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Clonotypic Structure of the Human CD4⁺ Memory T Cell Response to Cytomegalovirus¹

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High steady-state frequencies of CMV-specific CD4⁺ memory T cells are maintained in CMV-exposed subjects, and these cells are thought to play a key role in the immunologic control of this permanent infection. However, the essential components of this response are poorly defined. Here, we report the use of a step-wise application of flow cytometric and molecular techniques to determine the number and size of the TCR V β -defined clonotypes within freshly obtained CMV-specific CD4⁺ memory T cell populations of four healthy, CMV-exposed human subjects. This analysis revealed a stable clonotypic hierarchy in which 1–3 dominant clonotypes are maintained in concert with more numerous subdominant and minor clonotypes. These dominant clonotypes accounted for 10–50% of the overall CMV response, and comprised from 0.3 to 4.0% of peripheral blood CD4⁺ T cells. Two subjects displayed immunodominant responses to single epitopes within the CMV matrix phosphoprotein pp65; these single epitope responses were mediated by a single dominant clonotype in one subject, and by multiple subdominant and minor clonotypes in the other. Thus, the CMV-specific CD4⁺ T cell memory repertoire in normal subjects is characterized by striking clonotypic dominance and the potential for epitope focusing, suggesting that primary responsibility for immunosurveillance against CMV reactivation rests with a handful of clones recognizing a limited array of CMV determinants. These data have important implications for the understanding of mechanisms by which a genetically stable chronic viral pathogen such as CMV is controlled, and offer possible insight into the failure of such control for a genetically flexible pathogen like HIV-1. *The Journal of Immunology*, 2001, 167: 1151–1163.

A number of viral pathogens—herpes family viruses, retroviruses, among others—have the capacity to establish chronic, often permanent, infection in which effective host immunity serves to contain the pathogen and prevent disease, but does not eliminate the infection (1). Although primary responsibility for immune effector activity against these pathogens is usually attributed to Ab or CD8⁺ CTL responses, there is increasing appreciation for the role of CD4⁺ T cells in 1) supporting high affinity Ab production, 2) initiating, and particularly, maintaining CTL numbers and function, and 3) performing direct effector activity, largely via elaboration of cytokines (2–6).

Among chronic viral infections of the human, the importance of CD4⁺ T cells in viral control is best appreciated for the β -herpes family virus CMV. This virus infects >60% of adults, usually following clinically benign primary infection in either early childhood or adolescence, and remains as a latent or low level infection without end organ disease in immunocompetent individuals (7, 8). Reactivation and disease occurs most commonly in the setting of post transplant immunosuppression and in late HIV-1 infection, the latter usually when peripheral blood CD4⁺ T cells drop below ~50 cells/ μ l (9–11). The close association between the degree of

CD4⁺ T cell deficiency and CMV disease in HIV-1 infection is consistent with a crucial role for CD4⁺ T cells in control of CMV reactivation—an hypothesis that is sustained by the recent specific correlation of end organ CMV disease in the AIDS setting with loss of CMV-specific CD4⁺ T cell responses (12). In addition, a clinical trial in which adoptive transfer of CMV-specific CD8⁺ CTL clones was used to treat post transplant CMV infection demonstrated that persistence of transferred cytolytic activity correlated with the availability of CMV-specific CD4⁺ T cell help (13). The potential importance of CD4⁺ T cells in CMV control is also suggested by the tremendous investment of overall CD4⁺ memory T cell “resources” in CMV. Among normal CMV seropositive individuals, CD4⁺ memory T cells specific for CMV determinants included in the virions themselves or crude viral lysates average about 2.0% of total CD4⁺ T cells (14), frequencies that are generally an order of magnitude greater than that of CD4⁺ memory T cells specific for nonpersistent viruses such as influenza, measles, mumps, or adenovirus (L. Picker, unpublished data). Moreover, in progressive HIV-1 infection, CMV-specific CD4⁺ T cells are often selectively preserved relative to other specificities, and CMV-specific frequencies >5% of CD4⁺ T cells are not uncommon (12, 15, 16).

However, observations to date have not defined either the precise role of CD4⁺ T cells in protection against CMV, or the essential component(s) of a “protective” CD4⁺ T cell response. Given the genetic complexity of the CMV genome (>200 open reading frames) and the ability of this virus to infect a variety of cell types throughout the body, to achieve latency, and to subvert the immune response (7), the requirements for protection are likely to be complex, involving coordinate activity of individual memory T cell clonotypes with heterogeneous specificity and perhaps function. Here, we begin to address this issue by “dissecting” the presumably protective CMV-specific CD4⁺ T cell responses of

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healthy CMV seropositive subjects down to the level of the T cell clonotype, operationally defined as the cohort of CMV epitope-specific CD4⁺ T cells bearing the same TCR-V β -chain. Although clonotypic complexity has been explored for virus-specific CD8⁺ T cells in the human (17–23), there is little information on the parallel CD4⁺ T cell responses (20). Using T cells taken directly from the blood, we demonstrate that CMV-specific CD4⁺ T cell memory is characterized by a hierarchical pattern of clonal dominance in which 1–3 clones dominate the response, but are maintained in concert with a cohort of subdominant clones and numerous minor clones. These findings define a clonotypic structure for CMV-specific CD4⁺ T cell memory, which has significant implications for the mechanisms controlling T cell memory in the human, and the design of vaccines aimed at providing protection against CMV and other chronic viral infections.

Materials and Methods

Cell preparation and Ag stimulation

PBMC were isolated from heparinized or citrated venous blood by density gradient sedimentation using Ficoll-Hypaque (Histopaque-1077; Sigma, St. Louis, MO). Cells were then washed twice in HBSS (Ca²⁺/Mg²⁺-free, Cellgro/Mediatech; Fisher Scientific, Federal Way, WA) and resuspended in RPMI 1640 medium (HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated FCS (HyClone), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), and 50 μ M 2-ME (Sigma). Ag stimulations for intracellular cytokine staining were performed as follows: PBMC were placed in 17 \times 100-mm polypropylene tissue culture tubes (Sarstedt, Newton, NC) at 1 \times 10⁶ cells/ml complete medium (1–10 ml/tube) with appropriately titered “whole” CMV viral preparations (~40 μ l preparation/ml), recombinant CMV pp65 (1 μ g/ml), CMV pp65 15 mer peptide(s) (see below) or no Ag as a negative control (previously shown to be equivalent to mock virus preparations; Ref. 15), and the costimulatory mAbs CD28 and CD49d (1 μ g/ml each; these mAbs provide exogenous costimulation so as to allow the total cohort of Ag-specific cells to respond in this assay; Ref. 14). The cultures were routinely incubated at a 5° slant at 37°C in a humidified 5% CO₂ atmosphere for 6 h with the final 5 h including 10 μ g/ml of brefeldin A (Sigma). After incubation, cells were harvested by washing in cold (4°C) Dulbecco's PBS (dPBS³; Life Technologies, Rockville, MD) with 0.1% BSA (Roche Molecular Biochemicals, Indianapolis, IN) and processed for immediate staining. Ag stimulation for cell surface CD69 and CD40 ligand (CD40L) staining and surface IFN- γ capture were performed similarly, except brefeldin A was not included, and the cultures were incubated for a total of 5 h. For surface IFN- γ capture analysis, harvested cells were labeled with IFN- γ capture reagent, and then incubated for an additional 45 min at 37°C per the manufacturer's instructions (Miltenyi Biotech, Auburn, CA).

Immunofluorescent staining and flow cytometric analysis and sorting

For intracellular cytokine analysis, stimulated cells were first stained on the cell surface with directly conjugated TCR-V β or CD3 mAbs (30 min at 4°C), washed once with cold dPBS/BSA before resuspension in fixation/permeabilization solution (BD Biosciences, San Jose, CA) at 2 \times 10⁶ cells/ml, and incubated for 10 min at room temperature in the dark. Fixed and permeabilized cells were washed twice with cold dPBS/BSA and then incubated on ice (protected from light) with directly conjugated cytokine, CD69, and CD4 mAbs for 30 min. For cell surface staining only, the fixation/permeabilization steps were omitted. After staining, the cells were washed, resuspended in 1% paraformaldehyde in dPBS (for analysis) or dPBS/BSA (for sorting), and then kept protected from light at 4°C until analysis or sorting on the flow cytometer. Five- or 6-parameter flow cytometric analysis was performed on a 2-laser FACSCalibur instrument using FITC, PE, PerCP, and allophycocyanin as the 4 fluorescent parameters. List mode multiparameter data files (each file with forward scatter, orthogonal scatter, and 3–4 fluorescent parameters, and including 30,000–250,000 events after gating on CD4⁺ small T cells) were analyzed using the PAINT-A-GATE^{Plus} software program (BD Biosciences). In some instances, live gating on TCR-V β ⁺, CD4⁺ or γ -IFN⁺/CD69⁺, CD4⁺ T cell subsets (with collection up to 10,000 gated events) was performed to enhance quantification of small populations. These procedures and criteria

for delineating and quantifying responding (CD69⁺/cytokine⁺) vs nonresponding T cells have been previously described in detail (14–16).

Five-parameter fluorescence-activated cell sorting was performed using a two-laser FACSVantage SE flow cytometer (BD Biosciences). Viable CMV-reactive cells (required for RT-PCR) were sorted on the basis of cell surface expression of CD4 (FITC), CD69 (allophycocyanin), and either CD40L (PE; subjects 1–3) or surface IFN- γ (PE; subject 4). For PCR analysis, cells can be analyzed after fixation/permeabilization; therefore, cells were sorted on the basis of intracellular expression of IFN- γ (FITC), CD69 (PE), and CD4 (allophycocyanin). Sorted populations were used immediately for RT-PCR, or stored at –80°C for PCR analysis.

