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Research Article

Major contribution of codominant CD8 and CD4 T cell epitopes to the human cytomegalovirus-specific T cell repertoire

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Abstract. Human cytomegalovirus (HCMV) infection or reactivation is a cause of morbidity and mortality in immunocompromised individuals. In immunocompetent individuals, in contrast, HCMV is successfully controlled by specific CD8 and CD4 T cells. Knowledge of CD8 and CD4 T cell epitopes from HCMV and their immunodominant features is crucial for the generation of epitope-specific T cells for adoptive immunotherapy and for the development of a peptide-based HCMV vaccine. Therefore, we investigated the natural frequencies of a large number of CD8 and CD4 T cell epitopes, including 10 novel ones. We determined several epitopes as immunodominant. Surprisingly, no clear hierarchies were found for CD8 T cell epitopes, indicating codominance. These results will be valuable for adoptive transfer strategies and support initiatives towards development of a peptide-based HCMV vaccine.

Key words. HCMV; immunodominance; antiviral T cells; T cell epitopes; adoptive immunotherapy; peptide-based HCMV vaccine.

Introduction

Human cytomegalovirus (HCMV) infection is normally asymptomatic in immunocompetent individuals [1]. However, primary HCMV infection [1, 2] or reactivation of HCMV from latency [3] are causes of significant morbidity and mortality in immunocompromised individuals such as transplant recipients. In non-immunocompromised seropositive individuals, HCMV-specific cytotoxic T lymphocytes (CTLs) are present in high frequencies in the peripheral blood [4], and there is a direct correlation between the recovery of HCMV-specific CTL responses with an improved outcome of HCMV disease [5]. The concept of adoptive immunotherapy was derived [6], and the adoptive transfer of HCMV-specific CTL clones [7, 8] or HCMV-specific T cell lines [9] has successfully protected patients at risk from HCMV disease. The importance of T helper cells (T_H) in primary HCMV infection [10] as well as for expansion of memory CD8 T cells [11] has lately been emphasized. Furthermore, several studies have outlined the significance of antiviral effector functions of T_H cells in maintaining CTL responses after adoptive transfer [12, 13] and their capacity to produce antiviral cytokines [10, 14]. These findings underline the importance of T cell immunity in the control of HCMV infection and the relevance of T cell based approaches in therapeutic settings.

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Knowledge of HCMV epitopes is crucial not only for monitoring of antiviral immunity but also for the in vitro generation of antiviral CTLs for possible application in adoptive immunotherapy. Several studies have already been successful in the identification of HCMV epitopes [14–29]. According to Sercarz et al. [30], immunodominant epitopes are defined as those that account for the bulk of the global specific T cell response within one individual. However, we use this term to refer to the frequency of responders among individuals carrying the restricting allele, while we refer to codominance as the responsiveness of a single donor against more than one epitope derived from a given protein. Identification of a sufficiently large number of CTL and T_H epitopes as well as knowledge of immunodominance of individual epitopes and epitope hierarchies on individual MHC alleles is pivotal for development of an HCMV vaccine. In addition, the use of well-defined peptides for the generation of HCMV-specific T cells for adoptive immunotherapy is preferential to the use of ill-defined viral lysates, which have so far mostly been employed for this purpose [9, 31].

We performed large-scale screenings of HCMV-seropositive donors for determination of immunodominance of known CTL and T_H epitopes and for identification of novel CTL and T_H epitopes. Our experiments focused on pp65 as the major target of human CTL reactions, but we also included other HCMV antigens with reported CTL responses. In the course of this study, we were able to determine the immunodominant features of a great number of CTL and T_H epitopes. Furthermore, we identified six novel CTL and four novel T_H epitopes.

Materials and methods

Donors

Buffy coats were obtained from healthy blood bank donors of known major histocompatibility complex (MHC) class I and II types and of known HCMV serostatus. The local Ethics Committee approved this study. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats using standard gradient separation (Lymphocyte Separation Medium, PAA Laboratories, Pasching, Austria) and cryopreserved in fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) with 10% DMSO (Merck, Darmstadt, Germany) at –80 °C until further use.

Peptides, recombinant MHC molecules and fluorescent tetramers

Peptides from HCMV pp65, pp50 and IE1 were synthesized by standard Fmoc chemistry using a Synergy Personal Peptide Synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) or alternatively using the Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). HLA class I restricted peptides were either selected according to epitope prediction using the SYFPEITHI database [32] or according to published CTL epitope sequences. A panel of 15–20mer peptides constituting potential HLA-DR (Human leukocyte antigen class II DR) epitopes from pp65 and IE-1 (immediate-early protein 1) for CD4 T cell epitope screening was selected according to several HLA-DR peptide motifs [32], http://www.syfpeithi.de]. Peptide pools for CD4 T cell epitope screening consisted of three to five peptides with single peptide concentrations of 5 μg/ml. Peptides were dissolved at 10 mg/ml in DMSO (Merck), diluted 1:10 in ddH_2O and aliquots stored at –80 °C.

