



Immune response to HHV-6 and implications for immunotherapy

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Most adults remain chronically infected with HHV-6 after resolution of a primary infection in childhood, with the latent virus held in check by the immune system. Iatrogenic immunosuppression following solid organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) can allow latent viruses to reactivate. HHV-6 reactivation has been associated with increased morbidity, graft rejection, and neurological complications post-transplantation. Recent work has identified HHV-6 antigens that are targeted by the CD4+ and CD8+ T cell response in chronically infected adults. T cell populations recognizing these targets can be expanded *in vitro* and are being developed for use in autologous immunotherapy to control post-transplantation HHV-6 reaction.

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Introduction

The increasing clinical importance of HHV-6 demands effective treatment options. Currently, individuals with complicated HHV-6 infection or reactivation are treated with ganciclovir or similar drugs approved for managing other viral infections [1]. However, these drugs have significant toxicity [2] and in some cases are ineffective against HHV-6 [3]. Immunotherapies based on antibodies, expanded T cells, or vaccines potentially could provide an alternative or adjunctive approach to controlling HHV-6 infection. Immunotherapy for human herpesviruses has been in development since the early 1990s [4], and has

been shown to be a safe and practical approach to controlling related human herpesviruses human cytomegalovirus (HCMV) [5], Epstein-Barr virus (EBV) [6,7] and herpes simplex virus (HSV) [8]. For HHV-6, little is known about the immune mechanisms that control infection, and current understanding is based largely on a few studies and extrapolation from HCMV [9]. Here we review recent progress in characterizing the immune response to HHV-6 and discuss implications for development of immunotherapies in immunocompromised patients.

Challenges to characterizing the immune response to HHV-6

The lack of a basic understanding of the immune response to HHV-6 has delayed the development of HHV-6 specific immunotherapies. Several aspects of HHV-6 biology interfere with straightforward application of conventional approaches to characterizing antiviral immunity. First, two closely related viruses HHV-6A and HHV-6B have been treated as a single species until very recently [10]. Mounting evidence suggests important differences in the biology of these two viruses and the immune response that they induce [11], but in general they have not been distinguished in studies of the immune response to HHV-6. Second, antibody titers to HHV-6 and frequencies of T cells recognizing HHV-6 are low, making detection of these responses challenging [12**]. Blood samples obtained during active viremia might exhibit higher antibody titers or T cell responses, but symptomatic viremia occurs primarily in young children or immunosuppressed patients from whom sufficient blood samples are difficult to obtain. Third, HHV-6 is a lymphotropic virus that prefers T cells for replication, but also is capable of infecting a variety of antigen presenting cells [1,13]. Profound effects on the normal function of both infected T cells and infected antigen presenting cells have been demonstrated [14–17], and these effects interfere with *ex vivo* analyses. Finally, HHV-6 infection is restricted to humans and closely related primates [18,19], so the lack of a small animal model has inhibited detailed mechanistic studies. Despite these limitations, recently there have been notable advances in defining HHV-6-specific T cell responses and in developing approaches to adoptive immunotherapy.

HHV-6B protective immunity

The observation that primary HHV-6B infection is a mild febrile disease from which most children recover rapidly without complications suggests that protective HHV-6B

immune responses are commonly elicited. After primary infection, HHV-6B is able to persist as a chronic or latent infection controlled by the adaptive immune response. The virus can reactivate under conditions of deficient cell-mediated immunity [20]. Although immunity to HHV-6B could evolve over time, there is evidence that lifelong responses to HHV-6B are imprinted very early after the first onset of HHV-6B infection [21]. Neonates are usually protected from HHV-6B infection by maternal-derived antibodies until titers wane over 3–9 months after birth, making older children susceptible to infection [22]. Primary infection occurs usually before the second year of age, and induces antibodies that persist throughout life [22]. Evidence that T cells are required to control HHV-6B replication is inferred from persistent HHV-6B viral replication in immunosuppressed patients who do not have proliferative T cell responses [20].

