Direct Measurement of CD4+ and CD8+ T-Cell Responses to CMV in HIV-1-Infected Subjects

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Data from murine models of chronic viral infection suggest that CD4+ T-cell responses to viral pathogens are important in sustaining the number and/or function of CD8+ cytotoxic T-cell (CTL) effectors. In this study, we used cytokine flow cytometry (CFC), staining with HLA-A*0201-peptide tetramers, and peptide stimulation with epitopic peptides to study functional CD4+ and CD8+ T-cell responses to cytomegalovirus (CMV) in human subjects coinfected with CMV and the human immunodeficiency virus, type 1 (HIV-1). We show that strong CD4+ and CD8+ T-cell responses to CMV antigens are sustained over time in HIV-1-infected individuals. Those who maintain a strong CD4+ T-cell response to CMV are also likely to maintain higher frequencies of CD8+ T cells capable of binding to HLA-A*0201-CMV pp65 (A2-pp65) tetramers as well as responses to pp65 peptide stimulation with effector cytokine production. These data support the hypothesis that declines in frequencies of CD4+ T-cell responses to CMV are associated with an inability to sustain high levels of CMV-specific CD8+ T-cell responses in HIV-1-infected subjects. These declines may precede the onset of CMV-associated end organ disease.

INTRODUCTION

The cytotoxic T-cell response to viral pathogens is marked by four phases: (1) activation and expansion of CD8+ antigen-specific clones; (2) reduction of viral load through effector mechanisms, including cytolyis; (3) contraction, by apoptosis, of effector populations; and (4) maintenance of long-lived antigen-specific memory (for reviews, see Ahmed and Gray, 1996 and Kalams and Walker, 1998). The role of antigen-specific CD4+ T cells in the initiation and maintenance of these CD8+ CTL responses has been evaluated in murine models of chronic viral infection (e.g., with lymphocytic choriomeningitis virus or murine herpesviruses). A protective CTL response can be mounted through the initial phase of expansion (Rahemtulla et al., 1991; von Herrath et al., 1996) without the need for CD4+ T-cell help; thereafter, the number and/or function of CTLs may not be sustained and control of viral replication may be impaired if CD4+ T-cell help is absent (Battegay et al., 1994; Cardin et al., 1996; Matloubian et al., 1994; Zajac et al., 1998).

Our understanding of T-cell responses during chronic viral infections has been greatly facilitated by the recent introduction of sensitive and specific methods to enumerate and to characterize the function of CD4+ and CD8+ T cells. Direct flow cytometric visualization of CTLs using tetrameric complexes consisting of HLA class I, β2-microglobulin, and epitopic peptide (reviewed in Ogg and McMichael, 1998) revealed that T cells responding to a single antigen may expand dramatically (e.g., to frequencies of >40% of peripheral CD8+ T cells) in the setting of experimental murine infections (Murali-Krishna et al., 1998) and during acute human Epstein-Barr virus infection (Callan et al., 1998). In individuals infected with HIV-1, the frequency of CD8+ T cells specific for immunodominant HLA-A*0201-restricted Gag and Pol epitopes (detected using HIV-1-specific tetramers) was found to be inversely correlated with plasma HIV-1 viral load, implicating HIV-1-specific CTL in the control of viral replication and disease progression (Ogg et al., 1998). Flow cytometric methods have also enabled the characterization of antigen-specific CD4+ T-cell subsets, detecting specific responses to CMV and HIV-1 by intracellular detection of cytokines after antigen stimulation (Pitcher et al., 1999; Waldrop et al., 1997). Using such a cytokine flow cytometry (CFC) assay for CMV, we demonstrated that CMV-specific CD4+ T-cell responses...
were diminished in HIV-1-infected subjects who had CMV end organ disease (EOD) but preserved in HIV-1-infected subjects without a history of such EOD (Komanduri et al., 1998). In other studies using a CFC assay for HIV-1 (Pitcher et al., 1999), HIV-specific CD4+ T-cell responses were preserved in subjects treated soon after infection with HIV-1 but were undetectable in the majority of subjects after prolonged (>47 weeks) suppression of viremia.

