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REGULATORY T CELLS AND THE RISK OF CMV END-ORGAN DISEASE IN AIDS PATIENTS

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Abstract

Objectives—Cytomegalovirus (CMV)-specific T-cell effectors (CMV-Teff) protect against CMV end-organ disease (EOD). In HIV-infected individuals, their numbers and function vary with CD4+ cell numbers and HIV load. The role of regulatory T cells (Treg) in CMV-EOD has not been extensively studied. We investigated the contribution of Treg and Teff towards CMV-EOD in HIV-infected individuals independently of CD4+ cell numbers and HIV load and controlling for CMV reactivations.

Design—We matched 43 CMV-EOD cases to 93 controls without CMV-EOD, but with similar CD4+ cell numbers and HIV plasma RNA. CMV reactivation was investigated by blood DNA PCR over 32 weeks preceding the CMV-EOD in cases and preceding the matching point in controls.

Methods—CMV-Teff and Treg were characterized by expression of IFNγ, IL2, TNFα, MIP1β, granzyme B (GrB), CD107a, TNFα, FOXP3 and CD25.

Results—Sixty-five% cases and 20% controls had CMV reactivations. In multivariate analyses that controlled for CMV reactivations, none of the CMV-Teff subsets correlated with protection, but high CMV-GrB ELISPOT responses and CMV-specific CD4+FOXP3+%, CD4+TNFα+% and CD8+CD107a+% were significant predictors of CMV-EOD.

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Conclusions—Since both FOXP3 and GrB have been previously associated with Treg activity, we conclude that CMV-Treg may play an important role in the development of CMV-EOD in advanced HIV disease. We were not able to identify a CMV-Teff subset that could be used as a surrogate of protection against CMV-EOD in this highly immunocompromised population.

Keywords

Cytomegalovirus; HIV1 infection; CMV end-organ disease; AIDS; regulatory T cells; effector T cells

INTRODUCTION

The incidence of cytomegalovirus (CMV)-end-organ disease (EOD) in HIV-infected individuals has decreased since the introduction of highly active antiretroviral therapy (HAART) in 1996, but has remained stable in recent years at 2 to 20% of its pre-HAART incidence depending on underlying risk factors[1–3]. Furthermore, CMV reactivation continues to be a frequent event in patients with HIV infection even among those receiving HAART, as demonstrated by the 20 to 38% rate of positive CMV-DNA results in blood monitoring studies[1,2,4].

The risk of CMV-viremia and CMV-EOD depend on the integrity of the host's immune system. CD4-[5,6] and CD8-mediated[7,8] effector T-cell responses have each been associated with control of CMV infection [8–10]. The current paradigm is that CMV-specific Th1 CD4+ and/or CD8+ memory and/or early effectors rise in response to CMV active replication and prompt the clearance of cells harboring the virus. In addition to effector-memory characteristics, the magnitude, fine specificity and breadth of IFNγ–measured CMV T-cell responses have also been ascribed critical importance for protection against EOD in various populations[7,8,11,12]. We have previously shown that in HIV-infected individuals there is a negative correlation of CMV-EOD, viremia and death with CMV-specific IFNγ responses measured by ELISPOT or ELISA[13,14]. However, both CMV-EOD and CMV-specific IFNγ responses of HIV-infected individuals were highly associated with CD4+ cell numbers and with plasma HIV load (HIV-VL), confounding the interpretation of the results.

The role of regulatory T cells (Treg) in the development of CMV-EOD has been insufficiently explored. Treg contribute to viral persistence in human and mouse chronic infections such as hepatitis C and lymphocytic choriomeningitis viruses, respectively[15–17]. In addition to CD4+ Treg, CD8+ Treg have also been demonstrated[18–20]. Natural Treg originate in the thymus and are characterized by FOXP3 and high CD25 expression as well as low CD127 [21]. Adaptive Treg can be generated in the periphery from CD25- or CD25+ T cells, but less is known about the exact process. Treg use several mechanisms of action, including stimulation through CTLA4, TNFα and IL10 secretion, granzyme (GrB) production and ATP deaminase [22–27]. We have previously demonstrated that CMV-stimulated Treg express high levels of GrB, TGFβ and PD-1, in addition to FOXP3 and that their regulatory activity could be blocked by anti-TGFβ neutralizing antibodies and GrB inhibitors[28].