Antibody responses

Most studies of the antibody response to HHV-6 have aimed to develop diagnostic methods that differentiate between the three closely related roseoloviruses, HHV-6A, HHV-6B and HHV-7. Little is known about the range of antigens targeted by antibodies recognizing these viruses [23,24]. A few HHV-6 antigens prominently targeted by the antibody response have been identified. These include the major antigenic virion protein U11 [25], the major glycoproteins gH (U48) [26,27] and gQ (U100) [28], the polymerase processivity factor (U27) [29], the late antigen U94 [30], and the tail-anchored membrane protein U24 [31,32] (Figure 1). Serological assays that utilize U11 as antigen are in development for the definition of roseolovirus-specific antibodies. Neutralizing antibodies have been found mainly after primary infection and in transplant recipients but also in some healthy adult donors, which might indicate subclinical reactivation of the virus [33]. Monoclonal neutralizing antibodies targeting gH [34–36], gQ [37] and gB [38] have been described.

T cell responses

The T cell response to HHV-6 mostly has been characterized using peripheral blood from healthy adults. Responding T cells, measured as the number of IFN- γ producing cells, are present at low frequency (on the order of a few cells per 1000 (or <0.2%) [12^{••},39^{••},40^{••}], which contrasts sharply with the stronger responses that are observed for HCMV (up to 4%) [41]. T cell responses have been reported to be higher in HSCT patients [42], but few studies have focused on this population. Despite the low frequency of responding T cells in healthy adults, strong CD4⁺ and CD8⁺ T cell proliferative responses are observed [43–45], primarily restricted to memory cells. Proliferation of HHV-6-specific T cells in response to viral antigen allows these low-frequency T cell populations to be expanded *in vitro* for detailed study. The expanded population consists mainly of CD4⁺ T cells

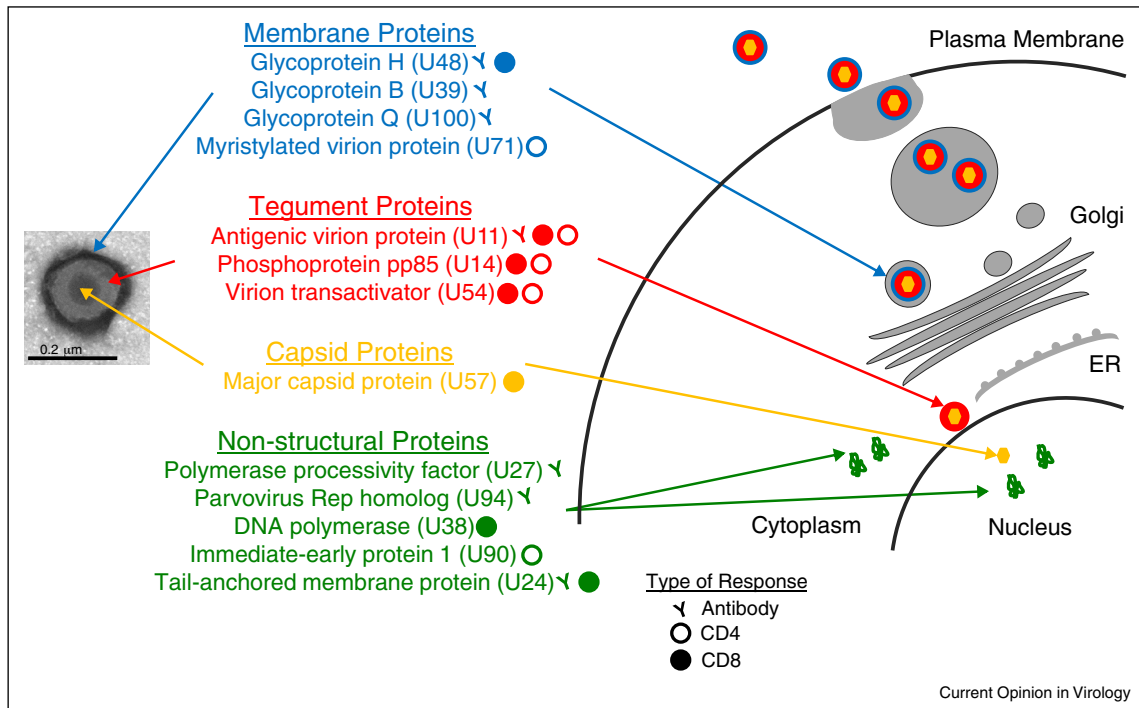
that secrete IFN- γ and exhibit cytotoxic capacity [12^{••},39^{••},46]. A prominent subpopulation secretes IL-10 [12^{••},47[•]]. IL-4 and low amounts of IL-2 also are also produced [12^{••}], a profile similar to that reported in serum of children with roseola [48^{••}]. The reason for CD4⁺ T cell skewing and limited CD8⁺ T cells in expanded populations is not clear. The lower frequency of responding CD8⁺ T cells in blood ($\sim 10^{-5}$) certainly is a factor [40^{••}], but viral evasion mechanisms also might be responsible.

