Human CD8+ T Cell Memory Generation in Puumala Hantavirus Infection Occurs after the Acute Phase and Is Associated with Boosting of EBV-Specific CD8+ Memory T Cells

Tamara Tuuminen, Eliisa Kekäläinen, Satu Mäkelä, Ilpo Ala-Houhala, Francis A. Ennis, Klaus Hedman, Jukka Mustonen, Antti Vaheri and T. Petteri Arstila


http://www.jimmunol.org/content/179/3/1988

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**  This article cites 46 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/179/3/1988.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human CD8\(^{+}\) T Cell Memory Generation in Puumala Hantavirus Infection Occurs after the Acute Phase and Is Associated with Boosting of EBV-Specific CD8\(^{+}\) Memory T Cells\(^{1}\)

Tamara Tuuminen,\(^{2,\star}\) Eliisa Kekäläinen,\(^{2,\dagger}\) Satu Mäkelä,\(^{\Pi}\) Ilpo Ala-Houhala,\(^{\Pi}\) Francis A. Ennis,\(^{\dagger}\) Klaus Hedman,\(^{\star}\) Jukka Mustonen,\(^{\dagger\dagger}\) Antti Vaheri,\(^{*}\) and T. Petteri Arstila\(^{3,\dagger\dagger}\)

The induction and maintenance of T cell memory is incompletely understood, especially in humans. We have studied the T cell response and the generation of memory during acute infection by the Puumala virus (PUUV), a hantavirus endemic to Europe. It causes a self-limiting infection with no viral persistence, manifesting as hemorrhagic fever with renal syndrome. HLA tetramer staining of PBMC showed that the CD8\(^{+}\) T cell response peaked at the onset of the clinical disease and decreased within the next 3 wk. Expression of activation markers on the tetramer-positive T cells was also highest during the acute phase, suggesting that the peak population consisted largely of effector cells. Despite the presence of tetramer-positive T cells expressing cytoplasmic IFN-\(\gamma\), PUUV-specific cells producing IFN-\(\gamma\) in vitro were rare during the acute phase. Their frequency, as well as the expression of IL-7R\(\alpha\) mRNA and surface protein, increased during a follow-up period of 6 wk and probably reflected the induction of memory T cells. Simultaneously with the PUUV-specific response, we also noted in seven of nine patients an increase in EBV-specific T cells and the transient presence of EBV DNA in three patients, indicative of viral reactivation. Our results show that in a natural human infection CD8\(^{+}\) memory T cells are rare during the peak response, gradually emerging during the first weeks of convalescence. They also suggest that the boosting of unrelated memory T cells may be a common occurrence in human viral infections, which may have significant implications for the homeostasis of the memory T cell compartment. The Journal of Immunology, 2007, 179: 1988–1995.

Immunological memory rests on a population of long-lived T and B lymphocytes, which are both quantitatively and qualitatively different from naive cells (for review, see Refs. 1 and 2). Despite the theoretical and practical importance of memory T cells, many aspects of their development and survival are inadequately understood especially in humans, in whom individual differences in the genetic background and disease history pose additional problems of interpretation. The longer lifespan, outbred nature, and varied environmental exposures of humans make it obvious that results obtained in the mouse on long-term phenomena such as immunological memory should be verified before extrapolating them to humans. It is quite likely that the maintenance of T cell memory in mice over periods of months may have different requirements than the often decades-long immunity encountered in humans. Studies in murine models indicate that the memory phenotype is gained gradually following clearance of the pathogen, with measurable changes occurring in the T cell population for up to 40 days postinfection (3). This suggests that the first weeks after the acute phase of an infection, when the declining Ag load, the disappearance of most effector cells, and the effect of prosurvival cytokines shape the emerging memory population, are crucial in determining the characteristics of T cell memory.

Little is known of memory T cell differentiation in humans during the important postinfection period. Based on the differential behavior and tissue homing, the memory T cells have been divided into two main subsets: the CCR7\(^{+}\) central memory T cells found predominantly in the lymphoid tissues and the CCR7\(^{-}\) effector memory T cells found in peripheral nonlymphoid tissues (4). In this scenario, it remains unclear whether one of the memory subsets is a precursor for the other or whether the subsets develop independently. Others, however, have argued that instead of two distinct memory phenotypes, the human CD8\(^{+}\) T cell memory acquisition is a linear process, with the Ag-experienced cells gaining gradually a more differentiated phenotype and simultaneously approaching proliferative senescence (5). How far along this scheme of differentiation the cells proceed seems to depend on the type of infection. Thus, hepatitis C or influenza-specific memory T cells have an early memory phenotype, retaining partly the expression of naive cell markers

---

1 This work was supported by grants from the Academy of Finland, the Sigrid Juselius Foundation, the Finnish Technology Advancement Fund, the Finnish Cancer Research Foundation, the Helsinki University Central Hospital Research and Education Fund, and the Medical Research Fund of Tampere University Hospital, European Commission Contracts QLK2-CT-1999-01119 and QLK2-CT-2002-01358, and National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant U19 AI057319.