The goal of the current study was to identify immunologic markers of protection against CMV-EOD in HIV-infected individuals that are independent of CD4+ T-cell numbers and HIV-VL and to investigate the contribution of Treg in the development of CMV-EOD in this population.

SUBJECTS AND METHODS

Study design

This was a case-control study that used stored specimens from subjects enrolled in the following trials conducted by the AIDS Clinical Trials Group (ACTG): 360[1], 384[29,30], 388[31], 398[32], A5001[33], A5030[34], A5095[35] and A5164[36]. Cases were subjects who developed CMV-EOD while participating in the above-mentioned studies. All CMV-EOD diagnoses met published diagnostic criteria. Immunologic assays were performed on peripheral blood mononuclear cells (PBMC) archived at the last CMV aviremic visit (defined as CMV-DNA <1000 copies/ml of plasma or whole blood) prior to the development of CMV-EOD. For subjects enrolled in studies that did not include CMV-DNA monitoring, we measured CMV-DNA by PCR in stored plasma.

Control subjects were matched to the cases based on their characteristics at the visit when PBMC for immunologic assays were obtained. Controls were CMV-seropositive, had no history of CMV-EOD and had CMV-EOD and death-free follow-up, including normal routine eye exams, while participating in the ACTG study. Controls were matched 2:1 to the corresponding case by ACTG study, sex, CD4 category (≤25, 26–50, 51–100, 101–150, 151–200, 201–250, 251–300, 301–350, 351–400, 401–450, 451-<500, and ≥501 cells/μL) and HIV-VL category (<400, 400-<1000, 1000-<10000, 10000-<100000, and ≥100000 RNA copies/mL). Controls had event-free follow-ups after the matching point at least as long as the interval between the matching point and the diagnosis of CMV-EOD in the corresponding cases. Exclusion criteria were immunosuppressive medication or immunosuppressive disease other than HIV and CMV infections, systemic opportunistic infection in the preceding 4 weeks and CMV antiviral therapy. All ACTG studies were approved by the site institutional review boards and all participants provided written informed consent.

Additional PBMC samples from 11 HIV- and CMV-infected de-identified subjects were used for phenotypic characterization of GrB-producing PBMC.

CMV-DNA measurements

In ACTG 360[1] and A5030[34] studies, CMV-DNA was prospectively measured at regular intervals using the COBAS Amplicor CMV Monitor Test (Roche Molecular Systems) with a limit of detection of 400 copies/mL of plasma and/or the Hybrid Capture 2CMV-DNA Test (Digene) with a limit of detection of 200 copies/mL of whole blood. Both assays were performed as per manufacturers' instructions. For subjects in all other ACTG protocols, CMV-DNA was measured in banked plasma using a CMV Real-Time PCR assay with a limit of detection of 100 copies/mL. DNA was extracted from 200 μl of specimen using the MagNApure instrument (Roche Molecular Systems) and DNA extraction kit (Qiagen). Five μl of extracted DNA were added to 15 μl of CMV-DNA PCR master mix containing LightCycler FastStart DNA reaction mix (Roche), Eco R1 region D CMV primers GGCAGCTATCGTGACTGG and GATCCGACCCATTGTCTAAG (0.5 μM each) and probes CGACGGTGATTCGTGGTCGT-fluorescein and LC Red640 – CCAACTGGTGCTGCCGGTCG-phosphate elongation block (0.2 μM each) and $MgCl_2$ (3 mM). The reaction developed over 45 cycles in the LightCycler™ apparatus (Roche). The number of CMV-DNA copies/mL was calculated by comparison with CMV standards containing a previously defined number of DNA copies (Advanced Biotechnology Inc.) amplified in parallel with the test samples.

Immunologic Assays

Cryopreserved PBMC were stored and shipped in liquid nitrogen. Cells were thawed and assays were performed without knowledge of the subject's case/control group. Functional assays were performed on cells with ≥66% viability based on our previous studies[37].

ELISPOT assays

IFNγ ELISPOT was performed as previously described[14]. A positive result was defined by differences ≥2-fold between CMV- and mock-infected control stimulated wells; and ≥20 spot forming cells (SFC)/10^6 PBMC (representing mean+2S.D. of results in CMV-seronegative adults) in CMV-stimulated wells. GrB ELISPOT used Granzyme B ELISpot ALPD kit (Mabtech) with the following specific conditions: 100,000 PBMC/well in duplicate wells were infected for 48 h with a clinical strain of CMV (to allow presentation through MHC class I and II), mock-infected control and PHA. SFC were revealed following the manufacturer's instructions and read with an ImmunoSpot Series 3B Analyzer (C.T.L. Cellular Technologies, Ltd). A positive result was defined by differences ≥2-fold between CMV- and mock-stimulated wells; and ≥60 SFC/10^6 PBMC (representing mean+2S.D. of results from CMV-seronegative adults) in CMV-stimulated wells.

