Immunotherapeutic strategies to prevent and treat human herpes virus (HHV) 6 reactivation post allogeneic stem cell transplant

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Abstract

Human herpesvirus (HHV) 6 causes substantial morbidity and mortality in the immunocompromised host and has no approved therapy. Adoptive transfer of virus specific T cells has proven safe and apparently effective as prophylaxis and treatment of other virus infections in immunocompromised patients; however, extension to subjects with HHV6 has been hindered by the paucity of information on targets of cellular immunity. We now characterize the cellular immune response from 20 donors against five major HHV6B antigens predicted to be immunogenic and define a hierarchy of immunodominance of antigens based on the frequency of responding donors and the magnitude of the T cell response. We identified specific epitopes within these antigens and expanded the HHV6 reactive T cells using a GMP-compliant protocol. The expanded population comprised both CD4+ and CD8+ T cells that were able to produce multiple effector cytokines and kill both peptide-loaded and HHV6B wild type virus-infected target cells. Thus, we conclude that adoptive T cell immunotherapy for HHV6 is a practical objective, and that the peptide and epitope tools we describe will allow such cells to be prepared, administered and monitored in human subjects.
Introduction
Adoptive T cell immunotherapy can successfully prevent and treat otherwise fatal infections in the immunocompromised host. At present, these benefits have been restricted to adenovirus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV)^1^9. However, it is increasingly apparent that a distinct herpesvirus, human herpesvirus 6 (HHV6) is also a significant cause of morbidity and mortality, particularly after hematopoietic stem cell transplantation (HSCT). To date, there have been no controlled studies on the use of antivirals as treatment for HHV6, but foscarnet, ganciclovir, and cidofovir have been used clinically, with variable results^10^-^14^.

HHV6 is a member of the β-herpesvirus subfamily and exists as two species, HHV6A and HHV6B, which share 75-95% nucleotide sequence identity^15^. Primary infection occurs in >90% of individuals before the age of 2 years and is associated with clinical symptoms including acute febrile illness and roseola infantum or exanthem subitum^16^. The virus subsequently persists in latent form, in an analogous way to other herpesviruses. Although latency is usually asymptomatic, HHV6 reactivates in >50% of allogeneic HSCT recipients and can produce clinically significant manifestations including encephalitis, delayed engraftment and an increased rate of GVHD, substantially increasing mortality^10^;^11^;^14^;^17^-^19^.

Thus, while HHV6 might be a suitable candidate virus to benefit from adoptive immunotherapy, such treatments can only be developed if there is evidence that T cells recognizing the virus exist in (or can be generated from) the peripheral blood of healthy seropositive individuals. If such cells exist, then it is important to know the antigens and HLA-restricted epitopes they recognize, so that the most effective T cells can be identified and prepared.

We now demonstrate that HHV6-reactive CD4+ and CD8+ T cells do indeed exist in the peripheral blood of healthy subjects and HSCT recipients and that these cells can be expanded ex vivo. We have also analyzed the antigens recognized by these HHV6-specific T cells, and identified CD8+ epitopes within those antigens that are most immunodominant. Finally, we show that HHV6 peptide-specific T cells can react to, and kill, virus-infected target cells. We anticipate such cytotoxic T lymphocytes (CTLs) will prove of value in the prevention and treatment of HHV6 infections in the immunocompromised.
Materials and Methods
Donors and Cell Lines
Peripheral blood mononuclear cells (PBMCs) from healthy volunteers and HSCT patients were obtained with informed consent per the Declaration of Helsinki on IRB-approved protocols. PBMCs were used to generate CTL lines and PHA blasts. PHA blasts were generated from PBMCs (2×10^6/ml) using PHA (5μg/ml) and maintained in CTL media (RPMI 1640, 45% Click’s (Irvine Scientific, Santa Ana, CA), 2mM GlutaMAX TM-I (Invitrogen, Carlsbad, CA), and 5% Human AB Serum (Valley Biomedical, Inc., Winchester, VA)) supplemented with IL2 (100U/ml, NIH, Bethesda, VA), which was replenished every 3 days.

CTL generation – Peptide stimulation
Peptides/Pepmixes
For stimulation we used either commercially available or custom-ordered pepmixes (15 mers overlapping by 11aa) spanning U54 (JPT Technologies, Berlin, Germany), U90, U11, U14, and U71 (Genemed Synthesis Inc., San Antonio, Texas). Control pepmixes spanning adenovirus-Hexon, CMV-pp65, and CMV-IE1 were also purchased from JPT. For epitope mapping, peptides were pooled into 25 (U14) and 33 (U90) mini-pools (Figure 3A and Supplementary Figure 2) such that each 15mer was represented in 2 mini-pools. To identify minimal epitopes, we generated a series of 9mers overlapping by 8aa spanning immunogenic sequences of U14 aa597-610 VARRLTEMMNDARL and U90 aa53-71 CDVSFESLLFPELEAFDLF and aa29-51 RMQNYHPDPVVEESIKEILESL, which were used as a stimulus in IFNγ ELIspot. Lyophilized peptides were reconstituted at 5mg/ml in DMSO.

PBMC stimulation
PBMCs were isolated from whole blood by ficoll gradient, pelleted and pulsed for 30-60min at 37°C with peptides/pepmixes, individually or pooled (100ng/peptide/15x10^6 PBMCs). After incubation cells were resuspended in CTL media with IL7 (10ng/ml) + IL4 (1000U/ml) (R&D Systems, Minneapolis, MN) and plated in a 24-well plate (2x10⁶/well). Media and cytokines were replenished on day 5, and cultures split at a density >3x10^6 cells/well. On day 9-11 CTLs were harvested, counted and used for phenotypic/functional studies.

