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# Role of Specific CD8$^+$ T Cells in the Severity of a Fulminant Zoonotic Viral Hemorrhagic Fever, Hantavirus Pulmonary Syndrome[1]

**Elizabeth D. Kilpatrick,* Masanori Terajima,* Frederick T. Koster,‡ Michelle D. Catalina,† John Cruz,* and Francis A. Ennis[2]***

**We report on the role of specific CD8$^+$ T cells in the pathogenesis of a highly lethal human viral disease, hantavirus pulmonary syndrome (HPS). HPS is a zoonotic disease caused by transmission of Sin Nombre virus (SNV) from chronically infected deer mice. In humans, this fulminant infection is characterized by lung capillary leakage, respiratory failure, and cardiogenic shock. Individuals with HLA-B*3501 have an increased risk of developing severe HPS, suggesting that CD8$^+$ T cell responses to SNV contribute to pathogenesis. We identified three CD8$^+$ T cell epitopes in SNV presented by HLA-B*3501 and quantitated circulating SNV-specific CD8$^+$ T cells in 11 acute HPS patients using HLA/peptide tetramers. We found significantly higher frequencies of SNV-specific T cells in patients with severe HPS requiring mechanical ventilation (up to 44.2% of CD8$^+$ T cells) than in moderately ill HPS patients hospitalized but not requiring mechanical ventilation (up to 9.8% of CD8$^+$ T cells). These results imply that virus-specific CD8$^+$ T cells contribute to HPS disease outcome. Intense CD8$^+$ T cell responses to SNV may be induced by the encounter of the unnatural human host to this zoonotic virus without coevolution. This may also be the immunopathologic basis of other life-threatening human virus infections.** ***The Journal of Immunology,*** **2004, 172: 3297–3304.**

In contrast to certain animal models of acute viral infections (1–4), there is little information about the roles of CD8$^+$ T cells in the pathogenesis of severe or fatal acute viral diseases in humans. Elevated levels of virus-specific CD8$^+$ T cells have been reported during the acute infection phase of a few primary human viral infections including EBV (5), HIV (6), hepatitis C virus (HCV)[3] (7), and hepatitis B virus (HBV) (8). However, these virus infections are rarely fatal during the acute phase and generally establish lifelong chronic infections. Very recently, patients with secondary dengue virus (DV) infections were reported to have circulating levels of virus-specific CD8$^+$ T cells that correlated with disease severity (9). We hypothesize that virus-specific CD8$^+$ T cells are important in the pathogenesis of viral hemorrhagic fevers, many of which are primary fulminant zoonotic infections with high case fatality rates in humans. Examples include hantavirus pulmonary syndrome (HPS) caused by Sin Nombre virus (SNV) (10), Lassa fever caused by Lassa virus (11), and Ebola hemorrhagic fever caused by Ebola virus (12). Similar mechanisms may also play a role in the pathogenesis of other severe zoonotic virus infections with high case fatality rates in humans such as severe acute respiratory syndrome caused by a newly identified coronavirus (13). In the present report, we quantitate CD8$^+$ epitope-specific T cells in HPS patients during acute primary infection with SNV.

SNV is an emerging human pathogen that causes a chronic, asymptomatic infection in its natural host, the deer mouse (*Peromyscus maniculatus*). SNV is spread to humans following exposure to infected rodents. Zoonotic SNV infections resulting in HPS have been documented in 353 humans in North America since its discovery in 1993 (www.cdc.gov/ncidod/diseases/hanta/hps/noframes/epislides/episl6.htm). In contrast to rodents, human SNV infection causes HPS, an acute fulminant illness, and is quickly cleared. The HPS case fatality rate is ~40%, and no antiviral therapy is available (10). SNV primarily infects human lung endothelial cells (14, 15). Clinically, HPS is characterized initially by nonspecific flu-like symptoms and thrombocytopenia rapidly followed by a capillary leak syndrome focused in the lung. In severe cases, patients experience extreme pulmonary distress and cardiogenic shock requiring mechanical ventilation and occasionally extracorporeal membrane oxygenation (ECMO) therapy (16).

Although SNV is one of the most lethal of acute human viral infections, the mechanisms of HPS pathology are poorly understood. Both in vivo (14, 15) and in vitro (17, 18) observations suggest that SNV is not directly cytopathic to infected cells. Instead, immunopathological mechanisms involving CTLs have been suggested (19). Lung tissues obtained at necropsy from HPS patients contain abundant large immunoblasts consisting of CD4$^+$ and CD8$^+$ T cells (14, 15), and high numbers of cytokine-producing cells (20). Several of the cytokines detected in the lung tissues, including TNF-$\alpha$, IL-2, and IFN-$\gamma$, are produced by T cells and could mediate capillary leakage. In addition, preliminary evidence suggests that, in SNV infection, HLA-B*3501 is associated with severe HPS, implying involvement of CD8$^+$ T cells (21). These observations led us to hypothesize that quantitative and/or qualitative aspects of SNV-specific T cell responses contribute to the pathology of HPS.

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; HBV, hepatitis B virus; DV, dengue virus; HPS, hantavirus pulmonary syndrome; SNV, Sin Nombre virus; ECMO, extracorporeal membrane oxygenation; B-LCL, B lymphoblastoid cell line; YFV, yellow fever virus; DHF, dengue hemorrhagic fever.



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A system is established at the University of New Mexico Health Science Center to facilitate the clinical management and research in patients who are hospitalized for moderate or severe HPS. In the present study, we collected PBMC samples from HPS patients at first presentation for medical care and during hospitalization to study cellular immune responses to SNV. We defined three HLA-B*3501-restricted CD8$^+$ T cell epitopes in SNV. Using HLA-B*3501/peptide tetramers to quantitate CD8$^+$ T cell responses to three SNV epitopes in PBMC from 11 HLA-B*3501$^+$ HPS patients during acute infection, we demonstrate very high frequencies of SNV-specific CD8$^+$ T cells compared with the frequencies reported in the literature in other acute human viral infections (22). Furthermore, the magnitude of virus-specific T cell responses were significantly higher in the patients with clinically severe HPS (patients who met clinical criteria requiring mechanical ventilation) than in patients with moderate disease (hospitalized but not requiring mechanical ventilation), implying that SNV-specific CD8$^+$ T cells contribute to HPS disease outcome.

