

Broadly targeted human cytomegalovirus-specific CD4⁺ and CD8⁺ T cells dominate the memory compartments of exposed subjects

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Human cytomegalovirus (HCMV) infections of immunocompetent hosts are characterized by a dynamic, life-long interaction in which host immune responses, particularly of T cells, restrain viral replication and prevent disease but do not eliminate the virus or preclude transmission. Because HCMV is among the largest and most complex of known viruses, the T cell resources committed to maintaining this balance have never been characterized completely. Here, using cytokine flow cytometry and 13,687 overlapping 15mer peptides comprising 213 HCMV open reading frames (ORFs), we found that 151 HCMV ORFs were immunogenic for CD4⁺ and/or CD8⁺ T cells, and that ORF immunogenicity was influenced only modestly by ORF expression kinetics and function. We further documented that total HCMV-specific T cell responses in seropositive subjects were enormous, comprising on average ~10% of both the CD4⁺ and CD8⁺ memory compartments in blood, whereas cross-reactive recognition of HCMV proteins in seronegative individuals was limited to CD8⁺ T cells and was rare. These data provide the first glimpse of the total human T cell response to a complex infectious agent and will provide insight into the rules governing immunodominance and cross-reactivity in complex viral infections of humans.

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Abbreviations used: CFC, cytokine flow cytometry; dPBS, Dulbecco's PBS; HCMV, human cytomegalovirus; IE, immediate-early; ORF, open reading frame.

Human cytomegalovirus (HCMV) is a member of a distinct, widely distributed subgroup of β -herpesviruses that share common growth properties, characteristic cytopathology, salivary gland tropism, and a capacity to establish permanent infection (1). A large portion of humanity harbors this virus; infection rates range from 40% to >90% depending on socioeconomic status. For almost all infected persons, both acute and persistent infection are benign. However, HCMV can cause severe disease in the setting of cellular immune deficiency or immaturity, including transplant recipients, individuals with late-stage HIV infection, and congenitally infected neonates (2), and studies in both humans and animal models have demonstrated a critical role for both CD4⁺ and CD8⁺ T cell immunity in limiting viral replication and preventing the clinical manifestations of progressive infection (3–5). Given the capacity

of unrestrained infection to cause disease, the biology of HCMV infection in immunocompetent populations can be conceptualized as an evolutionarily “negotiated” balance between viral mechanisms of pathogenesis, persistence, and immune evasion and the host cellular immune response.

The immunologic basis of this balance has not been characterized completely. In particular, the nature and threshold level of HCMV-specific T cell responses required for long-term HCMV containment remain to be defined. Such information would facilitate identification of highly susceptible individuals and provide a specific target for immunotherapeutic approaches designed to establish, maintain, or restore immunologic protection against this virus. Moreover, recent data closely link HCMV exposure and expansion of HCMV-specific CD8⁺ T cells to senescence of the immune system in the elderly (6, 7), suggesting that there are clinical consequences to an overly robust HCMV-specific T

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cell response. An obvious prerequisite for a better understanding of what constitutes insufficient or excessive HCMV-specific T cell immunity is the ability to evaluate the overall HCMV-specific T cell response in infected individuals. However, the complexity of this virus, which includes a double-stranded DNA genome encoding ~200 open reading frames (ORFs) for proteins >80 amino acids in length (1), has been a major obstacle to the comprehensive analysis of the HCMV-specific T cell response necessary for such evaluation.

Previous work by many groups has defined CD4⁺ and/or CD8⁺ T cell responses to whole viral lysates, virally infected cells, a subset of individual ORFs, and various MHC-restricted epitopes (8–19), but the extent to which these measurements reflect the overall T cell response to HCMV has not been determined. To resolve this issue definitively, we selected the inclusive, empirical approach of synthesizing overlapping 15mer peptides encompassing all 213 known or predicted HCMV ORFs and applying these peptides in a validated cytokine flow cytometry (CFC) assay to a large cohort of HLA-disparate, HCMV-seropositive subjects and to a control cohort of HCMV-naïve individuals. These experiments provide the first complete picture of the structure of the human T cell response to a complex, persistent infectious agent, including definition of (a) the frequency and extent to which these HCMV gene products are recognized by human CD4⁺ and CD8⁺ T cells; (b) the relationship between the immunogenicity of each gene product and its function, expression kinetics, abundance, and variability; (c) total HCMV-specific CD4⁺ and CD8⁺ responses in HCMV-infected subjects, and (d) the extent to which T cells in HCMV-naïve individuals are cross-reactive with HCMV-encoded determinants.

RESULTS

Experimental approach to the pan-genomic evaluation of HCMV immunogenicity

Based on the HCMV sequence information available in June 2000, we synthesized consecutive 15mer peptides, overlap-

ping by 10 amino acids, for 213 predicted HCMV proteins, including 191 from the HCMV laboratory strain AD169 and an additional 22 from the Toledo or Towne HCMV strains (see Materials and methods and Table S1, available at <http://www.jem.org/cgi/content/full/jem.20050882/DC1>). These 13,687 peptides were then arranged in ORF-specific mixes or, for very large ORFs (those yielding >200 peptides), sub-ORF mixes. The 232 resulting peptide mixes were used in individual CFC assays (20), along with AD169 lysate, a negative control (costimulation alone), and a positive control (Staphylococcal enterotoxin B), for a total of at least 235 determinations per subject. These assays were performed on PBMCs from 33 HCMV-seropositive and 10 HCMV-seronegative adult subjects who were selected to maximize ethnic and HLA diversity (Table I). Note that the HCMV-seropositive cohort includes a heterogeneous sampling of HLA alleles (15 HLA-A, 26 HLA-B, 13 each HLA-C and HLA-DRB1, and 5 HLA-DQB1), including most of those commonly found in most human populations (21). All subjects were healthy volunteers with no HCMV viremia detectable by real-time PCR.

Identification of immunogenic HCMV ORFs in natural infection

Fig. 1 A illustrates a typical analysis of an HCMV-seropositive subject. CD4⁺ or CD8⁺ T cells specifically reactive with peptides within the peptide mixes respond by induction of CD69 and γ -IFN. Negative controls indicated the extent of background activation, and these frequencies were subtracted from the peptide mix result to provide a net response frequency. Because this background subtraction is subject to stochastic fluctuation, we defined a positive net response as $\geq 0.06\%$ for CD4⁺ T cells and $\geq 0.08\%$ for CD8⁺ T cells, based on statistical analysis of the responses of the HCMV-seronegative cohort (see Materials and methods). Even by these conservative criteria, the HCMV genome was found to be remarkably immunogenic for T cells (Fig. 1 B). Of the 213 HCMV ORFs assessed, 151 elicited at least one CD4⁺ and/

Table I. Cohort demographics and HLA types

	Number screened	Number enrolled	Gender		Race				Age (yr)		
			Female	Male	White	African American	Asian American	Other	19–29	30–39	40–55
HCMV serostatus											
Positive	58	33	16	17	22	3	5	3	7	12	14
Negative	72	10	5	5	6	0	2	2	3	2	5
HLA type											
Alleles represented in HCMV-seropositive (all) and -seronegative (*) cohorts											
A	01*, 02*, 03*, 11*, 23*, 24*, 26, 29, 30, 31*, 32, 33, 66*, 68, 74										
B	07*, 08*, 13*, 14*, 18*, 27, 35*, 38*, 39, 44*, 46, 47, 49*, 51*, 53, 56*, 57, 58, 59, 60*, 61, 62, 63*, 70, 75, 81										
Bw	04*, 06*										
Cw	01*, 02, 03*, 04*, 05*, 06, 07*, 08*, 12*, 14, 15*, 16, 18										
DRB1	01*, 03*, 04*, 07, 08*, 09*, 10, 11*, 12, 13*, 14, 15*, 16										
DRB 3/4/5	3*, 4*, 5*										
DQA1	01*, 02, 03*, 04*, 05*										
DQB1	02*, 03*, 04*, 05*, 06*										

or CD8⁺ T cell response. Fig. 1 C shows the rank order of all ORFs recognized by CD4⁺ T cells (top) or CD8⁺ T cells (bottom) with respect to the frequency of recognition in the HCMV seropositive cohort. Note that 40 ORFs (19%) were recognized by CD4⁺ T cells in at least 4/33 subjects, and five ORFs (UL55, UL83, UL86, UL99, and UL122) were recognized by more than half of the study subjects. For CD8⁺ T

cells, 33 ORFs (15.5%) were recognized by four or more subjects, and three ORFs (UL48, UL83, and UL123) were recognized by more than half the subjects. Although the recognition hierarchies for CD4⁺ and CD8⁺ T cell responses were different, there was considerable overlap: 8 of the top 15 most recognized ORFs were common to both lineages (UL55, UL83, UL122, UL48, UL32, UL123, UL99, and UL82).