Flow cytometric enumeration of T-cell subpopulations

The following mAbs were used for Treg and T-cell effector (Teff) measurements: TNFα-FITC (Becton Dickinson), CD3-PerCP, PE or APC-Cy7 (Becton Dickinson), CD25-FITC (Becton Dickinson), IL-2-FITC (Becton Dickinson) CD27-FITC (Becton Dickinson), CD28-FITC (Becton Dickinson), CD107a-PE-Cy5 (Becton Dickinson) or PE (Myltenyi Biotec), FOXP3-PE (eBioscience) or AlexaFluor 647 (Becton Dickinson), MIP1β-PE (eBioscience), CD4-PE-Cy7 (Beckman Coulter), TGFβ-PE (Cedarlene), anti- GrB-FITC (Becton Dickinson), CD16-PE (Becton Dickinson), CD161-PE-Cy5 (Becton Dickinson), γδ-APC (Becton Dickinson).

Circulating Tregs were measured in freshly thawed PBMC, which were washed, counted and stained with the appropriate monoclonal antibodies. Events were counted with Guava EasyCyte (Millipore) and analyzed with FlowJo (Treestar). T-cell subsets were expressed as percentages of CD4+ or CD8+ parent populations.

CMV-specific Teff and Treg were measured after in vitro stimulation. PBMC were incubated for 4 days at 37°C and 5% CO_2 with CMV- or mock-infected lysate at the pre-optimized concentration of 1:200 after which cells were washed and stained with the appropriate fluorochrome-conjugated mAbs. Preliminary optimization assays showed the following: 1) CMV lysate provides potent CD4+ and CD8+ T-cell stimulation equal to live viral in vitro infection and more potent than pp65 or IE1 overlapping peptide mixtures; 2) peak cytokine production in HIV-infected individuals occurs after 4 days of CMV in vitro stimulation. Fig 1S shows the gating strategy for these assays.

For the phenotypic characterization of GrB-producing cells, PBMC were infected with a clinical strain of CMV in tissue culture tubes following the same procedure used for the GrB ELISPOT. After 48 h, cells were washed and stained with the appropriate mAbs.

Statistical analysis

Descriptive statistical analyses (median, 25^{th} and 75^{th} percentile) were used to summarize patient characteristics and immunologic responses. Statistical comparisons between cases and controls were done using conditional logistic regression with strata based on parent study, CD4+ cells and HIV RNA levels. For the adjusted odds ratios (OR) a simplified model with 15 strata based on CD4+ cells and HIV RNA was used. Sensitivity analyses

(data not shown) generally showed consistent results for varying stratification approaches. All OR are scaled by interquartile range (IQR). ORs for continuous predictors are presented in terms of a one IQR (interquartile range) difference in the value of the predictor; the IQR was obtained pooling cases and controls. No adjustments were made in the univariate analysis for multiple comparisons, but multivariate analyses were subsequently performed for immunological results that yielded significant differences in the univarate analyses.

RESULTS

Characteristics of study participants

The study used PBMC and plasma cryopreserved between 1997 and 2007 from 136 ACTG study participants, including 43 cases with CMV-EOD and 93 matched CMV-seropositive controls without CMV-EOD. CMV-EOD included 27 cases of retinitis, 3 colitis, 5 esophagitis, 3 pneumonitis, 2 gastroenteritis and 1 each of encephalitis, proctitis and mucocutaneous ulcers.

Cases and controls had similar demographic and HIV-disease characteristics by design (Table 1) including CD4+ cell numbers (median=23 cells/μL for all subjects), HIV-VL (median=141,032 RNA copies/mL) and sex (12% females). Race, age (median=39 years), CD8+ cell numbers and use and duration of ART were also similar in the two groups. However, cases had a 65% incidence of CMV-viremia in the 32 weeks preceding the CMV-EOD diagnosis and 40% before the PBMC for immunologic assays were obtained. In contrast only 20% of the controls had CMV-viremia detected in the 32 weeks preceding the matching point (p=0.008).