Ags and Abs

CMV Ag preparations were obtained from BioWhittaker (Walkersville, MD), used in subjects 1–3, and Microbix Biosystems, (Toronto, Ontario, Canada), used in subject 4 (both preparations provided equivalent results). Recombinant CMV pp65 was obtained from Austral Biologicals (San Ramon, CA). CMV pp65 peptides (consecutive 15 mers overlapping by 11 aa) were custom synthesized by Dr. D. Stoll (Natural and Medical Sciences Institute of the University of Tuebingen, Tuebingen, Germany) based on the pp65 sequence of CMV strain AD169. Peptide sequences were confirmed by electrospray mass spectroscopy. To prepare total pp65 mixes, the 138 overlapping peptides were individually solubilized in DMSO (Sigma) at 100 mg/ml, and mixed together so that the final concentration of each individual peptide was 0.72 mg/ml. Two microliters of this mixture were used per milliliter of cell stimulation medium (1.45 μ g/ml final concentration of each peptide). Such peptide mixes efficiently reveal the CD4⁺ T cell response to protein Ags as shown by the strong correlation of frequencies observed with these peptide mixes vs whole recombinant protein, and by the observation that the sum of responses to individual epitopes within the Ag in question closely approximates the responses observed with the total mix preparation.⁴ A matrix of 24 overlapping peptide pools, each containing 12 peptides, was constructed and used for rapidly identifying the specific epitopes responsible for the overall pp65 response, as previously described (24). Each peptide in these pools and single peptides were used at a final concentration of 2 μ g/ml. mAbs SK3 (CD4; PerCP-, allophycocyanin-conjugated), SK7 (CD3; PerCP, allophycocyanin); L78 (CD69; PE, PerCP, allophycocyanin), L293 (CD28; unconjugated), L25.3 (CD49d; unconjugated), 89-76 (CD40L or CD154; PE), 25723.11 (anti-IFN- γ ; FITC, allophycocyanin), 5344.111 (anti-IL-2; FITC), and IgG1 and IgG2 isotype-matched controls were obtained from BD Biosciences. TCR V β mAbs (V β 1, 2, 3, 5.1, 5.2, 5.3, 7, 8.1/8.3, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 21.3, 22, and 23) were obtained from Coulter/Immunotech (Hialeah, FL). The anti-IFN- γ mAb (PE) used for surface IFN- γ staining was obtained from Miltenyi Biotech.

RT-PCR spectrotyping and clonotype characterization

Equivalent numbers of sorted CMV-reactive and nonreactive CD4⁺ T cells (subjects 1–3) were washed and resuspended in 5.0 μ l of dPBS to which 5 μ l GeneReleaser solution (BioVentures, Murfreesboro, TN) was added. The lysate was then processed in a Perkin-Elmer (Norwalk, CT) 9600 thermocycler for a series of heating and cooling cycles per the manufacturer's protocol, and finally was clarified by centrifugation (3000 rpm \times 5 min at 4°C). In some experiments (subject 4), total RNA was isolated by TRIzol reagent, per manufacturer's instructions (Life Technologies). RT-PCR mix (RT-PCR buffer with 1.5 mM MgCl₂; Roche Molecular Biochemicals), 0.2 mM dNTP mix (Roche Molecular Biochemicals), 5.0 μ l [α -³²P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ), 5 mM DTT (Roche Molecular Biochemicals), 10 U RNAGuard (Amersham Pharmacia Biotech), 0.5 μ M 5' primer, 0.5 μ M 3' primer, and 1.0 μ l Titan enzyme mix (AMV and Expand High Fidelity PCR-System; Roche Molecular Biochemicals) was added to either GeneReleaser lysate or total RNA, and reverse transcription was conducted at 50°C for 30 min followed by 5 min of inactivation at 95°C. This was in turn followed by 35 cycles of PCR (denaturation at 94°C for 0.5 min, annealing at 60°C for 0.5 min, and extension at 68°C for 0.5 min) with a final extension at 68°C for 2 min.

Six microliters of the final RT-PCR volume was added to 4 μ l of formamide/dye stop solution, heated at 95°C for 2 min, and applied to a 4% acrylamide sequencing gel (Zaxis, Hudson, OH). Autoradiography was performed on dried gels, and after scanning, spectratyping bands were quantified by densitometry using NIH Image 1.62 software. The DNA from the dominant bands was eluted from the dry gel by precisely cutting and

⁴ H. T. Maecker, H. S. Dunn, M. A. Suni, E. Khatamzas, C. J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T.-M. Fu, E. Sinclair, B. M. Bredt, J. M. McCune, V. C. Maino, F. Kern, and L. J. Picker. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *Submitted for publication.*

³ Abbreviations used in this paper: dPBS, Dulbecco's PBS; CD40L, CD40 ligand.

placing the dried gel band in a microfuge tube. One hundred microliters water was added to each tube, and the tubes were heated at 100°C for 10 min and then microcentrifuged at $13,000 \times g$ for 5 min. The supernatant was removed, and 20 μ l from each eluted DNA sample was used in a separate PCR using the appropriate V β and C β specific primers. The PCR products were purified on a 2% agarose gel. Each band was cut out of the agarose gel, DNA was extracted (Concert Matrix Gel Extraction System; Life Technologies) and cloned into pGEM vector (Promega, Madison WI), and JM109 High Efficiency Competent Cells (Promega) were transformed. White colonies were picked, and plasmid DNA was isolated (Promega) and submitted for sequencing. Analysis of sequence data was performed using MacVector software (Oxford Molecular, Madison, WI).

The contribution (%) of each identified clonotype to the total population of peripheral blood CMV-specific CD4⁺ memory T cells was estimated as follows: (% contribution of each TCR-V β family to the total response by cytokine flow cytometry) \times (the density of the clonotype containing spectratyping band/total density of all spectratyping bands for that V β) \times (the fraction of clonotype sequences/total sequences identified within that band). Individual clonotypes were defined as dominant, subdominant, or minor if they accounted for ≥ 10 , 3–10, and $< 3\%$ of the total CMV response, respectively.

Clonotype-specific and VB-JB PCR

Clonotype-specific and JB-specific primer pairs were designed/selected for quantitative PCR and/or semiquantitative PCR such that the 3' primers

anneal in the CDR3 or JB region and the 5' primers anneal upstream in the V β region (Table I). Whole PBMC or sorted CMV-reactive and nonreactive CD4⁺ T cells were aliquoted in microfuge tubes and pelleted by centrifugation at $13,000 \times g$ for 5 min. Fifty microliters of 10 mM Tris-HCl, pH 7.4, containing PCR Grade Proteinase K (50 μ g/ml; Roche Molecular Biochemicals) was added to the cell pellets, and the lysate was incubated overnight at 56°C. Proteinase K was then inactivated at 95°C for 10 min. For semiquantitative PCR, 1 μ l DNA (1 μ g/1 μ l for whole PBMC or containing the number of cells designated in the figures for sorted populations) from each sample was combined with PCR mix containing PCR buffer (20 mM Tris-HCl, pH 8.0, and 50 mM KCl), 0.2 mM dNTP mix (Roche Molecular Biochemicals), 1.5 mM MgCl₂, 0.5 μ M 5' primer, 0.5 μ M 3' primer, 5.0 μ Ci [α -³²P]dCTP (Amersham Pharmacia Biotech), and 2.5 U Platinum *Taq* DNA Polymerase (Life Technologies). Generally, the PCR protocol included denaturation at 94°C for 1 min, 35 cycles of PCR (denaturation at 94°C for 0.5 min, annealing at 60°C for 0.5 min, and extension at 72°C for 0.5 min), and a final extension at 72°C for 2 min. The PCR conditions were optimized for each primer pair by varying annealing time, temperature, and extension time. The PCR products were applied to a 6% polyacrylamide gel (Invitrogen, Carlsbad, CA) and visualized by exposing a phosphor screen (Amersham Pharmacia Biotech). Analysis of data was performed using ImageQuant software (Amersham Pharmacia Biotech). For VB-JB PCR, analysis required cloning the products into bacteria and sequencing, as described above.

Table I. PCR primer sequences

Subject	5' Primer	3' Primer
	CB	CB
	AATGGAAGGAGGTGCACAG	ATAGAGGATGGTGGCAGAC
	VB	CB
	5.1 GATGAATGTGAGCACCTTGAG	AGATCTCTGCTTCTGATGGCTC
	7 CCTGAATGCCCAACAGC	AGATCTCTGCTTCTGATGGCTC
1	12 CTCTGGAGTCCGCTACCAGT	AGATCTCTGCTTCTGATGGCTC
	17 ACTGTGACATCGGCCAAAA	TGTGCACCTCCTTCCCATTCA
	18 GGATCCAGCAGGTAGTGCG	AGATCTCTGCTTCTGATGGCTC
	7 CCTGAATGCCCAACAGCTCTC	TGTGCACCTCCTTCCCATTCA
2	16 AGAGTCTAACAGGATGAGTCC	TGTGCACCTCCTTCCCATTCA
	17 ACTGTGACATCGGCCAAAA	TGTGCACCTCCTTCCCATTCA
	1 CACAACAGTTCCTGACTTGCA	TGTGCACCTCCTTCCCATTCA
3	8 TCTGGTACAGACAGCCATGAT	TGTGCACCTCCTTCCCATTCA
	13.1 GACCAAGGAGAAGTCCCAA	TGTGCACCTCCTTCCCATTCA
	2 ATACGAGCAAGGCGTCGAGAAG	TGTGCACCTCCTTCCCATTCA
4	5.1 GAGTGAGACACAGAGAAACAAA	TGTGCACCTCCTTCCCATTCA
	22 AAGTGATCTTGCCTGTGTCCCA	TGTGCACCTCCTTCCCATTCA
	VB	CDR3
	5.1 TCCCTGGTCGATTCTCAGGGCG	GTTGCCCCAGCCTCCTGTCCC
1	12 TGGATGCTGGAATCACCCAGAGCC	GCCCGAAGTACTGGGCTCTTAGGTC
	17 GGTACAGCTCTCTCGGGAG	GAACTCCCTGTCCCAAGA
	7 CTAAACCTTACCTACACGCC	GTATCTGTTCTCCGAATCGATC
2	16 CCCAGCCACAGCGTAATAGAG	TGCCATTGTACCCTGCCIGAGG
	17 CTGAAGGTTACAGCGTCTCT	TGTAGCCATAGTTATCCCGG
	8 TCTGGTACAGACAGCCATGAT	GTCTCTTGGAGCCCTGTCCCGC
3	13.1 TGACAAGACCCAGGCATGG	TGCTGGGGCTGATTACCCTG
	VB	JB
	18 GGATCCAGCAGGTAGTGCG	1.3 CAACTTCCCTCTCCAAAATAT
1	18 GGATCCAGCAGGTAGTGCG	2.3 AGCACTGTCAGCCGGGTG
	1 CACAACAGTTCCTGACTTGCA	2.7 ACCGTGAGCCTGGTGTCC

For quantitative PCR, an internal standard was constructed that was identical with the target TCR template (i.e., same primer binding sequences) except that internal sequences were deleted so as to make the standard 20% shorter than the endogenous product. The internal standard and the endogenous DNA amplification efficiency were determined to be equivalent, as described (25, 26). To a series of serial dilutions of endogenous DNA Q-PCR mix containing PCR buffer (20 mM Tris-HCl, pH 8.0, and 50 mM KCl), 0.2 mM dNTP mix (Roche Molecular Biochemicals), 1.5 mM MgCl₂, 0.5 μM 5' primer, 0.5 μM 3' primer, 5.0 μCi [α -³²P]dCTP (Amersham Pharmacia Biotech), 100 molecules internal standard, and 2.5 U Platinum *Taq* DNA Polymerase (Life Technologies) was added. PCR included denaturation at 94°C for 1 min, followed by 35 cycles of PCR (denaturation at 94°C for 0.5 min, annealing at 65°C for 0.5 min, and extension at 72°C for 0.5 min) and a final extension at 72°C for 2 min. The PCR products were applied to a 6% polyacrylamide gel (Invitrogen) and visualized by exposing a phosphor screen (Amersham Pharmacia Biotech). The template band intensities were then compared with intensities of the internal standard bands using ImageQuant software (Amersham Pharmacia Biotech) and, where equivalent (after correcting for any differences in GC content between standard and template), represent the dilution of cells containing 100 copies of clonotype DNA, and therefore 100 clonotype⁺ cells.

