reported in chronic NK cell lymphocytosis [42, 43], where functional studies have shown decreased NK cell cytotoxicity [43]. Moreover, murine models of chronic NK cell lymphocytosis [44] and β₂-integrin deficiency [45] support an association between decreased CD11b expression and accumulation of immature NK cells with functional hyporesponsiveness. The decreased expression of CD11b in our patient appears to reflect a maturation block in NK cell development associated with decreased degranulation and cytolyis, although it is possible that low CD11b expression directly contributed to his NK cell functional impairment (via loss of cell-cell adhesion and downstream signaling events).

Unfortunately, this patient was lost to follow-up, and we were unable to perform further analysis of his NK cells.

Poor NK cell function was also noted in the context of hyporesponsiveness to activating stimuli in 3 patients, 2 of whom were a mother and son with similar clinical and NK cell functional phenotypes. In addition to poor enhancement of cytolysis and IFN-γ production following IL-2 stimulation, NK cells from these first-degree relatives also failed to upregulate activation markers following exposure to IL-15, an essential cytokine for NK cell development and homeostasis [46, 47]. Given the central roles of IL-2 and IL-15 in NK cell physiology, insufficient responses to either of these cytokines could contribute to these patients' vulnerability to HSV. The third patient in this subset had a more extensive herpesvirus history, and her NK cells displayed a more...
A profound defect in IL-2 stimulation, suggesting that her herpesvirus susceptibility was associated with defective IL-2 responsiveness.

NK cells from a patient with recurrent HSV encephalitis revealed an interesting phenotypic and functional correlation, wherein cells evaluated shortly after an episode of HSV-1 encephalitis were ILT-2hi and had poor K562 cell cytolysis, whereas NK cells obtained 7 months later were ILT-2lo with normal cytolytic function. ILT-2 (LILRB1/LIR-1/CD85j) is an inhibitory receptor that recognizes classic and nonclassic major histocompatibility complex class I molecules and UL18, a viral ligand produced by cytomegalovirus [48, 49]. Interestingly, following incubation with HIV-infected dendritic cells, NK cells downregulated ILT-2 [50]. It is possible that a robust inhibitory signal generated by ILT-2 engagement in the context of HSV-1 infection may have contributed to the abnormal killing that was observed in patient 6, although we have no direct evidence to support this hypothesis.

Although 94% of our patient cohort experienced recurrent or severe HSV infections, there was heterogeneity in their infections that may have limited our ability to identify global defects in the cohort. Another potential limitation was the fact that the NK cells analyzed in this study were from peripheral blood rather than from affected tissues (eg, facial skin and nerves, esophagus, and brain), which might have been more informative. However, obtaining adequate samples from these areas is impractical.

In summary, this study identified no global differences in target cell killing, degranulation, IFN-γ production, or stimulated surface receptor upregulation in patients with recurrent and/or severe herpesvirus infections. Minor differences of unclear clinical significance were seen at the cohort level in

![Figure 4](https://academic.oup.com/jid/article-abstract/207/3/458/876349)
the basal expression of NKG2D, NKP44, granzyme A, and perforin. However, we identified potentially relevant NK cell functional and phenotypic abnormalities in 28% of patients (5 of 18) with recurrent and severe herpesvirus infections. These findings suggest that a substantial fraction of patients with particularly severe and/or recurrent herpesvirus infections may have previously uncharacterized functional NK cell deficits.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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