Targeting and exploiting cytomegalovirus for vaccine development

Panagioti, E.

Chapter 4

The spontaneous response to Human Cytomegalovirus immediate early protein 2 is focused on 5 highly immunogenic regions and predominantly comprises polyfunctional type 1 CD4$^+$ T cells

Eleni Panagioti[1], Ierry-Ann Lourens[1], Ilina Ehsan[1], Jan W. Drijfhout[2], Marij JP. Welters[1], Ramon Arens[2] and Sjoerd H. van der Burg[1,*]

[1]Department of Medical Oncology, Leiden University Medical Center, Leiden, The Netherlands and [2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.

Manuscript submitted

ABSTRACT

Human Cytomegalovirus (HCMV) is an omnipresent pathogen that is associated with increased morbidity and mortality of immunocompromised individuals. Studies of T-cell immunity to HCMV primarily reflect anti-HCMV pp65 or immediate early (IE) antigen 1 (IE1) activity. Recent evidence highlights the importance of the major immediate-early 2 (IE2) protein, which is expressed early after HCMV infection and reactivation, for triggering the lytic cycle of HCMV infection. In this study, we assessed IE2 HCMV T-cell responses in the peripheral blood of 15 HCMV-seropositive and 6 HCMV-seronegative healthy donors using IE2 synthetic long peptide (SLP) pools and cytokine flow cytometry. The response was dominated by $CD4^+$ T cells as IE2-specific $CD8^+$ T-cell reactivity was measured in only 3 donors. Surprisingly, an IE2 HCMV specific T-cell response was detected in 3 out of 6 donors who were screened negative for HCMV antibody. Most of the donors recognised chiefly the $IE2_{351-434}$ residues, revealing a remarkably immunogenic area of the protein. Numerous HLA class I- and II-restricted IE2 T-cell epitopes were identified, which significantly advances the existing evidence. Functional characterization of the IE2 T-cell responses uncovered 5 highly immunogenic IE2 SLPs, which induced polyfunctional Th1 cytokine (IFN-γ^+/ TNFα^+/ IL-2^+) responses. The testing of these 5 highly immunogenic IE2 SLPs as potent candidates for T-cell based vaccine platforms aiming to inhibit CMV infection by targeting the expression of IE genes is warranted.

INTRODUCTION

The β-herpesvirus Human Cytomegalovirus (HCMV) is a widespread pathogen that infects a large proportion of the population worldwide. After primary infection, the virus establishes lifelong latency within its host. HCMV infection is usually benign, with no clinical disease manifestations in immunocompetent individuals but it can lead to severe complications in susceptible individuals including newborns, allograft recipients, and HIV patients [1-4].

Despite extensive research, a licensed vaccine intervention to treat or prevent CMV infection remains elusive [5,6]. Studies in patients concur on the importance of T cells for establishment of immunity and long-lasting memory against HCMV [7-11] Recently, T-cell based vaccines designed to induce $CD4^+$ and/or $CD8^+$ T-cell responses have received significant research attention. Evidence from various models of mouse CMV infection (MCMV) suggests that vaccine-induced T-cell responses may successfully control CMV replication without the aid of antibodies [12-14].

Many of the HCMV proteins are recognized by $CD4^+$ and $CD8^+$ T cells in healthy HCMV-seropositive subjects [15]. While T cells most frequently target the antigens pp65 and immediate-early 1 (IE1), the dominance and magnitude of the T-cell response to IE1 is associated with protection from HCMV-induced disease [16,17]. Interestingly, IE1 but also the major immediate-early 2 (IE2) protein are abundantly expressed and play a key role in initiating lytic cycle virus gene expression and replication [18]. Deletion of the IE1 gene partially inhibits viral DNA replication [19], whereas IE2 gene is indispensable for expression of early lytic genes and virus replication [20,21]. This makes the IE antigens promising HCMV immune targets for vaccine approaches that aim to attack viral infection at an early phase and prevent wide dissemination [22]. While vaccines against IE1 are already tested [23-26], less is known about the immune response to IE2. Characterization of IE2-specific T-cell immunity in protected healthy individuals will reveal its immunogenicity and may foster the development of vaccines targeting indispensable proteins of CMV.

In this study, we aimed to identify novel HLA class I- and II-restricted IE2 T-cell epitopes. We mined the Immune Epitope Database (IEDB) to record previously identified IE2 epitopes and examined IE2-specific T-cell reactivity, directly *ex-vivo*, in the peripheral blood of 15 HCMV seropositive and 6 seronegative healthy donors, using overlapping synthetic long peptides (SLPs) covering the entire IE2 amino acid sequence. Interestingly, IE2-specific T-cell reactivity could be detected directly *ex-vivo* in more than half of all screened individuals. The response was dominated by $CD4^+$ T cells and IE2-specific $CD8^+$ T-cell reactivity was found in only 3 donors. A number of previously identified but also new T-cell epitopes were found. Among all immunogenic SLPs there were five, which were frequently recognized among healthy donors, containing epitopes recognized by $CD4^+$ T cells and/or $CD8^+$ T cells, and considered to be useful in vaccine development.

RESULTS

Ex vivo detection of CD4$^+$ and CD8$^+$ T-cell responses against various HCMV IE2 peptide pools

To identify new HCMV IE2 MHC class I and MHC class II restricted T-cell epitopes a traditional high-throughput and well established screening strategy using overlapping SLPs was performed [27]. Importantly, the use of overlapping SLPs accelerates prediction of the exact peptide sequence recognized when a positive response is monitored. Synoptic illustration of the approach is presented in Figure 1.

A cohort of 21 healthy human volunteers (15 HCMV-seropositive and 6 HCMV-seronegative), aged between 34-65 years, were recruited irrespective of gender, ethnic and educational background criteria (Table 1). To capture virus-primed T-cell reactivity, the directly *ex vivo* detectable IE2-specific cellular response against each of the peptide pools, was evaluated based on the percentage of antigen-specific IFN-γ and TNFα producing CD4$^+$ and CD8$^+$ T cells.

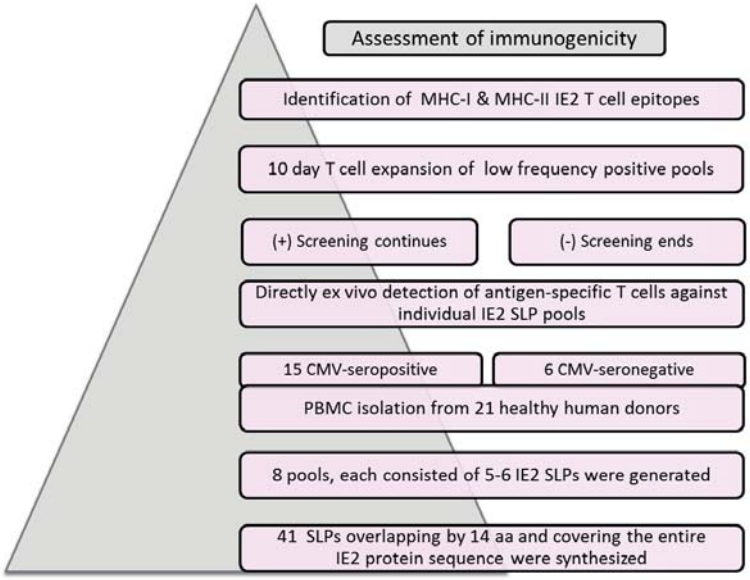


Figure. 1. Overview of the screening strategy to discover IE2-specific T cell epitopes. Forty-one '28-mer SLPs overlapping by 14 aa and covering the whole IE2 HCMV protein were synthesized. Eight pools each consisted of 5-6 SLPs. PBMCs from 21 healthy adult human individuals of which 15 were HCMV-seropositive and 6 HCMV-seronegative were screened by *ex vivo* ICS for direct recognition of IE2 SLP pools. Next, T cells that recognized peptide pools were cultured *in vitro* for 10 days and subsequently expanded T cells were analysed for IE2-specific CD4$^+$ and CD8$^+$ T cells using ICS and flow cytometric analysis. Positive responses against individual SLPs were confirmed and IE2-specific MHC class I and II epitopes were identified. A set of 5 IE2 peptides that were frequently recognized by CMV seropositive healthy donors was identified.