## Materials and Methods

### *HPS patient sample collection*

Patients were recruited for this study from those referred to University of New Mexico Health Science Center for the treatment of suspected HPS. Informed consent was obtained from the patients, and the human experimentation guidelines of the U.S. Department of Health and Human Services and the authors' institutions were followed in the conduct of this research. Clinical diagnosis of HPS was confirmed by serology (23). PBMC were separated from samples of arterial blood using Hypaque-Ficoll, and cryopreserved at −70°C. HLA typing was performed using the HLA-B High Resolution Typing System (Applied Biosystems, Foster City, CA).

HPS disease severity was defined using the following criteria. HPS is considered severe if the patient experiences pulmonary edema severe enough to warrant mechanical ventilation (ratio of partial pressure of arterial oxygen/fraction of inspired oxygen of <100 (16)). Many of these patients also exhibit decreased cardiac function and shock, warranting the use of ECMO therapy. Eight patients in this study met the clinical criteria for severe HPS, and two of the eight died. HPS is considered moderate if the patient requires hospitalization and experiences mild pulmonary edema requiring nasal $O_2$ therapy, but not mechanical ventilation. Three patients in this study met the criteria for moderate HPS. HPS is considered mild if the patient experiences fever and prodrome, but has no pulmonary edema on chest x-ray. Only one HPS patient with HLA-B*3501$^+$ has been documented with mild disease, but PBMC samples from that patient were not available for the present study. SNV seroprevalence is very low, even among high-risk populations in endemic areas (24), suggesting that subclinical infections are rare.

HPS is a rare disease that occurs sporadically in rural areas; nevertheless, we were able to study ~50% of both the severe (8 of 17 cases) and moderate (3 of 6 cases) cases of HPS in individuals with HLA-B35.

### *Identification of SNV epitopes and cell culture*

T cell epitopes within SNV proteins were identified as has been described (19). Briefly, PBMC were cultured and stimulated nonspecifically every 2 wk with an anti-CD3 mAb (12F6; kindly supplied by Dr. J. Wong (Harvard Medical School, Boston, MA)). Limiting dilution cloning was performed, and lines were initially screened in CTL assays using autologous B lymphoblastoid cell lines (B-LCLs) infected with recombinant vaccinia viruses expressing the SNV N, G1, or G2 proteins (19, 25). The SNV epitopes were defined in CTL assays using B-LCL target cells pulsed with synthetic peptides. HLA restriction of isolated CTL lines was determined using partially HLA-matched allogenic B-LCLs as target cells.

### *HLA-peptide tetramers*

The HLA/peptide tetramers were generated according to the method originally described by Altman et al. (26). The HLA-B*3501 and human $\beta_2$-microglobulin cDNA constructs used in this study were generously provided by Dr. M. Takiguchi (Kumamoto University, Kumamoto City, Japan), Dr. A. McMichael (University of Oxford, Oxford, U.K.), and Dr. D. Garboczi (National Institutes of Health, Rockville, MD).

For analysis by flow cytometry, the PBMC samples were thawed, washed, and immediately stained with tetramer and FcR blocking reagent (Miltenyi Biotec, Auburn, CA) at room temperature for 20 min. Cells were then stained with mAbs to CD3, CD4, CD8, and CD38 (BD PharMingen, San Diego, CA) at 4°C for 30 min, washed, and fixed with paraformaldehyde. Flow cytometry was performed using a FACSVantage (BD Biosciences, San Jose, CA) flow cytometer at the University of Massachusetts Medical Center Flow Cytometry core facility.

### *Statistical analysis*

We compared the frequencies of SNV-specific CD8$^+$ T cells between severe HPS and moderate HPS patient groups using the Wilcoxon rank sum test. Values of $p < 0.05$ were considered significant.

## Results

### *Identification of T cell epitopes in SNV*

We have previously defined three SNV epitopes recognized by human CD8$^+$ and CD4$^+$ T cells by cloning SNV-specific CTL lines (19). We now report two additional CD8$^+$ T cell epitopes in the SNV G2 protein (aa 664–673 and aa 746–755) presented by HLA-B*3501. CTL lines were generated from acute PBMC samples from two HPS patients, line NM7 3.1 and line NM3 10c27, by limiting dilution cloning at 3 or 10 cells per well. We initially performed CTL assays using autologous B-LCLs infected with recombinant vaccinia viruses expressing SNV proteins. Subsequently overlapping 20-mer peptide-pulsed target cells were used, and these lines were specific for two separate epitopes within the SNV G2 protein (localized to aa 745–764 and 657–676, respectively). The precise epitopes were defined in further CTL assays using autologous B-LCLs pulsed with truncated peptides at several titrations (Table I). Interestingly, three of the four CD8$^+$ T cell epitopes in SNV that we have defined to date are presented by HLA-B*3501 (Table II). The epitopes fit the HLA-B*3501 binding motif, which includes anchor residues at position 2 (proline, alanine, or valine) and the C terminus (tyrosine, phenylalanine, methionine, leucine, or isoleucine) (27). When comparing SNV to other hantaviruses (Table II), there is little variation at the HLA-B*3501 anchor residues in the regions of these three epitopes. Outside the anchor residues, these regions are also well conserved, especially among the New World hantaviruses that cause disease.