2 T.T. and E.K. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. T. Petteri Arstila, Haartman Institute, Department of Immunology, PB21, University of Helsinki, Finland. E-mail address: petteri.arstila@helsinki.fi

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00
such as CD27, CD28, and CCR7. In infections by EBV or HIV, CD28 and CCR7 are largely down-regulated, whereas CMV infection seems to induce the most highly differentiated late-stage memory T cells (5).

One problem in reconciling these different views of human CD8\(^+\) T cell memory may be the nature of the pathogens studied. In the case of the influenza virus it is unlikely that what is studied truly represents a primary infection, given the repeated reinfections by this pathogen. The other pathogens mentioned establish a chronic, persistent infection, so it is not possible to study T cell memory generation during virus clearance. Moreover, some of these viruses, most obviously HIV and EBV that infect CD4\(^+\) T cells and B cells, respectively, have profound pathological effects on the immune system, further confounding the interpretation of T cell dynamics following the acute phase. Attempts to circumvent these problems have involved experiments with live or killed vaccines, making it possible to control the kinetics of the response. However, it is unclear whether a vaccine exposure, which by definition should be harmless, reliably reproduces all the immunological phenomena associated with an infection caused by a virulent pathogen. The relatively short half-life of T cell memory reported in some vaccine studies may thus reflect qualitatively or quantitatively insufficient effector activation. In support of this, the pattern of T cell expansion and memory generation after vaccination is often highly variable (6).

Puumala virus (PUUV),\(^4\) a zoonotic pathogen carried by rodents, is a member of the Hantaviridae and is endemic in large areas of Europe (7–9). It causes a self-limiting and relatively mild form of hemorrhagic fever with renal syndrome termed nephropathia epidemica (NE). The disease usually follows a characteristic course with a sudden onset of high fever in the absence of respiratory symptoms accompanied by headache, hemodynamic changes, and acute renal failure that sometimes necessitates hemodialysis treatment. During the acute phase, viral RNA can be detected in the blood and urine of many patients, but only until 9 days from the onset of the symptoms (10, 11). Reinfection by PUUV has not been reported, yet Abs have been detected for decades after the infection (12). We have previously shown that PUUV-specific CD8\(^+\) memory T cells also persist for more than a decade after the acute infection in the absence of viral persistence or reinfection (13). This, combined with the readily diagnosable acute syndrome, makes PUUV infection an excellent model of immunopathology (9). Hantaviral infections may also serve as a model of immunopathology (9).

Puuvla-specific CD8\(^+\) T cells correlates with disease severity (16). These data suggest that the T cell response in the acute phase may contribute to the pathogenesis of the disease.

In this report, we characterize the dynamics of the Ag-specific CD8\(^+\) T cell population in patients during primary PUUV infection and the immediate recovery period. Our results show that following the contraction of the effector population, memory T cells appear during the first weeks after the clinical disease followed by an attrition of unknown kinetics. Our data also reveal an instance of simultaneous boosting of unrelated antiviral T cell memory, a phenomenon that may be of importance to the maintenance of immunological memory but may also predispose to an imbalance of T cell repertoire and immunological senescence.

### Materials and Methods

#### Patients

Patients (16 altogether) presenting with acute PUUV infection at the Tampere University Hospital (Tampere, Finland) were partially HLA-typed for HLA-A2 and B8 haplotypes with Abs purchased from One Lambda. Nine patients with either or both haplotypes and positive PUUV serology at any point of the acute disease (one woman, eight men, age 36–72 years, mean 58 years) were enrolled. PBMC, sera, and plasma were collected daily during hospitalization and after discharge on two follow-up visits, roughly 3 and 6 wk after the onset of symptoms. The group of healthy controls was recruited from students and staff with negative PUUV serology and consisted of six men, mean age 31 years. All patients gave written, informed consent and the study was approved by the ethics committee of the Tampere University Hospital.