Results

Cytokine flow cytometry reveals pronounced skewing of TCR-V β usage by CMV-specific CD4⁺ memory T cells

We have previously demonstrated the efficient and specific quantification of CMV-specific CD4⁺ memory T cells by short-term (6 h) *in vitro* activation of secretion-inhibited PBMC with CMV Ag preparations in the presence of optimal exogenous costimulation (CD28 and CD49d mAbs), intracytoplasmic staining for IFN- γ , CD69, and CD4, and multiparameter flow cytometric analysis (14–16). This technique, termed cytokine flow cytometry, allows detection of CMV-specific T cells before either Ag-induced proliferation or cell death, and therefore offers the potential of delineating the clonotypic content of CMV-specific CD4⁺ T cells as it exists *in vivo*, unaltered by long-term *in vitro* culture (27).

Because precise delineation of CMV-responsive CD4⁺ T cells requires three fluorescent parameters (cytokine, CD69, and CD4), the fourth parameter of four-color flow cytometry remains available for the phenotypic dissection of CMV-specific CD4⁺ T cell response complexity by cell surface staining with a panel TCR V β family and subfamily mAbs. Fig. 1 illustrates a typical example of such analysis in a healthy CMV seropositive individual. Although the CD3/TCR down-regulation that accompanies TCR ligation and downstream signaling (14, 28, 29) diminishes the level of TCR V β expressed on the surface of the Ag-activated cells, the contribution of each TCR V β family and subfamily to the overall response is clearly evident. In Fig. 2, complete analysis of four additional CMV seropositive individuals shows that 1–3 TCR V β families or subfamilies dominate the CD4⁺ T cell CMV response (comprising 9–50% of the overall response each), with the remaining TCR V β families and subfamilies examined making minor contributions or, in many instances, no significant contribution at all. Given the fact that the available panel of TCR V β mAbs only covers 62–73% of the total TCR V β repertoire (Fig. 2), these results indicate a profound skewing of TCR V β usage in the CMV-specific CD4⁺ T cell memory population. This general pattern of TCR V β skewing was similar in a total of eight subjects examined, but in each of these subjects, the dominating TCR V β families or subfamilies were different. In two subjects, we had the opportunity to re-evaluate TCR V β usage among circulating CMV-specific CD4⁺ T cells over time. As shown in Fig. 3, the hierarchy of dominant and subdominant TCR V β families and subfamilies remained unchanged over a period of 20–25 months in these normal subjects, even though in one subject (subject 3), the overall frequency of CMV-specific CD4⁺ T cells in peripheral blood increased by almost 4-fold over the period of observation.

CMV + CD28 and CD49d

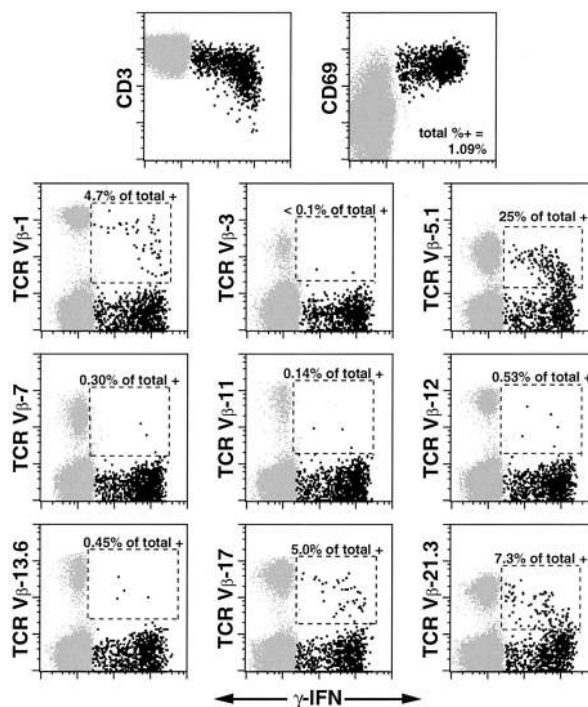


FIGURE 1. Determination of TCR-V β usage among CMV-specific CD4⁺ memory T cells by cytokine flow cytometry. PBMC from a CMV seropositive subject (44-year-old female; DR 9, 17; DRw, DQ: 52, 53, 2, 3) were stimulated with CMV Ag + CD28 and CD49d for 6 h in the presence of the secretion inhibitor brefeldin A for the final 5 h, and then examined for their correlated expression of cell surface CD3 or TCR-V β (stained before fixation and permeabilization) and intracellular IFN- γ , CD69, and CD4 (stained after fixation and permeabilization). One hundred thousand events, gated on CD4⁺ small lymphocytes, are shown with the events within the total responding population (IFN- γ ⁺ and CD69⁺) enlarged and colored black. The top two dot plots demonstrate the response within the overall CD4⁺ population as a function of CD3 expression with the total %+ shown in the *right* profile (total %+ was essentially identical in all analyses; mean \pm SD = 1.09 \pm 0.05%; range = 0.98–1.15%). The remaining dot plots illustrate the distribution of the total response among various mAb-defined TCR-V β families or subfamilies with the fraction of the total CMV response attributable to each TCR-V β provided in each dot plot. As would be expected (14, 28, 29), TCR (and CD3) expression is substantially down-regulated in the responding population, but the intensity of staining for TCR remains sufficient to delineate the contribution of each V β family/subfamily to the overall response (see dashed boxes).

Determination of the clonotypic composition of the dominant and subdominant TCR V β families and subfamilies comprising the human CMV-specific CD4⁺ memory T cell repertoire

The marked skewing of TCR V β usage among CMV-reactive CD4⁺ memory T cells suggest a substantial limitation on the number of major T cell clonotypes involved in this response, and indirectly, on the number of CMV epitopes to which these clonotypes are directed. To confirm this suggestion, we sought to define molecularly the participating clonotypes on the basis of their specific TCR β CDR3 region sequences (corresponding to the Ag combining site of the TCR β -chain). The most direct approach to achieve this goal would be the isolation of total CMV-specific CD4⁺ T cells by FACS, followed by the analysis of the TCR β repertoire of these cells by RT-PCR spectratyping using TCR-CB and -VB primers (30, 31), and finally the cloning and sequencing of the RT-PCR product(s). Prior definition of the major TCR V β families involved in the response by cytokine flow cytometry

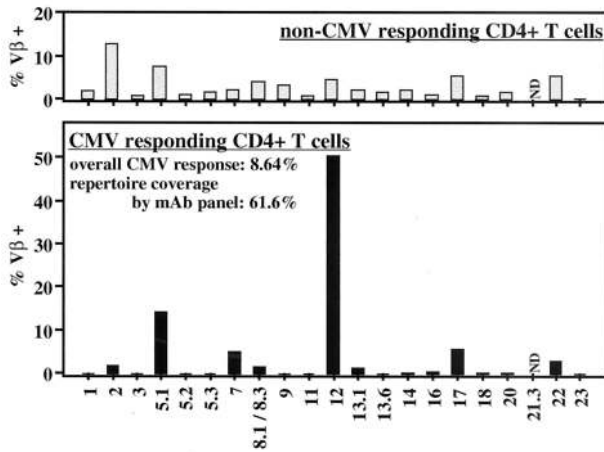
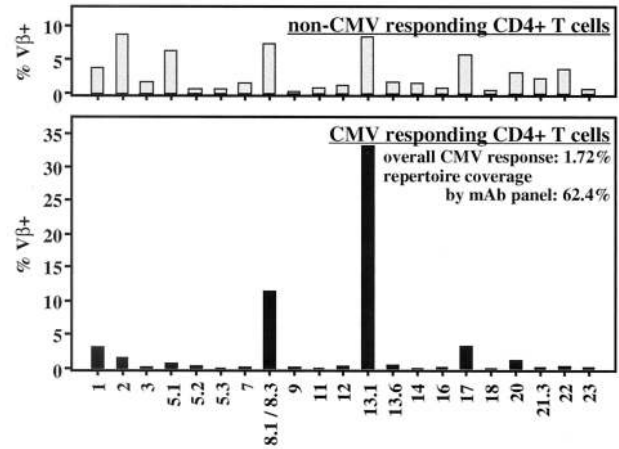
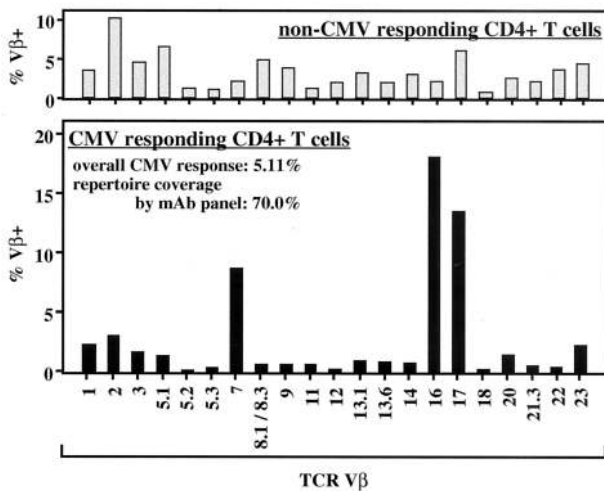
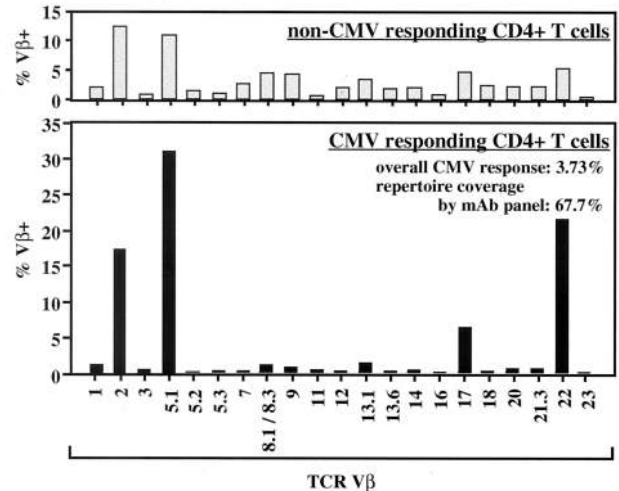
Subject 1 (55 y.o. male; DR 11, 17; DRw, DQ: 52, 2, 3)**Subject 3 (40 y.o. male; DR 1, 7)****Subject 2 (25 y.o. female; DR 13, 16; DRw, DQ: 51, 52, 3, -)****Subject 4 (22 y.o. female; DR 17, -; DRw, DQ: 52, 2)**

