Functional Exhaustion of CD4$^+$ T Lymphocytes during Primary Cytomegalovirus Infection

Pierre Antoine, Véronique Olislagers, Ariane Huygens, Sandra Lecomte, Corinne Liesnard, Catherine Donner and Arnaud Marchant

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Human CMV is a member of the herpesviridae family that establishes lifelong persistence after primary infection. CMV infection is usually asymptomatic in immunocompetent subjects but can lead to severe clinical consequences in immunocompromised hosts and fetuses (1, 2). CD4+ T lymphocytes play a central role in the control of CMV infection by expressing antiviral functions and by promoting B and CD8+ T lymphocyte responses. CD4+ T cell deficiency is correlated with CMV reactivation in patients with HIV infection (3) and with solid organ transplantation (4). Delayed appearance of functional CMV-specific CD4+ T lymphocytes is associated with symptomatic infection in kidney transplanted patients with primary infection (5). Following allogeneic stem cell transplantation, an early CD4+ T cell response to CMV is inversely correlated with the risk of CMV viremia (6), and the survival of adoptively transferred CMV-specific CD8+ T cell clones is correlated with the detection of an antiviral CD4+ T cell response (7, 8).

During the chronic phase of CMV infection, virus-specific CD4+ T cells undergo large oligoclonal expansions and include a variable fraction of cells expressing a late differentiation phenotype characterized by the loss of expression of two costimulatory receptors, CD27 and CD28 (9). This phenotype is typical of CMV-specific cells (10) and is associated with a high capacity to produce effector cytokines (IFN-γ and TNF-α) and with the acquisition of MHC class II-restricted cytolytic activity (11).

The differentiation of CD4+ T lymphocytes and their acquisition of effector functions during primary CMV infection remains poorly characterized. CMV-specific CD4+ T cells producing effector cytokines can be detected rapidly during the course of primary infection (12). In contrast, the proliferative capacity of CD4+ T cells is very low during the first months of infection when viral excretion in urine and saliva can be detected (13, 14). This suggests that active CMV replication interferes with the acquisition of at least some of CD4+ T lymphocytes effector functions. This interference could have significant clinical implications because low proliferative responses of CMV-specific T cells are associated with in utero transmission of CMV following primary infection in pregnancy (15–17) and with an increased risk of CMV retinitis in HIV-infected patients (18). Previous studies suggest that the low proliferative responses of CD4+ T cells observed during primary CMV infection is related to their differentiation into effector T cells with a reduced capacity to produce IL-2 (19, 20). Optimal IL-2 production by T lymphocytes is dependent on the signals provided by the costimulatory receptor CD28. Indeed, CD8–CD4+ and CD8–CD8+ T cells produce lower levels of IL-2 than their CD8+ counterparts following polyclonal stimulation (21). In addition, CD28 transduction of CD8–CD8+ T cells restores their capacity to produce IL-2 (22). Taken together, these observations suggest that active viral replication associated with primary CMV infection selectively impairs the capacity of CD4+ T cells to produce IL-2 by promoting their differentiation into CD28– effector cells. The aim of our study is to test this hypothesis and to comprehensively assess the functional capacity of CD4+ T cells and its link with cell differentiation in pregnant women diagnosed with primary CMV infection and healthy adults with chronic infection.
Materials and Methods

Study population

This study was approved by the Ethics Committee of the Université Libre de Bruxelles. Pregnant women referred with a diagnosis of primary CMV infection to the fetal medicine outpatient clinic of the Hôpital Erasme were recruited. Diagnosis of primary infection was based on documented IgG seroconversion or, in case of unknown status at the beginning of the pregnancy, Tit2s and/or CMV IgM of CMV-specific IgM. A total of 48 of 51 women were recruited in the study. The median delay between the diagnosis of primary infection and blood collection for this study was 32 d (range, 6–132 d). Twenty-two healthy subjects chronically infected with CMV were recruited as controls. Following written informed consent, a 30 ml aliquot of heparinized blood was collected. Viremia was assessed using a qualitative in-house diagnostic PCR assay targeting the pp150 CMV gene. Fifty-three percent of pregnant women were CMV PCR positive at the time of analysis. Analysis of CD4+ T cell responses to CMV and control Ags were similar in patients who were viremic or nonviremic at the time of analysis (data not shown). In addition, samples from 21 pregnant women diagnosed with primary CMV infection and participating in the ongoing GlaxoSmithKline Biologicals-sponsored study (NCT01251744) were analyzed in agreement with the study protocol and consent form, to complement the results.

