

Generation of Cytomegalovirus-Specific Human T-Lymphocyte Clones by Using Autologous B-Lymphoblastoid Cells with Stable Expression of pp65 or IE1 Proteins: a Tool To Study the Fine Specificity of the Antiviral Response

CHRISTELLE RETIÈRE,¹ VIRGINIE PROD'HOMME,¹ BERTHE-MARIE IMBERT-MARCILLE,²
MARC BONNEVILLE,¹ HENRI VIÉ,¹ AND MARIE-MARTINE HALLET^{1*}

*Institut National de la Santé et de la Recherche Médicale U463¹ and Laboratoire de Virologie,²
Institut de Biologie, 44093 Nantes Cedex 1, France*

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Cytotoxic T lymphocytes (CTLs) play a central role in the control of persistent human cytomegalovirus (HCMV) infection in healthy virus carriers. Previous analyses of the specificity of HCMV-reactive CD8⁺ CTLs drawn from in vitro models in which antigen-presenting cells were autologous fibroblasts infected with laboratory HCMV strains have shown focusing of CTL responses against the major tegument protein, pp65. By contrast, the 72-kDa major immediate-early protein (IE1) was identified as a minor target for this response. Here we have studied the fine specificity and T-cell-receptor features of T-cell clones generated against autologous B lymphoblastoid cell lines stably transfected with HCMV cDNA coding for either pp65 or a natural variant of IE1. This strategy allowed efficient generation of T-cell clones against IE1 and pp65 and led to the identification of several new IE1 and pp65 epitopes, including some located in polymorphic regions of IE1. Such an approach may provide relevant information about the characteristics of the CTL response to IE1 and the effect of viral polymorphism on the immune response against HCMV.

Human cytomegalovirus (HCMV), the largest of the beta-herpesviruses, infects between 40 and 60% of individuals in developed countries and nearly 100% in developing countries. After primary infection, which is asymptomatic in immunocompetent hosts, the virus persists lifelong under the control of the immune system in latent sites, including cells of the myeloid lineage (20, 32, 34, 35). The maintenance of this latent state depends on the cellular immune response, and cytotoxic T lymphocytes (CTLs) have been shown to play a major role in the control of HCMV reactivation and replication (25, 29, 30, 38).

Due to inhibition of the major histocompatibility complex (MHC) class I antigen presentation pathway by a set of viral proteins acting in a cooperative and multistep process during the HCMV replication cycle (1, 3, 13, 15–18, 39, 41, 42), the CTL response against HCMV appears to be targeted against a small set of proteins (for a review, see reference 5). Among these have been identified, on the one hand, structural proteins which are delivered to the cytosol by the infecting virion and rapidly presented to CD8⁺ CTL by MHC class I before blockade of their processing and, on the other hand, the immediate-early proteins, the presentation of which depends on *de novo* synthesis very early after virus infection (15).

Early analyses of the CTL response in seropositive individuals have suggested that the 72-kDa immediate-early protein IE1 was a dominant target for CD8⁺ CTLs (4). Nevertheless, more recent observations, drawn from an in vitro experimental model with autologous fibroblasts infected with the cytopathic AD169 viral strain as a stimulator to propagate HCMV-specific CD8⁺ CTLs, have designated the major tegument protein

pp65 as a dominant target and have shown a highly focused peptide-specific memory CTL response against few dominant epitopes (21, 28, 40, 43).

In the present study, we have developed a novel strategy allowing analysis of the composition and fine specificity of the CTL response of HCMV-seropositive healthy donors against selected immunogenic HCMV proteins. B lymphoblastoid cells (BLCs) were derived by Epstein-Barr virus (EBV) transformation from peripheral blood mononuclear cells (PBMCs) and stably transfected with plasmidic vectors coding for either pp65 or IE1 proteins. These cells were then used as targets to stimulate a specific autologous T-lymphocyte response and to amplify reactive T-cell subsets. After cloning, the cells recognizing the recombinant HCMV protein targets were selected, the characteristics of their TCR repertoire was studied by sequencing their T-cell-receptor β -chain junctions, and their fine specificity and HLA restriction were determined. This study led to the identification of several new class I MHC-restricted CTL epitopes against which were focused the anti-pp65 and -IE1 responses. Since pp65 and IE1 cDNA used in this study were cloned from a clinical strain, the IE1-expressed recombinant protein was a genetic variant which diverged by several amino acids from corresponding products of the laboratory strains Towne and AD169, classically used for analysis of memory CTL response to HCMV infection (4, 21, 28, 40, 43). Interestingly, several polymorphic residues were found within three of the four epitopes recognized by HCMV-specific T-cell clones generated against the clinical IE1 variant from a single donor (i.e., 22 of 22 clones restricted by HLA-A2 molecules and 23 of 25 characterized in the HLA-B18 context). Thus, the present experimental system represents a powerful tool for characterizing new target epitopes of the anti-HCMV T-cell response and should allow in-depth analysis of the effect of viral polymorphism on the modulation of the host repertoire.