Table 1. Donor demographic characteristics and summary of the total pp65 and IE2 pool responses measured by *ex vivo* ICS. 21 healthy human volunteers were tested for $CD4^+$ and $CD8^+$ T-cell recognition of HCMV pp65 and IE2 SLP pools. Donor's demographic characteristics including the donor's number, age, gender (male = M/female = F) and HCMV status are depicted. HLA-A/B/C and HLA-DR/DQ/DP restrictions of each donor is indicated when available. The *ex vivo* positive (+) or negative (-) $CD4^+$ and/or $CD8^+$ T-cell responses against the IE2 and pp65 SLP pools are shown for each donor.

				Ex vivo T cell response		Class I			Class II	
Donor #	**Age**	**Gender**	**CMV status**	**pp65 CD4/CD8**	**IE2 CD4/CD8**	**HLA-A**	**HLA-B**	**HLA-C**	**HLA-DR**	**HLA-DQ**
1	65	M	+	+/+	+/+	2, 11	55, 35	3, 4	1, 15	5, 6
2	56	M	+	+/-	+/+	2, 3	44, 6	3, -		
3	62	M	+	-/+	+/-	2, 3	27, 35	2, 4	1, 4	
4	48	M	+	-/-	-/-	2, 3	7, 35	4, 7	1, 15	1
5	53	F	+	-/-	+/-	2, 3	7, 35	4, 7		
6	46	F	+	+/+	+/-					
7	64	F	+	+/+	+/-	1, 2	44, 56			
8	51	F	+	+/-	+/-					
9	56	F	+	-/-	-/-	2, 29	7, 44	7	4, 7	2, 8
10	51	M	+	+/+	+/+					
11	42	M	+	+/+	+/-					
12	44	F	+	+/+	+/-	2, 24	62, 38	3	4, 14	5, 3
13	45	M	+	-/-	-/-					
14	61	M	+	-/+	-/-					
15	55	F	+	-/-	+/-	2, 3	44, 62	3	4, 7	2, 3
16	54	M	-	-/-	+/-	31, 68	51, 39		11, 14	5, 7
17	34	M	-	-/-	+/-					
18	54	M	-	-/-	-/-	1, 3	39, 35	4		
19	53	F	-	-/-	-/-					
20	46	M	-	-/-	+/-	23, 68	51, 44	4		
21	58	M	-	-/-	-/-	2, 24	14, 14	6		

Interestingly, IE2-specific $CD4^+$ T-cell responses were detected in 11 of the 15 HCMV-seropositive donors and IE2-specific $CD8^+$ T cell responses were detected in 3 of these donors (Table 1, Figures 2A and B). Surprisingly, IE2-specific $CD4^+$ T-cell reactivity was also detected in 3 HCMV-seronegative donors (donor 16, 17 and 20). Peptide pool 6 ($IE2_{351-434}$) was the most frequently recognized immunogenic region (9/21 positive responders), followed by pool 1 ($IE2_{1-84}$) and pool 7 ($IE2_{421-504}$). Peptide pools 3, 4 and 5 ($IE2_{141-364}$) were barely recognized (Figures 2A and B). Notably, the magnitude of the *ex vivo* detected response was low and a phenotypic analysis was difficult.

Typical examples of HCMV-seropositive subjects (donor 1 and 6) and HCMV-seronegative subjects (donor 16) who displayed IFN-γ^+/TNFα^+ $CD4^+$ T-cell reactivity upon stimulation with IE2 peptide pools are provided in Figure 3.

In parallel, the response to the pp65 peptide pool was measured as this allowed us to compare and interpret the magnitude and impact of the IE2 T-cell immune response during HCMV infection. Although the level of the responses fluctuated among donors, most of the HCMV seropositive donors showed $CD4^+$ T-cell reactivity against both antigens (Table 1, Figure 2 and Figure 4A). The size of the pp65 $CD4^+$ T-cell responses, however, did not exhibit a direct correlation with the equivalent IE2 peptide pool responses (Figure 4B). Furthermore, while IE2-specific $CD8^+$ T-cell reactivity was infrequently detected, the pp65 SLP pool was recognized by $CD8^+$ T cells in 8 out of 15 seropositive donors and none of the seronegative donors (Figure 4C, Table 1 and Figure 2B).

Taken together, *ex-vivo* immune reactivity testing showed that the HCMV IE2 protein has a high propensity to activate $CD4^+$ T cells comparable to pp65. Especially, the amino acid sequence covered by the IE2 peptide pool 6 is recognized by the majority of the donors responding to IE2, revealing a remarkably immunogenic area of the protein. In contrast to pp65, the IE2 protein does not efficiently stimulate *ex vivo* detectable $CD8^+$ T-cell reactivity.

Identification of MHC class I- and II- restricted IE2 T-cell epitopes

Responding donor PBMCs were stimulated with each of the individual peptides present in the pool to confirm the *ex vivo* detected IE2-specific T-cell responses and to identify the peptides recognized in each of the recognized IE2 peptide pools. The responding T cells were first expanded for 10 days *in vitro*, then stimulated with each individual

Figure. 2. *Ex vivo* IFN-γ^+/TNFα^+ $CD4^+$ (A) and $CD8^+$ (B) T-cell responses measured in each donor following stimulation with different peptide pools. Donor's identification number (ID) and HCMV status are depicted. Responses to the pp65 and IE2 pools are also shown. Unstimulated samples (negative) response measured present the background response. Cellular cytokine responses >2 fold higher than the background responses were considered positive (light grey) and the peptide response was further evaluated. Positive $CD4^+$ T-cell responses were measured in 14 donors and positive $CD8^+$ T-cell responses in 3 donors.

A

Ex vivo IFN-γ⁺/TNFα⁺ cytokine producing CD4⁺ T cell responses

Donor #	pool 1	pool 2	pool 3	pool 4	pool 5	pool 6	pool 7	pool 8	negative (%)	pp65 pool	IE2 pool	CMV status
1									0.0034			+
2									0.0062			+
3									0.0053			+
4									0.0041			+
5									0.0008			+
6									0.0009			+
7									0.0019			+
8									0.0031			+
9									0.0008			+
10									0.0027			+
11									0.0013			+
12									0.0004			+
13									0.0041			+
14									0.0471			+
15									0.0009			+
16									0.0017			-
17									0.0007			-
18									0.0212			-
19									0.0046			-
20									0.0431			-
21									0.0562			-

<2 >2 >4 >10

B

Ex vivo IFN-γ⁺/TNFα⁺ cytokine producing CD8⁺ T cell responses

Donor #	pool 1	pool 2	pool 3	pool 4	pool 5	pool 6	pool 7	pool 8	negative (%)	pp65 pool	IE2 pool	CMV status
1									0.0096			+
2									0.0091			+
3									0.0009			+
4									0.0022			+
5									0.0025			+
6									0.0068			+
7									0.0022			+
8									0.0043			+
9									0			+
10									0.0086			+
11									0.0102			+
12									0.0201			+
13									0.0191			+
14									0.0101			+
15									0			+
16									0			-
17									0			-
18									0			-
19									0			-
20									0.0102			-
21									0.0051			-

<2 >2 >4 >10

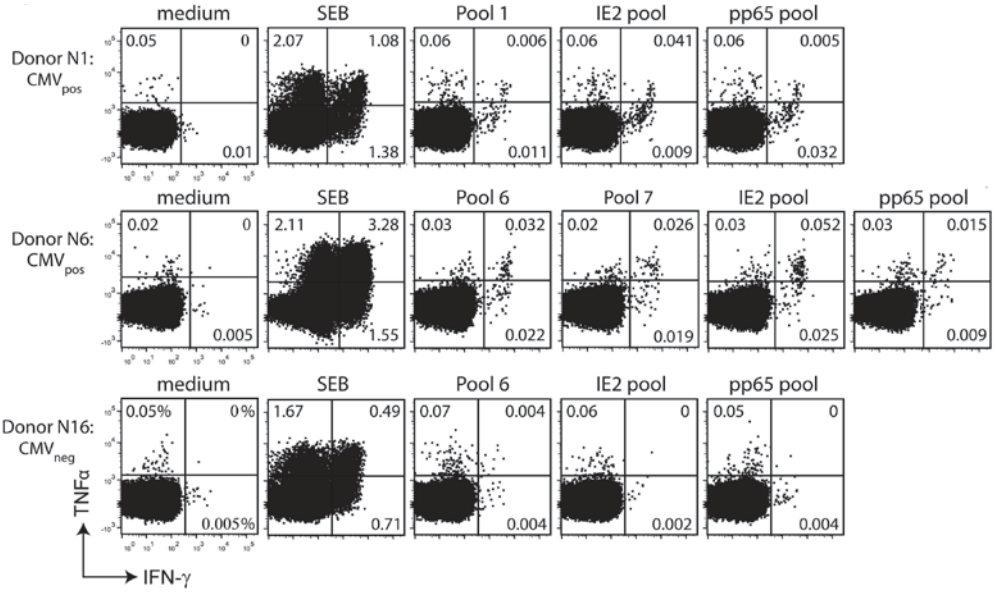


Figure. 3. CD4⁺ T-cell recognition of IE2 SLP pools in HCMV-seronegative and seropositive subjects. Donor's PBMCs were stimulated with 8 different IE2 peptide pools (each pool consists of 5-6 SLPs) and T-cell reactivity was measured by ICS assay and analysed by flow cytometry. Typical examples of *ex vivo* IFN-γ⁺/ TNFα⁺ CD4⁺ T-cell reactivity captured in HCMV-seropositive (donor 1 and 6) and HCMV-seronegative individuals (donor 16) are displayed. The background response was measured in unstimulated PBMCs (medium) and SEB served as positive control. The x-axis of the fluorescent intensity plots indicates percentages of IFN-γ and the y-axis TNFα cytokine production within CD4⁺ T cells.