Proliferation assay

PBMC were purified from fresh peripheral blood by gradient centrifugation using Lymphoprep (Nycomed Pharma) and were cultured in RPMI 1640 medium containing 5% human serum, penicillin/streptomycin, and glutamine in 96-well plates (200,000 cells/well). Cells were stimulated with a lysate of CMV-infected fibroblasts (1 μg/ml) (Viruses), a pool of 15 aa peptides overlapping by 11 and derived from the CMV pp65 tegument protein (75 μg/ml) (BD Pharmingen), a pool of 20 aa peptides overlapping by 10 and derived from the CMV glycoprotein B (2 μg/ml), tetanus toxoid (TT) (1 μg/ml), or a preparation of split Jiangsu influenza virus (1 μg/ml) (all provided by GlaxoSmithKline Biologicals) and cultured for 6 or 7 d at 37°C in the presence of 5% CO2. The role of IL-2 or inhibitory receptors was explored by adding rIL-2 (5 U/ml) (R&D Systems), anti–programmed death-1 (PD-1) blocking Ab, anti–Tim3 blocking Ab, or isotype control (5 μg/ml; all from BioLegend) to the culture medium. Cells were pulsed with BrdU (BD Biosciences) for the last 18 h of stimulation. Cells were stained according to the manufacturer’s protocol with the following Abs: CD3 PerCP, CD4 Pacific Blue, CD8 PE-Cy7, Ki67 FITC, and BrdU allophycocyanin (all from BD Biosciences). Proliferating cells were defined as Ki67+ and BrdU+. Data were obtained on a Cyan ADP LX9 cytometer and analyzed using the Summit 4.3 software (DakoCytomation) or FlowJo 9.0.1 software (Tree Star).

Cytokine production and cell phenotype

Fresh PBMC were cultured in RPMI 1640 medium containing 10% FCS, penicillin/streptomycin, and glutamine with or without addition of anti-Fas agonist Ab (1 μg/ml) (Millipore). Cells were then stained with the following Abs: CD3 Cascade Yellow, CD4 V450, CD8 PE-Cy7, CD28 allophycocyanin, Annexin V FITC (all from BD Biosciences, except CD3 from DakoCytomation; CD8 from Beckman Coulter) and with 7-aminoactinomycin D (BD Biosciences), according to the manufacturer’s instructions. Apoptotic cells were defined as Annexin-V+ and 7-aminoactinomycin D+. Data were obtained on a Cyan ADP LX9 cytometer (DakoCytomation) and analyzed using the FlowJo 8.8.6 or 8.8.7 software (Tree Star).

Statistical analyses

Data are presented as individual results, medians and interquartile ranges or means and SEs on the mean. Specific fluorescence intensity of Bcl-2 expression was calculated using the following formula: specific fluorescence intensity = (MFI[Bcl-2] − MFI[fluorescence minus one])/MFI[fluorescence minus one]. Multiple parameter comparisons of primary and chronic infections were performed with the two-way ANOVA test. When significant differences were observed, data obtained from primary and chronic infections were compared for each parameter using the Mann–Whitney U test. Statistical significance was defined at p < 0.05. GraphPad Prism 5 was used to perform the analyses.

Results

Primary CMV infection induces high frequencies of cytokine-producing CD4+ T cells with limited proliferative capacity

To assess the magnitude of the CD4+ T lymphocyte response to primary CMV infection, frequencies of cells producing at least one of three cytokines (IFN-γ, TNF-α, and IL-2) following short-term stimulation with CMV or control Ags were measured (Fig. 1A). During primary infection, median frequencies of cytokine-
producing cells ranged between 0.11 and 0.45% of CD4+ T cells, depending on the Ag tested. Similar frequencies of CMV-specific cells were detected in chronically infected subjects. Also, subjects with primary or chronic CMV infection had similar frequencies of CD4+ T cells producing cytokines in response to TT and influenza Ags. Proliferative responses to CMV and control Ags were measured using the BrdU incorporation assay (Fig. 1B). As previously reported (13, 14), proliferative responses of CD4+ T cells to CMV Ags were significantly lower during primary as compared with chronic infection. In contrast, subjects with primary or chronic CMV infection had similar proliferative responses to control Ags. Taken together, these data indicate that primary CMV infection induces the expansion of high frequencies of cytokine-producing CD4+ T cells with a limited proliferative capacity. The defective proliferation appears to selectively affect CMV-specific cells.

