Effect of Cytomegalovirus-Induced Immune Response, Self Antigen–Induced Immune Response, and Microbial Translocation on Chronic Immune Activation in Successfully Treated HIV Type 1–Infected Patients: The ANRS CO3 Aquitaine Cohort

Linda Wittkop,1,2,3,4 Juliette Bitard,4,6 Estibaliz Lazaro,7 Didier Neau,7 Fabrice Bonnet,7 Patrick Mercie,7 Michel Dupon,7 Mojgan Hessamfar,7 Michel Venture,5,6 Denis Malvy,7 François Dabis,1,2,3 Jean-Luc Pellegrin,7 Jean-François Moreau,4,6 Rodolphe Thiebaut,1,2,3 and Isabelle Pellegrin,7 for the Groupe d’Épidémiologie Clinique du SIDA en Aquitaine8

1University Bordeaux, ISPED, Centre INSERM U897-Epidemiologie-Biostatistique, 2INSERM, ISPED, Centre INSERM U897-Epidemiologie-Biostatistique, 3CHU de Bordeaux, Pôle de Santé Publique, Service d’Information Medicale, 4CHU de Bordeaux, Laboratoire d’Immunologie et Immunogénétique, University Bordeaux, 5University Bordeaux, CIRID, UMR 5164, 6CNRS, CIRID, UMR 5164, 7CHU de Bordeaux, Service de Médecine Interne et Maladies Infectieuses, 8CNRS, Microbiologie Fondamentale et Pathogénicité UMR 5234, University Bordeaux, and 9University Bordeaux, CNRS UMR 5234, Bordeaux, France

We evaluated the impact of cytomegalovirus (CMV)–induced immune responses, autoimmune-induced immune responses, and microbial translocation on immune activation in 191 human immunodeficiency virus type 1–infected patients from the ANRS CO3 Aquitaine Cohort. All enrolled subjects had achieved long-term virological suppression during receipt of combination antiretroviral therapy (cART). HLA-DR+/CD38+ expression was 16.8% among CD8+ T cells. Independent of age, CD4+ T-cell count, 16S ribosomal DNA load, and regulatory T-cell count, positive results of Quantiferon CMV analysis (P = .02), positive results of CMV-pp65 enzyme-linked immunosorbent spot analysis (P = .01), positive results of CMV-pp65–specific CD8+ T-cell analysis (P = .05), and CMV seropositivity (P = .01) were associated with a higher percentage of CD8+ T cells that expressed HLA-DR+/CD38+. Autoimmune response and microbial translocation were not associated with immune activation. Therefore, the CMV-induced immune response seems to be associated with chronic immune activation in cART recipients with sustained virological suppression.

Keywords. chronic immune activation; CMV-induced immune response; microbial translocation; autoimmune-induced immune response; HIV-infection; antiretroviral therapy.

In human immunodeficiency virus (HIV)–infected individuals, chronic immune activation predicts progression to AIDS, independently of HIV RNA load [1, 2]. Chronic immune activation is multifactorial: the presence of HIV leads to an adaptive immune response mediated by HIV–specific T and B cells, microbial products translocated from the intestinal lumen to the systemic circulation may cause broad activation of the immune system, infections due to environmental or opportunistic pathogens (eg, cytomegalovirus [CMV] [3]) may reactivate more frequently and stimulate large numbers of specific T cells, and autoantigens from apoptotic cells could activate the dendritic cell system, stimulate interferon α (IFN-α) production, and stimulate large-scale activation of CD8+ T lymphocytes [4]. The contribution of each of these mechanisms to chronic immune activation is not well evaluated. Most studies were designed to understand the participation of a specific and unique cause [5–7]. We hypothesized that CMV could be a leading factor in chronic immune activation in these patients because of its prevalence, the intensity of the immune response to CMV [8], and the frequency of reactivation of CMV in immunocompromised patients. We assessed the impact of the CMV-induced immune response, the autoimmune-induced immune response, and microbial translocation on chronic immune activation, defined by the percentage of CD8+ T cells that expressed HLA-DR+/CD38+ in patients who have been receiving successful combination antiretroviral therapy (cART) for at least 2 years.

