Protective Immunity to Cytomegalovirus (CMV) Retinitis in AIDS Is Associated with CMV-Specific T Cells That Express Interferon-γ and Interleukin-2 and Have a CD8+ Cell Early Maturational Phenotype

Elizabeth Sinclair,1,3 Qi Xuan Tan,1 Margaret Sharp,1,3 Valerie Girling,1 Chungkee Poon,1 Mark Van Natta,6 Douglas A. Jabs,6,7 Margaret Inokuma,5 Holden T. Maecker,5 Barry Bredt,1,3 and Mark A. Jacobson,2,4 for the Studies of Ocular Complications of AIDS Research Group

1Division of Experimental Medicine and 2Positive Health Program, Department of Medicine, and 3General Clinical Research Center at San Francisco General Hospital, University of California, San Francisco, and 4The Medical Service, San Francisco General Hospital, San Francisco, and 5BD Biosciences, San Jose, California; 6Department of Epidemiology, The Johns Hopkins University Bloomberg School of Public Health, and 7Departments of Ophthalmology and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland

To determine potential correlates of immune recovery from AIDS-related cytomegalovirus retinitis (CMVR), multiparameter flow cytometry was used to characterize CMV-specific T cells from subjects with CMVR. Individuals with active retinitis were compared with those who had been clinically immunoresurred by antiretroviral therapy and had ≥2 years of ophthalmologic follow-up without anti-CMV therapy or retinitis reactivation or progression. In comparison with patients with active retinitis, immunoresurred patients had higher circulating CD4+ and CD8+ T cells expressing interleukin-2 and interferon-γ in response to combined CMV pp65 and IE1 peptide pool stimulation. CD4+ T cell responses were predominantly to pp65, whereas CD8+ T cell responses were predominantly to IE. Immunoresurred patients, compared with patients with active retinitis, had increased levels of circulating CMV-specific CD8+ T cells with “early” (CD27+CD28+CD45RA+ or CD27+CD28+CD45RA-) and “intermediate” (CD27+CD28+CD45RA-) phenotypes. Recovery from AIDS-related CMVR after the initiation of antiretroviral therapy may be mediated by CMV-specific CD4+ and CD8+ T cells capable of promoting antigen-specific CD8+ T cell proliferation.

Before the availability of highly active antiretroviral therapy (HAART) regimens, cytomegalovirus (CMV) end-organ disease, primarily retinitis (CMVR), was a common complication of AIDS, occurring in 40% of patients with absolute CD4+ T cell counts <50 cells/μL [1–3]. Before HAART, retinitis progressed despite systemic anti-CMV drug therapy [4]. CMVR incidence has dropped substantially in Western countries where HAART became widely available [5,6], although it subsequently stabilized at ~25% of its pre-HAART incidence [5–7]. Most patients with prevalent CMVR whose absolute CD4+ T cell counts were immunoresurred by HAART have been able to discontinue anti-
CMV therapy without further retinitis progression [8–10]. Nevertheless, CMVR remains an important complication of AIDS internationally, being reported in up to 8.5% of patients with AIDS in parts of Africa [11], 15% in Thailand [12], and 17% in India [13]. Thus, interventions that could prevent or more cost effectively treat CMVR could have worldwide impact.

The ability to differentiate patients at high risk for incident retinitis or progression of prevalent retinitis from those at low risk would allow treatment to be delivered to patients at high risk, whereas those at low risk could be spared the significant drug toxicities and costs associated with long-term anti-CMV treatment. Because CMV-specific T cells may be important in controlling replication of CMV, numerous studies have investigated their role in controlling disease, both in patients with AIDS and in transplant recipients [14–23]. Furthermore, several groups, including ours, have investigated whether CMV-specific T cell responses could potentially be used to inform clinicians when to initiate or discontinue treatment for patients with AIDS with CMVR [14–20]. Thus, to identify potential immune correlates of protection against AIDS-related CMV end-organ disease that might subsequently be tested for clinical utility in patients at risk for developing AIDS-related CMVR or progression of prevalent CMVR, we measured the frequency of CMV-specific, interferon (IFN)–γ– and interleukin (IL)–2–expressing CD4+ and CD8+ T cells by cytokine flow cytometry (CFC) and simultaneously examined the maturational status of CMV-specific CD8+ T cells in patients with a history of CMVR. These measurements were compared in 2 groups of patients with CMVR—one with clear evidence of absent CMV-protective immunity (i.e., patients with active CMVR) and one with clear evidence of restored CMV-protective immunity (i.e., patients with CMVR clinically immunorestored by ART who were able to discontinue anti-CMV therapy without further retinitis reactivation or progression).