SLP followed by polychromatic ICS. Peptide-specific CD4⁺ and CD8⁺ T-cell reactivity was detected in 11 and 3 donors, respectively, confirming the *ex vivo* measured responses. Representative examples of robust reactivity detected in the peptide-expanded PBMC of HCMV-seropositive and seronegative donor's T cells are depicted in Figure 5. Few donors displayed reactivity to one SLP but most of them reacted to 2 or more SLPs with donor 6 showing a CD4⁺ T-cell response to 13 different SLPs (Supplementary Table 2).

In the end, the *in vitro* culture revealed many more IE2-specific T-cell epitopes than appreciated from the *ex vivo* ICS where IE2 peptide pools were tested. A summary of all CD4⁺ and CD8⁺ T-cell responses measured against individual SLPs is provided in Tables 2 and 3, respectively.

The Immune Epitope Database (IEDB) site was used to search for all known MHC class I and MHC class II T-cells epitopes of the IE1 (UL123) and IE2 (UL122) HCMV viral proteins till June 2016. A chart depicting the exact position and HLA restriction of all the previously identified IE1 and IE2 T-cell epitopes (including the T-cell epitopes identified in this study) is given in Supplementary Fig. 2. Importantly, we were able to confirm most of the previously reported MHC class I- and class II-restricted IE2 specific

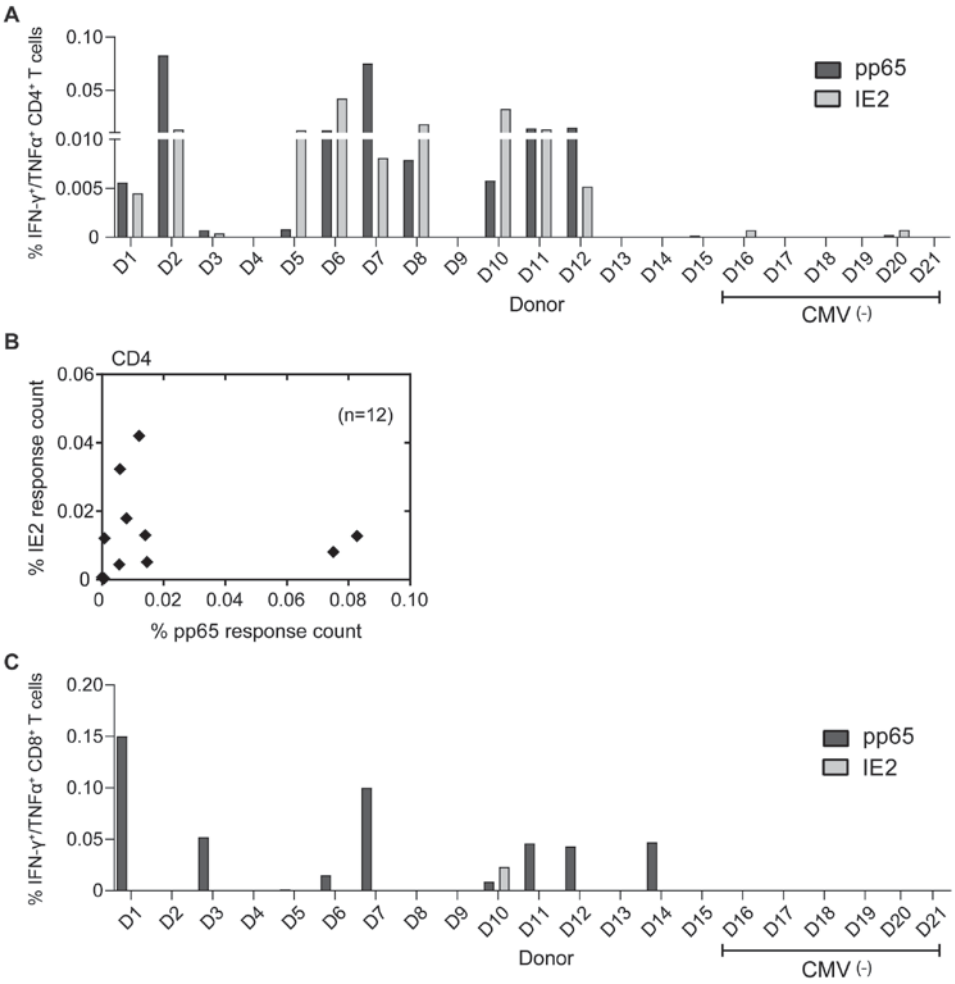


Figure. 4. Characterization of the total IE2 and pp65 HCMV-specific CD4⁺ and CD8⁺ T-cell responses measured in HCMV-seropositive and seronegative subjects. Donor PBMCs were screened by *ex vivo* ICS for direct detection of IE2 and pp65 pool specific CD4⁺ and CD8⁺ T-cell reactivity. In total 21 donors were tested, of whom 15 were HCMV-seropositive and 6 HCMV-seronegative **(A)** Percentages of the total pp65 and IE2 pool IFN-γ⁺/ TNFα⁺ CD4⁺ T-cell responses detected across the whole population sample are shown. **(B)** No correlation between the magnitude of the pp65 and IE2 pool CD4⁺ T-cell responses was observed in responder donors (n=12 and $p > 0.055$). **(C)** Frequency of IFN-γ⁺/ TNFα⁺ cytokine producing CD8⁺ T-cell responses measured *ex vivo* against the pp65 and IE2 peptide pools I (n=21). CD8⁺ T-cell reactivity against the IE2 peptide pool was detected only in donor 10, whereas pp65 specific CD8⁺ T-cell reactivity was measured in 8 out of 15 HCMV-seropositive donors.