METHODS

Study Population

Patients enrolled in the ANRS CO3 Aquitaine Cohort, a prospective, hospital-based cohort of HIV-1–infected patients in...
southeastern France [9], were consecutively included in this cross-sectional study. The study protocol was approved by the local ethics committee, and all patients provided written informed consent.

HIV-infected patients who initiated cART between 2005 and 2008, had no modification in their current cART regimen for at least 2 years, and had an HIV-1 RNA load below the detection limit of 50 copies/mL for at least 2 years were included. Exclusion criteria were 2 consecutive HIV-1 RNA load measurements of >50 copies/mL, hepatitis C virus or hepatitis B virus coinfection, and signs of acute infection.

**Laboratory Measurements**

**Flow Cytometry**

Stained whole fresh blood samples were analyzed in a 10-color flow cytometer (Navios, Beckman Coulter, Villepinte, France). All monoclonal antibodies except anti-HLA-DR-V450 and anti-HLA-A*0201-PE (Becton Dickinson, Pont de Claix, France) were purchased from Beckman Coulter (Villepinte, France). Nine panels were used for each sample. For HLA-A*0201-positive patients, enumeration of CMV-pp65-specific and self-antigen-specific CD8+ T-cells was determined with appropriate HLA-A*0201 tetramer staining (class I iTag major histocompatibility complex MHC Tetramer, Immunomics-Beckman, Marseille, France; Supplementary Table 1). A minimum of 30,000 CD3+CD8+ events were collected. An isotypic control iTag-PE tetramer (Neg-iTag-PE) was used to determine tetramer-nonspecific staining.

**CMV Status**

CMV seropositivity was assessed by detecting immunoglobulin G and immunoglobulin M antibodies to CMV late antigens (Dade Behring, Deerfield, IL). CMV DNA quantification was performed in whole blood (lower limit of detection, 300 copies/mL; Roche, Meylan, France). The CMV-specific immune response was determined using Quantiferon CMV analysis (Cellestis, Australia), CMV-pp65 enzyme-linked immunosorbent spot (Elispot) analysis, and CMV-pp65-specific tetramers.

Quantiferon CMV analysis was performed according to the manufacturer’s instructions. Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed and analyzed for CMV reactivity in an IFN-γ Elispot (Supplementary Table 3). A positive CMV-pp65 Elispot result was defined as detection of >16 spot-forming cells (SFCs) per 2.5 × 10^5 PBMCs. The positivity threshold for CMV-pp65-specific CD8+ T-cells, based on HLA-A*0201 tetramer enumeration, was 0.04% of CD8+ T-cells.

**Self Antigen–induced Immune Response**

Antinuclear antibody (ANA) titers were measured by indirect immunofluorescence staining on an HEP-2 cell substrate (DiaMed, Marnes-la-Coquette, France). We considered titers of ≥1:250 as indicating a positive finding. All sera were screened for 13 antibodies specific to extractable nuclear antigens (anti-ENA), using the FIDIS Connective Profile kit (Biomedical Diagnostics, Marne-la-Vallee, France). The positivity threshold for self-antigen–specific CD8+ T-cells, based on HLA-A*0201 tetramer enumeration, was 0.04% of CD8+ T-cells.

**Microbial Translocation**

We measured the level of bacterial 16S rRNA, as a direct marker of microbial translocation, and the level of soluble CD14 (sCD14), as a marker of monocyte activation and a surrogate marker of microbial translocation. Levels of sCD14 were quantified using an enzyme-linked immunosorbent assay (Diaclone, CT). The plasma 16S rRNA load was measured by quantitative polymerase chain reaction (PCR). Bacterial DNA was extracted from 200-µL plasma samples in a PCR-free clean room, using the DNeasy Blood and Tissue Kit (Qiagen, Courtabœuf Cedex, France), according to the manufacturer’s instructions. Degenerate forward and reverse primers (8F: 5'-AGAGTTTGATYMTGGCTCAG-3'; 361R: 5'-CGYC-CATTGGBAAADATTCC-3') and a TaqMan probe (338P: 5'-FAM-TACGGGAGGCACAGT-BHQ1-3') for 16S rRNA were synthesized by Eurofins MWG Biotech (Ebersberg, Germany). A standard curve was created from serial dilutions of a linearized plasmid DNA (pCR2.1 TOPO-16S rDNA) containing known copy numbers of the template. Our control group of 10 healthy volunteers had a mean 16S rDNA level of <2.69 log_{10} copies/mL (median level, 2.15 log_{10}/copies/mL).