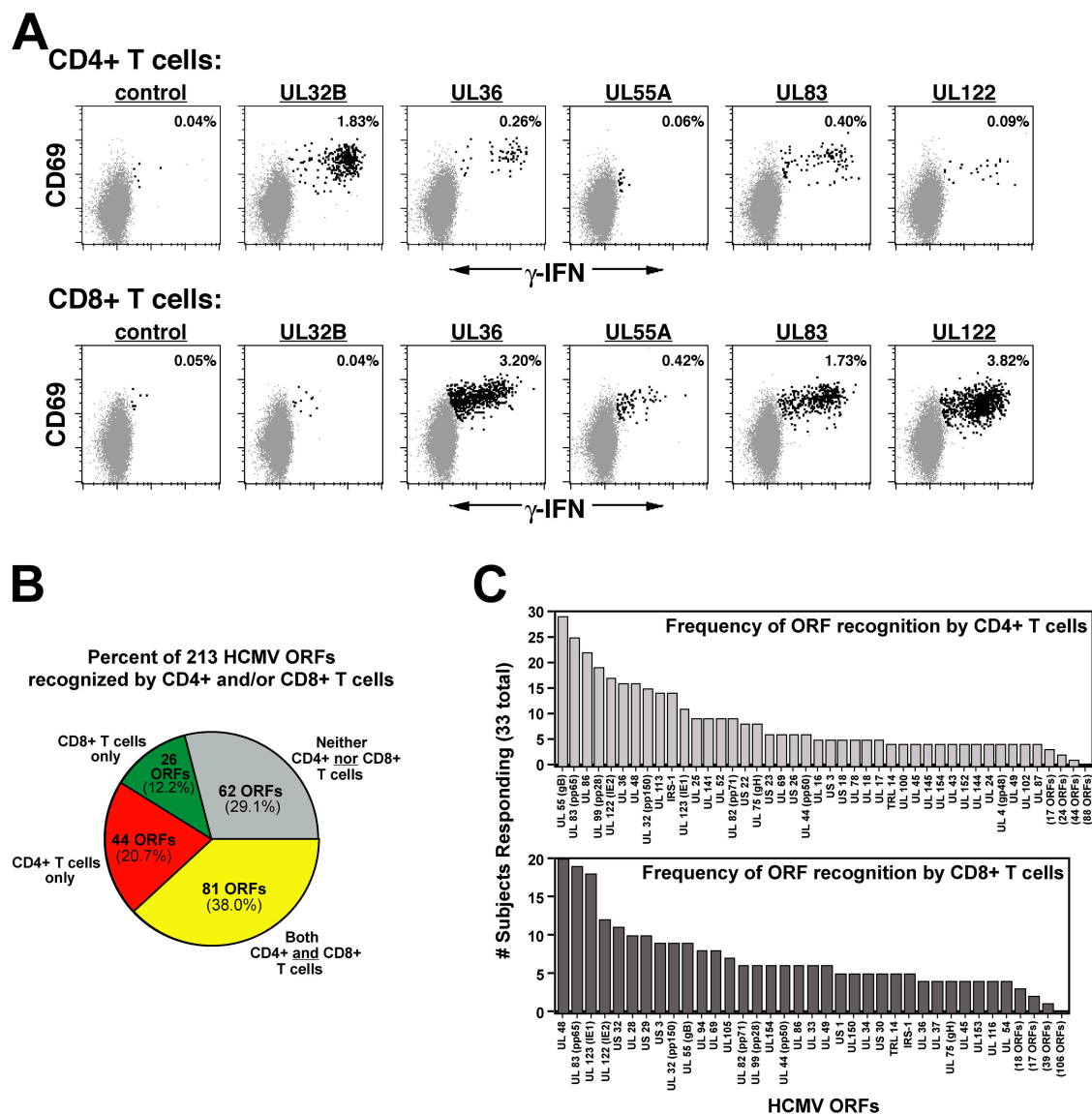


Figure 1. Identification of HCMV ORF-specific CD4⁺ and CD8⁺ T cell responses in HCMV-seropositive adults. (A) PBMCs from HCMV-seropositive subject P6 were stimulated with costimulation plus mixtures of consecutive, overlapping 15mer peptides comprising complete HCMV ORFs (UL36, UL83, and UL122) or large ORF fragments (UL32B, UL55A) or with costimulation alone (negative control) and then were examined by CFC for their correlated expression of surface CD3 and CD4 and intracellular CD69 and γ -IFN. The profiles shown were gated on CD3⁺/CD4⁺ small lymphocytes (CD4⁺ T cells) or CD3⁺/CD4⁺ small lymphocytes (CD8⁺ T cells), with the responding (CD69⁺/ γ -IFN⁺) T cells depicted in bold in upper right corner of each profile and the nonresponding T cells in gray.

The results shown are typical of seropositive donors in which certain ORFs generated clusters of responding cells over and above the spontaneous activation noted in controls. (B) The pie chart shows the fraction of the tested 213 HCMV ORFs that are able to stimulate at least one positive CD4⁺ and/or CD8⁺ T cell response (as defined in Materials and methods) in 33 HCMV-seropositive donors. (C) The number of times that each HCMV ORF is recognized by CD4⁺ T cells (top) or CD8⁺ T cells (bottom) in the 33 HCMV-seropositive donors is shown, from the most to least frequently recognized (note: the CD4 and CD8 analyses are independent, reflecting all the responses of a given lineage, irrespective of the response of the other lineage).

T cell recognition of HCMV ORFs in HCMV-seronegative subjects

T cells from the 10 HCMV-seronegative subjects manifested a very different reactivity pattern to the HCMV peptide mixes. For the vast majority of these analyses (2,320/2,320 CD4; 2,313/2,320 CD8), the responses to the HCMV pep-

