

Cytomegalovirus (CMV) Phosphoprotein 65 Makes a Large Contribution to Shaping the T Cell Repertoire in CMV-Exposed Individuals

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Antigen-specific, cytokine flow cytometry was used to analyze the prevalence and frequency of CD4 and CD8 memory T cells specific for the abundantly expressed cytomegalovirus (CMV) phosphoprotein 65 (pp65) in healthy CMV IgG-seropositive individuals. Stimulation of peripheral blood mononuclear cells with peptide pools and individual peptides derived from the pp65 amino acid sequence in 40 donors revealed that 63% of donors had a detectable CD4 T cell response and that 83% of donors had a detectable CD8 T cell response against this protein. The overall frequencies of T cells directed against pp65 were analyzed for 20 donors by stimulation with peptide pools covering the complete pp65 protein and were as high as 2 in 1000 and 9 in 1000 (median) peripheral blood CD4 and CD8 T cells, respectively. In addition, a comparison between CD4 responses to a CMV lysate containing various CMV proteins and pp65-specific responses in 9 donors indicated that pp65 was a dominant target of the CMV-specific CD4 T cell response in some, but not all, donors. Several new T cell epitopes were identified.

The cytomegalovirus (CMV) genome has >200 open-reading frames potentially coding for as many proteins [1]. The lower matrix phosphoprotein 65 (pp65), a structural protein that is abundant throughout CMV infection [2], is an important subject of CMV research. It is widely accepted as being the immunodominant target of the CD8 T cell response against CMV [3]. In addition, in a comparative study of 14 CMV proteins that was carried out in 1995, pp65 was the dominant antigen recognized by CD4 T cells [4]. At present, however, no more than 2 or 3 helper T cell epitopes, of ~15 CMV helper T cell epitopes, are known to be in pp65 [5–7]. Until 1996, the published frequencies of antigen-specific T cells (including CMV) were usually estimated indirectly by cytotoxicity testing and proliferation studies combined with limiting dilution [3, 8, 9]. With the development of major histocompatibility complex (MHC) class I tetramer/peptide complexes in 1996, the first direct method for

measuring the frequencies of antigen-specific CD8 T cells became available [10]. It was not until 1997, however, that flow-cytometric multiparameter analysis of antigen-specific CD4 T cells was described [11]. This method was based on the stimulation of peripheral blood mononuclear cells (PBMC) with recombinant protein antigens or lysates from pathogens, followed by multiparameter cytokine flow cytometry [12]. Antigen-specific CD4 T cells were visualized directly by intracellular cytokine staining. Because antigen presentation in this assay is mainly MHC class II-associated [11], it did not lend itself very well to the detection of antigen-specific CD8 T cells. However, a recently developed modification of the original assay allows for the study of antigen-specific CD4 and CD8 T cells in the same test and even in the same test tube [13, 14]. This approach is based on the use of peptides and peptide pools, instead of protein antigens or pathogen lysates, for PBMC stimulation. It was used successfully for the study of the CD8 T cells reactive to the CMV immediate-early 1 and pp65 proteins (including the determining of new epitopes) in the past [15]. Because the same parameter for T cell activation (i.e., intracellular interferon [IFN]– γ production) can be used for both CD4 and CD8 T cells, the responses pertaining to each subset can be differentiated simply by including the respective lineage antigens (CD4 or CD8) into the multiparameter analysis. In this way, the frequencies of antigen-specific CD4 and CD8 T cells can be measured under identical conditions and by the same standard. Because the assay is fast and the handling of cells is minimal, artifacts occurring in long-term culture are avoided.

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Written informed consent was obtained from all blood donors. The guidelines of the US Department of Health and Human Services were followed in the conduct of the research. The research was approved by the Charité Ethics Committee, and the pertinent guidelines were followed.

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Here, we used this recently developed technique to determine the percentage of healthy CMV-exposed (i.e., CMV IgG-seropositive) individuals who have a detectable CD4 and CD8 T cell response to peptides from pp65. We also measure the magnitude (percentage of responding cells in the respective population) of the responses directed at a pool of peptides representing all T cell epitopes in pp65 (the "pp65 total peptide pool"). Finally, we compare the CD4 T cell response to the pp65 total peptide pool with the response induced by a CMV lysate (containing many CMV proteins) in 9 donors. Our results show, first, that a majority of healthy CMV-exposed donors have a CD4 and CD8 T cell response to peptides from the pp65 protein; second, that the frequencies of these T cells range from almost undetectable to several percent; and, third, that pp65 dominates the CMV-specific CD4 response in some, but not all, donors.