### *Quantitation of SNV-specific CD8$^+$ T cells in PBMC samples*

We generated HLA/peptide tetramers for the three SNV epitopes presented by HLA-B*3501, and a negative control tetramer using an epitope in the yellow fever virus (YFV) NS1 protein that is presented by HLA-B*3501 (28). The specificity of each tetramer was confirmed by flow cytometry using SNV- and YFV-specific CD8$^+$ CTL lines, and by staining PBMC from an HLA-B3501$^+$ individual with no known exposure to either SNV or YFV (Fig. 1). We used the tetramers to directly quantitate the CD8$^+$ T cells specific for each epitope in the PBMC from 11 patients with acute HPS. PBMC samples were obtained within 1 day of the onset of pulmonary edema from all but one patient. In the 11 patients, between 2.9 and 44.2% of the CD8$^+$ T cells were specific for the three SNV epitopes in combination (Fig. 2, Table III). We also stained the PBMC samples for CD38 expression to determine the activation state of the tetramer-positive cells. CD38 is a surface glycoprotein expressed on many cell types including mature T cells during activation (29). In the HPS patients, most of the tetramer-positive cells were also CD38$^+$ (Fig. 2, *B* and *C*), indicating an activated phenotype. In nearly all of the patients studied early during illness, the frequencies of CD8$^+$ T cells specific for each of the two epitopes within the SNV G2 protein were higher than for the epitope within the SNV N protein, but the hierarchy varied from patient to patient (Table III). Acute plasma samples were available for determining viremia levels by quantitative RT-PCR from 8 of the 11 patients. Viremia levels ranged from $10^{4.9}$ to $10^{8.0}$

Table I. *SNV epitope identification: specificity and peptide dose-response analysis*

| Peptide Amino Acid Sequence | 25 μg/ml | 2.5 μg/ml | 0.25 μg/ml | 25 ng/ml | 2.5 ng/ml | 0.25 ng/ml | 25 pg/ml |
|---|---|---|---|---|---|---|---|
| Cell line NM7 3.1 (SNV G746-755 specific) | | | | | | | |
| YPWQTAKCFFEK | 94.3[a] | 71.4,[a] 67.8[b] | 20.5,[a] 23.9[b] | −0.4[a] | | | |
| WQTAKCFFEKDY | 1.3[a] | 2.0,[a] 0.9[b] | 1.1,[a] −0.9[b] | −2.4[a] | | | |
| PWQTAKCFFEK | | 2.4[b] | 1.4[b] | | | | |
| YPWQTAKCFFEK | 72.4[a] | 31.4,[a] 38.6[b] | 3.3[a], 6.3[b] | −2.2[a] | | | |
| YPWQTAKCFFEK | 83.8[a] | 45.7[a] 37.7[b] | 7.2,[a] 5.6[b] | 2.8[a] | | | |
| (−) | −1.5,[a] −1.2[b] | | | | | | |
| Cell line NM3 10c27 (SNV G664-673 specific) | | | | | | | |
| MESGWSDTAHGVGIIPMKTD | 98.9,[c] 102.7,[d] 76.4[e] | | | | | | |
| ESGWSDTAHGVGII | −1.1[c] | | | | | | |
| WSDTAHGVGIIPMK | 92.3[c] | | | | | | |
| TAHGVGIIPMKTD | 81.7[c] | | | | | | |
| TAHGVGIIP | 93.5,[d] 63.4[e] | 64.5[e] | 56.0[e] | 23.5[e] | 10.0[e] | −2.1[e] | −3.6[e] |
| AHGVGIIPM | 99.8,[d] 56.8[e] | 65.5[e] | 69.1[e] | 45.6[e] | 9.7[e] | 0.1[e] | −2.7[e] |
| HGVGIIPMK | 2.5[d] | | | | | | |
| (−) | 2.6,[c] 1.6,[d] −2.2[e] | | | | | | |

[a,b,c,d,e] Separate experiments.

viral RNA copies/ml of plasma in the 7 patients who had detectable levels (30), but did not correlate with either disease severity nor the tetramer frequencies in this limited study (Table III), which may be due to the small number of patients analyzed.

### *Kinetic analysis of T cell responses and clinical parameters of disease*

For two of the HPS patients, we were able to collect PBMC at three or more time points during hospitalization, thereby allowing a kinetic analysis of the T cell responses to SNV. In these patients, we compared the kinetics of the T cell responses to two clinical parameters of disease, hematocrit and platelet counts (Fig. 3). Elevated hematocrit is an indication of hemoconcentration and capillary leakage, and pronounced thrombocytopenia is an early and consistent laboratory abnormality in the early stage of HPS; both of these clinical parameters correlate with HPS disease severity (31).

For patient NM21 (Fig. 3*A*), the peak hematocrit (55.5%) and platelet count nadir ($31 \times 10^3/\mu$l) coincided with the onset of respiratory distress and shock (day 0). This patient was placed on mechanical ventilation and ECMO therapy on day +1. Viremia was measured by RT-PCR (30) at two acute time points: $10^{8.0}$ viral RNA copies/ml of plasma were detected on day −1, and viremia was below the limit of detection ($<10^5$ copies/ml) on day +9. PBMC samples were obtained from this patient on days +1, +2, +8, and +9. The percentages of $CD8^+$ T cells specific for the three combined SNV epitopes measured by tetramer staining were 11.9, 15.4, 10.9, and 8.4% at these time points, respectively.

For patient NM26 (Fig. 3*B*), the peak hematocrit (54.6%) and platelet count nadir ($30 \times 10^3/\mu$l) occurred on days +1 and +2, respectively. The patient met the criteria for requiring mechanical ventilation beginning on day +2, but declined this treatment. Viremia was measured on day +1, at which time $10^{8.0}$ viral RNA copies/ml of plasma were detected. The percentages of $CD8^+$ T cells specific for the three combined SNV epitopes measured by tetramer staining were 7.4, 9.4, and 14.0% on days +1, +2, and +5, respectively.