To better define the interdependence of CD4+ and CD8+ T-cell responses to chronic viral infections in human subjects, we used antigen-specific MHC tetramers and the CFC assay to assess CMV-specific T-cell responses in subjects infected with both CMV and HIV-1. Our results show that the magnitude of CMV-specific CD4+ T-cell responses is positively associated with the frequency of CMV-specific CD8+ T cells in HIV-1-infected subjects, confirming predictions derived from murine models of chronic viral infection.

RESULTS

Flow cytometric quantitation of CD4+ and CD8+ T-cell responses to CMV

Figure 1 shows T-cell responses to CMV detected in the peripheral blood of an HIV-1-infected, CMV-seropositive (HLA-A*0201+) subject (Patient 4 in Table 1). To quantitate functional CD4+ T-cell responses to CMV, freshly isolated PBMC were stimulated either with a CMV lysate (Fig. 1A, left) or with a matched control antigen (Fig. 1B, right). As previously described (Komanduri et al., 1998), the frequency of CMV-specific cells was determined by gating on the fraction of CD4+ T cells expressing the cell surface activation marker CD69 and intracellular TNFα. To determine whether CD8+ T cells specific for CMV were present in the peripheral blood of this patient, cells were stained with PE-conjugated HLA A2-pp65 tetramers (Fig. 1B). The fraction of CD3+CD8+ T cells specific for this immunodominant CMV epitope (5.3%) was determined by quantitating tetramer-binding cells by flow cytometry. By contrast, few (0.03%) CD3+CD8+ T cells in the peripheral blood of this patient bound to an HLA-A*0201-restricted HIV-1 Gag epitope. CD8+ T cells responsive to the CMV pp65 epitope were also enumerated after direct stimulation in the CFC assay. 2.3% of CD8+ T cells were found to upregulate CD69 and produce intracellular TNFα after stimulation with this peptide. As expected, activation of CD4+ T cells was minimal under these conditions (e.g., 0.10% in Fig. 1C, right).

These data confirm previous observations (Komanduri et al., 1998; Pitcher et al., 1999; Waldrop et al., 1997) that flow cytometry may be used to identify responsive CD4+ T cells and that CMV-specific CD4+ T cells are present in very high frequencies in some HIV-1-infected individuals. Additionally, MHC tetramer staining reveals high frequencies of CD8+ T cells specific for the immunodominant CMV pp65 antigen in a subset of HIV-1-infected individuals. Since CD8+ T cells within these patients can also respond to stimulation with the CMV pp65 peptide, it is likely that the tetramer-binding subpopulation contains functional effector cells.

Stability of antigen-specific CD4+ and CD8+ T-cell responses

To study the maintenance of antigen-specific CD4+ T-cell responses within individuals over time, we prospectively analyzed the peripheral blood CD4+ CMV-specific CFC responses of 12 HIV-1-infected CMV-seropositive subjects over a period of approximately 1 year. Ten of 12 subjects had received HAART for greater than 1 year at the initiation of observation; two had initiated HAART less than 6 months prior to the first assessment. The frequency of CMV-specific CD4+ T cells by CFC was determined for each subject (Fig. 2A). Overall, CD4+ T-cell responses to CMV were very stable, without significant variation over time (median slope = −0.002%/day; range −0.012 to +0.009%/day).