Methods

Blood donors. The present study comprises data on the percentage of individuals with CD4 and CD8 T cells reactive to pp65-derived peptides and peptide pools among 40 healthy subjects, including data on the antigenic determinants (epitopes) recognized (Group A). The study also encompasses a comparison between the frequencies of CD4 and CD8 T cells responsive to a peptide pool representing the pp65 total peptide pool in 20 healthy subjects (Group B). Finally, there is a comparison among 9 healthy subjects between the CD4 T cell reactivity to a purified virus lysate and the pp65 total peptide pool (Group C). Groups A–C overlap, because some of the donors donated blood for >1 of these investigations. All blood donors were CMV seropositive by ELISA. The donors were 20–60 years old (median, 30 years old). Five additional healthy CMV-seropositive blood donors were recruited for determining or confirming the HLA molecules presenting new epitopes. Blood (10–40 mL) was drawn by peripheral venous puncture. Sodium citrate was used as anticoagulant.

PBMC preparation. After standard Ficoll-Paque gradient (Pharmacia), cells were washed with sterile PBS (Gibco BRL), resuspended in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum (FCS) and 2 mM L-glutamine (RPMI/FCS), and adjusted to the required concentrations for each series of experiments. RPMI 1640 medium, FCS, and L-glutamine were purchased from Biochrom.

Group A: stimulation with peptides for T cell epitope identification. Except in PBMC from 6 donors (see below), cell concentrations were adjusted to 5×10^6 cells/mL; 400 μ L of this cell suspension was transferred into Falcon 2052 tubes. After the addition of 100 μ L of peptide solution containing 10 μ g/mL of each used peptide in RPMI/FCS, all tubes were incubated in a standard incubator (at 37°C in a humidified 5% CO₂ atmosphere at a 5° slant). After 2 h, 500 μ L of RPMI/FCS containing 20 μ g/mL of Brefeldin A (BFA; Sigma) was added, increasing the volume to the final 1 mL. In some experiments, RPMI 1640 medium supplemented with 0.1% (wt/vol) bovine serum albumin (BSA; Biochrom) was used instead of RPMI/FCS during the first 2 h. In these experiments, 500 μ L of RPMI 1640 medium supplemented with 20% (vol/vol) FCS and 10 μ g of BFA was added at 2 h. For 6 donors, the incubation was

begun in a volume of 2 mL from the beginning in RPMI/FCS. In these experiments, 20 μ L of BSA working solution (1 mg/mL) was added at 2 h. In all experiments, BSA was added at 2 h, and the end concentrations of FCS and BSA were 10% (vol/vol) and 10 μ g/mL, respectively.

Group B: stimulation with the pp65 total peptide pool for measuring CD4 and CD8 T cell frequencies. Cell concentrations were adjusted to 5×10^6 cells/mL in RPMI/FCS; 400 μ L of cell suspension and 100 μ L of peptide solution (10 μ g/mL per peptide in RPMI 1640 medium supplemented with BSA) were transferred into Falcon 2052 tubes and incubated in a standard incubator (at 37°C in a humidified 5% CO₂ atmosphere at a 5° slant). After 2 h, 500 μ L of RPMI 1640 medium containing 10% (vol/vol) FCS (Biochrom), 2 mM L-glutamine, and 20 μ g/mL BFA were added, increasing the volume to 1 mL. End concentrations of FCS and BSA were 10% (vol/vol) and 10 μ g/mL, respectively.

Group C: comparing pp65 total peptide pool to virus lysate stimulation. We first tested stimulation with 0.1, 1, 5, and 10 mg/mL of virus lysate (CMV purified virus lysate; ABI) in PBMC from 5 donors in triplicate assays, to determine the optimum concentration for stimulation. This was found to be 5 mg/mL, whereas 10 mg/mL was inhibitory for 2 donors, and responses were not increased or were only marginally increased for the remaining 3 donors. We also tested stimulation with 0.1, 1, and 5 mg/mL per peptide of the pp65 total peptide pool in triplicate for 5 donors. These concentrations were chosen on the basis of previously established dose/response curves with individual peptides (authors' unpublished data). Results indicated that 1 mg/mL per peptide was the optimum concentration, with only a very marginal increase for 2 donors when using 5 mg/mL (data not shown). However, to exclude the possibility that higher responses to virus lysate simply could result from greater amounts of relevant pp65 peptides supplied by the lysate, 5 mg/mL per peptide of the pp65 total peptide pool (i.e., 690 mg/mL of peptide in total) and 5 mg/mL of virus lysate were used when comparing the responses for 9 donors. All experiments were done in triplicate. To avoid dimethyl sulfoxide (DMSO) toxicity, stimulation was begun with 2×10^6 PBMC/mL in 1 mL of RPMI/FCS. Tubes were incubated in a standard incubator (at 37°C in a humidified 5% CO₂ atmosphere at a 5° slant). Ten milliliters of BFA working solution (1 mg/mL) was added after 2 h. End concentrations of FCS and BSA were 10% (vol/vol) and 10 μ g/mL, respectively.

