Determination of Antigen-specific Memory/Effector CD4+ T Cell Frequencies by Flow Cytometry

Evidence for a Novel, Antigen-specific Homeostatic Mechanism in HIV-associated Immunodeficiency

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Abstract

The highly regulated secretion of effector cytokines by CD4+ T cells plays a critical role in immune protection against pathogens such as cytomegalovirus. Here, we directly compare the frequency and functional characteristics of cytomegalovirus-specific CD4+ memory/effector T cells in normal and HIV+ subjects using a novel, highly efficient multiparameter flow cytometric assay that detects the rapid intracellular accumulation of cytokine(s) after short-term (6 h) in vitro antigen stimulation. Responses in this assay correlate precisely with independent measures of sensitization history (e.g., seroreactivity), and allow the simultaneous assessment of multiple cytokines in single effector T cells. Healthy HIV− individuals manifested an average of 0.71, 0.72, 0.38, and 0.06% CD4+ T cells responding to cytomegalovirus with γ-IFN, TNF-α, IL-2, and IL-4 production, respectively, with the simultaneous production of γ-IFN, TNF-α, and IL-2 being the most common effector phenotype. Significantly, overall cytomegalovirus-specific CD4+ effector frequencies were markedly higher among 40% of HIV+ subjects (2.7–8.0%), and demonstrated a predominately polarized γ-IFN+/TNF-α+/IL-2−/IL-4− phenotype. In contrast, CD4+ effector frequencies for heterologous, nonubiquitous viruses such as the mumps virus were low or absent in the HIV+ group. These data suggest the existence of homeostatic mechanisms in HIV disease that selectively preserve memory T cell populations reactive with ubiquitous pathogens such as cytomegalovirus—likely at the expense of T cell memory to more sporadically encountered infectious agents. (*J. Clin. Invest.* 1997. 99:1739–1750.) Key words: T cells • cytokines • flow cytometry • HIV immunodeficiency

Introduction

In the vast majority of cases, the natural history of infection with HIV is characterized by the progressive loss of immunologic function and the ultimate development of severe, unremitting infections with organisms that the normal immune system readily controls (1, 2). Although immunologic abnormalities in HIV-infected individuals are diverse, the available evidence strongly suggests that the most basic defect involves the progressive destruction and functional deterioration of the CD4+ memory/effector T cell subset (2–8), resulting in the decline and ultimate loss of all the myriad functions performed by these cells in host defense. HIV is thought to mediate this effect by the direct, selective infection of the CD4+ T cells themselves, and by infection of critical accessory cells required for the normal differentiation and maintenance of the CD4+ T cell subset (2–8). However, the pathophysiologic "link" between measurable effects on CD4+ T cell number and function and the increased susceptibility of HIV+ patients to opportunistic infections is poorly understood. Moreover, there is little understanding of the putative homeostatic mechanisms that likely work to counteract viral-associated immune damage, and to preserve protective immune effector responses until relatively late in the course of the disease.

Although CD4+ T cells in HIV infected patients have been subjected to extensive experimental scrutiny in the years since the original description of AIDS, the techiques employed for the majority of these studies have been limited in their ability to provide precise, quantitative information on functionally defined, pathogen-specific CD4+ memory/effector cells. Since the clone or collection of clones of memory/effector T cells that respond to a particular pathogen or antigen (Ag)[1] constitute the fundamental functional unit of the CD4+ T cell lineage (9), we felt that determination of the fate of these "units" during the course of HIV disease might provide new insight into the complex relationship between HIV and the immune system, and the consequences of this relationship with regard to the development of opportunistic infection.

To this end, we have developed a novel approach to the assessment of Ag-specific T cells that quantitates and characterizes these cells with unprecedented clarity. This approach involves a protocol for the rapid (6 h), highly efficient, Ag-specific activation of secretion-inhibited CD4+ (memory/effector) T cells, followed by the quantitation and characterization of these Ag-

The contributions of the first two authors to this work should be considered equal.

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1. *Abbreviation used in this paper:* Ag, antigen; APC, allophycocyanin; BDIS, Becton Dickinson Immunocytometry Systems; CMV, cytomegalovirus; C.V., coefficient of variation; PE, phycoerythrin; ELISPOT, enzyme-linked immunospot assay; LD, limiting dilution; PerCP, peridinin chlorophyl protein; PPD, purified protein derivative.

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specific T cells using multiparameter ("4-color") flow cytometric, immunofluorescent detection of one or more intracellular cytokines and the early activation Ag CD69, in combination with one or more T cell subset-defining phenotypic markers. We report the use of this assay in the quantitative and qualitative analysis of Ag-specific CD4+ T cell effectors in HIV− and + subjects, particularly those effectors reactive with the important opportunistic pathogen cytomegalovirus (CMV). Our results demonstrate that many HIV+ subjects increase their frequencies of CMV-specific, dual TNF-α- and γ-IFN-producing memory/effector CD4+ T cells at the apparent expense of T cells reactive with more sporadically encountered agents such as the mumps virus. These findings suggest the existence of a novel Ag-specific homeostatic mechanism that counters the general decline of CD4+ T cell number and function in HIV disease by the preferential preservation of functionally polarized CD4+ T cells reactive with important, continuously encountered pathogens such as CMV.

Methods

Cell preparation and Ag stimulation. Peripheral blood mononuclear cells (PBMC) obtained from HIV− or HIV+ patient samples were isolated from heparinized venous blood by density gradient sedimentation using Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, MO) or CPT blood collection tubes (Becton Dickinson VACUTAINER Systems, Franklin Lakes, NJ). Cells were then washed twice in HBSS (Gibco BRL, Gaithersburg, MD) and resuspended in medium, as appropriate (see below), for either in vitro stimulation, or immunofluorescence staining of freshly isolated cells.

For Ag-specific cytokine responses, 1×10^6 PBMC were placed in 16 × 125 mm polystyrene tissue culture tubes (Corning Costar Corp., Cambridge, MA) containing 2 ml of RPMI 1640 media (Gibco BRL) supplemented with 10% heat inactivated FCS (Hyclone, Logan, UT), 100 U/ml penicillin (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), and 2 mM L-glutamine (Gibco BRL), appropriately titered Ag preparations and in most instances (see below), 1 μg anti–CD28 mAb. Culture tubes were incubated at a 5° slant at 37°C in a humidified 5% CO_2 atmosphere for a total of 6 h, with the last 5 h including a final concentration of 10 μg/ml of Brefeldin A (a relatively nontoxic, but potent, inhibitor of intracellular transport that prevents secretion of any produced cytokines; 10). After incubation, cells were harvested by washing once in cold d (Dulbecco's) PBS, resuspended in dPBS containing 0.02% EDTA, incubated at 37°C for 15 min and then washed once in cold dPBS. These cells were then fixed in 4% paraformaldehyde in dPBS for 5 min at 37°C, and then washed once with cold dPBS with 1% BSA prior to resuspension at 5×10^6 cells/ml in freezing medium (10% dimethyl sulfoxide in dPBS with 1% BSA). Finally, the cells were frozen in 2 ml polypropylene cryule® vials (Wheaton Co., Millville, NJ) at −70°C in a freezing chamber (Nalge Co., Nalgene Labware Div., Rochester, NY).

Immunofluorescent staining. Cell preparations frozen as described above were rapidly thawed in a 37°C water bath and then washed once with cold dPBS prior to resuspension in fixation/permeabilization solution (Becton Dickinson Immunocytometry Systems, San Jose, CA) (BDIS) at 10^6 cells/500 μl, and incubation for 10 min at room temperature in the dark. These cells (or fresh PBMC for cell surface immunophenotyping) were washed twice with dPBS with BSA and sodium azide, and then were incubated protected from light on ice with directly conjugated mAbs for 30 min. In some experiments (e.g., experiments involving CO45RA or CO45RO staining), the freezing step was omitted, and cells freshly harvested after Ag activation were cell surface-stained first, then fixed/permeabilized/washed, and then stained for intracytoplasmic Ags. After staining, the cells were washed, refixed in 1% paraformaldehyde in dPBS, and then kept protected from light at 4°C until analysis on the flow cytometer.