Biotinylated recombinant MHC class I molecules and fluorescent MHC tetramers for immunodominance studies and verification of predicted CD8 T cell epitopes were produced as described earlier [33]. Briefly, fluorescent tetramers were generated by coincubating biotinylated HLA monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, the Netherlands) at a 4:1 molar ratio. For all HLA-B*44 peptides, HLA-B*4405 monomers were used. The used HLA-B*2705 tetramers had a cysteine-to-serine mutation in position 67.

Reagents and media

T cell medium consisted of RPMI 1640 containing HEPES and L-glutamin (Gibco, Paisley, UK) supplemented with 10% heat-inactivated human serum (PAA, Cölbe, Germany), 50 U/ml penicillin, 50 μg/ml streptomycin and 20 μg/ml gentamicin (all BioWhittaker, Verviers, Belgium). PBE (PBS/BSA/EDTA) was PBS (phosphate-buffered saline) (BioWhittaker, Verviers, Belgium) containing 0.5% of bovine serum albumin (Sigma Aldrich) and 2 mM EDTA (Roth, Karlsruhe, Germany). PFEA (PBS/FCS/EDTA/NaN_3) was PBS supplemented with 2% heat inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM EDTA (Roth) and 0.01% sodium azide (Merck, Darmstadt, Germany). TSB (tetramer staining buffer) was PBS (BioWhittaker/Cambrex) containing 50% FCS (PAA), 2 mM EDTA (Sigma Aldrich) and 0.02% NaN_3 (Merck).

Peptide stimulation and intracellular IFNγ staining

Cryopreserved PBMCs were thawed, washed two times in PBE, resuspended at 10^7 cells/ml in T cell medium and cultured overnight to reduce unspecific interferon-γ (IFNγ) production [34]. On the next day, PBMCs were stimulated with 5 μg/ml peptide for 6 h in the presence of Golgi-Stop (Becton Dickinson) for the final 4 h of incubation.

Intracellular IFNγ staining was performed for the analysis of CD4 T cell responses because it allows for combining IFNγ and CD4 staining. Cells were analysed using a Cytofix/Cytoperm Plus kit (Becton Dickinson, Heidel-

berg, Germany) plus Abs IFNγ-PE (Becton Dickinson), CD4-fluorescein isothiocyanate (FITC) (Immunotools, Friesoythe, Germany) and/or CD8-PerCP clone SK1 (Becton Dickinson). After staining, cells were analysed on a three-color FACSCalibur (Becton Dickinson).

Tetramer staining

2×10^6 thawed PBMCs were stained with CD8-PerCP clone SK1 antibody (Becton Dickinson) and incubated at 4 °C for 20 min in the dark, followed by 30 min incubation with fluorescent MHC tetramers at 4 °C in the dark. After washing, cells were resuspended in 1% paraformaldehyde in PFEA. Cells were analysed by flow cytometry on a four-color FACSCalibur cytometer (Becton Dickinson).

Combined tetramer staining/intracellular IFNγ-staining

Two different conditions were used to perform this assay provided enough PBMCs were available. PBMCs were stained with tetramer before and after stimulation with peptide (condition 1), or with tetramer only after stimulation with peptide (condition 2), as reported previously [35]. Staining procedures were performed as described above using IFNγ-FITC (Becton Dickinson), CD8-PerCP clone SK1 (Becton Dickinson) and fluorescent MHC tetramer-PE.

Phenotyping of antigen-specific CD8$^+$ T cells

Thawed and washed PBMCs were stained with CD8-PerCP clone SK1 (Becton, Dickinson) plus either CD45RA-FITC (Becton Dickinson) or CD27-FITC, CD28-FITC, CD45RO-FITC, CD57-FITC, CD62L-FITC, respectively (all Coulter-Immunotech, Hamburg, Germany). For staining with CCR7, cells were first labelled with rat hybridoma supernatant 3D12 (kindly provided by R. Förster, Anova). After washing, cells were incubated with FITC-labelled donkey anti-rat $F(ab')_2$ fragments (Jackson Immunoresearch Laboratories, West Grove, PA), and blocking with heat-inactivated mouse serum (CC pro) was performed.

After staining with antibodies, cells were washed and labeled with tetramers diluted to 2 µg/ml in TSB (Tetramer Staining Buffer). Stained cells were washed and fixed in PFEA containing 1% formaldehyde (Merck). Cells were analysed on a three-color FACSCalibur (Becton Dickinson).