Shortly after the discovery of HHV-6, the immunosuppressive properties of this virus were recognized. Initially, it was reported that HHV-6 arrests IL-2 synthesis and T cell proliferation [49,50]. Subsequent studies identified immune modulation by effects in both infected and non-infected cells (reviewed in [51]). In infected CD4⁺ T cells, HHV-6 induces apoptosis [52], inhibition of IL-2 synthesis [14], cell cycle arrest [16], and TCR and MHC-I down modulation [11]. In antigen-presenting cells, HHV-6 induces MHC-I down-modulation [53] and reduces the ability of these cells to present antigens and activate T cells [11]. In addition, IL-10 secreted by CD4⁺ T cells responding to HHV-6 modulates proliferation of other T cell populations [47[•]]. Different subsets of regulatory T cells (Tregs) have been observed *in vitro* after HHV-6-specific expansion or cloning [12^{••},39^{••},54[•]].

Targets of the T cell response to HHV-6

The HHV-6 genome encodes ~ 100 proteins, and many of them are >1000 amino acids in length, making the identification of immunodominant epitopes a laborious and time consuming task. Information on the particular peptide epitopes recognized by T cells is required for identification, characterization, and modulation of T responses specific to HHV-6 as compared to closely related viruses. Approaches used to limit the number of antigens/peptides to screen have focused on HHV-6 proteins present at high levels in virus preparations [12^{••}], or on HHV-6 homologues of antigens defined for HCMV [39^{••},40^{••}]. Our group used the first approach to define 11 CD4⁺ T cell epitopes [12^{••}]. These derived from 4 virion proteins (the major capsid protein U57, the tegument proteins U11 and U14, and the glycoprotein U48) and from a non-structural protein (DNA polymerase U38) (Table 1). CD4⁺ T cells expanded with peptides corresponding to these epitopes responded to APC treated with virus preparations and produced IFN- γ and expressed markers associated with cytotoxic potential. Using the second approach, Martin *et al.* were able to expand CD8⁺ T cell lines and clones that showed reactivity to peptides from tegument proteins U11 and U54 and showed pro-inflammatory and cytotoxic capabilities [40^{••}]. Also, using the second approach and T cell lines expanded *in vitro*, Gerdemann *et al.* demonstrated T cell responses to peptide epitopes derived from the immediate-early protein U90, the tegument proteins U11, U14

Figure 1



Targets of the adaptive immune response to HHV-6. Antibody and T cell responses target the viral surface membrane, tegument, and capsid components of the virion as well as non-structure proteins expressed in infected cells. Inset (left) shows a purified viral particle with components indicated, and cartoon (right) shows intracellular locations for the expected stepwise viral assembly process.