Flow cytometric analysis. Up to six parameter analysis was performed on a modified FACSort flow cytometer equipped with a second 532 nm line diode laser (BDIS) using FITC, phycoerythrin (PE), and peridinin chlorophyl protein (PerCP), and allophycocyanin (APC) as the four fluorescent parameters. Methods of cytometer set up and data acquisition have been described previously (10, 11). For each analysis, 50,000 events were acquired, gated on CD4 expression and a light scatter gate designed to include only viable lymphocytes (most files required "fine-tuning" of gating during analysis on these same parameters, leaving 48,000 events for the final profiles). In some analyses, additional "live" gating based on a fluoresecent parameter (for example, CD69 reactivity) was performed to enhance the sampling of small populations. List mode multiparameter data files (each file with forward scatter, orthogonal scatter, and 3–4 fluorescent parameters) were analyzed using the PAINT-A-GATE^{Plus} program (BDIS). Isotype-matched negative control reagents were used to verify the staining specificity of experimental antibodies, and as a guide for setting markers to delineate "positive" and "negative" populations.

Antibodies/antigens. MAbs Leu4 (CD3; FITC, PE, PerCP, APC*), Leu3a (CD4; FITC, PE, PerCP, APC*); Leu23 (CD69; PE; PerCP*), Leu28 (CD28; unconjugated), Leu45RO (CD45RO; PE), anti–LFA-1β (CD18; unconjugated), anti–γ-IFN* (clone 25723.11; FITC; PE), anti–IL-2* (clone 5344.11; FITC; PE), anti–IL-4* (clone 3010.2.4; PE), anti–TNF-α* (clone 1106; FITC; PE); G1CL (mouse IgG1 control; FITC, PE, PerCP, APC*), G2GL (mouse IgG2 control; FITC, PE, PerCP) were obtained from BDIS. MAbs or conjugates marked with * were produced in the research laboratories of BDIS, and may or may not be commercially available. MAb 2H4 (CD45RA; PE) was obtained from Coulter Immunology (Hialeah, FL), the anti-T cell receptor Vβ8 and Vβ12 mAbs from Immunotech (Westbrook, ME), the anti–B7.2 (CD86) mAb IT2.2, a control mouse IgG1, and a control rat IgG2a from PharMingen (San Diego, CA), and the anti–B7.1 mAb BB1 (CD80) from Ancell Corporation (Bayport, MN). Mouse myeloma proteins MOPC 21 and 141 (used as IgG2a and 2b controls, respectively) were obtained from Sigma Chemical Co. The mouse mAbs TS2/9 (anti–LFA-3; CD58), GAP8.3 (CD45), and L3B12 (CD45) were produced in our laboratory from hybridomas obtained from the American Type Culture Collection (Rockville, MD). MAb L243 (anti–HLA-DR) was a kind gift of Dr. Laurie Davis (UT Southwestern Med. Ctr., Dallas, TX).

CMV Ag; mumps viral Ag, measles virus Ag, and the matched control Ags for each of these viruses were obtained from BioWhittaker, Inc. (Walkersville, MD). The matched control Ags represent mock isolated virus from the same (uninfected) substrate used to grow the virus. Mycobacterial Ags—whole lysates of *Mycobacterium tuberculosis*, strain H37Ra, and *Mycobacterium avium intracellular*, strain 101—were kindly prepared for our use by Dr. Luis Bermudez (Kuzell Institute, San Francisco, CA). The superAgs staphylococcal enterotoxin (SE) A and B were purchased from Toxin Technology (Sarasota, FL). All Ags were used at optimal stimulatory concentrations as determined by preliminary titration experiments.

CMV seroreactivity. Serum anti–CMV IgG was evaluated by microparticle enzyme immunoassay with an IMX System kit (Abbott Labs, Abbott Park, IL) per the manufacturer's instructions. All subjects identified as seropositive demonstrated > 130 Au/ml of reaction mixture, whereas seronegative subjects demonstrated Au/ml < 15.

Results

Establishment of a flow cytometric assay of Ag-specific T cell cytokine responses. We have previously demonstrated the feasibility of detecting and quantitating T cell cytokine responses to mitogens (e.g., phorbol ester plus ionomycin) and superAgs (the staphylococcal enterotoxin superAgs SEA and SEB) using a protocol based on intracytoplasmic staining of cytokine in short-term activated, secretion-inhibited T cells and multi-

parameter flow cytometric analysis (10). Preliminary studies indicated that with modification this approach would identify and quantitate T cells producing cytokine in response to nominal Ags as well. However, Ag responses differed from mitogen and superAg responses in several key areas. First, the geometry of the T cell/accessory cell interaction was critical for Ag responses; maximal responses were observed in slant tubes that allowed close proximity of T cells and accessory cells, but still allowed adequate media access to responding cells. Second, responses were maximized when Brefeldin A was omitted from the initial hour of interaction (likely to allow optimal Ag processing), and when exogenous co-stimulation was provided (see below). Third, precise detection of responding T cells was enhanced with inclusion of CD69 assessment in the multiparameter protocol. CD69 is upregulated on activated T cells prior to cytokine production (10), and thus allows more definitive "clustering" of the true responding fraction. Background staining, when present, is often only present in the CD69 negative fraction, and thus can be excluded from consideration. CD69 can also be used as an additional live gating parameter to enhance collection of the cytokine producing cells, yet still allow back calculation of responding cell frequency to the overall CD4+ population. Finally, because of the relatively small size of the Ag-specific populations, accurate assessment of these responses required the routine collection and analysis of 45,000–50,000 (CD4+ T cell gated) events per determination.

Fig. 1 *A* demonstrates a typical CD4+ T cell response to CMV versus control Ag using the optimized assay (without exogenous co-stimulation; see below). The enlarged, red colored dots in the dot plot histograms represent those CD4+ T cells in the "response" zone of the flow cytometric profiles (e.g., CD69+ and cytokine +), whereas the blue dots represent nonresponding CD4+ T cells. Note that responses to the mock purified control Ag (see Methods) are negligible and nonspecific, whereas distinct clusters of responding cells are noted in samples stained with γ-IFN, IL-2, and TNF-α specific mAbs, but not a control mAb. This flow cytometric assay demonstrated excellent technical reproducibility; on parallel tested aliquots from the same specimen, coefficients of variation (CV) generally ranged from 5–15% (Fig. 1 *B*).

As would be expected of a true Ag-specific response, the responses observed with the flow cytometric assay were consistently inhibitable with blocking mAbs against Ag-presenting HLA-DR class II MHC determinants, or against co-stimulatory/adhesion molecules that are known to participate in T cell responses to Ag (12), including CD18 (LFA-1β chain), CD80 and CD86 (the major CD28 ligands on antigen presenting cells), and CD58 (LFA-3) (Fig. 2 *A*). Providing exogenous co-stimulation in the form of an anti–CD28 mAb augmented the response in the presence of specific Ag (Fig. 2 *B*), but had no effect by itself (Fig. 2 *C*). Among nine independent determinations of CMV-specific γ-IFN producing effectors, the degree of augmentation provided by anti-CD28 was 2.1±0.2 (SEM) fold. Importantly, the anti-CD28 enhanced responses showed