IFNγ ELISpot assay

IFNγ ELISpot assays were performed for the analysis of CD8 T cell responses if no tetramers of the respective HLA molecule were available. 96-well nitrocellulose plates (MHABS4510, Millipore, Bedford, MA) were coated with a mouse anti-human IFNγ antibody (5 µg/ml, Becton Dickinson). 10^6 thawed PBMCs/well were stimulated with 5 µg/ml peptide for 24 h, and secreted IFNγ was detected using biotinylated anti-IFNγ mAb (1 µg/ml), streptavidin-alkaline phosphatase conjugate (all Becton Dickinson) and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate toluidine and nitroblue tetrazolium, Sigma Aldrich, Steinheim, Germany). The plate was washed with water and dried overnight. IFNγ-producing cells were detected as purple spots on the nitrocellulose membrane of each well. Plates were analysed using the Immunospot Image Analyzer (series 1) and ImmunoSpot Software Version 3.2e (both Cellular Technology, Cleveland, OH).

Results

Immunodominance of CD8$^+$ T cell epitopes

PBMCs of 67 healthy HCMV seropositive donors were tested ex vivo with respect to the frequency of specific T cells in their peripheral blood. Reactivity of CD8$^+$ T cells to peptides was determined by IFNγ-ELISpot, and specificity of T cell receptors by tetramer staining. From our experience, frequencies down to 0.01% among CD8$^+$ T cells can be clearly distinguished from background by tetramer staining and 0.01–0.001% by IFNγ-ELISpot, respectively.

HLA tetramers of several allotypes were prepared containing HCMV peptides derived from pp65, pp50 and IE-1, respectively, to determine the frequency of responders among donors positive for the restricting HLA allele. Epitopes were considered immunodominant if more than 75% of seropositive donors showed a T cell response with at least six or more donors tested; epitopes with values detected below this average were regarded as subdominant. Peptides tested are listed together with references in table 1. Here we determined five CTL epitopes presented by three different HLA alleles (HLA-A*0101, HLA-A*0201 and HLA-B*0702) to be immunodominant. Among 15 HCMV-seropositive donors expressing HLA-A1, 14 showed CD8 T cells specific for pp65 363–373 and 8/10 donors had high frequencies of CTLs specific for peptide pp50 245–253 (examples in fig. 1A). T cells specific for NLVPMVATV (pp65 495–503) were observed in all HLA-A2$^+$, HCMV-seropositive donors tested (examples in fig. 1B), but not in HCMV-seronegative donors, indicating this epitope to be the most immunodominant CTL epitope. T cells restricted to pp65 120–128, also presented by HLA-A*0201, were detected in 5/10 donors (examples in fig. 1B). Furthermore, immunodominance was determined for two epitopes presented by HLA-B*0702 (pp65 417–426, pp65 265–275) as can be seen in fig. 1C. Apart from these immunodominant epitopes, we also noticed six CTL epitopes which occurred in less than 50% of donors tested (A*0201: MLNIPSINV, VLEETSVML; A*0301: TTVYPPSSTAK;

Table 1. Summary of CTL epitopes analysed.

HLA	Protein	Position	Sequence	Reference	n[c] Tetramer	n[c] IFNγ
A*0101	**pp65**	**363–373**	**YSEHPTFTSQY**[d]	**[26]**	**14/15**	n.t.
	pp50	**245–253**	**VTEHDTLLY**[d]	**[17]**	**8/10**	n.t.
	pp50	274–253	RGDPFDKNY	predicted	0/6	n.t.
A*0201	**pp65**	**495–503**	**NLVPMVATV**[d]	**[29]**	**13/13**	n.t.
	pp65	120–128	MLNIPSINV	[28]	5/10	n.t.
	pp65	522–530	RIFAELEGV	[24]	0/6	n.t.
	IE1	316–324	VLEETSVML	[22]	2/6	n.t.
A*0301	pp150	945–955	TTVYPPSSTAK	[26]	1/12	n.t.
A*1101	pp65	16–24	GPISGHVLK	[20]	0/5	n.t.
	pp65	501–509	ATVQGQNLK	[24]	0/3	n.t.
A*2402	pp65	341–349	QYDPVAALF	[15]	0/6	n.t.
A*6801	pp65	186–196	FVFPTKDVALR	[26]	1/5	n.t.
	pp65	183–191	TSAFVFPTK[b]	this paper		see table 4
	pp150	794–802	VTSTPVQGR	predicted	0/5	n.t.
A*69	pp65	86–94	EVENVSVNV[a]	this paper		see table 4
B*0702	**pp65**	**417–426**	**TPRVTGGGAM**[d]	**[20]**	**11/13**	n.t.
	pp65	**265–275**	**RPHERNGFTVL**[d]	**[26]**	**10/11**	n.t.
	IE1	310–317	RVLCCYVL	predicted	0/19	n.t.
B*13	pp65	211–219	TRATKMQVI[a]	this paper		see table 4
B*1501	pp65	355–363	LLLQRGPQY	predicted	0/3	0/3
	pp65	73–81	NQLQVQHTY	predicted	0/3	0/3
	pp65	361–369	PQYSEHPTF	predicted	0/3	0/3
	pp65	505–513	GQNLKYQEF	predicted	0/3	0/3
	pp65	502–510	TVQGQNLKY	predicted	0/3	0/3
	pp65	319–327	LLMNGQQIF	predicted	0/3	0/3
	pp65	74–82	QLQVQHTYF	predicted	0/3	0/3
	pp65	215–223	KMQVIGDQY	[24]	0/3	0/3
	pp65	173–181	NQWKEPDVY	predicted	n.t.	0/3
	pp65	223–231	YVKVYLESF	predicted	n.t.	0/3
B*2705	pp65	539–547	RRRHRQDAL	predicted	0/2	0/2
	pp65	373–381	YRIQGKLEY	predicted	n.t.	0/2
	pp65	264–272	MRPHERNGF	predicted	n.t.	0/2
	pp65	39–47	TRLLQTGIH	predicted	n.t.	0/2
	pp65	257–265	TRNPQPFMR	predicted	n.t.	0/2
	pp65	358–366	QRGPQYSEH	predicted	n.t.	0/2
	pp65	418–426	PRVTGGGAM	predicted	n.t.	0/2
	pp65	3–11	SRGRRCPEM	predicted	n.t.	0/2
B*40	pp65	42–50	KEVNSQLSL[a]	this paper		see table 4
B*44	pp65	511–521	QEFFWDANDIY	predicted	0/10	1/10
	pp65	267–275	HERNGFTVL	[24]	0/10	0/10
	pp65	232–240	CEDVPSGKL	[24]	0/10	0/10
	pp65	364–373	SEHPTFTSQY	[24]	1/10	5/10
B*51	pp65	116–123	LPLKMLNI[a]	this paper		see table 4
	pp65	114–123	YALPLKMLNI[a]	this paper		see table 4