### *Comparison of T cell responses in patients with severe vs moderate HPS*

Next, we wished to determine whether the frequency of tetramer-positive cells in the PBMC samples correlated with disease severity in the 11 HPS patients. For this analysis, the HPS patients were characterized as having either severe (meeting clinical criteria requiring mechanical ventilation) or moderate (hospitalized but not requiring mechanical ventilation) disease as described in *Materials and Methods*. For the statistical analyses, we used the combined frequency of $CD8^+$ T cells specific for the three SNV epitopes measured. First, we compared the combined SNV frequencies observed at the earliest available time point during hospitalization in the 10 patients from whom PBMC were collected within 24 h of the onset of pulmonary edema (Fig. 4*A*). The frequencies were significantly higher ($p < 0.05$) in the severe HPS patients (7.4–44.2%) than in the moderate HPS patients (2.9–9.8%). Second, we

Table II. *SNV T cell epitopes presented by HLA-B*3501 and conservation among hantaviruses*[a]

| Virus | Epitope G746-755 | Epitope G664-673 | Epitope N131-139 | GenBank Accession Nos. |
|---|---|---|---|---|
| Sin Nombre | YPWQTAKCFF | TAHGVGIIPM | LPIILKALY | L25783, L25784 |
| New York | ---------- | -------V-- | I--------- | U36801, U09488 |
| Black Creek Canal | ---------- | ------D--- | ---V----- | L39950, L39949 |
| Bayou | -----T---- | ------D--- | ---V----- | L36930, L36929 |
| Andes | -----S---- | ------E--- | I--------- | NC_003467, NC_003466 |
| Prospect Hill | ---------L | ----A-V-L- | --------H | X55129, M34011 |
| Hantaan | ---H----HY | N------SV- | V-L------ | M14627, M14626 |
| Seoul | ---H----H- | N------SV- | V--------- | M34882, M34881 |
| Puumala | ------G--V | -----S---- | ---------- | X61034, X61035 |
| Dobrava | ---H----H- | N------VV- | V-L------ | L33685, L41916 |

[a] Amino acid letters indicate variation from SNV; dashes indicate conservation.