The stability of CD8+ T-cell responses were evaluated for one HLA-A*0201+ individual for whom banked PBMC were available for several time points over a 450-day period of follow-up. Consistent with the results for other subjects, this subject's CD4+ T-cell responses measured by the CFC assay (Fig. 2B) were stable following stimulation with staphylococcal enterotoxin B (means ± SD = 29.53 ± 1.83%) or by CMV (21.55 ± 3.81%). Similarly, the frequency of CD8+ T cells specific for CMV remained stable, measured either using HLA A2-pp65 tetramers (5.23 ± 0.64%) or after stimulation in the CFC assay with the CMV pp65 peptide (2.64 ± 0.32%). These data indicate that even HIV-1-infected individuals can maintain high and stable frequencies of CMV-specific CD4+ and CD8+ T cells.

Association between functional CMV-specific CD4+ T-cell responses and CD8+ T-cell responses to CMV pp65 antigen

To better define the association between CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects, we identified a group of 21 HLA-A*0201+ individuals who were coinfected with HIV-1 and CMV, and who did not have a prior history of CMV-associated EOD. CMV serostatus, HLA typing, and T-cell counts (e.g., total, CD4+ and CD8+ T-cell counts/μl) were obtained for each individual (Table 1). Functional CD4+ T-cell responses in the peripheral blood of these individuals were determined using the CMV-CFC assay; specific CD8+ T-cell responses were visualized using HLA A2-pp65 tetramers. CD4+ CMV-specific frequencies ranged from 0.6 to 46% (means = 5.86%), while HLA A2-pp65 tetramer-specific CD8+ T-cell frequencies ranged from 0.45 to 5.13% (means = 1.23%). Paired CD4+ and CD8+
FIG. 1. Quantitation of CMV- and HIV-1-specific CD4+ and CD8+ T-cell responses in an HLA-A*0201+ individual coinfected with HIV-1 and CMV.

(A) CMV-specific CD4+ T-cell responses are shown for an HIV-1- and CMV-coinfected individual. PBMC were stimulated with lysates of CMV antigens (left panel) or a matched control antigen (right panel) in the presence of antibody to CD28 and brefeldin A. Following stimulation, cells were fixed, permeabilized, and stained with monoclonal antibodies specific for CD4 (conjugated to FITC), CD69 (conjugated to PerCP), and TNFα (conjugated to PE). The frequency of cells responsive to CMV or the control antigen was determined by the fraction of CD4+ cells which upregulated CD69 and expressed detectable amounts of intracellular TNFα (upper right quadrants).

(B) CD8+ T-cell responses to CMV and to HIV-1 measured by tetramer staining. PBMC were stained with antibody to CD8 (conjugated to tricolor) and with a PE-conjugated HLA A2-tetramer containing either the CMV pp65 NLVPMAATV epitope (left panel) or the HIV-1 Gag SLYVTATL epitope (right panel).

(C) CD8+ functional responses to CMV measured by peptide stimulation and intracellular cytokine staining. PBMC were stimulated with the CMV pp65 NLVPMAATV peptide in the presence of antibody to CD28 and brefeldin A. The frequency of cells functionally responsive to CMV was determined by flow cytometry, with responsive cells determined by double staining with antibodies specific for CD69 (conjugated to PerCP) and for TNFα (conjugated to PE) within lymphocyte populations demarcated by staining with antibodies specific for CD8 (conjugated with FITC, left panel) or CD4 (conjugated with APC, right panel).
CMV-specific responses (analyzed by CFC and by tetramer staining, respectively) are shown in Fig. 3A. To examine the quantitative relationship of CD4+ T-cell responses to CD8+ T-cell responses, we stratified the 21 HLA-A*0201+ subjects into quartiles based on percentile rank (see Materials and Methods). Using these quartiles as a basis for comparison, we then examined the relationship between CD8+ CMV-specific T-cell responses and CD4+ CMV-specific responses in the HLA-A*0201+ individuals. Individuals with the lowest (Quartile 1) CD4+ T-cell responses by CFC had significantly lower frequencies (Fig. 3B) and total numbers of cells (after adjustment with the total CD8+ T-cell count) (Fig. 3C) capable of binding to the A2-pp65 tetramer relative to those with the highest (Quartile 4) responses ($P = 0.009$ in each case). Reciprocally, increasing CD4+ CMV-specific responses (grouped in Quartiles 2–4) were associated with a higher frequency and total number of CD8+ CMV-specific cells as measured by A2-pp65 tetramer staining.