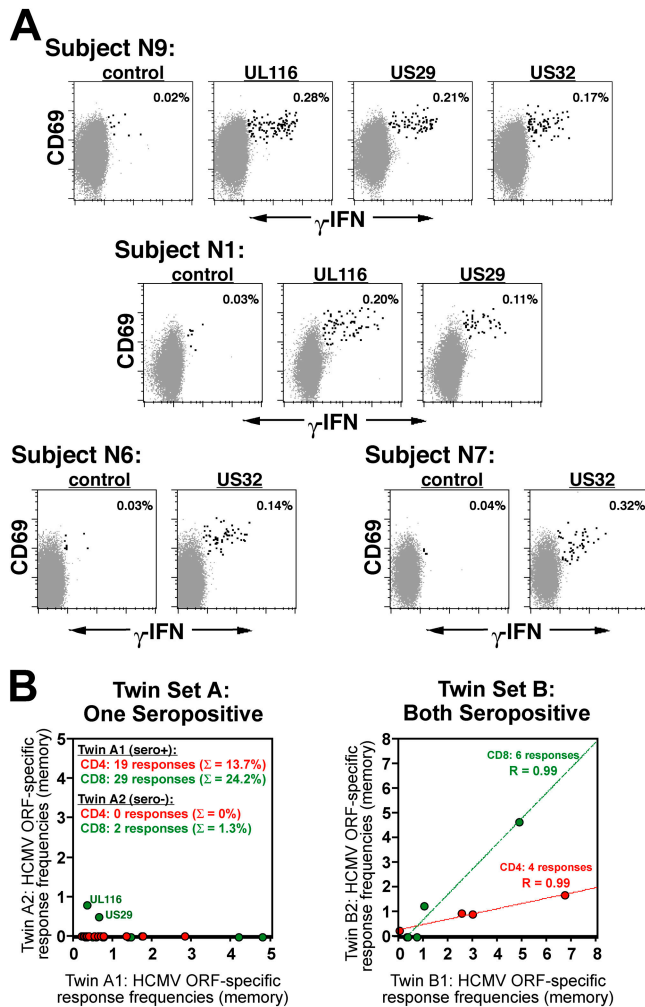


Figure 2. T cell recognition of HCMV ORFs in HCMV-seronegative subjects and in identical twins that are concordant or discordant for HCMV seroreactivity. (A) CFC was used to assess the CD4⁺ and CD8⁺ T cell responses of 10 HCMV-seronegative donors to 15mer peptide mixes encompassing all 213 HCMV ORFs. The figure demonstrates the 7 assays of 4,640 total performed on these subjects (2,320 each for CD4⁺ and CD8⁺ T cell responses)—all CD8⁺—that gave reproducible clusters of responding cells that were clearly distinct from background ($n = 3$ for all seven responses, including at least one repeat with CD3⁺/CD8⁺ gating). (B) Two sets of identical twins, one (Twin Set A) discordant for HCMV seroreactivity, the other (Twin Set B) concordant positive, were evaluated for peripheral blood CD4⁺ and CD8⁺ T cell responses to 213 HCMV ORFs by CFC. The figure shows all identified responses (CD4⁺ T cell responses in red; CD8⁺ T cell responses in green), presented as memory-corrected response frequencies. The two responses (UL116 and US29) of the seronegative twin of set A (subject N1) were two of the seven cross-reactive responses noted in Fig. 2 A.

tide mix were not quantitatively or qualitatively different from background. However, four HCMV-seronegative subjects demonstrated seven clear-cut, reproducible CD8⁺ T cell responses to three HCMV ORF mixes: three subjects responded to US32, and two each responded to US29 and UL116 (Fig. 2 A). One of the HCMV-seronegative subjects with HCMV ORF-specific CD8⁺ T cell responses (subject N1) was an identical twin whose HCMV-seropositive sibling was also studied (subject P33). These twins manifested quantitatively similar responses to US29 and UL116 (Fig. 2 B: twin set A), but the seropositive twin manifested an additional 19 CD4⁺ and 27 CD8⁺ ORF-specific responses. In contrast, the HCMV ORF-specific response profiles of a pair of HCMV-seropositive identical twins were strikingly concordant (twin set B), strongly suggesting that the ability of the HCMV-seronegative twin of set A to respond to the two HCMV ORFs resulted from cross-reactive recognition of HCMV epitopes by memory T cells originating from distinct (e.g., non-HCMV) antigenic exposures.

Viral factors influencing T cell recognition of HCMV ORFs

To evaluate the influence of viral gene regulation and function on T cell recognition of HCMV ORFs, we first classified the tested ORFs with respect to expression kinetics, function, and virion localization (Table S1). We then determined the relative contribution of each ORF class to the overall set of HCMV-specific responses in the HCMV-seropositive cohort (number of CD4⁺ or CD8⁺ responses to a given ORF class divided by the total number of CD4⁺ or CD8⁺ responses in the 33 HCMV-seropositive subjects). Finally, we compared this relative contribution of each ORF class to overall T cell recognition with the representation of that ORF class in the HCMV genome (Table II). This analysis demonstrated that immunogenic ORFs span all kinetic and functional categories and that, in general, the observed contribution of each ORF class to the total response was commensurate with the coding space of that ORF class in the HCMV genome. However, there were several noteworthy deviations from this proportional distribution. Immediate-early (IE) gene products were recognized 2.3- and 3.0-fold over their representation in the HCMV genome by CD4⁺ and CD8⁺ T cells, respectively. Additionally, the frequencies of CD8⁺ T cells reactive with IE gene products in peripheral blood were 2.4- to 4.0- fold higher than those reactive to gene products of other kinetic classes. Also notable was the preferential recognition of primary immune evasion proteins by both CD4⁺ and CD8⁺ T cells and of viral tegument and glycoproteins by CD4⁺ T cells.

As a group, virion components were recognized only slightly more frequently than their representation in the genome by both CD4⁺ and CD8⁺ T cells (Table II), but these proteins vary tremendously in abundance. As shown in Fig. 3 A, there was a significant correlation between the relative abundance of virion proteins determined by proteomic analysis (22) and the frequency of ORF recognition by the CD4⁺ and CD8⁺ T cells of HCMV-seropositive individuals

Table II. ORF kinetics and function vs. T cell recognition

Category ^a	Number of HCMV ORFs (% of 213)	Genomic coding space	CD4 ⁺ T cells			CD8 ⁺ T cells		
			Positive responses ^b (n = 499)	Ratio ^c	Average positive response ± SEM ^d	Positive responses ^b (n = 378)	Ratio ^c	Average positive response ± SEM ^d
Kinetics								
Immediate-early	8 (3.8)	4.6	10.4**	2.3	0.58 ± 0.09	13.8**	3.0	2.23 ± 0.45
Early	56 (26.3)	29.3	29.5	1.0	0.85 ± 0.11	23.3*	0.8	0.55 ± 0.09
Early-late	41 (19.2)	22.9	19.2	0.8	0.73 ± 0.15	20.9	0.9	0.71 ± 0.09
Late	47 (22.1)	22.3	27.9**	1.3	0.75 ± 0.10	27.5*	1.2	1.35 ± 0.21
Function								
Capsid	7 (3.3)	8.1	9.8	1.2	0.79 ± 0.20	9.5	1.2	0.70 ± 0.26
Matrix/tegument	23 (10.8)	17.0	27.1**	1.6	0.74 ± 0.09	20.4	1.2	1.46 ± 0.26
Glycoprotein	19 (8.9)	8.6	13.8**	1.6	1.03 ± 0.19	7.4	0.9	0.50 ± 0.11
DNA/regulatory	23 (10.8)	18.5	15.8	0.9	0.50 ± 0.07	24.6**	1.3	1.42 ± 0.27
Immune evasion ^e	27 (12.7)	10.5	20.0**	1.9	0.82 ± 0.11	18.0**	1.7	1.25 ± 0.24
Localization								
Dense bodies	17 (8.0)	17.0	27.0**	1.6	0.88 ± 0.09	22.4*	1.3	1.16 ± 0.17
Virion	60 (28.2)	43.7	55.3**	1.3	0.84 ± 0.08	50.0*	1.1	1.13 ± 0.13

^aSee Table S1 for specific ORF assignments to each category.

^bp-value from comparison of percentage of genomic coding space vs. percentage of positive response values: <0.05 (*) or <0.005 (**).

^cRatio: percentage of positive responses/percentage of genomic coding space.

^dPercentage of peripheral blood memory compartment.

^eORFs implicated in immune evasion that are not essential for optimal in vitro growth (may overlap with other functional categories).

