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Specific CD8$^+$ T cells recognize human herpesvirus 6B

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The importance of human herpesvirus 6 (HHV-6) species as human pathogens is increasingly appreciated. However, we do not understand how infection is controlled in healthy virus carriers, and why control fails in patients with disease. Other persistent viruses are under continuous surveillance by antigen-specific T cells, and specific T-cell repertoires have been well characterized for some of them. In contrast, knowledge on HHV-6-specific T-cell responses is limited, and missing for CD8$^+$ T cells. Here we identify CD8$^+$ T-cell responses to HHV-6B, the most widespread HHV-6 species, in healthy virus carriers. HHV-6B-specific CD8$^+$ T-cell lines and clones recognized HLA-A2-restricted peptides from the viral structural proteins U54 and U11, and displayed various antigen-specific antiviral effector functions. These CD8$^+$ T cells specifically recognized HHV-6B-infected primary CD4$^+$ T cells in an HLA-restricted manner, produced antiviral cytokines, and killed infected cells, whereas HHV-6A-infected cells were not recognized. Thus, HHV-6B-specific CD8$^+$ T cells are likely to contribute to control of infection, overcoming the immunomodulatory effects exerted by the virus. Potentially, HHV-6-associated disease could be addressed by active or passive immunotherapy that reconstitutes virus-specific CD8$^+$ T-cell responses.

Keywords: CD8$^+$ T cells · human herpesvirus 6B · infectious diseases · virology

Introduction

Human herpesvirus 6 (HHV-6) species are widespread pathogens, and more than 90% of humans are seropositive. The two species HHV-6A and HHV-6B have close sequence homology but differ in their epidemiology and pathogenicity [1, 2]. Primary infection with HHV-6B, the more widespread species, usually takes place in early childhood and is often associated with a self-limiting illness known as three-day fever or exanthema subitum [3, 4]. After primary infection, HHV-6 remains in a latent state in its immunocompetent host [5], and occasional reactivations are normally asymptomatic. However, HHV-6 (in most cases HHV-6B) can reactivate in immunosuppressed individuals. HHV-6B reactivation is observed in 40–50% of patients receiving stem cell transplantation, and viral reactivation is associated with delirium and cognitive decline, severe encephalitis, graft-versus-host disease, transplant failure, and overall mortality [6–9]. Apart from the immunosuppressed host, HHV-6 has been involved in a variety of diseases involving the CNS, such as febrile seizures, encephalitis, epilepsy, and multiple sclerosis [2, 10].

Healthy humans frequently carry a number of other persistent viruses that may reactivate under immunosuppression, including the herpesvirus family members Epstein-Barr virus (EBV) and cytomegalovirus (CMV) [11]. It is assumed that these viruses are under continuous control by antigen-specific T cells, and viral reactivation results from a deficiency in virus-specific T cells caused by therapy-related immunosuppression [11]. In accordance with this notion, adoptive transfer of virus-specific T cells prevents and

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Table 1. Predicted HLA-A*0201-restricted HHV-6B epitope peptides examined in this study

HHV-6 antigen	HHV-6B peptide sequence[a)]	Homologous HHV-6A sequence[b)]	Amino acid position	Establishment of peptide-specific T-cell clones	Recognition of infected targets
U11					
	YLVTSINKL	YL**I**TSINKL	188	No	
	SLMSGVEPL	SLMSGVE**SP**[c)]	381	Donor 2	Not tested
	YLGKLFVTL	YLGKLF**LA**L	198	No	
	GILDFGVKL	GILDF**N**VK**F**	413	Donors 1 and 2	Yes
	DLSRDLDSV	DLSR**E**LDS**A**	750	No	
	MLWYTVYNI	MLWYTVYNI	171	Donors 1 and 2	No
	DVVNGLANL	DVVNG**F**ANL	450	No	
U54					
	LLCGNLLIL	LLCGNLLIL	195	Donor 1	Yes
	ILYGPLTRI	**VI**YGPLTRI	129	Donors 1 and 2	Yes
	SMGIFLKSL	**N**MGIFL**S**SL	392	No	
U89/90					
	LVLEQLGQL	L**G**LEQL**S**QL	149	No	
	KLDKEMEAV	None	637	No	

[a)] Strain Z29 (GenBank accession code NC_000898.1).
[b)] Strain U1102 (GenBank accession code X83413.1).
[c)] Amino acids in HHV-6A which differ from HHV-6B are displayed in boldface.

eliminates EBV- and CMV-associated disease after transplantation [12–14].

However, it has remained in doubt whether specific T cells play a similarly important role in controlling HHV-6 infection. In contrast to other human herpesviridae, HHV-6-specific T cells have been difficult to characterize. Target antigens of virus-specific $CD4^+$ T cells are now being defined [15], but HHV-6-specific $CD8^+$ T cells have not been identified so far, in spite of their potentially important role in control of infection.

Certain biological properties of HHV-6 might have discouraged researchers from investigating classical antigen-specific $CD8^+$ T-cell responses to these viruses. HHV-6 has a repertoire of immunosuppressive functions in vitro [16] that might interfere with the establishment or function of such T-cell responses in healthy carriers or patients. These immunomodulatory effects include direct infection and lysis of T cells [17], functional inhibition of T cells [18], and inhibition of APCs [19].

Thus, it has remained in doubt whether virus-specific T-cell responses contribute to control of HHV-6 infection and replication, and the knowledge required to develop passive or active immunotherapy has been thus far lacking. Here, we address the question whether functional virus-specific $CD8^+$ T cells exist in healthy carriers in spite of viral immunomodulation, and show that this is the case. We established HHV-6B-specific $CD8^+$ T-cell lines and clones, assessed their antigen and epitope specificity, and analyzed their function. We identified HHV-6B-specific $CD8^+$ T cells that recognized HLA-A2-restricted peptides from the viral structural proteins U54 and U11. These T cells mobilized a repertoire of antiviral effector functions upon specific recognition of productively HHV-6B-infected target cells, thus overcoming the immunosuppressive properties of the virus.

Results

Choice of potential $CD8^+$ T-cell antigens of HHV-6B

At the outset of our study, $CD8^+$ T-cell antigens of HHV-6B were unknown. We chose to study HHV-6B tegument proteins U11 and U54 and immediate early protein U89/90, which are functional or positional homologs to known $CD8^+$ T-cell antigens pp150, pp65, and IE-1 from human CMV. We defined a set of 12 peptides that were putative ligands of the widespread human HLA class I allotype HLA-A*0201 (Table 1).