Ending incubation, permeabilization of cells, and staining. After 6 h of incubation (i.e. 4 h after BFA addition), cells were washed with cold PBS, resuspended in PBS with 1 mM EDTA (Merck), incubated for 10 min at 37°C (water bath), and washed again with cold PBS. Cells then were fixed in PBS containing 4% (wt/vol) paraformaldehyde (Merck) for 5 min at 37°C, washed with PBS, and permeabilized (permeabilizing solution; Becton Dickinson), according to the manufacturer's instructions.

After staining with fluorescent-conjugated monoclonal antibodies in a total staining volume of 100 μ L, cells were washed in PBS with 0.1% BSA and analyzed on a FACScalibur flow cytometer using CellQuest software (both from Becton Dickinson). Unstimulated samples were analyzed to verify the effect of stimulation. Data files were analyzed with Cell-Quest or Paint-a-Gate software (both from Becton Dickinson).

Data evaluation. In each sample, the response to peptides or virus lysate was assessed by visual cluster analysis. It was compared with the response in the respective control sample (no stimulant and/or irrelevant peptide), and a “positive response region” was defined. Results are reported as the percentage of the gated reference T cell population (CD4 or CD8) falling into this region minus the percentage of the same population falling into this region in the corresponding control sample. The smallest response classified as being positive in this study encompassed 0.03% of the respective reference T cell population. Smaller responses could not be discriminated from “background noise” and were rated as negative. T cells from CMV-seronegative individuals do not respond to stimulation with CMV antigens in this assay, as reported elsewhere [14–16]; that is, the T cell responses to CMV antigens (i.e., frequencies of IFN- γ -producing T cells after stimulation with CMV antigens expressed as a percentage of the total reference population) in these individuals are approximately equivalent to those measured in unstimulated control samples and generally in the order of 0.01% to 0.02% of the reference T cell population.

Antibodies and peptides. Fluorescein isothiocyanate-conjugated anti-IFN- γ ; phycoerythrin-conjugated anti-CD69; peridinin chlorophyll protein-conjugated anti-CD3, anti-CD4, and anti-CD8; allophycocyanin-conjugated anti-CD3, anti-CD4, and anti-CD8; and the corresponding isotype and fluorochrome-conjugated matched control reagents were purchased from Becton Dickinson. Peptides were purchased from NMI or were produced in our laboratory. In brief, peptides were synthesized on solid phase (50 μ mol scale) on a Tentagel SRam resin (Rapp Polymere), using benzotriazole-1-yl-oxy-Tris-pyrrolidino-phosphonium hexafluorophosphate activation and a standard Fmoc chemistry-based protocol of an AMS 422 Peptide Synthesizer (Abimed). Mass spectroscopy was performed on a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (Laser BenchTopII; Applied Biosystems). The purity of the products was characterized by analytical high-pressure liquid chromatography. All peptides were stored in DMSO stock at 8 or 80 mg/mL. Pools were generated from DMSO stock.

The peptide set we used contained 138 15-aa peptides (11 overlaps between subsequent peptides) covering the complete sequence of pp65 (Swiss-Prot accession no. P06725). Because of the size of the overlap, this set of peptides contained all possible “core epitopes” not exceeding 12 aa in length. Since the normal length of T cell core epitopes is ~9 or 10 aa [17], flanking amino acids would also be included in most cases (potentially important for CD4 T cells). Such peptide mixes induce CD4 T cell responses that are equivalent to those observed with the whole recombinant protein expressed in engineered yeast cells [18]; however, they also provide for CD8 T cell responses equivalent to those induced by the corresponding individual peptides [14, 18]. Of the complete set of 138 peptides, individual stimulating peptides were identified by the results obtained with overlapping peptide pools of 11 or 12 peptides (“mapping pools”). These had been compiled in such a way that every single peptide was contained in exactly 2 pools (figure 1, *top left*). Because each peptide was contained in 1 “vertical” pool (pools 1–12) and 1 “horizontal” pool (pools 13–24), any peptide inducing a T cell response would result in 1 vertical and 1 horizontal pool inducing a T cell response. Conversely, each intersection

of a horizontal and a vertical pool that both had induced a T cell response defined 1 candidate peptide. If >2 pools induced a T cell response, there would be several candidate peptides to be tested. The stimulation with the mapping pools was performed on day 1, and candidate peptides were tested individually on day 2 using PBMC from the same donor. PBMC had been kept in a standard incubator overnight. Control samples (stimulation with non-candidate peptides or no antigen) were used to rule out unspecific stimulation. This procedure was described in detail elsewhere [14].