FIGURE 2. TCR-V β usage among CMV-specific CD4⁺ memory T cells is highly skewed. The contribution of individual TCR-V β families or subfamilies to the total CMV-specific CD4⁺ memory T cell response is shown for four additional CMV seropositive subjects (■; corresponding to the black colored events in the Fig. 1 dot plots). For comparison, the TCR-V β distribution among non-CMV-responsive CD4⁺ T cells in each subject is also provided (□; corresponding to the gray colored events in the Fig. 1 dot plots). The TCR-V β families or subfamilies shown represent all those to which mAbs are currently available. The percentage of the CD4⁺ T cell repertoire covered by this panel were calculated for each subject by determining the sum of the % + for all mAbs in this panel, and are indicated for each subject in the upper corner of the CMV responding T cell plots, along with the overall percentage of CD4 T cells responding to CMV. The four subjects in this figure and the separate subject in Fig. 1 are representative of eight such individuals examined in this manner. ND, No data.

would allow us to restrict our focus to the dominant and subdominant responses in each examined subject.

Because the paraformaldehyde fixation and detergent permeabilization required for cytokine flow cytometry negatively affect cellular mRNA (via loss or chemical modification) and therefore preclude RT-PCR-based analysis of RNA in cytokine-stained cells (Ref. 32 and data not shown), we first needed to develop criteria for the identification and sorting of viable CMV-specific CD4⁺ T cells. In essence, we needed a cell surface phenotype that would serve as a “surrogate” for cytokine expression. We ultimately used two approaches to accomplish this goal. The first approach involved the coordinate analysis of cell surface expression of CD69 and CD40L (CD154), the latter being a key functional cell surface molecule on Ag-activated CD4⁺ T cells, which shares transcriptional regulatory elements with IL-2, and is up-regulated with similar kinetics after TCR-mediated activation (33, 34). As shown in a representative analysis of subject 1 (Fig. 4A), direct comparison of the cellular expression of CD40L, CD69, and cytokine in CD4⁺ T cells after CMV stimulation (performed with secretion inhibition

by brefeldin A) confirmed that these three markers delineate a largely overlapping CMV-reactive population. CD40L is up-regulated in concert with CD69 on almost all CMV-reactive cells capable of making IL-2, and $\geq 72\%$ of those capable of making IFN- γ (like cytokine, CD40L is expressed on $< 0.05\%$ of control-treated cells; data not shown). In keeping with this, cell surface expression of CD69 and CD40L on CD4⁺ T cells stimulated with CMV in the absence of brefeldin A delineates a clear responsive population, accounting for 76% of the responsive fraction defined by IFN- γ synthesis (Fig. 4B). The second approach involved a new technique of capturing secreted IFN- γ on the cell surface of the stimulated cell with a proprietary reagent (Miltenyi Biotec). Used in combination with CD69, this surface IFN- γ approach reveals 50–100% of the responding cells identified by intracellular staining (Fig. 4C and data not shown).

Using the first approach on subjects 1–3 and the second on subject 4, PBMC were stimulated with CMV + costimulatory mAbs and then FACS purified into 1) CMV-responsive (CD69⁺/CD40L⁺ or CD69⁺/surface IFN- γ ⁺) and 2) CMV-nonresponsive

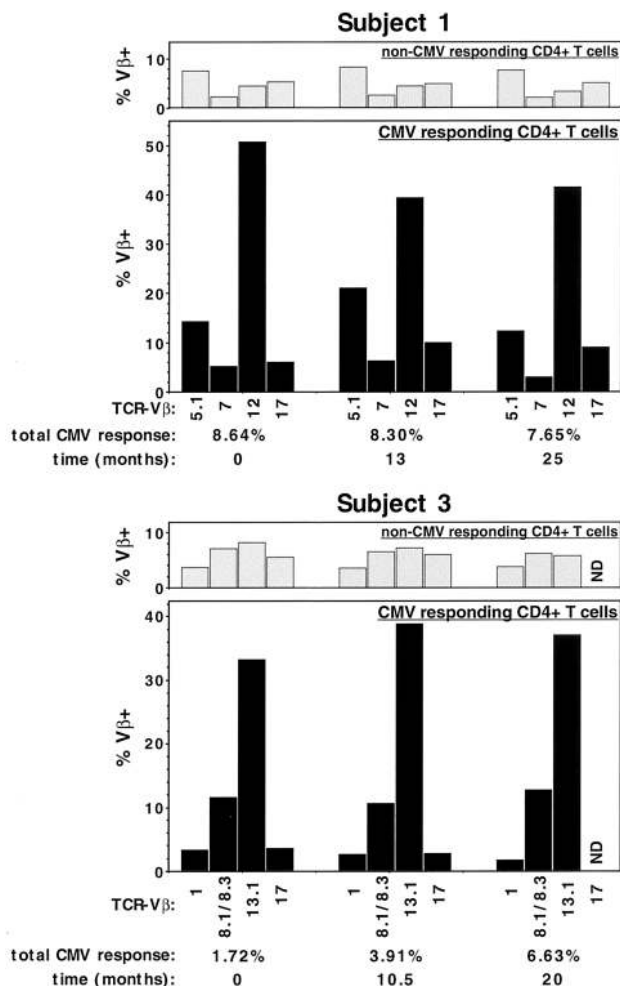


FIGURE 3. Patterns of TCR-V β usage among CMV-specific CD4⁺ T cells are stable over time. As described in Figs. 1 and 2, cytokine flow cytometry was used to determine the overall CMV-specific CD4⁺ T cell response of subjects 1 and 3, and the relative contribution of dominant and subdominant V β families or subfamilies to this overall response (■; and for comparison, the nonresponding CD4⁺ T cell population, □) at the time intervals shown.

(CD69⁻/CD40L⁻ or CD69⁻/surface IFN- γ ⁻) CD4⁺ T cell subsets. These isolated subsets were processed for RT-PCR analysis using CB and select VB primers corresponding to all dominant and subdominant V β families/subfamilies for these subjects, and for comparison, one minor V β family, V β 18 of subject 1 (Table I). RT-PCR products were then visualized on sequencing-type polyacrylamide gels (Fig. 5). Given enough cells in the reaction, such spectratyping analysis of highly diverse T cell populations will reveal a gaussian distribution of ~6–8 size species for each V β region, each differing in size from the others by multiples of 3 bp, whereas T cell populations with limited TCR heterogeneity will show one or a few prominent bands (30, 31). Although the number of sorted cells available for these analyses (4,000–30,000) were too few to always manifest the classic polyclonal pattern, the sorted CMV-nonreactive cells did indeed demonstrate either multiple distinct bands, a smear of bands with gaussian features, or no bands at all (Fig. 5, “–” lanes). In contrast, the CMV-reactive population (Fig. 5, “+” lanes) demonstrated 1–2 dominant bands for each VB analyzed (designated by arrows and small case letters in the figure).

To characterize individual clonotypes, the dominant bands for each V β family examined were eluted from the gels, cloned into

bacteria, and sequenced. As shown in Fig. 6, the majority of the major bands were clonal or biclonal, including those within the dominant V β families of subject 1–3 (V β 12, V β 16, and V β 13.1, respectively), many subdominant V β families (subject 1, V β 5.1 and V β 17; subject 2, V β 7; subject 3, V β 1 and V β 8; subject 4, V β 2), and the one minor V β family (subject 1, V β 18) examined in detail. It should be noted that both the major and closely associated minor band in the V β 16 analysis of subject 2 (Fig. 6, bands *b* and *c*) demonstrated the same sequence, suggesting the slightly larger band *b* is an artifactual modification (likely *Taq* polymerase-related; see legend) of the dominant band *c*. Interestingly, this highly restricted clonality was not universal: the V β 7 response in subject 1, the V β 17 response in subject 2, the V β 5.1 response in subject 4, and the V β 22 response in subject 4 demonstrated a total of 8, 11, 7, and 13 sequences, respectively (Fig. 6; see legend).

These data indicate that although a strong participation by a particular V β family in the overall CMV response does not always indicate a dominant clonotype, all subjects did in fact demonstrate such dominant clonotypes, and the major clonotypes (dominant and subdominant) did make up a substantial portion of the overall response. Combining the flow cytometric data (Fig. 2), densitometry of the spectratyping bands (Fig. 5), and sequence frequencies (Fig. 6) allows estimation of the contribution of each major clonotype to the overall CMV response. As shown in Table II, in the four subjects examined in detail, the dominant single clonotypes contribute 13–47%, and the top three clonotypes together 28–60% of the total response. This clonotypic dominance is even more striking when it is recalled that these results were based on the use of a panel of TCR-V β mAbs covering only 62–73% of the memory repertoire (Fig. 2); thus, the true denominator of these clonotypic frequencies is not the total response, but rather 62–73% of the response that was analyzed.

CD4⁺ T cell clonotypes identified by CD69 and CD40L expression are also found in the IFN- γ -producing subset

To confirm the CMV specificity of the clonotypes identified by sorting on the basis of CD69 and CD40L, we developed a semi-quantitative clonotype-specific PCR assay that would allow assessment of clonotype within the rearranged genomic DNA of CD4⁺ memory T cells responding to CMV with (intracellular) IFN- γ production (fixation/permeabilization does not affect PCR analysis of genomic DNA). 3' PCR primers were designed to incorporate the specific features of each specific CDR3 region and were matched to 5' primers in the associated TCR VB region of that clonotype (Fig. 6 and Table I). As shown in Fig. 7, PCR using these primers yielded significant product only in the individual from which they were derived, confirming the specificity of the clonotype-specific PCR and the uniqueness of each clonotype in these individuals.