Activation of CMV-specific CD4+ T lymphocytes during primary infection

During primary CMV infection, active viral replication is expected to induce more intense T cell activation as compared with chronic infection (23). The state of activation of CMV-specific CD4+ T cells was assessed by measuring the expression of the membrane molecules CD38 and MHC class II as well as the intracellular content of the antiapoptotic molecule Bcl-2 following short-term stimulation with CMV or control Ags and gating on cytokine-positive cells (Fig. 2). During primary infection, CMV-specific CD4+ T cells expressed high levels of CD38, whereas low levels of expression were detected in chronically infected subjects (Fig. 2A). Similar results were obtained with HLA class II expression, although significant differences where only observed for pp65-specific cells. T cell activation during primary viral infections is associated with reduced expression of the anti-apoptotic Bcl-2 protein and with an increased susceptibility to apoptosis (24). In subjects with primary infection, CMV-specific CD4+ T cells expressed low levels of Bcl-2 as compared with chronically infected subjects (Fig. 2B, 2C). In contrast, similar levels of Bcl-2 were detected in TT and influenza-specific cells from subjects with primary or chronic infection. The biological consequence of Bcl-2 downregulation was assessed by measuring the susceptibility of CD4+ T cells to spontaneous or Fas agonist-induced apoptosis (Fig. 2D). In these experiments, CD28 negativity was used as a marker of CMV-specific cells to avoid Ag stimulation (10). Susceptibility of CD28+ CD4+ T cells to spontaneous apoptosis following overnight incubation was significantly higher during primary as compared with chronic infection. Incubation with anti-Fas agonist Abs further increased the apoptosis of CD28+ CD4+ T cells in subjects with primary infection but not in chronically infected subjects. Taken together, these data indicate that during primary infection CMV-specific CD4+ T cells express a phenotype of activation that is more marked than that of cells of other specificities or CMV-specific cells during chronic infection. This state of activation further validates the diagnosis of primary CMV infection.

Defective production of IL-2 by CMV-specific CD4+ T cells during primary infection

To identify the mechanisms underlying the defective proliferative responses of CD4+ T cells associated with primary CMV infection, we measured the capacity of CMV-specific cells to produce IL-2. The proportions of cells producing IL-2 among the total population of cytokine-producing CD4+ T cells was assessed following stimulation with CMV or control Ags (Fig. 3A, 3B). During primary infection, CMV-specific CD4+ T cells included low frequencies of cells producing IL-2 as compared with chronic infection. In contrast, similar frequencies of TT and influenza-specific cells producing IL-2 were detected in the two study groups. The effect of addition of exogenous IL-2 on the proliferative responses of CD4+ T cells to CMV Ags was then assessed (Fig. 3C). In subjects with primary CMV infection, addition of low doses of IL-2 significantly increased the proliferation of CMV-specific CD4+ T cells, whereas no significant effect was observed in chronically infected subjects. Proliferative responses to CMV lysate and to pp65 peptide pool in the presence of IL-2 were comparable in subjects with primary and chronic infection, whereas the responses to the gB peptide pool remained lower in primary as compared with chronic infection. These results show that, during primary infection, the defective proliferative response of CMV-specific CD4+ T lymphocytes is associated with a decreased capacity to produce IL-2 and that addition of IL-2 alone can restore cell proliferation to levels similar to that observed during chronic infection.

Role of CD28 downregulation in the defective production of IL-2 during primary CMV infection

The capacity of T lymphocytes to produce IL-2 depends on their expression of the costimulatory molecule CD28 (25). To gain insight into the mechanisms involved in the downregulation of IL-
IL-2–producing cells was assessed within the CD28 regulation in the defective production of IL-2, the proportion of were detected among TT and influenza-specific cells during pri-

The observation that the defective IL-2 production affected CD28+

Primary CMV infection is associated with a defective per cell production of IFN-γ and TNF-α by CD4+ T cells