**Association between CMV pp65 tetramer-binding and the magnitude of functional responses to CMV pp65 peptide stimulation in CD8+ T-cells**

To determine whether the HLA A2-pp65 tetramer-binding cells were capable of functional responses, PBMC obtained from 21 HLA-A*0201+ HIV-1-infected, CMV-
seropositive subjects were stimulated with the pp65 peptide and analyzed for CD8+ T-cell responses using the CFC assay. A strong positive correlation was observed between the frequency of HLA A2-pp65 tetramer-binding cells and of CD69+ TNFα+ CD8+ T cells, which responded to pp65 peptide stimulation (R² = 0.85, P = 0.001, Fig. 3B). Notably, the absolute number of CD8+ T cells that was functionally reactive in the HLA A2-pp65 CFC assay was always less than the number of CMV pp65 tetramer-binding cells, raising the possibility that some of the CD8+ tetramer-binding cells in these individuals may be nonresponsive (as measured by CD69 upregulation and TNF production). The ratio of peptide-responsive cells by CFC to A2-pp65 tetramer-binding cells was similar from subject to subject and averaged 55% overall (slope of regression = 0.55, Fig. 4A).

**Association between CD4+ functional responses to CMV and CD8+ functional responses to CMV pp65 peptide stimulation**

We next determined the relationship between the frequency of CMV-specific CD4+ T cells responsive in the CFC assay and the frequency of CD8+ T cells responding to the CMV pp65 peptide. CD4+ functional responses to CMV were studied in 21 HLA-A*0201+ subjects and compared to CD8+ T-cell responses elicited after stimulation with the pp65 peptide. The pp65 CD8+ CFC responses were again stratified by CD4+ response quartiles, as described above. Subjects with the lowest CD4+ CMV-specific T-cell responses (Quartile 1) had significantly lower total numbers of CD8+ pp65-responsive cells by CFC than those in Quartiles 2–4 (P = 0.04, Fig. 4B). A trend toward significance was also observed (P = 0.08, data not shown) when frequencies of CD8+ pp65-stimulated CFC responses (fraction of CD8+ cells) were similarly stratified based on CD4+ CMV-specific responses.

**Subjects with low CD4+ CMV-specific responses have lower total CD8+, but not CD4+, T-cell counts**

To determine whether deficiencies in CD4+ antigen-specific T-cell responses were associated with trends in overall CD4+ or CD8+ T-cell counts, we compared total T-cell counts in groups stratified by their CD4+ CMV-specific T-cell response measured by CFC. No statistically significant differences were observed in total CD4+ T-cell counts between groups stratified by CD4+ T-cell responses (Fig. 5A, P > 0.6 for all quartile comparisons). When total CD8+ T-cell counts were similarly compared, groups of subjects with higher CD4+ CMV-specific T-cell responses had higher total CD8+ T-cell counts (Fig. 5B, Quartile 1 vs 3, P = 0.04, Quartile 1 vs 4, P = 0.009, Quartile 3 vs 4, P = 0.03).