CTL generation – HHV6 z29-infected PBMC
HHV6B z29 supernatant
MOLT3 cells were maintained in cell culture media (RPMI-1640+10% FBS) (Hyclone, Waltham, MA) and 2mM GlutaMAX. For infection, MOLT3 cells were resuspended in a 15ml falcon at 5x10^6/ml in HHV6 z29 supernatant and incubated for 90-120min at 37°C. Subsequently, infected cells were resuspended at 0.8x10^6 cells/ml in cell culture media, cultured for 10-12 days, and split when >80% confluence was reached. On day 10-12, when 90% of the cells showed cytopathic effects, cells were collected, pelleted (10min/1000xg) and virus-containing supernatant was harvested and frozen at -80°C.

**CTL generation using HHV6B z29**

PBMCs were isolated, resuspended at 1x10^6 cells/ml in a 1:1 mix of viral supernatant and CTL media with IL4+7, and cultured in a 24-well plate (2x10^6 cells/well). On day 5-6 cultures were replenished with media + cytokines and on day 9-10 harvested, counted and used for phenotypic/functional analyses.

**Immunohistochemistry**

Infected cells were resuspended in PBS, mounted onto glass slides (Shandon Cytospin 4 Cytocentrifuge; Thermo Scientific, Waltham, MA) and then placed in the steamer for 10min in target retrieval solution (DAKO). Slides were incubated in preblock/diluent for 30min followed by incubation with mouse-anti-human HHV6B mAb (monoclonal antibody MAB8535, 101kDa protein, clone C3108-003 - Chemicon International Inc., Temecula, California) diluted 1:50 in diluent for 1hr at RT. HRP/ biotin-conjugated anti-mouse-Ig was used to detect positive cells, followed by enzymatic conversion using the chromogenic substrate 3,3 diaminobenzidine.

**Mapping studies**

Epitope mapping were performed using CD8-enriched T cell lines, produced by depletion of CD4/CD56/CD16 cells using MACS depletion (Miltenyi Biotec, Bergisch Gladbach, Germany) as per manufacturers’ instructions. The purity of the CD8-enriched CTL fraction was evaluated using FACS; cells were then counted and used for functional studies.

**Flow Cytometry**

**Immunophenotyping**

CTLs were surface-stained with antibodies to: CD3, CD4, CD8, CD14, CD16, CD56, CD27, CD45RO, and CD62L (BD, Franklin Lakes, NJ). Cells were washed once with PBS+2% FBS (Sigma, St Louis, MO), pelleted, and 10μl antibody added. After 15mins at 4°C in the dark, cells
were washed and analyzed. Approximately 20,000 live cells were analyzed. Samples were acquired on a FACSCalibur equipped with Cell Quest software (BD).

Intracellular cytokine staining
CTLs were harvested, resuspended at 5x10^6/ml in CTL media, plated at 200μl/well in a 96-well U-bottom plate, and stimulated with 100ng of test or control peptide/pep mix in Brefeldin A (1μg/ml), CD28 and CD49d (1μg/ml) (BD) for 5-7 hrs. Subsequently, CTLs were washed with PBS+2% FBS, pelleted, and surface stained with CD3, CD8 and/or CD4 (10μl/tube). After 15mins, cells were washed twice, pelleted, fixed and permeabilized with cytofix/cytoperm (BD) for 20mins at 4°C, then washed twice with PBS/2%FBS+0.1% saponin (Calbiochem, EMD Chemicals, NJ) and incubated with 20μl IFNγ and/or TNFα antibodies (BD) for 30min at 4°C in the dark. Cells were then washed twice with cold PBS+2%FBS+0.1% saponin and at least 100,000 events analyzed.

FoxP3 staining
FoxP3 staining was performed using the e-Bioscience FoxP3 kit. Briefly, CTLs were rested in CTL media for 48hrs, then 1x10^6 cells were resuspended in PBS+2% FBS and surface stained for CD3/25/4. After washing cells were resuspended in 1ml fixation/permeabilization solution, incubated for 1hr (4°C), then washed, resuspended in permeabilization buffer and incubated with 0.2μl isotype or 10μl FoxP3 antibody (Clone PCH101) for 30mins at 4°C then washed and acquired using a FACSCalibur.

Functional studies
Multiplex
1x10^5 CTLs were stimulated using 100ng/peptide HHV6B, pp65 or control pep mixes. After 16hrs supernatant was collected and the cytokine profile assessed using the MILLIPLEX High Sensitivity Human Cytokine Panel (Millipore, Billerica, MA). Specifically, 50μl supernatant was incubated overnight at 4°C with beads, then washed and incubated for 1hr at RT with the biotinylated detection antibody. Finally Streptavidin-Phycoerythrin was added for 30min at RT, and duplicate samples were washed and analyzed using the Luminex 200.

Enzyme-linked Immunospot Assay
We used IFNγ and Granzyme B ELIspot to quantify antigen-specific T cells. Triplicate samples were serially diluted from 4x10^6-2.5x10^4 cells/well, and specificity measured after direct
peptide/pepmix stimulation. Unstimulated or cells stimulated with an irrelevant pepmix served as controls. After 16-18hrs incubation, plates were developed, dried, and sent to Zellnet Consulting for quantification. Spot-forming cells (SFC) and input cell numbers were plotted, and a linear regression calculated after excluding plateau data points. The frequency of antigen-specific cells was expressed as SFC/input cell number.