Results

Two different approaches were used to analyze the T cell response to CMV pp65. First, to identify target amino acid sequences (epitopes) of responsive T cells, the 138 pp65-derived peptides were divided into 24 peptide pools, each consisting of 11 or 12 15-aa peptides. These pools were organized in a matrix that allowed for easy identification of individual target peptides (figure 1, *top left*). These individual peptides then were used for confirmation (figure 1A–1F). This approach was also suitable for determining whether an individual did or did not have a CD4 or CD8 T cell response to pp65 (without considering the magnitude of this response).

Second, to determine the frequencies of all CD4 or CD8 T cells reactive to epitopes contained in pp65, we used the pp65 total peptide pool of all overlapping peptides for stimulation (i.e., 138 peptides in 1 pool) [14]. Since IFN- γ is made by both CMV-specific CD4 and CD8 T cells after antigen recognition, both populations could be analyzed simultaneously (figure 1G and 1H) [18]. In these experiments, we refer to CD4⁺CD3⁺ T cells as CD8 T cells. Rather than using the additional lineage marker (CD8), we used the activation marker (CD69), which facilitates the discrimination of truly activated IFN- γ -positive events [14, 15, 18]. This approach may have resulted in a small error in estimating the total size of the CD8 T cell subset, because CD8⁺CD4⁺ T cells cannot be discriminated from CD8⁺CD4⁺ (double negative) T cells. These cells, however, represent no more than 5% on average (median) of peripheral blood T cells (authors' unpublished observation of 76 healthy blood donors).

Responsiveness and frequencies. A CD8 T cell response to peptides from pp65 was detected in 33 (83%) of 40 donors, and a CD4 T cell response was detected in 25 (63%) of 40 donors. In total, 23 (58%) of 40 donors had detectable CD4 and CD8 T cell responses to pp65, 10 (25%) responded only with CD8 T cells, 2 (5%) responded only with CD4 T cells, and 5 (13%) had no detectable T cell response to pp65.

The frequencies of all CD4 or CD8 T cells responsive to epitopes contained in pp65 were measured by stimulation of PBMC from 20 healthy donors with the pp65 total peptide pool (representing all T cell epitopes in pp65). Figure 2 shows the distribution of the CD4 and CD8 T cell responses in all donors, including nonresponders. Considering responsive donors only, the frequen-