**DISCUSSION**

Most HIV-1-infected subjects are also coinfected with CMV, a human gammaherpesvirus that remains a major cause of morbidity and mortality in immunocompromised subjects (e.g., in AIDS and in the setting of myeloablative chemotherapy) (Cunningham and Margolis, 1998; Ljungman, 1998). The study of CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects offers an opportunity to extend previous studies in animal models of chronic persistent viral infection (e.g., LCMV), examining the relationship between CD4+ and CD8+ T-cell responses to viral pathogens. Until recently, it was difficult to enumerate the frequencies of CD4+ or CD8+ T cells responding to viral pathogens in human subjects. The development of HLA-peptide tetramers to study T-cell responses (McHeyzer-Williams et al., 1996; Ogg and McMichael, 1998) and cytokine flow cytometry-based methods to quantitate CD4+ antigen-specific T cells (Picker et al., 1995; Waldrop et al., 1997) led to a reevaluation of the precursor frequencies of CD4+ and CD8+ T cells arising in the setting of viral infection (Butz and Bevan, 1998; Murali-Krishna et al., 1998). In addition to these two methods, the responses of human CD8+ T
cells may be quantitated by flow cytometry following stimulation with epitopic peptides (Kern et al., 1998). In this study, we utilized these methods to study CD4+ and CD8+ T-cell responses to CMV in 21 HLA-A*0201+ HIV-1-infected subjects.

Previous studies (Waldrop et al., 1997; Komanduri et al., 1998) demonstrated that the CMV-specific CD4+ T-cell response to HIV-infected individuals is dominated by TH1 polarization. This response consists primarily of cells that produce both TNFα and IFNγ, with strong correlations between CMV-specific T-cell frequencies derived via measurement of TNFα and IFNγ production (Komanduri et al. unpublished results). It was also previously established that most CMV-specific CD8+ T cells in HIV-1-infected subjects (identified by HLA A2-pp65 tetramer staining) produce both TNFα and IFNγ following epitopic peptide stimulation (Appay et al., 2000). For this reason, we confined our analyses of CMV-specific T-cell frequencies to those determined using intracellular TNFα production as an endpoint.

We first confirmed the observation (Komanduri et al., 1998; Pitcher et al., 1999; Waldrop et al., 1997) that CMV-specific CD4+ T cells are present in high frequencies in HIV-1-infected subjects. The magnitude and stability of CMV-specific CD4+ T-cell responses were examined over a period of 12 months in 12 HIV-1-infected subjects receiving highly active antiretroviral therapy. CD4+ T-cell responses following stimulation with CMV remained stable during this interval in all subjects, with only a slight median decline (median slope = −0.002%/day) noted overall for CD4+ CMV-specific responses. In one HLA-A*0201+ subject analyzed longitudinally for CD4+ and CD8+ CMV-specific T-cell responses, similar stability was also observed in the proportion of CD8+ T cells responsive to CMV measured both by HLA A2-pp65 tetramer staining and by the CMV pp65 CFC assay. A recent cross-sectional study suggested that some HIV-1-infected subjects maintained on prolonged (>47 weeks) antiretroviral therapy have reduced frequencies (but unchanged absolute counts/µl) of CD4+ T cells responding to CMV relative to untreated subjects (Pitcher et al., 1999). These data suggest that long-term suppression of HIV-1 is not always associated with loss of T-cell responses against other viral pathogens or intermediate normalization of skewing in the repertoire in subjects with high frequency T-cell responses to viruses such as CMV.

Substantial evidence from animal models supports the hypothesis that CD4+ T cells are important in sustaining the CTL effector response in the setting of chronic viral infection (reviewed in Kalams and Walker, 1998). These data predict that a similar association between the CD4+ and CD8+ T-cell response might be observed in the setting of chronic persistent viral infections in human subjects. Since a hallmark of HIV-1 infection is CD4+ T-cell depletion, we asked whether the expected association between CD4+ and CD8+ T-cell responses to CMV and HIV-1 would be found even in the setting of chronic HIV-1-induced CD4+ T lymphopenia. To answer this question, we identified a group of 21 HLA-A*0201+ individuals and measured both CD4+ and CD8+ T-cell responses to CMV. It was previously shown that the CTL response to CMV is dominated by the response to the CMV structural protein pp65 (McLaughlin-Taylor et al., 1994; Wills et al., 1996). It was also shown that most HLA-A*0201+ individuals studied have a dominant response to a single pp65 peptide epitope (Wills et al., 1996). For this reason, we correlated CMV-specific CD4+ T-cell responses following stimulation with viral lysates to CD8+ T-cell responses directed at the immunodominant HLA-A*0201-restricted pp65 epitope.