[a/b] Epitopes were identified by intracellular IFNγ staining[(a)] or tetramer staining[(b)] in donors who had reacted to corresponding long peptides (see table 4). n.t., not tested.
[c] n refers to the number of individuals showing positive response against this epitope/total number of individuals with corresponding allele tested.
[d] Entries in bold letters are considered immunodominant.

A*6801: FVFPTKDVALR; B*44: QEFFWDANDIY, SEHPTFTSQY). For several peptides, including published CTL epitopes [15, 20, 24], no CTL response could be detected in the HCMV-seropositive donors tested (table 1).

Investigation of hierarchy among immunodominant epitopes

Frequencies of CTLs specific for immunodominant epitopes were compared in donors having at least two of the alleles HLA-A1, -A2 or -B7 in common. As can be seen in table 2, T cells specific for different CTL epitopes were detected in parallel. Moreover, strong responses to one epitope did not prevent the detection of lower frequencies of CTLs specific for other epitopes, as can be seen in donor 5 and donor 16. The detection limit of tetramer staining is described as 0.01%. Nethertheless, in two donors we observed distinct $CD8^+$ T cell populations lower than 0.01%. Overall, no clear hierarchy was observed in that CTL populations specific for several im-

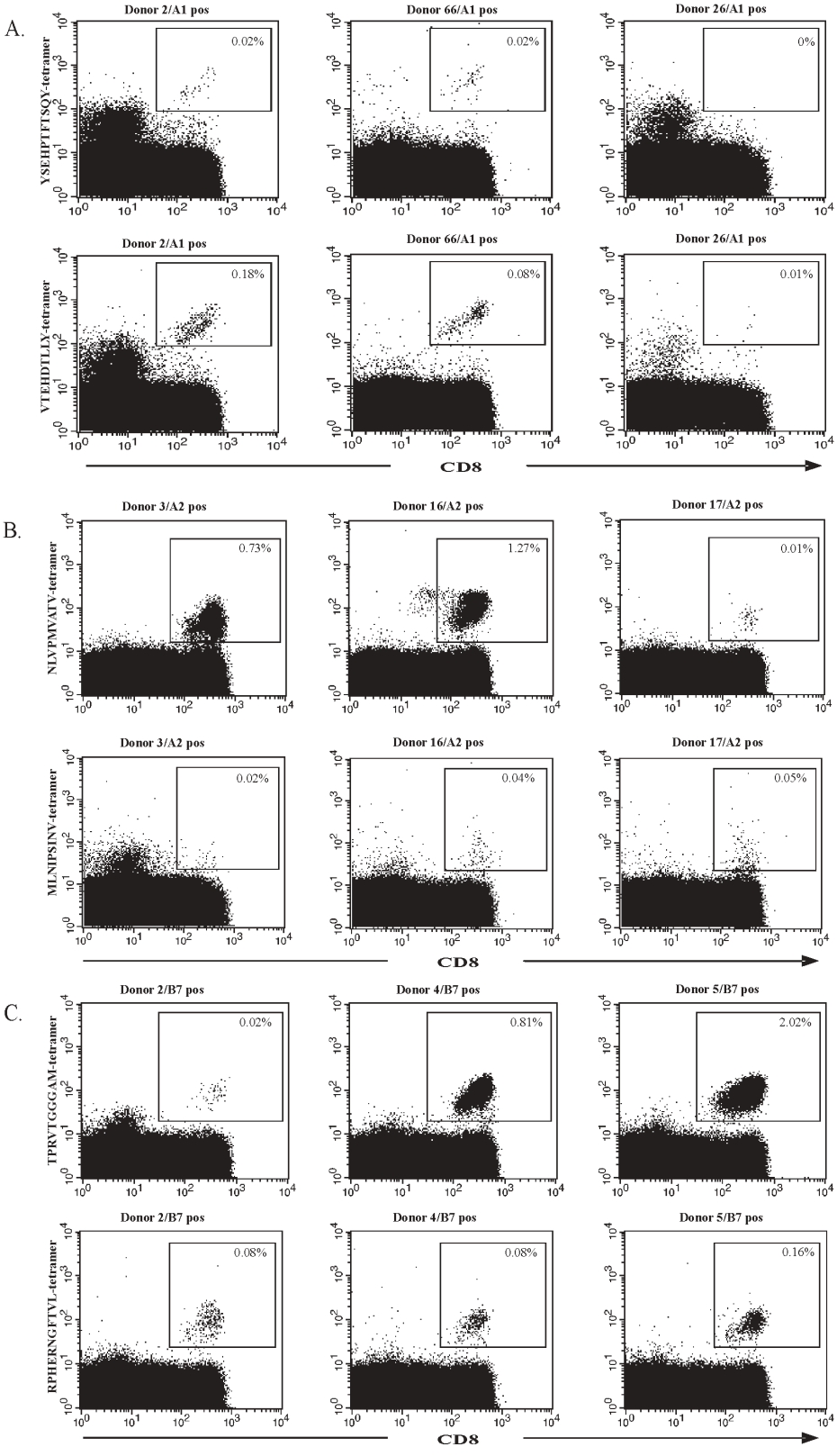

Figure 1. Representative tetrameric analyses for alleles HLA-A*0101 (*A*), HLA-A*0201 (*B*) and HLA-B*0702. (*C*) PBMCs were stained with HCMV-tetramer-PE, CD8-PerCP and CD19-FITC. Percentages refer to tetramer$^+$ T cells within the CD8$^+$ population. (*A*) Binding to HLA-A*0101 tetramers YSEHPTFTSQY (pp65 363–373) and VTEHDTLLY (pp50 245–253) in same donors. (*B*) Binding of HLA-A*0201 NLVPMVATV-tetramer (pp65 495–503) and MLNIPSINV-tetramer (pp65 120–128) in HLA-A2 positive donors. (*C*) Parallel detection of T cells binding to HLA-B*0702 tetramers TPRVTGGGAM (pp65 417–426) and RPHERNGFTVL (pp65 265–275).