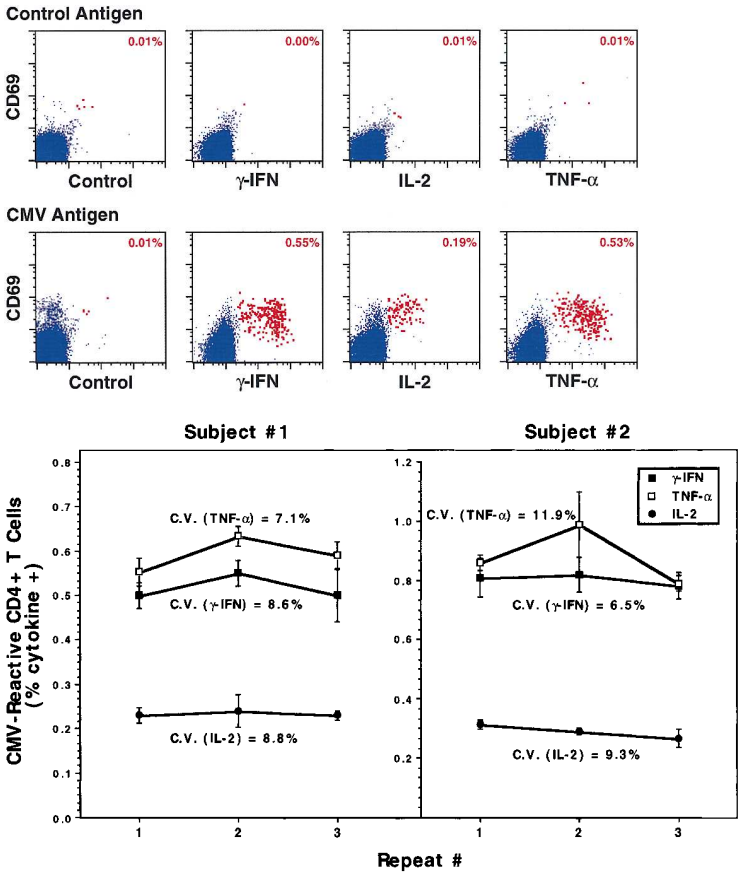

Figure 1. Detection and characterization of CMV-specific CD4+ T cells by multiparameter flow cytometric detection of CMV Ag-induced CD69 upregulation and cytokine synthesis. (*A, top*) Normal PBMC were stimulated with CMV Ag versus control Ag preparations for 6 h in the presence of the secretion inhibitor Brefeldin A for the last 5 h, and examined as described in Methods for their correlated expression of intracellular cytokine (*γ-IFN*, *IL-2*, *TNF-α* versus *Control*) versus the activation antigen CD69 versus CD4. 48,000 events, gated on viable CD4+ lymphocytes, are shown in each plot. Events in the cytokine+, CD69+ "response" region of the profiles are colored red (with the percentage of these cells given in the upper right hand corner), whereas events in the "nonresponding" region are colored blue. The responses shown for control Ag (*top*) were identical to that observed in the absence of any added Ag (Brefeldin A alone). The experiment shown is representative of > 10 independent experiments. (*B, bottom*) To demonstrate the reproducibility of the results obtained using this assay, PBMC from two unselected (HIV−) subjects were divided into three aliquots (Repeat No.s 1–3) and assayed independently. Each aliquot was stimulated with CMV in the presence of Brefeldin A (as above) and then stained and analysed as demonstrated in (*A*) in triplicate, with mean±SD for these triplicate values provided for each repeat of each cytokine in the figure. The total coefficient of variation (*CV*) for each cytokine (effector frequency) is also shown.

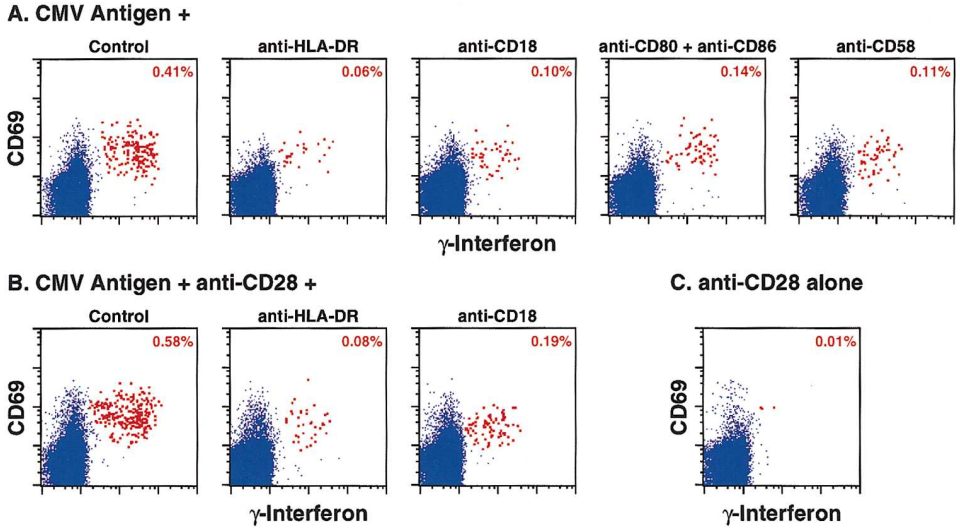

Figure 2. Inhibition of CMV-specific CD4+ T cell effector cytokine responses by class II MHC and costimulation/adhesion blockade, and augmentation of these responses by the provision of exogenous co-stimulation. PBMC (HIV−) were stimulated with CMV Ag preparations in the presence of (*A*) (5–10 μg/ml) blocking mAbs to HLA-DR, CD18, CD80 + CD86 (B7.1 and B7.2), and CD58 versus appropriate isotype-matched control mAb(s); (*B*) (1 μg/ml) anti–CD28 and (5–10 μg/ml) blocking mAbs to HLA-DR and CD18 versus appropriate isotype-matched control mAb(s); or (*C*) (1 μg/ml) anti-CD28 in the absence of CMV Ag. As described in Fig. 1, these cells were examined for their correlated expression of intracellular cytokine (γ-*IFN* versus *Control*) versus CD69 versus CD4 with 48,000 events gated on viable CD4+ lymphocytes shown in each plot, and colored red for responding events and blue for nonresponding events. The net percentage of the responding (red colored) events (after subtracting out the background events determined with an isotype-matched control mAb for the anti–γ-IFN mAb; Fig. 1) are shown in upper right corner of each plot. Although control mAbs of different isotypes corresponding to the test mAbs were examined separately, these all showed essentially identical results, and only one representative plot is presented. The anti–CD45 mAbs L3B12 and GAP8.3 (binding "controls") produced only minimal inhibition in these assays (10–20%; not shown). The experiment shown is representative of 3–5 independent experiments using different subjects. The overall mean % inhibition (±SEM; compared to cultures treated with class-matched control mAbs) for each blocking mAb for these experiments were as follows: anti–HLA-DR: 80±5% (n = 4); anti-CD18: 68±3% (n = 4); anti-CD80 + CD86: 58±4% (n = 3); and anti-CD58: 55±6% (n = 5).

the same susceptibility to inhibition by class II MHC and adhesion molecule (CD18) blockage as the response to Ag alone (Fig. 2 *B*). These results indicate that anti-CD28 optimizes cytokine responses resulting from T cell receptor crosslinking without compromising the Ag-specificity of the response. Although Fig. 2 illustrates these results for γ-IFN, essentially identical results have been observed for IL-2, TNF-α , and IL-4 (data not shown). Moreover, augmentation of detectable effector frequencies with anti-CD28 inclusion has been observed for all Ags tested to date (including a variety of viral and mycobacterial Ag preparations). These results prompted our routine inclusion of anti-CD28 in all subsequent assays, with control groups having anti-CD28 alone or anti-CD28 added to control Ag preparations.

Validation of the flow cytometric assay of Ag-specific T cell cytokine responses. Previous reports have established that both the proliferative and cytokine responses of CD4+ T cells to specific Ag are largely confined to the $CD45RA^{low}$/$CD45RO^{high}$ memory/effector subset (9, 10, 13, 14). Fig. 3 shows a representative experiment demonstrating that the same restriction applies to the Ag-specific cytokine responses detected in this assay (nonspecific staining would not be expected to show such a restriction). More definitive data confirming the specificity of the responses measured in this assay are shown in Figs. 4 and 5. Since CMV provokes a strong antibody response in immunocompetent individuals, seroreactivity has been used as a sensitive indicator of immunologic exposure to CMV, and has also been shown to correlate with T cell proliferative responses to this Ag (15). Here, we demonstrate that CMV-induced cytokine production in our flow cytometric assay also correlates precisely with seroreactivity—no responses were detectable in seronegative subjects, whereas an average of 0.71% γ-IFN and 0.36% IL-2 producing CD4+ T cells were detected in normal seropositive subjects (as shown in Fig. 5, similar results were observed with TNF-α). The lack of response to CMV among these seronegative subjects was not because of a nonspecific degradation of cell viability or response capabilities, as we observed that all these subjects manifested a demonstrable response to either another Ag (e.g., mumps or measles virus) or to superAgs (SEA+SEB) (data not shown). A statistically significant correlation ($P < .005$; Student's t test) was also observed between cytokine responses to whole lysed mycobacteria measured in our assay and the cutaneous delayed-type hypersensitivity response to purified protein derivative (PPD) (Fig. 5). Among nine subjects with a history of PPD skin test reactivity, the frequency of CD4+ T cells producing either or both of γ-IFN and IL-2 in response to mycobacterial Ag averaged 0.17%, whereas among 10 PPD negative subjects, the mean mycobacterial Ag-specific effector frequency for these cytokines was only 0.03%. Note from these data and the examples shown in Fig. 5 that unlike the complete lack of responses manifested by CMV seronegative individuals, subjects with PPD skin test non-reactivity did manifest a very low level, but specific, cytokine response to these whole mycobacteria preparations. Since we observed that responses to *M. tuberculosis* and *M. avium-intracellular* preparations were virtually identical in all cases, it is possible that environmental exposure to ubiquitous mycobacteria might produce cross reactive responses that are detectable in our assay. Alternatively, the PPD exposure provided by the PPD skin test itself might be sufficient to induce low level CD4+ T cell priming to this Ag.