**Statistical Analysis**

Qualitative variables were described using numbers (and percentages), and quantitative variables were described using medians (and interquartile ranges [IQRs]). Concordance between tests for CMV-induced immune response was assessed by k coefficients. The Mann–Whitney U test was used to compare quantitative variables between groups. For each CMV assay and marker of autoimmune-induced immune response, a multivariable linear regression model was adjusted to assess its association with chronic immune activation, measured by HLA-DR+/CD38+CD8+ T cells. All multivariable models were adjusted for age, CD4+ T-cell count, regulatory T-cell count, and 16S rDNA load. The response variable was log_{10} transformed for normality and homoscedasticity of residuals. All analyses were performed with SAS 9.13/SAS 9.3 (SAS Institute, Cary, NC).

**RESULTS**

**Patient Characteristics**

Of 200 patients included in the ACTHIV study, 191 were considered for the current analysis (Supplementary Table 2);
9 patients were excluded because they fulfilled exclusion criteria. Median CD4⁺ and CD8⁺ T-cell counts were 517 cells/µL (IQR, 387–720 cells/µL) and 676 cells/µL (IQR, 480–977 cells/µL), respectively. Patients had a nadir CD4⁺ T-cell count of 188 cells/µL (IQR, 89–275 cells/µL; Supplementary Table 2). Seventy percent of patients had a CD4⁺/CD8⁺ T-cell ratio of <1 (only 13% and 6% of patients had a ratio of >1.5 and >2, respectively). The expression of HLA-DR⁺/CD38⁺ was 16.8% among total CD8⁺ T cells (Supplementary Table 2).

Results of CMV serologic analysis were positive for 93% of patients. Only 1 patient had a positive whole blood CMV PCR result (Supplementary Table 3). Results of the Quantiferon CMV test were positive for 78% of patients, with a median IFN-γ level of 6 IU/mL. Of 188 patients with an available CMV-pp65 EliSpot assay result, 89% had a positive response to CMV-pp65 peptides, with a median of 424 SFCs per 2.5 × 10⁵ PBMCs. Of the 87 HLA-A02–positive patients, 83% (67 of 81) had CMV-pp65–specific CD8⁺ T cells (median percentage of CD8⁺ T cells, 1.5%). κ coefficients for results of Quantiferon CMV analysis and CMV-pp65 EliSpot analysis, results of Quantiferon CMV analysis and CMV-pp65–specific CD8⁺ T-cell analysis, and results of CMV-pp65 EliSpot analysis and CMV-pp65–specific CD8⁺ T-cell analysis were 0.5 (95% confidence interval [CI], .3–.6), 0.4 (95% CI, .2–.7), and 0.5 (95% CI, .2–.8), respectively.

Factors Associated With Chronic Immune Activation
A positive result of CMV serologic analysis was associated with a higher level of immune activation, as measured by the median percentage of CD8⁺ T cells that expressed HLA-DR⁺/CD38⁺: 17.1% (IQR, 11.5%–23.7%) in seropositive patients, compared with 9.4% (6.7%–15.9%) in seronegative patients (P = .0053; Figure 1A). The percentage of CD8⁺ T cells that expressed HLA-DR⁺/CD38⁺ was significantly higher for patients with a positive result of the Quantiferon CMV assay, compared with those who had a negative result (17.3% [IQR, 11.6%–26.0%] vs 13.0% [IQR, 8.7%–18.6%]; P = .0043; Figure 1B). Patients with a positive CMV-pp65 EliSpot result had a median HLA-DR⁺/CD38⁺/CD8⁺ T-cell percentage of 17.1% (IQR, 11.3%–24.4%), compared with a median value of 10.7% (IQR, 7.1%–13.4%) among patients with a negative result (P = .0022; Figure 1C). The median percentage of CD8⁺ T cells that expressed HLA-DR⁺/CD38⁺ among patients with a positive result of the CMV-pp65–specific CD8⁺ T-cell assay was 16.5% (IQR, 11.7%–23.2%), compared with a median value of 12.4% (IQR, 8.1%–18.6%) among patients with a negative result (P = .1058; Figure 1D). Most CMV-pp65–specific CD8⁺ T cells were terminally differentiated effector memory T cells and HLA-DR⁺ (Supplementary Table 3).