Enrichment of HHV-6B-specific T cells from healthy carriers

We tried to detect functional $CD8^+$ T cells specific for our set of 12 HHV-6B peptides by IFN-γ-ELISPOT assay in PBMCs from five healthy HLA-A*0201-positive, HHV-6-seropositive donors loaded with each peptide individually. No specific reactivity was detected (data not shown), indicating that the frequency of IFN-γ-secreting $CD8^+$ T cells specific for any of these peptides was below 1 in 10^5 cells in PBMCs.

Therefore, we attempted to enrich HHV-6B-specific T cells from peripheral blood. We modified a restimulation procedure capable of enriching rare antigen-specific $CD8^+$ T cells [20, 21]. PBMCs from two arbitrarily chosen HLA-A*0201-positive healthy HHV-6 carriers were initially stimulated with a mixture of the 12 HHV-6B peptides and then weekly with peptide-loaded autologous CD40-activated B cells. After 50 days of cultivation, T-cell lines from donors 1 and 2 were analyzed for reactivity to each HHV-6B

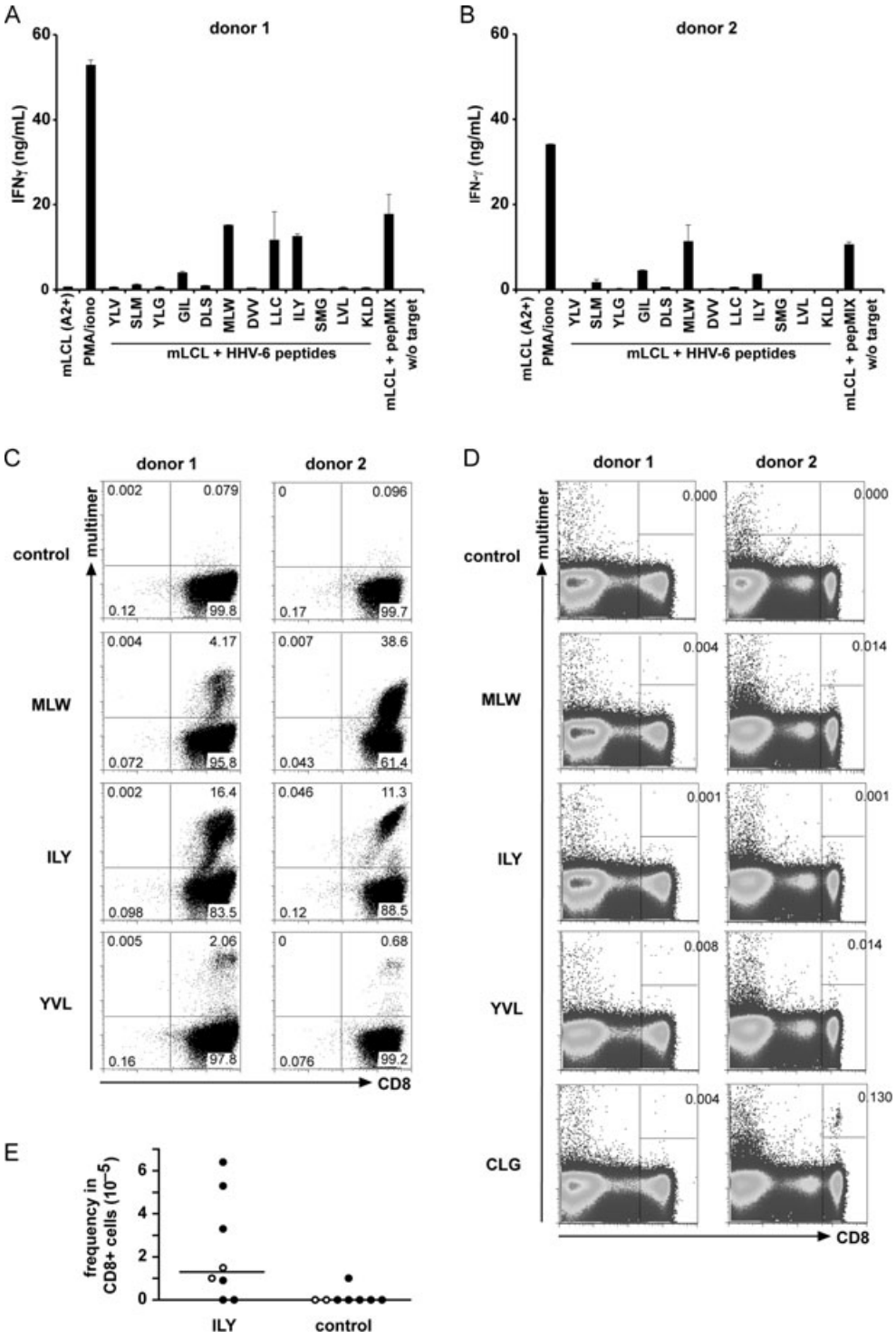

Figure 1. Enrichment of HHV-6B peptide-specific T cells. T-cell cultures from (A) donor 1 and (B) donor 2 were tested for reactivity to individual HHV-6B peptides after 50 days of stimulation by IFN-γ ELISA. An HLA-A*0201-matched mini-LCL (mLCL) served as antigen-presenting cell. Polyclonal activation with PMA and ionomycin (PMA/iono) served as positive control. One of the two experiments (three replicates and SD) is shown; data displayed as mean + SD of $n = 3$ replicates. (C) $CD8^+$ T cells specific for the HHV-6B peptides MLW or ILY, or the EBV peptide YVL in complex with HLA-A*0201 were enumerated by HLA/peptide pentamer staining in T-cell cultures from donors 1 and 2 after 61 days of stimulation. Numbers indicate the percentage of cells within each quadrant. Day 61 is one of the five analyzed time points. (D) PBMCs from donors 1 and 2 were analyzed ex vivo by fluorescent HLA-A*0201/peptide pentamer staining for the presence of $CD8^+$ T cells specific for the HHV-6B peptides MLW or ILY or the EBV peptides YVL and CLG. Numbers indicate percent of $CD8^+$ lymphocytes. (E) PBMCs from eight randomly selected healthy adult HLA-A*0201-positive HHV-6-positive individuals were stained with A*0201/ILY pentamer. Open circles, donors 1 and 2. One to three stainings were performed for each of the eight donors, one value per donor is shown, bar represents median.