* Corresponding author. Mailing address: INSERM U463, Institut de Biologie, 9 quai Moncousu, 44093 Nantes Cedex 1, France. Phone: (33) 02-40-08-47-12. Fax: (33) 02-40-35-66-97. E-mail: mmhallet@nantes.inserm.fr.

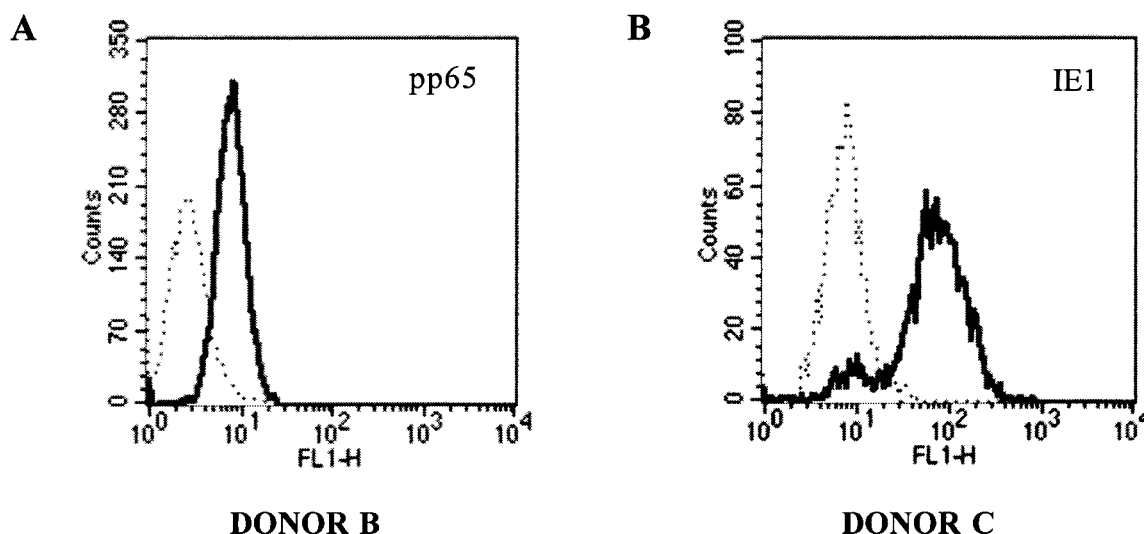


FIG. 1. Flow cytometry analysis of pp65 expression in BLCs of donor B stably transfected with pRc/CMV-pp65 ([Mab 1C3 plus AYM-1]-FITC) (A) and IE1 expression in BLCs of donor C stably transfected with pRc/CMV-IE1 (Mab E13-FITC) (B). The BLC lines transfected with pRc/CMV mock were used as negative controls (dotted lines).

MATERIALS AND METHODS

Cells. PBMCs were prepared from fresh heparinized peripheral blood of three CMV⁺ healthy donors expressing known HLA class I haplotypes (donor A, A1A1 B8B35 Cw4Cw7; donors B and C, A2A25 B18B44 Cw5Cw12) by Ficoll-Hypaque (Eurobio, les Ulis, France) density gradient centrifugation. HLA class I genotyping was established in the HLA typing Laboratory of Centre Régional de Transfusion Sanguine de Nantes by PCR amplification techniques (6, 36). There was no parental link between donors B and C. All donors carried antibodies against EBV. BLC lines were established from PBMCs by EBV transformation as previously described (24).