After adjustment for age, CD4⁺ T-cell count, regulatory T-cell count, and 16S rDNA load, a positive result of the CMV

Figure 1. Association between CD8⁺ T-cell activation, as measured by the percentage of CD8⁺ T cells that expressed HLA-DR⁺/CD38⁺, and result of cytomegalovirus (CMV) serologic analysis (A), result of Quantiferon CMV analysis (B), result of CMV-pp65 enzyme-linked immunosorbent spot analysis (C), and result of CMV-pp65–specific CD8⁺ T-cell analysis (D). The Mann-Whitney U test was used to compare percentages of CD8⁺ T cells that expressed HLA-DR⁺/CD38⁺ between patients with a positive and those with a negative result of CMV analysis.
serologic assay (P = .006), as well as a CMV-induced immune response detected by Quantiferon CMV assay (P = .0238) and the CMV-pp65 EliSpot assay (P = .0141), were still significantly associated with a higher level of immune activation, as measured by the percentage of CD8+ T cells that expressed HLA-DR+/CD38+ (Table 1). Patients with a positive result of a CMV-pp65–specific CD8+ T-cell assay showed a tendency for higher percentages of CD8+ T cells that expressed HLA-DR+/CD38+ in adjusted analysis, compared with patients who tested negative for CMV-pp65–specific CD8+ T cells (P = .0538). However, models adjusted for results of CMV serologic analysis, Quantiferon CMV analysis, CMV-pp65 EliSpot analysis, and CMV-pp65–specific CD8+ T-cell analysis explained 16%, 14%, 14%, and 20% of the variation of the percentage of CD8+ T cells that expressed HLA-DR+/CD38+, respectively.

The percentage of CD8+ T cells that expressed HLA-DR+/CD38+ was not correlated with the sCD14 level or the 16S rDNA level (Spearman correlation coefficients: r = −0.03 [P = .6904] and r = −0.13 [P = .1050], respectively).

A positive ANA-Hep-2 test was observed in 43 patients (25.6%), of whom 36 had a titer of 1:250. ANA positivity was not associated with the percentage of CD8+ T cells that expressed HLA-DR+/CD38+ (P = .3336) or age (P = .5224). Anti-ENA (SSA-52kd and SSA-60kd) was detected in 2 patients without symptoms of autoimmune disease. The percentage of CD8+ T cells that expressed HLA-DR+/CD38+ was associated with actin–specific CD8+ T cells (18.9% in tetramer–actin–positive patients vs 13.6% in tetramer–actin–negative patients; P = .0150) but not with vimentin–specific or lamin–specific CD8+ T cells. Actin–specific CD8+ T cells were not associated with chronic immune activation in multivariable analysis (Table 1).

EBV PCR positivity (33 of 190 patients [17%]) was not associated with higher percentages of CD8+ T cells that expressed HLA-DR+/CD38+ (P = .5894). Adjustment for EBV PCR seropositivity had no impact on the association between CMV and immune activation.

The CD4+/CD8+ T-cell ratio (P < .0001), the nadir CD4+ T-cell count (P = .0106), and the natural killer cell count (P = .0001) were associated with immune activation in univariable analysis. Adjustment for the nadir CD4+ T-cell count, the CD4+/CD8+ T-cell ratio (instead of the CD4+ T-cell count), and natural killer cell count had no impact on the association between CMV assays and immune activation. In addition to the CMV assays, older age was always independently associated with a higher level of immune activation.