(CD8 > CD4). We also investigated whether strain-to-strain variability in HCMV protein sequence affected either T cell recognition or our ability to identify responses with a single set of peptides. At the time of this writing, sequence data for six different HCMV strains—two laboratory strains (AD169 and Towne) and four clinical strains (Toledo, FIX, PH, and TR)—were available for 160 ORFs (23). Based on these data, we classified these ORFs into three sequence variability categories: high (<80% amino acid identity), intermediate (80–95% identity), and low (>95% identity). As shown in Fig. 3 B, almost all frequently recognized HCMV proteins were in the low-variability category, and frequency of recognition declined with increasing sequence heterogeneity. Peptide mixes encompassing the most variable HCMV proteins (<80% identity) were recognized very infrequently, suggesting that sequence differences between the HCMV strains infecting our subjects and the reference strains used as a template for peptide synthesis affected our ability to detect such responses. However, the influence of this effect on our overall analysis is probably quite small, because these highly variable proteins constituted only 8% of the total HCMV proteins assessed.

Characterization of total HCMV-specific T cell responses

Given the extraordinary degree of HCMV ORF immunogenicity documented, it is not surprising that, when the individual ORF-specific responses of seropositive subjects were totaled, the overall HCMV-specific responses were complex and often enormous. As shown in Fig. 4 A, the median total

response frequencies for the overall CD4⁺ and CD8⁺ peripheral blood T cell populations were 4.0% and 4.6% (average, 5.4% and 7.6%), respectively, corresponding to 9.1% and 10.2% (average, 10.7% and 12.8%), respectively, of the memory compartment. Ten of 33 HCMV-seropositive subjects manifested total HCMV-specific CD4⁺ and/or CD8⁺ T cell responses of ≥20% of their circulating memory repertoire. CD4⁺ and CD8⁺ T cells from HCMV-seropositive subjects recognized a median of 12 and 8 ORFs, respectively, but the interindividual heterogeneity was considerable, with the number of ORF-specific CD4⁺ or CD8⁺ responses per person ranging from as few as 1 to as many as 39 (Fig. 4 B). If CD4⁺ and CD8⁺ responses are considered together, we found that individual subjects recognized between 5 and 55 different HCMV ORFs (median, 21). The average size of the CD8⁺ ORF-specific responses in the blood memory compartment of each individual was approximately twice that of the CD4⁺ ORF-specific responses overall, and the range of these averages was significantly larger for the CD8⁺ compartment (Fig. 4, C and D). Moreover, the average size of an ORF-specific CD4⁺ or CD8⁺ T cell population in blood was unrelated to the frequency of ORF recognition in the overall seropositive cohort (Fig. 4 D). Finally, we did not identify a significant relationship between the total HCMV-specific responses within the CD4⁺ vs. CD8⁺ memory compartments, nor was the total HCMV-specific response found within either of these compartments significantly linked to any particular HLA haplotype (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20050882/DC1>).

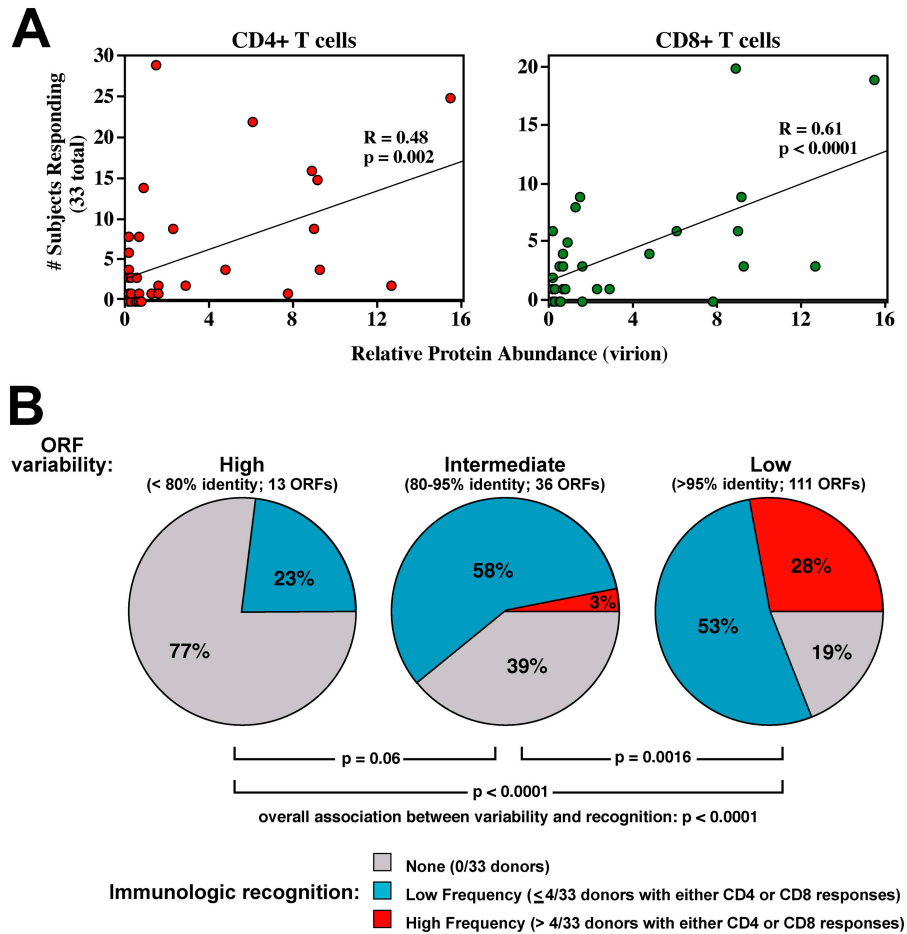


Figure 3. Determinants of T cell recognition of HCMV ORFs: abundance of ORF products in virions and ORF variability among different HCMV strains. (A) The relative abundance of the 25 HCMV proteins found in highly purified HCMV virions, as determined by proteomic analysis (22), is plotted against the frequency of recognition of the corresponding ORF peptide mixes by CD4⁺ and CD8⁺ T cells in the cohort of 33 HCMV-seropositive donors. Note that although virion protein abundance correlates positively with both CD4⁺ and CD8⁺ ORF recognition frequencies, the strength (correlation coefficient, R) and statistical significance (p-value) of this association is considerably stronger for CD8⁺ T cell responses. (B) To determine the impact of HCMV ORF sequence variability on the measurement of T cell immunogenicity of these ORFs, we compared sequence information for the 160 HCMV ORFs that have been sequenced in six

different HCMV strains (AD169, Toledo, Towne, FIX, PH, and TR) and divided these ORFs into three categories: high variability (<80% amino acid identity; 13 ORFs), intermediate variability (80–95% identity; 36 ORFs), and low variability (>95% identity, 111 ORFs). The pie charts show the relative fraction of the ORFs within each of these categories that are frequently recognized by either CD4⁺ or CD8⁺ T cells or both (>4/33 donors; red), infrequently recognized (1–4 donors; blue), or not recognized at all (gray). The statistical significance of the differences in frequency of T cell recognition among three groups of ORFs (high, intermediate, and low variability) was determined by chi square analysis with the p-values between each pair of groups shown. The p-value for the overall strength of the association between ORF amino acid sequence variability and T cell recognition frequencies was <0.0001.

