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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*

Human CMV establishes lifelong persistence after primary infection. Chronic CMV infection is associated with intermittent viral reactivation inducing high frequencies of CD4+ T lymphocytes with potent antiviral and helper properties. Primary CMV infection is characterized by an intense viral replication lasting for several months. The impact of this prolonged exposure to high Ag loads on the functionality of CD4+ T cells remains incompletely understood. In pregnant women with primary CMV infection, we observed that CMV-specific CD4+ T lymphocytes had a decreased capacity to proliferate and to produce IL-2. A very large proportion of CMV-specific CD4+ T cells had downregulated the expression of CD28, a costimulatory molecule centrally involved in the production of IL-2. Unexpectedly, both CD28− and CD28+CD4+ T cells produced low levels of IL-2. This defective production of IL-2 was part of a larger downregulation of cytokine production. Indeed, CMV-specific CD4+ T cells produced lower amounts of IFN-γ and TNF-α and showed lower functional avidity during primary as compared with chronic infection. Increased programmed death-1 expression was observed in CD28+ CMV-specific CD4+ T cells, and programmed death-1 inhibition increased proliferative responses. These results indicate that primary CMV infection is associated with the exhaustion of CMV-specific CD4+ T cells displaying low functional avidity for viral Ags.   *The Journal of Immunology*, 2012, 189: 000–000.

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, CD28−CD4+ and CD28−CD8+ T cells produce lower levels of IL-2 than their CD28+ counterparts following polyclonal stimulation (21). In addition, CD28 transduction of CD28−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, increased titers of CMV-specific IgM. A total of 41 patients 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. 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 fibrobasts (1 μg/ml) (Virusys), a pool of 15 aa peptides overlapping by 11 and derived from the CMV pp65 tegument protein (1.75 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% CO$_2$. 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 Ki-67$^+$ 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 and were stimulated with a lysate of CMV-infected fibroblasts (5 μg/ml), a pool of CMV pp65 overlapping peptides (1.75 μg/ml), a pool of CMV gB overlapping peptides (2 μg/ml), TT (10 μg/ml), or a preparation of split Jiangsu influenza virus (1 μg/ml) for 18 h at 37°C in the presence of 5% CO$_2$. Brefeldin A (5 μg/ml) (Sigma-Aldrich) was added to the medium for the last 16 h of stimulation. The role of inhibitory receptors was assessed by adding anti–PD-1 blocking Ab, Tim3 blocking Ab, or isotype control (5 μg/ml; all from BioLegend) to the culture medium. Ag-specific CD4$^+$ T cells were identified on the basis of the production of at least one among three cytokines (IFN-γ, TNF-α, and IL-2). The phenotype of Ag-specific cells was characterized using the following Abs: CD3 FITC, CD4 allophycocyanin-H7 or V500 or V450, CD8 PerCP, CD14 PE, CD16 PE, CD19 PE, CD56 PE, CD45RO PE-Cy7, CD27 V450, CD28 ECD, MHC class II FITC, CD38 AF700, IFN-γ PE-Cy7 or AF700, TNF-α Pacific Blue or eFluor 450 or AF700 or allophycocyanin, IL-2 allophycocyanin or PE, MIP-1β PE-Cy7, Bcl-2 FITC, Tim3 PE, and PD-1 allophycocyanin (all from BD Biosciences, except CD28 from Beckman Coulter; TNF-α Pacific Blue or eFluor 450 from eBioscience; Tim3 and PD-1 from BioLegend). CD3, cytokines, and Bcl-2 were stained intracellularly after permeabilization with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Data were obtained on a Cyan ADP LX9 cytometer (DakoCytomation) and analyzed using the FlowJo 8.8.6, 8.8.7, or 9.0.1 software (Tree Star). To provide consistent measurements of mean fluorescence intensities (MFI) between experiments, flow cytometer settings were standardized using SPHERO Rainbow Beads (BD Biosciences). The voltage of each photomultiplier tube was set to detect the beads with the same median of fluorescence before every data acquisition. A single batch SPHERO Rainbow Beads was used for the duration of the study.