**PATIENTS, MATERIALS, AND METHODS**

**Study participants.** Frozen peripheral-blood mononuclear cell (PBMC) specimens were obtained from Longitudinal Studies of the Ocular Complications of AIDS (LSOCA)—a prospective, multicenter, observational study of patients with AIDS [24, 25]. Two patient groups were studied. Group 1 (active CMVR) was composed of subjects with a white opacified retinal lesion (consistent with CMVR) or new CMVR progression (defined as movement of a retinitis border by ≥750 μm along a front ≥750 μm wide or development of a new area of retinitis ≥750 μm in diameter). Group 2 (immunorestored CMVR) was composed of subjects with a history of CMVR diagnosis who were no longer receiving anti-CMV treatment and had been receiving HAART for ≥6 months, whose absolute CD4+ T cell counts were ≥200 cells/μL and who had ≥2 years of ophthalmologic follow-up at the time of the study without receiving anti-CMV therapy or having retinitis reactivation or progression. Informed consent was obtained from all patients participating in the study. The human experimentation guidelines of the US Department of Health and Human Services and of participating institutions were followed in conducting this research.

**PBMC samples.** PBMCs, cryopreserved from acid citrate dextran whole blood as described elsewhere [15], were obtained from the LSOCA specimen repository.

**CMV-specific multiparameter CFC assay.** For each subject, 1 aliquot of 10 × 10^6 cryopreserved PBMCs were rapidly thawed into warm RPMI 1640 with 10% fetal bovine serum (Invitrogen), washed, and rested overnight in slanted 15-mL conical tubes with loosened caps in 3 mL of the same medium, in a CO2 incubator at 37°C. PBMCs were then washed and resuspended for counting using the Viacount assay on a Guava Personal Cell Analysis system (Guava). Samples with <50% viability or <10% recovery were discarded.

Remaining samples were plated at 1 × 10^6 to 2 × 10^6 cells/mL and stimulated with pp65 or IE1 peptide pools or the combined pools. Peptides (15-aa residues, overlapping by 11 aa) spanning the pp65 (SynPep) and IE-1 (Jerini AG) proteins of CMV were dissolved in dimethyl sulfoxide at a concentration of 0.7 mg/mL/peptide and used at a final concentration of 1.7 μg/mL/peptide (1:400 dilution). For each sample, a nonstimulated control was set up. If <4 × 10^5 cells were recovered, a single combined pp65/IE1 stimulation was set up. Data from the independent pp65 and IE1 stimulations were summed for each subject and considered as combined pp65/IE1 stimulation data. Data were background corrected by subtracting the control assay results and normalized to cytokine-expressing cells per milliliter by multiplying the percentage of cytokine-expressing cells by the absolute number of CD4+ or CD8+ T cells per milliliter.