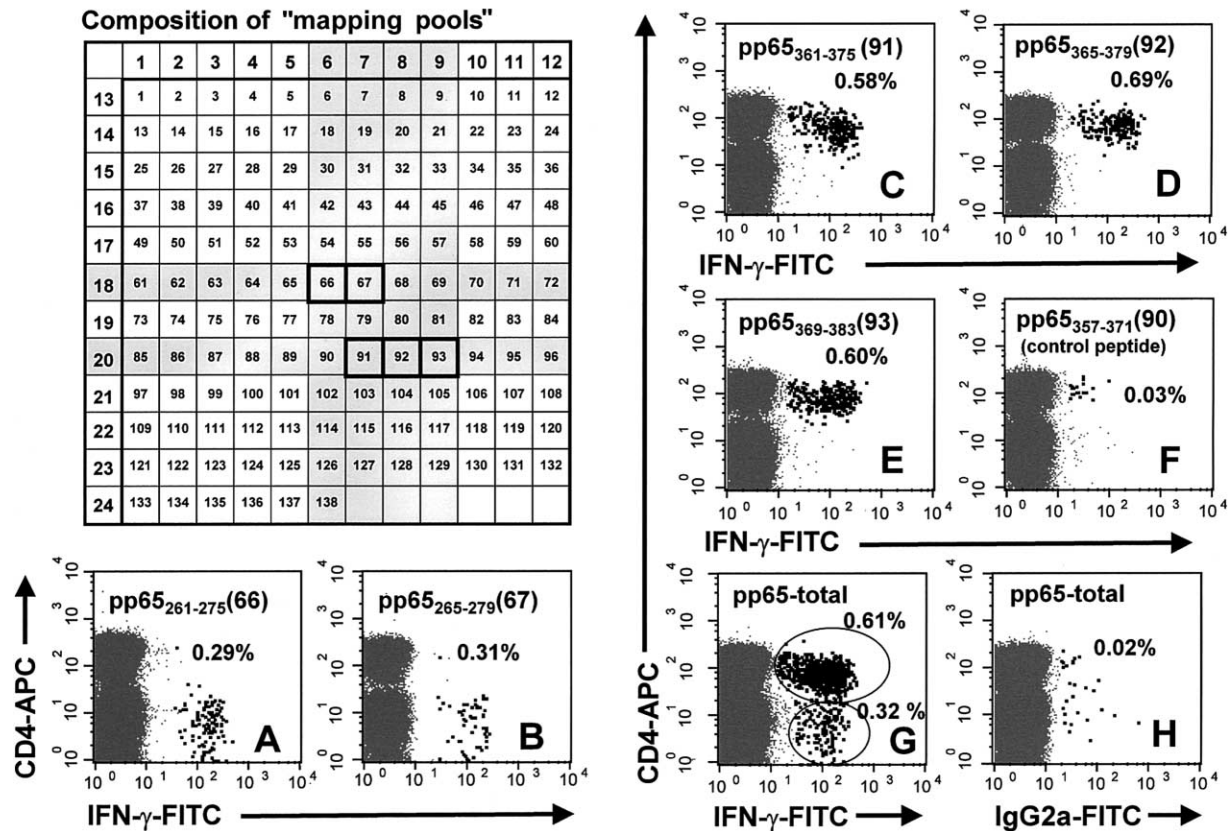


Figure 1. Composition of peptide pools and determination of candidate stimulating peptides. One hundred thirty-eight 15-aa peptides (with 11 overlaps between consecutive peptides) were derived from cytomegalovirus (CMV) phosphoprotein 65 (pp65). For epitope mapping, 24 peptide "mapping pools" were compiled. These are represented by the numbered columns (1–12) and rows (13–24) in the table (*top left*). Peripheral blood mononuclear cells (PBMC) were stimulated with peptide pools on day 1, and individual "candidate" stimulating peptides were stimulated on day 2. The shaded areas in the table correspond to the "mapping pools" inducing T cell stimulation (rapid interferon [IFN]– γ induction) in the shown experiment on day 1. Although pools 6 and 18 stimulated only CD8 T cells, pools 8, 9, and 20 stimulated only CD4 T cells. Pool 7 stimulated both CD4 and CD8 T cells. The candidate peptides for stimulating CD8 T cells were, therefore, peptides 66 (pp65_{261–275}) and 67 (pp65_{265–279}), whereas candidates for stimulating CD4 responses were peptides 91 (pp65_{361–375}), 92 (pp65_{365–379}), and 93 (pp65_{369–383}). *A–E*, Positive responses to individual candidate peptides tested on day 2. *F*, Control stimulation with a noncandidate single peptide. Stimulation with all 138 peptides from pp65 at the same time (*G*) resulted in stimulation of both CD4 and CD8 (i.e., CD4⁺CD3⁺) T cells. *H*, Isotype control staining corresponding to panel *G*. At least 20,000 CD3 T cells are displayed in each dot plot. PBMC were stained with anti-IFN– γ –fluorescein isothiocyanate (FITC), anti-CD69–phycoerythrin, anti-CD3–peridinin chlorophyll protein, and anti-CD4–allophycocyanin (APC). Axes show log fluorescence intensity.

cies of pp65 total peptide pool–reactive CD4 T cells ranged from 0.03% to 1.95% (median, 0.19%; $n = 16$), and the frequencies of pp65 total peptide pool–reactive CD8 T cells ranged from 0.04% to 2.38% (median, 0.85%; $n = 16$). If the test was performed ≥ 2 times in the same donor, the median sizes of the responding CD4 and CD8 T cell subsets were considered.

We also compared stimulation with the pp65 total peptide pool and a virus lysate (an extract from CMV-infected fibroblasts, representing many CMV proteins, likely also including pp65). The exact composition of the virus lysate is not known; however, higher CD4 T cell responses to the virus lysate than to the pp65 total peptide pool would at least indicate the presence of other proteins that may be relevant CD4 T cell targets, putting the pp65 peptide–specific response in proportion (CD8 cells are

not effectively stimulated by the virus lysate, and responses therefore cannot be compared). Dose/response curves indicated that 5 mg/mL of virus lysate gave maximum responses. For these experiments only, the pp65 total peptide pool also was used at a concentration of 5 mg/mL per peptide. In this way, we were able to definitely exclude that a relevant pp65 peptide was supplied in a higher concentration by the virus lysate than by the peptide pool. In general, the response to virus lysate was higher (0.13%–1.04% of CD4 T cells) than the response to the pp65 peptide mix (0%–0.85%) in 9 tested donors. The ratio between these responses (response to pp65:response to virus lysate) varied greatly among donors, indicating that, in some donors, the response to pp65 was only a fraction of the CD4 response to CMV, whereas, in others, it represented most of that response (data not