Apoptosis

Fresh PBMC were cultured for 18 h at 37°C with 5% CO$_2$ 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, AnnexinV 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-
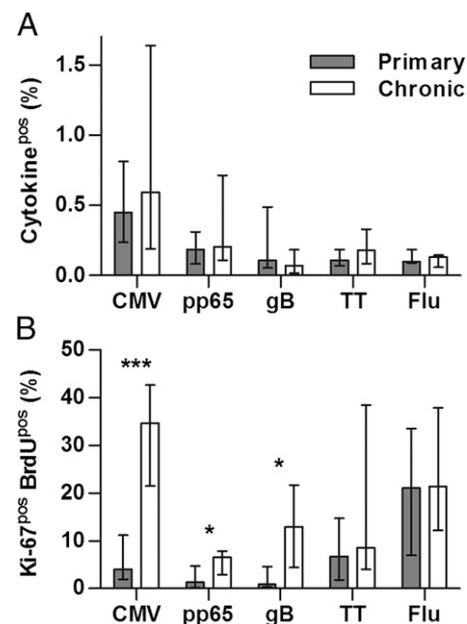

FIGURE 1. Frequencies and proliferative responses of CMV-specific CD4$^+$ T cells during primary and chronic infection. (**A**) Frequencies of total cytokine-producing CD4$^+$ T cells (producing at least one cytokine among IFN-γ, TNF-α, and IL-2) were measured among CD4$^+$ T cells after overnight stimulation with whole CMV lysate, pp65 peptide pool, gB peptide pool, TT, and influenza split virus. (**B**) CD4$^+$ T lymphocytes proliferative responses to CMV and third-party Ags were measured using the BrdU incorporation assay. Figures show medians ± interquartile ranges of 6–21 subjects depending on the Ag tested and readout. *$p < 0.05$, ***$p < 0.001$.

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
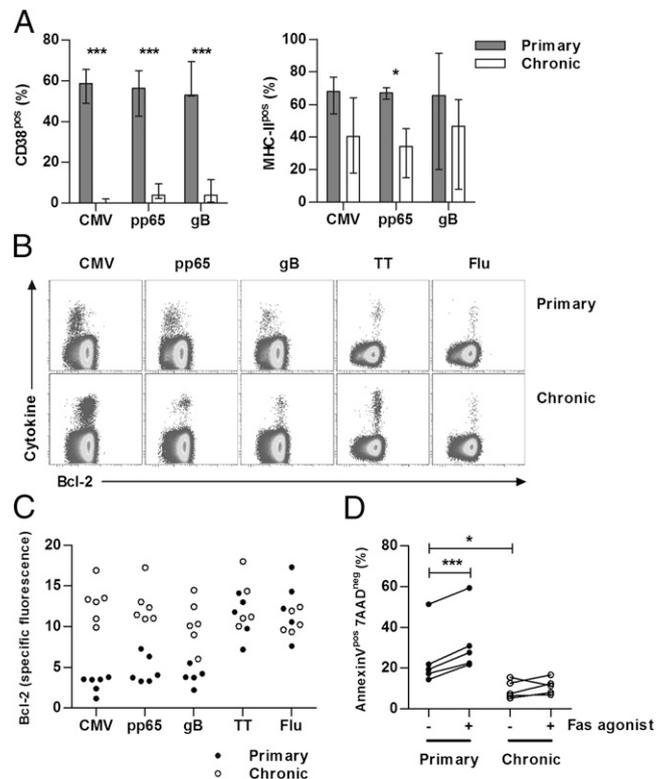

FIGURE 2. Activation of CMV-specific CD4+ T cells during primary infection. (**A**) Expression of CD38 (*left panel*) and MHC class II (*right panel*) was measured at the surface of CMV-specific CD4+ T lymphocytes producing at least one cytokine after overnight Ag stimulation (medians ± interquartile ranges of five to six subjects per Ag tested). (**B**) Representative dot plots of Bcl-2 expression by Ag-specific CD4+ T cells. For this two-parameter representation, dominant cytokines were selected (IFN-γ for CMV-specific cells and IL-2 for TT and influenza-specific cells). (**C**) Quantification of Bcl-2 expression by CD4+ T cells producing at least one cytokine in response to stimulation with CMV or control Ags in subjects with primary (closed symbols) or chronic infection (open symbols). (**D**) Apoptosis of CD28−CD4+ T cells was measured after overnight incubation in the absence and presence of Fas agonist Abs in subjects with primary (closed symbols) or chronic infection (open symbols). *p < 0.05, ***p < 0.001.