peptide in IFN-γ-ELISA (Fig. 1A and B). We observed reactivities to five HHV-6B peptides, MLW, GIL, and SLM from U11, and ILY and LLC from U54. Each cell line recognized four of these five peptides, suggesting an overlapping repertoire of HHV-6B specificities in different healthy A*0201 donors. Peptides from U89/U90 were not recognized. HLA-A*0201/peptide pentamers were synthesized for two peptides, U11-derived peptide MLW, and U54-derived peptide ILY. Staining with these pentamers confirmed that $CD8^+$ T cells specific for MLW and ILY in complex with HLA-A*0201 were present at significant frequencies in peptide-stimulated T-cell lines (Fig. 1C). T cells specific for the EBV-derived "supporter" peptide YVL were detectable but less frequent (Fig. 1C). To determine primary frequencies of HHV-6 peptide-specific T cells, PBMCs of donors 1 and 2 were stained with MLW pentamers (Fig. 1D), and PBMCs of these and six additional A*0201-positive healthy HHV-6 carriers with ILY pentamers (Fig. 1D and E). MLW-specific $CD8^+$ T cells were detected at frequencies of 4 and 14 $\times$ 10^{-5} within $CD8^+$ cells; ILY-specific $CD8^+$ T cells had a median frequency of 1.3 $\times$ 10^{-5} (range, 0–6.4 $\times$ 10^{-5}). Thus, HHV-6 peptide-specific $CD8^+$ T cells are detectable at low frequency in peripheral blood.

Functional analysis of HHV-6B-specific $CD8^+$ T-cell clones

We established $CD8^+$ T-cell clones by limiting dilution of the polyclonal T-cell lines of donors 1 and 2. From donor 1, we obtained 80 T-cell clones; an initial screen for specific IFN-γ secretion showed that they were specific for GIL (three clones), MLW (seven clones), LLC (18 clones), and ILY (six clones). From donor 2, we obtained 53 clones, specific for SLM (seven clones), GIL (one clone), MLW (27 clones), and ILY (nine clones). Thus, all major HHV-6B peptide specificities within the two polyclonal T-cell lines were obtained as T-cell clones. For each HHV-6B peptide specificity, one to three well-proliferating T-cell clones from donor 1 or 2 were further analyzed. All were $CD8^+CD3^+CD4^-$, and ILY- and MLW-specific T-cell clones gave a bright and homogenous staining with the corresponding HLA-A*0201/peptide multimers (data not shown). Figure 2 shows the reactivity pattern of representative T-cell clones specific for each of the five HHV-6B peptides. When challenged with autologous or HLA-A*0201-matched B cells loaded with graded concentrations of peptide, T-cell clones specifically secreted IFN-γ, TNF-α, and granzyme B in a peptide dose-dependent manner (Fig. 2A), indicating that they had antiviral, pro-inflammatory, and cytotoxic effector functions typical for virus-specific $CD8^+$ T cells. We further analyzed the secretion of IFN-γ and GM-CSF (Fig. 2B) by T-cell clones after contact with autologous, HLA-A*0201-matched and mismatched B cell lines (mLCLs). All T-cell clones secreted these two cytokines in strict dependence on peptide loading and HLA-A*0201 expression of the target cell. Different T-cell clones secreted similar amounts of GM-CSF but different maximal amounts of IFN-γ (Fig. 2B), suggesting a degree of functional diversification.

Recognition of intracellularly processed HHV-6B antigen

To study whether HHV-6B peptide-specific T cells recognize intracellularly processed HHV-6B antigens, we transfected HLA-A*0201-positive 293T human kidney cells with plasmids coding for U54 or U11. T-cell clones specific for the U11 epitopes GIL, MLW, and SLM did not recognize transfected 293T cells, in accordance with western blot analyses which indicated that U54, but not U11, was well expressed in transfected 293T cells (data not shown). In contrast, T-cell clones specific for U54 epitopes ILY and LLC specifically recognized U54-transfected 293T cells (Fig. 3). Recognition could be blocked by an HLA-A/B/C-specific antibody. The response of ILY-specific T-cell clones to transfected 293T cells was at least as strong as their response to exogenously peptide-loaded cells (Fig. 3A and B), suggesting that the target HLA/peptide complex was very efficiently produced by endogenous processing of U54. Unexpectedly, LLC-specific T cells had a much higher reactivity to transfected than to exogenously peptide-loaded target cells. Possibly, the LLC peptide was inefficiently loaded onto cells from the soluble phase due to its high hydrophobicity (six aliphatic but no charged side chains). Alternatively, the efficiently presented endogenous peptide may carry a post-translational modification, for example at cysteine 3.

Recognition of HHV-6B-infected target cells

We now addressed the question whether HHV-6B-specific $CD8^+$ T cells recognize target cells infected with the virus. Because productive replication of HHV-6B takes place mainly in $CD4^+$ T cells [22], we infected phytohemagglutinine (PHA) stimulated primary $CD4^+$ T cells from peripheral blood for use as targets in T-cell assays. After 6 days of infection, cells were readily recognized by HHV-6B-specific $CD8^+$ T cells specific for the U11 epitope GIL or the U54 epitopes ILY and LLC, resulting in specific secretion of IFN-γ (Fig. 4A), whereas MLW-specific cells did not recognize infected cells (data not shown). Only infected cells from HLA-A*0201-positive donors were recognized, confirming classical HLA-A*0201-restricted recognition. We also analyzed the potential cytotoxic effector function of an ILY-specific T-cell clone by measuring secretion of the cytotoxic mediator granzyme B (Fig. 4B) and by direct determination of target cell lysis (Fig. 4C). Although baseline cytotoxicity was relatively high, granzyme B secretion and cytotoxicity to target cells were specifically elevated when the targets were both HLA-A*0201-positive and infected with HHV-6B. These results suggest that HHV-6B infection is subject to cytotoxic control by virus-specific $CD8^+$ T cells.

Recognition of infected cells over time

Our previous experiments did not exclude the possibility that the specific $CD8^+$ T-cell clones recognized HHV-6B antigens that were presented by $CD4^+$ T cells after antigen transfer by nonreplicative