**DISCUSSION**

We included patients without long-term active HIV replication and without hepatitis virus coinfection. Our study population represents patients receiving cART who are currently in routine clinical care in developed countries. We found an association between chronic immune activation and CMV-induced immune response but not with microbial translocation or autoimmune-induced immune responses.

Chronic immune activation was independently associated with a CMV-induced immune response detected by CMV-pp65 EliSpot analysis and Quantiferon CMV analysis, suggesting that a CMV-specific immune response may play a role in chronic immune activation in HIV-infected patients. This finding is in agreement with former studies suggesting that CMV-specific immune response alters T-cell reconstitution [3] and could participate in chronic immune activation [6]. CMV was described as driving the acquisition of immune signatures associated with immunosenescence. The accumulation of CD8+ T cells, the enrichment in terminally differentiated effector memory T cells among total CD8+ and CMV-pp65–specific CD8+ T cells, and the inverted CD4+/CD8+ ratio mimic the immune risk profile seen in elderly individuals, in whom CMV is believed to play a major role in immunosenescence [10]. The absence of detectable levels of CMV does not rule out the possibility of a CMV burst during the course of the disease. A greater proportion of patients had an elevated anti-CMV–specific immune response measured by CMV-pp65–specific CD8+ T cells, compared with healthy CMV-seropositive subjects (from our group or [11]). We observed a high proportion of patients with a positive CMV-pp65 EliSpot result whose anti-CMV–specific immune response had a magnitude similar to that of healthy, HIV-negative, CMV-seropositive subjects [8, 12]. The high frequency of CMV-specific responses found using Quantiferon CMV analysis in our patients is in agreement with a study by Singh et al [13]. Quantiferon CMV analysis represents a surrogate assay that evaluates a broader anti-CMV immune response in vitro (against pp65, pp50, IE1, IE2, and gB proteins).

Whatever the assay used to measure the anti-CMV immune response, there is a link between CMV and immune activation. Measuring the cellular response specifically does not add to the results provided by the assessment of the humoral response, which might be sufficient to link CMV to the immune activation status. However, this conclusion may be hampered by the small size of the CMV-seronegative control group. Moreover, the serologic result would only represent the immunological “scar,” rather than the current specific cellular immune response against CMV reactivation, which is related to immune activation of CD8+ T cells.

Alteration of the regulatory T-cell compartment has been reported as a factor associated with chronic immune activation, but this was not observed in our analyses. The frequency of positive results among ANA assays was lower than that for healthy individuals [14]. The barely detectable level of self-antigen–specific T cells indicated a limited or no role in chronic immune activation.