At the time when the PBMC for the immunologic assays were obtained, all subjects had undetectable CMV-DNA with the exception of 2 with CMV-EOD (5%) and 6 controls (6%) who had CMV-DNA above the level of detection, but <1000 c/mL. Cases developed CMV-EOD at a median (Q1-Q3) of 0.52 years (0.15–1.22) after the matching time point, when PBMC were obtained, which was exceeded by the CMV-EOD- and death-free interval from the matching time point to the end of follow-up that controls had on their parent ACTG study [median; Q1-Q3 of 1.97 years (0.77–2.99)].

CMV GrB ELISPOT responses and the risk of CMV-EOD

Subjects with CMV-EOD had median (Q1- Q3) GrB ELISPOT values of 128 (15–375) SFC/10^6 PBMC, whereas controls had 20 (5–150) SFC/10^6 PBMC (Table 2). Furthermore, 65% cases and 35% controls had positive values defined by ≥60 SFC/10^6 PBMC, the threshold previously established by comparing results of CMV-seropositive and seronegative healthy adults. CMV GrB ELISPOT responses were associated with a 3.87 higher odds ratio (OR) of developing CMV-EOD (p<0.01). Results (ORs) were similar when separately analyzing the CMV retinitis and non-retinitis cases (Tables 1S and 2S).

Because of the strong association of high CMV GrB SFC with increased risk of CMV-EOD, we sought to determine the cell type responsible for the GrB production. Using PBMC from 11 HIV-infected de-identified donors (not included in the study cohort), we determined that CD8+ cells accounted for an average of 60% of the lymphocytes that secreted GrB in response to CMV stimulation, followed by CD4+ (12%), NK (9%), NKT (9%) and γδ cells (5%; Fig 2S).

CMV-IFNγ ELISPOT responses

IFNγ ELISPOT values were low both in cases [median (Q1- Q3) of 1 (0- 7) SFC/10^6 PBMC] and controls [4 (0- 71) SFC/10^6 PBMC; Table 2]. Only 18% of the cases and 34%

of the controls had positive IFNγ ELISPOT values defined as ≥20 SFC/10^6 PBMC, the threshold previously established by comparing results of CMV-seropositive and seronegative healthy adults. CMV-IFNγ ELISPOT responses were associated with a 0.44 OR for CMV-EOD (p=0.17). Results (ORs) were similar when separately analyzing the CMV retinitis and non-retinitis cases (Tables 1S and 2S).

Circulating Treg

CD4+CD25+FOXP3+%, CD8+CD25+FOXP3+%, total CD4+FOXP3+% and total CD8+FOXP3+% cells were measured in freshly thawed PBMC (Table 3). In the univariate analysis, higher circulating CD4+FOXP3+% were predictive of CMV-EOD with OR = 1.45 (p=0.03, Table 3).

CMV-specific T-cell responses

The CMV-EOD predictive value of the T-cell subpopulations stimulated by CMV or mock-infected control is presented in Table 4. Higher frequencies of CD4+ lymphocytes that expressed IL2, TNFα, FOXP3 or both FOXP3 and CD25 in response to CMV stimulation were significantly associated with CMV-EOD in the univariate analysis (OR of 2.3 to 4.7; p of 0.01 to 0.05). However, higher CD4+IL2+% and CD4+FOXP3+% were also observed in mock-stimulated cultures of PBMC obtained from cases compared with controls and were associated with OR of 4.3 and 1.8, respectively (p of 0.02 and 0.04, respectively) for CMV-EOD. Among CD8+ subpopulations, higher frequencies of cells expressing IL2, TNFα or CD107a in response to CMV in vitro stimulation were predictive of CMV-EOD in the univariate analysis (OR of 1.5 to 4.3, p of <0.01 to 0.05). Of note, mock-stimulated PBMC of study subjects had activated CD4+ and CD8+% manifold higher than control PBMC from HIV-uninfected donors (Fig 3S).