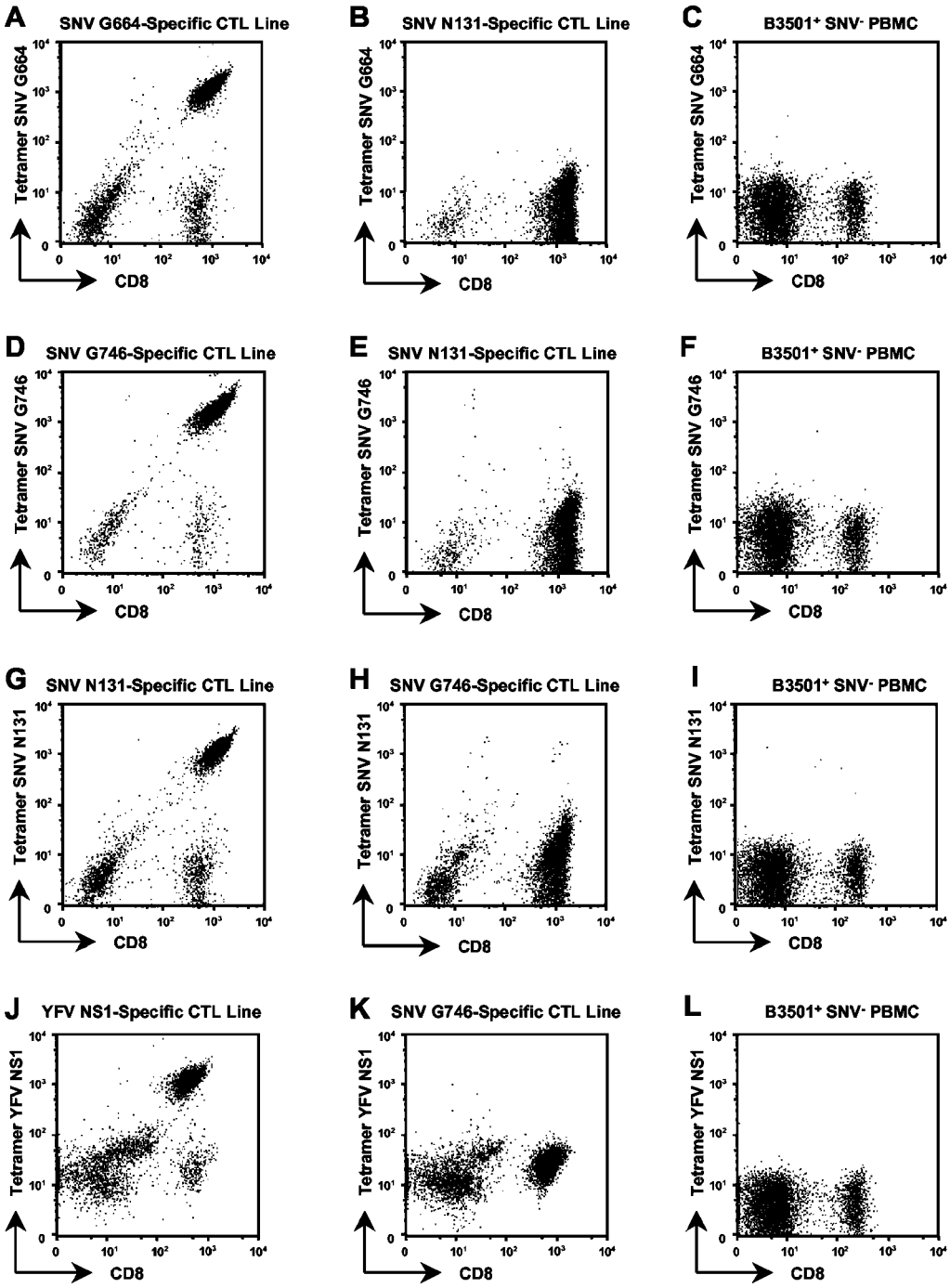


**FIGURE 1.** Demonstration of the specificity of the HLA-B*3501/peptide tetramers. HLA-B*3501-restricted CD8$^+$ CTL lines specific for SNV or YFV epitopes, and PBMC from an HLA-B*3501$^+$ control individual with no known exposure to SNV or YFV were stained with the four tetramers used in this study. *A–C*, Tetramer SNV G664 staining of line NM3 10c27 (G664 specific), line NM5 10.3 (N131 specific; Ref. 19), and control PBMC. *D—F*, Tetramer SNV G746 staining of line NM7 3.1 (G746 specific), line NM5 10.3 (N131 specific), and control PBMC. *G—I*, Tetramer SNV N131 staining of line NM5 10.3 (N131 specific), line NM7 3.1 (G746 specific), and control PBMC. *J—L*, Tetramer YFV NS1 staining of line YF 3.14 (YFV NS1 specific), line NM7 3.1 (G746 specific), and control PBMC.

compared the peak of the combined frequencies observed at any available time point during hospitalization in all 11 patients (Fig. 4*B*). When using these values, the frequencies were also significantly higher ($p < 0.05$) in the severe HPS patients (10.4–44.2%) than the moderate HPS patients (4.8–9.8%).

## Discussion

Our results demonstrate very high frequencies of SNV-specific CD8$^+$ T cells (up to 25% specific for a single epitope; Fig. 2) in the circulation of HPS patients during the acute phase of this zoonotic infection. Furthermore, in PBMC samples obtained early in disease, the frequencies of SNV-specific T cells were significantly higher in the patients with severe HPS (up to 44.2% of CD8$^+$ T cells) than in patients with moderate disease (up to 9.8% of CD8$^+$ T cells). These results are consistent with previous studies showing increased numbers of CD8$^+$ T cells (14, 15), and increased numbers of TNF-$\alpha$-, IL-2-, and IFN-$\gamma$-producing cells (20) in the lungs of patients who died from HPS, and support our hypothesis that