Assessment of cytokine production heterogeneity among Ag-specific CD4+ memory/effector T cells. Experiments using

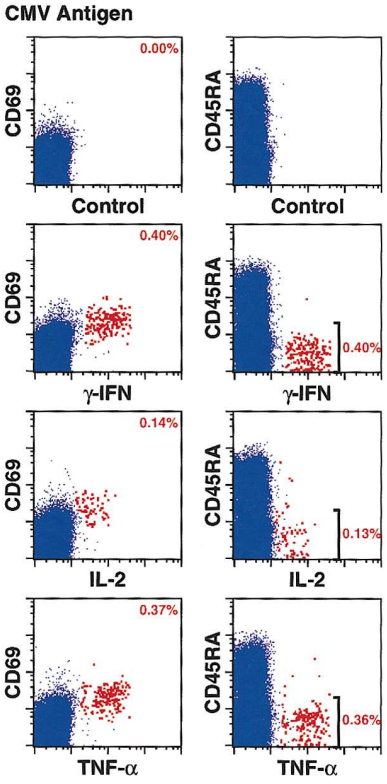

Figure 3. CD4+ T cells manifesting a cytokine response to CMV Ag display a CD45RA(−) memory/effector phenotype. PBMC (HIV−) were stimulated with CMV Ag + anti-CD28 and then examined for their correlated expression of intracellular cytokine (*γ-IFN*, *IL-2*, *TNF-α* versus *Control*) versus CD69 versus surface CD45RA versus CD4 with 48,000 events gated on viable CD4+ lymphocytes shown in each plot, and colored red for responding events and blue for nonresponding events. The overall percentage of responding cells is shown in the upper right corner of the left plots, whereas the percentage of responding cells with a CD45RA (-/low) phenotype is shown in the right plots adjacent to the brackets. Note the vast predominance of responding T cells show low expression of CD45RA. This low CD45RA expression reflects the original, resting phenotype of the responding cells, as previous studies have demonstrated that even after optimal mitogen stimulation, naive T cell downregulation of CD45RA requires a minimum of 2 d (versus the 6 h stimulation in these assays), and moreover, is preceded by a transient upregulation (11). Separate determinations indicated, as expected, that the CD45RA(-/low) responding cells expressed high levels of the RO CD45 isoform. The experiment shown is representative of three independent experiments.

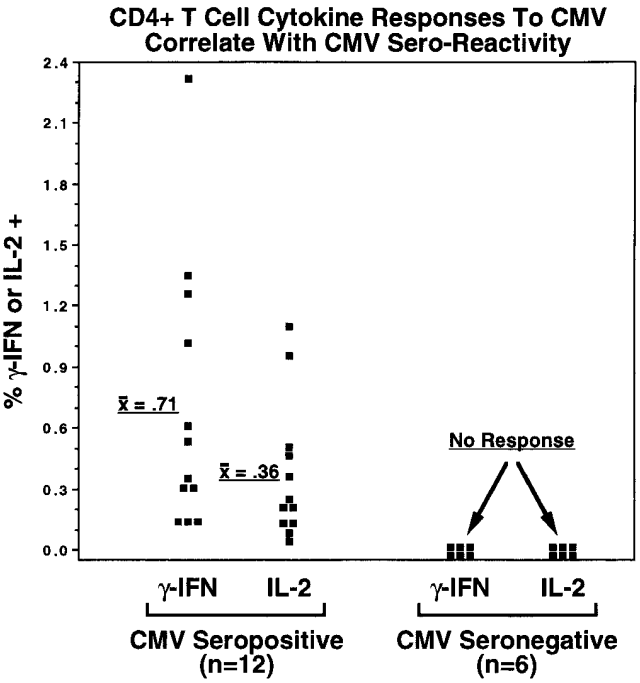

Figure 4. CMV-specific CD4+ T cell responses detected by flow cytometry correlate precisely with serologic evidence of immunologic exposure to CMV. PBMC from 18 (HIV−) subjects (12 CMV seropositive; 6 CMV seronegative; see Methods) were stimulated with CMV Ag + anti-CD28 and then examined for their correlated expression of intracellular cytokine (*γ-IFN*, *IL-2* versus *Control*) versus CD69 versus CD4. Net responding cells for each cytokine were determined as described in Fig. 2. CMV specific responses were not detected in any of the seronegative individuals, although all displayed reactivity to one or more other Ags (e.g., mumps or measles virus) and/or to the superAgs SEA and SEB, indicating their T cells were fully capable of manifesting detectable responses in this assay.

cloned T cells in both rodent and human systems have demonstrated that Ag-specific CD4+ T cells manifest marked potential heterogeneity in their cytokine synthesis function, with individual cells, depending on conditions of priming, potentially producing completely different sets of cytokines in response to

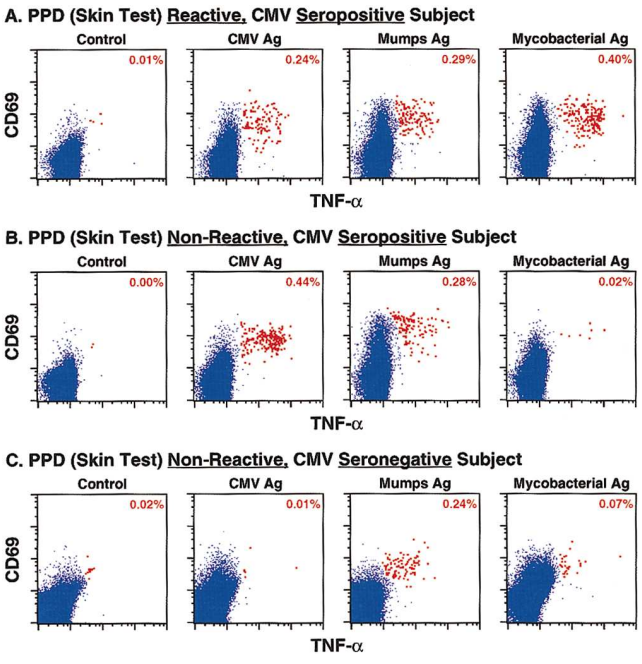

Figure 5. Profiling of Ag-specific T cell responses in CMV seropositive versus seronegative and PPD (*skin test*) + versus PPD− subjects. PBMC from (HIV−) subjects with the independent evidence of sensitization to CMV and mycobacterial Ags as shown were stimulated with CMV Ag versus Mumps virus Ag versus *M. tuberculosis* Ag (all with anti-CD28), and then examined for their correlated expression of intracellular cytokine (*TNF-α* versus *Control*) versus CD69 versus CD4. 45,000–48,000 events gated on viable CD4+ lymphocytes are shown in each plot, colored red for responding events (the percentages of the red colored events are provided in the upper right corner of each plot), and blue for nonresponding events. All subjects manifested a mumps virus response, consistent with the nearly universal sensitization to this virus in our donor population. Illustrative of the data in Fig. 4, only the CMV seropositive individuals manifested a CMV-specific response. Mycobacterial responses (essentially identical for *M. tuberculosis* versus *M. avium intracellulare* Ag preparations) were on average 5.7-fold stronger in subjects with a history of PPD skin test reactivity versus those without such history ($n = 9$ PPD+ versus 10 PPD− subjects; $P < .005$; Student's t test) (see text).

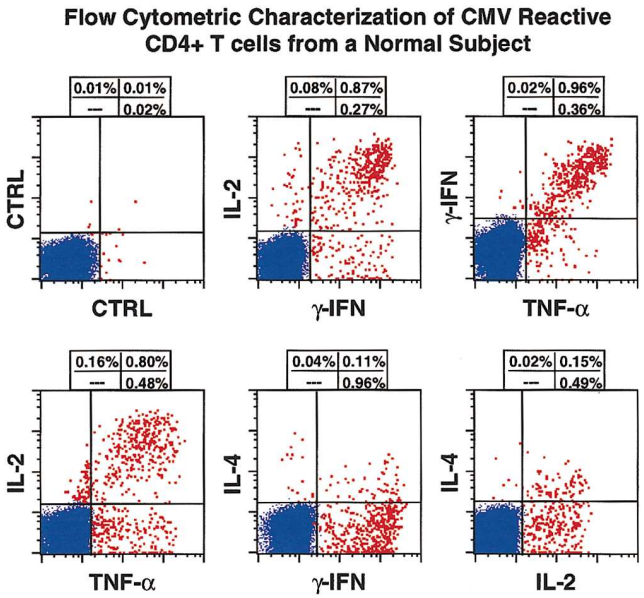

Figure 6. The single cell cytokine synthesis phenotype of CMV-specific CD4+ T cells from normal subjects is heterogeneous, but dominated by cells producing γ-IFN, TNF-α , and IL-2, but not IL-4. PBMC (HIV−) were stimulated with CMV Ag + anti-CD28 and then examined for their correlated expression of two intracellular cytokines (all paired combinations of γ-IFN, IL-2, TNF-α, and IL-4, as well as isotype-matched controls) versus CD69 versus CD4 with 48,000 events gated on viable CD4+ lymphocytes shown in each plot, and colored red for responding events and blue for nonresponding events. Although CD69 expression is not shown in this figure, this activation marker was used in conjunction with cytokine synthesis to define the red colored events as in all previous figures. The net percentage of the responding (*red colored*) cells which display a +/−, +/+, or −/+ phenotype for the cytokines being examined are shown above the profiles. The experiment shown is representative of the results obtained with nine normal subjects (for all cytokines).