Multivariate analysis

Because CMV-viremia was a very strong predictor of CMV-EOD, it was further used as a covariate in multivariate analyses of the relationship of ELISPOT, Treg and Teff with CMV-EOD. In the CMV-specific Teff and Treg multivariate analyses, both CMV- and mock-infected control-stimulated conditions were included in the analysis of each T-cell subset. After adjustment for CMV-viremia in the 32 weeks prior to the PBMC collection in cases and controls, GrB ELISPOT positive values remained significantly associated with CMV-EOD (OR=4.73, p<0.01; Fig 1) and the relationship for IFNγ ELISPOT with CMV-EOD remained nonsignificant (OR=0.45, p=0.19; Fig 1). Other T-cell subsets including CMV-stimulated CD4+FOXP3+% (OR=2.4; p=0.05), CD4+TNFα+% (OR=6.2; p=0.02) and CD8+CD107a+% (OR=7.1; p=0.03) remained significant predictors of CMV-EOD.

DISCUSSION

This study showed that CMV-specific Treg may play an important role in the development of CMV-EOD in HIV-infected individuals with low CD4+ T-cell numbers. Among the T-cell subsets with previously described Treg characteristics, CMV-specific CD4+FOXP3+% and GrB ELISPOT responses remained significantly associated with increased risk of CMV-EOD after controlling for CMV reactivation. FOXP3 is a transcription regulatory factor necessary for the initiation of the T-cell regulatory program[38]. More recent studies have shown that activated conventional T cells, may also transiently express FOXP3[39] such that not all FOXP3+ T cells may truly represent Treg. However, the FOXP3+ conventional T cells have lower and delayed cytokine production when compared with FOXP3- cells and may evolve into Treg [40–42]. Other phenotypic characteristics that in conjunction with FOXP3 expression provide a more precise identification of Treg include high CD25 expression. In this study, high proportions of CMV-specific CD4+CD25+FOXP3+ T cells

were significantly associated with CMV-EOD in the univariate, but not in the multivariate analysis.

GrB was originally described as a mediator of cytotoxicity used by effector T cells and NK cells, but more recently its role in immune regulation has been recognized[23,28,43,44]. GrB stimulates the intrinsic pathway of apoptosis by activating Bid, which releases the inhibition of Bax and Bad imposed by the Bcl-2 family of molecules. Recent evidence suggests that GrB may activate additional mechanisms that result in programmed cell death. Historically, the first described Treg were CD4+. However, it was soon recognized that other cell types, including CD8+ T, CD19+ B, and dendritic cells may acquire regulatory function and use GrB as a mediator[23,43–45]. In this study, CD8+ T cells accounted for the majority of the CMV-specific GrB production. It is interesting to note that CD4 T-cell depletion in mice in the context of a chronic viral infection results in differentiation of viral-specific CD8+ Teff into Treg[46]. CD4 T-cell depletion is also the hallmark of AIDS and might contribute to Treg differentiation. Others have previously shown that HIV-specific Treg are abundant in chronic HIV infection and may contribute to the downregulation of protective T cell responses against HIV[22,26,47–50].

High CMV-specific CD8+CD107a+% was also associated with increased risk of CMV-EOD. CD107a is a lysosomal membrane component that is typically found on the cell surface as a consequence of cytotoxicity-associated degranulation. The expression of CD107a may overlap with production of GrB, since both are components of cytotoxicity. We found that in HIV-infected and uninfected CMV-seropositive donors, roughly 25% of the CMV-stimulated CD3+GrB+ cells co-expressed CD107a and vice versa (data not shown). It is possible that CMV-stimulated CD8+CD107a+ cells may include a Treg subset, similarly to the GrB+ T cells, but this hypothesis still needs to be tested.

We did not find significant associations between CD8+FOXP3+% or CD8+CD25+FOXP3+% subsets and CMV-EOD. However, in the univariate analysis CMV-specific CD8+TGFβ+% were associated with an increased risk of CMV-EOD. It is well known that IL10+ and TGFβ+ regulatory T cells, also known as Tr1 and Th3 cells, downregulate FOXP3 expression[51]. Less is known about the kinetics of FOXP3 expression in GrB+ Treg, an aspect that needs to be further evaluated.

Another T cell subset that positively correlated with the development of CMV-EOD was the CMV-CD4+TNFα+%. TNFα is an inflammatory cytokine traditionally associated with Th1 responses. Although Th1 responses have a critical role in immune protection against CMV, TNFα has not been described as an important contributor to anti-CMV defenses. The function of CD4+TNFα+ cells in the context of CMV infection needs further elucidation.