Cells were stimulated for 6 h at 37°C in 0.5 mg/mL brefeldin A in a temperature-controlled plate incubator (INHECO) and held at 18°C after the incubation period (holding the sample at 18°C until staining was found to give better results than holding at 4°C; data not shown). The following day, cells were treated with 2 mmol/L EDTA, then washed and resuspended in PBS with 0.5% bovine serum albumin (BSA). Cells were then stained with CD4-AmCyan, CD8-Alexa 700, CD45RA-PE-CY7, CD27-APC, and CD28-APC-CY7 (all from BD Biosciences) in the presence of 5 μg/mL ethidium monoazide bromide (EMA; Molecular Probes) and incubated for 50 min in the dark at 4°C followed by exposure to a 40-W fluorescent light for 10 min at room temperature, to cross-link the EMA. Cells were washed and then fixed and permeabilized by 10-min incubation in FACS Lyse and 10 min incubation in FACS Perm (both from BD Biosciences). Cells were then washed and stained with IFN-γ–fluorescein isothiocyanate, IL-2–phyco-
Table 1. Baseline characteristics of evaluable patients with cytomegalovirus retinitis (CMVR).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (active CMVR)</th>
<th>Group 2 (immunorestored CMVR)</th>
</tr>
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<tbody>
<tr>
<td>Age, median (range), years</td>
<td>40 (26–62)</td>
<td>42 (32–61)</td>
</tr>
<tr>
<td>Male</td>
<td>87.5</td>
<td>94</td>
</tr>
<tr>
<td>Race/ethnicity</td>
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<tr>
<td>White</td>
<td>58</td>
<td>56</td>
</tr>
<tr>
<td>Black</td>
<td>21</td>
<td>12</td>
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<tr>
<td>Hispanic</td>
<td>12.5</td>
<td>23.5</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Absolute T cell count, ( \text{median (range), cells/µL} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁰</td>
<td>28 (1–102)</td>
<td>439 (206–882)</td>
</tr>
<tr>
<td>CD8⁰</td>
<td>516 (14–1800)</td>
<td>1183 (309–3546)</td>
</tr>
</tbody>
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**NOTE.** Data are percentage of patients, unless otherwise indicated.

\* On the date that peripheral-blood mononuclear cell specimen was obtained.

RESULTS

Viability and recovery of cells from archived PBMC samples. PBMC samples from each of 49 patients with active CMVR (group 1) and 51 patients with CMVR who were immunorestored (group 2) were thawed for CFC. Of these, 37 group 1 and 38 group 2 patients had adequate viability and recovery by the Guava PCA Viacount assay. After EMA staining, 24 group 1 and 34 group 2 samples had adequate viability for evaluation. Sufficient viable PBMCs permitted independent stimulations with the pp65 and IE1 peptide pools in specimens from 10 of 24 group 1 patients and 24 of 34 group 2 patients. The remaining samples were stimulated with the combined pp65/IE1 peptide pool.

Baseline characteristics of evaluable subjects. Baseline characteristics of evaluable subjects are summarized in table 1.

CD4⁺ and CD8⁺ T cell IFN-\(\gamma\)- and IL-2 expression in response to stimulation with pp65 and IE1. Representational flow analyses of CD4⁺ and CD8⁺ T cell expression of IFN-\(\gamma\)- and IL-2 in figure 1A–B. CD4⁺ T cell cytokine responses to pp65 or combined pp65/IE1 were significantly higher in the immunorestored group 2 patients than in the active CMVR group 1 patients for both IFN-\(\gamma\)- and IL-2 expression \((P<.0001)\) and IFN-\(\gamma\)- and IL-2 responses to IE1 alone were essentially absent in both groups (figures 1 and 2A–B). CD4⁺ T cell cytokine responses to combined pp65/IE1 were also significantly higher in group 2 for expression of both IFN-\(\gamma\)- and IL-2 cells \((P=.0001)\) and IFN-\(\gamma\)- and IL-2 cells \((P=.0015)\) (figure 2C–D). However, in contrast to the absence of CD4⁺ T
cell responses to IE1, there was a trend toward group 2 having higher CD8+ T cell responses to IE1 than group 1 for IFN-γ’IL-2+ expression (P = .0072) (figures 1 and 2C). There was no significant difference between the 2 groups in response to pp65 alone (P = .697, for IFN-γ’IL-2+ expression; P = .954, for IFN-γ’IL-2+ expression) (figures 1 and 2C–D), suggesting that, for combined pp65/IE1 stimulation, higher responses in group 2 were largely due to IE1 responses. Notably, differences in the number of CMV-specific CD8+ T cells between the 2 groups was most pronounced when we measured IFN-γ’IL-2+ cells (figure 2C–D). CMV-specific CD4+ and CD8+ T cell IFN-γ’IL-2+ responses were very low and were not included in this analysis.