The level of sCD14 and the 16S rDNA load were not associated with CD8+ T-cell activation, which is in agreement with
Table 1. Univariable and Multivariable Linear Regression Analysis of Variables With a Potential Influence on Chronic Immune Activation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariable</th>
<th>Multivariable (n = 167), ( R^2 = 0.16 )</th>
<th>Multivariable (n = 165), ( R^2 = 0.14 )</th>
<th>Multivariable (n = 164), ( R^2 = 0.14 )</th>
<th>Multivariable (n = 70), ( R^2 = 0.20 )</th>
<th>Multivariable (n = 58), ( R^2 = 0.17 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reg Coeff(^a)</td>
<td>SD</td>
<td>Stand Coeff(^b)</td>
<td>P</td>
<td>( R^2 )</td>
<td>Reg Coeff(^a)</td>
</tr>
<tr>
<td>Result of CMV serologic analysis, pos vs neg</td>
<td>0.226</td>
<td>0.070</td>
<td>0.23</td>
<td>0.014</td>
<td>0.05</td>
<td>0.215</td>
</tr>
<tr>
<td>Result of Quantiferon CMV analysis, pos vs neg</td>
<td>0.130</td>
<td>0.045</td>
<td>0.21</td>
<td>0.0042</td>
<td>0.04</td>
<td>0.106</td>
</tr>
<tr>
<td>Result of CMV-pp65 EliSpot analysis, pos vs neg</td>
<td>0.162</td>
<td>0.025</td>
<td>0.22</td>
<td>0.0025</td>
<td>0.05</td>
<td>0.105</td>
</tr>
<tr>
<td>Result of CMV-pp65-specific CD8(^+) T-cell analysis, pos vs neg</td>
<td>0.144</td>
<td>0.079</td>
<td>0.20</td>
<td>0.0017</td>
<td>0.04</td>
<td>0.123</td>
</tr>
<tr>
<td>Result of actin-specific CD8(^+) T-cell analysis, pos vs neg</td>
<td>0.098</td>
<td>0.064</td>
<td>0.18</td>
<td>0.1290</td>
<td>0.03</td>
<td>0.010</td>
</tr>
<tr>
<td>Age, per 1-y increase</td>
<td>0.005</td>
<td>0.002</td>
<td>0.22</td>
<td>0.0026</td>
<td>0.05</td>
<td>0.004</td>
</tr>
<tr>
<td>CD4(^+) T-cell count, per 100-cells/μL increase</td>
<td>-0.023</td>
<td>0.006</td>
<td>-0.26</td>
<td>0.003</td>
<td>0.07</td>
<td>-0.015</td>
</tr>
<tr>
<td>16S rDNA load, per 1-log(_{10})-copies/mL increase</td>
<td>-0.081</td>
<td>0.055</td>
<td>-0.11</td>
<td>0.1431</td>
<td>0.01</td>
<td>-0.045</td>
</tr>
<tr>
<td>Regulatory T-cell count, per 1000-cell/μL increase</td>
<td>-2.595</td>
<td>0.793</td>
<td>-0.23</td>
<td>0.013</td>
<td>0.05</td>
<td>-0.627</td>
</tr>
</tbody>
</table>

Abbreviations: CMV, cytomegalovirus; EliSpot, enzyme-linked immunosorbent spot; neg, negative; pos, positive; rDNA, ribosomal DNA; reg coeff, regression coefficient; stand coeff, standardized regression coefficient.

\(^a\)Indicates the difference in the log\(_{10}\) percentage of CD8\(^+\) T cells that expressed HLA-DR+/CD38+ with respect to variables adjusted for in the model. For example, consider Result of Quantiferon CMV analysis, pos vs neg. In a comparison of 2 patients of the same age and with the same 16S ribosomal DNA load and regulatory T-cell count, a patient with a CD4\(^+\) T-cell count that is 100 cells/μL greater than the count for the other patient has 0.015 log\(_{10}\) lower percentage of CD8\(^+\) T cells that expressed HLA-DR+/CD38+.

\(^b\)Computed by multiplying the regression coefficient by the sample SD of the variable, and dividing the quotient by the SD of the outcome variable. The standardized coefficient is interpretable as the average change in the outcome variable, in SDs, for a 1-SD increase in an explanatory variable, holding constant the other explanatory variables. If there are just 2 variables (ie, the outcome variable and 1 explanatory variable), then the standardized coefficient equals the correlation coefficient. Thus, standardized regression coefficients reveal the relative importance of each of the explanatory variable included in the model to explain the outcome variable.
results from a substudy of the CORAL trial [15]. Mechanisms linking microbial translocation and chronic immune activation may be more complex, involving numerous factors directly or indirectly stimulating immune cells. In patients with long-term suppression, although CMV-induced immune responses (humoral and cellular) were associated with chronic immune activation in adjusted analyses, all models explained only a part of the HLA-DR+/CD38+/CD8+ T-cell variability. Other putative causes of chronic immune activation (ie, regulatory T-cell count, response to self-antigens, or microbial translocation) did not significantly participate in the explanation of the variation of the percentage of CD8+ T cells that expressed HLA-DR+/CD38+. Factors associated with chronic immune activation may fluctuate during the course of HIV infection and treatment. As in elderly individuals, CMV seems to be associated with immunosenescence, but the consequence of this observation on patient care remains to be established.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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