Expression vectors. IE1 and pp65 full-length coding sequences were amplified by reverse transcription (RT)-PCR from MRC-5 fibroblasts infected with a CMV strain selected from a clinical isolate, cloned in KS⁺ vectors (Stratagene, La Jolla, Calif.) and sequenced. The IE1 coding sequence used subsequently for expression exhibited nucleotide variation when compared to the corresponding nucleotide sequence of AD169 and Towne laboratory strains. All of these mutations were also found in two other IE1 PCR products independently amplified from the same clinical strain and then were due to natural variation. Such a polymorphism was not found in the pp65 coding sequence. IE1 and pp65 cDNAs were excised from KS⁺ and subcloned in pRc/CMV expression vector (Invitrogen Corp., San Diego, Calif.) under the control of CMV promoter.

Expression vectors containing various genomic DNA or cDNA encoding HLA class I alleles were used to determine the HLA restriction of IE1 or pp65 peptide recognition. The genomic HLA-A*0101 was kindly provided by J. Girdlestone (Medical Research Council, Cambridge, United Kingdom). HLA-B*0801 and -B*3501, a gift of L. Statz (Laboratory of Immunogenetics, Hospital de Clinicas Jose de San Martin, Buenos Aires, Argentina), and HLA-B18, a gift of E. Scotet (INSERM U463, Institut de Biologie, Nantes, France), were previously cloned into pcDNA3 (31). Genomic HLA-A*0201 cloned into pSV2 vector and HLA-B*4402 cDNA cloned into pcDNA3 were kindly supplied by T. Boon and P. Coulie (Ludwig Institute, Brussels, Belgium). The cDNA HLA-Cw4 cloned into pBK-RSV and the genomic DNA HLA-Cw7 cloned into pSV2neo were kindly furnished by M. Colonna (Basel Institute for Immunology, Basel, Switzerland).

Transfection of BLCs. Five million actively dividing BLCs were resuspended in 0.8 ml of complete medium, mixed with 15 μ g of vector, incubated for 1 min at room temperature, and electroporated in an Easyject Plus apparatus (Eurogentec, Seraing, Belgium). One pulse of 250 V/cm using a 1,500- μ F capacitor was delivered. The cells were immediately plated with 5 ml of fresh medium. The same protocol was repeated three times to obtain 1.5×10^7 electroporated BLCs for each transfection assay. Selection with G418 (0.8 mg/ml) started 2 days after electroporation. After 1 week, dead cells and debris were eliminated by centrifugation through a Ficoll-Hypaque gradient, and the cells from the same three electroporations were pooled.

Generation of specific T lymphocytes. Anti-pp65 and anti-IE1-specific T lymphocytes were amplified against BLCs expressing stably these viral recombinant proteins using the following protocol: donor PBMCs were plated at 2×10^6 cells per well in 24-well plates containing RPMI 1640 supplemented with 10% pooled human serum and 1 mM L-glutamine and then stimulated with 5×10^4 irradiated (30 Gy) autologous BLCs expressing either pp65 or IE1. After 9 days of coculture, 5×10^5 cells per well were restimulated with 1.25×10^5 autologous

recombinant irradiated BLCs. Nine days later, recombinant interleukin-2 was added to the culture (20 IU/ml). Twenty days after the second stimulation, cells were cloned from the amplified lines by limiting dilution as previously described (37) at 0.3 cells per well. Growing colonies with a probability of monoclonality of >95% were kept for further analysis and expanded *in vivo* using standard conditions (9). Cells were maintained for 3 to 4 weeks without restimulation prior to functional analysis in order to decrease their spontaneous tumor necrosis factor (TNF) release and their nonspecific cytotoxic activity.

Flow cytometry analysis and functional assays. Expression of pp65 and IE1 recombinant proteins was checked by flow cytometry in stably transfected BLCs permeabilized and then fixed as described previously (14). (1C3 plus AYM-1)-fluorescein isothiocyanate (FITC) monoclonal antibodies (MAbs) (Argene) were used to detect pp65, and E13-FITC MAb (Biosys) was used to detect IE1.

T cells were phenotyped by indirect fluorescence as previously described (37). Anti-coreceptor and -TCR MAb were purchased from Immunotech-Coulter Corp. (Marseille, France).

The proliferative and cytotoxic activity of T-cell clones against autologous irradiated BLCs stably transfected with pp65, IE1, or mock vector were estimated as previously described (8, 37).

HLA restriction characterization. HLA restriction of T cells was characterized, as previously described (31), by testing their TNF content when added to COS cells cotransfected with an expression vector coding for their target viral protein and an expression vector coding for one of the HLA.