a given Ag (reviewed in references 10 and 16). Here, we extend these observations to Ag-specific memory/effector T cells taken directly from the body and examined within 6 h. Fig. 6 demonstrates a typical multi-cytokine analysis of CMV-specific CD4+ T cells from a normal subject. Note that the predominant population of these Ag-specific memory/effector cells is characterized by the simultaneous production of γ-IFN, TNF-α , and IL-2, although smaller subsets producing these cytokines and IL-4 in virtually all possible combinations are also present. Although the overall number of the T cells responding to CMV varies from normal subject to normal subject, this pattern of cytokine secretion—the relative predominance of classical "TH1-like" cells, but with considerable overall heterogeneity—was similar among all normal (CMV seropositive) subjects examined to date ($n = 9$ for all cytokine pairs).

Comparison of CMV-specific CD4+ T cells in HIV− versus HIV+ subjects. CMV is one of the most important pathogens of immunodeficiency of all types, but particularly of AIDS patients (reviewed in references 17 and 18). Thus, delineation of the number and functional status of CMV-specific memory/effector T cells in HIV-infected immune systems is of great clinical interest. Among a diverse group of HIV+ subjects, ranging from asymptomatic individuals to individuals with histories of

Table I. Characteristics of the HIV+ Patients Studied

Characteristic	No.
Race	
White	16 (76.2%)
Black	3 (14.3%)
Hispanic	2 (9.5%)
Risk factor for HIV disease	
Homosexual/Bisexual	15 (71.4%)
Heterosexual	1 (4.7%)
Injection drug use	1 (4.7%)
Unknown	4 (19.0%)
Mean CD4 count	278 (range 6–652)
Patients with CD4 from:	
0 to 100	5 (23.8%)
100 to 350	8 (38%)
350	8 (38%)
Patients with documented opportunistic infections (OIs)	
# OIs	
0	16 (76%)
1	4 (19%)
2	1 (5%)
OI Types	
CMV retinitis	1
Mycobacterium avium complex	1
Cryptococcal meningitis	1
Pneumocystis pneumonia	1
Esophageal candidiasis/thrush	1
Eosinophilic folliculitis	1

*21 men; mean age 41 yr (21–61 yr).

one or more opportunistic infections (Table I), we observed (Fig. 7) that CMV-specific, CD4+ effector frequencies for γ-IFN and TNF-α were significantly increased over normal ($P < 0.03$ and 0.01, respectively; Mann-Whitney U test). The number of CD4+ T cells producing IL-2 and IL-4 in response to CMV were only modestly increased in HIV+ subjects versus normal (and these differences did not achieve statistical significance; $P = 0.15$ and 0.10, respectively). Multiparameter assessment clearly demonstrated the basis for the differences between the γ-IFN and TNF-α-defined, and IL-2- and IL-4-defined CMV effectors, showing that the CMV responses in HIV+ subjects were largely polarized to the γ-IFN and TNF-α producing, IL-2 and IL-4 nonproducing phenotype (Fig. 8; compare with the normal, prominent IL-2-producing pattern in Fig. 6). In contrast to what was observed with CMV, CD4+ effector frequencies for the nonopportunistic mumps and measles virus were not increased (Fig. 7, and data not shown). Indeed, mumps-specific (γ-IFN producing) T cells were not detectable at all in 6 of 10 HIV+ patients tested with this virus (versus detectable responses in all normal subjects tested), and were low in the remainder.

Although, as mentioned above, the frequencies of γ-IFN and TNF-α producing, CMV-specific CD4+ effectors for the overall HIV+ cohort was significantly increased compared to the HIV− population, the data in Fig. 7 suggest the posssiblity of a bimodal distribution of these frequencies among HIV+ subjects rather than a general, uniform shift towards higher frequencies. Among the 21 HIV+ individuals tested, 8 dis-

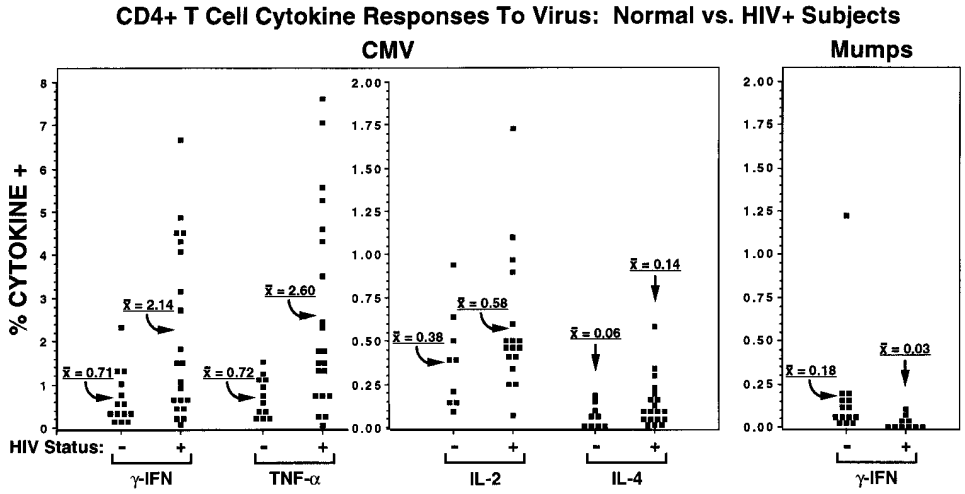

Figure 7. CMV-specific, but not mumps virus-specific, effector T cell frequencies are frequently increased in HIV+ subjects. PBMC from HIV+ and HIV− subjects were stimulated with CMV Ag or mumps virus Ag + anti-CD28 and then examined for their correlated expression of cytokine (*γ-IFN*, *IL-2*, *TNF-α*, and *IL-4* for CMV; *γ-IFN* for mumps virus) versus CD69 versus CD4 with net effector frequencies determined as described in Fig. 2. Each solid box represents a single (separate) subject studied at a single timepoint for the cytokine in question, with the mean percentage for each group of subjects designated by the arrows. In these studies, HIV− and HIV+ samples were examined in parallel, using identical reagents for all determinations of a given cytokine. For the normal subjects and all but two of the HIV+ subjects the provided data points represent the mean of duplicate or triplicate determinations for each cytokine. In the remaining two subjects, cell recovery was insufficient for such replicate analysis and the provided data point was determined by a single stain for each cytokine. It should be noted that the C.V. between replicate analyses on the same specimen is relatively small (< 15%; Fig. 1 *B*). Spontaneous cytokine secretion (i.e., the appearance of events in the "response" region of the flow cytometric profiles among cells cultured in the absence of Ag, either Brefeldin A alone or Brefeldin A + anti-CD28) was negligible in both HIV− and HIV+ specimens.

played a greater than threefold increase in the frequency of both γ-IFN- and TNF-α-producing CMV-specific effectors over the mean effector frequency observed in HIV− subjects for these cytokines, whereas the effector frequencies in the remaining HIV+ subjects were within what could be considered the "normal" range (although even among these subjects, the mean responses were still slightly elevated over the mean of HIV− subjects; 1.10% versus 0.72% for TNF-α). Significantly, CMV-reactive, γ-IFN-producing T cells in the eight subjects with elevated percentages of these cells averaged 4.4% of overall CD4+ T cells, which corresponds to 6.0% of CD45RA−/RO+ memory/effector T cells (correcting for the average of 72% memory/effector CD4+ cells in this group).