**Maturational status of CMV-specific IFN-γ’IL-2’ CD8’ and IFN-γ’IL-2’ CD8’ T cells.** CMV-specific CD8’IFN-γ’IL-2’ and CD8’IFN-γ’IL-2’ cells were subsetted, using the maturation markers CD45RA, CD27, and CD28, into each of the possible 8 subpopulations for each cytokine population. An example of a subject from each group is shown in figure 3A. Figure 3B shows the absolute numbers of each subpopulation plotted in approximate order of differentiation, with the presumed earliest subpopulations to the left and the more mature subpopulations to the right. Although the majority of both CMV-specific IFN-γ’IL-2’ and IFN-γ’IL-2’ cells have a more mature phenotype, in group 2 subjects, the distribution appears shifted toward less mature phenotypes. Of note, group 2, compared with group 1, had significantly higher numbers of CMV-specific IFN-γ’IL-2’ CD27’CD28’CD45’ cells (median [range], 53.5 [0–711] vs. 7.5 [0–113] cells/mL, respectively; P < .0001) and IFN-γ’IL-2’ CD27’CD28’CD45RA− cells (median [range], 442 [28–8155] vs. 34.5 [0–881] cells/mL, respectively; P = .0002). Although the absolute numbers of CMV-specific CD8’IFN-γ’IL-2’ CD27’CD28’ T cells did correlate with absolute CD8+ T cell counts (data not shown), the difference between groups in this subset was independent of differences between the groups in absolute CD8+ T cell counts, as evidenced by a significant difference in the percentage of total CD8+ T cells that were CD8’IFN-γ’IL-2’ CD27’CD28’ (median [range], 0.07% [0%–0.66%] in group 2 vs. 0.01% [0%–0.14%] in group 1; P = .0003). There was also a trend in group 2 of having higher numbers of cells expressing the CD27’CD28’RA− phenotype (median [range], 320.5 [0–2235] vs. 12 [0–616] cells/mL, respectively; P = .0026). Among the CD8’IFN-γ’IL-2’ subsets, none of the differences between the groups were significant.

Because these data suggested that CD27 and CD28 expression by CMV-specific cells may be important in immune recovery from CVMR, we examined the relative importance of each marker to identify parameters that might not require 9-color analysis. We compared expression of total CD28, CD27, and both CD27 and CD28 on CMV-specific CD8+ cells stimulated with combined pp65/IE1 and observed that group 2 had significantly higher numbers than group 1 of all 3 marker combinations—total CD8’IFN-γ’CD27’ (P = .0037), CD8’IFN-
Figure 2. Cytomegalovirus (CMV)–specific T cell cytokine responses in patients with active CMV retinitis and patients with CMV retinitis with clinical evidence of a restored, protective immune response to CMV retinitis to stimulation with pp65, IE1, or combined pp65/IE1 peptide pools. The no. of circulating CD4+ T cells expressing both interferon-γ (IFN-γ) and interleukin (IL)-2 (A) or IFN-γ alone (B) and of CD8+ T cells expressing both IFN-γ and IL-2 (C) or IFN-γ alone (D) are shown. P values were adjusted for multiple comparisons using the Bonferroni correction. *Borderline P value in the .0019–.01 range for any pairwise comparison (Mann-Whitney U test); **P < .0001.

CD4+IFN-γ+IL2+ ( ), and CD8+IFN-γ+CD27+CD28+ ( ), and CD8+IFN-γ+CD27+CD28+ ( ), and CD8+IFN-γ+CD27+CD28+ ( ).