and U54, and the myristylated virion protein U71 [39**]. The expanded T cells produced IFN- γ and TNF- α and killed antigen-pulsed autologous target cells. Although the expanded T cell populations consisted mainly of CD4+ T cells, epitopes were identified only for the minor component of CD8+ T cells. Additional CD8+ T cell epitopes in U90 were identified recently by Iampietro *et al.* [55]. Overall, twelve CD8+ T cell epitopes were defined: three from U11, two from U54 [40**], six from U90 and one from U14 [39**], to complement the eleven CD4+ T cell epitopes described above. In agreement with earlier reports, many of the mapped T cell responses are crossreactive between HHV-6A and HHV-6B, and so cannot be used as markers of virus-specific responses. Non-crossreactive responses have been reported for capsid antigens, but specific epitopes were not defined [56]. Additionally, three putative HHV-6 epitopes have been defined by virtue of cross-reactivity with human self-antigens (Table 1). T cell responses to a HHV-6 U24 peptide that shares homology with the multiple-sclerosis autoantigen myelin basic protein (MBP) have been reported, but any significance of this crossreactive response in multiple sclerosis is controversial [32,57]. CD4+ T cells recognizing the diabetes-associated glutamic acid decarboxylase islet autoantigen GAD95 were shown to cross-react with a similar peptide sequence from

HHV-6A U95 [58], but whether these cells recognize naturally processed antigens is not known. A summary of HHV-6 proteins targeted by CD4+ and CD8+ T cell responses is shown in Figure 1, with specific epitopes listed in Table 1.

HHV-6B reactivation after solid organ transplantation

In the period following immunosuppression, transplant recipients are highly susceptible to common viruses such as herpesviruses, adenoviruses, and seasonal viruses like influenza. HHV-6 reactivation, mostly by HHV-6B [59], occurs in over 40% of HSC T and in up to 60% of solid organ transplant (SOT) recipients [59] during the first weeks after transplantation. HHV-6 reactivation in HSC T patients is associated with graft-versus-host disease, delayed engraftment [60], and CNS dysfunction, including encephalitis that may have long term effects [61*,62*,63**]. In SOT patients, HHV-6B reactivation is associated with prolonged anti-HHV-6 immunosuppression [64], fever, rash, hepatitis, pneumonitis, encephalitis and colitis [59]. In the months after transplantation, T cell proliferative responses to HHV-6 are absent in most individuals. By comparison, proliferative responses to HCMV develop over several weeks (although they do not reach levels observed in healthy donors) [64]. Autologous immune