**Chromium (Cr$^{51}$) release assay**

Cytotoxicity was measured in a 4-6hr Cr$^{51}$ release assay, using E:T ratios of 40:1, 20:1, 10:1, and 5:1. As targets we used autologous HHV6B z29-infected or peptide/pepmix-pulsed monocytes. Autologous mock-infected monocytes served as controls. To identify HLA restriction, we used autologous and partially HLA-matched peptide-pulsed PHA blasts as targets. Percentage specific lysis was calculated as specific lysis \[ \frac{(\text{experimental release}-\text{spontaneous release})}{(\text{maximum release}-\text{spontaneous release})} \times 100\].

**Coculture experiments**

CD14-isolated monocytes were infected by culture in a 1:1 mix of HHV6B z29 viral supernatant and CellGenix media (CellGenix, Antioch, IL), 2 mM L-GlutaMAX (1x10$^6$ cells/ 24-well) with 800U/ml GM-CSF (Sargramostim Leukine, Immunex Corp.,Seattle, WA) and 1,000U/ml IL4 for 24hrs. Subsequently, infected or mock-infected monocytes were mixed with HHV6B or control CTLs at a 1:2 monocyte:T cell ratio in CTL media with IL4+7 and after 24hrs were collected, counted, stained (CD3 and CD11c), and analyzed by FACS.
Results

Identification of immunogenic HHV6B antigens

HHV6 shares approximately 70 conserved and co-linear genes with CMV, in which the immediate early 1 (UL123) and tegument proteins (UL32: pp150, UL25:pp85, UL83:pp65 and UL99:pp28) are immunodominant T cell targets. Since little is known about the T cell immune response to HHV6B, we assessed whether the corresponding HHV6B genes (respectively U90, U11, U14, U54 and U71 – Table 1) had equivalent immunogenicity in seropositive individuals. We isolated PBMCs from healthy donors, stimulated them with pepmixes (peptide libraries of 15mers overlapping by 11aa) spanning each antigen and evaluated the frequency of antigen-specific T cells in peripheral blood. The frequency directed against our target antigens was low (median <6.5 SFC/4x10⁵ PBMC) as evaluated by IFNγ ELIspot (Figure 1A), in sharp contrast to responses against CMV, in which circulating T cells with reactivity against pp65 (HHV6 homolog = U54) and IE1 (HHV6 homolog = U90) may form 1-10% of the total T cell population (21-25, Figure 1A).

To determine whether the apparent paucity of HHV6-reactive T cells in peripheral blood reflected a frequency below the limit of detection of the ELIspot, we stimulated PBMCs with each of the HHV6B pepmixes and cultured the cells for 9-10 days in vitro in the presence of IL4+7 to support the expansion of antigen-specific CTLs26. When we analyzed the specificity of the expanded lines by IFNγ ELIspot, we now readily detected immunity to all stimulating antigens. These expanded HHV6-reactive cultures comprised CD3+ T cells (mean 86.9±0.3%), with a majority of helper (CD4+) T cells (59.6±0.6%) and a minor CD8+ T cell component (31.8±0.8%) (n=14). A subset of the expanded CTLs expressed the memory markers CD62L (36.5±17%), CD27 (60.7±22%) and CD45RO (50.6±14%) (n=4) (Figure 1B). These data imply that the expanded HHV6 response was derived from a memory population. There was no evidence of regulatory T cell outgrowth, as assessed by CD4/CD25/FoxP3+ staining (Supp. Figure 1).

Based on the frequency of IFNγ-secreting cells and the proportion of responding donors, we identified a hierarchy of immunodominance. Thus all 14 donors (100%) responded to U90, U14, U54 and U11 (mean 327.8±20.6, 285.4±14, 218.2±11, and 174.3±4.8 SFC/1x10⁵, respectively), while only 8/14 (57%) responded to U71 (mean 61.1±5.4 SFC/1x10⁵) (Figure 1C).

To confirm that the expanded, cytokine-producing cells truly reflected expanded HHV6B-specific cells and not cross-reactive CMV-specific T cells expanded by HHV6-derived peptides, we evaluated whether T cells reactive for our chosen HHV6B antigens could be expanded from
CMV seronegative donors. Results from 6 donors in Figure 1D show a profile similar to lines generated from CMV+/HHV6+ donors, with activity against U90, U14, U54 and U11 (mean 397.8±53.5, 297.5±23.2, 176.3±19.5, and 154.5±11.5 SFC/1x10^5) detected in all (100%), whereas only 3/6 (50%) lines reacted to U71 (mean 31.3±3.8 SFC/1x10^5).

Characterization of HHV6B-specific T cells
To evaluate whether HHV6B specificity was mediated predominantly by the major population of CD4+ or the minor CD8+ T cell component, we used ICS and gated on IFNγ-producing CD3/CD8+ or CD3/CD8- (CD4+) T cells. For all antigens in 5 donors studied, specificity was detected predominantly in the CD4+ compartment, with a minor CD8+ component. Figure 2A shows a representative FACS plot while summary data from all 5 donors is shown in Figure 2A, right panel panel.

Production of multiple proinflammatory cytokines may correlate with improved cytolytic function and contribute to in vivo activity27-29. Therefore we assessed whether the reactivated CTL produced more than one cytokine in response to stimulation. For all but U54, we found that the majority of IFNγ-producing cells also produced TNFα (detailed results from one donor; Figure 2B; summary of 5 Figure 2B, right panel). Supplementary Figure 1 shows that, in addition to IFNγ and TNFα, the lines produced GM-CSF as well as granzyme B (Supplementary Figure 2) with production of Th2 cytokines including IL5, IL10 and IL13 (Supplementary Figure 1) detected at levels similar to pp65-specific CTLs. Thus, our expansion of HHV6-reactive cells induced polyclonal, Th1-polarized cells that produce a similar spectrum of effector cytokines as CMVpp65-specific CTLs.