Table 2. Frequencies of immunodominant pp65 epitopes in HCMV-seropositive donors as determined by tetramer staining.

Donor	HLA type of donors tested	YSEHPTFTSQY (A*0101)	NLVPMVATV (A*0201)	TPRVTGGGAM (B*0702)	RPHERNGFTVL (B*0702)
BD 1	A1 A3 B7 B8	0.01		<0.01	0.12
BD 2	A1 A28 B7 B49	0.02		0.02	0.08
BD 3	A1 A2 B8 B35	0.01	0.70		
BD 4	A2 B7 B13		0.85	0.81	0.08
BD 5	A1 B7 B55	0.01		2.02	0.16
BD 8	A11 A24 B7			0.21	0.69
BD 9	A2 A3 B7 B51		0.03	1.31	0.42
BD 10	A1 A2 B18 B62	0.04	0.03		
BD 11	A1 A23 B7 B49	0.07		<0.01	<0.01
BD 13	A3 B7 B35			1.10	0.14
BD 15	A3 A11 B7 B62			0.03	0.03
BD 16	A1 A2 B8 B44	0.32	1.26		
BD 17	A2 A3 B7 B35		0.01	0.29	0.57

munodominant T cell epitopes in parallel were frequently observed. Therefore, codominance among these epitopes, especially for B*0702 restricted ones, appeared to be a common feature.

Functional and phenotypic characterization of CD8$^+$ T cells restricted to immunodominant epitopes

CD8$^+$ T cells specific for immunodominant epitopes were analysed for their functional capacity to produce IFNγ. Therefore, tetramer staining in combination with intracellular IFNγ staining was performed with PBMCs of donors already tested with the tetramers. As shown in table 3, tetramer$^+$ CD8$^+$ T cells recognizing epitopes presented by HLA-A*0101, -A*0201 or -B*0702 were often capable of producing IFNγ. Surprisingly, no IFNγ secretion by T cells specific for the immunodominant epitope pp65 363–373 presented by HLA-A*0101 could be detected in three independent experiments.