#### IFN-\(\gamma\) ELISPOT

PBMC were harvested with Ficoll-Paque (Amersham Biosciences) gradient centrifugation and cryopreserved in 90% heat-inactivated FCS and 10% DMSO (Sigma-Aldrich) until use. ELISPOT was performed in duplicate using the ELISpotPRO human IFN-\(\gamma\)-kit (Mabtech) according to the manufacturer’s instructions. Control Ags were tested in single wells. As a viability control the cells were stimulated with phytohemagglutinin (Sigma-Aldrich) (7 \(\mu\)g/ml) and heat-killed (30 min at 65°C) Candida albicans (2.5 \(\mu\)g/ml). Inter assay stability was monitored by testing multiple aliquots of a single buffy coat isolation of human PBMC (Finnish Red Cross Blood Service, Helsinki, Finland) simultaneously with the patient samples.

The antigenic PUUV peptides from the nucleocapsid (N) protein (N\(_{204–212}\) is HLA-A2 restricted; N\(_{243–248}\) and N\(_{173–181}\) are HLA-B8 restricted) have been previously described (13). They were synthesized by the Peptide and Protein Laboratory of the Haartman Institute, University of Helsinki (Helsinki, Finland) with standard methods. The HLA-A2- and HLA-B8-restricted control peptides were from the BMLF-1 (GLCTLVAML) and EBNA-3 (FLRGRAYGL) proteins of EBV and the Env (KTLPLCVTL) and Gag (GEIYKRWII) proteins of HIV, respectively. EBV peptides were used as a positive control and HIV peptides as a negative control and both were purchased, together with the HLA-A2-restricted influenza matrix epitope peptide GILGFVFTL, from Proimmune. All peptides were also tested, with no detectable spot formation, in the ELISPOT assay plates without cells.

#### HLA tetramer analysis and flow cytometry

Customized, PE-labeled HLA-A2 tetramers with the PUUV N\(_{204–212}\) peptide were purchased from Beckman Coulter and Proimmune, and the PE-labeled HLA-A2 tetramers with the EBV epitope GLCTLVAML were from Proimmune. To minimize nonspecific background, the cells were also stained with anti-CD3 and anti-CD8 mAbs (BD Biosciences) and gated to the CD3\(^+\)/H11001 T cell population before analysis. mAbs to CD122 and Ki-67 were purchased from eBioscience according to the manufacturer’s instructions. Control Ags were tested in single wells. As a viability control the cells were stimulated with phytohemagglutinin (Sigma-Aldrich) (7 \(\mu\)g/ml) and heat-killed (30 min at 65°C) Candida albicans (2.5 \(\mu\)g/ml). Inter assay stability was monitored by testing multiple aliquots of a single buffy coat isolation of human PBMC (Finnish Red Cross Blood Service, Helsinki, Finland) simultaneously with the patient samples.

### Table 1. The highest values of the laboratory findings measured during hospital care

<table>
<thead>
<tr>
<th>Laboratory Finding</th>
<th>Median (Range)</th>
<th>Days after the Onset of Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum plasma creatinine ((\mu)mol/L)</td>
<td>445 (79–1285)</td>
<td>6–8</td>
</tr>
<tr>
<td>Maximum plasma C reactive protein (mg/L)</td>
<td>152 (30–269)</td>
<td>3–7</td>
</tr>
<tr>
<td>Maximum blood leukocyte count (10(^3)/L)</td>
<td>12.9 (8.5–26.8)</td>
<td>4–8</td>
</tr>
</tbody>
</table>

\(^4\)Abbreviations used in this paper: PUUV, Puumala virus; N, nucleocapsid protein; NE, nephropathia epidemica.
Quantitative real-time PCR

Total RNA was isolated by using the TriPure reagent (Roche Diagnostics), and first-strand cDNA was synthesized with oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Finnzymes). Quantitative PCR was done in duplicate with commercially available primer-probe sets for human IL7-Rα and IL-15Rα and the TaqMan master mix, all purchased from Applied Biosystems. The results were normalized against β-actin mRNA levels in the samples, and relative quantitation was achieved by comparison with a standard dilution curve. Reactions were performed by using the ABI7700 instrument (Applied Biosystems).

Determination of TNF-α by ELISA

Human TNF-α was determined from patients’ sera collected during the hospital stay and after recovery. TNF-α was also measured from supernatants harvested from cell cultures stimulated with the various PUUV peptides. Analysis of cytokine levels was done by using a commercial enzyme immunoassay for human TNF-α as instructed (Mabtech).