Finally to determine whether the polyclonal, HHV6B-directed CTL were cytolytic we incubated specific T cells (confirmed by ELIspot - Figure 2C) with autologous CD14-selected monocytes alone (control) or pulsed with U14, U90, U54, U11 and U71 pepmixes, and measured Cr51 release after 6hrs. At an effector:target (E:T) ratio of 40:1, HHV6-specific CTLs killed U14-, U90-, U54- and U11-pulsed targets (mean 30±16%, 24±8%, 21±6%, and 21±6%, respectively). In contrast, lysis mediated by U71-specific CTLs was weak (mean 13±8%; 40:1), likely due to the low frequency of reactivated cells (median 22.3, range 8-53.5 IFNγ SFC/1x10^5CTL). Control target cells were not recognized (6±1%) (Figure 2C).

Identification of T cell epitopes
We assessed the breadth of epitope specificity within U90 and U14, the two most immunogenic antigens. We measured responses to 267 and 150 15mers (overlapping by 11aa) spanning U90
and U14, respectively, and arranged these into 33 and 25 pools such that each peptide was represented in 2 pools (Figure 3A and Supplementary Figure 3). CTL lines with specificity for these individual antigens were exposed to the relevant minipools and positive responses were detected by IFNγ ELISPOT. Figure 3A (lower panel) shows the results from U90-specific CTLs generated from 5 donors with disparate HLA types. Each line recognized multiple mini-pools [minimum 9 (donor 3), maximum 27 (donor 2)]. All mini-pools except #19 were recognized by at least 1 donor, demonstrating that the CTL lines were polyclonal and that immunogenic T cell epitopes were present throughout the antigen.

We next defined the minimal CD8+ epitopes and HLA class I-restricting alleles associated with CD8+ responses. We depleted CTL lines of CD4/CD56/CD16+ cells using magnetic beads, achieving a purity of >85% CD8+ T cells, and then re-exposed these CD8-enriched cells to the mini-pools. Results for a representative donor (donor 1 - HLA-A2; B40; B07) are shown in Figure 3B. We mapped CD8+ T cell reactivity to 4 mini-pools (9, 14, 15, and 18), which intersected to identify 2 stimulatory 15mers (#9 – aa33-47 YHPDPVVEESIKEIL and #14 – aa53-67 CDVSFESLLFPELEA). To map the minimal epitopes we synthesized a panel of 9mer peptides (overlapping by 8aa) starting from aa29 (RMQNYHPDP) to aa51 (IKEILEESL) to encompass all potential CD8+ epitopes within the 15mer #9. Similarly, we generated a panel of 9mers spanning aa53-71 CDVSFESLLFPELEAFDLF (corresponding to 15mers #14 and #15). The minimal epitope recognized in peptide #9 corresponded to aa39-47 VEESIKEIL (VEE), while reactivity against peptides #14 and 15 reflected recognition of aa57-65 FESLLFPEL (FES). Recognition of these peptides by CD8+ T cells was confirmed by ICS following peptide stimulation (Figure 3C, left panel).

To determine the HLA restricting alleles of VEE and FES, we used autologous (HLA-A2; B7; B40) and partially HLA-matched peptide-pulsed PHA blasts as targets. Detailed results are shown in Figure 3C. T cells reactive against FES and VEE recognized autologous and allogeneic targets matched at HLA-B40 with no significant reactivity detected against targets matched at other alleles (Figure 3C, right panel). We were subsequently able to raise epitope-specific T cells from at least 2 other donors sharing the restricting HLA allele (not shown), confirming our mapping results.

Using the same approach, we analyzed the breadth of specificity to U14. Figure 3D shows results from 5 donors, with recognition of 7-17 of the 25 mini-pools. All pools except for #23 were recognized by at least 1 donor. By rescreening CD8-enriched T cells and epitope mapping we identified one novel HLA B40-restricted epitope peptide, aa602-610 TEMMNDARL (TEM), by ICS and a cytotoxicity assay using autologous and partially HLA-matched peptide-
pulsed PHA blasts (Figure 3E). Table 2 shows a summary of the 3 novel CD8+ epitope peptides identified in U90 and U14 with amino acid coordinates and identified restricting HLA allele.

**Multiantigen-targeted CTLs kill virus-infected targets**

Since adoptive immunotherapy should target multiple viral antigens to prevent immune escape, we sought to generate CTL lines reactive against multiple HHV6B antigens simultaneously. We pulsed PBMCs with a mastermix of pepmixes spanning U90, U11, U14, U54 and U71 and then expanded the cells for 9-10 days in IL4+7 (Figure 4A – schematic). The resulting CTLs were specific for all 5 stimulating antigens (Figure 4B) at frequencies similar to those achieved using a single antigen stimulus (Figure 2C). Next we evaluated the cytolytic ability of these multiantigen-specific CTLs using, as targets, i) HHV6B pepmix-pulsed monocytes and ii) HHV6 wild-type virus-infected monocytes. Eighty percent of monocytes were HHV6-infected, as assessed by IHC (Figure 4C), and confirmed by analyzing cells for the presence of HHV6 transcripts associated with active infection (not shown). In a 6hr Cr release assay, multiantigen-specific CTLs specifically lysed pepmix-pulsed (37±7%, 40:1) and virus-infected targets (28±5%, 40:1), with no recognition of mock-infected targets (6±1%, 40:1) (n=5) (Figure 4D). Cytolytic ability was confirmed in a 24hr co-culture assay of virus- or mock-infected monocytes with HHV6B-specific or control T cells. Surface staining for CD11c and CD3 was used to discriminate monocytes from T cells. At initiation T cells and monocytes were co-cultured at a 2:1 ratio. This T cell:monocyte ratio remained stable in all 3 control groups; (i) non-infected monocytes + control T cells (31%:69%), (ii) HHV6B-infected monocytes + control T cells (26%:74%), and (iii) mock-infected monocytes + HHV6B-specific CTLs (30%:70%). However, coculture of HHV6B-specific CTLs with infected monocytes for 24hrs eliminated 66% of monocytes (with the 10% residual cells likely representing non-infected targets) (Figure 4E). Figure 4F summarizes these results from 4 donors in whom HHV6B-specific CTLs killed infected (12.5±2.5% residual monocytes) more effectively than mock-infected targets (27.8±7.7%, p=0.01) and than co-culture of control T cells with virus-infected monocytes (32.6±7.7%, p=0.05).