In this study, multiple T-cell subsets with both Teff and Treg phenotypic characteristics were associated with higher risk of CMV-EOD in the univariate, but not in the multivariate analysis that controlled for CMV-viremia. These subsets included CMV-specific and/or nonspecific CD4+IL2+%, CD8+IL2+%, CD4+FOXP3+%, CD4+TNFα+%, CD8+TNFα+%, CD4+CD25+FOXP3+% and CD8+TGFβ+%. This observation suggests that CMV reactivation may play an important role in the differentiation or selection of these CMV-specific and/or nonspecific Treg and Teff subsets. This is an important hypothesis to be further investigated, because the activation of both Treg and Teff may contribute to some of the indirect adverse effects ascribed to CMV infection, such as increased risk of death in HIV-infected[4] and very old individuals[52]; increased frequency of opportunistic infections in transplant recipients[53]; graft rejection[54]; and atherosclerosis[55,56].

In contrast to previous studies[8,14], we did not find a CMV-specific Teff subset that correlated with protection against CMV-EOD. Our study differs from most previous ones in

that we matched the cases and controls by CD4+ cell numbers and plasma HIV-VL, thus minimizing the potential analytic bias of these very strong determining variables. Jacobson et al. also showed in a smaller study that CMV-specific CD69+IFNγ+% Teff did not predict protection against CMV retinitis in a CD4-matched case-control investigation of HIV-infected individuals[57]. In the current report, we expand on those findings by studying a larger number of subjects and using a more comprehensive Th1 panel. Recent studies in transplant recipients, who also have a high risk of developing severe CMV infection, showed that the balance between Treg and Teff ultimately determines the risk of CMV-EOD CMV-EOD[58]. In our study participants with advanced HIV-infection, the protective role of Teff was completely obfuscated.

This raises the question of what mechanisms allow Treg to outlive or outperform Teff in individuals with AIDS. Several scenarios can be postulated including that CD4+ Treg survive longer than CD4+ Teff in the context of chronic HIV infection, perhaps due to lower permissivity to HIV infection [59]. Furthermore, Treg may increase with HIV replication and/or disease progression[60,61]. Other possibilities are that in persons with low CD4+ cell numbers, CD4-mediated help, which is necessary for the CD8+ Teff function, is not available, whereas Treg function may not require the same amount of CD4+ help[19], or that CD4+ and/or CD8+ Teff are exhausted and function poorly in patients with AIDS.

A limitation of this study was the number of cells available for functional analyses and the low viability of some PBMC preparations, which precluded functional assays (sample sizes shown in the tables). The flow cytometric analysis did not use a vital dye to exclude dead cells, which may bind mAbs in a nonspecific fashion and inflate the frequency of positive events. However, this equally affected cases and controls without skewing the results of the comparisons between the 2 groups. For interpretation of the effect of immunologic measures, which had varying frequencies in the CD4+ and CD8+ cell populations, we presented OR in terms of a difference of one IQR in the immunologic parameter. Since there was differing precision in estimating the OR, there was not a direct relationship between the magnitude of the OR and the level of significance.

Overall, our data support the hypothesis that Treg play a role in the development of CMV-EOD in HIV-infected patients. Our results complement previous studies that showed associations of high Treg frequencies with progression of HIV and hepatitis C virus infections[17,50,62,63]. It has been suggested that a low absolute number of Treg in the early stages of HIV infection may be responsible for high levels of CD4+ T-cell activation[64,65] and faster disease progression. Our data suggest that in advanced stages of HIV infection, it may be beneficial to have lower proportions of Treg, thus decreasing the risk of CMV-EOD and, perhaps, of other opportunistic infections. As new immune modulators are evaluated in the context of HIV infection[66], including agents that act on Tregs, it is important to be aware of the multiple potential effects of Treg manipulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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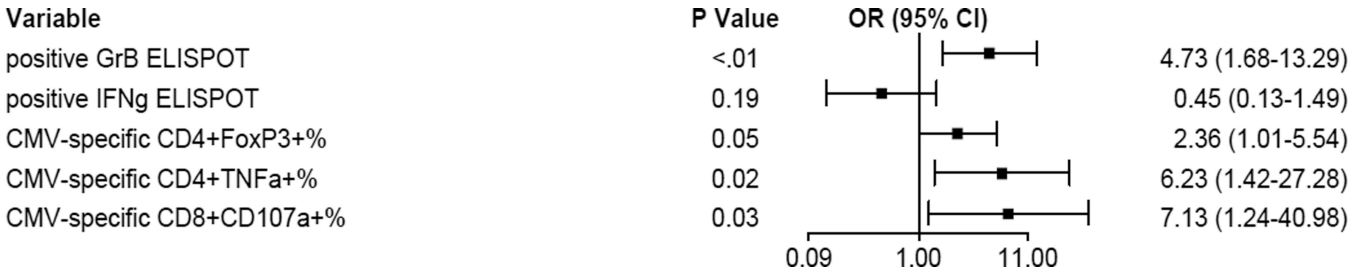