Table 1

CD4 and CD8 T cell epitopes defined for HHV-6

Epitope	HLA ^a	ORF ^b	Protein	Evidence ^c	Specificity ^d	Reference
GILDFGVKL	A2	U11	Antigenic virion protein	Cyto; grzB	6B	[40**]
MLWYTVYNI	A2	U11	Antigenic virion protein	Cyto; grzB; Tet	6B	[40**]
SLMSGVEPL	A2	U11	Antigenic virion protein	Cyto; grzB	6B	[40**]
ILYGPLTRI	A2	U54	Virion transactivator	CTL; Cyto; grzB; Tet	6B	[40**]
LLCGNLLIL	A2	U54	Virion transactivator	Cyto; grzB	6B	[40**]
TEMMNDARL	B40	U14	Phosphoprotein pp85	CTL; Cyto	n.d.	[39**]
FESLLFPEL	B40	U90	Immediate-early protein 1	CTL; Cyto	n.d.	[39**]
VEESIKEIL	B40	U90	Immediate-early protein 1	CTL; Cyto	n.d.	[39**]
CIQSIGASV	A2	U90	Immediate-early protein 1	CTL; Cyto	6A/6B	[55]
CYAKMLSGK	A3	U90	Immediate-early protein 1	CTL; Cyto	6B/6A?	[55]
STSMFILGK	A3	U90	Immediate-early protein 1	CTL; Cyto	6B/6A?	[55]
NPEISNKEF	B7	U90	Immediate-early protein 1	CTL; Cyto	6B/6A?	[55]
SLESYSASKAFSVPENG	DR1	U11	Antigenic virion protein	Cyto	6A/6B	[12**]
RDNSYMPIALSLHENG	DR1	U14	Phosphoprotein pp85	Cyto	6A/6B	[12**]
VVGKYSLQDSVLVRLF	DR1	U38	DNA polymerase	Cyto; Tet	6A/6B	[12**]
GIYYIRVVEVRQMQYDN	DR1	U48	Glycoprotein H	Cyto; Tet	6A/6B	[12**]
VDEEYRFISDATFVDET	DR1	U48	Glycoprotein H	Cyto; Tet	6A/6B	[12**]
TRPLYITMKAQKNSRI	DR1	U54	Virion transactivator	Cyto; Tet	6A/6B	[12**]
FKSLYINENTKILEVE	DR1	U57	Major capsid protein	Cyto; Tet	6A/6B	[12**]
IRHHVIGIEKPNPSEGEA	DR1	U57	Major capsid protein	Cyto	6A/6B	[12**]
SLLSIMTLAAMHSLKSP	DR1	U57	Major capsid protein	Cyto; Tet	6A/6B	[12**]
TTNPWASLPGSLGDILY	DR1	U57	Major capsid protein	Cyto; Tet	6A/6B	[12**]
DPSRYNISFEALLGIYS	DR1	U57	Major capsid protein	Cyto; Tet	6A/6B	[12**]
KELLQSYVSKNNN	DR53	U95	Immediate-early protein	Cyto	6A?; GAD95	[58]
MDRPRTPPPSYSE	n.d.	U24	Tail-anchored mb. protein	Prolif; Cyto	6A/B; MBP	[32]
RPRTPPPSY	n.d.	U24	Tail-anchored mb. protein	Prolif; CTL	6B?; MBP	[68]

^a HLA restriction. HLA-A2, HLA-A3, HLA-B7, and HLA-B40 are class I MHC proteins recognized by CD8+ T cells. HLA-DR1 and HLA-DR53 are class II MHC proteins recognized by CD4+ T cells. n.d., not defined.

^b HHV-6 open reading frame.

^c CTL, cytotoxicity (cell killing) assay; Cyto, cytokine release; GrzB, granzyme B release; Prolif, cell proliferation assay; Tet, MHC tetramer staining.

^d Specificity for HHV-6A or HHV-6B, or self antigens, where defined.?, presumptive specificity; n.d. not defined.