It is clear from these data that a single ORF-specific T cell response, or even several of these responses, will not be representative of the overall status of an individual’s HCMV-specific T cell response. We thus sought to determine the minimal combination of ORF-specific responses that would reliably predict a subject’s total HCMV-specific response. The most successful strategy for this prediction was based on the ranking of ORFs with respect to their total cumulative CD4⁺ or CD8⁺ T cell responses in the HCMV-seropositive cohort (Table S3, available at <http://www.jem.org/cgi/content/full/jem.20050882/DC1>) and then comparing by regression analysis the total CD4⁺ and CD8⁺ T cell re-

sponses to 213 ORFs with the summed responses of the top 1, 2, 3, 4, 5, 6 . . . 212 ORFs using this ranking system. As shown in Fig. 5 A, despite the variable sizes of these ORFs, the sequential addition of the next-ranked ORF resulted in a fairly linear accumulation of peptides. Fig. 5 B demonstrates how the slope of these regression lines increased with the inclusion of additional ORFs. A perfect correlation is reflected by a slope of 1, and slopes <1 reflect the relative fraction of the total available information that is captured with each analysis. Most relevant to our purpose is the Pearson correlation coefficient, which reflects how well each subtotal analysis correlates with the total analysis. Note in Fig. 5 C that the

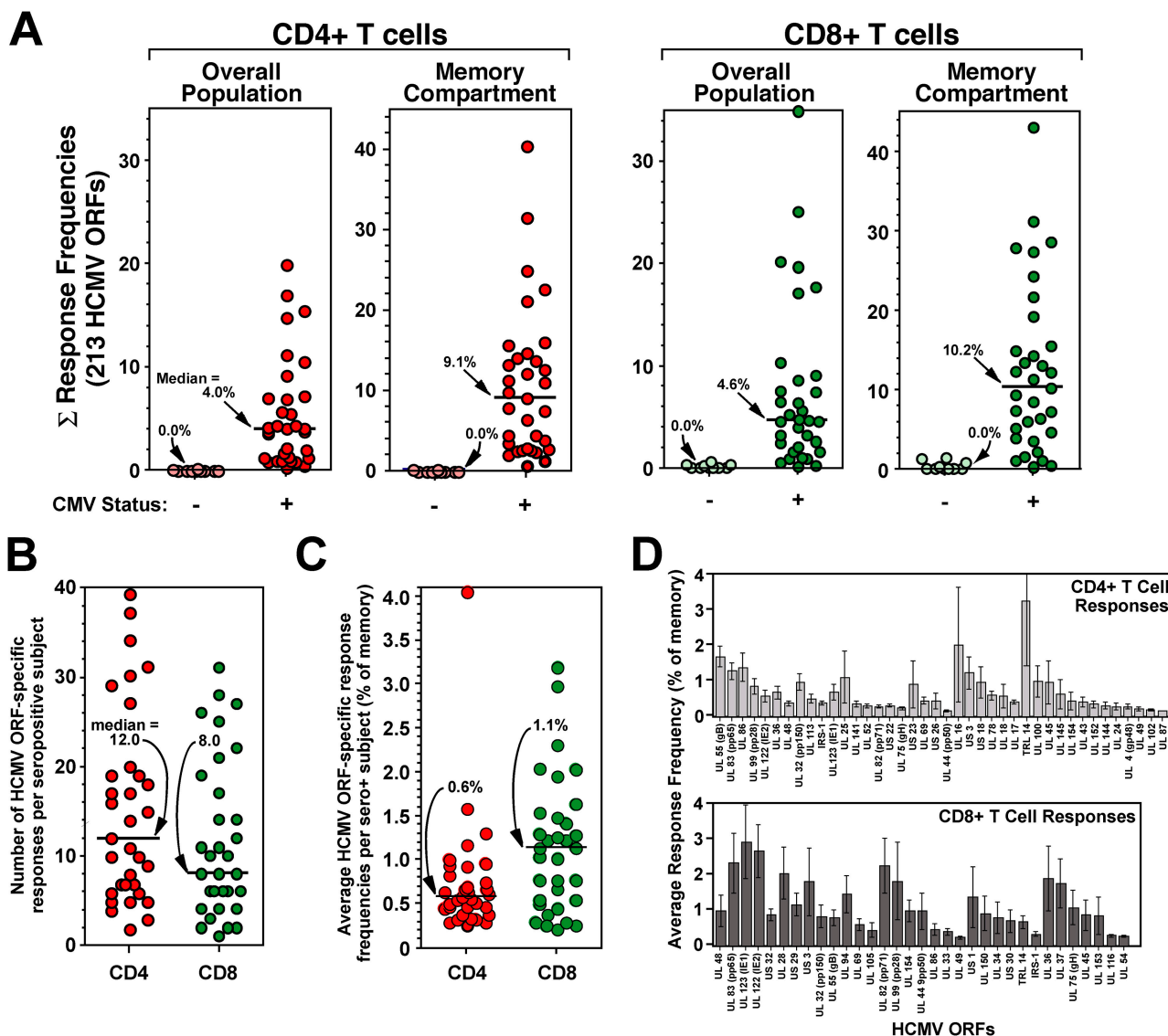


Figure 4. Characterization of total HCMV-specific T cell responses in seropositive subjects.

(A) For each seropositive and seronegative donor, all positive peripheral blood responses to 213 HCMV ORFs were summed (Σ response frequencies) to determine the total HCMV-specific response within the overall or memory CD4⁺ and CD8⁺ peripheral blood T cell populations. The horizontal lines and accompanying arrows and percentages indicate the median total response for the 10 seronegative or 33 seropositive donors. Note that the total Σ HCMV CD4⁺ T cell responses was zero for all 10 seronegative donors. As indicated in Fig. 2, the total Σ HCMV CD8⁺ T cell responses was positive for 4/10 donors, but the median of this distribution remained zero. (B) The number of ORFs recognized by the CD4⁺ or CD8⁺ T cells of each HCMV-seropositive donor is shown. The horizontal line indicates the median value of the

overall population of 33 donors. (C) For each HCMV-seropositive individual, the total HCMV ORF-specific CD4⁺ or CD8⁺ T cell responses in the memory compartment (Σ response frequencies) were divided by the number of responses contributing to that total to arrive at the average size of HCMV ORF-specific responses in each donor (as a fraction of the peripheral blood memory population). The figure shows the distribution of these average sizes with the horizontal line indicating the median value of the overall population of 33 seropositive donors. (D) The figure shows the average size (\pm SEM) of the memory-corrected CD4⁺ or CD8⁺ T cell response to each individual ORF in the individuals that manifested a positive response to that ORF. Only ORFs with responses in at least 4 of 33 donors are shown in descending order with respect to ORF recognition frequency (see Fig. 1).

increase in this coefficient with inclusion of additional ORFs was neither linear nor similar for CD4⁺ and CD8⁺ responses, reflecting the documented differences in the size and variability of ORF-specific CD4⁺ vs. CD8⁺ responses. Achieving a correlation coefficient of 0.9 required analysis of the top 15 ORFs for CD8⁺ responses (1,518 total peptides)

but only the top 6 ORFs for CD4⁺ responses (860 peptides). Fig. 5, D and E shows the actual regression analysis comparing the summed responses of these numbers of ORFs/peptides with the total responses for CD8⁺ and CD4⁺ T cells, respectively. It is noteworthy that CD4⁺ responses to whole HCMV lysates (Fig. 5 F) also correlated