EBV serology and detection

IgG and IgM class Abs to EBV were determined with the Enzygnost anti-EBV IgG and IgM kits by using the Behring ELISA processor. The IgG avidity was determined as described (17). Real-time quantitative PCR for EBV DNA in plasma was performed as published (18).

Results

Kinetics of the T cell response in acute PUUV infection

The typical incubation period of PUUV infection is 2–4 wk, and all of our nine patients were seropositive at presentation. The clinical picture was typical of NE in all patients. A summary of the main laboratory findings is shown in Table I, which also includes the periods at which the highest values were measured in different patients. Serum creatinine concentrations were elevated in all but one patient and three needed transient hemodialysis treatment. All patients had proteinuria and hematuria. Seven of nine patients had thrombocytopenia. One patient was hypotensive (blood pressure 95/60 mmHg), but none had a clinical shock. By the end of hospitalization (8–12 days after onset) the laboratory findings had normalized.

The frequency of circulating CD8+ T cells during the acute phase and convalescence (p < 0.05 between d1 and c2; Student’s two-tailed t test). Comparison of the frequency of HLA-A2/PUUV N204–212 tetramer-reactive (circle) and IFN-γ producing PUUV N204–212-specific cells at different stages of the infection. d1, Presentation; c1, first control, on average 3 wk after the onset. c2, second control, 6 wk after the onset. In A only CD3+ cells are shown, while the results in B and C are shown as mean ± SD.

FIGURE 1. Cytotoxic T cell response to an acute infection by PUUV. A, Kinetics of a HLA-A2 restricted, PUUV N204–212-specific response in three representative patients as measured by HLA tetramers. B, The frequency of circulating CD8+ T cells during the acute phase and convalescence (p < 0.05 between d1 and c2; Student’s two-tailed t test). C, Comparison of the frequency of HLA-A2/PUUV N204–212 tetramer-reactive (circle) and IFN-γ producing PUUV N204–212-specific (square) cells at different stages of the infection. d1, Presentation; c1, first control, on average 3 wk after the onset. c2, second control, 6 wk after the onset. In A only CD3+ cells are shown, while the results in B and C are shown as mean ± SD.

FIGURE 2. Frequency of PUUV-specific, IFN-γ-producing CTLs during acute PUUV infection and convalescence. Responses against HLA-A2-restricted N204–212 (triangle) and HLA-B8 restricted N173–181 (square) PUUV peptides were measured by using an IFN-γ ELISPOT assay. Non-specific background was subtracted from the results, and only responses higher than twice the background were considered positive.

Image figure 1 and 2 describe the kinetics of the T cell response to an acute infection by PUUV, showing the frequency of CD8+ T cells during the acute phase and convalescence. The graphs illustrate the comparison of HLA-A2/PUUV N204–212 tetramer-reactive cells and IFN-γ producing PUUV N204–212-specific cells at different stages of the infection (presentation and first control). The results are shown as mean ± SD.

Image figure 2 shows the frequency of PUUV-specific, IFN-γ-producing CTLs during acute PUUV infection and convalescence. The responses were measured against HLA-A2-restricted N204–212 and HLA-B8 restricted N173–181 PUUV peptides using an IFN-γ ELISPOT assay. Non-specific background was subtracted from the results, and only responses higher than twice the background were considered positive.
FIGURE 3. Changes in the expression of IL-7Rα mRNA during PUUV infection. cDNA was synthesized from total RNA by using an oligo(dT) primer and IL-7Rα levels were measured by quantitative PCR. The results were normalized against human β-actin levels and relative quantitation was achieved by comparing the results with a standard dilution curve. The mean expression level of six healthy controls is indicated by a dotted line. The values are shown in arbitrary units.

50% and decreased significantly at later sampling days to 35% (Fig. 1B). ELISPOT analysis of the frequency of T cells secreting IFN-γ in response to three different PUUV N peptides (N204-212, N243-251, and N173-181), including the A2-restricted tetramer epitope (Fig. 1C), produced different results. Measured by the ELISPOT assay, the frequency of reactive T cells at presentation was low, ranging from 300 to <50 cells/million PBMC. Thus, the increased CD8+ T cell population at presentation consisted to a large degree of cells that can be visualized by direct HLA tetramer staining but are not readily detectable by an in vitro functional assay such as IFN-γ ELISPOT.