Because each clonotype-bearing T cell would contain one rearranged (clonotype⁺) TCRB allele, this approach would allow the direct comparison of the frequency of clonotype⁺ cells among CMV-responsive vs nonresponsive CD4⁺ T cells sorted on the basis of IFN- γ and CD69 (Fig. 8A). As shown in Fig. 8B, such semiquantitative analysis of clonotype in subjects 1–3 revealed either exclusive detection or marked enrichment of clonotype in the CMV-responding (IFN- γ -producing) subset. Some “residual” clonotype was detected in CMV-nonresponsive cells, most prominently for the major V β 12/JB2.5 clonotype of subject 1. To better assess the extent of clonotype reactivity among nonresponding cells, an internal standard was constructed for the V β 12/JB2.5 template (see *Materials and Methods*), and the same sorted populations were evaluated by quantitative PCR (25, 26). In the sort

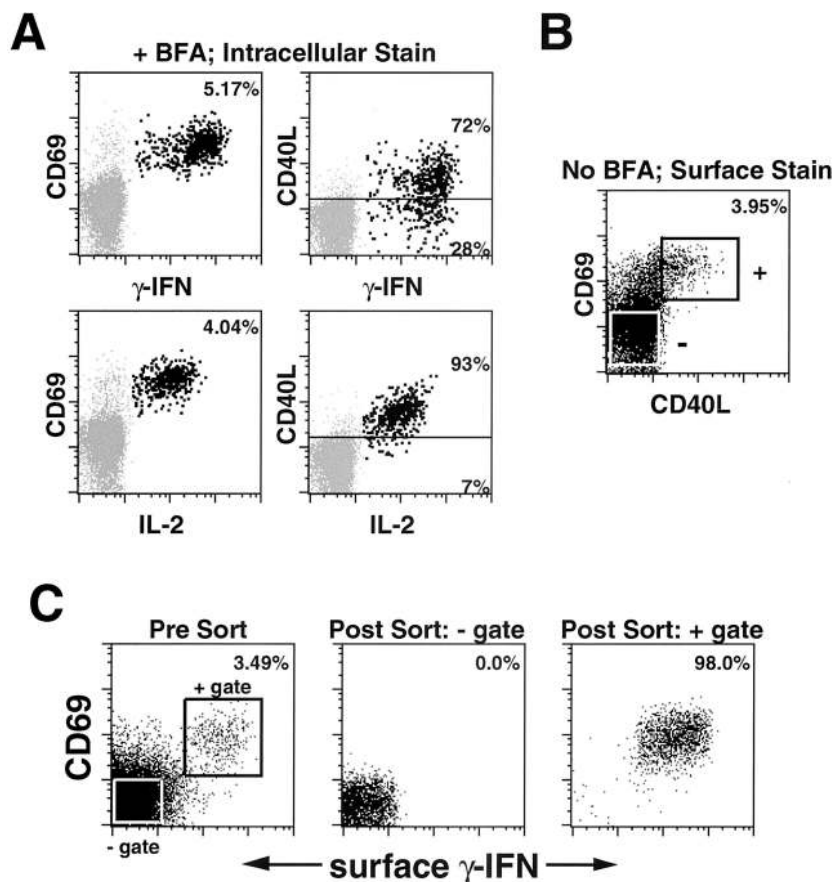


FIGURE 4. Cell surface expression of CD69 and either CD40L or surface captured IFN- γ delineates the majority of CMV-specific CD4⁺ T cells identified by intracellular cytokine expression, and allows viable cell sorting of this population. *A*, PBMC from subject 1 were stimulated with CMV + CD28 and CD49d for 6 h in the presence of brefeldin A for the final 5 h, fixed and permeabilized, and then examined for their correlated (intracellular) expression of IFN- γ or IL-2, CD69, CD40L, and CD4. Ten thousand events, gated on CD4⁺ small lymphocytes, are shown with the events within the CMV responding population (cytokine⁺ and CD69⁺) enlarged and colored black (total % + for IFN- γ and IL-2 expression provided in the upper right corners of the dot plots on the upper and lower left, respectively). *Right* dot plots, fraction of the cytokine-producing CMV-responsive cells expressing CD40L. Note that 72% of CD69⁺/IFN- γ ⁺ cells and almost all of CD69⁺/IL-2⁺ cells express this molecule. In the absence of Ag stimulation, both cytokine and CD40L expression is essentially absent (<0.05%; data not shown). Identical studies performed on subjects 2 and 3 demonstrated similar results: CD40L expression delineated 78 and 92%, respectively, of IFN- γ ⁺ and essentially all IL-2⁺ cells in these two subjects. *B*, PBMC from subject 1 were similarly stimulated with CMV in the absence of brefeldin A and then examined for their correlated expression of cell surface CD4, CD69, and CD40L. Ten thousand events are shown, gated on CD4⁺ small lymphocytes, with sort gates for responding and nonresponding cells delineated by the boxes designated “+” and “-,” respectively. Total % responding (“+” box) is provided in the upper right corner of the profile. Similar profiles were observed for subjects 2 and 3. *C*, PBMC from subject 4 were stimulated with CMV + CD28 and CD49d for 5 h (without brefeldin A), labeled with surface IFN- γ capture reagent, and then incubated for an additional 45 min for surface IFN- γ capture, after which the cells were examined for their correlated expression of surface IFN- γ , CD4, and CD69. *Left*, 10,000 events, gated on CD4⁺ small lymphocytes, with sort gates for nonresponding and responding cells delineated by the boxes designated “-” and “+,” respectively. *Middle and right*, Same profiles following cell sorting (3000 events for - sort; 1400 events for +). The percentage of cells in the + region is provided in the upper right corner of each plot.

shown, this analysis revealed that 40.1% of the CD69⁺/IFN- γ ⁺ subset were clonotype⁺ vs 5.2% in the CD69⁻/IFN- γ ⁻ subset. Moreover, in an independent sort, 54.6% of the CD69⁺/IFN- γ ⁺ subset vs 1.2% of the CD69⁻/IFN- γ ⁻ subset were clonotype⁺.

We also used VB/JB PCR followed by sequence analysis on the same sorted (CD69⁺/IFN- γ ⁺ vs CD69⁻/IFN- γ ⁻) samples to evaluate the CMV specificity of three additional minor or small subdominant clonotypes: subject 1, VB18/JB1.3 and VB18/JB2.3, and subject 3, VB1/JB2.7 (see Fig. 6). In these experiments, VB/JB PCR revealed similar bands in both + and - sort populations (data not shown), but the sequences contained within these bands were vastly different. Ten of 10, 10/10, and 9/9 of the clones from the CMV-responsive population recapitulated the original CDR3 region sequence for the VB18/JB1.3, VB18/JB2.3, and VB1/JB2.7 clonotypes (Fig. 6), respectively, whereas the CDR3 regions of 14/14 VB18/JB1.3, 13/15 VB18/JB2.3, and 12/12 VB1/JB2.7 se-

quences from the CMV-nonresponsive population were diverse and distinct from this original clonotype (data not shown). Thus, across subjects 1–3, all 11 clonotypes examined in these sorting experiments (four dominant, five subdominant, and two minor) were highly enriched in the CD69⁺/IFN- γ ⁺ subset after CMV stimulation, a finding strongly supporting the notion that the clonotypes identified in these subjects are indeed CMV specific.

The dominant V β 12/J β 2.5 clonotype of subject 1 was also analyzed by quantitative PCR in total PBMC. At the first time point examined, 1.54% of total PBMC (~4.5% of CD4⁺ T cells) were clonotype⁺ by this analytic approach (in reasonably close agreement with the 4.0% estimate of this clonotype frequency in the CD4⁺ subset provided in Table II). Twelve months later, 1.73% of PBMC were clonotype⁺. These data confirm both the striking frequency of this dominant CMV-specific CD4⁺ T cell clonotype, and its stability over time.

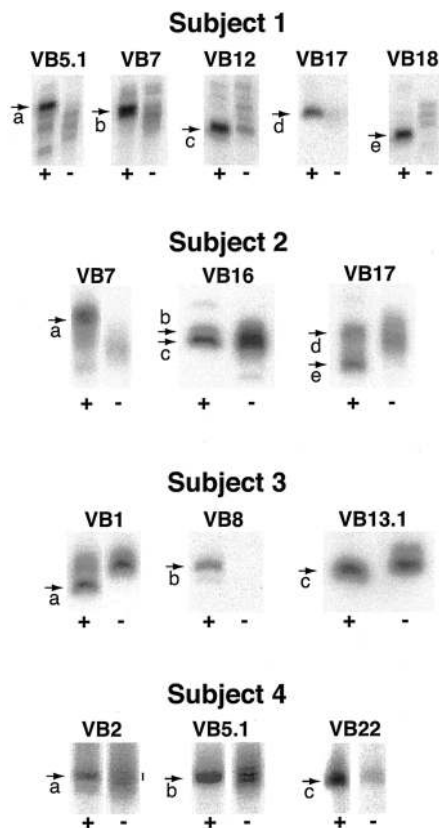


FIGURE 5. RT-PCR spectratyping analysis of sorted CMV-reactive and nonreactive CD4⁺ T cells. PBMC from subjects 1–3 were stimulated with CMV + CD28 and CD49d for 5 h in the absence of brefeldin A, stained for CD4, CD69, and CD40L, and sorted as shown in Fig. 4B. Similarly stimulated PBMC from subject 4 were processed and stained for surface IFN- γ , CD69, and CD4 and sorted as shown in Fig. 4C. The sorted CMV-reactive (+) and nonreactive (–) CD4⁺ T cells were subjected to RT-PCR spectratyping analysis for the TCRVB shown using the primers listed in Table I. Each reaction contained RNA from 30,000 cells for subject 1, 10,000 cells each for subjects 2 and 3, and 4,000 cells for subject 4. The arrows and small case letters designate dominant bands derived from CMV-reactive cells that were cloned into bacteria for sequencing (see Fig. 6).