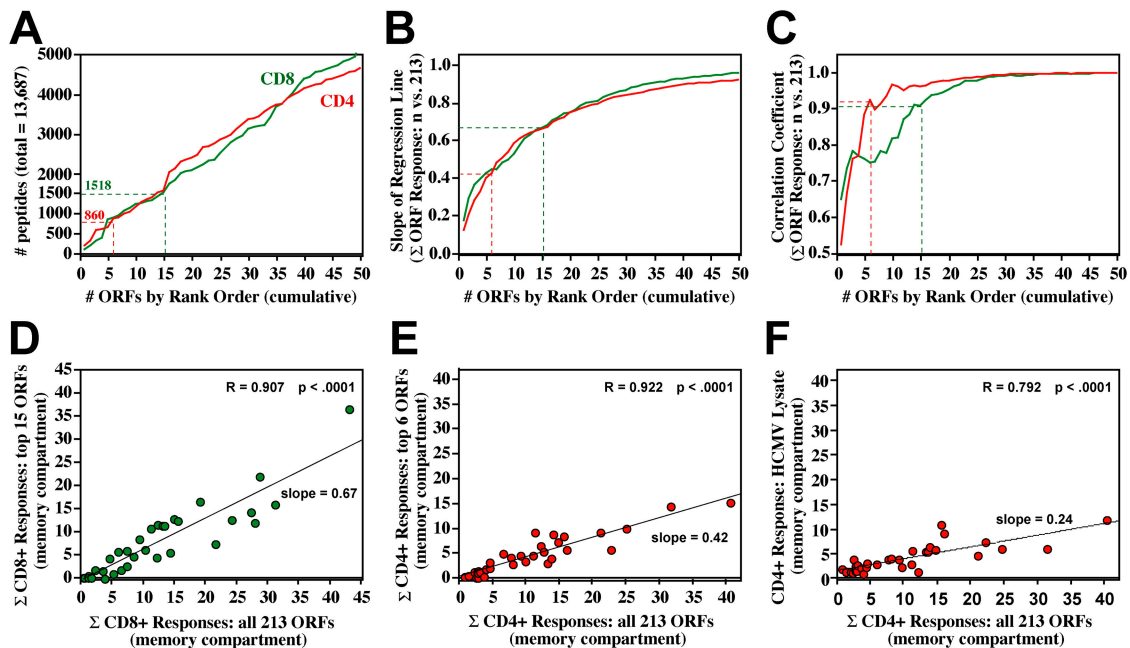


Figure 5. Modeling of the total HCMV-specific T cell response for practical clinical evaluation. (A–C) To ascertain a practical surrogate for the Σ response frequencies to 213 HCMV ORFs, we ranked the HCMV ORFs by their total response in the 33 seropositive donors (e.g., Σ response frequencies in the overall cohort; see Table S3 for ranking) and then performed linear regression analysis comparing the Σ response frequencies of the 213 HCMV ORFs with the Σ response frequencies of the top 1 ORF, the top 2 ORFs, the top 3, top 4, and so on. The x-axis of these graphs indicates the increasing number of ORFs added to each analysis (of 213 total, only 1–50 are shown). In plot A, the number of peptides added to the analysis with each added ORF is shown (of 13,687 total). Plot B

shows the slope of the regression line as a function of increasing ORFs. Plot D shows the relationship between increasing ORFs and the Pearson correlation coefficient. The dotted lines in plot D indicate the minimum number of ORFs required to achieve a correlation coefficient of 0.9; in plots A and B, the corresponding number of peptides and regression slope are indicated. (D and E) Regression analysis comparing Σ response frequencies using 213 HCMV ORFs with the Σ response frequencies of the top 15 ORFs for CD8⁺ responses and the top 6 ORFs for CD4⁺ responses. (F) Regression analysis comparing Σ CD4⁺ response frequencies using all 213 HCMV ORFs with the CD4⁺ response frequencies obtained using whole HCMV lysates as the stimulating antigen.

significantly with total ORF responses, but both the slope and correlation coefficient value of this relationship were significantly less than observed for the summation of the top six CD4⁺ ORFs.

DISCUSSION

In this report, we provide the first comprehensive assessment of human CD4⁺ and CD8⁺ memory T cell responses to the overall HCMV proteome. We demonstrate that normal, HCMV-exposed individuals devote a median of 9.1% and 10.2% of their circulating CD4⁺ and CD8⁺ memory T cell repertoires, respectively, to this virus, recognizing a median of 21 different HCMV ORFs by CD4⁺ T cells, CD8⁺ T cells, or both. With the exception of infrequent CD8⁺ T cell cross-reactivity, HCMV-seronegative, presumably unexposed, subjects lack these responses, highlighting the enormous, permanent influence HCMV infection has on the memory T cell compartment. Moreover, our data may under-represent the actual extent and complexity of the HCMV-specific T cell responses in our subjects because (a) γ -IFN production is not an all-inclusive marker of memory T cell responsiveness (9, 24); (b) 15mer peptides are not optimal agonists for MHC class I-restricted responses (20, 25); (c)

some epitopes may be excluded using a 10–amino acid overlap between the consecutive peptides of our ORF mixes; and (d) strain-specific sequences, recently described ORFs, and ORFs <80 amino acids (26, 27) were not evaluated.

Although there are few comparative data for pan-proteome immunogenicity analysis of other human viral infections, previous reports assessing T cell responses to viral lysates, infected cells, or immunodominant epitopes strongly suggest that the frequencies of HCMV-specific T cells in healthy subjects considerably exceed those observed for other common viruses, including measles, mumps, influenza, adenovirus, poxvirus, and even other persistent Herpes family viruses (e.g., Herpes simplex, Herpes zoster [9, 11, 15, 28–30]) and are comparable with frequencies of HIV-specific T cells in active HIV infection (31). The properties of HCMV that engender such large, sustained T cell responses in the absence of overtly active infection have not been established definitively, but the observation that the high-frequency T cell responses characteristic of CMV infection in mice, nonhuman primates, and humans evolve during the first year of infection ([12, 32–34] and L. Picker, unpublished observations) suggests that the nature of antigen delivery to the immune system during primary infection is a criti-

cal parameter. The relatively slow, prolonged, and limited viral replication of CMV primary infection may simply allow more efficient recruitment of CMV-specific T cells into the memory compartment than do the antigen-presentation patterns of other viruses.

The extent to which these relatively high frequencies of HCMV-specific T cells are required for protection against progressive HCMV infection and disease remains unclear. The rarity of HCMV disease in immunocompetent individuals suggests that even within the lowest decile of HCMV-specific responsiveness (frequencies in blood about 10-fold lower than the median), most exposed individuals have sufficient responses to protect them from HCMV disease throughout their lifetime. At the other end of the response spectrum, our observation that ~30% of adult subjects have HCMV-specific populations comprising >20% of their circulating CD4⁺ and/or CD8⁺ memory T cell repertoire suggests that HCMV-specific T cell responsiveness might be excessive in some individuals, perhaps with detrimental clinical effects. Indeed, current thinking on the pathophysiology of immune senescence supports this notion (6, 7). Elderly individuals with this condition demonstrate high-frequency, oligoclonal CD8⁺ effector-memory T cell responses to HCMV epitopes with concomitant loss of both naive and non-HCMV-specific memory CD8⁺ T cells, and it has been hypothesized that expanded HCMV-specific T cell populations crowd out or otherwise dysregulate immune responses to other potential pathogens (7, 35–38). We did not include elderly subjects in this study but note that it is easy to envision that small changes in the relative survival of CMV-specific vs. non-CMV-specific CD8⁺ memory T cells over a period of years could lead eventually to an overwhelming preponderance of CMV-specific cells and consequent immune dysfunction.

The strikingly high representation of HCMV-reactive memory T cells in HCMV-seropositive subjects contrasts sharply with the almost nonexistent HCMV reactivity of T cells from HCMV-seronegative subjects. We found no evidence of CD4⁺ T cell reactivity with HCMV epitopes in HCMV-seronegative subjects, extending previous work that demonstrated a lack of reactivity to HCMV whole viral preparations in such subjects (9, 39). With respect to CD8⁺ T cell reactivity, we identified an average of 0.7 HCMV ORF-specific responses, comprising 0.39% of memory T cells in blood, per seronegative subject—a total that was ~3% of the total HCMV-specific CD8⁺ T cell reactivity of HCMV-seropositive subjects. These responses focused on only three HCMV ORFs (UL116, US29, US32), suggesting a very limited set of potentially cross-reactive epitopes among the many hundreds of total HCMV epitopes. Experimental studies in mice have suggested that cross-reactivity is common and plays a major role in molding the memory T cell repertoire and in determining the outcome of pathogen encounters (40). Anecdotal observations have demonstrated the existence of such CD8⁺ T cell cross-reactivity in humans (40, 41), but the extent to which cross-reactivity con-

tributes to the configuration of the overall human memory T cell repertoire has remained unclear. As the first comprehensive assessment of this question in humans, our data suggest that cross-reactivity is rare to nonexistent for CD4⁺ T cells and is uncommon for CD8⁺ T cells. Therefore, cross-reactivity does not seem to be a major determinant of the human memory T cell repertoire. However, this finding does not mean that CD8⁺ T cell cross-reactivity is clinically insignificant. With respect to HCMV, it would be of particular interest to determine whether preexistent cross-reactive responses to UL116, US29, or US32 affect the course of primary HCMV infection.