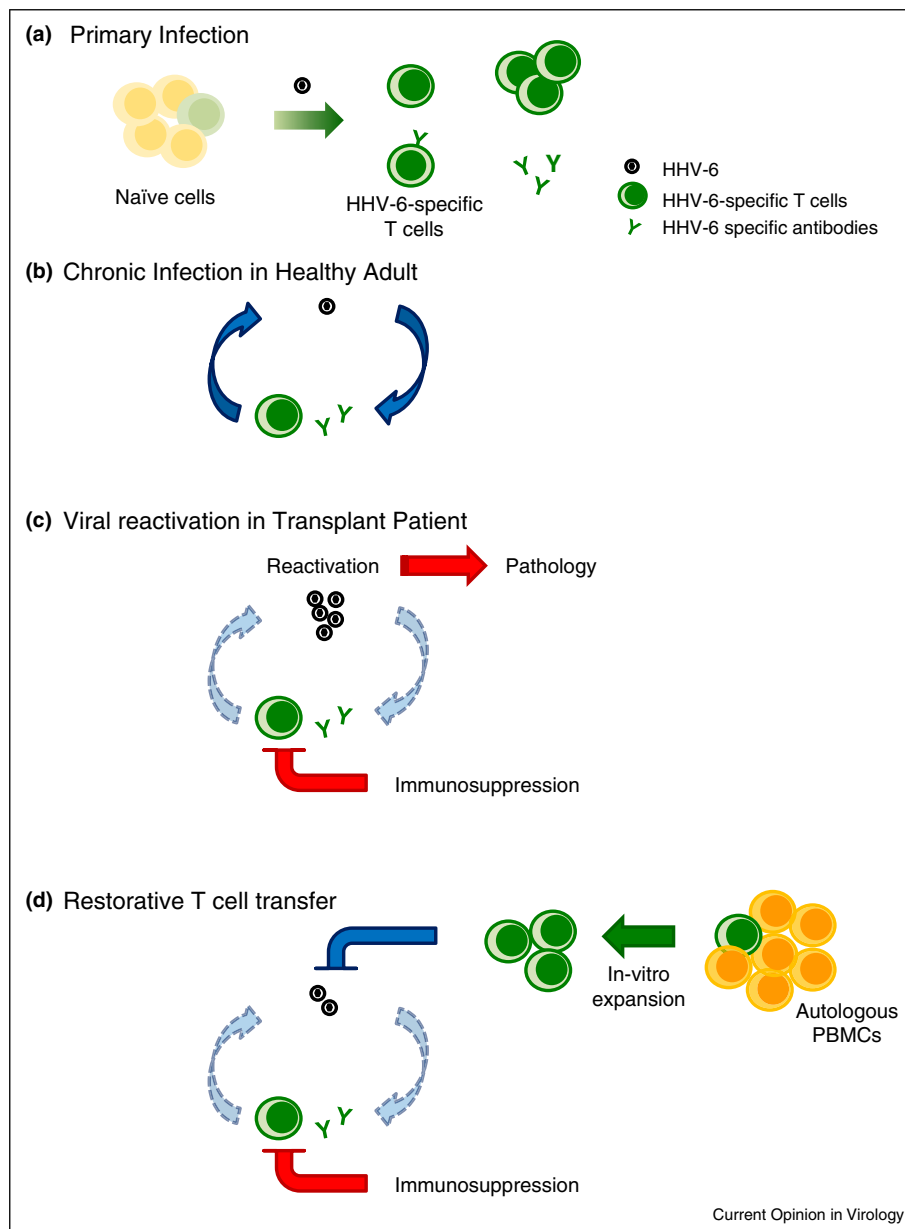
enhancement therapy, in which pre-transplantation T cell populations are expanded *ex vivo* and re-introduced (Figure 2), could provide a way to boost the post-transplantation T cell response and help control HHV-6 reactivation.

Autologous immune enhancement therapy

Susceptibility to HHV-6B and other common viruses is associated with deficiency of T cell responses in immunosuppressed individuals [20,65]. Restoration of protective T cell responses against other herpesviruses such as HCMV and EBV has been achieved by transfer of T cells expanded *in vitro* using purified virus, recombinant proteins, or synthetic versions of known immunodominant antigens to stimulate T cell proliferation [5]. A turning point in the immunotherapy to HHV-6B has been the identification of immunodominant antigens and the demonstration that HHV-6B-specific T cells can be expanded in large numbers if cytokines that support T cell expansion are provided in culture [12**,39**,66*]. This knowledge has been rapidly transferred to a small clinical trial that attempted the reconstitution of immune responses to HHV-6B, HCMV, BK virus, EBV, and adenovirus by expansion of PBMCs

with a mixture of peptides from these viruses [67**]. The Phase I clinical trial showed that adoptive transfer of peptide-expanded T cells for these viruses was safe, did not induce high levels of cytokines, and did not induce allo-specific responses. Expansion of HHV-6B T cells was performed with overlapping peptides of the immediate early protein 1 (U90) and the tegument proteins U11 and U14 shown to be important targets of the HHV-6B T cell response [12**,39**,40**]. The transferred T cell population consisted of ~60% CD4+ and 35% CD8+ T cells. Although T cell lines were generated from a relatively small number of cells (15 million), and a large number of peptides were included (opening the possibility of peptide competition for binding to MHC proteins), almost 30% of the developed T cell lines had responses to all 5 viruses and 70% to at least 3 viruses. More important and striking was that virus levels were reduced after adoptive transfer of T cells, and this reduction was accompanied by an increase in the number of IFN- γ producing cells. Moreover, three patients that received expanded T cells as prophylaxis were protected from virus reactivation beyond 3 months after the adoptive transfer. However, as indicated by the authors, the clinical trial