Cellular immune responses to HIV-1 in a subset of nine of these subjects were markedly diminished (data not shown), confirming previous observations in subjects chronically infected and effectively suppressed with antiretroviral therapy (Autran et al., 1997; Ogg et al., 1999; Pitcher et al., 1999). In contrast, all 21 individuals studied had detectable CD4+ and CD8+ T-cell responses against CMV antigen. Recently, Zajac et al. (1998) demonstrated that in the setting of CD4+ depletion, CD8+ T cells specific for a class I-restricted peptide epitope (based on tetramer staining) may be functionally silenced, either by deletion or anergy, in an epitope-specific manner. Here, we observed that the frequency of CD8+ T cells binding to an HLA A2-pp65 tetramer was strongly associated with the frequency of CD8+ T cells responding functionally to stimulation with the pp65 epitopic peptide. While the absolute numbers (per µl

FIG. 3. Association of CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects. (A) CD4+ and CD8+ T-cell responses to CMV in 21 HIV-1-infected subjects. Responses for all 21 HLA-A*0201+ subjects studied are illustrated. Solid lines connect CD4+ and CD8+ T-cell responses in individual subjects. (B) Individuals with low CD4+ T-cell responses to CMV antigens are likely to have lower CD8+ T-cell responses to CMV pp65 by tetramer staining. CD4+ T-cell responses to CMV (by the CFC assay) in 21 HIV-1-infected subjects were stratified based on response quartiles. The quartile boundaries were as follows: Quartile 1 <1.47%; Quartile 2 1.47% and <1.99%; Quartile 3 >1.99% and <2.00%; Quartile 4 >2.00% and <2.95%; Quartile 5 >2.95%. CD8+ T-cell responses to CMV pp65 (determined by staining with the HLA A2-pp65 NLYPMATV tetramer) are illustrated for individuals with respect to quartile rank based on responses to CMV in the CD4+ T-cell compartment. CD8+ T-cell responses are shown as the frequency of A2-pp65 tetramer-binding cells (in %). (C) The association of CD4+ CMV-specific T-cell responses by CFC and the total number of circulating CD8+ cells capable of binding the A2-pp65 tetramer (adjusted for the total CD8+ T-cell count and measured in cells/µl). Statistical significance was determined by the Mann–Whitney U test (One subject was studied by A2-pp65 tetramer staining within 3 weeks of CD4+ CFC assessment; determinations for all other subjects were done at coincident time points.)
peripheral blood) of functionally reactive cells were always lower than those of tetramer-binding cells (suggesting that some tetramer-binding cells may be nonresponsive), our data prove that high frequencies of CMV pp65-responsive cells are both present and responsive, and therefore likely functional, in most HLA-A*0201 subjects.

These experiments do not directly measure cellular cytotoxicity of CD8+ T cells specific for CMV. It was previously shown, however, that the frequency of HLA A2-pp65 tetramer-binding cells in HIV-1-infected subjects correlated closely with CD8+ cytotoxicity, as measured by a standard chromium-release assay (Appay et al., 2000). Furthermore, detailed immunophenotypic analyses of a subset of the patients presented here demonstrated that the A2-pp65 tetramer-binding cells were CD57+ perforin+, a phenotype that is closely associated with cytotoxicity in standard assays (Roederer et al., unpublished results).