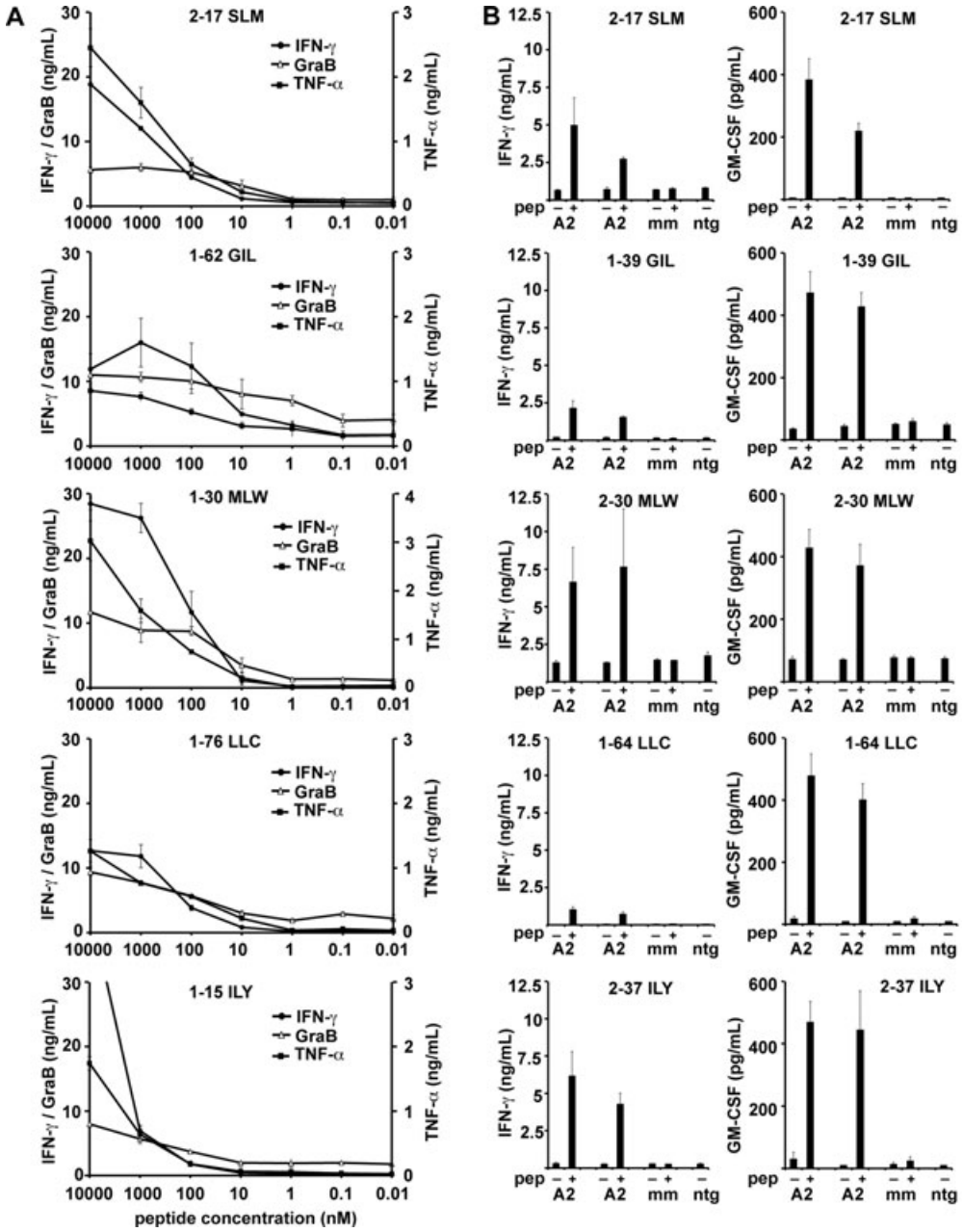

Figure 2. Function of HHV-6B peptide-specific CD8$^+$ T cells. (A) Secretion of IFN-γ, granzyme B (GraB) and TNF-α in response to graded concentrations of peptide loaded on HLA-A*0201-matched mLCLs was assessed for five CD8$^+$ T-cell clones specific for HHV-6B peptides SLM, GIL, MLW, LLC, or ILY. Every T-cell clone was analyzed for reactivity to its specific target peptide. ELISA was performed after overnight cocultivation of 20 000 T cells and 50 000 peptide-loaded B cells per replicate. IFN-γ and GraB, left Y-axis; TNF-α, right Y-axis. (B) HLA-restricted secretion of IFN-γ (left) and GM-CSF (right) by CD8$^+$ T-cell clones specific for five HHV-6B peptides (SLM, GIL, MLW, LLC, ILY) in response to peptide-loaded HLA-A*0201-matched mini-LCLs (A2) or HLA-A*0201-nonexpressing mini-LCLs (mismatched, mm). As a negative control, T-cell clones were incubated in the absence of target cells (no target, ntg). ELISA was performed after overnight cocultivation of 20 000 T cells and 20 000 peptide-loaded stimulator cells per replicate. (A, B) Mean and range of two replicates from one representative experiment out of one (SLM), two (GIL, LLC), or four experiments (MLW, ILY) is shown for each T-cell clone.

particles or other components of the virus preparation, but not after de novo antigen synthesis in cells replicating HHV-6B. To address this question, we analyzed the recognition of infected cell cultures over time in the absence or presence of inhibitors of late HHV-6B gene expression, foscarnet or ganciclovir [5]. CD8$^+$ T cells specific for the U54 ILY epitope did not recognize HHV-6B-infected cells on day 1 after infection (Fig. 5A), but recognition continuously increased thereafter, peaking on day 6 (Fig. 5B). CD8$^+$ T-cell recognition was fully inhibited by ganciclovir or foscarnet treatment of infected cells, demonstrating that the CD8$^+$ T cells recognized U54 that was expressed, processed, and presented by HHV-6B-replicating cells in late lytic cycle. An increase