A dissociation between staining and functional assays has been demonstrated in many experimental models and can be due to the susceptibility of effector cells to activation-induced cell death (19). The ELISPOT assay also showed lower responses to the polyclonal stimulator PHA during the acute phase than at later sampling days (not shown), perhaps because of the tendency of the effector population to respond to stimulation by apoptosis. A similar transient decrease in PHA responses has also been observed in other acute viral infections (20, 21). To test the susceptibility of the isolated cells to activation-induced apoptosis, we stimulated them overnight in vitro with an immobilized anti-CD3 mAb and then performed annexin-V staining to detect apoptotic cells. The results showed that cells isolated during the acute phase were more prone to apoptosis than cells isolated during the convalescence, with 16.3% (range 7.2–23.9%) and 7.3% (range 5.7–10.0%) annexin-positive cells, respectively.

During follow-up the frequency of IFN-γ-producing T cells started to increase, reaching in some subjects nearly 2,800 reactive cells per million PBMC (Fig. 2), while at the same time the frequency of both CD8+ T cells and A2 tetramer-positive cells decreased and the results from the different methods of analysis drew closer to each other (Fig. 1C). These changes thus reflected the contraction phase of the acute response, suggesting that the increase of the IFN-γ-producing population may have corresponded to the emergence of a memory T cell population, peaking between the days 20 to 45 after the onset of the symptoms. Interestingly, the highest frequencies of IFN-γ-producing cells at this convalescence stage were roughly an order of magnitude higher than those of memory T cells found in individuals with old immunity (13).

We have previously shown that patients with the HLA-B8-DR3 haplotype have a more severe course of PUUV infection (14, 15). This has been suggested to be due to exaggerated T cell responses and subsequent immunopathology. In our patient cohort we observed no clear difference in the frequency of HLA-B8-restricted T cells as compared with HLA-A2-restricted cells, but because only three of our patients had the B8 haplotype this result should be assessed with caution. However, the same conclusion was also drawn from an earlier analysis of the frequency of PUUV-specific memory T cells (13).

Expression of IL-7Rα and IL-15Rα

In addition to the Ag-specific signals, several cytokines have been shown in murine studies to be important for the generation of CD8+ T cell memory (22). Some of these, especially IL-7 and IL-15, are produced constitutively so that the regulation of their effects takes place on the receptor level. We analyzed the expression of IL-7Rα (CD127) and IL-15Rα mRNA during the acute phase and convalescence by using the average expression in six healthy volunteers as a baseline. Analysis of PBMC by quantitative PCR showed that the expression levels of IL-7Rα mRNA were low in the acute phase but increased in most patients during the follow-up (Fig. 3). These data are in accordance with previous reports that have shown that IL-7Rα is down-regulated during T cell activation but re-expressed at the transition to the memory phenotype (23). This provides further support for the view that the increase of IFN-γ-producing T cells during the follow-up corresponded to the emergence of the specific memory population. IL-15Rα has been reported to be up-regulated in activated T cells and maintained in memory T cells (24), but in our patients the expression of IL-15Rα showed no apparent change and was comparable to that in noninfected controls (data not shown).

We also measured the production of TNF-α, reported to be high during the acute phase of PUUV infection and suggested to be important in pathogenesis (25). In our hands the concentrations of TNF-α in the patient sera were all below the detection level of the kit (8 pg/ml). However, TNF-α was detectable in supernatants collected from the cultures of T cells stimulated with PUUV Ags in amounts proportional with the frequency of IFN-γ producing cells. This suggests that local production of TNF-α may play a role in the clinical disease.
Expression of activation markers on PUUV-specific T cells

To verify the results described above and to further characterize the composition of the PUUV-specific CD8\(^+\) T cell population, we used flow cytometry to analyze the activation status of the PUUV tetramer-positive cells in seven HLA-A2\(^+\) patients, comparing an early sample taken from each patient during hospitalization with the first control sample. The average time between the two samples was 15 days (range 9–20). In contrast to the ELISPOT results obtained after in vitro stimulation, when analyzed directly ex vivo by intracellular cytokine staining the frequency of cells producing IFN-\(\gamma\) was higher in the acute phase than during convalescence in all seven patients (15.2% vs 5.1% on the average; Fig. 4). Similarly, the frequency of cells expressing the activation markers HLA-DR and CD122 (the high-affinity IL-2R\(\alpha\)-chain) was higher in the acute phase in most of the patients. We also analyzed the expression of the cell cycle-associated intracellular Ki-67 Ag in five patients and found that in four of them the frequency of Ki-67\(^+\) cells among the HLA-A2 tetramer-positive population decreased sharply during the transition to convalescence. Taken together, these data show that the population detected by the tetramers in the acute phase consists of highly activated cells undetectable by the IFN-\(\gamma\)-ELISPOT assay.