HCMV tetramer$^+$ CD8$^+$ T cells were also investigated for their phenotypic attributes with several surface markers and found to represent so-called TEMRA cells (CD45RA$^+$ effector memory T cells) which display phenotypic features that are intermediate between naive and effector T cells [36, 37] (data not shown). Similar phenotypes have already been described for circulating memory CTLs specific for other persisting viruses such as Epstein-Barr virus (EBV) [38]. These cells were observed to be functional CTLs as determined by ^{51}Cr-assay after polyclonal expansion (data not shown).

Table 3. IFNγ responses to immunodominant HCMV epitopes.

HLA tested	Protein/position	Sequence	n[a]
A*0101	pp65 363–373	YSEHPTFTSQY	0/6
	pp50 245–253	VTEHDTLLY	3/6
A*0201	pp65 495–503	NLVPMVATV	7/8
B*0702	pp65 417–426	TPRVTGGGAM	7/9
	pp65 265–275	RPHERNGFTVL	7/9

[a] n refers to number of individuals showing IFNγ response against this epitope/total number of individuals with corresponding alleles tested.

Identification of novel CD8 T cell epitopes

Among several strategies for identification of CD8 T cell epitopes, both verification of predicted epitopes and epitope identification by large-scale screens with partially overlapping 15–20mer peptides [39] have been successfully employed. As these two strategies may complement each other, we utilized both for identification of CD8 T cell epitopes. In this context, the nature of non-CD4 T-cell-mediated reactions observed during screens with 15–20mer peptides was further investigated as to their identity as CD8 T cell responses directed against shorter peptides included in longer ones, probably after in vitro processing. Non-CD4-mediated reactions were observed after stimulation of PBMCs from 14 HCMV-seropositive donors, but not with PBMCs from HCMV-seronegative donors. Altogether, IFNγ-producing non-CD4 T cells specific for 14 peptides from pp65 and IE-1 were detected (data not shown). As some of these long peptides contained known CD8 T cell epitopes, we assumed that the observed non-CD4-mediated T cell reactions were CD8 T cell responses. Therefore, considering the MHC class I alleles of reacting donors, potential CD8 T cell epitopes were selected from 15–20mer peptides according to peptide motifs of HLA allotypes [32]. Depending on the availability of the heavy chains of MHC class I alleles for tetramer synthesis, which can be restrictive, short peptides were either used for tetramer synthesis and tetramer staining plus anti CD8-PerCP antibody, or tested for their capacity to give rise to specific CD8 T cell mediated reactions by intracellular IFNγ-staining plus CD8-PerCP antibody. Peptides capable of eliciting non-CD4 T cell

Table 4. Non-CD4 reactions to 15–20mer peptides from pp65 and IE-1 and CD8 reactions to corresponding 8–11mer peptides.

Peptide sequence	Protein/position	(Potential) MHC class I restriction	Reference
	pp65		
ISVLGPISGHVLKAV	12–26		
GPISGHVLK	16–24	A*1101	[20]
EVENVSVNVHNPTGR	86–100		
EVENVSVNV [a]	*86–94*	*A*69*	*this paper*
MSIYVYALPLKMLNI	109–123		
LPLKMLNI [a]	*116–123*	*B*51*	*this paper*
YALPLKMLNI [a]	*114–123*	*B*51*	*this paper*
PLKMLNIPSINVHHY	117–131		
IPSINVHHY	123–131	B*3501	[19]
VYYTSAFVFPTKDVA	180–194		
TSAFVFPTK [b]	*183–191*	*A*6801*	*this paper*
TSAFVFPTKDVALRH			
FVFPTKDVALR	183–197		
FPTKDVAL	186–196	A*6801	[26]
	188–195	B*3502	
VCSMENTRATKMQVI	205–219		
TRATKMQVI [a]	*211–219*	*B*13*	*this paper*
GPQYSEHPTFTSQYRI	360–375		
YSEHPTFTSQY	363–373	A*0101	[26]
HPTFTSQYRIQGKLE	366–380		
FTSQYRIQGKL	369–379	A*2402	[26]
	IE1		
QTMLRKEVNSQLSLG	37–51		
KEVNSQLSL [a]	*42–50*	*B*40*	*this paper*
RVLCCYVLEETSVMLAKRPLI	310–330		
VLEETSVML	316–324	A*0201	[22]

[a] Specific CD8 T cells were detected by intracellular IFNγ-staining.
[b] Specific CD8 T cells were detected by tetramer staining.
Novel CD8 T cell epitopes with their position in protein and potential MHC class I restriction are printed in italic.