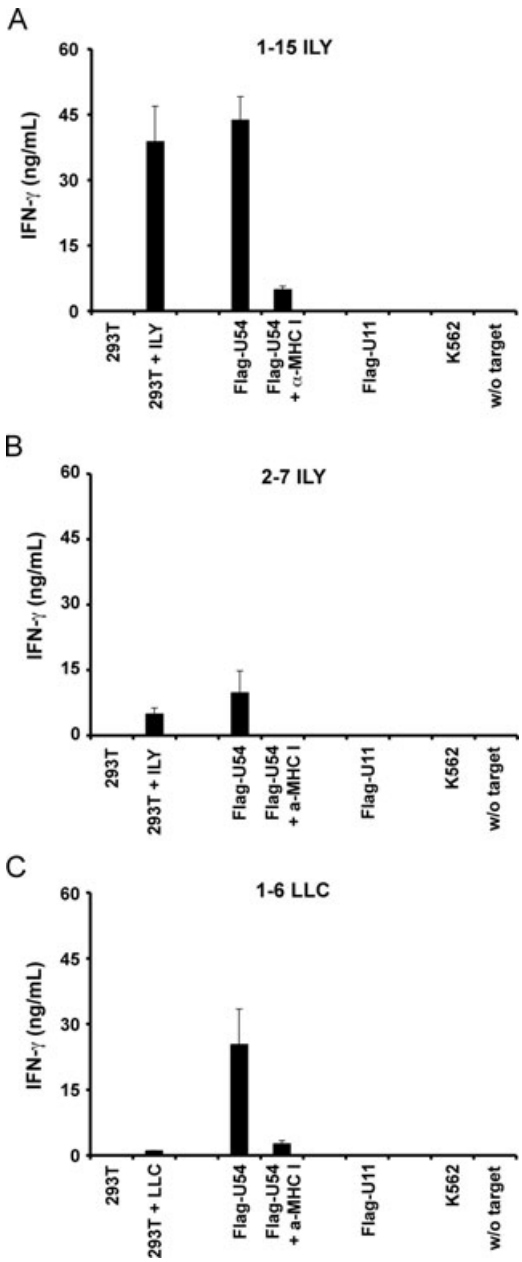

Figure 3. Recognition of endogenously expressed antigen U54 by HHV-6-specific CD8$^+$ T cells. The reactivity of three different CD8$^+$ T-cell clones specific for U54-derived peptides (A, B) ILY and (C) LLC in response to 293T cells loaded with peptide (293T+ILY, 293T+LLC) or transiently transfected with plasmid vectors coding for Flag-tagged HHV-6B antigens U54 (Flag-U54) or U11 (Flag-U11) was assessed. To block MHC class I-restricted recognition of antigen, anti-MHC class I-antibody (α-MHC I) was added to Flag-U54-transfected cells. K562 cells served as an additional negative control target. Mean and range of two replicates from one of the two experiments is shown.

of recognition from day 3 to 6 after infection was also noted for a CD8$^+$ T-cell clone specific for the U54 LLC epitope (Fig. 5C); limited availability of this T-cell clone prevented the analysis of more time points. Neither of the CD8$^+$ T-cell clones recognized targets infected with HHV-6A. This was unexpected for the LLC epitope, which is identical in HHV-6A and 6B (Table 1), and raises the possibility that HHV-6A interferes more strongly with CD8$^+$ T-cell recognition than HHV-6B. Expression of U11 and U54 mRNA in infected cells increased in parallel with CD8$^+$ T-cell recognition (Fig. 5D) and remained high after day 6, whereas T-cell recognition decreased after day 6 (Fig. 5B). Taken together, specific CD8$^+$ T cells recognize processed antigens from HHV-6B structural proteins on infected cells in late lytic cycle. However, viral functions potentially interfere with recognition of HHV-6A-infected cells and of long-term HHV-6B-infected cells.

Discussion

In this study, we identify HHV-6B-specific CD8$^+$ T cells. We show that CD8$^+$ T cells specific for three HLA-A*0201-restricted peptides from the antigens U11 and U54 are present at low frequency in healthy virus carriers, can be obtained as T-cell clones in vitro, recognize productively virus-infected cells, and mobilize a variety of antiviral effector functions. While the specificities we describe may only be part of a much wider repertoire, our data establish that HHV-6B-specific CD8$^+$ T cells recognize infection. The methods we describe for preparation and functional analysis of such T cells may be useful in further studies. We conclude that HHV-6B-specific CD8$^+$ T cells likely contribute to control of infection in vivo.

To our knowledge, this work is the first to describe CD8$^+$ T cells specific for an HHV-6 species. Since its discovery in 1986 [23], relatively few studies have been dedicated to HHV-6-specific cellular immunity, and these have involved CD4$^+$ T cells or uncharacterized effectors from blood. Early studies have shown that PBMCs from most healthy carriers proliferate in response to HHV-6 lysates [24]. CD4$^+$ T-cell clones specific for total viral antigen could be established from lysate-stimulated cultures, and some of these clones specifically killed HHV-6A-infected lymphocytes [25]. Analyses of a large set of HHV-6-specific CD4$^+$ clones showed that most of them recognized both variants A and B [26]. HHV-6-specific CD8$^+$ T cells were not observed in these studies, possibly because exogenous viral antigen is not efficiently processed for presentation by HLA class I molecules [27]. In contrast, our approach involved stimulation of blood cells with peptide-loaded CD40-activated B cells, an efficient way to expand CD8$^+$ T cells of low frequency [20, 21]. The definition of T-cell target antigens of HHV-6 was first attempted in studies of responses of blood cells to subsequences of two HHV-6 proteins, U11, and U24 [28, 29]. These antigens were chosen because U11 is a major target of virus-specific antibodies [30], and the U24 sequence contains a 7-amino acid stretch that is identical to a subsequence of human myelin basic protein, giving rise to the idea that cross-reactive T cells might be involved in the pathogenesis of multiple sclerosis [29]. However, the study on U11 analyzed only short-term proliferative responses of blood cells; in the study on U24, specific reactivity was only analyzed using exogenous peptide, leaving open the question of specificity for intracellularly processed antigen. A recent, more comprehensive approach identified several viral epitope peptides that were recognized by virus-specific CD4$^+$