γ’CD27’CD28’ (P < .0001), and CD8’IFN-γ’CD28’ (P < .0001). This suggests that expression of CD28 by CMV-specific CD8+ T cells may be as sensitive for distinguishing between group 1 and 2 patients as combined CD27 and CD28 expression.

Optimizing the potential clinical utility of the assay.

Receiver operator curves were inspected to determine optimal cutoff values for a positive result with combined pp65/IE1 stimulation (insufficient data were available for analysis of pp65 or IE1 stimulation alone). Optimal cutoff values were >50 cells/mL for CD4’IFN-γ’IL-2’ T cells, >70 cells/mL for CD4’IFN-γ’IL-2’ T cells, >100 cells/mL for CD8’IFN-γ’IL-2’ T cells, and >4000 cells/mL for CD8’IFN-γ’IL-2’ T cells. Zero (0%) of 24 active group 1 patients with CMVR, compared with 26 (76%) of 34 immunorestored group 2 patients with CMVR, had a positive CD4’IFN-γ’IL-2’ result; 0 (0%) of 24 group 1 patients, compared with 27 (79%) of 34 group 2 patients, had a positive CD4’IFN-γ’IL-2’ result; 8 (33%) of 24 group 1 patients, compared with 29 (85%) of 34 group 2 patients, had a positive CD8’IFN-γ’IL-2’ result; and 7 (29%) of 24 group 1 patients, compared with 26 (76%) of 34 group 2 patients, had a positive CD8’IFN-γ’IL-2’ result.

Because there were substantial false-positive CD8’IFN-γ’IL-2’ and CD8’IFN-γ’IL-2’ results in patients with active CMVR (33% and 29%, respectively), we examined whether the maturational status of these CMV-specific CD8+ T cells might improve assay discrimination between the 2 patient groups. Exploratory analysis revealed that CMV-specific CD8’IFN-γ’T cells expressing CD28 and CD27 (independent of CD45RA and IL-2 expression) had the maturational profile that maximally reduced false-positive results. Receiver operator curves revealed that the optimal cutoff value for a positive result was >1100 CD8’IFN-γ’CD27’CD28’ T cells/mL. By combining this parameter with CD4’IFN-γ’IL-2’ and CD4’IFN-γ’IL-2’ T cell results, we observed that none (0%) of the 24 subjects with active CMVR had absolute numbers of CMV-specific CD8+ T cells above the respective cutoff values, whereas 29 (85%) of 34 of the immunorestored patients with CMVR had at least 1 positive value, and 25 (74%) had positive results by at least 2 of these measurements. These CMV-specific assays are more likely to have clinical utility than measurements with higher false-positive rates because, from a treatment perspective, there is more potential for irreversible harm in not treating patients who will develop active CMVR (i.e., loss of vision) than in...
Figure 3. Expression of maturational markers on cytomegalovirus (CMV)–specific CD8+ T cells. Flow analysis of CD45RA and CD28 expression was gated on CD8+ interferon (IFN)–γ–CD27+ (A, left panels) and CD8+IFN–γ–CD27+ (A, right panels) for CD8+ T cells stimulated with combined pp65/IE peptide pools. pp65/IE–specific cells are shown in the foreground (black dots) overlayed on the total CD8+ cell population (gray dots). Representative plots from a patient with active CMV retinitis (CMVR) (A, top panels) and a patient with CMV retinitis with clinical evidence of a restored, protective immune response to CMV (A, bottom panels) are shown. Percentages of cytokine-expressing CD8+ T cells were converted to total cells per milliliter for each population. Median and SE of each population are displayed for both group 1 active (B, top panel) and group 2 patients with immunorestored CMVR (B, bottom panel). Only subjects with >50 cytokine-positive events were included in the analysis. P values were adjusted for multiple comparisons using the Bonferroni correction. *Borderline P value in the .0019–.01 range for any pairwise comparison (Mann-Whitney U test); **. P < .0018 treating patients who will not develop active CMVR (i.e., cost and toxicities of treatment).