Figure 1. Odds Ratio (OR) for CMV-EOD, from conditional logistic regression models, adjusted for CMV-viremia

ELISPOT ORs are for comparison of positive versus negative responses. For the CMV-specific T-cell subpopulations, OR are for the association with a one interquartile range (Q3-Q1) higher level; these models additionally adjust for mock-stimulated PBMC results.

Table 1

Demographics and clinical characteristics at the time of testing

Variable	Cases (N=43)	Controls (N=93)
Age [median (Q1; Q3) years]	38 (33; 43)	39 (34; 45)
Sex (F:M)	5:38	11:82
Race (B:H:W:O)	11:11:20:1	36:26:28:3
CD4+ [median (Q1; Q3) cells/μL)]	20 (10; 54)	23 (10; 52)
CD8+ [median (Q1; Q3) cells/≤μL)]	466 (263; 766)	579 (323; 784)
Plasma HIV RNA [median (Q1; Q3) c/mL)]	135,423 (44,261; 324,630)	141,040 (21,238; 363,049)
Subjects on HAART (%)	32 (74%)	59 (63%)
Time since first ART use (median; Q1-Q3 years)	1.44 (0.36–3.41)	1.05 (0.08–3.21)
Subjects with CMV-viremia in the 32 weeks preceding CMV-EOD or matching point (%)*	**26 (65)**	**19 (20)**

Bold-facing indicates significant differences ($p<0.05$) based on conditional logistic regression.

* Three cases were excluded from this analysis because their last available CMV-viremia data were earlier than 32 weeks before the CMV-EOD diagnosis. 17 (40%) of the subjects with CMV-EOD and valid viremia information had documented CMV-viremia prior to the matching point.

Table 2

GrB and IFNγ ELISPOT responses in cases with CMV-EOD and CMV-seropositive matched controls without EOD.

Parameter	Cases			Controls			OR	p Value
	N	*Median (Q1-Q3) SFC/10^6 PBMC*	*N pos (%)*	*N*	*Median (Q1-Q3) SFC/10^6 PBMC*	*N pos (%)*		
GrB	**34**	**127.5 (15- 375)**	**22 (65)**	**63**	**20 (5- 150)**	**22 (35)**	**3.87**	**<0.01**
IFNγ	33	1 (0- 7)	6 (18)	61	4 (0- 71)	21 (34)	0.44	0.17

Odds ratios (OR) are for comparison of positive vs. negative ELISPOT responses, from unadjusted conditional logistic regression models.

Table 3

Nonspecific circulating Treg (%*) of CMV-EOD cases and matched controls.

Parameter	Cases		Controls		OR#	p value
	N	*Median (Q1- Q3)*	*N*	*Median (Q1- Q3)*		
CD4+CD25+FOXP3+	28	2.33 (0.37- 8.11)	68	2.91 (0.79- 5.65)	1.21	0.18
CD4+FOXP3+	**28**	**6.35 (1.94- 20.31)**	**68**	**7.63 (2.98 -11.32)**	**1.45**	**0.03**
CD8+CD25+FOXP3+	37	0.36 (0.18- 0.71)	79	0.54 (0.24- 1.01)	0.94	0.48
CD8+FOXP3+	37	2.10 (1.04- 4.45)	79	2.89 (1.83- 3.84)	1.07	0.53

*Percentages were calculated in relation to the CD4+ or CD8+ parent gate.

#Unadjusted conditional logistic regression models associated with one interquartile range (IQR; Q3-Q1) higher level.

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Table 4

CMV- and mock-stimulated Teff and Treg (%*) of CMV-EOD cases and matched controls.