Because the bulk analysis of IL-7Ra mRNA levels indicated an increase during convalescence, we next analyzed IL-7Ra surface expression on the tetramer-positive cells (Fig. 4). In six of seven patients the frequency of IL-7Ra\(^+\) cells increased during the follow-up, consistent with the emergence of a memory population. Finally, we measured the mean fluorescence intensity of tetramer staining to determine the amount of surface TCR expressed by the tetramer-positive cells. T cell activation is associated with rapid TCR down-modulation, whereas on memory T cells the levels are restored (reviewed in Ref. 26). When compared with the samples taken during the acute phase, the level of surface TCR decreased during convalescence in all seven patients, on average by 80% (range 15–258%).

**EBV-specific T cell response and viral reactivation**

For specificity control of our ELISPOT assay we used antigenic peptides of EBV and HIV. As expected, there was no detectable response to HIV peptides in our HIV-negative patients, whereas some reactivity to EBV peptides was observed in most patients. Unexpectedly, however, during the follow-up seven of our nine patients also showed a clear increase in the frequency of EBV-reactive T cells. The EBV-specific response followed closely the kinetics of the PUUV-specific response as measured by the ELISPOT assay (Fig. 5). During the acute phase the responses were low (25–225 specific cells per million PBMC) but increased during the follow-up to 165-1845 cells per million PBMC. To exclude the possibility of an immunosuppressive effect during the acute phase, we also used HLA-A2 tetramers bound to the same EBV epitope to directly measure the frequency of EBV-specific T cells in six patients. The results were in close agreement with the ELISPOT data, with an average of 129 (range 0–207) specific cells per million PBMC in the acute phase and 328 (range 107–613) in the first control sample.

We then used the ELISPOT assay to test the response of 6 HLA-A2\(^+\) patients to an immunodominant A2-restricted influenza matrix epitope in the acute phase and in the first control sample. A response was detected in five of the patients, but the frequency of responding cells was low (≤60 specific cells per million PBMC). An increase was observed in only one patient, from 15 specific cells in the acute phase to 60 specific cells per million PBMC in the control sample, and on the average there was no change. It is thus unlikely that the increase in EBV-specific T cells would have been due to nonspecific, generalized memory T cell activation caused by cytokines or putative virus-induced lymphopenia.

Serological analysis showed that all nine patients had pre-existing immunity to EBV and were thus carriers of a latent infection. To determine whether the EBV-specific T cell response was accompanied by viral reactivation, we used quantitative PCR to measure the number of EBV DNA copies in the patients’ plasma. In three patients EBV DNA was detected during the acute phase of the PUUV infection but not during follow-up. Strikingly, two of the three patients with a transient viremia had no measurable EBV-specific T cell response while only one of the seven patients with an EBV-specific T cell response had detectable EBV DNA, suggesting a link between the T cell response and the suppression of EBV reactivation. Thus, all nine patients had either a measurable EBV-specific T cell response or transient EBV viremia. It is also noteworthy that in five of the seven patients with an EBV-specific T cell response the levels of specific CD8\(^+\) T cells remained higher at the last control sampling than at the beginning of the study. These results suggest that the PUUV-associated EBV reactivation and the resultant immune response also served to boost the EBV-specific T cell memory.

**Discussion**

The requirements for the maintenance of T cell memory have been the object of a lengthy debate, with special attention given to the question of whether contact with the original or a cross-reactive Ag is needed. In one extreme, some experimental models have indicated that memory may be a form of chronic, low-grade stimulation rather than a latent state of more or less resting T cells (27). In other studies, MHC-deficient mice without any T cells were...
grafted with memory T cells and the survival of the transferred cells was monitored in the absence of any possible TCR-mediated stimulus (28). Although the transferred T cells seemed to survive for months, their functional properties have been reported to decline in the absence of boosting (28, 29). Even in murine models, the need for antigenic boosting therefore remains an open question. In humans, where genetic manipulations are impossible, it is very difficult to obtain a reliable answer. Both anecdotal and experimental evidence suggest that human T cell memory can be maintained for years and even decades in the apparent absence of boosting (13, 30–32), but it is difficult to exclude all forms of antigenic stimulus. Our data allow several putative conclusions to be drawn. First, the human memory T cell population seems to develop during a period of a few weeks after the clearance of the pathogen. Second, the resulting population is significantly larger than the memory population detectable several years later. Third, the immune system seems to take advantage of the acute infection to boost the memory population specific to an unrelated pathogen.