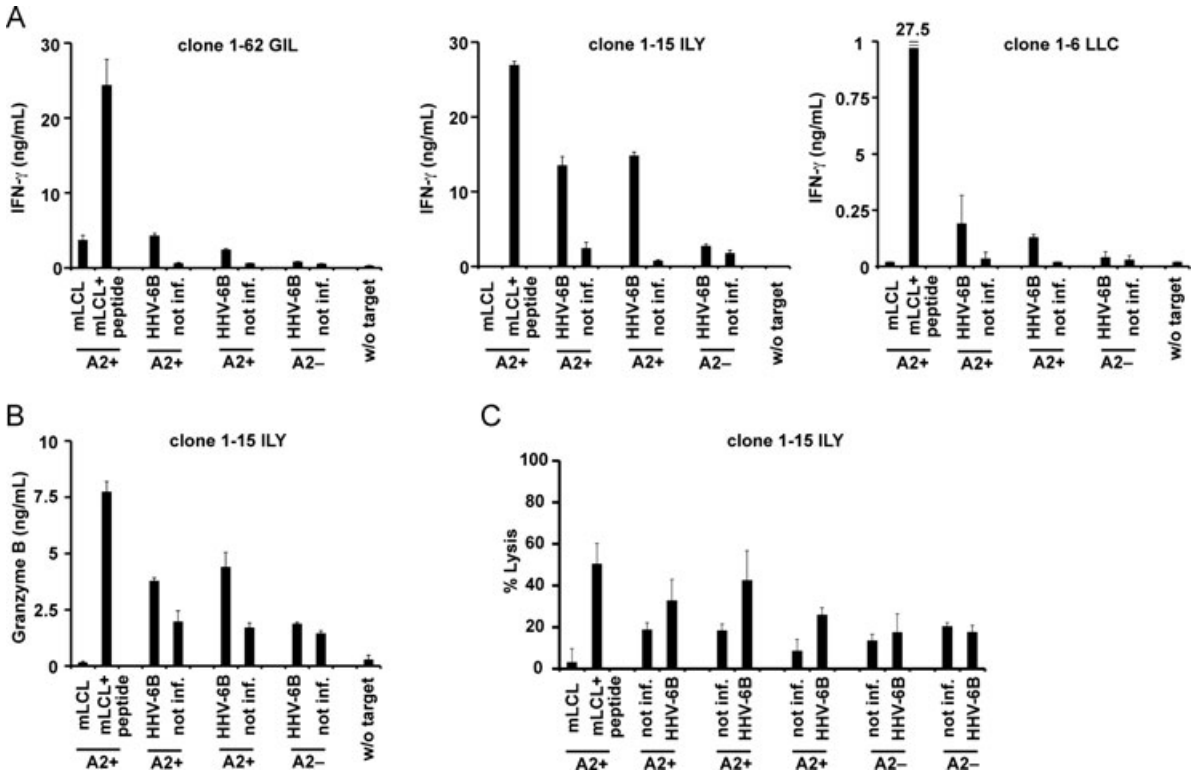

Figure 4. Reactivity of HHV-6B-specific CD8$^+$ T cells to infected target cells. PHA-activated primary CD4$^+$ T cells from HLA-A*0201-positive or negative donors were infected with HHV-6B, or not infected, and cocultivated with CD8$^+$ T-cell clones specific for different peptides from antigens U11 (GIL) and U54 (ILY and LLC) as indicated. Additional control targets were autologous or HLA-A*0201-matched mini-LCLs, untreated or preloaded with the relevant peptide. Reactivity of the T-cell clones was assessed by measuring (A) IFN-γ secretion, (B) release of granzyme B, and (C) direct cytotoxicity toward target cells. Data are shown as mean and range of two replicates from three of ten (A) and one of two (B, C) experiments.

T cells after processing of exogenous viral antigen [15]; most of these epitopes were from structural proteins including U11 and U54. All identified specificities could be efficiently expanded in vitro but were very rare in peripheral blood [15], suggesting that the HHV-6-specific CD4$^+$ T-cell response may consist of many different specificities, each of low frequency. Our results are compatible with the hypothesis that the HHV-6B-specific CD8$^+$ T-cell repertoire might be similarly structured, but more comprehensive analyses of CD8$^+$ T-cell specificities will be required to test this.

For CD8$^+$ T cells, it was unknown which of the close to 100 genes of HHV-6B [31] encode potential target antigens. We hypothesized a similarity between the antigenic repertoires of HHV-6 and its better-studied human beta-herpesvirus family member CMV. Therefore, we searched for potential T-cell epitopes in putative HHV-6B homologs [32] of well-characterized immunodominant CMV CD8$^+$ T-cell antigens pp150, pp65, and IE-1 [33]. Our hypothesis was based on the idea that similar function or expression might predispose the HHV-6 homologs of these CMV antigens for presentation to T cells. Of note, we did not expect any homology of our selected HHV-6 and CMV antigens on the epitope level; their sequences are too divergent to expect cross-reactive T-cell responses. For example, U54 and pp65 share only 20% of amino acid identity, uniformly distributed over the sequence; this value is close to random similarity.

A much higher likelihood existed for cross-reactivity of HHV-6B-specific T cells with HHV-6A. In fact, one of the three HHV-6B epitopes shown to be presented by infected cells, LLC, is identical in HHV-6A. Nevertheless, LLC-specific CD8$^+$ T cells were specific for HHV-6B and did not recognize HHV-6A infection (Fig. 5B), for reasons that remain speculative. Interestingly, the HHV-6A immunoevasin U21 downregulates surface MHC class I on astrocytes more efficiently than its HHV-6B counterpart [34]. Considering that CMV interferes with MHC class I presentation in a highly antigen-dependent [35] and MHC class I allele-specific manner (Ameres et al., unpublished), interference of HHV-6 with CD8$^+$ T-cell recognition may be more intricate than currently known.

Our present findings may contribute to a reconsideration of previous ideas on the interactions of HHV-6 with the immune system. In marked contrast to herpesviruses such as CMV and EBV, the interaction of HHV-6 and the immune system was interpreted predominantly in terms of the immunosuppressive effects of the virus [16]. Because HHV-6 may inhibit T cells [17, 18, 36] and APCs [19, 37] by a variety of mechanisms, some of them operative only in infected cells but some also in bystander cells, HHV-6 has been described as an immunosuppressive virus [16]. This has left open the question how healthy carriers control HHV-6 infection, and how virus-specific T cells may contribute to control, although