Characterization of target peptides. Synthetic peptides (23-mer overlapping by 12 residues) encompassing the whole primary amino acid sequence of pp65 and IE1 were obtained from Chiron Technologies (Pty Ltd., Clayton, Victoria, Australia). The pp65 peptides are numbered 1 to 50, and IE1 peptides are numbered 1 to 44 from the N terminus to the C terminus. Minimal 9- or 10-mer peptides corresponding to proposed MHC-restricted epitopes were obtained from Genosys (Pampisford, United Kingdom).

Peptides recognized by T-cell clones directed against pp65 and IE1 were characterized by determining the TNF release in an autresentation assay. Ten-thousand T cells were incubated for 2 h with 10 μ M pp65 or IE1 individual synthetic peptides. The quantification of TNF release was determined as described earlier (31).

Analysis of TCR β transcripts. T-cell-receptor β (TCR β) cDNAs were amplified by RT-PCR from total RNA of clones cDNAs as previously described (31) and then directly sequenced.

RESULTS

Amplification and cloning of anti-pp65 or IE1 CTLs. BLCs expressing stably pp65 were generated from donors A and B. BLCs expressing stably IE1 were generated from donor C (see, for example, Fig. 1). They were subsequently used as stimulators to amplify specific T cells from autologous PBMCs. The presence of specific CTLs in bulk cultures was checked using a technique in which their TNF release against COS cells co-

TABLE 1. Amino acid sequence of TCR β -chain junction of T-cell clones derived from three HCMV⁺ donors and recognizing either pp65 or IE1^a

Donor	HLA restriction	Amino acid position in:		TCR β -chain sequence					No. of clones
		IE1	pp65	V β	CAS	CDR3	J β		
A	A1		353–375	V β 1S1	CAS	SPRGFDAGGYWLD	LRF	J β 1.2	1
Total									1
B	A2		485–507	V β 6S4A1	CAS	SYGQNPLNTE	AFF	J β 1.1	1
B	A2		485–507	V β 14S1	CAS	SLEGYTE	AFF	J β 1.1	1
Total									2
C	A2	309–331		V β 1S1	CAS	SVDGTGGALGNT	IYF	J β 1.3	1
C	A2	309–331		V β 4S1	CSV	EXSPPGEEWWLT	LRF	J β 1.6	1
C	A2	309–331		V β 210S1	CAW	SWG DSTNSP	LHF	J β 1.6	19
C	A2	309–331		V β 21S3	CAS	SLVGGTGSVSY	QFF	J β 2.1	1
Total									22
C	B18	188–210, 199–221		V β 3S1	CAS	SLLMATNEK	LFF	J β 1.4	1
C	B18	188–210, 199–221		V β 6S5	CAS	SYRANYE	QYF	J β 2.7	2
C	B18	188–210, 199–221		V β 9S2	CAS	SQAVASGGGRSE	QFF	J β 2.1	1
C	B18	188–210, 199–221		V β 22S1	CAS	VTGSTDT	QYF	J β 2.3	7
C	B18	188–210, 199–221		V β 22S1	CAS	SDGTAYE	QYF	J β 2.7	10
C	B18	265–287		V β 7S1	CAS	SLARNQP	QHF	J β 1.5	1
C	B18	265–287		V β 17S1	CAS	GGADMKTE	AFF	J β 1.1	1
C	B18	375–397		V β 2S1	CSA	NPDWGLQLWLH	LRF	J β 2.1	1
C	B18	375–397		V β 6S4	CAS	SPGGGKET	QYF	J β 2.5	1
C	B18	ND		V β 6S4	CAS	RLRGSSSYNE	QFF	J β 2.1	1
Total									26

^a The recognized epitope and restricting HLA molecule are given for each clone. The total number of anti-pp65 or IE1 T-cell clones restricted by a given HLA context for each donor is in boldface. ND, not determined.

transfected with pRc/CMV-pp65 or -IE1, and the DNA coding for individual HLA class I alleles was measured. Cells amplified from donors A and B were reactive against COS cells coexpressing pp65 and HLA-A1 or -A2 molecules, respectively. Furthermore, the bulk CTL line from donor C recognized COS cells expressing IE1 and either HLA-A2 or HLA-B18. Cell lines were cloned by limiting dilution, and colonies were tested for their reactivity against target cells expressing or not expressing recombinant pp65 or IE1 proteins.