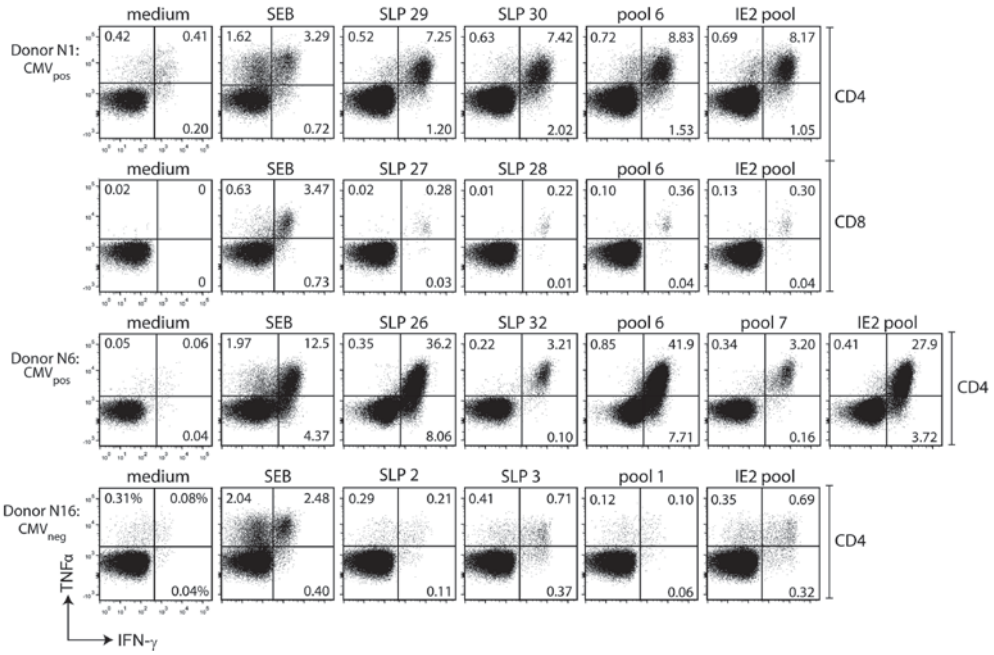


Figure. 5. CD4⁺ and CD8⁺ T-cell epitope identification in HCMV-seropositive and seronegative subjects. *Ex vivo* positive peptide pools for CD4⁺ and CD8⁺ T-cell reactivity PBMCs were expanded following 10 days *in vitro* culture and subsequently stimulated with each SLP contained in the relevant pools. Characteristic plots of T-cell epitope validation ICS results are shown for 2 HCMV-seropositive individuals (donor 1 and 6) and a HCMV-seronegative donor (donor 16). For donor 1, CD4⁺ and CD8⁺ T-cell reactivity to SLPs 29 and SLP 30 was measured indicating the presence of MHC class I and II epitopes. Responses to individual SLPs, peptide pools and the IE2 pool are depicted. As a negative control, unstimulated PBMCs (medium) were used and as a positive control SEB. The x-axis of the fluorescent intensity plots indicates percentages of IFN-γ and the y-axis TNFα cytokine production within CD4⁺ or CD8⁺ T-cell populations.

T-cell epitopes [28-37], but we also identified a total of 6 new CD4⁺ T-cell epitopes and 1 new CD8⁺ T-cell epitope.

Polyfunctional cytokine profiling of T-cell responses against the most immunogenic IE2 epitopes

The number of individuals responding to a peptide is a reflection of its immunogenicity. From the 41 individual IE2 SLPs there were 5 of which a unique sequence is present that was recognized by the CD4⁺ T cells (SLPs #3, 26, 28, 30 and 32) or CD8⁺ T cells (SLP #27/28) of the majority of tested donors (peptide *sequences marked in bold in* Table 2 and 3). As the functional traits of the T-cell response responding to such a peptide is an important determinant for its potential protective efficacy against HCMV infection, the cytokine profile of all the IE2-specific T-cell epitopes was examined. All selected peptide epitopes elicited a polyfunctional cytokine response as indicated by the percentage of T cells

Table 2. Summary of all identified IE2-specific CD4⁺ T-cell epitopes. CD4⁺ T-cell responses of individual IE2 pools (i.e. pool 1= p1) and the corresponding numbers of SLPs in each pool are depicted. SLP amino acid residues and sequence are also presented. The number of the responder donors and the frequency of the IFN-γ-producing CD4⁺ T cells measured in individual donors by ICS assay are displayed. Epitopes which have been described for the first time in this study are shown in red and all previously reported epitopes are shown in black.

SLP #		Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
p1	1	$IE2_{1-14}$	MESSAKRKMDPDNPDEGPSSKVPRPETP	1/5	16	0.083			This paper
2		$IE2_{15-42}$	DEGPSSKVPRPETPVTKATTFLQTMLRK	1/5	16	0.13			This paper
3		$IE2_{29-56}$	**VTKATTFLQTMLRKEVNSQLSLGDPLFP**	4/5	2	0.57			This paper
					11	0.13			
					12	0.091			
					16	0.81			
4		$IE2_{43-70}$	EVNSQLSLGDPLFPELAEESLKTFEQVT	1/5	16	0.12			This paper
5		$IE2_{57-84}$	ELAEESLKTFEQVTEDCNENPEKDVLAE	1/5	16	0.06			This paper
p2	6	$IE2_{71-98}$	EDCNENPEKDVLAELGDILAQAVNHAGI	1/3	10	0.48	$IE2_{86-100}$	GDILAQAVNHAGIDS	(30), this paper
7		$IE2_{85-112}$	LGDILAQAVNHAGIDSSSTGPTLTTHSC	1/3	10	0.56	$IE_{286-100}$	GDILAQAVNHAGIDS	(30), this paper
10		$IE2_{127-154}$	VAVTNTPLPGASATPELSPRKKPRKTTR	1/3	12	0.15			This paper
p3	11	$IE2_{141-168}$	PELSPRKKPRKTTRPFKVIIKPPVPPAP	1/2	11	0.18			(30), this paper
12		$IE2_{154-182}$	PFKVIIKPPVPPAPIMLPLIKQEDIKPE	1/2	11	0.13			(30), this paper
p6	26	$IE2_{351-378}$	**TPNVQTRRGRVKIDEVSRMFRNTNRSLE**	4/8	1	0.50	$IE2_{356-370}$	TRRGRVKIDEVSRMF	(30), this paper
					5	0.16			
					6	42.82			
					20	0.12			
27		$IE2_{365-392}$	EVSRMFRNTNRSLEYKNLPFTIPSMHQV	4/8	1	0.54	$IE2_{383-397}$	PFTIPSMHQVLDEAI	(30), this paper
					5	0.10			
					6	16.4			
					10	0.32			
28		$IE2_{379-406}$	**YKNLPFTIPSMHQVLDEAIKACKTMQVN**	8/8	1	0.91	$IE2_{383-397}$	PFTIPSMHQVLDEAI	(30), this paper

Table 2. (continued)

SLP #	Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
				5	1.01			
				6	13.9			
				7	0.31			
				10	1.12			
				15	1.62			
				17	0.78			
				20	0.10			
29	$IE2_{393-420}$	LDEAIKACKTMQVNNKGIQIIYTRNHEV	6/8	1	7.54	$IE2_{408-422}$	KGIQIIYTRNHEVKS	(30), this paper
				5	6.69	$IE2_{412-420}$	IIYTRNHEV	
				6	6.18			
				10	10.62			
				16	0.015			
				20	0.10			
30	$IE2_{407-434}$	**NKGIQIIYTRNHEVKSEVDAVRCRLGTM**	6/8	1	8.17	$IE2_{408-422}$	KGIQIIYTRNHEVKS	(30), this paper
				5	5.13	$IE2_{412-420}$	IIYTRNHEV	
				6	3.13			
				10	14.2			
				17	0.74			
				20	0.12			
p7 31	$IE2_{421-448}$	KSEVDAVRCRLGTMCNLALSTPFLMEHT	2/5	6	1.95	$IE_{2438-452}$	ALSTPFLMEHTMPVT	(30), this paper
				10	0.17			
32	$IE2_{435-462}$	**CNLALSTPFLMEHTMPVTHPPEVAQRTA**	4/5	3	0.23	$IE2_{438-452}$	ALSTPFLMEHTMPVT	(30), this paper
				6	3.19	$IE2_{443-457}$	FLMEHTMPVTHPPEV	
				7	0.27			
				10	6.98			

Table 2. (continued)

SLP #	Peptide	Sequence	Responders/ donors tested	Responding donor	ICS (%)	Identified epitope	Identified epitope sequence	Reference
33	$IE2_{449-476}$	MPVTHPPEVAQRTADACNEGVKAAWSLK	1/5	10	0.07			This paper
34	$IE2_{463-480}$	DACNEGVKAAWSLKELHTHQLCPRSSDY	1/5	10	0.59			This paper
35	$IE2_{477-504}$	ELHTHQLCPRSSDYRNMIIHAATPVDLL	2/5	2	0.3	$IE2_{493-507}$	MIIHAATPVDLLGAL	(33), this paper
				3	0.26			
p8 36	$IE2_{481-518}$	RNMIIHAATPVDLLGALNLCLPLMQKFP	1/3	6	0.28	$IE2_{493-507}$	MIIHAATPVDLLGAL	(34), this paper
37	$IE2_{505-532}$	GALNLCLPLMQKFPKQVMVRIFSTNQGG	2/3	6	1.53			This paper
				8	3.23			
38	$IE2_{519-546}$	KQVMVRIFSTNQGGFMLPIYETAAKAYA	2/3	6	0.11	$IE2_{523-537}$	VRIFSTNQGGFMLPI	(30), this paper
				8	3.17			
39	$IE2_{533-560}$	FMLPIYETAAKAYAVGQFEQPTETPPED	2/3	6	0.15	$IE2_{550-558}$	FEQPTETPP	(31-33, 35), this paper
				8	1			
40	$IE2_{547-574}$	VGQFEQPTETPPEDLDTLSLAIEAAIQD	2/3	6	0.059	$IE2_{550-558}$	FEQPTETPP	(31-33, 35), this paper
				8	1.27	$IE2_{558-572}$	PEDLDTLSLAIEAAI	
41	$IE2_{561-580}$	LDTLSLAIEAAIQDLRNKSQ	2/3	6	0.12	$IE2_{558-572}$	PEDLDTLSLAIEAAI	(30), this paper
				8	1.63	$IE2_{563-577}$	TLSLAIEAAIQDLRN	

Table 3. Summary of all identified IE2-specific CD8$^+$ T-cell epitopes. Characteristics of the detected CD8$^+$ T-cell responses are shown. For all the immunogenic MHC class I epitope containing SLPs, submer peptides were synthesized and their binding status was validated in an ICS assay. In total 2 IE2 MHC class I T-cell epitopes were identified.