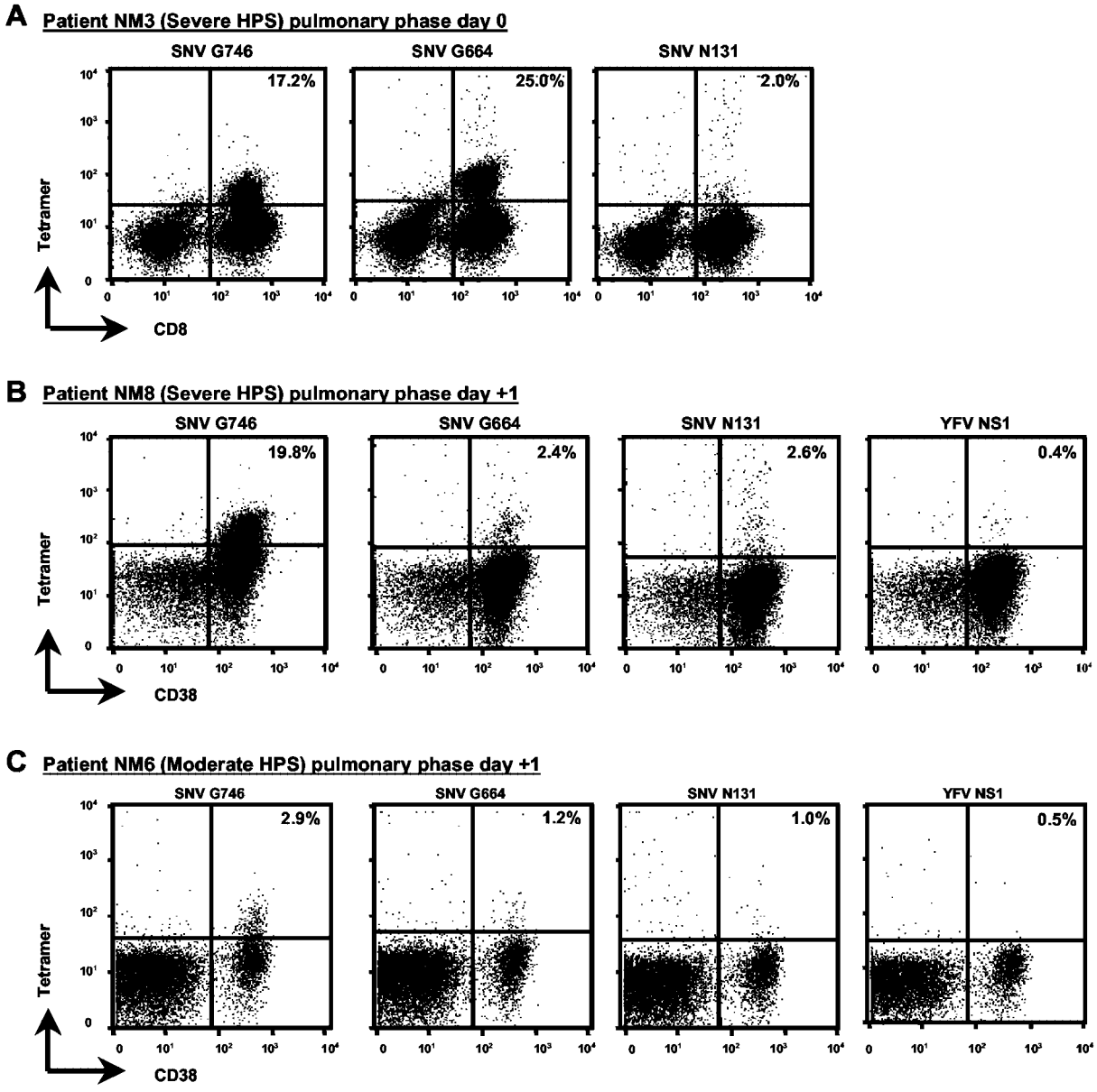


**FIGURE 2.** High frequencies of activated SNV-specific $CD8^+$ T cells in the PBMC of acute HPS patients. *A*, Tetramer and CD8 staining of PBMC from acute severe HPS patient NM3, gated on $CD3^+$ lymphocytes. *B*, Tetramer and CD38 staining of PBMC from acute severe HPS patient NM8, gated on $CD8^+CD3^+$ lymphocytes. *C*, Tetramer and CD38 staining of PBMC from acute moderate HPS patient NM6, gated on $CD8^+CD3^+$ lymphocytes. In all plots, the frequencies indicate the percentage of $CD8^+CD3^+$ lymphocytes that stain positively with the tetramer listed above the plot.

virus-specific $CD8^+$ T cells contribute to HPS disease outcome. This is the first report quantitating virus-specific T cells during an acute hantavirus infection. Previously, we demonstrated the presence of high levels of virus-specific memory T cells up to 15 years postinfection for Puumala virus, an Old World hantavirus that generally causes mild kidney disease and is subsequently cleared (32).

Experimental infection models in mice have proven invaluable in the study of T cell responses to viruses. The murine lymphocytic choriomeningitis virus model is perhaps the best studied (1, 2, 4), and in this model, up to 70% of $CD8^+$ T cells in the spleen are virus specific during primary infection (3), and $CD8^+$ T cell-mediated immunopathology has been demonstrated after intracerebral inoculation of lymphocytic choriomeningitis virus in mice (33). However, SNV does not cause disease in infected mice. Some strains of New World hantaviruses, although not SNV, have been shown to cause disease in Syrian golden hamsters (34, 35), but few immunologic reagents exist for studying hamsters. Thus, it is necessary to study HPS in patients infected with SNV. Due to the fact that HPS is a rare disease that occurs sporadically in remote rural areas, progresses rapidly, and has a high mortality rate, the window of time for obtaining patient specimens can be as short as a few hours. It was a major challenge to obtain PBMC from a reasonable number of HPS patients with HLA-B35 for these studies. In addition, because 71% of HPS patients with HLA-B35 have severe disease (F. T. Koster, unpublished data), it was even more difficult to obtain samples from $HLA\text{-}B35^+$ patients with moderate disease. Nevertheless, the individuals described in this study represent ~50% of the $HLA\text{-}B35^+$ defined patients with HPS in this endemic area as described in *Materials and Methods*.

In recent years, virus-specific $CD8^+$ T cells have been quantified using HLA/peptide tetramers during the acute phase of several human viral infections including EBV, HIV, HCV, HBV, vaccinia

Table III. *Quantitation of CD8$^+$ T cell responses to individual SNV epitopes*

| Donor | Disease Severity | Pulmonary Phase Day | Viremia[a] (Copies/ml) | % SNV[b] G746 | % SNV[b] G664 | % SNV[b] N131 | % Combined[b] SNV |
|---|---|---|---|---|---|---|---|
| NM30 | Severe (fatal) | +1 | | 5.7 | 3.8 | 0.9 | 10.4 |
| NM31 | Severe (fatal) | 0 | | 4.0 | 7.0 | 0.3 | 11.3 |
| | | +1 | | 5.2 | 7.1 | 0.2 | 12.5 |
| NM3 | Severe | 0 | $10^{4.9}$ | 17.2 | 25.0 | 2.0 | 44.2 |
| NM5 | Severe | +1 | $10^{4.9}$ | | | | |
| | | +10 | ND | | | | |
| | | +24 | | 2.9 | 1.3 | 7.4 | 11.6 |
| NM21 | Severe | −1 | $10^{8.0}$ | | | | |
| | | +1 | | 4.2 | 6.4 | 1.3 | 11.9 |
| | | +2 | | 5.1 | 8.1 | 2.2 | 15.4 |
| | | +8 | | 4.1 | 4.5 | 2.3 | 10.9 |
| | | +9 | ND | 3.4 | 3.4 | 1.6 | 8.4 |
| NM2 | Severe | +1 | $10^{5.3}$ | 7.6 | 1.7 | 1.2 | 10.5 |
| | | +18 | ND | 3.4 | 0.7 | 9.0 | 13.1 |
| NM8 | Severe | +1 | $10^{4.9}$ | 19.8 | 2.4 | 2.6 | 24.8 |
| | | +5 | ND | 4.2 | 0.9 | 0.9 | 6.0 |
| NM26 | Severe | +1 | $10^{8.0}$ | 3.2 | 3.3 | 0.9 | 7.4 |
| | | +2 | | 6.1 | 2.8 | 0.5 | 9.4 |
| | | +5 | | 7.7 | 5.1 | 1.2 | 14.0 |
| NM7 | Moderate | +1 | $10^{7.3}$ | 1.5 | 4.7 | 3.6 | 9.8 |
| | | +5 | ND | | | | |
| | | +195 | | 5.2 | 1.1 | 1.2 | 7.5 |
| NM32 | Moderate | 0 | ND | | | | |
| | | +1 | | 0.5 | 2.1 | 0.3 | 2.9 |
| | | +2 | | 2.0 | 2.5 | 0.3 | 4.8 |
| NM6 | Moderate | +1 | | 2.9 | 1.2 | 1.0 | 5.1 |

[a] Reported in Ref. 30; ND, Not detectable.
[b] Frequencies indicate the percentage of CD8$^+$CD3$^+$ lymphocytes that stain positively with each tetramer.