Thus, virtually all the HIV+ subjects either preserved or increased CD4+ effector frequencies for CMV with about 40% of subjects showing dramatic increases. These increased frequencies did not appear to be secondary to acute clinical events. In the patients repeatedly tested over time, the increased CMV-specific CD4+ T cell effector frequencies were stable over weeks to months (Fig. 9). Moreover, there were no

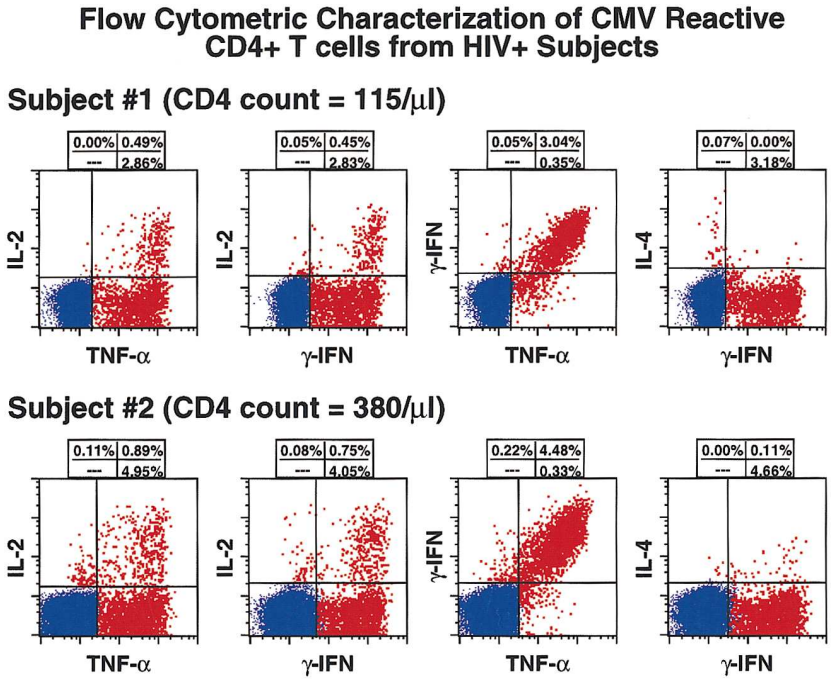

Figure 8. The increased CMV-specific, effector CD4+ T cells in HIV+ subjects display a polarized γ-IFN+, TNF-α+, IL-2−, IL-4− phenotype. PBMC from two HIV+ subjects were stimulated with CMV Ag + anti-CD28 and then examined for their correlated expression of two intracellular cytokines (*IL-2* versus *TNF-α*, *IL-2* versus *γ-IFN*, *γ-IFN* versus *TNF-α*, and *IL-4* versus *γ-IFN*) versus CD69 versus CD4 with 48,000 events gated on viable CD4+ lymphocytes shown in each plot, and colored red for responding events (cytokine+, CD69+) and blue for nonresponding events. The net percentage of the responding (red colored) cells which display a +/−, +/+, or −/+ phenotype for the cytokines being examined are shown above the profiles. In keeping with the differences in profiles between HIV− subjects (Fig. 6) and HIV+ subjects (shown here), the ratio of CD4+ γ-IFN-producing effectors to IL-2-producing effectors was 1.6:1 for the overall HIV− cohort (n = 9 evaluable subjects), 3.7:1 for the overall HIV+ cohort (n = 17), and 5.8:1 for the HIV+ cohort (n = 6) with γ-IFN-producing effector frequencies over 2.0%.

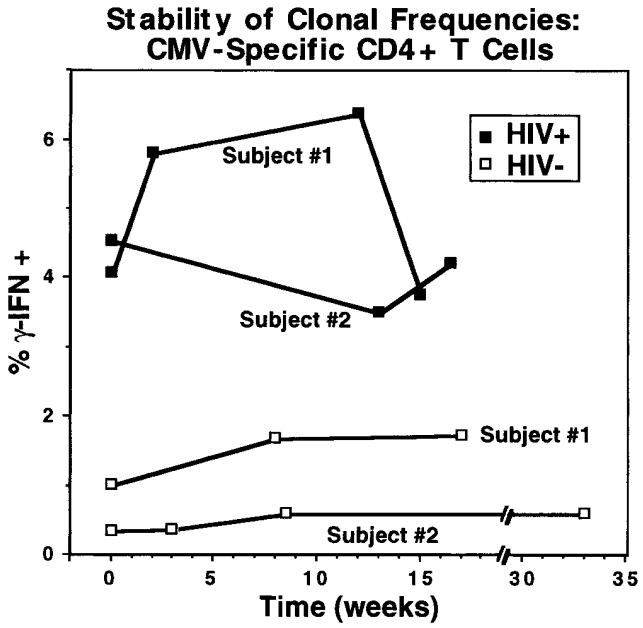

Figure 9. CMV-specific CD4+ T cell effector frequencies are stable over time. PBMC from 2 HIV+ and 2 HIV− subjects were stimulated with CMV Ag + anti-CD28 on 3–4 occasions over a 15–33 wk period, and then examined for γ-IFN-defined, CMV-specific CD4+ T cell effector frequencies at each time point. Note that the high effector frequencies of the 2 HIV+ subjects remained in the elevated range throughout the period of study. Virtually identical results were observed with TNF-α-defined CMV-specific CD4+ T cell effector frequencies in these subjects (data not shown).

clinical or routine laboratory characteristics of these patients that correlated with or predicted those subjects with increased CMV-specific CD4+ effector frequencies, including overall CD4 count, history of opportunistic infection, or viral load (data not shown). Only one patient had clinical evidence of overt CMV infection at the time of testing (retinitis). This patient had an extremely low overall CD4 count (6 cells/μl), but displayed one of the highest frequencies of CMV-specific CD4+ T cells (% CMV-specific γ-IFN producers = 4.5%; TNF-α producers = 7.1%).

Previous work has suggested that CMV may encode a T cell superAg for T cells expressing the Vβ12 T cell receptor (TCR) β-chain (19). Such a superAg could not account for the observed CMV responses in normal individuals as there was no relationship between Vβ12-expressing T cells and CMV responses. For example, CMV-nonreactive, seronegative subjects had equivalent numbers of Vβ12-expressing T cells as CMV reactive subjects (data not shown). However, given that HIV+ immune systems may have greater overall exposure to CMV, and thus to this putative superAg than normal immune systems, it seemed possible that HIV infection may unmask the activity of this superAg, and that the marked increase in CMV-specific T cells observed in some HIV+ subjects could be a superAg-mediated effect. However, as illustrated in Fig. 10, among HIV+ subjects with elevated γ-IFN-producing effectors to whole CMV Ag preparations, few of these CMV reactive T cells were Vβ12-expressing.

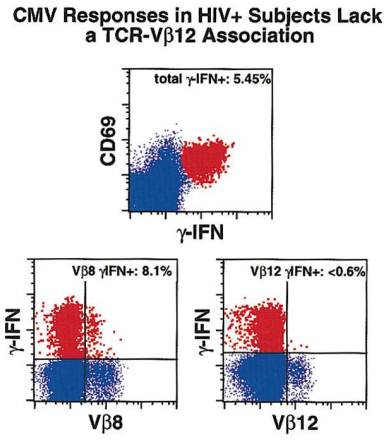

Figure 10. Increased CMV-specific CD4+ T cell effector frequencies in HIV+ subjects is not due to the putative CMV-associated T cell receptor Vβ-12 stimulating superAg. HIV+ PBMC were stimulated with CMV Ag + anti-CD28 and then examined for their correlated expression of intracellular cytokine (*γ-IFN*) versus CD69 versus CD4 versus either Vβ8 or anti-Vβ12. 48,000 events gated on viable CD4+ lymphocytes are shown in each plot, and colored red for responding events and blue for nonresponding events. Note that whereas CMV responding T cells are slightly enriched among Vβ8+ T cells (8.1% > 5.45%), they are prominently underrepresented among Vβ12+ T cells (0.6% < 5.45%). The experiment shown is representative of results obtained with 4 different HIV+ subjects.

Discussion

In this report, we describe a novel method that uses sophisticated multiparameter flow cytometry to quantitate and, both phenotypically and functionally, characterize Ag-specific CD4+ memory/effector T cells in the human with unparalleled sensitivity. This technique has several important advantages over both traditional measures of Ag-specific T cell reponses such as Ag-induced proliferation, and other single cell quantitative techniques such as limiting dilution (LD) and enzyme-linked immunospot (ELISPOT) assays. First of all, this assay identifies and quantitates Ag-responsive, CD4+ memory/effector T cells by their most basic—indeed, defining—function, the synthesis of effector cytokines (including, potentially, any combination of T cell cytokines), and does so in a time period that largely precedes the onset of activation-induced apoptosis. In this regard, it is now well documented that many, if not most, memory/effector T cells respond to Ag with an effector response (e.g., cytokine release, or in the case of CD8+ cells, cytotoxicity) and then succumb to activation-induced apoptosis prior to any possible proliferative response (20, 21). Thus, any technique requiring "extended" in vitro incubation (activation-induced apoptosis, as measured by DNA strand breaks, can initiate in as few as 12–18 h; 22) may underestimate total responding cells. This potential for apoptosis to reduce measured T cell responses is likely an even greater issue for the evaluation of such responses in HIV disease, as it has been clearly documented that memory/effector T cells from HIV+ individuals display an increased general susceptibility to apoptosis (23–26), and indeed, show enhanced Ag-induced apoptosis in vitro (26).