SLP #	Peptide	Sequence	Responder/ donors tested	Responding donors	ICS (%)	Identified epitope	Identified epitope sequence	Reference
p1 3	$IE2_{29-56}$	VTKATTFLQTMLRKEVNSQLSLGDPLFP	1/5	2	0.38	$IE2_{42-50}$	KEVNSQLSL	(30, 36-39), this paper
p6 27	$IE2_{365-392}$	**EVSRMFRNTNRSLEYKNLPFTIPSMHQV**	2/7	1	0.29	$IE2_{382-390}$	LPFTIPSMH	This paper
				10	0.17			
28	$IE2_{379-406}$	YKNLPFTIPSMHQVLDEAIKACKTMQVN	2/7	1	0.23			
				10	0.13			

co-secreting all three, IFN-γ^+/ TNFα^+/ IL-2$^+$, antiviral cytokines. In conclusion, 5 highly immunogenic IE2 SLPs, capable of inducing polyfunctional type 1 cytokine T-cell responses (Figures 6A-F) were identified.

DISCUSSION

Controlling viral infection at an early phase may prevent wide dissemination and full blown infection. Reinforcement of the T-cell response to highly immunogenic antigens expressed immediately after infection could be key in the development of protective vaccines. The IE2 protein, which is expressed early after HCMV infection and reactivation, is likely to be a particularly valuable target for vaccine strategies against HCMV. In this study, we assessed the immunogenicity of the HCMV IE2 protein to identify amino acid stretches that were frequently targeted by T cells of healthy HCMV-protected donors. This led to the identification of numerous new and previously found [28-37] MHC class I- and II-restricted epitopes, to which T-cell reactivity could be detected directly *ex vivo*. The majority of the identified epitopes are located across the $IE2_{351-434}$ residues, stressing that this is a very immunogenic area. Furthermore, we could single out five particularly interesting SLPs that were frequently recognized by donors with different HLA restriction elements. These SLPs contained epitopes recognized by CD4$^+$ T cells and/or CD8$^+$ T cells [28,34-37] and could form the core of a IE2-targeting T-cell based vaccine.

Unexpectedly, IE2 specific CD4$^+$ T cell responses were detected *ex vivo* and in the 10 day cultures from 3 out of 6 HCMV seronegative donors. This is not uncommon among individuals who are immune against viral infections. For instance, HIV-specific memory T cells accompanied with a complete lack of humoral immunity has also been observed in seronegative highly HIV-1-exposed individuals [38,39]. This finding supports the idea that CMV exposure does not inevitably lead to persistent infection and that heterogeneity in infection susceptibility could be due to natural protective immunity to CMV. One potential contributor to this difference may be the size of the initial infectious inoculum as it dramatically influences the antibody and T-cell response to the virus [40,41]. Phenotypic and functional characterization of cellular responses in HCMV resistant individuals or donors, who elicit merely virus-specific T-cell reactivity but lack humoral immune responses, might reveal distinct T-cell features and biomarkers of protection. However, the very low response rate in this study did not allow us to determine the T-cell activation status and to distinguish between effector or memory T-cell populations.

Analysis of the T-cell response against IE2 revealed a predominance of *ex vivo* detectable CD4$^+$ T-cell mediated reactivity (11 out of 15 seropositive donors) whereas CD8$^+$ T-cell reactivity was seen in 3 donors only. This is consistent with a recent study [28] and not a technical problem since pp65-specific reactivity was readily detected *ex vivo* in the CD8$^+$ T-cell population in parallel. A concomitant induction of CD4$^+$

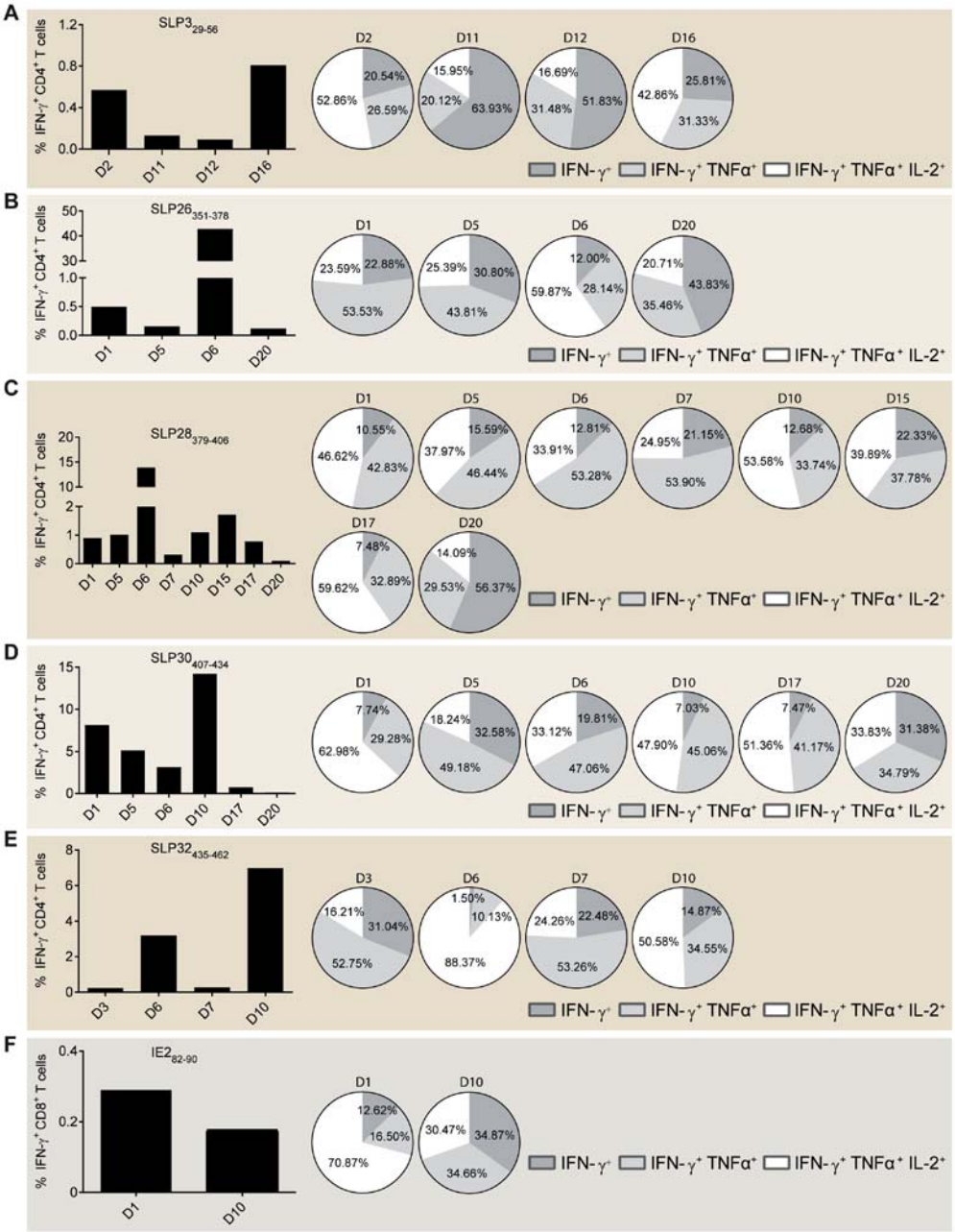


Figure. 6. Functional profiling of the most immunogenic MHC class I and II IE2 T-cell epitopes identified. (A-E) Percentages of IFN-γ$^+$ producing CD4$^+$ and **(F)** CD8$^+$ T cells elicited by responder donors following re-stimulation with relevant peptide. Cytokine production was measured in (*in vitro)* expanded donor T cells by polychromatic ICS assay. Pie charts show the percentages of the single (IFN-γ$^+$), double (IFN-γ$^+$/ TNFα$^+$) and triple (IFN-γ$^+$/ TNFα$^+$/ IL-2$^+$) cytokine producers within each antigen-specific CD4$^+$ or CD8$^+$ T-cell population and across all the responder donors.

and $CD8^+$ T-cells responses of a good magnitude, breadth and functional profile are crucial preconditions of vaccine efficacy [42-45]. Indeed, in the mouse model for CMV prophylactic vaccination with a mixture of SLPs comprising MHC class I- and II-restricted T-cell epitopes derived from different immunogenic proteins resulted in the best containment of virus dissemination after a challenge with lytic MCMV [12,14]. The detection of several immunodominant T-cell epitopes in the most immunogenic HCMV open reading frames (ORFs proteins) makes the translation of these findings in human clinical vaccine applications a valid and promising research target. Interestingly, while IE2 predominantly activates a strong *ex vivo* detectable $CD4^+$ T-cell response, the IE1 protein largely triggers an *ex vivo* measurable response to $CD8^+$ T cells [28]. These data would argue that an optimal SLP vaccine targeting the early phase of HCMV infection might require a mixture of IE1 and IE2 SLPs to achieve induction of a balanced $CD4^+$ and $CD8^+$ T-cell response. Of note, the SLP3 is shared between IE1 and IE2. Potentially the processing and presentation of this epitope is stronger in infected cells, however, whether such an epitope is more protective remains to be elucidated.