Previous work on HCMV-specific T cell immunity has often focused on a very restricted group of HCMV ORFs, principally UL83 (pp65) and UL123 (IE-1), with the underlying assumption that such responses are immunodominant and representative of the total response to this virus. A recent study examining responses to epitopes predicted by HLA-binding algorithms has cast some doubt on this assumption, suggesting that CD8⁺ T cell recognition is broader than anticipated, including responses to functionally and kinetically diverse ORFs (14). This study extends these observations, definitively demonstrating that T cell recognition of HCMV is complex, often very broad, and poorly approximated by responses to any one or two HCMV ORFs. We found that 151 (70%) of the 213 HCMV ORFs examined were immunogenic for CD4⁺ T cells, CD8⁺ T cells, or both, with 40 ORFs for CD4⁺ T cells and 33 for CD8⁺ T cells recognized by at least 12% of subjects. Significantly, of the 62 ORFs that failed to generate a response in our seropositive subjects, 26 were on a list of 34 ORFs that newer annotation approaches indicate have low coding potential and therefore are unlikely to represent bona fide HCMV ORFs (27). Thus, our 151 immunogenic ORFs may in fact derive from a total of only 187, yielding an actual ORF immunogenicity rate for T cells of 86%. Whether the remaining 14% of ORFs truly lack immunogenicity remains an open question. It is possible that further screening of more subjects or the use of more sensitive assays would demonstrate recognition of all expressed HCMV ORFs; on the other hand, restricted expression, processing restrictions, and/or a paucity of HLA-presentable epitopes might efficiently prevent immunogenicity of a small minority of HCMV-encoded proteins.

Immunogenic ORFs were found in all kinetic and functional categories of HCMV proteins, and, in general, the frequency with which T cells recognized these ORF categories was in proportion to their representation in the HCMV proteome. However, for some ORF classes, the frequency of immune recognition did show statistically significant deviation from what would be expected if epitope selection was random (Table II), most notably, the ~3-fold preferential recognition of IE gene products by CD8⁺ T cells. Because expression of HCMV-encoded MHC class 1 down-regulatory genes initiates in the IE phase (42), it is possible that reduced MHC class I expression in early- to late-stage infected cells might have impeded CD8⁺ T cell recognition of early

and late HCMV proteins, leading to preferential recognition of IE proteins. However, 86% of HCMV ORF-specific CD8⁺ T cell responses were not directed at IE gene products (Table II), confirming recent data by Manley et al. (43) suggesting that MHC class I down-regulation is not a major impediment to afferent CD8⁺ T cell recognition (perhaps because uninfected DCs have a major role in the cross-presentation of HCMV epitopes during CD8⁺ T cell response development). Indirect antigen presentation is the norm for MHC class II-restricted responses, and the preferential recognition of IE genes by CD4⁺ T cells also suggests that factors other than MHC down-regulation contributed to the preferential recognition of IE gene products. One potential contributor to differential HCMV protein recognition is simply the relative expression level of these proteins during *in vivo* infection. Although the overall representation of HCMV proteins during *in vivo* infection is unknown, our observation that protein abundance in virions was significantly associated with the frequency of protein recognition by both CD4⁺ and CD8⁺ T cells supports the importance of this factor. Finally, it is noteworthy that HCMV genes that have a primary function in immune evasion are preferentially immunogenic (Table II). The retention of such genes in the HCMV genome is clear evidence that the likely “cost” of these proteins to viral fitness in terms of enhanced immunogenicity is more than compensated by their immune modulatory activity.

The extraordinary complexity of HCMV-specific T cell responses complicates efforts to understand the basis of the HCMV-immune balance and, in clinical practice, to determine the thresholds that define the boundary between controlled vs. progressive HCMV infection in immune-compromised subjects and between normal and excessive HCMV-specific immunity in the elderly. Total HCMV-specific CD4⁺ and CD8⁺ T cell frequencies are a fundamental measure of the HCMV-specific cellular immune response and serve as an appropriate starting point for determining these thresholds in clinical cohorts. Qualitative aspects of the total responses would almost certainly contribute to protection as well, but these characteristics must still be considered in the context of the quantitative response. Because it is impractical to perform the comprehensive 213-ORF analysis of this report in a clinical setting, we sought to identify a limited combination of ORF-specific responses that, when added together, would efficiently approximate the total response. For CD4⁺ T cell responses, this goal was achieved using only the six most immunogenic ORFs (UL55, UL83, UL86, UL99, UL153, and UL32), requiring 860 15mer peptides. Notably, responses with crude HCMV viral lysates also correlated closely with the total response, but the correlation coefficient was lower (0.79), and the slope of the correlation line (a measure of the total information captured by the analysis) was considerably less (0.24 vs. 0.42, respectively). Modeling the total HCMV-specific CD8⁺ T cell response was more difficult. Whole HCMV lysates are poorly stimulatory for MHC class I-mediated responses (20), and such responses

do not correlate at all with pan-ORF responses (unpublished data). Moreover, because HCMV ORF-specific CD8⁺ T cell responses were more variable than their CD4⁺ counterparts, we needed to sum the response of the top 15 ORFs (UL123, UL83, UL122, UL28, UL48, US3, UL151, UL82, UL94, US29, UL99, UL103, US32, US24, and UL36; 1,518 peptides) to achieve an acceptable correlation coefficient. Because UL83 and UL99 are common to both the CD4⁺ and CD8⁺ ORF lists, analysis of 19 ORF peptide mixes (2,231 total peptides) would provide a very good approximation of an individual's total HCMV-specific T cell response. Using CFC and judicious construction of the peptide mixes (180–200 peptides per test), such analysis could be performed with 15×10^6 PBMCs, a scope well within the range of practical clinical application.

MATERIALS AND METHODS

Subjects. This study was approved by the Institutional Review Board of Oregon Health and Science University. 130 healthy adult human volunteers (all subjects recruited after obtaining written informed consent) were screened for seroreactivity to HCMV using the CMVScan passive latex agglutination test (Becton Dickinson). Of 58 seropositive subjects, 26 were selected for inclusion in the study group based on maximizing the HLA and ethnic diversity. An additional seven separately identified seropositive subjects were added to the study population (for a total of 33), four to increase the breadth of HLA allele and ethnic representation and three as part of identical twin sets (one set concordant for HCMV seroreactivity and the other discordant). Nine seronegative subjects were selected randomly as controls, with the CMV-seronegative twin from the discordant twin set added as the tenth control. In keeping with previous reports (9, 39), CFC assays assessing CD4⁺ T cell responses to whole HCMV lysates were positive (with net response frequencies $\geq 0.3\%$) in all 33 seropositive subjects and were negative in all 10 seronegative subjects ($\leq 0.02\%$). Real-time PCR analysis of HCMV DNA in PBMCs was negative in all studied subjects. All subjects were HLA genotyped by the Puget Sound Blood Center in Seattle, WA.