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-
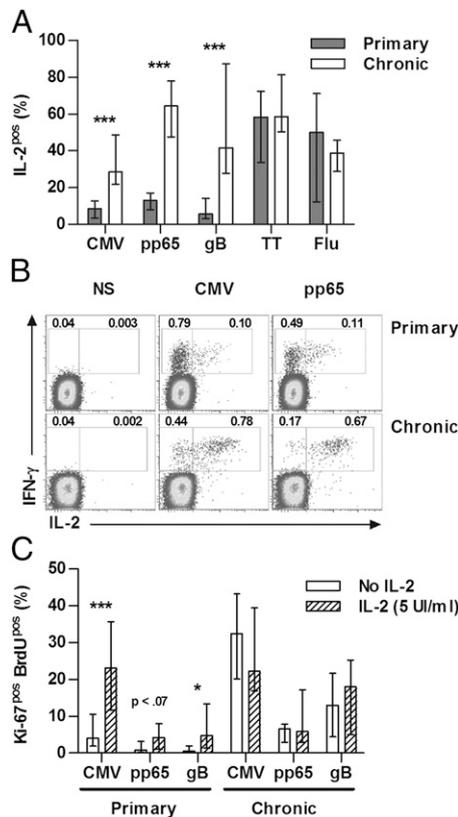
FIGURE 3. Defective production of IL-2 and proliferative responses of CMV-specific CD4+ T lymphocytes during primary CMV. (**A**) The proportion of IL-2–producing cells among total cytokine-producing CD4+ T cells was measured after overnight stimulation with CMV or control Ags (medians ± interquartile 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.

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.

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 downregulation 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.

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

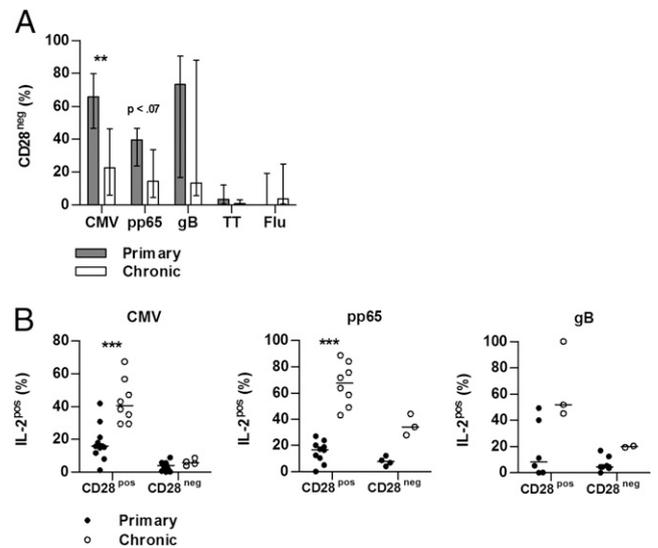The observation that the defective IL-2 production affected CD28+ T lymphocytes suggested that the functional impairment of CMV-specific CD4+ T cells may not be restricted to IL-2. Because the proportion of CD4+ T cells producing either IFN-γ or TNF-α was similar in subjects with primary or chronic infection, 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-α MFI in subjects with primary infection as compared with chronic infection (Fig. 5C). To further characterize the capacity of CMV-specific CD4+ T cells to produce effector cytokines during primary infection, their functional avidity was assessed by measuring the impact of Ag titration on cytokine responses (Fig. 6). Percentage of cytokine-producing CD4+ T cells following stimulation with optimal concentrations of pp65 and gB peptide pools was defined as 100%. Ag titration resulted in a significantly sharper decline of cytokine responses, indicating lower functional avidity of CD4+ T cells, in subjects with primary infection as compared with subjects with chronic infection. Similar results were obtained with total cytokine or with IFN-γ responses following stimulation with either gB or pp65 peptide pools. Taken together, these data indicate that during primary CMV infection CD4+ T cells are impaired in their capacity to produce multiple cytokines and have a lower functional avidity as compared with chronic infection.