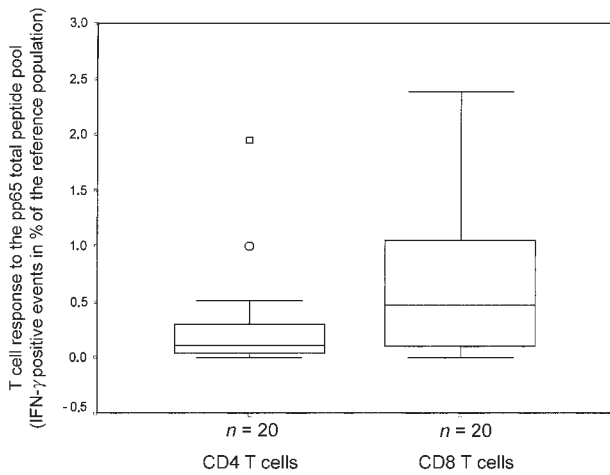


Figure 2. Prevalence and magnitude of T cell responses directed at peptides from cytomegalovirus (CMV) phosphoprotein 65 (pp65). Box plots show the magnitude of CD4 and CD8 T cell responses (interferon [IFN]- γ -positive CD4 or CD8 T cells in percentage of the total reference T cell population) directed at a peptide pool containing 138 overlapping 15-aa peptides (11-aa overlap) derived from CMV pp65. This pool represented all possible CD4 and CD8 T cell epitopes in pp65. Peripheral blood mononuclear cells were stimulated with the peptide pool for 6 h prior to flow-cytometric analysis. Box plots show range, median, 25th and 75th percentiles, and outliers (\square and \circ).

shown). Box plots in figure 3 show the frequencies of CD4 T cells (in percentage of the respective CD4 reference T cell population) responding to the pp65 total pool and virus lysate.

Confirming known and defining new epitopes. Epitope mapping (including CD4 and CD8 responses) was performed for a total of 40 healthy donors. The number of epitopes to which individuals responded varied from 1 to 4 for CD8 T cells and from 1 to 3 for CD4 T cells. Most individuals responded to 2 epitopes each with their CD4 and CD8 T cells. The procedure for identifying T cell epitopes is illustrated in figure 1.

Several previously known CD4 and CD8 T cell epitopes were confirmed in this study. Confirmed CD8 T cell epitopes included, for example, the peptides NLVPMVATV (pp65₄₉₅₋₅₀₃) and TPRV-TGGGAM (pp65₄₁₇₋₄₂₆), presented by HLA-A2 and HLA-B7, respectively [3], and the HLA-B7-presented peptide RPHER-NGFTV (pp65₂₆₅₋₂₇₄) [19]. Compared with CD8 T cell epitopes, the CD4 T cell epitopes known in CMV seem less well defined. In addition, fewer binding motifs are known for MHC class II alleles than for MHC class I alleles [17]. Table 1 lists CD4 T cell stimulating peptides identified in this study, along with the corresponding and largely overlapping sequences that were previously reported to be CD4 T cell epitopes.

New CD4 T cell epitopes against which responses were identified in several donors are represented by peptides 11 and peptides 71-72 (table 2). The 2 donors responding to peptide 11 shared HLA-DR15 and HLA-DQ6. An additional HLA-DR15- and HLA-DQ6-positive donor recruited from the blood bank donor pool also responded to this peptide. Some ligands for HLA-

DR15 are known [17], whereas, to our knowledge, no definite ligands for HLA-DQ6 were reported, and binding motifs are not known [17]. The donors responding to peptides 71-72 shared HLA-DR53 and HLA-DQ3. According to Rammensee et al. [17], binding motifs for HLA-DR53 (HLA-DRB4) are also not known, whereas reported binding motifs for HLA-DQ3 (DQB1*0301) do not seem to favor binding of these 2 peptides.

We also discovered 2 new CD8 T cell epitopes. These were contained in peptides 38-39 and peptides 43-44 (table 2). The most likely presenting HLA allele for the epitope contained in peptide 38-39 was determined to be HLA-B52, because the only donor responding to this peptide was also the only donor found to be HLA-B52 positive. Because the length of MHC class I-presented peptides is usually 9 aa [17], the 7 possible 9-aa peptides (each corresponding to a potential epitope) contained in the original 15-aa peptide were synthesized and tested on the same donor. The epitope was found to be contained in the sequence QMWQARLTV. This was also the most likely recognized sequence, as predicted by an HLA-binding prediction algorithm for peptides described by Parker et al. [20]. Two additionally recruited HLA-B52-positive donors also had a CD8 T cell response to this peptide (the only HLA allele shared by all 3 responding donors was HLA-B52). The donor responding to peptide 43-44 shared all HLA molecules with various other donors who did not respond to this peptide. We believe that the presenting allomorph must be one with a high number of different