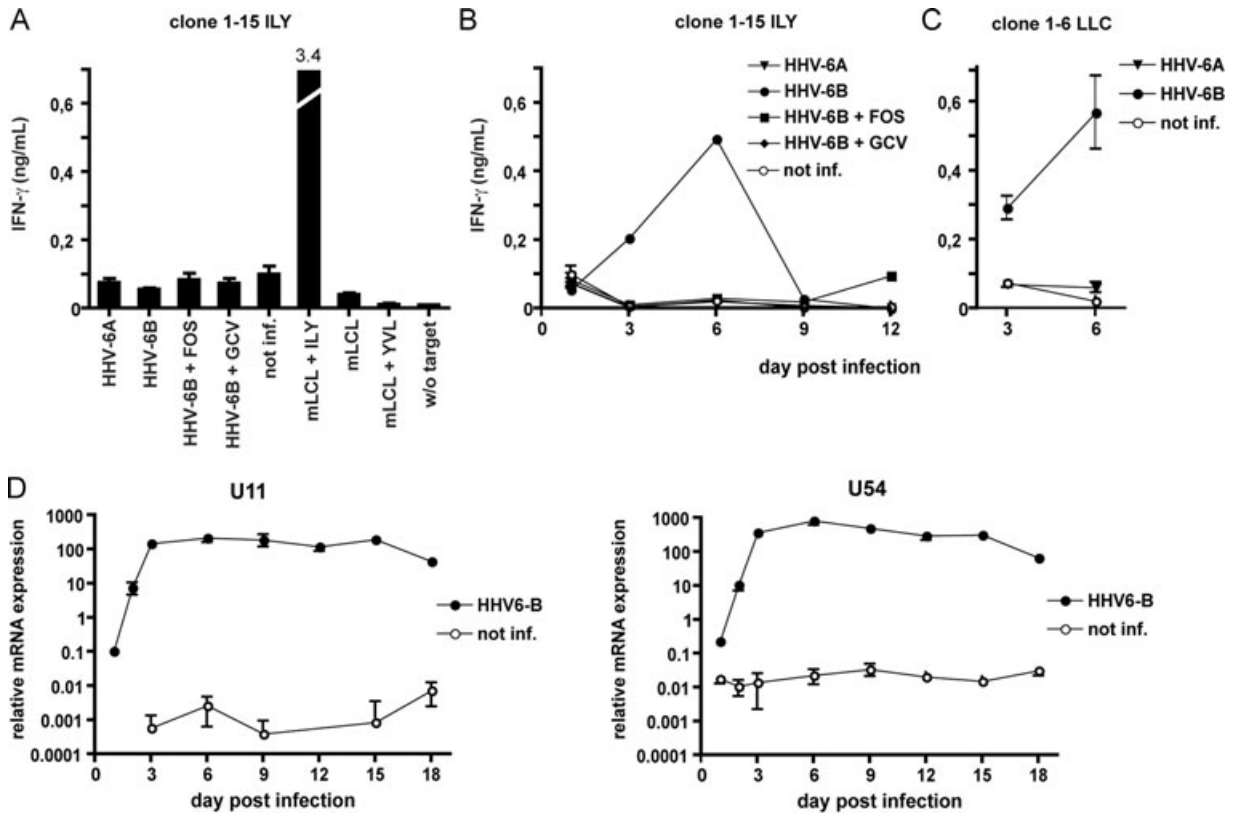

Figure 5. T-cell recognition of HHV-6A/6B-infected cells over time. HLA-A*0201-positive CD4$^+$ T cells were infected with HHV-6A or -6B, and their recognition by ILY- or LLC-specific CD8$^+$ T-cell clones was tested at (A) 1 day or (B, C) various time points after infection by ELISA. Foscarnet (FOS) or ganciclovir (GCV) was added at the time of infection where indicated. Controls included mLCLs loaded with relevant (ILY) or irrelevant (YVL) peptide. Data are shown as mean and range of duplicates from one of two (A, B) or a single (C) experiment. (D) Transcript levels of U11 and U54 were determined by pRT-PCR and normalized against glucuronidase beta. Data are shown as mean + SD of triplicates from one of the two experiments.

such knowledge is essential for an understanding of HHV-6 disease and the development of new (immuno) therapies. For example, in patients after stem cell transplantation, HHV-6 and CMV reactivation were associated with lymphopenia and reduction in proliferative responses to HHV-6 and CMV [38, 39]. However, these results were interpreted in contrasting terms for the two herpesviruses: HHV-6 was supposed to be the causal agent for an immunosuppression that permitted reactivation of this virus and of CMV [38, 39]. Only recently, the straightforward alternative explanation has been offered that lymphopenia-associated deficiencies in T cells specific for either virus could be the common cause why either virus may reactivate in transplant patients [40]. Accordingly, reconstitution of antigen-specific T cells might help control HHV-6 infection, in analogy to therapeutic strategies for CMV [12, 27] or EBV [13, 14], especially in patients with impaired overall T-cell reconstitution after allogeneic stem cell transplantation. If the frequencies of HHV-6-specific T cells prove too low in donor blood to permit their direct isolation, they could be manufactured using T-cell receptor gene transfer, in analogy to procedures proposed to obtain CMV-specific T-cells from nonimmune donors [41]. Future studies of HHV-6-specific CD8$^+$ T-cell responses in patients with different HHV-6-associated diseases will help understand how this still enigmatic virus is controlled, how it causes disease, and how its therapy may be improved.

Materials and methods

Ethics statement

PBMCs from anonymous healthy adult donors were obtained from the Institute for Transfusion Medicine, University of Ulm, Germany. The institutional review board (Ethikkommission, Klinikum der Universität München, Munich, Germany) approved the use of these anonymized samples. All work was conducted according to the principles expressed in the Helsinki Declaration.

Cells and cell lines

Standard cell culture was performed as described [41]. PBMCs were obtained by centrifugation on Ficoll/Hypaque (Biochrom). High-resolution HLA typing was performed by PCR-based

methods (IMGM). HLA types of the donors whose T-cell clones are described in this study are as follows: donor 1, HLA-A*0201, A*2501, B*1503, B*51, C*1203, C*1402, DRB1*07, DRB1*08; donor 2, HLA-A*0201, A*29, B*44, C*05, DRB1*04, DRB1*15. Both donors were seropositive for HHV-6 and EBV, donor 2 was additionally seropositive for CMV (Max-von-Pettenkofer Institute, Munich, Germany). Cell lines and cultures from these and other HLA-typed donors were used as APCs in T-cell assays. Mini-lymphoblastoid cell lines (mLCLs) are continuously proliferating activated B-cell lines transformed by a lytic replication-deficient mini-EBV, and were generated as described [42, 43]. CD40-activated B-cell cultures were established and maintained as described [20]. Human embryonic kidney 293T cells (partial HLA type: HLA-A*0201, B*0702) were obtained from ATCC (CRL-11268).

Peptides

Nonameric peptides from HHV-6B antigens, putatively presented by HLA-A*0201 (Table 1), were chosen for study based on the following criteria: (i) a high score in the SYFPEITHI algorithm [44]; (ii) the presence of hydrophobic aliphatic amino acids (L/V/I/M) in positions 2, 6, and 9; (iii) the presence of such amino acids in positions 2, 7, and 9. For U54, the three top SYFPEITHI-scoring nonamers (scores 29, 28, 26) all fulfilled criterion (ii) and were chosen for study. For U89/90, among nonamers that fulfilled criterion (ii), the two with the best SYFPEITHI scores (24 and 22) were chosen. For U11, five peptides fulfilling (ii) (scores 29, 29, 25, 23, 21) and two peptides fulfilling (iii) (scores 27 and 27) were chosen. Peptides were synthesized to >70% purity by JPT (Berlin). EBV-derived peptides matched to the donors' HLA types were used as "supporter peptides" in the first rounds of T-cell stimulation as described below. Their short designations (amino acid sequences, antigens of derivation, and HLA restrictions) are as follows: YVL (YVLDHLIVV, BRLF1, HLA-A*0201); PYYV (PYYVVDLSVRGM, BHRF1, HLA-DR4); NEIF (NEIFLTKKMTEVC, BALF4, HLA-DR8); TDAW (TDAWRFAMNYPRNPT, BNRF1, HLA-DR15). All of these EBV epitopes were described previously [45, 46]. Peptides YVL and NEIF were used for donor 1, peptides YVL, PYYV, and TDAW for donor 2.