DISCUSSION

The availability of cryopreserved PMBCs from subjects with active CMVR and quiescent CMVR with restored CMV-protective immunity after HAART, combined with multicolor flow cytometric assays, enabled testing of the hypothesis that recovery from AIDS-related CMVR is mediated, in part, by CMV-specific CD4+ and CD8+ T cells capable of promoting antigen-specific CD8+ T cell proliferation. A 9-color CFC assay was used to simultaneously measure the expression of IFN–γ and IL-2 and to characterize the maturational phenotype of CMV-specific CD8+ T cells. Although many published studies have compared either CD4+ or CD8+ T cell responses in patients with active and immunorestored CMVR, only 2 that we know of have compared CMV-specific CD4+ and CD8+ T cell responses in the same patients [19, 20], and neither report examined IL-2 expression or the maturational status of cytokine-positive CD8+ T cells.

Consistent with most previous reports, we observed that patients with active retinitis had significantly lower CD4+ and CD8+ T cell IFN–γ responses than patients who were clinically immunorestored [14–16, 18–20]. However, we also observed significantly lower numbers of CMV-specific T cells that also expressed IL-2 in subjects with active CMVR, findings not previously reported in the context of AIDS-related CMV disease. These findings are consistent with both murine CMVR (MCMVR) and HIV studies. In experimental MCMVR, systemic immunotherapy with IL-2 provided protection against murine AIDS-related MCMVR by stimulation of the perforin-mediated pathway of cytotoxicity used by NK cells and cytoxic CD8+ T cells to kill virus-infected cells [26]. Furthermore, lack of HIV-specific CD4+IL-2+IFN–γ+ cells has been associated with HIV-related disease progression [27, 28], whereas HIV–1–specific CD8+IL-2+ T cells have been shown to promote CD8+ T cell proliferation, independently of CD4+ T cell help. The lack of IL-2+ T cells may account for the reduced ability of HIV–1–specific CD8+ T cells from HIV disease progressors, compared with those from nonprogressors, to proliferate [29, 30]. CMV-specific CD8+IL-2+ T cells may play a similar role in patients with AIDS with CMV end-organ disease.
Among specimens stimulated with individual pp65 or IE1 pools, we observed significantly higher pp65-specific CD4+ T cell responses among the immunorestored CMVR group, whereas both groups lacked an IE1-specific CD4+ T cell response. In contrast, although there was no significant difference between the 2 groups in the number of CD8+ T cells that responded to pp65, the immunorestored group had higher numbers of CD8+ T cells that responded to IE. This latter finding is consistent with that of Sacre et al., who observed a broader diversity of CD8+ T cell IFN-γ responses to IE1 peptides by ELISPOT assay in subjects with CMVR who had been immunorestored by HAART within 2 years than in patients with active CMV end-organ disease [16]. IE1-dominant CD8+ T cell IFN-γ responses have also been reported to be associated with protection from developing CMV disease in HIV-negative transplant recipients [21]. We also recently reported that CMV Towne vaccine induces an IE1-dominant CD8+ T cell IFN-γ and proliferation response [31]. This vaccine, although not capable of preventing infection, was able to reduce the incidence and severity of CMV disease in renal transplant recipients [32]. IE1 is one of the first CMV proteins expressed after cell infection [33], and expression of IE1 on the surface of recently infected cells in the context of major histocompatibility complex class I may allow infected cells to be killed before they produced new CMV virions, thus reducing the spread of CMV and promoting recovery from CMV disease.