**In vitro reactivation of HHV6B-specific T cells using virus-infected APCs**

To assess the frequency of T cells specific for our predicted antigens in an *in vitro* model of HHV6 reactivation we expanded cells directed against our target antigens using virus-infected, rather than pepmix-pulsed, APCs. We infected healthy donor PBMCs with the HHV6B Z29 wild-
type strain, which has tropism for monocytes, dendritic cells and CD4+ T cells within PBMCs. To expand antigen-activated T cells we cultured the infected PBMCs in IL4+7 for 9-10 days and subsequently evaluated the phenotype and specificity of the lines.

Flow cytometry demonstrated that, similar to pepmix-stimulated T cells, these lines were almost exclusively CD3+ T cells (93.3±1.6%) (Figure 5A), and dominated by the CD4+ subset (54.9±6.9%); CD8+ T cells formed 40.2±6.5% of the total (Figure 1D). To evaluate the specificity of these CTLs we stimulated them with pepmixes spanning U11, U14, U54, U71 and U90 and measured responses by IFNγ ELIspot. We confirmed reactivity against all antigens previously identified as immunogenic [U11 (56.5±3.7 SFC/1x10^5 cells), U14 (67.4±8.3 SFC/1x10^5 cells), U54 (116.6±17.9 SFC/1x10^5 cells) and U90 (73.2±5.1 SFC/1x10^5 cells)]. No or minimal activity was detected after stimulation with U71 pepmix (4.8±1.1 SFC/1x10^5 cells). In contrast, mock-infected PBMCs showed no evidence of activity for these antigens (Figure 5B). Thus, HHV6B virus-infected APCs induce T cells with reactivity for the antigens U11, U14, U54 and U90, confirming that our chosen antigens are both immunologically and physiologically relevant in considering T cell targeting against HHV6-infected cells.

**Detection of HHV6B-specific T cells after in vivo viral reactivation**

To assess the in vivo relevance of HHV6B antigen-specific T cells for viral elimination, we investigated whether allogeneic HSCT recipients had evidence of circulating T cells directed against our identified immunodominant target antigens after HHV6B reactivation. First, we measured the frequency of HHV6-reactive T cells in the peripheral blood of patients. In one patient who presented with an active viral HHV6B reactivation (day 29 post-transplant), we were able to measure HHV6B-specific T cells directed against U11 (21 SFC/1x10^5 cells) and U90 (47 SFC/1x10^5 cells) (Figure 6A), even without the ex vivo expansion steps invariably required for normal donors. To ensure that the cytokine-producing T cells detected in our patients’ PBMCs were specifically induced by HHV6B, we also confirmed lack of expansion of T cells reactive against other viral antigens including CMV-pp65 and adenovirus-Hexon. Four additional patients who recovered from HHV6B reactivation (ranging from day 25-82 post-transplant) had reactive CD4+ and CD8+ T cells following ex vivo expansion (Figure 6B), which were specific for at least one of our chosen antigens (Figure 6C), further confirming the in vivo relevance of U11, U14, U54 and U90-specific T cells for viral elimination.
Discussion

In this study we characterized the cellular immune response to five HHV6B antigens predicted to be immunogenic and defined a hierarchy of immunodominance with respect to both the frequency of responding donors and the magnitude of reactive T cells. U90, U14, U54 and U11 were recognized by all 20 donors screened with progressively smaller frequencies of reactive T cells, while U71 induced the lowest frequency of specific cells and was immunogenic in just over half of the donors. This profile of anti-viral activity was subsequently confirmed in patients with active/recent HHV6 reactivations, demonstrating the in vivo relevance of our chosen antigens.

We show that T cells reactive against these antigens, which can be expanded ex vivo using either peptides or virus-infected monocytes, were able to produce multiple effector cytokines and kill both peptide-pulsed and HHV6B virus-infected targets. Finally, we were able to successfully map 3 novel CD8+ epitope peptides in U90 and U14, the first identified in HHV6, and define their HLA class I-restricting allele.