Generation of HHV-6B-specific T-cell lines and clones

HHV-6B-specific T cells were enriched from PBMCs from donors 1 and 2 by stimulation with a mixture of 12 HHV-6B peptides (Table 1). At day 0, PBMCs were coincubated with the pool of 12 HHV-6B peptides and two to three EBV peptides (1 μg/mL for each peptide) at 37°C for 2 h. To remove excess peptides, cells were washed repeatedly with PBS and plated at 5×10^6 cells in 2 mL per well of a 12-well plate. Ten to 14 days later, cells were pooled, counted using Trypan blue staining, and replated at 3×10^6 cells in 2-mL medium per well, adding freshly irradiated (50 Gy) autologous CD40-activated B cells previously loaded with peptides to an effector:stimulator ratio of 4:1, and 25–50 U/mL recombinant IL-2 (Novartis). Cells were restimulated every following week with peptide-loaded CD40-activated B cells in the same manner; the IL-2 concentration was successively increased to 100 U/mL. Between stimulations, the T-cell cultures were expanded using fresh IL-2-containing culture medium as required. EBV "supporter peptides" were included in the first three stimulations; all later stimulations were performed only with the HHV-6B peptides. Preliminary experiments had indicated that EBV "supporter peptides" improved the survival of T-cell cultures in their early phase of cultivation, presumably due to paracrine effects mediated by transiently coexpanded EBV-specific T cells.

For single-cell cloning, 0.5 or 3 T cells/well were seeded into 96-well round-bottom plates. A 2×10^4/well irradiated (50 Gy) HLA-A2-positive mini-LCLs loaded with the 12 HHV-6B peptides (Table 1), 3×10^5 cells/well of a mixture of irradiated (50 Gy) allogeneic PBMCs from three different donors, and 1000 U/mL IL-2 were added. Outgrowing T-cell clones were expanded in 96-well round-bottom plates by restimulating every 2 weeks under the same conditions. Clones with known specificity were restimulated in an analogous manner but using only the single specific peptide.

Flow cytometry

HLA-A*0201/peptide pentamers containing the HHV-6B peptides ILY and MLW and the EBV peptides YVL and CLG (CLGGLLTMV from LMP2) [45] were synthesized by Proimmune. Synthesis of pentamers for HHV-6B peptide LLC failed for unknown reasons. Pentamer staining was performed by incubating T cells for 10 min at room temperature with unlabeled HLA/peptide pentamer. The cells were counterstained on ice for 15 min with anti-CD4-FITC, anti-CD3-PE-Cy5, anti-CD8 allophycocyanin antibodies (all BD Pharmingen), and Pro5 Fluorotag R-PE (Proimmune). Directly after staining, the cells were resuspended in 1.6% formaldehyde (Carl Roth) for fixation. In negative control stainings, pentamer was omitted in the first step. Cells were analyzed on a Becton Dickinson FACSCalibur. Data analysis was performed using FlowJo 8.8.4 software (Tree Star). Viable lymphocytes were gated according to forward/sideward scatter; no other gates were applied. All dot plots shown span, on both coordinates, a range from 1 to 10 000 arbitrary fluorescence units in a logarithmic scale.

Endogenous expression of the HHV-6B antigens U11 and U54 in 293T cells

To clone HHV-6B antigens, RNA was extracted from strain HST-infected PHA-activated $CD4^+$ T cells (RNeasy Mini Kit, Qiagen). cDNA was synthesized using murine leukemia virus reverse

transcriptase and oligodeoxythymidylate primer (MBI-Fermentas). Sequences coding for HHV-6B proteins U54 and U11 were amplified from cDNA using specific primers containing 5′-*Not*I and 3′-*Bam*HI sites, and cloned into pCMV2-FLAG for transient expression in N-terminally FLAG-tagged form. HLA-A*0201-positive 293T cells were transfected by calcium phosphate precipitation. Twenty-four hours later, cells were harvested, washed with PBS, and used as targets in T-cell assays.

T-cell effector assays

HHV-6B-specific T-cell clones and lines were analyzed for specific cytokine secretion in ELISA or ELISpot assays, or for specific cytotoxicity in calcein release assays. For ELISA, effector cells (10^4, unless noted otherwise) were co-cultivated overnight with target cells (2×10^4, unless noted otherwise) in 200 μL per well of a 96 V-well plate at 37°C and 5% CO_2. Then supernatants were harvested, and an ELISA specific for IFN-γ, GM-CSF, granzyme B, or TNF-α was performed (Mabtech). For ELISpot, effector cells (10^3) were co-cultivated overnight with target cells (2×10^4) in 200 μL per well of a Multiscreen-HA plate (Millipore) at 37°C and 5% CO_2. Spots were developed according to the manufacturer's protocol (Mabtech). Specific cytotoxicity was determined in 4 h calcein release assays as described [20].

HHV-6A and -6B infection of PHA-activated T cells

HHV-6A (strain U1102) and HHV-6B (strain HST) purchased from NCPV, UK, were serially propagated on PHA-activated cord blood mononuclear cells. Fresh or cryoconserved cord blood cells at 2×10^6 cells in 2 mL per well of a 24-well plate were stimulated with 5 μg/mL PHA-M (Calbiochem). Two to four days later, cells were expanded 2-fold and infected with 230 μL virus suspension per well. After 5–7 days, cytopathic effect appeared maximal, cell cultures were harvested, cells were pelleted by centrifugation at 300 × *g* for 10 min, and supernatants were stored in aliquots at −80°C.

Peripheral blood from adult donors with known HLA types was used to prepare HHV-6B-infected target cells for the analysis of T-cell function. $CD4^+$ T cells were positively isolated from PBMCs using anti-CD4-coupled paramagnetic beads (Miltenyi Biotec), and 2×10^6 $CD4^+$ cells were activated in 2 mL of a 24-well plate using 5 μg/mL PHA. After 3 days, the cells were pooled, counted, replated at 2×10^6 cells/well, and infected with 230 μL per well of HHV-6A or HHV-6B virus stocks. Six days after infection (unless noted otherwise), the cells were used as targets for HHV-6-specific T-cell clones in cytokine secretion or cytotoxicity assays. In selected experiments (Fig. 5), foscarnet (100 μg/mL, Sigma) or ganciclovir (20 μg/mL, Roche) was added immediately before infection. Viral mRNAs in HHV-6B-infected PHA-activated $CD4^+$ T cells were quantified by real-time RT-PCR (Roche LightCycler). Data were normalized to levels of glucuronidase-beta.

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Abbreviations: **HHV-6**: human herpesvirus(es) 6 · **mLCL**: mini-lymphoblastoid cell line

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