The observation that the defective IL-2 production affected CD28+

specific CD4+ T cells may not be restricted to IL-2. Because the proportion of CD4+ T cells producing either IFN-γ or TNF-α was similar in subjects with primary or chronic infection, the amount of cytokines synthesized on a per cell basis was measured (Fig. 5). During primary infection, CMV-specific CD4+ T cells produced significantly lower amounts of IFN-γ and TNF-α, as indicated by lower MFI, as compared with chronic infection (Fig. 5A). This difference was specific to CMV as TT and influenza-specific cells expressed similar IFN-γ, TNF-α, and IL-2 MFI in the two study groups (Fig. 5A, 5B). The expression of CD28 by CMV-specific CD4+ T cells did not influence the amount of cytokines produced as both CD28+ and CD28− cells expressed lower IFN-γ and TNF-

FIGURE 3. Defective production of IL-2 and proliferative responses of CMV-specific CD4+ T lymphocytes during primary CMV. (A) The proportion of IL-2–producing cells among total cytokine-producing CD4+ T cells was measured after overnight stimulation with CMV or control Ags (medians ± interquartile ranges of 4–13 subjects). (B) Representative dot plots of IFN-γ and IL-2 production during primary (top panel) or chronic infection (bottom panel). (C) Influence of exogenous IL-2 on proliferative responses of CD4+ T cells to CMV Ags was studied using the BrdU incorporation assay (medians ± interquartile ranges of 6–20 subjects). *p < 0.05, **p < 0.001.

2 production by CD4+ T cells during primary CMV infection, the differentiation phenotype of CMV-specific cells was assessed (Fig. 4A). During primary infection, CMV-specific CD4+ T cells included very large proportions of CD28− cells. These proportions were significantly higher than those observed during chronic infection. In contrast, low and similar frequencies of CD28− cells were detected among TT and influenza-specific cells during primary and chronic infection. To evaluate the role of CD28 down-regulation in the defective production of IL-2, the proportion of IL-2–producing cells was assessed within the CD28+ and CD28− subsets of CMV-specific CD4+ T cells (Fig. 4B). As expected, very low frequencies of IL-2–producing cells were detected within the CD28− subset of CMV-specific cells during both primary and chronic infection. Interestingly, CD28+ CMV-specific CD4+ T cells were markedly less able to produce IL-2 during primary as compared with chronic infection. Taken together, these results indicate that the defective production of IL-2 by CD4+ T cells during primary CMV infection involves both the rapid differentiation of high frequencies of CD28− cells as well as a decreased capacity of CD28+ cells to produce this cytokine.

PD-1 CMV-specific CD4+ T cells express increased levels of PD-1 during primary infection

The reduced capacity of CMV-specific CD4+ T cells to proliferate and to produce cytokines during primary infection suggests a state of functional exhaustion. Functional exhaustion of T cells is observed in patients with chronic hepatitis or HIV infection and is associated with increased expression of inhibitory receptors, in-

FIGURE 4. Differentiation of CMV-specific CD4+ T cells and production of IL-2 during primary infection. (A) The proportion of CD28− cells was measured among CD4+ T lymphocytes producing at least one cytokine in response to CMV or control Ags (medians ± interquartile ranges of 4–13 subjects). (B) The proportion of cells producing IL-2 was measured among CD28− and CD28+ CD4+ T cells producing at least one cytokine (graphs show individual results and medians). **p < 0.01, ***p < 0.001.
including PD-1 and Tim-3 (26). To gain insight into the mechanisms regulating the function of CMV-specific CD4+ T cells, the expression of PD-1 and Tim-3 was measured at the level of CMV and TT-specific CD4+ T during primary and chronic infection. Primary infection was associated with increased expression of PD-1 by CMV-specific CD4+ T cells (Fig. 7A). This increased expression was observed only in CD28+ cells, whereas CMV-specific CD28−CD4+ T cells expressed similar levels of PD-1 during primary and chronic infection. As shown in Fig. 7A, TT-specific CD4+ T cells expressed low and similar levels of PD-1 during primary and chronic infection. In contrast to PD-1, low and similar levels of Tim-3 were observed in CMV and TT-specific cells in the two study groups (Fig. 7B). Addition of anti–PD-1 blocking Ab increased the proliferative responses of CMV-specific CD4+ T cells during primary infection but had no impact on the responses measured during chronic infection. In contrast to PD-1, low and similar levels of Tim-3 were observed in CMV and TT-specific cells in the two study groups (Fig. 7B). Addition of anti–PD-1 blocking Ab increased the proliferative responses of CMV-specific CD4+ T cells during primary infection but had no impact on the responses measured during chronic infection (Fig. 7C). In contrast, addition of anti–Tim-3 blocking Ab did not significantly influence proliferative responses to CMV Ags in primary or chronic infection. PD-1 or Tim-3 inhibition did not influence the proliferative responses of TT-specific or influenza-specific CD4+ T cells (data not shown). Analyses of cytokine production (percentage of positive cells and MFI) by total, CD28+, or CD28−CMV-specific CD4+ T cells did not reveal any impact of PD-1 or Tim-3 inhibition (data not shown). These results indicate that the defective proliferative responses associated with primary CMV infection involve the upregulation of PD-1 expression by a subset of CMV-specific CD4+ T cells.