Participation of the major CMV-specific clonotypes in the response to immunodominant epitopes within the CMV matrix phosphoprotein pp65

Each CD4⁺ T cell clonotype likely corresponds to a single CMV epitope; therefore, the number of major clonotypes identified provides a ceiling for the number of dominant epitopes recognized by the CD4⁺ memory population. However, because it is possible that a single epitope may be recognized by multiple clonotypes, the actual complexity of the response might be even less than suggested by patterns of clonotype dominance. Investigating this issue is difficult due to the sheer size of the CMV genome (>200 open reading frames of 100 aa or more) and the consequent plethora of potential epitopes available for T cell recognition. However, several studies have suggested that the pp65 lower matrix phosphoprotein is commonly a major target of the CD4⁺ T cell response (35, 36). Thus, we asked the question whether pp65 epitopes were the target of any of the CMV-specific clonotypes identified in subjects 1–4. As shown in Table III, all four subjects demonstrated a definitive response to pp65, but in subjects 2 and 3, this response was relatively minor, with the overall pp65 response comprising <10%, and the response to any single optimal pp65 epitope <6% of the total CMV response. In contrast, the pp65 responses in sub-

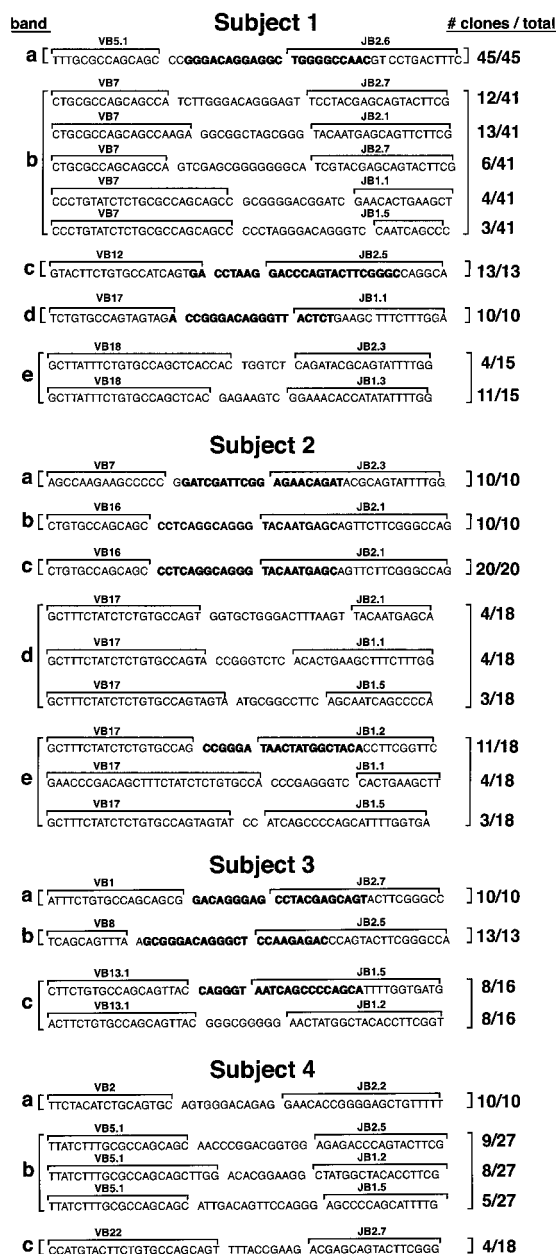


FIGURE 6. Sequence analysis of dominant bands derived from the CMV-reactive CD4⁺ T cells of subjects 1–4. The small case letters on the left correspond to the designated bands in Fig. 5. Only sequences that were identified in three or more clones are listed with the number of clones containing the indicated sequence and the total number of clones analyzed for that band designated on the right. Thus, when the number of clones found for each sequence do not sum to the total analyzed (subject 1 band b; subject 2 band d; subject 4 bands b and c), the difference represents sequences identified in only one or two clones (there were three single repeated additional sequences for subject 1, band b; five additional sequences—two double and three single repeats—for subject 2, band d; four additional sequences—three double and one single repeats—for subject 4, band b; and 12 additional sequences—two double and 10 single repeats—for subject 4, band c). Note that bands b and c of subject 2 display identical sequences, indicating that these two bands actually represent a single clone (the slightly higher m.w., less intense band b, ~1 nucleotide larger, likely represents a Taq polymerase-mediated addition of a terminal A to a portion of the PCR product). The sequence shown in bold indicates the areas of the CDR3 region used for clonotype specific primers. In subject 4, bands in the VB2 spectratyping of the CMV-nonresponsive cells corresponding in size to band a (filled bar in Fig. 5) were also cloned and sequenced. Of 13 clones analyzed, 11 diverse sequences were identified, none corresponding to the sequence found in band a (data not shown).

Table II. Frequencies of major clonotypes

Subject	Clonotype	% of Total CMV Response ^a	% of CD4 ⁺ T Cells ^b
1	VB12/JB2.5	46.7	4.03
	VB5.1/JB2.6	8.2	0.71
	VB17/JB1.1	5.5	0.48
	VB7/JB2.1	1.2	0.10
	VB7/JB2.7	1.1	0.10
	Total	62.7	5.42 (8.64) ^b
2	VB16/JB2.1	17.6	0.90
	VB7/JB2.3	6.2	0.32
	VB17/JB1.2	4.3	0.22
	Total	28.1	1.44 (5.11) ^b
3	VB13.1/JB1.5	16.0	0.28
	VB13.1/JB1.2	16.0	0.28
	VB8/JB2.5	10.4	0.18
	Total	42.4	0.74 (1.72) ^b
4	VB2/JB2.2	12.7	0.47
	VB5.1/JB2.5	8.6	0.32
	VB5.1/JB1.2	7.7	0.29
	VB5.1/JB1.5	4.8	0.18
	Total	33.8	1.26 (3.73) ^b

^a See Materials and Methods for calculation.

^b Total CMV-specific CD4⁺ T cell frequencies at the time of initial V β analysis were used in these calculations (provided in parenthesis); note for subjects 3 and 4, these frequencies increased (to 6.63 and 6.43%, respectively) at later timepoints with maintenance of the same relative V β profiles.

jects 1 and 4 were large, accounting for 38 and 31%, respectively, of the total CMV response, and the vast preponderance of these responses were attributable to single epitopes (aa 489–503 for subject 1; aa 509–523 for subject 4).

These data suggested that the pp65^{489–503} and pp65^{509–523} epitopes are likely targets for one or more of the major clonotypes identified in responses of these subjects to whole CMV. To address this question, we used cytokine flow cytometry to assess TCR-V β family usage by subject 1's pp65^{489–503}- and subjects 4's pp65^{509–523}-specific CD4⁺ T cells. As shown in Fig. 9, the dominant V β 12

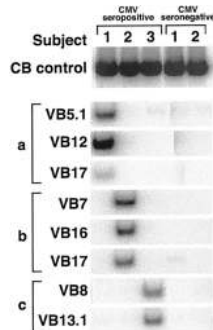


FIGURE 7. Clonotype-specific primers amplify product from genomic DNA only in the subjects in which the clonotypes originated. PBMC from CMV-seropositive subjects 1–3 and two CMV seronegative controls were lysed and genomic DNA (1 μ g/reaction) was subjected to PCR analysis using the CDR3 region specific 3' primers and the 5' V β region primers listed in Table I. As a control, the same samples were analyzed for total DNA using TCR β primers (Table I). Reactions with each primer set were performed on the five subject's DNA in parallel under identical conditions. All subjects demonstrated equivalent CB amplification, consistent with the matched amount of total DNA in each reaction. Clonotype-specific PCR resulted in discrete bands only in the subjects from which they were originally identified.

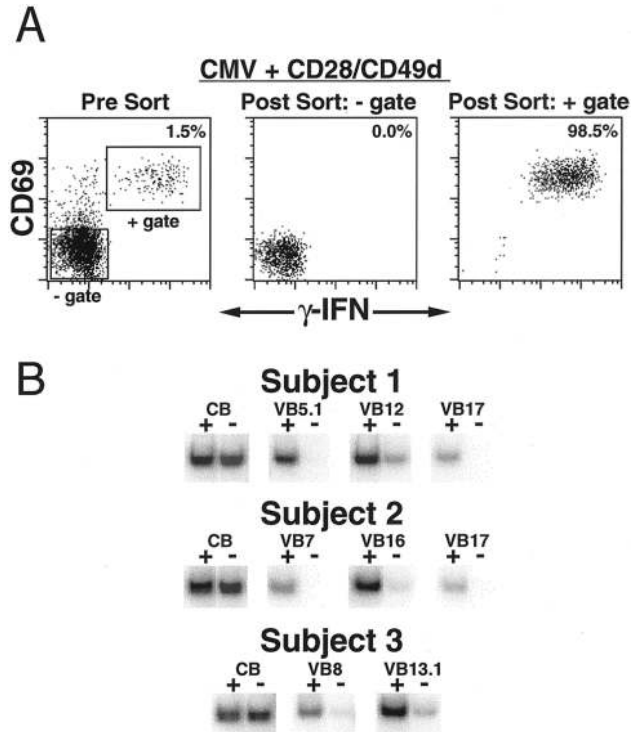


FIGURE 8. Semi-quantitative PCR reveals striking enrichment of clonotype in CD4⁺ T cells producing IFN- γ after stimulation with CMV + CD28 and CD49d. *A*, PBMC from CMV seropositive subject 2 were stimulated with CMV Ag + CD28 and CD49d for 6 h in the presence of the secretion inhibitor brefeldin A for the final 5 h. Harvested cells were fixed and permeabilized, stained for IFN- γ , CD69, and CD4, and then CD4⁺ small lymphocytes were sorted on the basis of CD69 vs IFN- γ expression as shown. The dot plots illustrating presort, - gate post sort, and + gate post sort analysis show 3000, 1000, and 811 events, respectively, all gated on CD4⁺ small lymphocytes; the percentage of CD69⁺/IFN- γ ⁺ events are provided in the upper right corner. Sorts for subjects 1 and 3 were performed identically. The frequency of responding CD4⁺ cells (CD69⁺/IFN- γ ⁺) in the - and + sorted populations were 0.11 and 96.3% for subject 1, respectively, and 1.14 and 77.3% for subject 3. *B*, Both CD69⁺/IFN- γ ⁺ (+) and CD69⁻/IFN- γ ⁻ (-) sorted cells from subjects 1–3 (7400, 3400, and 5200 cells from both + and - populations, respectively) were lysed, and genomic DNA was subjected to semi-quantitative PCR analysis for clonotype using CB as a control, as described in Fig. 7.

clonotype of subjects 1 did not recognize pp65^{489–503}, as there was virtually no contribution by this V β family to the response to this epitope. However, somewhat surprisingly, all three of this subject's subdominant V β families/subfamilies (V β 5.1, 7, and 17) made a substantial contribution to the pp65^{489–503} response, indeed, a larger contribution than that observed for whole CMV. In contrast, the V β family including subject 4's dominant whole CMV-specific clonotype (V β 2; Table II) accounted for the majority of this subject's pp65^{509–523} response, with little to no contribution by the other major V β families involved in the overall CMV response of this subject.