Cell preparation, antigen stimulation, and staining. Citrated peripheral blood was collected from each donor and processed into PBMCs, as previously described (8). These PBMCs (minimum 500×10^6 for each subject) were cryopreserved in 10% DMSO in FCS at 20×10^6 cells/ml using a Forma Scientific Model 7404 CryoPlus 3 cell freezer (Forma Scientific, Inc.). For each analysis, PBMC aliquots were rapidly thawed at 37°C, diluted with RPMI-1640 medium with 10% heat-inactivated FCS, washed once, and resuspended at 2×10^6 cells in 0.5 ml media in 12×75 -mm polystyrene tubes (Becton Dickinson). An additional 0.5 ml containing 1 μ g each of the costimulatory mAbs CD28 and CD49d (BD Biosciences) and no antigen, titered whole HCMV lysates (AD169 strain, Microbix Biosystems, Inc.), 0.2 μ g Staphylococcal enterotoxin B, or HCMV ORF peptide mixes was added to each tube. These cultures were incubated at a 5° slant from horizontal at 37°C in a humidified 5% CO₂ atmosphere for 6 hr with the final 5 hr including 10 μ g/ml of the secretion inhibitor Brefeldin A (Sigma-Aldrich). After incubation, cells were harvested with Dulbecco's PBS containing 0.5% BSA and 0.1% sodium azide (dPBS/BSA) and were washed before incubation in titered PerCP-Cy5.5-conjugated CD3 mAb (clone SK7, BD Biosciences) for 30 min at room temperature. Cells then were washed once in dPBS/BSA, were fixed and permeabilized with FACSLysing solution (BD Biosciences), and then, after being washed twice in dPBS/BSA, were incubated with FITC-anti-IFN- γ (clone 25723.11), PE-CD69 (clone L78), and APC-CD4 (clone SK3, all mAbs from BD Biosciences). After 30 min incubation at RT, cells were washed twice with cold PBS/BSA, resuspended in 1% paraformaldehyde in dPBS, and then stored at 4°C until analysis. Each PBMC sample was also assessed for the fraction of memory T cells within the CD4⁺ and CD8⁺ T cell populations by cell surface staining with mAbs (a

FITC-CD45RO/PE-CD27/PerCP-CD8/APC-CD95 and (b) FITC-CD45RO/PE-CD27/PerCP-CD4/APC-CD95 (all from BD Biosciences).

Preparation of HCMV ORF peptide mixes. As of June 2000, the initiation of this project, the only annotated, complete genetic sequence for HCMV was for the laboratory strain AD169, and this sequence served as the starting point for our peptide synthesis, providing 191 ORFs, including several alternative splice variants. AD169 had been shown to be missing numerous ORFs compared with other CMV strains (44), so we added 19 ORFs from Toledo and 3 ORFs from Towne strains to our list for a total of 213 ORFs (Table S1). Sequence data were obtained from GenBank/EMBL/DBJ (protein database) site and from the SWISS-PROT database via the EXPASY (expert protein analysis system) site (see Table S1 for accession numbers). ORFs were annotated with regard to expression kinetics and function as described in Table S1. Consecutive 15mer peptides, overlapping by 10 amino acids, were synthesized for each ORF by Mimotopes Pty., Ltd., using solid-phase peptide synthesis methods, employing an Fmoc synthesis strategy, with simultaneous side-chain deprotection and cleavage techniques incorporating scavengers (to maximize purity and yield), to liberate the peptides from solid phase. Peptides with free acid C-termini and free-amine N-termini were lyophilized in 96-tube racks, each with two controls that were analyzed for quality control by mass spectrometry, HPLC, and amino acid analysis. Lyophilized peptides were reconstituted in 5–40% acetic acid in acetonitrile. Peptides associated with each of the 213 ORFs were pooled, lyophilized, and reconstituted in pure DMSO, so that each peptide was at a minimum concentration of 0.5mg/ml. 4 μ l of these preparations was added to PBMC so that final stimulatory peptide concentrations were always ≥ 2 μ g/ml per peptide.

Flow cytometry and data analysis. Six-parameter flow cytometric analysis was performed on a two-laser FACSCalibur instrument (BD Biosciences) using CellQuest v3.3 software (BD Biosciences). List-mode multi-parameter data files (each file with forward scatter, orthogonal scatter, and four-fluorescent parameters) were analyzed using the PAINT-A-GATE Plus software program (BD Biosciences), as described (8). Files were gated on CD3⁺, CD4⁺ and separately on CD3⁺, CD4⁻ small lymphocytes with responding populations defined as CD69⁺/IFN- γ ⁺. Approximately 20% of positive CD4⁻ T cell responses were re-examined using a panel including a CD8 mAb, and in all cases the CD3⁺, CD4⁻ responses were confirmed as CD8⁺. A minimum of 20,000 gated events and background reactivity <0.06% (e.g., in the absence of peptides) was required for an acceptable data point. Analyses not meeting these criteria were repeated. To establish a precise definition of positive responses, we analyzed the net (e.g., minus background) CD4⁺ and CD8⁺ T cell responses to the 213 HCMV ORFs in the HCMV-seronegative subjects. With the exception of seven CD8⁺ responses (which were excluded from this analysis; see Results), the flow cytometric profiles of these analyses lacked clear-cut response clusters, and net response frequencies of both the CD4⁺ and CD8⁺ analyses formed a normal distribution with a mean of 0, and 4 SD equaling 0.06% for CD4⁺ analyses and 0.08% for CD8⁺ analyses. These 4-SD frequencies were adopted as our cut-off for a positive response to rule out stringently any spurious responses in our overall data analysis. Thus, net response frequencies of <0.06% for CD4⁺ T cells and <0.08% for CD8⁺ T cells were counted as zero response. To ensure accuracy, we repeated all analyses within 0.02% of the cut-off, and if two consecutive repeat analyses showed net response frequencies above the cut-off, the mean of these responses was counted. When ORFs were analyzed in parts, a positive response by these criteria by any ORF sub-mix constituted an ORF-specific response, and if one or more such positive responses was identified, these responses were summed to calculate a total ORF-specific response frequency. For memory correction, total ORF-specific response frequencies were divided by the fraction of the CD4⁺ or CD8⁺ T cells with a memory phenotype, as previously described (8). Note that the term “memory” is used to denote the overall compartment of previously activated, peripheral T cells, including “effector” or “effector memory” populations.

Statistical analyses. Linear regression analysis was used to determine the correlation between (a) the response profiles of identical twins, (b) ORF recognition vs. ORF abundance, (c) CD4 vs. CD8 responses, and (d) Σ 213 ORF responses to Σ 1–212 ORF responses. ANOVA was used to assess the association between the average positive response and ORF kinetics/function. A one-sample test for a proportion was used to compare the frequency among positive responses with the expected frequency from the genome coding space. Because we performed multiple tests within each class, the Bonferroni adjustment was used to control the overall type 1 error of 5%. Thus, a p-value < 0.05/K is considered significant, where K is the number of categories in each class. A chi-square test was used to assess an association between the recognition frequencies and ORF variability. Statistical tests were performed with Statistical Analysis System Version 9.0 or Statview 5.0 (SAS Institute).

Online supplemental materials. Table S1 provides the amino acid sequences of, and the functional and kinetic annotations for, the HCMV ORFs studied in this report, as well as the original source material for this information. Table S2 provides demographic information, HLA types, and a complete listing of the HCMV ORFs recognized by CD4⁺ and CD8⁺ T cells (listed separately) for each of the HCMV-seropositive subjects. Table S3 provides the complete database for the response modeling shown in Fig. 5. All HCMV ORFs were rank ordered with respect to their total immunogenicity in the HCMV-seropositive cohort (Σ of the total response frequencies in the memory compartments of all 33 seropositive subjects), and then the total response of each subject to all 213 tested ORFs (Σ 213 ORF response frequency) was sequentially compared with the top ORF, top 2 ORFs, top 3 ORFs, and so forth (Σ 1 – 212 ORF response frequencies). The correlation coefficients, the slope of the regression lines, and the 95% confidence limits of these statistical analyses are indicated, as well the number of peptides corresponding to each analysis. Online supplemental material available at <http://www.jem.org/cgi/content/full/jem.20050882/DC1>.

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