In acute viral infections the specific T cell response typically reaches its peak by 2 wk after the infection. In our patients, although the exact time of infection cannot be established, the highest frequency of circulating CD8+ T cells and PUUV-specific tetramer-positive cells was observed at presentation, suggesting that the peak response is roughly concurrent with the appearance of clinical symptoms. A large fraction of these cells also expressed activation markers, and a clear decrease was observed during the follow-up. It is thus likely that the cells forming the peak response were largely effector cells and were not readily detectable by in vitro stimulation, probably because of their susceptibility to activation-induced cell death (19). Interestingly, despite the presence of cells with cytoplasmic IFN-γ, during the acute phase the frequency of cells detectable by ELISPOT was very low or even below the sensitivity of the method in every patient, and in all nine patients the frequency increased during convalescence. Thus, despite the strong response detectable by direct staining, the population responsible for the in vitro IFN-γ production induced by the same epitope at later stages was absent at the peak of the acute response. This suggests that the circulating effector population contained very few fully differentiated memory cells, at least if judged by the functional criteria that could be used to identify them years after the primary infection (13). Consistent with this scenario, the kinetics of IL-7Ra expression also followed closely the appearance of PUUV-specific IFN-γ producing cells, suggesting that they represented the emergent memory population (23). Previously, a study of acute lymphocytic choriomeningitis virus infection in mice showed that the gene expression pattern associated with T cell memory is obtained gradually (3), a finding in good agreement with our results in regard to acute infection of humans. Whether the survival of some effectors and their further development into memory cells is linked to TCR affinity as suggested by some experimental models (33, 34), or if it happens stochastically, remains unclear. Our results in the human system with a natural infection by a virulent pathogen show that the generation of memory takes place only after the peak effector response has subsided. Thus, either the induction of memory T cells is independent of and significantly slower than the induction of effector cells, or, more likely, the PUUV-specific memory cell generation reflects an endpoint of T cell activation (5).

Another significant observation was that roughly a month after the acute phase the frequency of PUUV-reactive T cells was an order of magnitude higher than that observed several years after infection (13). Although this comparison was made between two different patient cohorts, the results are likely to indicate a significant decline in the memory T cell population during the years following its induction. The kinetics of this decline is not known, but it does suggest that the maintenance of high levels of memory cells may require occasional boosting. Such boosting may come from Ags persisting in the host or from reinfection, whether clinical or subclinical. A further possibility is that in some cases an immune response against one pathogen may also boost the memory response against another. This might be due to heterologous immunity induced by a cross-reaction, homologous immunity induced by the reappearance of latent pathogens or microbial components, or nonspecific lymphopenia-driven or cytokine-driven boosting. Such bystander maintenance of T cell memory has been demonstrated in several experimental settings in mice, but clear examples from human systems are scarce (35). Our data putatively provide such an example, by showing an increase of EBV-reactive T cells in seven of nine patients with an acute PUUV infection.

 Reactivation of latent EBV is a well-known phenomenon, but as a systemic manifestation it is generally linked with situations of clinical immunosuppression such as organ transplantations and AIDS (36). In the absence of immunosuppression the EBV cycle becomes productive in the salivary glands and oropharyngeal mucosa, with occasional shedding into saliva contributing to viral transmission (37). In contrast, plasma drawn from healthy people very rarely contains EBV DNA (18, 38). Here, a systemic EBV reactivation or a T cell response suggestive of such reactivation was observed during an acute infection in otherwise healthy people in every one of the nine patients studied. During latency EBV persists in B cells, so the most likely explanation of our finding is that EBV gets reactivated within EBV-infected, PUUV-specific B cells participating in the acute immune response. It is also possible that the cytokines produced during the immune response induce viral reactivation in B cells not directly involved in the PUUV-specific response. In either case, it may be that the phenomenon is restricted to EBV and might also be a part of the viral survival strategy. However, increased expression of microbial constituents or even the appearance of viable organisms may be a more common feature of immune responses. In an earlier study on herpesvirus-specific B cell responses during acute CMV infection, the emergence or increment of Abs suggested the reactivation of EBV and also human herpesvirus 6, and in one patient the varicella virus also (39). More recent studies using quantitative PCR or gene arrays have provided direct evidence of the propensity of several herpesviruses to become reactivated at the same time (40, 41). Also, during acute HIV infection CD8+ T cells specific for EBV, CMV, and influenza start to express activation markers without detectable viral reactivation or infection (42). Cells of the immune system, especially phagocytic APCs like the macrophages, hosts to many kinds of microbes (43). Participation in an immune response against another pathogen might well have an impact on the presentation of Ags from pathogens encountered earlier, whether or not they remain alive. In this way the heterologous boosting of resting memory T cells could be a general feature of T cell responses, creating a kind of ripple effect that might help to preserve pre-existing T cell memory in the face of a massive response directed against the infecting pathogen.