**Discussion**

This study shows for the first time, to our knowledge, that the functional capacity of CMV-specific CD4+ T lymphocytes is selectively impaired during primary infection. In subjects with primary CMV infection, CMV-specific CD4+ T cells have a reduced capacity to produce IFN-γ, TNF-α, and IL-2 as compared with subjects with chronic infection, whereas the function of CD4+ T cells of other specificities is preserved. During primary infection, CMV-specific CD4+ T cells expressed an activation phenotype indicating recent antigenic exposure. We observed no correlation between CD4+ T cell response to CMV or control Ags and the detection of viremia at the time of analysis (data not shown). Excretion of CMV in urine and saliva lasts for several months following primary infection, whereas viremia is more transient (14, 27). Our results, therefore, suggest that the prolonged replication of CMV in tissues is an important source of Ags stimulating CD4+ T cells.

The reduced capacity of CMV-specific CD4+ T cells to produce IL-2 was associated with defective proliferative responses, and addition of exogenous IL-2 restored proliferative responses to the levels observed during chronic infection. These results are in line with the low frequencies of IL-2–producing CD4+ T cells ob-
primary CMV infection. Limited production of IL-2 by CD4+ T cells observed by Lilleri et al. (19) in HIV-seronegative subjects with advanced stage of CMV-specific CD4+ T cells occurs early during the course of primary infection. Because optimal production of IL-2 by T lymphocytes is dependent on the signals provided by the costimulatory receptor CD28 (25), the higher proportions of CD28− cells among CMV-specific CD4+ T lymphocytes during primary infection could have explained their defective production of IL-2. As expected, we observed that CD28− cells produce very low levels of IL-2 during both primary and chronic infection. Unexpectedly, the capacity of CMV-specific CD28+ cells to produce IL-2 was significantly lower during primary as compared with chronic infection. These results indicate that the defective production of IL-2 by CMV-specific CD4+ T cells during primary infection is only partly related to their advanced differentiation.

The fact that the defective capacity to produce IL-2 affected both CD28− and CD28+ CD4+ T cells suggested broader functional alterations of CD4+ T cells. In agreement with this hypothesis, we observed that although the frequencies of CMV-specific CD4+ T cells producing IFN-γ and TNF-α were comparable during primary and chronic infection, the per cell production of these cytokines was significantly lower during primary infection. This defective production of effector cytokines was independent on the expression of CD28 by CD4+ T cells. Because CMV-specific CD4+ T cells were detected following in vitro Ag stimulation, cells unable to produce the measured cytokines would have been excluded from our analyses, and functional exhaustion, therefore, may have been underestimated. Functional analyses of CMV-specific CD4+ T cells detected by MHC class II tetramer staining should provide a more sensitive evaluation of their functional potential.

An impairment of the production of multiple cytokines is characteristic of the T cell exhaustion phenotype that has been described in several models of chronic viral infections (39). Functional exhaustion of T cells is induced by prolonged exposure to high Ag loads and is characterized by the partial or complete loss of capacity to produce effector cytokines. Functionally exhausted CD8+ T cells have been characterized in details in several animal models and human diseases. Much less is known about this process in CD4+ T lymphocytes. Exhaustion of CD4+ T cells has been observed in the early phase of lymphocytic choriomeningitis virus infection in mice and in patients infected with HIV or HCV (40–42). T lymphocyte exhaustion can involve multiple mechanisms including an increased expression of inhibitory receptors, such as PD-1 and Tim-3, modulating TCR signaling (26, 43). We observed that CMV-specific CD4+ T cells expressed increased levels of PD-1 and similar and low levels of Tim-3 during primary as compared with chronic infection. These results are in line with those reported by Sester et al. (45) showing increased proliferation of CMV-specific CD4+ T cells following programmed death ligands 1 and 2 blockade in transplanted patients with CMV vi-