Selection of T-lymphocyte clones reactive against either pp65 or IE1 and characterization of their HLA-restricting allele. Two methods were used to screen for the specificity of T-cell clones derived from bulk cultures. Clones obtained from PBMCs stimulated against autologous BLCs expressing pp65 (donors A and B) were selected on the basis of their differential proliferative activities against autologous BLCs stably transfected with either pRc/CMV-pp65 or the empty pRc/CMV control. CD8⁺ CTL clones obtained from PBMCs stimulated against autologous BLCs expressing IE1 (donor C) were selected using a COS cell cotransfection assay which allowed us to characterize at the same time the HLA molecules restricting the presentation of HCMV peptides.

From 70 T-cell clones generated from donor A, a single clone (A18) proliferating against autologous targets expressing pp65 was isolated. Clone A18 recognized specifically pp65 when expressed in COS cells together with the HLA-A1 allele. From 75 clones generated from donor B, 2 clones (B3 and B20) were shown to recognize pp65-transfected BLCs. These two clones recognized pp65 in the HLA-A2 context, as indicated by COS screening assays. Of the 75 T-cell clones generated from donor C, 22 were reactive against IE1 in the HLA-A2 context and 26 were reactive in HLA-B18 context (Table 1).

All three anti-pp65 clones and the 48 anti-IE1 selected T-cell clones specifically lysed pp65- or IE1-transfected BLCs,

respectively, but not the mock-transfected autologous BLCs. As expected, when analyzed by flow cytometry, all clones selected from the three donors were TCR $\alpha\beta$ ⁺ and CD8⁺.

Analysis of the fine epitope specificity of pp65- and IE1-reactive CTL clones. The fine specificity of HCMV-reactive T-cell clones was studied by screening their reactivity to synthetic peptides encompassing the whole IE1 and pp65 proteins. The results from such screenings are summarized in Table 1. The donor A-derived clone A18 recognized the pp65_{353–375} peptide in the HLA-A1 context. The two clones B3 and B20 were specific for the HLA-A2-restricted pp65_{485–507} peptide. The anti-IE1 response of donor C-derived T cells was focused against the HLA-A2-restricted IE1_{309–331} peptide and a dominant B18-restricted peptide located within the IE1_{188–210}-IE1_{199–221} overlapping peptidic region, which was recognized by 21 of 26 T-cell clones. Nine-mer peptides recognized in the HLA-A2 and HLA-B18 contexts by T-cell clones from donor C were further characterized through screening of an additional set of peptides (Table 2).

TCR β -chain features of IE1- and pp65-reactive T-cell clones. TCR V β gene usage by anti-pp65 and -IE1 CTLs generated from the three HCMV⁺ donors was first studied by flow

TABLE 2. Nine-mer peptides recognized by the anti-IE1 clones generated from donor C

Amino acid sequence ^a	Amino acid position in IE1	Restricting HLA allele	No. of clones
YILEETSV	315–323	A2	22
ELKRKMIYM	199–207	B18	21
CVETMCNEY	279–287	B18	2
DEEDAIAAY	379–387	B18	2

^a Target residues for natural polymorphism are indicated in boldface.

cytometry using a panel of 22 TCR V β -specific MAbs (results are summarized in Table 1). The clone generated from donor A against pp65 expressed the V β 1 segment. Of the two clones generated from donor B against pp65, one expressed the V β 6 segment and the other expressed the V β 14 segment. Of the 48 clones generated from donor C, 19 of 22 clones (i.e., 86%) recognizing the 9-mer IE1₃₁₅₋₃₂₃ peptide in the HLA-A2 context expressed the TCR V β 20 gene segment and 17 of 26 clones (i.e., 63%) recognizing IE1 in the HLA-B18 context expressed the V β 22 gene segment. All of these V β 22⁺ T-cell clones recognized the IE1₁₉₉₋₂₀₇ epitope.

TCR β -chain sequencing of anti-pp65 and -IE1 CTL clones is presented in Table 1. All V β 20 T-cell clones reactive against the IE1-HLA-A2 epitope exhibited identical junctional characteristics (i.e., J β 1.6 segment and identical CDR3 sequence), suggesting that they were the progeny of a single clone. Similarly, of 17 CTL clones restricted by the HLA-B18 molecule and expressing the V β 22 gene segment, 10 exhibited the J β 2.7 segment and identical junctional features. The seven other clones used the J β 2.3 segment and showed identical junctional sequences. All of these 17 clones exhibited conserved length of their CDR3 loop and a shared NDN-encoded Gly at a conserved position within the CDR3 loop.