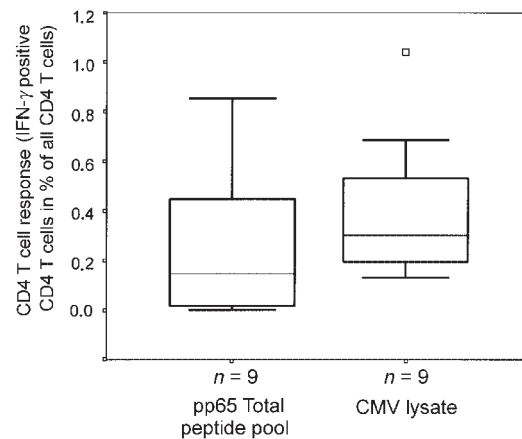


Figure 3. The CD4 T cell response directed at peptides from cytomegalovirus (CMV) phosphoprotein 65 (pp65), compared with the response against a lysate from CMV-infected fibroblasts. Box plots show the CD4 T cell response (interferon [IFN]- γ -positive CD4 T cells in percentage of the total CD4 T cell population) directed at the pp65 total peptide pool (*left*) and a CMV virus lysate (*right*) in 9 individuals. Although the peptide pool represents all possible CD4 T cell epitopes in pp65, the virus lysate represents all CMV proteins present in infected fibroblasts. Peripheral blood mononuclear cells were stimulated with peptide pool or virus lysate for 6 h prior to flow-cytometric analysis. Box plots show range, median, 25th and 75th percentiles, and outliers (\square).

Table 1. Confirmation of CD4 T cell epitopes in phosphoprotein 65 (pp65).

Peptide no.	Peptide position ^a	Amino acid sequence	Published sequence(s) ^b	Presenting HLA allele ^b	No. of responding donors (<i>n</i> = 40) ^c
91	pp65 _{361–375}	PQYSEHPTFTSQYRI	PQYSEHPTFTSQYRIQ and FTSQYRIQGKLEYRHT	DR11	8
92	pp65 _{365–379}	EHPTFTSQYRIQGKL			
93	pp65 _{369–383}	FTSQYRIQGKLEYRH			
128	pp65 _{509–523}	KYQEFFWDANDIYRI	QEFFWDANDIYRIFA	DR52	6
129	pp65 _{513–527}	FFWDANDIYRIFAEL			
123	pp65 _{489–503}	AGILARNLVPMVATV	PPWQAGILARNLVPMV	Unknown	1
124	pp65 _{493–507}	ARNLVPMVATVQGQN			

NOTE. The entries under “Published sequence(s),” “Presenting HLA allele,” and “No. of responding donors” that are listed on the same line as the first peptide of each peptide group (91–93, 128–129, and 123–124) refer to the whole group.

^a Corresponding to the pp65 sequence according to Swiss-Prot (accession no. P06725).

^b Khattab et al. [5].

^c Positive response to ≥ 1 of the listed peptides.

alleles with potentially different peptide binding requirements, such as B35 (the responding donor was HLA-B*3501 positive). A 9-aa sequence contained in both peptides has 1 known anchor (boldface) and 3 preferred residues (underlined) for binding HLA-B*3501: **NQW**KEPDVY [17]. Of the 9-aa sequences contained in peptides 43/44, this sequence also obtained the highest score for binding HLA-B*3501, using the HLA-peptide binding prediction algorithm described by Parker et al. [20].

Discussion

In this study, we determined the prevalence of CD4 and CD8 T cell responses to the pp65 protein in a cross-sectional study of 40 healthy CMV-exposed (CMV IgG–seropositive) donors. We also measured the frequencies of CD4 and CD8 T cells reactive to a peptide pool representing all T cell epitopes contained in pp65. We believe that these frequencies are representative of the frequencies of T cells responsive to the protein itself [14, 18].

Because T cells play an important role in the immune defense against CMV [21–26], it would be interesting to perform such measurements in patient populations in which CMV disease occurs frequently, such as bone marrow transplant recipients. At present, however, there are no clinical data showing that the frequencies of circulating pp65-specific T cells (CD4 or CD8) in any way correlate with the risk of developing complications from CMV infection. On the other hand, it is well known that CMV reactivation is linked to stress events [27, 28] and occurs frequently even in healthy nonimmunocompromised individuals [28]. Because of the absence of CMV disease in healthy but CMV-exposed individuals, one may assume that healthy individuals are somehow protected. In this regard, it is interesting to observe that some healthy CMV-exposed individuals had no (or no detectable) response to peptides from pp65 at all (neither CD4 nor CD8). In addition, even in individuals who were clearly responders, the frequencies of pp65 peptide-specific T cells ranged from almost zero to several percent (this is true for both CD4

Table 2. New T cell epitopes in phosphoprotein 65 (pp65).

Responding T cell subset, peptide no.	Peptide position ^a	Amino acid sequence	Epitope sequence	Presenting HLA allele	No. of responding donors (<i>n</i> = 40) ^b
CD4					
11	pp65 _{41–55}	LLQTGIHVRVSQPSL	Unknown	DR15 (DQ6) ^c	2
71	pp65 _{281–295}	IIKPGKISHIMLDVA	Unknown	DR53 (DQ3) ^d	2
72	pp65 _{285–299}	GKISHIMLDVAFTSH			
CD8					
38	pp65 _{149–163}	IHASKQMWQARLTV	QMWQARLTV	B52 ^e	2
39	pp65 _{153–167}	GKQMWQARLTVSGLA			
43	pp65 _{169–183}	TRQQNQWKEPDVYYT	Unknown	Unknown	1
44	pp65 _{173–187}	NQWKEPDVYYTSAFV			

NOTE. The entries under “Epitope sequence,” “Presenting HLA allele,” and “No. of responding donors” that are listed on the same line as the first peptide of the peptide groups (71–72, 38–39, and 43–44) refer to the whole group.