HHV6 is a lymphotropic virus (genus Roseolovirus) of the β-Herpesvirus family, which also contains CMV and HHV7. There are two species of HHV6, HHV6A and HHV6B. While the HHV6 genome is highly conserved between the species, significant differences are observed between their biology, immunology, and epidemiology. Primary infection with HHV6B presents with roseola infantum or exanthem subitum and occurs early in most individuals, resulting in a prevalence of nearly 100%. By contrast, the epidemiology and clinical symptoms of primary HHV6A infection remain unclear. Though not usually associated with disease in the immunocompetent, in immunocompromised individuals, such as solid-organ or HSCT recipients, reactivation of HHV6 can produce CNS disease (including encephalitis and meningitis), pneumonitis, transplant rejection, or delayed engraftment. Reactivation has also been associated with seizures, myocarditis, and pityriasis rosea. Beyond the immunocompromised host, HHV6 has been proposed to have a pathogenic role in multiple sclerosis, chronic fatigue syndrome, and drug-induced hypersensitivity syndrome but these associations remain controversial. While ganciclovir, cidofovir and foscarnet have been used to treat HHV6 infection and severe disease, these agents are associated with substantial toxicities and have produced variable results. Given the success of adoptive immunotherapy for other herpesvirus diseases in the immunocompromised, we set out to identify appropriate antigens against which an effector T cell response might be effectively generated and we focused our efforts specifically on HHV6B, which is responsible for >95% of reactivations detected in transplant patients.
A comprehensive understanding of virus immune control in healthy individuals and the identification of immunogenic/protective viral antigens are pre-requisites for developing any immunotherapeutic strategy. To date, there have been few analyses of the specificity of the immune response to HHV6, a complex virus expressing >100 genes, any or all of which could be potential T cell targets. Given that HHV6B shares approximately 70 conserved and collinear genes with the immunologically well characterized CMV virus, we focused on the HHV6B equivalents of 5 CMV antigens known to be important in viral recognition/protection by T cells, namely IE1 (UL123) and tegument proteins (UL25-pp85, UL99-pp28, UL32-pp150 and UL83-pp65). In HHV6, these antigens are expressed at immediate early (U90), early (U71 and U14) and late (U11 and U54) stages of the lytic cycle. Unlike CMV, in which the majority of seropositive individuals have circulating reactive T cells at frequencies readily detected in peripheral blood, the frequency of T cells reactive to our chosen HHV6B antigens was low/undetectable when assessed directly ex vivo (Figure 1). This result is consistent with data from others who used whole virus (including cell lysate and purified/disrupted virus) as the antigen source. Thus, we believe it unlikely that our failure to find measurable reactivity was due to inappropriate selection of targets but instead reflects the low frequency in peripheral blood of T cells reactive to this persistent herpesvirus. Notwithstanding this lack of measurable numbers of HHV6-directed T cells, we were able to expand specific populations by amplifying the scanty population of reactive cells with a single in vitro stimulation using overlapping peptide libraries spanning our target antigens or with virus-infected APCs.

In CMV seropositive donors there is a clear hierarchy of immunodominance with pp65 (analog of HHV6 U54) being the most frequently detected T cell population, followed by IE1 (analog of U90), UL99 (analog of U71), and UL32 (analog of U11). Finally UL25 (analog of U14) is immunogenic in a minority of donors and is recognized predominantly by CD4+ T cells, in contrast to the CD8+ responses favored by other viral antigens. HHV6B is different. All donors tested had detectable activity against 4 of the 5 antigens tested (U90, U14, U54 and U11), but the magnitude of the response induced was strongest against U90 (CMV analog = IE1), followed by U14 (UL25 analog) and U54 (pp65 analog). We were concerned that the homology, albeit limited, between our 5 HHV6 antigens and their CMV counterparts might lead to a specious hierarchy profile if CMV-reactive T cells were cross-reactive. However, neither the distinct pattern of target recognition nor the magnitude of the responses induced was influenced by whether HHV6-specific CTL lines were generated from CMV seropositive (n=14) or...
seronegative donors (n=6) (Figure 1C/D). However, the situation may be different with HHV6A, which shares 75-95% homology with HHV6B\textsuperscript{15}. Indeed, recent work by Nastke and colleagues reported that T cells raised against HHV6A cross-reactively recognized HHV6B and vice versa\textsuperscript{42}.

We next addressed whether recognition of HHV6B, like CMV, was mediated predominantly by CD8\textsuperscript{+} T cells\textsuperscript{24;29}. We found that all our target antigens, except for U14, induced a predominantly CD4\textsuperscript{+} effector population with a minor CD8\textsuperscript{+} component. It is possible that the profile we detect is an artifact related to our use of just 5 antigens as others, not examined in this study, might induce a predominantly CD8-mediated response. However, this explanation is unlikely since T cell lines generated with virus-infected monocytes as stimulators produced similarly CD4-polarized products - a finding that is in agreement with reports from others\textsuperscript{37;43;44;46}. The bias to CD4-reactive T cells might instead be influenced by HHV6-derived immune-modulating genes. Such immune modulation is a common feature of herpesviruses. For example CMV expresses six different viral genes (pp65, US2, US11, US3, US6 and US10) dedicated to inhibiting T cell recognition by enhancing HLA class I and II internalization, inducing HLA degradation or preventing peptide-loading\textsuperscript{47}. Though less well studied, it is likely that equivalent genes may alter HLA class I-associated antigen processing/presentation in HHV6. For example, in HHV6B the lytic glycoprotein U21 has been shown to divert HLA class I to the lysosomal compartment for degradation as a means of evading CD8-mediated elimination\textsuperscript{48}. Despite this bias to a CD4 response, we found that both the predominant CD4\textsuperscript{+} population as well as the minor CD8\textsuperscript{+} fraction produced Th1-polarized effector molecules including IFN\textgamma{}, TNF\alpha{}, and granzyme B and both subsets effectively killed both peptide-pulsed and virus-infected targets, suggesting a potential future clinical utility\textsuperscript{49;50}.

A more extensive and detailed characterization of HHV6 epitopes greatly enhances our ability to produce effective vaccines and to evaluate the in vivo effects of adoptively-transferred CTL lines. Such studies are difficult with epitopes presented on HLA class II and recognized by CD4\textsuperscript{+} T cells as they are often promiscuously presented in the context of multiple HLA alleles\textsuperscript{51}. We therefore depleted helper and NK cells from our lines and limited our mapping to the epitope-specificity of CD8\textsuperscript{+} T cells recognizing U90 and U14. Given both the prevalence of the HLA-A2 allele in Caucasians and the fact that it is an immunodominant allele for CMV epitopes\textsuperscript{22;23}, we initially focused on HLA-A2\textsuperscript{+} donors and analyzed CTL lines generated from 5 donors, 3 of whom were HLA-A2 homozygous and 2 heterozygous. In contrast to similar studies
of CMV minimal epitopes, we were unable to identify a single HLA-A2-restricted CD8+ T cell epitope in either U90 or U14. Instead, the 3 CD8+ epitopes we identified - the first described for HHV6 - were all found to be presented in the context of HLA-B40(60).