PBMC from subjects 1 and 4 were then stimulated with their respective pp65 peptides, and peptide-responsive (+) and -nonresponsive (-) CD4⁺ T cells were sorted on the basis of either surface CD69 and CD40L expression (subject 1) or CD69 and surface IFN- γ expression (subject 4). Sorted cells were then subjected to RT-PCR spectratyping/sequencing analysis (Fig. 10, A and C) as described in Figs. 4–6. In keeping with the cytokine flow cytometry results, the dominant VB12/JB2.5 clonotype of subject 1 was only identified in pp65^{489–503}-nonreactive

Table III. Contribution of pp65 epitopes to the CMV-specific CD4⁺ T cell response

Subject	Response to		
	Whole CMV ^a (%)	pp65 protein or total pp65 15mer mix ^{a,b} (%)	Single pp65 15mer peptides ^{a,c} (%)
1	7.86	2.96	aa (489–503) –2.74 (509–523) –0.56
2	8.87	0.50	(509–523) –0.35 (417–431) –0.08 (177–191) –0.04
3	3.90	0.35	(507–521) –0.22 (285–299) –0.09
4	6.42	1.95	(509–523) –1.49

^a The percentage of CD69⁺/IFN- γ ⁺ within the Ag/peptide-stimulated CD4⁺ T cell cultures; the % CD69⁺/IFN- γ ⁺ within the CD4⁺ T cell subset of control (no Ag)-stimulated cultures.

^b The total pp65 response was determined using purified pp65 protein in subject 2 and the pp65 peptide mix (138 15-mers overlapping by 11 aa) in subjects 1, 3, and 4.

^c Determined using overlapping pools of pp65 15-mer peptides followed by analysis of all candidate single peptides (see Ref. 24). Responses observed to adjacent overlapping peptides were considered a single response, with the 15-mer peptide providing the highest response considered the “optimal” epitope. All such optimal epitopes are listed.

cells. However, the major spectratyping bands observed for VB5.1 and VB7 in the pp65^{489–503}-reactive cells were essentially identical with those observed for whole CMV, and included the same clonal VB5.1/JB2.6 sequence, as well as the same three most common VB7 sequences (compare Fig. 10A with Figs. 5 and 6). In subject 4, the peptide-responsive cells yielded a single clonal sequence, identical with the dominant VB2/JB2.2 clonotype identified after stimulation with whole CMV. The clonotypic make-up of subject 1's pp65^{489–503} response was also evaluated by sorting on the basis of intracellular IFN- γ and CD69 and assessment by semi-quantitative clonotype-specific PCR (Fig. 10B). In keeping with the above results, the dominant VB12/JB2.5 clonotype was found exclusively among the peptide-nonresponsive cells, whereas the subdominant VB5.1/JB2.6 and VB17/JB1.1 clonotypes were only identified in the peptide-responsive subset. Taken together, these data indicate that CD4⁺ T cell recognition of immunodominant epitopes may be mediated by a single dominant clonotype (subject 4) or by a collection of subdominant and minor clonotypes (subject 1).

Discussion

Responsibility for protective cellular immune responses against chronic pathogens such as CMV rests on the cohort of pathogen-specific memory T cells, both CD4⁺ and CD8⁺, selected and differentiated from the thymus-derived naive T cell population during the first encounter with pathogen in secondary lymphoid tissues, and maintained as differentiated memory cells in the periphery (37). Although the development of appropriate effector function and homing capabilities by memory cells plays a key role in protection (37), perhaps the most fundamental determinant of the protective potential of a memory response is its TCR repertoire, the spectrum of distinct TCR expressed by the memory cohort coupled with the number and nature of pathogen determinants recognized by these TCR. Indeed, the TCR-defined clonotype, those cells sharing identical TCR and therefore identical epitope specificity, might be considered the basic unit of T cell memory with protection ultimately being a function of the number of these clonotypes, their frequency, Ag recognition properties, and functional potential.

Although much consideration has been given to the potential importance of the number and variety of epitopes recognized by T cells (primarily CD8⁺ T cells, Ref. 38) on the effectiveness of pathogen-specific immune responses, the impact of the clonotypic organization of these responses on the establishment and maintenance of protective immunologic memory has not been well defined. On one level, it is logical to suggest that the generation of numerous pathogen-specific memory clonotypes during an immune response would offer great flexibility in both pathogen recognition and the fine tuning of effector response profiles, and thus might be an optimal strategy for organizing memory responses. However, the likely possibilities that the peripheral immune system is limited in size, and that inclusion of particular clonotypes in the memory pool is competitive (39, 40), suggest that such large, complex responses may come at a price. Because the response to any given pathogen must be integrated with the response to all other pathogens, a size-limited immune system dictates that complex responses must either maintain each individual clonotype at low frequency or potentially undermine T cell memory directed at other pathogens. It thus remains to be determined whether (or in what settings) a clonotypic structure of high complexity/low frequency, low complexity/high frequency, or something in between offers the most efficient protective immunity against chronic viral infection. Here, we define the pattern of choice for one natural and

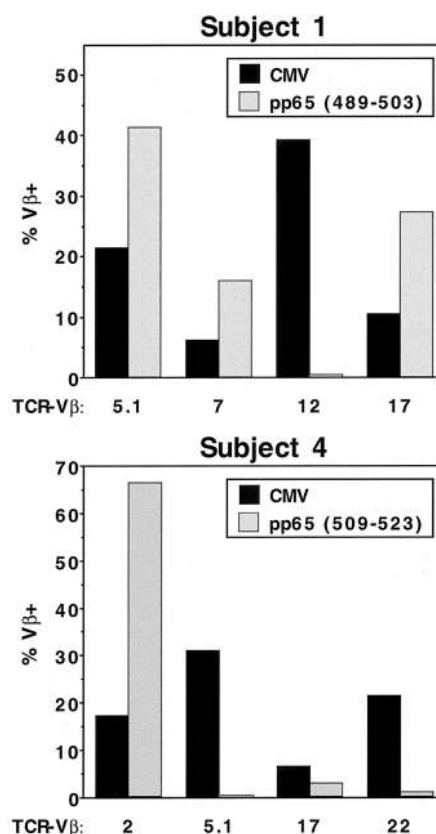


FIGURE 9. Flow cytometric analysis of TCR-V β family involvement in subject 1 and 4's CD4⁺ T cell response to a single immunodominant peptide from CMV pp65 lower matrix phosphoprotein. PBMC from subject 1 and 4 were stimulated in the presence of CD28 and CD49d with either whole CMV or these subject's specific pp65 immunodominant peptide, and the contribution of the TCR-V β families or subfamilies shown to the total CMV-specific and pp65 peptide-specific CD4⁺ memory T cell response was determined as described in Fig. 1 (e.g., % V β = % of CMV or pp65 peptide responding cells expressing each V β family/subfamily).

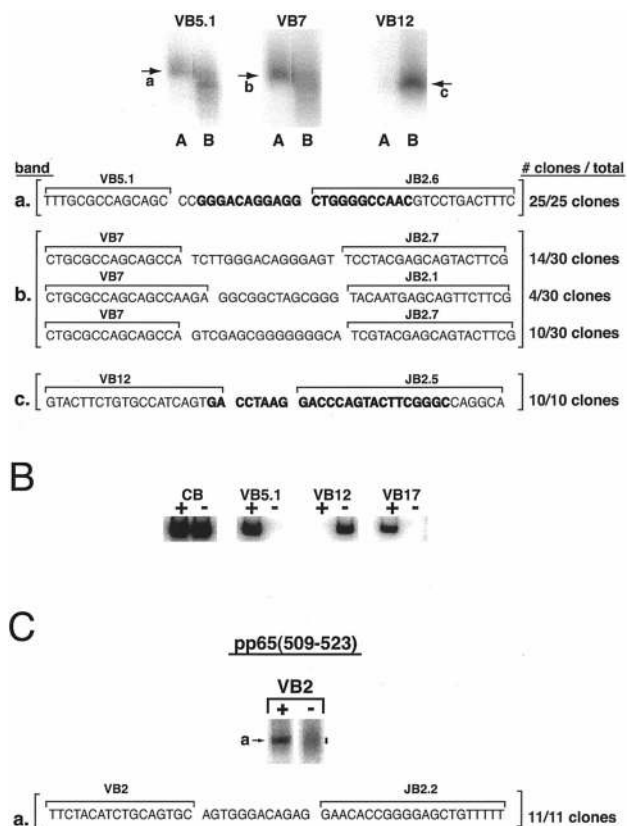


FIGURE 10. Molecular analysis of TCR-VB-defined clonotype in subject 1 and 4's CD4⁺ T cell response to a single immunodominant peptide from CMV pp65 lower matrix phosphoprotein. **A**, PBMC from subject 1 were stimulated with pp65⁴⁸⁹⁻⁵⁰³ + CD28 and CD49d for 5 h in the absence of brefeldin A, stained for CD4, CD69, and CD40L, and sorted as shown in Fig. 3*B*. Sorted CMV-reactive (+) and nonreactive (-) CD4⁺ T cells were subjected to RT-PCR spectratyping analysis for the TCRVB shown using the primers listed in Table I. Each reaction contained RNA from 26,000 cells. The arrows and small case letters designate bands that were cloned into bacteria for sequencing, and correspond to the sequences listed below. Note the VB5.1 and VB7 sequences in the CMV pp65⁴⁸⁹⁻⁵⁰³-responsive population here are identical with those identified the population responding to whole CMV (Figs. 5 and 6). In addition, two single VB7 sequences found in the peptide-responsive subset are not shown in the figure; one of these is identical with this subject's "whole CMV" VB7/JB1.5 sequence listed in Fig. 6; the other is unique. By cytokine flow cytometric analysis (Fig. 10), Vβ12-bearing CD4⁺ T cells do not participate in the pp65 peptide response, and in keeping with this, the dominant VB12 clonotype (band c; compare sequence to Fig. 6) remains in the nonresponsive cell population after peptide stimulation. **B**, PBMC from subject 1 were stimulated with pp65⁴⁸⁹⁻⁵⁰³ + CD28 and CD49d for 6 h in the presence of the secretion inhibitor brefeldin A for the final 5 h. Harvested cells were fixed and permeabilized, stained for IFN-γ, CD69, and CD4, and then CD4⁺ small lymphocytes were sorted on the basis of CD69 vs IFN-γ expression as illustrated in Fig. 8. The frequency of responding CD4⁺ cells (CD69⁺/IFN-γ⁺) in the - and + sorted populations were 0.33 and 98.8%, respectively. The CD69⁺/IFN-γ⁺ (+) and CD69⁻/IFN-γ⁻ (-) sorted cells (1500 cells each) were lysed, and genomic DNA was subjected to semiquantitative, clonotype-specific PCR analysis for subject 1's VB12/JB2.5, VB5.1/JB2.6, and VB17/JB1.1 clonotypes using CB as a control, as described in Fig. 8. In agreement with Figs. 9 and 10*A*, both VB5.1/JB2.6 and VB17/JB1.1 clonotypes are found exclusively in the pp65⁴⁸⁹⁻⁵⁰³-responsive subset, whereas the VB12/JB2.5 clonotype remains in the nonresponsive subset after stimulation with this peptide. **C**, PBMC from subject 4 were stimulated with pp65⁵⁰⁹⁻⁵²³ + CD28 and CD49d for 5 h, and then processed for surface IFN-γ capture and sorting, as described in Fig. 4*C*. The frequency of responding (surface IFN-γ⁺) CD4⁺ cells in the - and + sorted populations were 0.0 and 97.6%, respectively. The sorted CMV-reactive (+) and nonreactive (-) CD4⁺ T cells were subjected to RT-PCR spectratyping analysis for the TCRVB2 using the primers listed in Table I. Each reaction contained RNA from 2240 cells. The dominant band

likely protective human CD4⁺ memory T cell response—the response to CMV. Using a rigorous, multifaceted approach that identified, quantified, and confirmed the specificity of clonotypes comprising the CMV response among freshly isolated cells, we observed a striking clonotypic hierarchy in which 1–3 large clonotypes (each ≥10% of the total response) dominate the response, but always in coexistence with multiple subdominant (3–10%) and minor (<3%) clonotypes.