The development of efficient immunotherapeutic approaches against HCMV disease is an important research priority. The IE2 protein has never been explored in antibody or T-cell based vaccine platforms. We here showed that the spontaneous immune response to IE2 is characterized by highly polyfunctional $CD4^+$ T-cell reactivity that can be measured directly *ex vivo*. Moreover, the similar detection frequency of IE2- and pp65-specific T-cell reactivity validates IE2 as a strongly immunogenic HCMV target and identifies IE2 as a potential vaccine candidate. With that in mind, our study identified 5 highly immunogenic regions containing clusters of IE2 T-cell epitopes which could be exploited in vaccine development strategies but also in adoptive transfer therapies.

MATERIALS AND METHODS

Donors and sample

PBMCs from 15 HCMV-seropositive and 6 HCMV-seronegative subjects were isolated from buffy coats obtained after informed-consent (Sanquin, The Netherlands) by Ficoll (Ficoll-Amidotrizoaat, pharmacy LUMC) density gradient centrifugation. PBMC were cryopreserved in 80% Fetal Calf Serum (FCS; PAA laboratories) and 20% DMSO (Sigma) and stored in the vapour phase of the liquid nitrogen until further use. Handling and storage of donor PBMCs were performed according to the standard operating procedure (SOP) of the department of Medical Oncology at the Leiden University Medical Center [46].

Peptides and preparation of peptide pools

The IE2 amino acid sequence of the laboratory strain AD169 was used to synthesize 41 peptides of 28 amino acids in length and overlapping by 15 amino acids, together

covering the entire IE2 sequence, at the GMP-peptide facility of the LUMC. The purity (75-90%) of the synthesized peptides was determined by HPLC and the molecular weight by mass spectrometry. Additionally, a pool of peptides spanning the whole pp65 protein and a HLA A*0201-restricted $pp65_{495-503}$ (NLVPMVATV) peptide were included in the T-cell detection assays. The peptides were dissolved in DMSO at a stock concentration of 50 mg/ml and then further diluted to a concentration of 2 mg/ml and stored in -20°C. Subsequently, 8 peptide pools, each comprising either 5 or 6 SLPs, were made and stored under the same conditions as the individual peptides. All individual IE2 peptides, and IE2 peptide pools used in this study are listed in Supplementary Table 1.

Ex vivo detection of antigen-specific T cells

Direct *ex vivo* detection of HCMV-specific T cells was performed as described before [27]. Briefly, autologous plastic adherent monocytes were cultured in X-vivo 15 medium (Lonza) supplemented with granulocyte-macrophage colony-stimulating factor (800 IU/ml GM-CSF; Invitrogen) for 2-4 days at 37°C, 5% CO_2 in a humidified incubator. Then, cells were incubated for 5 h with each of the IE2 peptide pools, a pool of all IE2 peptides, a pool of all pp65 peptides or the pp65 short peptide (SP) in a final concentration of 50 µg/ml and for 24 h in the presence of poly (I:C) (25 µg/ml; Invivogen). A medium control sample served as negative control and *Staphylococcal* enterotoxin B (SEB; final concentration of 2 µg/ml; Sigma) was used as a positive control. Autologous donor PBMCs at a final concentration of 2-4 $\times$ 10^6 cells/well and Roferon (interferon-alpha 2a, 3 $\times$ 10^6 U/0.5 ml; Roche) were added to the cultured monocytes. An hour later, Brefeldin A (Sigma) was added and cells were incubated for 16-20 h (37°C, 5% CO_2) to allow the accumulation of intracellular cytokines. Subsequently, the cells were stained for live/dead marker (Yellow ARD, Life Technologies), CD3 (clone UCHT1), CD4 (clone SK3), CD8 (clone SK1), CD14 (M5E2), IFN-γ (B27), IL-2 (clone 5344.111), CD137 (clone 4B4-1), CD154 (TRAP1) all from BD and TNFα (clone MAb11), CD45RA (clone HI100) both from Biolegend according to SOP of the department of Medical Oncology. Samples were acquired at a LSRFortessa cytometer (BD Biosciences) and analysed using FlowJo-V10 software (Tree star). Flow cytometry gating strategies are shown in Supplementary Fig. 1. An immune response was considered positive when donor T cells produced both IFN-γ and TNFα and the response was ≥ 2 than the background (medium control).

In vitro expansion of low frequency antigen-specific T cells

To confirm the *ex vivo* detected T-cell responses against the IE2 SLP pools and to determine the reactivity to the individual SLPs within each pool, PBMC were subjected to a 10 day antigen-specific T-cell stimulation culture [47]. Briefly, donor PBMCs were thawed, resuspended in IMDM + 10% human AB serum (HAB, Life Technologies) and seeded in triplicate wells (3 ml/well) in a 6-wells plate (Costar). Tested peptide pools

were added to the PBMCs in a final concentration of 2.5 µg/ml and cells were cultured overnight at 37°C, 5% CO_2. The following day, 5 ng/ml IL-15 (Peprotech) and T cell growth factor (TCGF, Zepto Metrix) to a final concentration of 10% were added to the cultured T cells (referred to as bulk cultures) and cells were maintained for 10 days. In parallel on day 7, autologous donor PBMCs were thawed, seeded in 24-wells plates and monocytes were cultured for 2 days and loaded with indicated peptide pools and all individual SLPs that the pool contained in a concentration of 5 µg/ml. On day 10, bulk cultured T cells were harvested and $7 \times 10^5 - 1 \times 10^6$ cells/ml were added to the loaded monocytes. An hour later, Brefeldin A was added and cells were incubated for 16-20 h (37°C, 5% CO_2). The same positive and negative controls as in the *ex vivo* detection assay were also included. Subsequently, an intracellular cytokine staining (ICS) was performed and samples were measured and analysed as described above. An immune response was considered positive when donor T cells produced IFN-γ and the response was ≥ 2 than the background (medium control).

Statistical analysis

Statistical significance was calculated using the unpaired Student's t-test or ANOVA in GraphPad Prism software version 6 (GraphPad Software Inc., USA). Statistical significance levels were *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