Several lines of investigation indirectly support the hypothesis that development and maintenance of a CTL response in humans is dependent on a sustained antigen-specific CD4+ T-cell response (Brodie et al., 1999; Kalams and Walker, 1998; Riddell and Greenberg, 1995; Rosenberg et al., 1997; Tan et al., 1999). We directly compared functional CD4+ T-cell responses by cytokine
flow cytometry with HLA-tetramer and peptide stimulation-based quantitation of the antigen-specific CD8+ T-cell response in human subjects. We demonstrated that individuals with weak CD4+ T-cell responses to CMV are unlikely to sustain strong CD8+ T-cell responses measured either by A2-pp65 tetramer staining or by functional examination of CD8+ T cells by CFC following epitopic peptide stimulation with CMV pp65. It should be noted that we cannot formally establish a cause-and-effect relationship between the loss of CD4+ CMV-specific T-cell responses and an inability to sustain CD8+ T-cell responses based on these data. This association, now firmly established from work in murine models, may be difficult, if not impossible, to demonstrate in studies of human subjects.

Total CD8+ T-cell counts (in contrast to total CD4+
T-cell counts) were also lower in those subjects with diminished CD4+ CMV-specific responses, suggesting that an inability to maintain CMV-specific CD4+ T cells might be indicative of more global deficiencies in the functional T-cell receptor repertoire. If so, collective declines in aggregate antigen-specific CD8+ T-cell responses might be reflected in the lower total CD8+ T-cell counts observed in these subjects.

These data suggest that antigen-specific CD4+ and CD8+ T-cell responses are closely associated even in the setting of HIV-1-induced CD4+ T lymphopenia. In contrast to intact CD4+ and CD8+ T-cell responses specific for CMV, T-cell responses to HIV-1 were not detectable in a subset of the individuals that we studied. Possibly, the ability to maintain antigen-specific T-cell responses to one but not both of these coincident viral infections may be related to factors such as differences in antigenic load between CMV and HIV-1 (leading to earlier exhaustion of HIV-1-specific T cells) and/or of differential susceptibility to lytic or apoptotic death.

It is notable that diminished CD4+ T-cell responses to CMV may occur in association with clinically important CMV disease (Komanduri et al., 1998; Schrier et al., 1995). Our data suggest that such instances are likely to be associated with a concomitant decline in CD8+ T-cell responses to CMV. A better understanding of the mechanisms underlying this breakdown, as well as those responsible for the earlier loss of HIV-1-specific CD4+ and CD8+ T-cell responses, may lead to therapeutic strategies aimed at preserving or augmenting protective cellular immune responses in AIDS and other immunodeficiency states.

MATERIALS AND METHODS

Study subjects

Thirty-one HIV-1-infected individuals were studied, 13 of whom were evaluated previously for cellular immune responses to CMV (Komanduri et al., 1998). Subject 3, a female who was a prior injection drug user, was untreated at the time of study. The remaining subjects (all homosexual males) received combination antiretroviral therapy, generally consisting of at least one protease inhibitor, as directed by their primary physician. All patients gave informed consent for the studies, consistent with the policies of the institutional review boards at the University of California, San Francisco and the Aaron Diamond AIDS Research Center, New York.

Isolation of peripheral blood mononuclear cells

PBMC were isolated from heparinized whole blood by density-gradient centrifugation (Life Technologies, Rockville, MD) and washed twice in Dulbecco’s phosphate-buffered saline (dPBS) prior to use in functional studies, tetramer staining, or viable cryopreservation in 90% FCS (Sigma, St. Louis, MO).