mediated reactions and corresponding CD8 T cell epitopes are summarized in table 4. Potential CD8 T cell epitopes were tested in donors who had reacted to the corresponding long peptides and who were positive for the potentially restricting allele (one donor for each long peptide except two donors for pp65 109–123). IFNγ-producing CD8 T cells were observed in one donor for each novel CD8 T cell epitope, respectively. This refers to peptides pp65 86–94 (EVENVSVNV, potentially A*69 restricted), pp65 116–123 (LPLKMLNI) and pp65 114–123 (YALPLKMLNI, both potentially B*51 restricted), pp65 211–219 (TRATKMQVI, potentially B*13 restricted) and IE-1 42–50 (KEVNSQLSL, potentially B*40 restricted), while restriction could be assessed unequivocally by tetramer staining for peptide pp65 183–191 (TSAFVFPTK) for which an A*6801 tetramer had been produced.

Responses to known and novel CD4 T cell epitopes

PBMCs of 30 healthy HCMV-seropositive and 5 healthy HCMV-seronegative donors were stimulated with 15 peptide pools each containing three to five 15–20mer peptides from pp65 or IE-1 which represented potential HLA-DR epitopes. Reactivity to peptides was assessed by intracellular IFNγ-staining plus CD4-FITC antibody. Peptide specificity of reactions observed after stimulation with peptide pools was determined by splitting of pools and testing reactions to single peptides.

None of five seronegative donors showed any specific reaction after stimulation with peptide pools. Among 30 seropositive donors, 25 showed CD4 T cell reactions to at least one peptide. Most donors specifically reacted to one or two peptides; reactions to more than three peptides in a single donor were observed less frequently. Altogether, 15 peptides from pp65, but none from IE-1, were able to elicit CD4 reactions. Many of these peptides have previously been reported to constitute CD4 T cell epitopes and are listed in table 5. However, four of these peptides have not been described as CD4 T cell epitopes so far, pp65 109–123 (MSIYVYALPLKMLNI), pp65 191–205 (KDVALRHVVCAHELV), pp65 269–283 (RNGFTVLCPKNMIIK) and pp65 339–353 (LRQYDPVAALFFFDI). HLA-DR restriction for novel CD4 T cell epitopes was assigned according to overlapping expression of alleles in reacting donors. If no potential HLA-DR restriction could be assigned, as was the case for peptides pp65

Table 5. CD4 T cell reactions to 15mer peptides from pp65.

(Potential) DR restriction	Peptide position	Peptide sequence	Reference	n[b]
DRB1*01	117–131[a] (1)	PLKMLNIPSINVHHY	[25]	4/12
DRB1*01	166–180	LAWTRQQNQWKEPDV	[21, 25]	1/12
DRB1*01	510–524[a] (3)	YQEFFWDANDIYRIF	[18, 21, 25]	6/12
DRB1*03	510–524[a] (3)	YQEFFWDANDIYRIF	[18, 21, 25]	3/4
DRB1*01	512–524[a] (3)	EFFWDANDIYRIF	[18, 21, 25]	1/12
DRB1*03	512–524[a] (3)	EFFWDANDIYRIF	[18, 21, 25]	3/4
DRB1*03	250–264	VEEDLTMTRNPQPFM	[25]	1/4
DRB1*04	283–299[a] (1)	KPGKISHIMLDVAFTSH	[21, 25]	3/6
DRB1*07	283–299[a] (1)	KPGKISHIMLDVAFTSH	[21, 25]	3/5
DRB1*04	370–384	TSQYRIQGKLEYRHT	[25]	4/6
DRB1*13	370–384	TSQYRIQGKLEYRHT	[25]	4/10
*DRB1*07*	*109–123*	*MSIYVYALPLKMLNI*	*this paper*	*2/5*
DRB1*07	180–194	VYYTSAFVFPTKDVA	[25]	2/5
*DRB1*07*	*339–353*	*LRQYDPVAALFFFDI*	*this paper*	*3/5*
DRB1*11	360–375	GPQYSEHPTFTSQYRI	[18, 21, 23, 25]	5/12
DRB1*11	366–380	HPTFTSQYRIQGKLE	[18, 21, 23, 25]	6/12
DRB1*13	366–380	HPTFTSQYRIQGKLE	[18]	1/10
DRB1*15	39–53	TRLLQTGIHVRVSQP	[21, 23, 25]	4/5
not assigned	*191–205*	*KDVALRHVVCAHELV*	*this paper*	*(2)*
not assigned	*269–283*	*RNGFTVLCPKNMIIK*	*this paper*	*(1)*

[a] Peptides with (a) potential DR restriction(s) that cannot account for all of the observed responses are marked. The number of additional reacting donors negative for the potentially restricting DR allele(s) is given in brackets.
[b] n refers to the number of individuals showing responses against this epitope/total number of individuals with corresponding allele tested. Novel CD4 T cell epitopes with potential HLA-DR restriction are printed in italic.