Parameter	Cases		Controls		OR	p value
	N	*Median (Q1, Q3)*	*N*	*Median (Q1, Q3)*		
CMV CD4+IL2+	24	22.10 (11.76,45.88)	41	5.26 (2.89,11.11)	2.90	0.06
Mock CD4+IL2+	**22**	**26.79 (11.8,48.58)**	**40**	**7.5 (3.63,21.03)**	**4.28**	**0.02**
CMV CD4+MIP1β	24	25.91 (12.29,38.42)	43	17.24 (7.34,34.21)	1.50	0.34
Mock CD4+MIP1β	23	32.99 (18.08,43.59)	42	16.39 (8.46,27.27)	2.30	0.07
CMV CD4+TNFα+	**24**	**27.48 (9.12,43.52)**	**43**	**8.46 (4.23,20.0)**	**4.73**	**0.02**
Mock CD4+TNFα+	23	19.35 (11.11,33.73)	42	7.64 (4.21,18.19)	1.71	0.16
CMV CD4+CD107a+	22	39.45 (16.13,75.0)	42	19.84 (11.06,41.82)	2.28	0.17
Mock CD4+CD107a+	21	45.95 (27.94,69.76)	43	22.16 (11.11,48.48)	1.84	0.24
CMV CD4+TGFβ+	**24**	**14.68 (6.78,28.8)**	**43**	**4.03 (2.23,10.95)**	**3.05**	**0.05**
Mock CD4+TGFβ+	23	13.89 (2.8,25.39)	42	4.18 (2.06,14.38)	1.76	0.12
CMV CD4+FOXP3+	**24**	**20.73 (13.24,36.85)**	**43**	**7.83 (3.33,16.03)**	**4.13**	**0.01**
Mock CD4+FOXP3+	**17**	**19.74 (6.25,37.74)**	**41**	**7.05 (2.37,11.71)**	**1.84**	**0.04**
CMV CD4+CD25+FOXP3+	**24**	**14.84 (3.68,26.43)**	**43**	**4.35 (1.0,11.85)**	**2.33**	**0.03**
Mock CD4+CD25+FOXP3+	17	7.89 (0.68,27.08)	41	2.38 (0.67,7.1)	1.39	0.11
CMV CD8+IL2+	**27**	**6.71 (4.29,14.46)**	**50**	**4.41 (2.35,6.88)**	**1.53**	**0.05**
Mock CD8+IL2+	26	7.58 (3.94,13.84)	47	4.20 (2.76,6.36)	1.48	0.20
CMV CD8+MIP1β	27	10.47 (5.9,17.89)	53	9.35 (5.03,17.04)	0.83	0.47
Mock CD8+MIP1β	27	10.21 (4.78,21.38)	50	9.86 (6.44,17.02)	0.94	0.81
CMV CD8+TNFα+	27	9.07 (3.98,12.16)	53	4.32 (2.62,8.06)	1.76	0.09
Mock CD8+TNFα+	27	6.68 (4.27,10.91)	50	4.42 (2.96,8.7)	1.42	0.23
CMV CD8+CD107a+	**25**	**27.53 (14.17,33.18)**	**52**	**11.77 (7.05,25.11)**	**4.27**	**<0.01**
Mock CD8+CD107a+	25	26.47 (14.05,32.09)	50	15.41 (8.12,29.05)	2.19	0.07
CMV CD8+TGFβ+	**27**	**4.63 (2.4,7.05)**	**53**	**2.03 (1.31,3.26)**	**1.57**	**0.04**
Mock CD8+TGFβ+	27	3.29 (2.03,5.83)	50	1.94 (1.57,2.83)	1.37	0.17
CMV CD8+FOXP3+	27	6 (2.24,8.97)	52	3.99 (2.52,7.33)	1.21	0.40
Mock CD8+FOXP3+	23	2.52 (1.12,4.78)	49	2.67 (1.71,3.84)	1.31	0.09

Parameter	Cases		Controls		OR	p value
	N	***Median (Q1, Q3)***	*N*	***Median (Q1, Q3)***		
CMV CD8+CD25+FOXP3+	27	3.86 (0.97,6.34)	52	1.46 (0.57,3.25)	1.52	0.14
Mock CD8+CD25+FOXP3+	23	0.39 (0.28,1.11)	49	0.6 (0.24,1.01)	1.54	0.08

*Percentages were calculated using the parent CD4+ or CD8+ population in the denominator.

Odds ratios (OR) from unadjusted conditional logistic regression models associated with one interquartile range (IQR; Q3-Q1) higher level.

Bold-facing indicates significant differences