DISCUSSION

In this report, we present data on TCR β -chain usage and the fine specificity of anti-HCMV CD8⁺ CTL clones obtained through stimulation of PBMCs from HCMV-seropositive individuals against pp65- and IE1-BLC transfectants.

Although recombinant proteins were similarly expressed in the stably transfected BLC stimulator cells, generation of T-cell clones reactive against pp65 was less efficient than against IE1. Such a result is in disagreement with those obtained by other authors who used a fibroblast infection model to generate anti-CMV CTLs (21, 28, 40, 43), but it is consistent with more recent data (19, 31). In an infection model, (i) the substantial level of dense bodies that carry only tegument proteins in laboratory strains (for a review, see reference 23), (ii) blockade of IE1 processing and presentation due to its phosphorylation (12), and (iii) downregulation of MHC class I expression (1, 3, 13, 15-18, 39, 41, 42) may together lead to an underestimation of the importance of CTL response against IE1. However, we cannot exclude that, in our expression model, we underestimated the CTL response against pp65.

The TCR repertoire of IE1-specific T-cell clones identified in donor C was highly heterogeneous, showing that the response to CMV proteins is more diversified, as has already been suggested by others (40, 43). In our experiments, we only stimulated donors A and B with pp65 and donor C with IE1, but conclusive information should be obtained from further investigations by using our antigenic presentation strategy to generate CTLs against both IE1 and pp65 from PBMCs of an HCMV-seropositive individual.

The clones generated from donors A and B against autologous BLCs expressing pp65 that were not specific of this HCMV product proliferated mainly against autologous untransfected BLCs and were then probably directed against EBV antigens (data not shown). Such a result is consistent with a very recent report showing that BLCs transduced with a recombinant retrovirus encoding pp65 can be used as antigen-presenting cells to stimulate expansion of EBV- and CMV-specific CTLs simultaneously (33).

Amplification of anti-EBV CD4⁺ and CD8⁺ CTLs against BLC targets has been already described (reference 22 and our own results). Therefore, CD4⁺ anti-HCMV clones should be

theoretically generated against BLCs expressing recombinant HCMV proteins. Other reports have shown a specific CD4⁺ T-cell response against IE1 in HCMV-seropositive individuals (2, 4, 10). Further investigations are necessary to obtain conclusive data regarding the efficiency of CD4⁺ T-lymphocyte-specific amplifications when the antigens are nuclear proteins synthesized by presenting BLCs.

Of the six epitopes characterized in this study, four were new. The pp65₄₈₅₋₅₀₇ peptide recognized in the HLA-A2 context contained the 495-NLVPMTATV-503 9-mer epitope, which had been identified in previous studies as an immunodominant epitope (40, 43). This minimal epitope was recognized by the two clones generated from donor B against pp65 (data not shown). Concerning IE1, the 9-mer peptide 379-DEEDAIAAY-387, recognized in the B18 restricted context, was internal to a 12-mer epitope 378-SDEEEAIVAYTL-389 previously characterized in the same HLA context but which differed from its sequence by two residues (11).

The sequences of IE1 have long been assumed to be well preserved from genetic variation. Nevertheless, we and others recently described polymorphic regions in the coding sequence of this viral protein (7, 27, 44) which could interfere with presentation by MHC molecules and/or recognition by the immune system. Our data suggest that a large part of the memory CD8 CTL response against IE1 protein cloned from a clinical strain is directed against polymorphic epitopes restricted by two HLA molecules. A single residue exchange within a viral CTL epitope could later recognition or destruction by specific proteasomal cleavage, resulting in a lack of antigen presentation (26). Important information on the role possibly played by the IE1 natural polymorphism on the modulation of the anti-HCMV memory repertoire should be obtained from analysis of the T-cell-clone reactivity against target cells expressing IE1 proteins derived from various clinical strains or loaded with modified peptides.

In summary, the results presented here show that IE1-specific CTL clones can be efficiently generated through stimulation of PBMCs against autologous IE1 BLC transfectants. Study of the TCR and antigenic characteristics of CD8⁺ CTL clones obtained through this technique should help us analyze the role of IE1 in the control of viral reactivation and infection in HCMV-seropositive individuals with various HLA genotypes and to assess the impact of its peptidic polymorphism on the modulation of the host response against numerous HCMV isolates.

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