The clonotypic composition of virus-specific CD4⁺ memory T cell responses in the human has been not been extensively investigated (20), but a variety of studies have looked at this question for human anti-viral CD8⁺ T cell responses, including responses to pathogens such as CMV, EBV, HIV-1, and influenza A (17–23, 41). Conclusions from these investigations, which primarily used in vitro cultured T cell lines or clones as the Ag-specific CD8⁺ T cells analyzed for clonality, range from the examined responses being oligoclonal ("focused") to markedly polyclonal ("diverse"). These studies vary considerably with respect to the cellular substrate analyzed and methodologies used, and not surprising, the finding of diverse responses was more common when cell lines or large panels of clones were examined. It is important to note that interpretation of the physiologic significance of "oligo-" or "polyclonality" requires some appreciation for the relative frequencies of clonotype⁺ cells in the in vivo circulating pool, information that cannot be reliably provided by the study of in vitro expanded and maintained clones or lines due to the possibility of differential proliferation and/or survival during prolonged in vitro culture (27). Without such quantitative information, identification of multiple clonotypes might represent either a balanced polyclonality or a hierarchical (skewed) clonotypic organization as described here for CMV-specific CD4⁺ T cells. (For example, the finding of 13 distinct clonotypes in subject 1 in this study might simply be interpreted as a polyclonal response if one was not aware of the vast differences in frequency between them.) In support of the general applicability of the latter interpretation, the oligoclonal perturbations of peripheral blood CD8⁺ TCR repertoires noted in association with acute or chronic viral infection have now been shown to represent expansions of viral-specific memory/effector cells (42–45), and more recently, clonotypic analysis of SIV-epitope/MHC tetramer⁺ CD8⁺ T cells in SIV-infected rhesus macaques has revealed patterns of clonotypic dominance analogous to that described here for the human CD4⁺ memory T cell response to CMV (46).

Insight into the epitope specificity of dominant CMV-specific CD4⁺ T cell clonotypes was gained by observations made in two subjects who demonstrated immunodominant responses to the CMV matrix protein pp65 (accounting for approximately one-third of their response to whole CMV virus). In subject 4, not surprisingly, recognition of the immunodominant peptide was primarily mediated by the dominant clonotype of total CMV response (VB2/JB2.2). However, in subject 1, recognition of the immunodominant peptide did not involve the dominant VB12/JB2.5 clonotype of the total CMV response of this subject, but rather was mediated by a series of subdominant (VB5.1/JB2.6; VB17/JB1) and minor (VB7)

(designated by "a") was cloned into bacteria for sequence analysis. Note that the clonal sequence obtained with the peptide stimulation was identical with the single VB2/JB2.2 clonotype found after whole CMV stimulation (Fig. 6). Bands from the pp65⁵⁰⁹⁻⁵²³-nonresponsive population corresponding in size to band 'a' (bar) were similarly analyzed. Of 15 clones analyzed, 15 different sequences were found, none of which included the clonal sequence of band a (data not shown).

clonotypes. These observations lead to several significant conclusions. First, evaluation of response complexity requires understanding of both clonotype complexity and the epitope specificity of these clonotypes—a multiclonal response recognizing the same epitope has considerably different functional implications than when each of the individual clonotypes recognizes distinct epitopes. Second, response focusing may be achieved in two ways: generation of 1) a few dominant clonotypes recognizing different epitopes, or 2) a large cohort of smaller clonotypes responding to a single epitope. Subject 1 demonstrated a combination of these mechanisms to achieve a remarkable level of response focusing: ~82% of his response was accounted for by two epitopes, ~47% by the recognition of an unknown epitope by the dominant VB12/JB2.5 clonotype and ~35% by the multiclotype recognition of pp65^{489–503}.

Immunodominance of the magnitude observed in subjects 1 and 4 is a common feature of anti-viral CD8⁺ T cell responses (38), and thus, from either a clonotype or epitope recognition perspective, the organization of CD4⁺ and CD8⁺ T cell memory repertoires appears to be more similar than different. This similarity is somewhat surprising given the observation that clonal skewing within the overall population of human peripheral blood CD4⁺ T cells is only rarely found, whereas such skewing is relatively common among circulating CD8⁺ T cells, even in normal subjects (20, 43). One possibility is that the organization of CMV CD4⁺ memory response is anomalous for this lineage. More likely, in our opinion, is the possibility that the unusually large CMV response simply allows us to visualize a clonotypic hierarchy that is not as easy to discern in the considerably smaller cohorts of CD4⁺ memory T cells specific for other Ags. In contrast, frequencies of Ag-specific CD8⁺ memory cohorts are commonly in the range of the CD4⁺ response to CMV, and moreover, are robustly expanded by Ag (e.g., CD8⁺ T cells manifest large “burst” sizes) (20, 47). Thus, the overall CD4⁺ population may show a lower degree of skewing as compared with CD8⁺ T cells, not because the clonotypic architecture of Ag-specific memory T cell cohorts is substantially different from that of CD8⁺ memory cells, but rather because CD4⁺ Ag-specific cohorts are generally smaller and therefore more numerous, increasing the diversity on the population level.

The hierarchical organization of the CMV-specific, CD4⁺ T cell memory repertoire has important implications for both mechanisms of anti-viral immunity and long-term memory T cell homeostasis. With respect to the former, it is important to note that CMV is a widely disseminated virus, which is capable of both productive and latent infection of a variety of cell types. Reactivation of latent infection in macrophages is associated with proinflammatory cytokines (48, 49), and thus, it is likely that CMV may frequently reactivate at the cellular level in local inflammatory foci. The high incidence of prompt reactivation of CMV in the transplant setting (7, 48, 49), which is characterized by both proinflammatory cytokines and immune suppression, is consistent with such frequent inflammation-associated reactivation, and suggests that such foci are normally dealt with quickly by the immune system of healthy CMV-infected subjects. Dominant clonotypes, by virtue of their high frequency, would be likely candidates for mediating the efficient immunologic surveillance required for such control. Subdominant clonotypes might participate in this surveillance activity as well, but may also serve as a “back-up” if viral escape mutations nullify the function of the dominant clonotype. This back-up function would equally apply to cohorts of subdominant clonotypes recognizing the same epitope, as the structural basis of epitope recognition and sensitivity to mutation might vary among such clonotypes (note there was no apparent amino acid homology between the different TCRB expressed by subject 1’s

pp65^{489–503}-specific clonotypes). Alternatively or additionally, there is a precedent for differential distribution of function among clonotypes (50), and thus it is possible that dominant and subdominant clonotypes have distinct, but complementing, protective roles. In either scenario, a hierarchical clonotypic structure may provide the most efficient packaging of a protective memory cohort—certain clonotypes are maintained at high frequency for efficient surveillance, whereas others are maintained at low frequency, either as back-up or for distinct functions (perhaps immunoregulatory) requiring fewer responding cells.

The development of stable clonotypic hierarchies would appear to work best for genetically stable, chronic pathogens such as CMV. Whether a more complex pattern of pathogen-specific memory CD4⁺ TCR repertoires can develop and be maintained in infections with other chronic pathogens remains an open question, but as discussed above, the available data with CD8⁺ T cells certainly suggest that clonotypic hierarchies and epitope focusing are inherent features of the mammalian immune system. Indeed, given the exquisitely balanced, immune-dependent relationship between Herpes family viruses like CMV and their hosts, it might be argued that the tendency toward pronounced clonotypic hierarchies and epitope focusing evolved because of its efficiency for controlling CMV and analogous stable, chronic pathogens. However, such a system may not be optimal for highly replicating, genetically unstable pathogens like HIV-1 or SIV. These pathogens have the ability to mutationally “escape” effective CTL responses within a few weeks (51), and overall immune control of these infections is tenuous at best (52). Although there is the potential for clonotypic editing of a memory response (the recruitment and expansion of back-up clonotypes; Refs. 15, 39, 40, 46, 53, and 54), it remains to be seen whether such repertoire editing is sufficiently rapid to effectively counter lentiviral escape. Thus, the difficulty the human immune system has in controlling HIV-1 infection may, at least in part, be due to its inherent tendency toward dominant clonotypes and epitope focusing. Understanding the mechanisms responsible for this tendency, and the development of strategies to counteract it may prove necessary for the effort to develop a broadly effective HIV-1 vaccine.

In conclusion, we have demonstrated that human CMV-specific CD4⁺ memory T cells are characterized by a pronounced hierarchical pattern of clonotypic dominance, implying that the robust, long-term control of CMV mediated by these cells relies heavily on a handful of clones. How the number of clonotypes and their relative frequency translates into degree of protection remains to be determined, but it is likely that at some threshold—determined by the number of clonotypes, their frequency, and function—overall CMV-specific CD4⁺ T cell function will be insufficient to prevent foci of CMV reactivation from progressing, and end organ disease will ensue. Experience in HIV-1 disease, where CMV end organ disease is usually only found in individuals with marked CD4⁺ T cell deficiency, suggests that this threshold is set at a small fraction of the normal response in healthy subjects, but precise characterization has not been accomplished. The clonotypic architecture of the response provides a quantitative framework for such characterization. Application of the approaches used in this report to susceptible subjects may allow definition of protective threshold in terms of universal units (clonotype number, frequency, and function), and these data can then be used to predict risk of end organ disease in immunosuppressed subjects, and to define quantitative targets for vaccine development.

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