However, the consequences of EBV reactivation might not be all beneficial. One facet of heterologous T cell activation is its potential to trigger pathological responses that might be of importance to the pathogenesis of NE. The severity of acute PUUV infection is highly variable and can be modulated by genetic factors such as the HLA type of the patient (14, 15). It is possible that the simultaneous EBV reactivation may contribute to the symptoms, the more so because PUUV and EBV infections can cause similar complications including CNS inflammation, myocarditis, hepatitis, nephritis, and arthritis (8, 44). These complications...
might be the direct result of the reappearance of an infectious virus or be associated with the T cell response thereby induced, as has been suggested for PUUV and the pathogenesis of NE (9, 14, 15). The magnitude and composition of the EBV-specific memory response that results from the viral reactivation reflects individual and partly stochastic events during the primary infection and might thus have a highly variable impact on the clinical course of the PUUV infection. A clear example of such a detrimental viral infection was provided by a recent demonstration that immunity against an influenza-derived Ag can cross-react with hepatitis C virus structures. In a minority of patients with acute hepatitis C virus infection, this pre-existing, influenza-induced T cell population comes to dominate the T cell response and is associated with a severe form of hepatitis (45). Similarly, the patient’s pre-existing pattern of EBV-specific T cell memory might be partly responsible for the outcome of the acute PUUV infection.

More generally, the responses against latent infections may eventually grow to such a magnitude as to outcompete and crowd out memory cells with other specificities, and in this respect members of the herpesvirus group may be of special importance. For example, a global analysis of CMV-specific memory T cells in humans has shown that on the average they account for 10% of the total memory population, but in some individuals this fraction may be up to 40% (46). In the competition for limiting amounts of homeostatic survival signals, this may lead to a clinically significant loss of diversity in the T cell memory compartment and has been suggested to play a role in the immune senescence observed in some aging individuals (47, 48). This potential for undesirable consequences highlights the importance of resolving whether heterologous responses are exceptions or the rule in the human immune system.

In conclusion, our data establish the kinetics of the generation of human CD8+ T cell memory during an acute infection with a nonpersistent pathogen and suggest that at least part of the pre-existing, unrelated memory T cell population is boosted at the same time. The relative contribution of the primary response and subsequent boosting to the maintenance of human T cell memory and partly stochastic events during the primary infection might thus have a highly variable impact on the clinical course of the PUUV infection. A clear example of such a detrimental viral infection was provided by a recent demonstration that immunity against an influenza-derived Ag can cross-react with hepatitis C virus structures. In a minority of patients with acute hepatitis C virus infection, this pre-existing, influenza-induced T cell population comes to dominate the T cell response and is associated with a severe form of hepatitis (45). Similarly, the patient’s pre-existing pattern of EBV-specific T cell memory might be partly responsible for the outcome of the acute PUUV infection.

More generally, the responses against latent infections may eventually grow to such a magnitude as to outcompete and crowd out memory cells with other specificities, and in this respect members of the herpesvirus group may be of special importance. For example, a global analysis of CMV-specific memory T cells in humans has shown that on the average they account for 10% of the total memory population, but in some individuals this fraction may be up to 40% (46). In the competition for limiting amounts of homeostatic survival signals, this may lead to a clinically significant loss of diversity in the T cell memory compartment and has been suggested to play a role in the immune senescence observed in some aging individuals (47, 48). This potential for undesirable consequences highlights the importance of resolving whether heterologous responses are exceptions or the rule in the human immune system.

In conclusion, our data establish the kinetics of the generation of human CD8+ T cell memory during an acute infection with a nonpersistent pathogen and suggest that at least part of the pre-existing, unrelated memory T cell population is boosted at the same time. The relative contribution of the primary response and subsequent boosting to the maintenance of human T cell memory remains to be resolved.

Acknowledgments
We thank Dr. Staffan Paulie of Maltech AB, Sweden, for help with the ELISPOT technique, Piivi Norja and Lea Hedman for expert technical assistance, Dr. Heikki Repo for discussions on cytokine measurements, and Dr. Dan Libraty for critical comments on the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