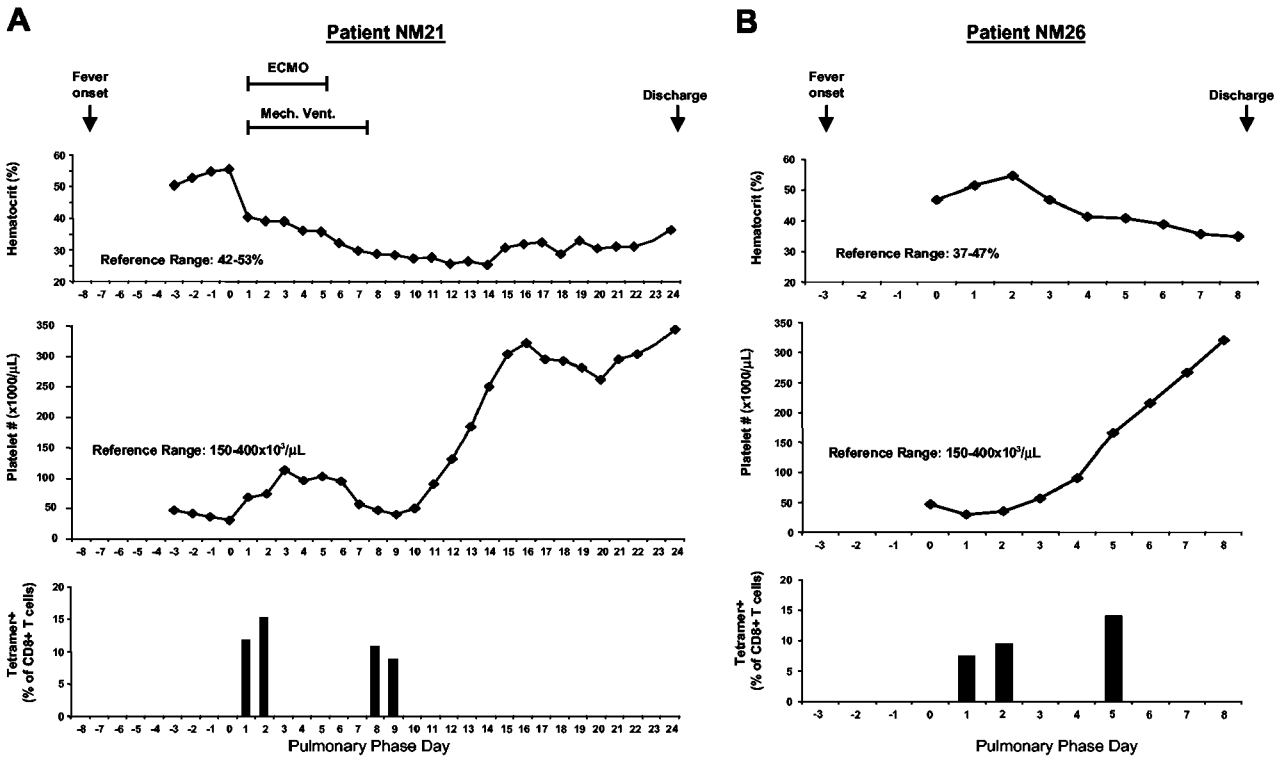


**FIGURE 3.** Kinetic analysis of T cell responses and clinical disease parameters in two acute severe HPS patients. *Top* and *middle panels* show daily hematocrit and platelet counts during hospitalization. *Bottom panels* show the combined percentages of CD8$^+$ T lymphocytes specific to the three SNV epitopes on the days that PBMC samples were available. The first day of respiratory distress is designated as pulmonary phase day 0. *A*, Patient NM21. *B*, Patient NM26.

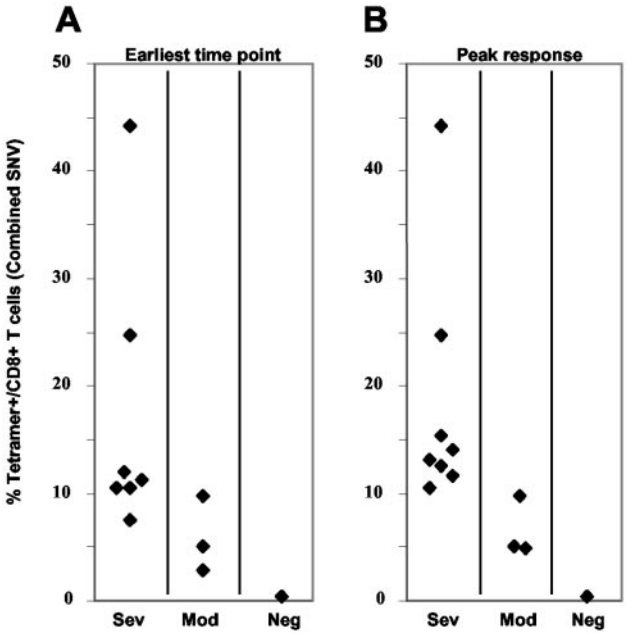


**FIGURE 4.** Comparison of SNV-specific $CD8^+$ T cell frequencies in severe (Sev) and moderate (Mod) HPS patients, and an SNV-negative (Neg) donor. *A*, The combined percentage of $CD8^+$ T lymphocytes specific to the three SNV epitopes at the earliest available time point during hospitalization in the 10 patients from whom PBMC were collected within 24 h of the onset of pulmonary edema, and an HLA-B*3501$^+$ SNV-negative donor. *B*, The highest combined percentage of $CD8^+$ T lymphocytes specific to the three SNV epitopes observed at any available time point during hospitalization in 11 patients, and an HLA-B*3501$^+$ SNV-negative donor.