Measurement of CD4+ and CD8+ T-cell functional responses

CMV-specific CD4+ T-cell frequencies were characterized as previously described (Komanduri et al., 1998). Briefly, PBMC were stimulated with either CMV lysates or matched control antigens (Bio-Whittaker, Walkersville, MD) or HIV-1 p24 or gp160 proteins (Protein Sciences, Meridian, CT) for 6 h in the presence of antibody to CD28 (1 μg/ml of clone L293, Becton–Dickinson Biosciences, San Jose, CA) in 96-well microtiter plates (Nunc, Rochester, NY). For CD8+ functional studies, PBMC were stimulated with HLA-A*0201-restricted epitopic peptides of CMV pp65 (residues 495–503: NLVPMVATV) or HIV-1 Gag (residues 77–85: SLYNTVATL) at a concentration of 10 μg/ml in the presence of antibody to CD28. The incubation was done at 37°C in 5% CO2 with the addition of brefeldin A (Sigma) at a final concentration of 10 μg/ml for the last 5 h. Following stimulation, cells were washed in dPBS, incubated for 5 min at 37°C in 0.02% EDTA and washed in dPBS. Cells were then fixed briefly in 1% paraformaldehyde (PFA; Sigma), washed in 1% BSA (Sigma), and frozen for at least 18 h in 1% bovine serum albumin (BSA) containing 10% DMSO (Sigma). Cells were then thawed, washed with 1% BSA, sequentially incubated for 10 min in FACSLyse and FACSPermit solutions (both from Becton–Dickinson Biosciences), and washed in 1% BSA. Finally, cells were stained with fluorescein isothiocyanate (FITC)- or allophycocyanin (APC)-conjugated antibodies against CD4, FITC- or APC-conjugated antibodies against CD8, peridinin chlorophyll protein (PerCP)-conjugated antibodies against CD69, and phycoerythrin (PE)-conjugated antibodies against TNFα (all antibodies were obtained from Becton–Dickinson Biosciences except CD4-APC, which was from Exalpha, Boston, MA). Cells were then fixed in 1% PFA, and collected on a FACSCalibur flow cytometry instrument using CellQuest software (both Becton–Dickinson Biosciences), as previously described (Komanduri et al., 1998). Flow cytometry data was analyzed and presented using FlowJo software (Tree Star, San Carlos, CA); 100,000 events were analyzed for each sample.

Analysis of CD8+ T-cell responses by MHC-tetramer staining

HLA-A*0201-restricted tetramers were produced using a method similar to that of Altman et al. (1996) and as previously described (Callan et al., 1998). Briefly, complexes of HLA class I heavy chains, β2-microglobulin, and epitopic peptides were biotinylated. The resulting MHC–peptide complexes were recovered by FPLC purification and ion-exchange chromatography. Multivalent complexes were made by incubation with streptavi-
din-PE (Sigma) at a molar ratio of 4:1. To determine the frequency of tetramer-binding CD8+ T cells, PBMC (either fresh or viably frozen, thawed, and incubated overnight in RPMI media supplemented with 10% FCS) were incubated with PE-labeled MHC–tetrmeric complexes in dPBS with 0.1% BSA for 20 min at 37°C, followed by an additional 30 min with FITC anti-CD3 and APC anti-CD8 antibodies (both from Becton–Dickinson Biosciences). Cells were then fixed in 1% PFA and analyzed on a FACSCalibur flow cytometer using CellQuest software for data collection and FlowJo (Tree Star) for data analysis and presentation. Consistency of analysis by tetramer staining was confirmed in a subset of individuals by staining with HLA A2-pp65 tetramers generated in two laboratories, with a strong correlation between measurements ($R^2 = 0.986$, $P < 0.0001$, data not shown).

The specificity of tetramers was confirmed using HIV-1- or CMV-specific CTL lines as positive controls; negative controls included PBMC from an HIV-1-seronegative donor (HIV-1 tetramer) or from an HLA-A*0201- individual (CMV tetramer) (data not shown).

**Statistical analyses**

Consistent with our prior observations that CD4+ CMV-specific T-cell responses are not normally distributed in healthy or HIV-1-infected subjects, linear regression analyses of CD4+ and CD8+ T-cell responses to CMV were not informative. Normal ranges for CD4+ T-cell responses in CFC assays have not been established and might depend on a number of methodological variables (e.g., stimulation time, permeabilization conditions, the presence or absence of costimulatory antibodies during stimulation). Quartile stratification based on CD4+ T-cell response frequencies was calculated based on percentile ranks within the overall group using Microsoft Excel (Microsoft, Redmond, WA). Statistical significance between groups was determined using the Mann–Whitney U test and calculated using StatView (Abacus Concepts, Berkeley, CA).

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