REFERENCES

1. Ross SA, Arora N, Novak Z, Fowler KB, Britt WJ, et al. (2010) Cytomegalovirus reinfections in healthy seroimmune women. J Infect Dis 201: 386-389.
2. Gandhi MK, Khanna R (2004) Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. Lancet Infect Dis 4: 725-738.
3. Pass RF (2005) Congenital cytomegalovirus infection and hearing loss. Herpes 12: 50-55.
4. Mussi-Pinhata MM, Yamamoto AY, Moura Brito RM, de Lima Isaac M, de Carvalho e Oliveira PF, et al. (2009) Birth prevalence and natural history of congenital cytomegalovirus infection in a highly seroimmune population. Clin Infect Dis 49: 522-528.
5. Schleiss MR, Heineman TC (2005) Progress toward an elusive goal: current status of cytomegalovirus vaccines. Expert Rev Vaccines 4: 381-406.
6. Krause PR, Bialek SR, Boppana SB, Griffiths PD, Laughlin CA, et al. (2013) Priorities for CMV vaccine development. Vaccine 32: 4-10.
7. Bronke C, Jansen CA, Westerlaken GH, De Cuyper IM, Miedema F, et al. (2007) Shift of CMV-specific CD4+ T-cells to the highly differentiated CD45RO-CD27- phenotype parallels loss of proliferative capacity and precedes progression to HIV-related CMV end-organ disease. Clin Immunol 124: 190-199.
8. Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, et al. (2002) Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood 99: 3916-3922.
9. Cwynarski K, Ainsworth J, Cobbold M, Wagner S, Mahendra P, et al. (2001) Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood 97: 1232-1240.
10. Gamadia LE, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, et al. (2003) Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. Blood 101: 2686-2692.
11. Peggs KS, Verfuerth S, Pizzey A, Khan N, Guiver M, et al. (2003) Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. Lancet 362: 1375-1377.
12. Panagioti E, Redeker A, van Duikeren S, Franken KL, Drijfhout JW, et al. (2016) The Breadth of Synthetic Long Peptide Vaccine-Induced CD8+ T Cell Responses Determines the Efficacy against Mouse Cytomegalovirus Infection. PLoS Pathog 12: e1005895.
13. Verma S, Weiskopf D, Gupta A, McDonald B, Peters B, et al. (2015) Cytomegalovirus-Specific CD4 T Cells Are Cytolytic and Mediate Vaccine Protection. J Virol 90: 650-658.
14. Panagioti E, Boon L, Arens R, van der Burg SH (2017) Enforced OX40 Stimulation Empowers Booster Vaccines to Induce Effective CD4+ and CD8+ T Cell Responses against Mouse Cytomegalovirus Infection. Front Immunol 8: 144.
15. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, et al. (2005) Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 202: 673-685.
16. Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, et al. (2005) Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. J Exp Med 201: 1031-1036.

17. Tormo N, Solano C, Benet I, Nieto J, de la Camara R, et al. (2011) Reconstitution of CMV pp65 and IE-1-specific IFN-gamma CD8(+) and CD4(+) T-cell responses affording protection from CMV DNAemia following allogeneic hematopoietic SCT. Bone Marrow Transplant 46: 1437-1443.
18. Paulus C, Nevels M (2009) The human cytomegalovirus major immediate-early proteins as antagonists of intrinsic and innate antiviral host responses. Viruses 1: 760-779.
19. Greaves RF, Mocarski ES (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. J Virol 72: 366-379.
20. Marchini A, Liu H, Zhu H (2001) Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J Virol 75: 1870-1878.
21. Heider JA, Bresnahan WA, Shenk TE (2002) Construction of a rationally designed human cytomegalovirus variant encoding a temperature-sensitive immediate-early 2 protein. Proc Natl Acad Sci U S A 99: 3141-3146.
22. van der Burg SH, Arens R, Melief CJ (2011) Immunotherapy for persistent viral infections and associated disease. Trends Immunol 32: 97-103.
23. Wang Z, La Rosa C, Li Z, Ly H, Krishnan A, et al. (2007) Vaccine properties of a novel marker gene-free recombinant modified vaccinia Ankara expressing immunodominant CMV antigens pp65 and IE1. Vaccine 25: 1132-1141.
24. Gallez-Hawkins G, Li X, Franck AE, Thao L, Lacey SF, et al. (2004) DNA and low titer, helper-free, recombinant AAV prime-boost vaccination for cytomegalovirus induces an immune response to CMV-pp65 and CMV-IE1 in transgenic HLA A*0201 mice. Vaccine 23: 819-826.
25. Wang Z, La Rosa C, Mekhoubad S, Lacey SF, Villacres MC, et al. (2004) Attenuated poxviruses generate clinically relevant frequencies of CMV-specific T cells. Blood 104: 847-856.
26. Kharfan-Dabaja MA, Boeckh M, Wilck MB, Langston AA, Chu AH, et al. (2012) A novel therapeutic cytomegalovirus DNA vaccine in allogeneic haemopoietic stem-cell transplantation: a randomised, double-blind, placebo-controlled, phase 2 trial. Lancet Infect Dis 12: 290-299.
27. Singh SK, Meyering M, Ramwadhdoebe TH, Stynenbosch LF, Redeker A, et al. (2012) The simultaneous ex vivo detection of low-frequency antigen-specific CD4+ and CD8+ T-cell responses using overlapping peptide pools. Cancer Immunol Immunother 61: 1953-1963.
28. Braendstrup P, Mortensen BK, Justesen S, Osterby T, Rasmussen M, et al. (2014) Identification and HLA-tetramer-validation of human CD4+ and CD8+ T cell responses against HCMV proteins IE1 and IE2. PLoS One 9: e94892.
29. Zhong J, Rist M, Cooper L, Smith C, Khanna R (2008) Induction of pluripotent protective immunity following immunisation with a chimeric vaccine against human cytomegalovirus. PLoS One 3: e3256.
30. Rist M, Cooper L, Elkington R, Walker S, Fazou C, et al. (2005) Ex vivo expansion of human cytomegalovirus-specific cytotoxic T cells by recombinant polyepitope: implications for HCMV immunotherapy. Eur J Immunol 35: 996-1007.
31. Walker S, Fazou C, Crough T, Holdsworth R, Kiely P, et al. (2007) Ex vivo monitoring of human cytomegalovirus-specific CD8+ T-cell responses using QuantiFERON-CMV. Transpl Infect Dis 9: 165-170.
32. Krishnan A, Wang Z, Srivastava T, Rawal R, Manchanda P, et al. (2008) A novel approach to evaluate the immunogenicity of viral antigens of clinical importance in HLA transgenic murine models. Immunol Lett 120: 108-116.

33. Crough T, Burrows JM, Fazou C, Walker S, Davenport MP, et al. (2005) Contemporaneous fluctuations in T cell responses to persistent herpes virus infections. Eur J Immunol 35: 139-149.
34. Nastke MD, Herrgen L, Walter S, Wernet D, Rammensee HG, et al. (2005) Major contribution of codominant CD8 and CD4 T cell epitopes to the human cytomegalovirus-specific T cell repertoire. Cell Mol Life Sci 62: 77-86.
35. Khan N, Best D, Bruton R, Nayak L, Rickinson AB, et al. (2007) T cell recognition patterns of immunodominant cytomegalovirus antigens in primary and persistent infection. J Immunol 178: 4455-4465.
36. Ameres S, Mautner J, Schlott F, Neuenhahn M, Busch DH, et al. (2013) Presentation of an immunodominant immediate-early CD8+ T cell epitope resists human cytomegalovirus immunoevasion. PLoS Pathog 9: e1003383.
37. Chang CX, Tan AT, Or MY, Toh KY, Lim PY, et al. (2013) Conditional ligands for Asian HLA variants facilitate the definition of CD8+ T-cell responses in acute and chronic viral diseases. Eur J Immunol 43: 1109-1120.
38. Shearer G, Clerici M (2010) Historical perspective on HIV-exposed seronegative individuals: has nature done the experiment for us? J Infect Dis 202 Suppl 3: S329-332.
39. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, et al. (1996) Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet 348: 1347-1351.
40. Asabe S, Wieland SF, Chattopadhyay PK, Roederer M, Engle RE, et al. (2009) The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. J Virol 83: 9652-9662.
41. Redeker A, Welten SP, Arens R (2014) Viral inoculum dose impacts memory T-cell inflation. Eur J Immunol 44: 1046-1057.
42. Seder RA, Darrah PA, Roederer M (2008) T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 8: 247-258.
43. Schluns KS, Lefrancois L (2003) Cytokine control of memory T-cell development and survival. Nat Rev Immunol 3: 269-279.
44. Gilbert SC (2012) T-cell-inducing vaccines - what's the future. Immunology 135: 19-26.
45. Lauvau G, Boutet M, Williams TM, Chin SS, Chorro L (2016) Memory CD8(+) T Cells: Innate-Like Sensors and Orchestrators of Protection. Trends Immunol 37: 375-385.
46. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, Lowik MJ, Berends-van der Meer DM, et al. (2010) Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc Natl Acad Sci U S A 107: 11895-11899.
47. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, et al. (2008) Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 14: 178-187.