To confirm that our chosen target antigens were clinically relevant and induced effective T cells, we used wild-type virus-infected cells to activate bulk HHV6-reactive T cells from healthy donors and demonstrated that the expanded populations had activity against our chosen antigens at comparable levels to those detected using peptide libraries as a stimulus. Next, in HSCT recipients with either active disease or evidence of recent HHV6 reactivation which was controlled, thus excluding the possibility that the patients had chromosomally-integrated HHV6, we detected HHV6-specific T cells for our chosen antigens. Finally, we generated single CTL lines specific for U90, U11, U14, U54, and U71 using pepmixes as a stimulus and showed that such lines were able to recognize and kill HHV6B Z29-infected monocytes but not control targets. Taken together this evidence suggests that HHV6-specific CTLs with specificity for U90, U11, U14, U54, and U71 will support viral control in vivo. Given these data and that our process for generating T cell lines targeting multiple HHV6-specific antigens is compatible with current Good Manufacturing Practices (cGMP), we conclude that adoptive T cell immunotherapy for HHV6B is a practical objective and our newly identified CD8+ T cell epitopes will provide valuable tools to better understand the behavior of the virus and transferred immunity in vivo.
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Authorship

This study was developed and designed by U.G. and A.M.L. and the manuscript was written by U.G., A.M.L., and M.K.B. U.G. developed the method for generating HHV6-specific CTLs using peptides and virus-infected targets. U.G., L.K. J.M.K., U.L.K., and C.T.Q.N. grew the CTL lines and performed phenotypic and functional studies. The data was analyzed by U.G. and A.M.L.. A.P. de P. provided critical reagents and technical expertise on expanding specific cells with virus-infected targets. C.A.R., A. K-N. and S. G. provided patient samples. H.E.H. and C.M.R. provided expertise in CTL generation. All co-authors critically reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
Reference List


Table 1
T cell immunogenic CMV antigens, their HHV6B equivalent and their gene function.

<table>
<thead>
<tr>
<th>CMV gene</th>
<th>HHV6B gene</th>
<th>Gene function</th>
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<tbody>
<tr>
<td>UL32 (pp150)</td>
<td>U11</td>
<td>major tegument phosphoprotein</td>
</tr>
<tr>
<td>UL25 (pp85)</td>
<td>U14</td>
<td>tegument phosphoprotein</td>
</tr>
<tr>
<td>UL83 (pp65)</td>
<td>U54</td>
<td>tegument phosphoprotein virion transactivator</td>
</tr>
<tr>
<td>UL99 (pp28)</td>
<td>U71</td>
<td>myristylated virion protein</td>
</tr>
<tr>
<td>UL123 (IE1)</td>
<td>U90</td>
<td>immediately early transactivator</td>
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Table 2
Peptide sequences and amino acid co-ordinates of novel HLA-B40-restricted CD8+ T cell epitopes identified in U90 and U14.

<table>
<thead>
<tr>
<th>HHV6B antigen</th>
<th>Peptide sequence</th>
<th>HLA restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>U90</td>
<td>VEESIKEIL (39-47)</td>
<td>B40(60)</td>
</tr>
<tr>
<td></td>
<td>FESLLFPEL (57-65)</td>
<td>B40(60)</td>
</tr>
<tr>
<td>U14</td>
<td>TEMMDARL (602-610)</td>
<td>B40(60)</td>
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Figure legends

Figure 1: HHV6B antigen-specific T cells can be expanded from peripheral blood

A. The frequency of antigen-specific T cells in peripheral blood was determined by IFNγ ELISpot assay after overnight stimulation of PBMCs with CMV (pp65, IE1) and HHV6 (U11, U14, U54, U71, U90) antigen-spanning pepmixes in 4 donors. Stimulation with Staphlococcus aureus was used as positive control and unstimulated PBMCs served as a negative control. Results are expressed as spot forming cells (SFC)/4x10^5 input cells with (– median SFC). B. PBMCs were stimulated with the HHV6 pepmixes in the presence of IL4+7. On day 9 after stimulation the phenotype (mean expression ± SD) was assessed (n=14). C. PBMCs from 14 donors were stimulated with HHV6 pepmixes in the presence of IL4+7 and specificity on day 9 was measured by IFNγ ELISpot. Results are expressed as SFC/1x10^5 input cells. D shows the specificity of CTLs generated from 6 CMV seronegative donors. IFNγ production was assessed on day 9 after stimulation and results are expressed as SFC/1x10^5 input cells. The negative control was IFNγ release detected in response to stimulation with an irrelevant pepmix.

Figure 2: HHV6B antigen-specific CTLs are polyfunctional

A shows IFNγ cytokine production, as evaluated by ICS, from CD3+/CD8- (CD4 helper – lower quadrant) and CD3+/CD8+ (cytotoxic – upper quadrant) CTLs on day 9 after stimulation in one representative donor (dot plots shown were gated on CD3+ cells) (left panel). Summary results from five donors (mean ± SEM) are shown in the right panel. B demonstrates polycytokine production by CTLs as assessed using ICS in one representative donor (left panel) and the summary results from 5 donors showing double-cytokine (IFNγ and TNFα) ■ or single cytokine-producing T cells IFNγ only □ or TNF α □ only) with specificity for U11, U14, U54, and U90 (right panel). U71 was omitted from the analysis due to the low frequency of reactive T cells detected. C demonstrates that HHV6-specific T cell lines generated from 4 donors (IFNγ ELISpot - upper panel) evaluated 9 days after pepmix stimulation were also cytotoxic (IFNγ ELISpot - lower panel), as evaluated by standard 4-6hr Cr51 release assay using pepmix-pulsed monocytes as targets. Results are presented as percentage specific lysis (mean ± SD, n = 4).