![FIGURE 7.](image-url)
remia. Our results indicated that PD-1 inhibition did not restore proliferative responses to the levels observed during chronic infection, suggesting that PD-1 upregulation is only one of the factors controlling CMV-specific CD4+ T cell proliferation. Also, our results suggest that the upregulation of PD-1 is not central to the control of cytokine production by CMV specific CD4+ T cells. Indeed, decreased cytokine responses were observed in both CD28+ and CD28- subsets, whereas PD-1 upregulation was specifically observed in CD28- cells. In addition, PD-1 inhibition did not influence the production of cytokines following short-term cell stimulation of CMV-specific CD4+ T cells. These results are in line those obtained by Serriari et al. (23) in transplanted patients with primary CMV infection and showing that PD-1 controls the proliferation of CMV-specific CD8+ T cells but not their capacity to produce effector cytokines. Taken together, these observations indicate that additional mechanisms to PD-1 upregulation and CD28 downregulation control the functions of CMV-specific T cells during primary infection.

The functional exhaustion of CMV-specific CD4+ T cells during primary infection was associated with a decreased functional avidity of these cells. Indeed, Ag dilution experiments revealed that CD4+ T cells were more sensitive to peptide titration during primary as compared with chronic infection. A similar association between functional avidity and production of cytokines was observed in CD8+ T cells from HIV-infected patients (46). CD8+ T cells with high functional avidity are detected in patients with slow HIV disease progression and exhibit increased polyfunctionality and clonal turnover, whereas low-avidity CD8+ T cells have defective cytokine production and proliferative capacity. The cellular mechanisms underlying these differences of functional avidity have not been fully characterized. Nonstructural mechanisms including inhibitory receptors, regulation of TCR signaling, transcriptional control of cytokine gene expression, or posttranscriptional control of cytokine synthesis associated with cellular stress could be involved (47, 48). The structural characteristics of the TCR could also play an important role as low-affinity interactions with the cognate MHC–peptide complexes would result in low functional avidity and low-intensity signaling. CD4+ T cells from HIV controllers were recently shown to express higher affinity TCRs than viremic or treated patients, suggesting that immune control of HIV replication may be favored by high-affinity CD4+ T cells (49). Further studies are needed to characterize the role of nonstructural and TCR-related mechanisms in the functional exhaustion of CD4+ T lymphocytes during primary CMV infection.

The mechanisms underlying the emergence of CD4+ T cells with higher functional capacity and avidity during the chronic phase of CMV infection remain to be elucidated. As viral replication decreases and Ag load is reduced, cells could recover from functional exhaustion and produce larger amounts of effector cytokines. Alternatively, new clones may emerge and acquire higher functional capacities when Ag load is reduced. The second hypothesis is supported by the observation that CMV-specific CD4+ T cells undergo intense clonal turnover between the primary and the chronic phase of infection in kidney transplanted patients (50). Differences in Ag load and in costimulatory signals at the time of priming would therefore determine the functional capacity and avidity of CMV-specific CD4+ T lymphocytes. According to this model, controlling Ag load in CMV-infected patients with antiviral therapy would allow the emergence of CD4+ T cell clones with more potent antiviral properties and the reconstitution of anti-CMV immunity. Longitudinal analysis of the functional capacities and repertoire of CD4+ T cell clones over the natural course of primary CMV infection and following anti-CMV therapy are needed to test this hypothesis.

In conclusion, our study shows that during primary CMV infection, CD4+ T lymphocytes express a phenotype of functional exhaustion, including reduced proliferative and cytokine responses and increased expression of the inhibitory receptor PD-1. These results indicate that CMV is part of the group of viruses including HIV and HCV that induce functional exhaustion of CD4+ T lymphocytes in humans. Exhaustion of CD4+ T lymphocytes during primary CMV infection may limit the control of CMV replication and therefore represent a target for therapeutic interventions.

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Disclosures
The authors have no financial conflicts of interest.

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