Second, the flow cytometric assay described here appears to have a significant sensitivity advantage over LD and ELISPOT assays, even after correcting for the different denominators of these assays (e.g., total mononuclear cells for LD and ELISPOT assays; CD4+ T cells for the flow cytometric assay). For example, reports in the literature using the ELISPOT and LD assays indicate precursor frequencies for CMV and mycobacteria-reactive T cells in appropriately exposed subjects on the order of 1/1,000 to 1/10,000 mononuclear cells (15, 27–29), whereas the flow cytometric assay described

here demonstrates precursor frequencies for these Ags in analogous subject populations to be in the 1/1,000 to 2/100 CD4+ T cell range. The increased sensitivity of the flow cytometric assay is likely the result of a combination of factors, including (*a*) the high sensitivity of fluorescence detection by modern flow cytometers (down to ~ 700 molecules/cell; V. Maino, unpublished observation); (*b*) the highly efficient capture of produced cytokine within the cytoplasm of the secretion-inhibited responding cell (as compared to only partial capture in ELISPOT-type assays); (*c*) the independence of culture (Ag stimulation) conditions and the single cell signal detection strategy, allowing these conditions to be set up with optimization of response as the only concern; and (*d*) the relatively short stimulation period (6 h), which as mentioned above, mitigates against the potential negative effect of activation-induced apoptosis on detection efficiency. The responses observed in our assay are clearly Ag-specific (Fig. 1), occur in the appropriate subset of CD4+ cells (e.g., the memory/effector subset; Fig. 3), show the expected dependence on class II MHC-mediated Ag presentation and appropriate co-stimulation (Fig. 2), and importantly, correlate with independent measures of sensitization history (Figs. 4 and 5). Bystander activation, a theoretically possible mechanism for inflating the number of responding cells, would not be expected to significantly contribute to responses in such short-term Brefeldin A-treated cultures (Brefeldin A efficiently prevents potentially stimulatory cytokines or cell surface adhesion molecules from being secreted or transported to the cell surface in these cultures; [10] and L. Picker, unpublished observations), and indeed, when specifically tested for among T cells stimulated with the SEB superAg, both CD69 and cytokine responses were restricted to appropriate TCR Vβ chain-defined subsets (e.g., Vβ12 and 17, but not Vβ8; L. Picker and V. Maino, data not shown).

Finally, the flow cytometric assay also has the significant advantages of being able to assess (*a*) Ag-specific responses in phenotypically defined T cell subsets; and (*b*) the synthesis of multiple cytokines in single Ag-responsive T cells. With respect to the subset delineation, we have used the multiparameter capabilities of this assay to precisely define Ag-specific responses within T cell subsets defined by CD4 versus CD8, TCR classes, homing receptors, and markers such as CD45RA or CD27 (this paper, and unpublished observations). Such subset assignment of the Ag-specific responses allows the separate evaluation of Ag-specific responses in distinct T cell subsets that are potentially independently regulated (e.g., CD4 versus CD8+ T cells), and greatly increases the precision and reproducibility of these assays by automatically correcting for potentially confounding changes in the representation of a particular T cell subset within the overall PBMC population. Obviously, this capability is particularly germane for the study of CD4+ T cell effector frequencies in HIV+ subjects, whose CD4 counts may vary tremendously.

With regard to the ability to assess multiple cytokines per cell, this assay is the only method described to date that allows the delineation of cytokine secretion patterns of freshly isolated T cells on an Ag-specific, single cell basis. Although the concept of cytokine synthesis heterogeneity among memory/effector T cell populations (e.g., the TH1 versus TH2 paradigm) has been well established by the study of cloned CD4+ T cells in both animals and humans (16, 30), it is unclear the extent to which the described cytokine synthesis phenotypes of cloned T cells represent the true spectrum of memory/effector T cell cytokine synthesis function in vivo. Cloned T cells, by definition, spend weeks to months in vitro prior to analysis, during which time functional biases may be introduced by clonal selection and/or inadvertent regulation of effector cell differentiation (10, 31). Here, we demonstrate the feasibility of determining cytokine synthesis patterns of Ag-specific T cells taken directly from the blood, and stimulated in vitro for only 6 h. While we report that the predominant pattern of cytokine production of CMV-specific CD4+ T cells from normal subjects is consistent with a classical TH1 phenotype (γ-IFN+/TNF-α+/IL-2+/L-4−), we also observed variably sized subsets of CD4+ T cells synthesizing all or most of the possible combinations of these four cytokines on a single cell basis (Fig. 6). These data support our previous conclusions (10) and those of others (32) that the ability of memory/effector T cells to synthesize different effector cytokines is independently regulated, and the spectrum of cytokine synthesis phenotypes is considerably more complex than suggested by the original TH0/TH1/TH2 model.

The capabilities of this new assay in defining Ag-specific T cell function offered the opportunity to approach one of the most challenging problems in clinical immunology today: the pathophysiology of HIV associated immunodeficiency. Recent advances in this area indicate that the interaction of HIV with the human immune system is highly dynamic, often characterized by high levels of viral replication and immune destruction which are counterbalanced, at least initially, by homeostatic mechanisms acting to reconstitute the viral-associated losses (33–38). With disease progression, however, continued damage to immune microenvironments (the sites of lymphocyte production and differentiation, and thus, of homeostasis) may lead to insufficient numbers or inappropriate function of critical immune effector cells, and thereby, to the failure of immune defense (5, 37, 38). Although a vast number of studies delineating numerous abnormalities in HIV+ immune systems have been published, and multiple potential mechanisms of immune dysregulation and destruction have been proposed, the key events leading to clinical immunodeficiency remain controversial. Moreover, little attention has been focused on the mechanisms of immune reconstitution that clearly operate early in this disease, only to ultimately fail. We reasoned that by delineating the number and function of key Ag-specific memory/effector T cells at different stages of HIV disease, we might gain insight into the mechanisms of both immune destruction and reconstitution. We decided to initially focus on CMV-specific T cells for two reasons. First, exposure to this virus is almost universal in the HIV+ population (18), and it thus serves as a good general model of an Ag-specific response. Second, CMV also happens to be the most common cause of life-threatening viral infection in AIDS (affecting up to 40% of such patients with either retinitis or other tissue manifestations; 17, 18), and delineation of the fate of CMV responsive T cells might lead to a better understanding of the factors responsible for the onset of opportunistic infection.

Since previous reports using a variety of methodologies have generally shown that CD4+ T cell responses to soluble Ag (e.g., tetanus toxoid, influenza virus, Candida Ags) are often preserved early in HIV disease and lost with disease progression (39–45), our original expectation was to find either normal or diminished CD4+ T cell responses to CMV, depending on disease stage. Somewhat surprisingly, our results

clearly demonstrated that the CD4+ T cells responsive to CMV were strikingly increased over normal levels (≥ 3-fold) in about 40% of the HIV+ subjects studied here (including subjects with relatively advanced disease; see below), and maintained at normal to slightly elevated levels in the remaining subjects. In the 40% of patients with increased CMV-specific effector frequencies, these frequencies averaged a remarkable 4.4% of total CD4+ T cells (which corresponds to 6.0% of memory/effector CD4+ T cells). We further demonstrated that the high frequencies of CMV-reactive T cells (*a*) were not related to the putative TCR Vβ12-stimulating CMV superAg reported by Dobrescu et al. (Fig. 10; 19); and (*b*) were stable over weeks to months, and therefore not attributable to acute clinical events. The normal to strikingly increased frequencies of CMV-specific effectors were in sharp distinction to the frequency of CD4+ T cells reactive with mumps, which similar to the previous studies of conventional recall Ags mentioned above, were either absent or diminished compared to normal in our HIV+ cohort. It is important to note that the CMV-specific CD4+ T cell cytokine effector reponses measured in this study varied independently from cytokine effector responses of the overall T cell population. We and others (10, 46, D. Peterson, C. Pitcher, and L. Picker, manuscript in preparation) have developed a pan T cell version of flow cytometric cytokine synthesis assay (e.g., stimulating the entire T cell population with stimuli such as phorbol ester and ionomycin) and have applied this assay to HIV disease. HIV+ patients frequently vary from normal in this assay (usually decreased IL-2 and γ-IFN producing, and normal to sometimes increased IL-4 producing CD4+ T cells), particularly those with more advanced disease. This pan-T cell test was applied to the HIV+ patients studied here, and while such abnormalities were identified, they did not correlate with the frequency of CMV-specific effector T cells (data not shown). This finding indicates that there must be considerable heterogeneity in the clonal representation of cytokine synthesis function within the overall memory/effector subset, and support our original contention that full understanding of T cell homeostasis in HIV disease will require delineation of changes in T cell function at the Ag-specific level for a wide variety of relevant antigenic specificities.