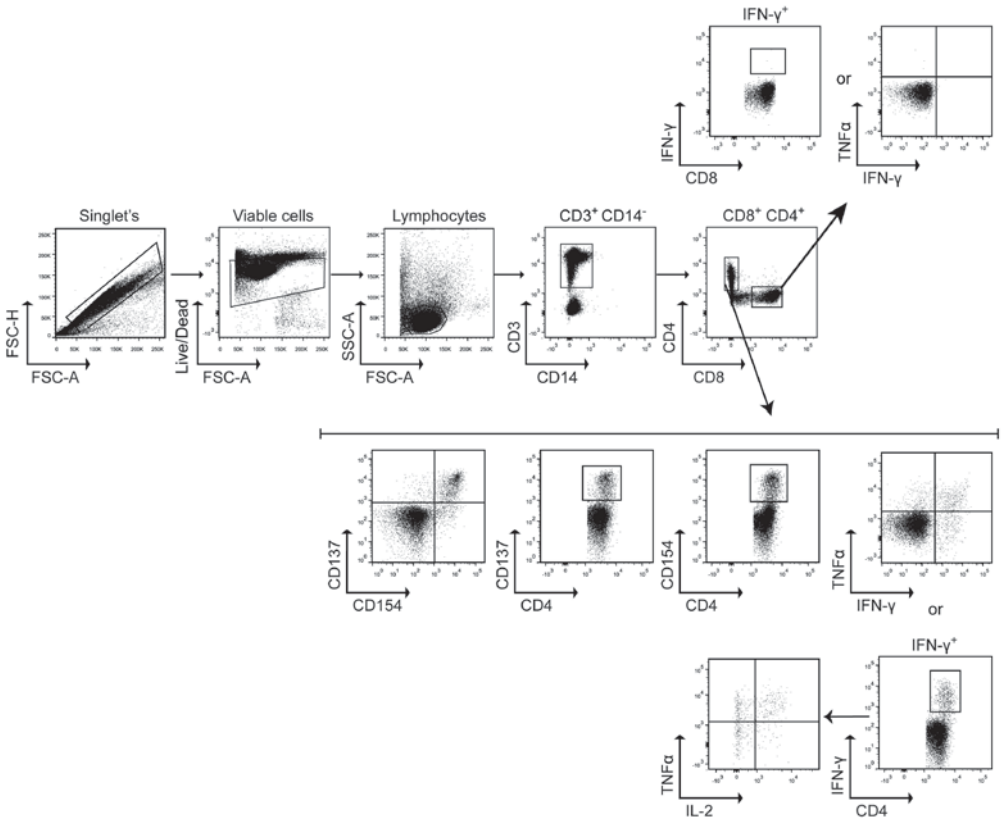
SUPPORTING INFORMATION

Supplementary Table 1. List of the IE2 SLP pools and individual SLPs used in this study.

	SLP #	Peptide residues (aa)	SLP Sequence
pool 1	1	$IE2_{1-14}$	MESSAKRKMDPDNPDEGPSSKVPRPETP
	2	$IE2_{15-42}$	DEGPSSKVPRPETPVTKATTFLQTMLRK
	3	$IE2_{29-56}$	VTKATTFLQTMLRKEVNSQLSLGDPLFP
	4	$IE2_{43-70}$	EVNSQLSLGDPLFPELAEESLKTFEQVT
	5	$IE2_{57-84}$	ELAEESLKTFEQVTEDCNENPEKDVLAE
pool 2	6	$IE2_{71-98}$	EDCNENPEKDVLAELGDILAQAVNHAGI
	7	$IE2_{85-112}$	LGDILAQAVNHAGIDSSSTGPTLTTHSC
	8	$IE2_{99-126}$	DSSSTGPTLTTHSCSVSSAPLNKPTPTS
	9	$IE2_{113-140}$	SVSSAPLNKPTPTSVAVTNTPLPGASAT
	10	$IE2_{127-154}$	VAVTNTPLPGASATPELSPRKKPRKTTR
pool 3	11	$IE2_{141-168}$	PELSPRKKPRKTTRPFKVIIKPPVPPAP
	12	$IE2_{154-182}$	PFKVIIKPPVPPAPIMLPLIKQEDIKPE
	13	$IE2_{169-196}$	IMLPLIKQEDIKPEPDFTIQYRNKIIDT
	14	$IE2_{183-210}$	PDFTIQYRNKIIDTAGCIVISDSEEEQG
	15	$IE2_{197-224}$	AGCIVISDSEEEQGEEVETRGATASSPS
pool 4	16	$IE2_{211-238}$	EEVETRGATASSPSTGSGTPRVTSPTHP
	17	$IE2_{225-252}$	TGSGTPRVTSPTHPLSQMNHPPLPDPLG
	18	$IE2_{239-266}$	LSQMNHPPLPDPLGRPDEDSSSSSSSSC
	19	$IE2_{253-280}$	RPDEDSSSSSSSSCSSASDSESESEEMK
	20	$IE2_{267-294}$	SSASDSESESEEMKCSSGGGASVTSSHH
pool 5	21	$IE2_{281-308}$	CSSGGGASVTSSHHGRGGFGGAASSSLL
	22	$IE2_{295-322}$	GRGGFGGAASSSLLSCGHQSSGGASTGP
	23	$IE2_{309-336}$	SCGHQSSGGASTGPRKKKSKRISELDNE
	24	$IE2_{323-350}$	RKKKSKRISELDNEKVRNIMKDKNTPFC
	25	$IE2_{337-364}$	KVRNIMKDKNTPFCTPNVQTRRGRVKID
pool 6	26	$IE2_{351-378}$	TPNVQTRRGRVKIDEVSRMFRNTNRSLE
	27	$IE2_{365-392}$	EVSRMFRNTNRSLEYKNLPFTIPSMHQV
	28	$IE2_{379-406}$	YKNLPFTIPSMHQVLDEAIKACKTMQVN
	29	$IE2_{393-420}$	LDEAIKACKTMQVNNKGIQIIYTRNHEV
	30	$IE2_{407-434}$	NKGIQIIYTRNHEVKSEVDAVRCRLGTM
pool 7	31	$IE2_{421-448}$	KSEVDAVRCRLGTMCNLALSTPFLMEHT
	32	$IE2_{435-462}$	CNLALSTPFLMEHTMPVTHPPEVAQRTA
	33	$IE2_{449-476}$	MPVTHPPEVAQRTADACNEGVKAAWSLK
	34	$IE2_{463-480}$	DACNEGVKAAWSLKELHTHQLCPRSSDY
	35	$IE2_{477-504}$	ELHTHQLCPRSSDYRNMIIHAATPVDLL
pool 8	36	$IE2_{481-518}$	RNMIIHAATPVDLLGALNLCLPLMQKFP
	37	$IE2_{505-532}$	GALNLCLPLMQKFPKQVMVRIFSTNQGG
	38	$IE2_{519-546}$	KQVMVRIFSTNQGGFMLPIYETAAKAYA
	39	$IE2_{533-560}$	FMLPIYETAAKAYAVGQFEQPTETPPED
	40	$IE2_{547-574}$	VGQFEQPTETPPEDLDTLSLAIEAAIQD
	41	$IE2_{561-580}$	LDTLSLAIEAAIQDLRNKSQ

Supplementary Table 2. Summary of IE1 and IE2 $CD4^+$ and $CD8^+$ T cell responses identified per donor. 21 donors were evaluated for $CD4^+$ and $CD8^+$ T-cell recognition of 28-mer IE2 peptides by ICS assay. Because the initial 85 amino acids of the IE1 and IE2 proteins are identical, the responses have been divided into three segments: shared IE1 and IE2 and unique IE2. The total number of the combined single and shared $CD4^+$ and $CD8^+$ T-cell responses measured is depicted. $CD4^+$ T-cell responses were confirmed in 11 donors, including 3 HCMV-seronegative (donors 16, 17 and 20). $CD8^+$ T-cell responses were detected in 3 HCMV-seropositive donors.

	IE1/IE2 shared segment*		Unique IE2 segment		IE2 total	
Donor #	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
1			5	2	5	2
2	1	1	1		2	1
3			2		2	
4						
5			5		5	
6			13		13	
7			2		2	
8			5		5	
9						
10			10	2	10	2
11	1		2		3	
12			1		1	
13						
14						
15			1		1	
16	5		1		6	
17			2		2	
18						
19						
20			4		4	
21						



Supplementary Figure 1. Gating strategy for intracellular cytokine staining to identify CD4$^+$ and CD8$^+$ IE2 T-cell responses. In sequential gating, cells were initially gated for singlet's (SSC-A vs. FSC-H) and then for viability (FSC-A vs. live/dead) and lymphocytes (FSC-A vs. SSC-A). Next lymphocytes were gated on CD3$^+$ CD14$^-$ to exclude monocytes and subsequently analysed for the expression of IFN-γ$^+$ producing CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T-cell subsets were further analysed for expression of TNFα and IL-2 cytokines and several activation markers (CD154, CD137).

Supplementary Figure 2. Chart of all the IE1 and IE2 HCMV T-cell identified epitopes.