191–205 and pp65 269–283, the number of reacting donors out of 30 HCMV-seropositive donors is given in brackets. In addition to determination of CD4 T cell epitopes, immunodominance values were evaluated for each of the CD4 T cell epitopes investigated in this study.

Discussion

We investigated the CD8 and CD4 T cell responses of 67 HCMV-seropositive donors against reported and predicted HCMV-derived peptides.

We identified six new HLA class I restricted and four new HLA class II restricted epitopes, and confirmed previously reported epitopes [15, 17, 20, 22, 24, 26, 28, 29]. We could determine immunodominance, as defined above, for each of two epitopes restricted to HLA-A*0101 and HLA-B*0702, respectively, and for one HLA-A*0201 restricted epitope. For HLA-A*0201 and for -B*0702, the detected immundominant epitopes have their origin in the same protein and were detected with similar frequencies in different donors. In view of this observation, we analysed the hierarchy among the frequently recognized epitopes detected in donors carrying HLA-A1, -A2 and/or -B7. Surprisingly, no clear hierarchy was observed among these epitopes tested. The observation that two different epitopes derived from the very same antigen and presented by the same HLA class I molecule appear to be codominant is in sharp contrast to hierarchy studies of viral CTL epitopes in mouse models [40].

The epitope pp65 364–373 was predicted according to the HLA-B*4402 motif. However, donors were only typed with low resolution (HLA-B44). Earlier reports suggested, that this epitope is restricted to HLA-B*4403 [24]. HLA-B*4402 and -B*4403 are two major HLA-B*44 subtypes in white individuals [41, 42] and HLA-B*4403 is the most frequent HLA-B*44 subtype in Japanese individuals [43–45]; the peptide repertoires presented by both subtypes are overlapping by 95% [46].

Furthermore, we were able to define six new CD8 T cell epitopes. Restriction of these novel epitopes could be determined unequivocally by tetramer staining for peptide pp65 183–191, which is A*6801 restricted. Studies aiming at the unambiguous determination of the five remaining epitopes' restriction and at confirming these peptides' identities as natural CD8 T cell epitopes from HCMV are currently in progress. Furthermore, due to rareness of restricting alleles, immunodominance values of individual novel CD8 T cell epitopes have not yet been assessed but are the subject of ongoing studies. This approach for identification of novel CD8 T cell epitopes from rare alleles may prove valuable for complementing other strategies, such as verification of predicted epitopes for frequent alleles.

We identified four novel CD4 T cell epitopes. However, the identity of these peptides as natural CD4 T cell epi-

topes still needs to be confirmed, and their HLA-DR restriction has to be assessed unequivocally. Both aspects are the subject of ongoing studies. All other peptides listed in table 5 have been reported previously as CD4 T cell epitopes [25].

In most cases, HLA-DR restriction of known CD4 T cell epitopes was in accordance with earlier reports [18, 21, 23, 25]. However, this was not the case for peptide pp65 370–384, where DRB1*04 and/or DRB1*13 restriction seems to be more plausible than the proposed DRB1*03 restriction. The reduction of immunodominance of peptide pp65 512–524 as compared with peptide pp65 510–524 on DRB1*01 (6/12 vs. 1/12) but not on DRB1*03 (3/4 vs. 3/4) may be explained by the binding motif of DRB1*01. DRB1*01 has a preference for hydrophobic residues in positions 1, 4, 6 and 9 which are provided by peptide pp65 510–524 but not by peptide pp65 512–524. On the other hand, peptide binding to DRB1*03 favours aspartate in position 4, which is supplied by D516 and is therefore independent of the first two amino acids in peptide pp65 510–524.

Immunodominance values were approximately equal to those determined in the study of [25]. In general, immunodominance values of CD4 T cell epitopes are considerably lower than those of CD8 T cell epitopes, some of which are capable of inducing responses in up to 100% of donors. In contrast to CD8 T cell epitopes, responses on one allele are not focused on a single epitope but seem to be spread over several epitopes, indicating a preference for codominance. In view of this fact, CD4 T cell epitopes with high codominance values were identified for nearly all frequent DR alleles. Such CD4 T cell epitopes should be valuable for therapy and vaccine development.

The observed CD4 and CD8 T cell responses were investigated for their interrelation. To this end, number and strength of CD4 and CD8 T cell responses were compared in 11 donors already tested. No obvious interrelationship could be assessed. CD8 T cell responses to various numbers of peptides and of varying strengths could be observed in donors independent of number and strength of CD4 T cell responses and even in donors in which no CD4 T cell responses could be observed. Reactions restricted to HLA-DQ and -DP were not the topic of this study.

In summary, we characterized six dominant CTL epitopes from both pp65 and IE-1. Furthermore, four new T_H epitopes from pp65 were found. For novel and reported CD8 T cell epitopes, as well as for CD4 T cell epitopes, levels of immunodominance and codominance were determined.

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