In examining maturational phenotypes of CMV-specific, cytokine-expressing CD8+ T cells, we observed that cells with an early maturational phenotype (CD27+CD28+CD45RA+ and CD28+-CD8+ T cells may retain the ability to expand and produce IL-2 in response to antigenic stimulation but may also require CD4+ T cell help. In the absence of less mature populations, the CD8+ T cell response against CMV may not be adequate to contain the replication and spread of CMV. This hypothesis is supported by data from Bronte et al., who reported the disappearance of CMV-specific CD4+ T cells 1 year before the onset of CMV end-organ disease in patients with AIDS [19]. Treatment of patients with AIDS-related CMVR by HAART may allow recovery of CMV-specific CD4+ T cells (presumably depleted by HIV-1 infection) as HIV-1 replication is suppressed. Our findings suggest that such immune restoration is associated with expression of both IFN-γ and IL-2 by CMV-specific CD4+ and CD8+ T cells and with expression of CD28 by CMV-specific CD8+ T cells. However, the current study does not rule out the possibility that the observed differences between the groups may be a consequence of differences in immunity to CMV. To determine whether the populations that were associated with immune restoration are actively involved in maintaining immunity, a prospective case-control study will need to be performed using PBMCs obtained before case patients develop incident CMVR or progression or reactivation of prevalent CMVR.

In conclusion, we identified 3 CMV-specific T cell parameters that, when combined, discriminated better between patients with active and immunorestored CMVR than CMV-specific T cell parameters reported in the past. CD8+IFN-γ+IL-2+, CD8+IFN-γ+IL-2-, and CD4+IFN-γ+IL-2+ T cells were low or absent in all 24 patients with active CMVR, whereas 29 (85%) of 34 of the patients with immunorestored CMVR had a positive value by at least 1 of these parameters. Because progression of untreated CMVR causes irreversible loss of retina and because the drugs used to treat CMVR have considerable risk of toxicity and are very expensive, there is a need for clinicians to know when it is optimal to initiate, discontinue, or reinstitute anti-CMV therapy. The CMV-specific T cell measurements identified in this exploratory analysis can now be tested in observational studies for their predictive value and potential clinical utility in defining CMV-protective immunity and guiding clinical management for HIV-infected patients with a history of CMVR or at risk for developing CMVR.

LONGITUDINAL STUDIES OF THE OCULAR COMPLICATIONS OF AIDS KEY PERSONNEL

Baylor College of Medicine, Cullen Eye Institute, Houston, Texas. Richard Alan Lewis (director), Larry Dillon, Victor Fainstein, Pamela Frady, Ronald L. Gross, Silvia Oreno-Nanjo, Tobias Samo, Laura Shawver, Benita Slight, Rachel Sotuyo, and Steven Spencer. Former members: Richard C. Allen, Stephen P. Travers, and James W. Shigley.

Emory University Eye Center, Atlanta, Georgia. Daniel F. Martin (director), Alex DeLeon, David Furukawa, Deborah
Gibbs, Baker Hubbard, Bob Myles, and James P. Steinberg. Former members: Denise Armenger, Antonio Capone, Jr., James Gilman, and Sandra Strittman.


**Louisiana State University Medical Center, New Orleans.** Bruce Barron (director), Robin Bye, Rebecca Clark, Larry Dillon, Deborah Elkins, Maxine Haslauer, Christine Jarrott, Audrey Lombard, Natalie Loyacano, and Lynn Otillo. Former members: John Bennett, Mandi Conway, and Gholam Peyman.

**New Jersey Medical School, Newark.** Ronald Rescigno (director), Rosa Paez-Boham, and Marta Paez-Quinde. Former member: Eileen Buroff.


**New York University Medical Center, New York, New York.** Dorothy N. Friedberg (director), Adrienne Addessi, Douglas Dieterich, Monica Lorenzo-Latkany, and Maria Pei. Former members: Richard Hutt and Alex McMeeking.

**Northwestern University, Chicago, Illinois.** Alice Lyon (director), Lori Kaminski, Robert L. Murphy, Frank Palella, and Jonathan Shankle. Former members: Daniel Andrews, Steve Grohmann, Robert Hirshsticker, Alexander Habib, Pamela Hulvey, Jill Koecher, Annmarie Mun, Peter Pertel, Michele Till, Jamie VonRoenn, David V. Weinberg, and James Yuhr.