^a Corresponding to the pp65 sequence according to Swiss-Prot (accession no. P06725).

^b Not including donors recruited additionally to determine the presenting allomorph.

^c Potential presenting alleles. In addition to DR15, both responding donors shared DQ6, for which no definite ligands have been reported.

^d Potential presenting alleles. In addition to DR53, both responding donors shared DQ3, for which the known binding motifs do not favor binding of this peptide.

^e Confirmed in 2 additional donors.

and CD8 T cells). We have recently suggested [29] that measurements of the anti-CMV T cell response should complement viral parameters in patients at risk of CMV disease, because the T cell response is a crucial factor in controlling the virus. Our findings in the present study, however, suggest that the absence of pp65 peptide-specific T cells in peripheral blood alone (whether CD4 or CD8) does not indicate the absence of protection from developing CMV disease, because 5 of 40 healthy CMV-exposed donors had no T cell response to peptides from this protein, yet they were not sick, and CMV probably was reactivated as frequently as in other healthy individuals. Furthermore, the results from comparing the T cell response to the pp65 total peptide pool and the response to virus lysate suggest that the pp65 peptide-specific reactivity in CD4 T cells does not permit conclusions as to the overall CD4 T cell response to CMV. Although stimulation of PBMC with virus lysate may be a reasonably good way of measuring "total" CD4 T cell reactivity to CMV even in a clinical setting, no comparable and equally practicable approach exists for CD8 T cells. The use of infected fibroblasts for stimulating CD8 T cells in this rapid *ex vivo* stimulation assay has not been successful in our laboratory, which may be due partly to virus-mediated MHC class I down-regulation [30, 31]. However, because of the complicated logistics that would be necessary for providing HLA-matched CMV-infected cell lines for a large number of patients, such an approach would also not be suitable for routine diagnostics.

Because pp65 is only one protein of many coded by the CMV genome, the high percentage of individuals with CD4 or CD8 T cell responses to pp65-derived peptides (63% and 83%, respectively) seems a little surprising. The high prevalence of CD4 T cell responses to pp65 may be explained by the fact that pp65 is an abundantly expressed tegument protein [2] and that peptides derived from abundant structural proteins are likely to be presented in a MHC class II context after endocytosis or phagocytosis of viral material. Regarding MHC class I presentation of pp65-derived peptides, it is known that pp65 may enter the endogenous pathway of antigen presentation in both the presence and absence of viral gene expression [32, 33].

Interestingly, in some individuals, we found CD8 responses to several (up to 4) different epitopes and CD4 responses directed against as many as 3 different epitopes. However, in other individuals, the response was concentrated on 1 epitope alone. Although this difference may be related to the HLA type, it is also possible that it is related to the time since primary infection, with the T cell response focusing on 1 epitope (possibly even with a single clone) late after primary infection [19]. Additional experiments will be dedicated to analyzing the composition of anti-CMV T cell responses, such as the number of recognized epitopes or the clonotypic distribution of responding cells in various clinical situations. The knowledge derived from such experiments may be helpful in vaccine design, as described elsewhere [34].

Knowledge of new epitopes, on the other hand, may be beneficial in several ways. Studying the responses to peptides of which the presenting HLA alleles are known in individuals positive for these alleles is only one option. Another very useful option seems to be the compilation of peptide pools containing all T cell epitopes ever determined in CMV proteins. Such pools could be used for testing T cell responses to CMV, potentially representing all epitopes in many proteins. For such pools, it will be essential to find as many epitopes as possible and clearly less important to know their presenting HLA alleles. For this purpose, we have recently begun analyzing many additional CMV proteins for the presence of T cell epitopes using cytokine flow cytometry.

Our finding that >60% of normal healthy donors have a CD4 T cell response and that >80% have a CD8 T cell response to peptides derived from pp65 shows that this protein is an important target for both CD4 and CD8 T cells in a majority of individuals. Approximate average frequencies of pp65-specific CD4 and CD8 T cells of 2 in 1000 and 9 in 1000 T cells, respectively, indicate, moreover, that this protein has great impact on shaping the T cell repertoire in exposed individuals. The frequencies of T cells "dedicated" to CMV pp65 are surprisingly high, considering, first, that this protein is only one of a large number of proteins potentially coded for by the CMV genome and, second, that CMV is only one of many pathogens that our immune system has to confront. Similar studies with other CMV proteins are in progress and should improve our understanding of the T cell immune response to this virus.

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