virus, and DV (5–9, 36). For the viruses studied to date, the frequencies of $CD8^+$ T cells specific for individual epitopes during the acute phase are generally much lower than what we report for SNV, and are often $<1\%$. Acute infectious mononucleosis during the acute phase of EBV infection is the only other human viral illness for which the frequencies of virus-specific $CD8^+$ T cells are comparable with those we report here for HPS. Callan et al. (5) demonstrated that 29–44% of the $CD8^+$ T cells in three HLA-B8$^+$ patients with acute infectious mononucleosis were specific for a single epitope presented by HLA-B8 in the EBV lytic protein BZLF1. We demonstrate intense $CD8^+$ T cell responses to specific viral epitopes, and report an association between the magnitude of the virus-specific T cell responses and the severity of the clinical disease. Despite comparable levels of virus-specific T cells during acute infectious mononucleosis due to EBV, that illness is rarely fatal. In addition to high levels of virus-specific T cells, HPS disease outcome is likely to be affected by other factors such as the primary infected target organ, which is the lung, and viral burden. The respiratory failure in HPS triggered by alveolar capillary leakage is probably the result of intense $CD8^+$ T cell and other immune responses in the lung (14, 15, 20), whereas EBV causes generalized lymphoid tissue inflammation and does not cause target organ failure.

DV is another human pathogen that can cause a hemorrhagic fever marked by capillary leakage, dengue hemorrhagic fever (DHF), which is usually observed in secondary, not primary, dengue infections. During HPS and DHF, very high levels of viremia are seen, and higher peak viral titers correlate with increased disease severity in both diseases (30, 37). We speculate that virus-specific T cells are likely to contribute to the capillary leak syndromes observed in HPS and DHF (20, 38, 39). The two viruses that cause HPS and DHF differ in several important aspects, as do the infectious syndromes. HPS has a significantly higher case fatality rate than DHF. Furthermore, SNV causes a fulminant life-threatening illness during primary infection; secondary infections with hantaviruses have not been documented. SNV has only one serotype, but DV has four serotypes. DV generally causes severe illness after secondary infection with a virus of a different serotype than the primary infection. During secondary DV infection, T cell activation is more intense, likely due to serotype cross-reactive T cells from the primary infection (38). Very recently, Mongkolsapaya et al. (9) quantitated virus-specific $CD8^+$ T cells during acute secondary DV infections. Similar to our study, frequencies of DV-specific $CD8^+$ T cells were found to correlate with disease severity, suggesting T cell-mediated immunopathology. However, in the present study, we report markedly higher frequencies of virus-specific $CD8^+$ T cells during primary SNV infection than were observed during secondary DV infection. In addition, in our study, the high levels of $CD8^+$ T cells were present early in disease, coinciding with peak clinical symptoms, rather than 14 days after defervescence as reported in secondary DV infections. Similar studies should be performed in other acute, fulminant virus infections, for example, severe acute respiratory syndrome.

Our results have important implications for the study of HPS. Currently, the only options available to patients are pulmonary and hemodynamic supportive therapies, in part because the mechanisms of HPS pathology are poorly understood. Although we observed high levels of epitope-specific T cells during acute HPS, more detailed analyses will be required to determine the kinetics of these $CD8^+$ T cell responses. Our observation that the magnitude of the SNV-specific $CD8^+$ T cell response correlates with disease severity suggests that suppressing cellular immune responses may be beneficial in the treatment of HPS. However, the fine balance between protective CTL responses, which are important for viral clearance, and the intense activation of the immune system, which may trigger immunopathology, will be complex to investigate. Improved diagnosis in the earliest phases of HPS would help to plan clinical investigations of potential therapies, including a short course of immunosuppressive therapy.

Excess TNF-$\alpha$ and IFN-$\gamma$ produced by activated SNV-specific T cells upon recognition of Ag on SNV-infected pulmonary endothelial cells likely contribute to the observed capillary leakage during HPS. We reported earlier that there were increased numbers of cytokine-producing cells in the lungs of patients who died from HPS (20). Increased reactive oxygen/nitrogen species are detected in acute HPS patients (40) and may be induced by high levels of TNF-$\alpha$ and IFN-$\gamma$ secreted by SNV-specific T cells. We plan to analyze freshly isolated PBMC from acute HPS patients to perform additional functional and phenotypic analyses that will allow us to examine qualitative aspects of the $CD8^+$ T cell response to SNV, such as cytokine secretion, that may be contributing to disease pathology.

There may be multiple reasons for greater expansion of virus-specific T cell responses in severe HPS. Higher levels of viremia have been found in patients with severe HPS (30). Certain HLA alleles may be associated with highly efficient SNV Ag presentation, as has been speculated in HIV progression (41). Alternatively, the *HLA-B*3501* allele could be linked to other genes associated with cellular immune responses, such as *TNF-α* (42).

In summary, we report epitope-specific T cell responses in patients with a frequently fatal, acute zoonotic viral infection. Unlike other virus infections, for example those caused by EBV, HIV, HCV, and HBV, SNV is cleared rapidly in HPS. We demonstrate the presence of very high levels of SNV-specific $CD8^+$ T cells early during disease in patients with HPS. There are significantly higher frequencies of these $CD8^+$ T cell responses to three peptide epitopes presented by HLA-B*3501 in patients with severe disease

than in patients with moderate disease. Presumably, there are additional SNV epitopes presented by this and other HLA alleles in these patients, so the present results are only a subset of the total CD8$^+$ T cell responses to SNV. Our results support the hypothesis that virus-specific CD8$^+$ T cells contribute to HPS disease pathology. SNV is an emerging human pathogen, discovered only a decade ago. The seroprevalence rate to this virus is very low, even in endemic areas (24). These observations suggest that humans have had little exposure to SNV. We speculate that a lack of evolutionary exposure to this zoonotic virus contributes to the very intense immune responses that are associated with severe HPS resulting in immunopathology and the high case fatality rate in the unnatural human host. Coevolution of viruses and hosts presumably results in lower case-fatality rates for most viral infections, which is advantageous to the virus as well as the infected host. This may also apply to other human viral hemorrhagic fevers. Improved understanding of the mechanism of pathogenesis of severe viral zoonoses will result in better treatment and prevention strategies.

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