**Rush University Medical Center, Chicago, Illinois.** Mathew W. MacCumber (director), Bruce Gaynes, Pamela Hulvey, Pauline Merrill, Denise Voskuil-Marre, and Allan R. Tenorio. Former members: Andrea Kopp, Nada Smith, Harold Kessler, and Frank Morini.

**University of California, Irvine.** Baruch D. Kuppermann (director), Marcia Alcolouloure, Donald N. Forthal, Jeff Grigalva, Faisal Jehan, Heikki Kostamaa, Rosie Magallon, and Brett Trump. Former members: Karen Lopez, Nader Moinfar, Santosh Patel, Mark Thomas, Randy Williams, and Melody Vega.


**University of California, San Diego.** William R. Freeman (director), Tom Clark, Denie Cochran, Randall L. Gannon, Victoria Morrison, Nicole Reigan, Michelle Shin, and Michelle Sittmann. Former members: Susan Chaidhawanqual, Lingyun Cheng, Mark Cleveland, Claudio Garcia, Patricia Garoutte, Daniel Goldberg, Marietta Karavellas, Brian Kosobucki, Mi-Kyoung Song, Francesca Torriani, Dorothy Wong, and Tekeena Young.


**University of North Carolina, Chapel Hill.** Travis A. Meridith (director), Debra Cantrell, Kelly DeBoer, M. Elizabeth Hartnett, Sandy Barnhart, Maurice B. Landers, and David Wohl. Former members: Stephanie Betran, David Eifrig, John Foley, Angela Jeffries, Roje Kacmaz, Jan Kylstra, Barbara Longmire, Sharon Myers, Kean T. Oh, Jeremy Pantell, Susan Pedersen, Cadmus Rich, Cecilia A. Sotelo, Charles van der Horst, and Samir Wadhanya.


**University of Southern California, Los Angeles.** Jennifer I. Lim (director), Lawrence Chang, Tom S. Chang, Alexander Charonis, Christina Plaxel, Jesus Garcia, Francoise Kramer, Lori Levin, Len Richine, A. Frances Walonker, and Zi Wui. Former members: John Cangano, Robert Equi, Tracy Nichols, Christopher Pelzek, Robert See, and Mark Thomas.

**University of South Florida, Tampa.** Peter Reed Pavan (director), Sandra Gompf, James Habib, Lori Mayor, Scott Paulter, Wyatt Saxon, and Nancy Walker. Former members: Bonnie Hernandez, JoAnn Leto, Sharon Millard, and Jeffrey Nadler.

**University of Texas Medical Branch, Galveston.** Helen K. Li (director), Susan Busch, John Horna, Wiline Jean, Zbtigniew Krason, Lan-Chi Nguyen, and David Paar. Former members: Robert Blem, Celia Hutchinson, Vivian Keys, Beverly B. Mizell, Michelle Onarato, Anne Stewart, and Sami H. Uwaydat.

**Chairman’s Office, The Johns Hopkins University School of Medicine, Baltimore, Maryland.** Douglas A. Jabs (study
chairman), Judith C. Southall, and Maria Stevens. Former members: Wanda M. Chaney, Nancy H. Davidson, Jacqueline Harden, Joan L. Klemstine, and Lana M. Kramer.


**Fundus Photograph Reading Center, University of Wisconsin, Madison.** Matthew D. Davis (director), Michael Altaweel, Jane Armstrong, Sheri Glaeser, Larry Hubbard, Dolores Hurburt, Jeffrey Joyce, Linda Kastorff, Michael Neider, Nancy Robinson, Therese Traut, Marilyn Vanderhoof-Young, and Hugh Wabers.

**National Eye Institute, Bethesda, Maryland.** Natalie Kurinij.

**National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.** Former member: Beverly L. Alston.

**Officers of the Study.** Douglas A. Jabs (chair), Matthew D. Davis, Janet T. Holbrook, Natalie Kurinij, and Curtis L. Meinert. Former member: James A. Tonascia.


**References**