Figure 3: Identification of U90 and U14 HLA-restricted CD8+ T cell epitopes

The breadth of T cell reactivity in pepmix-activated U90-specific CTLs generated from 5 donors was evaluated by IFNγ ELISpot on day 9 using a total of 33 mini peptide pools, each containing 15-17 peptides, representing all U90 peptides. Mini pools were arranged such that each peptide
was represented in 2 pools (A, upper panel). Each mini-pool that induced a specific response by IFNγ ELIspot was scored as 1 and the summary data from 5 lines screened is shown in A, lower panel. To specifically identify CD8+ T cell epitopes we depleted CD4 and NK cells from the CTL lines and rescreened using the U90 minipools. Panel B shows results from 1 donor screened who demonstrated specific activity against the minipools 9, 14, 15, and 18, which intersected at the 15mer peptides 9, 14 and 15 of U90 (B, upper panel). To identify the minimal epitope CTLs were rescreened against a panel of 9mers overlapping by 8aa spanning the sequence of the stimulatory 15mer peptides. The CD8+ epitope within peptide 9 corresponded to a single 9mer peptide, VEE (aa 39-47), while the stimulatory peptide contained within peptides 14 and 15 corresponded to the 9mer peptide FES (aa 57-65). Results of the ELIspot screening are presented as SFC/1x10^5 CTL. C, left panel confirms that these peptides indeed reflect stimulatory CD8+ epitopes, as assessed by ICS following CTL stimulation with the 9mer peptides. Afterward PBMCs were stimulated with the identified single peptide (Panel C). HLA restriction was evaluated by standard 4h Cr^51 release assay using autologous and partially HLA-matched PHA blasts alone or pulsed with the VEE and FES peptides as targets (C, right panel). The breadth of T cell reactivity directed against U14-specific CTLs was also evaluated by IFNγ ELIspot after exposure to 25 mini peptide pools representing all U14 peptides (Panel D). E, left panel confirms by ICS that the 9mer peptide TEM (aa 602-610) is recognized by CD8+ T cells, while the right panel shows that peptide-pulsed HLA-B40 matched targets are recognized in a 4hr Cr^51 assay.

**Figure 4: HHV6B multiantigen-specific CTLs kill HHV6B wild type virus-infected targets.** A Schematic of our multiantigen-specific HHV6 CTL generation process. B shows that by IFNγ ELIspot these multiantigen-specific lines were specific for the stimulating pepmixes. Results are expressed as SFC/1x10^5 input cells. C shows IHC analysis of CD14-selected monocytes infected with the HHV6B wild type virus strain z29 or the mock-infected controls. The IHC pictures were taken using 10x magnification at an exposure of 6ms on an Olympus BX41 microscope and images were captured using Q-capture software (Q-imaging). Autologous monocytes alone, or pepmix-pulsed or HHV6 wild type virus-infected cells were used as targets in a standard 4-6hr Cr^51 release assay (D). Mean ± SEM % specific lysis at an effector:target ratio of 40:1 is shown (n=4). E shows representative FACS results of a 24hr co-culture assay where either uninfected or HHV6 z29-infected autologous monocytes (CD11c+) were cultured with non-specific or HHV6 multiantigen-specific CTLs (CD3+). Summary results from 4 donors
are shown in F where the percentage of residual monocytes following treatment, as quantified by FACS analysis, are plotted (mean ± SD).

**Figure 5: HHV6B-specific CTLs induced using wild type virus-infected PBMCs.**

A shows the phenotype of CTL lines generated using HHV6B z29-infected PBMCs as stimulators (mean expression ± SD; n=6). In the same lines specificity was assessed by IFNγ ELISPOT (B). Results of HHV6B z29-stimulated CTLs (HHV6+) and MOCK stimulated CTLs (HHV6-) are expressed as SFC/1x10⁵ input cells.

**Figure 6: HHV6B-specific T cells are detected in the peripheral blood of HSCT recipients**

PBMCs isolated from a HSCT recipient at the time of acute HHV6 infection were tested for specificity against our panel of HHV6 antigens, using IFNγ ELISPOT as a readout. Unstimulated PBMCs was used as a negative control while stimulation with a cocktail of CMV-pp65 and Adv-Hexon pepmixes was used to assess the relative frequency of circulating T cells directed against other ubiquitous viruses. Results are expressed as SFC/1x10⁵ input PBMCs (A). PBMCs from 4 other HSCT recipients with recently controlled HHV6 reactivations were stimulated with HHV6B antigen spanning pepmixes and expanded for 9-10 days in presence of IL4+7. B shows the phenotype of the expanded cells assessed by flow cytometry (mean ± SD expression), while C shows their specificity by IFNγ ELISPOT assay - results are expressed as SFC/1x10⁵ input cells (C).
Figure 3

3A

3B

HHV8 U90 CTL (CD8+)

9 mer peptides overlapping 8aa spanning peptide # 9

VEESKIEIL

9 mer peptides overlapping 8aa spanning peptide # 14/15

FESLIFPBE

3C

3D

3E

specific (pts) (%)

specific (pts) (%)
Figure 6

6A

6B

6C
Immunotherapeutic strategies to prevent and treat human herpes virus (HHV) 6 reactivation post allogeneic stem cell transplant