Figure 2



Autologous immune enhancement therapy. **(a)** Primary infection with HHV-6 virus elicits antibody and T cell responses. **(b)** During chronic infection virus levels are controlled by antibody and T cell responses. **(c)** In transplantation patients, iatrogenic immunosuppression interferes with immune control of HHV-6 and allows virus reactivation with consequent pathology. **(d)** HHV-6-specific T cells expanded *in vitro* can be reintroduced after transplantation to control virus reactivation.

did not have enough participants to support a claim that reduction in virus levels was a result of infused of T cells rather than other host or viral factors. The T cell population transferred was highly enriched in CD4⁺ T cells with a minor component of CD8⁺ T cells, which may have been beneficial since allo-specific disease has been associated with higher frequency of HHV-6-specific CD8⁺ T cells [42].

Future directions

Although the outcome of the first human HHV-6 immunotherapy by transfer of *in vitro* expanded T cells was favorable, we do not know the epitope specificity of the transferred cells, how the transferred cells contributed to the virus control, or even if the antigens used to expand these cells are the major mediators of protective immune responses. Although a handful of both CD4⁺ and CD8⁺ T

cell epitopes have been identified, in most cases these were identified by reference to homologous HCMV antigens, and it is not clear how broadly these represent the overall HHV-6-specific response. A better understanding of the repertoire of peptides recognized by HHV-6-specific T cells would provide additional possibilities for *in vitro* T cell expansion for adoptive immunotherapy. In general studies of HHV-6 T cell epitopes have been performed using blood samples from healthy but chronically infected adults. Characterization of the T cell responses induced by primary infection in childhood, or by virus reactivation in transplantation patients where viremia is controlled, would be helpful to identify protective epitopes. Experiments using *in vitro* expanded T cell populations have identified both productive and suppressive responses, but these have not been associated with particular epitopes. Several *in vitro* studies have suggested that IL-10 produced by CD4+ T cells could play a major role in limiting the expansion of both CD4+ and CD8+ T cell responses. A better understanding of various types and functions of T cells recognizing HHV-6 antigens might allow beneficial responses to be preferentially expanded. New adoptive immunotherapy protocols incorporating this information might allow protection to be obtained with a lower number of transferred cells, limiting the time and resources needed for expansion and reducing the possibility of expansion of unwanted cell responses.

Conclusions

Important advances in defining the T cell response to HHV-6 have allowed the first clinical trial in HHV-6 immunotherapy. Although responses to HHV-6 antigens are present in PBMCs at low frequency, CD4+ and CD8+ T cell antigens have been identified from a variety of viral proteins. Sufficient numbers of cells for immunotherapies can be generated if cells are expanded in medium containing antigen and cytokines. Promising results from a clinical trial reported a decrease in virus load in patients with HHV-6 reactivation after transfer of *in vitro* expanded T cells, suggesting that immunotherapies for HHV-6 are possible without large numbers or antigenic specificities of responding cells. Whether the observed reduction in virus load was mediated by CD4+ or CD8+ T cells and the epitope specificity of these responses remains unknown. Since HHV-6 T cell reactivity has been associated with multiple sclerosis and other autoimmune diseases, future studies should distinguish protective and allo-specific epitopes to minimize potentially cross-reactive autoimmune responses. Nevertheless, efforts to improve the efficacy of HHV-6 therapies will greatly benefit the populations at risk of severe viral disease.

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- of special interest
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