Previous studies looking at CMV reactivity in HIV+ subjects have noted robust responses in some individuals (e.g., Ballet et al. [44] reported increased CMV proliferative responses compared to normal subjects in about 10% of HIV+ subjects with persistent generalized lymphadenopathy), but have not revealed the large differences in CMV reactivity that were discerned between normals and a substantial proportion of HIV+ subjects using our flow cytometric assay (44, 47). Part of this difference is clearly attributable to the nature of our assay itself, which as detailed above, is very different from conventional assays of T cell function in that it allows the very rapid quantitation of immediate, subset-specific effector responses (e.g., before significant apoptosis) on a per cell basis. However, the diminished IL-2 production by the "excess" CMV-specific T cells that we observed in HIV+ subjects may have contributed as well. Since maximal proliferative responses (and perhaps optimal survival in 72 h ELISPOT assays; 47) are IL-2 dependent, a decreased in vitro availability of this cytokine may act to blunt the observed CMV-specific responses in these subjects, and thereby lead to a (false) underestimation of the initial frequency of CMV-specific T cells.

The observation of increased frequencies of CMV-specific, CD4+ T cells in HIV disease can best be interpreted in the context of a recent model of T cell memory and homeostasis that suggests that, on a clonal level, the memory/effector T cell population is in constant flux, with the size and functional characteristics of any given antigen-specific T clone being continuously influenced by changing microenvironmental conditions in the tissues, and most significantly, by competition with other clones (38, 48, 49). This model implies that immunologic events seemingly unrelated to a particular Ag-specific clone can impact on the homeostasis of that clone, as each cell in that clone must compete for a finite number of survival "niches" with unrelated clones. Since Ag availability is one of the most potent (usually positive) influences on clonal survival, the number and extent of homologous versus heterologous Ag encounters subsequent to any given Ag will influence the homeostasis of the T cells responding to that given Ag. Indeed, ultimately, it is possible that T cell memory to a particular Ag may be lost entirely, if the memory T cells responsive to that Ag are "outcompeted" by memory cells arising in or expanded by subsequent Ag encounters.

In normal (HIV−) immune systems operating in the (relatively low level) microbial pathogen environment of Western society, this clonal turnover of memory T cells is likely slow, perhaps requiring years to decades to produce measurable changes in the memory repertoire. However, as indicated above, overall T cell turnover appears to be greatly accelerated in HIV infection, and significant microbial challenges more common—factors which together provide a situation favoring potentially rapid changes in memory T cell clonal representation (38). Given the above described model of T cell memory, it is significant that exposure to CMV Ags is likely continuous or at least chronic in most HIV+ subjects, as evidenced by the high rates of CMV viremia or viruria in this population, and its known capacity to maintain subclinical latent infections (18). In contrast, immune exposure to many other commonly studied recall Ags, including nonubiquitous viruses such as mumps and influenza, or protein Ags such as tetanus toxoid, is more likely to be episodic or low level. Our findings are thus consistent with the hypothesis that (in the context of an immune system being assaulted by HIV) this continuous exposure to CMV Ags favors the survival and/or expansion of CMV-specific memory T cells at the expense of T cells specific for nonubiquitous Ags (e.g., mumps virus). This effect must be particularly potent as it evidently increases the frequency of CMV-reactive memory T cells despite the reportedly increased susceptibility of activated T cells to productive HIV-infection, and thus potentially, to HIV-mediated or -associated cell death (3, 50, 51). It is important to emphasize, however, that exposure to CMV is probably not the only determinant of increased CMV-specific CD4+ effector T cell frequencies. The condition of the immune system of the individual subject would also be expected to play a major role. For example, in order for this relative increase in CMV-specific CD4+ effectors to become manifest, there likely must be sufficient ongoing immune damage and/or activity to increase T cell turnover, but not enough damage to abrogate the capacity of the immune system to respond to CMV.

Since total CD4+ T cells progressively decline in HIV infection, the preservation of T clones reactive with ubiquitous Ags might serve as a compensatory mechanism, preserving CD4+ T cell reactivity and therefore immunity to commonly

encountered microbes that are likely to be a significant threat to the host (e.g., opportunistic pathogens). In other words, protective immunity to some particularly common pathogens may be maintained in a setting of decreasing overall CD4+ T cells by increasing the relative clonal representation of T cells reactive with such pathogens. Ultimately, however, when total CD4+ T cells drop below a certain threshold, even a dramatically increased fraction of pathogen-specific effector T cells may be unable to provide a high enough absolute number of effector cells to combat infection. Thus, it is possible that the onset of progessive opportunistic infection with a particular pathogen occurs when absolute numbers of effector T cells reactive with this pathogen drop below this putative threshold (which may be different for each pathogen). In the case of CMV, the increased CMV-specific effector frequencies reported here might account for the relative freedom from clinically significant, tissue-invasive CMV infections until total CD4 T cell counts fall to < 50–$100/\mu l$ (17, 18; D. Peterson, personal observations).

However, it important to emphasize that the ability of the immune system to mount a protective CD4+ effector response is not only dependent on the number of Ag-specific memory T cells present, but also on the functional phenotype of these cells (7, 9, 16). For example, previous work has suggested an HIV-associated switch from a "TH1" to a "TH2" cytokine synthesis phenotype might qualitatively impair effector responses to microbial Ags, and thereby contribute to immune deterioration in AIDS (7, 8, 52). Our studies here failed to provide any evidence of such a phenotypic switch among CMV-reactive CD4+ T cells in HIV+ subjects (although this finding clearly does not rule out such a switch for other Ag specificities), but we did demonstrate that the expanded CMV-reactive CD4+ effector T cells in HIV+ subjects were altered in their cytokine synthesis characteristics compared to normal, showing a predominant, highly polarized TNF-α+, γ-IFN+, IL-2−, IL-4− phenotype (as opposed to the diverse, IL-2 predominant pattern described above for HIV− individuals). The role of this polarized phenotype in controlling CMV infection remains to be determined. Indeed, it is possible that the lack of IL-2 production by the majority of the increased CMV-specific CD4+ effector cells compromises, rather than benefits, the overall CMV effector response, limiting, for example, recruitment, differentiation, and expansion of CD8+ effector T cells or NK cells. In this scenario, the increased CMV-specific CD4+ effectors noted in this study would not constitute a beneficial "compensatory" response, but rather, a reflection of an abnormal or "burned-out" CD4+ effector T cell response.

In this relatively small cross sectional study, the 40% of the HIV+ cohort with markedly expanded CMV-reactive CD4+ T cells were indistinguishable from the remaining 60% with "normal" CMV-reactive T cell frequencies by routine clinical criteria, including CD4 count, history of opportunistic infection, and viral load. Although not examined here, it is possible that sophisticated measures of latent CMV infection (e.g., CMV antigenemia or viral load) may delineate these groups. However, it is also possible that a quantitative or qualitative difference in CMV exposure is not significantly different among these groups, and that the increased CMV effector frequencies observed in some HIV+ subjects might reflect fundamental differences in the operation of immune homeostatic mechanisms in these individuals. If this hypothesis is true, the frequency of CMV-specific CD4+ T cells (irrespective of their functional potential with regard to control of CMV disease) might tell us more about overall immune function in HIV+ subjects than would be expected from examination of a single antigenic specificity. Indeed, it might correlate (either positively or negatively) with overall immune damage in HIV disease, and therefore be predictive of clinical course. In this regard, it is interesting that Schrier et al. (53, 54) recently correlated relatively high CMV-induced T cell proliferative responses in HIV+ subjects with decreased incidence of CMV disease, but more rapid overall progression of the HIV disease. Clearly, long-term prospective clinical studies will be required to formally test this hypothesis.

In conclusion, we have demonstrated the capability of a new multiparameter flow cytometric technique to simultaneously quantitate and functionally, as well as phenotypically, characterize Ag-specific memory/effector T cells in the human. This technique has provided evidence of a novel Ag-specific homeostatic mechanism in HIV disease, the understanding of which will be critical to our efforts to model not only the mechanisms of immunocompromise in this disease, but also the equally important mechanisms of immune reconstitution. Indeed, it is these latter mechanisms that will ultimately determine the degree to which HIV-induced immunologic disease can be reversed when effective pharmacologic control of the virus is established.

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