CD28+ 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 5. Defective production IFN-γ and TNF-α by CMV-specific CD4$^+$ T cells during primary infection. (**A** and **B**) Per cell production of individual cytokines by CD4$^+$ T cells was measured following stimulation with CMV or control Ags and is expressed as MFI (medians $\pm$ interquartile ranges of 4–10 subjects). (**C**) Per cell production of cytokines by CMV-specific CD28$^+$ and CD28$^-$CD4$^+$ T cells was measured as in (A) and (B) and is expressed as individual results and medians. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
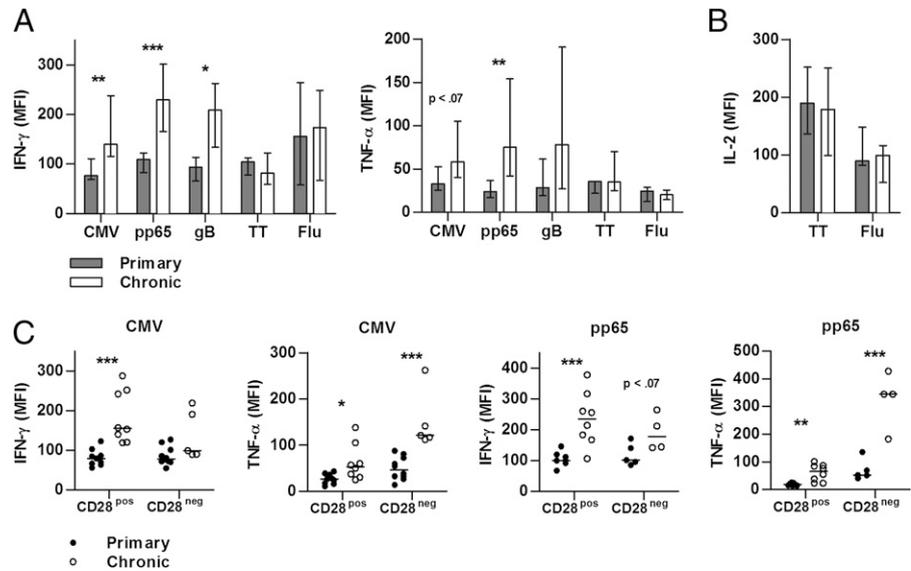
cluding PD-1 and Tim-3 (26). To gain insight into the mechanisms regulating the function of CMV-specific CD4$^+$ T cells, the expression of PD-1 and Tim-3 was measured at the level of CMV and TT-specific CD4$^+$ T during primary and chronic infection. Primary infection was associated with increased expression 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 (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-
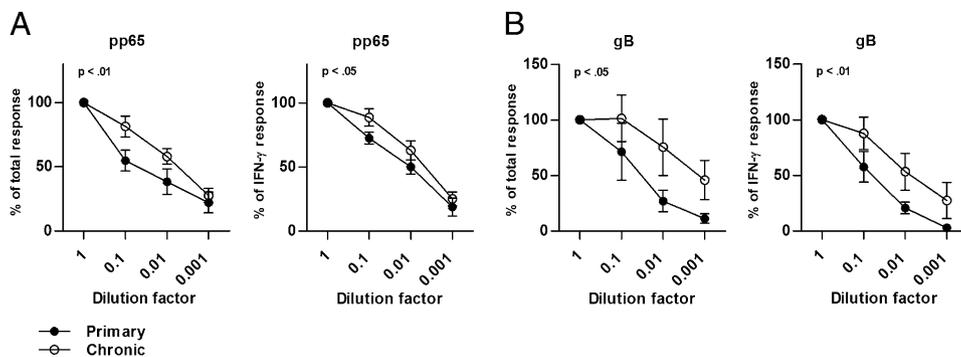

FIGURE 6. Reduced functional avidity of CMV-specific CD4$^+$ during primary infection. Frequencies of CD4$^+$ T cells producing one of three cytokines (*left panel*) or producing IFN-γ (*right panel*) in response to serial dilutions of optimal concentrations of pp65 (**A**) or gB (**B**) peptide pools were measured. Results are expressed as mean percentage ($\pm$SE on the mean) of the responses to optimal peptide pool concentrations. Figures show five to nine subjects depending on the Ag tested and the readout.
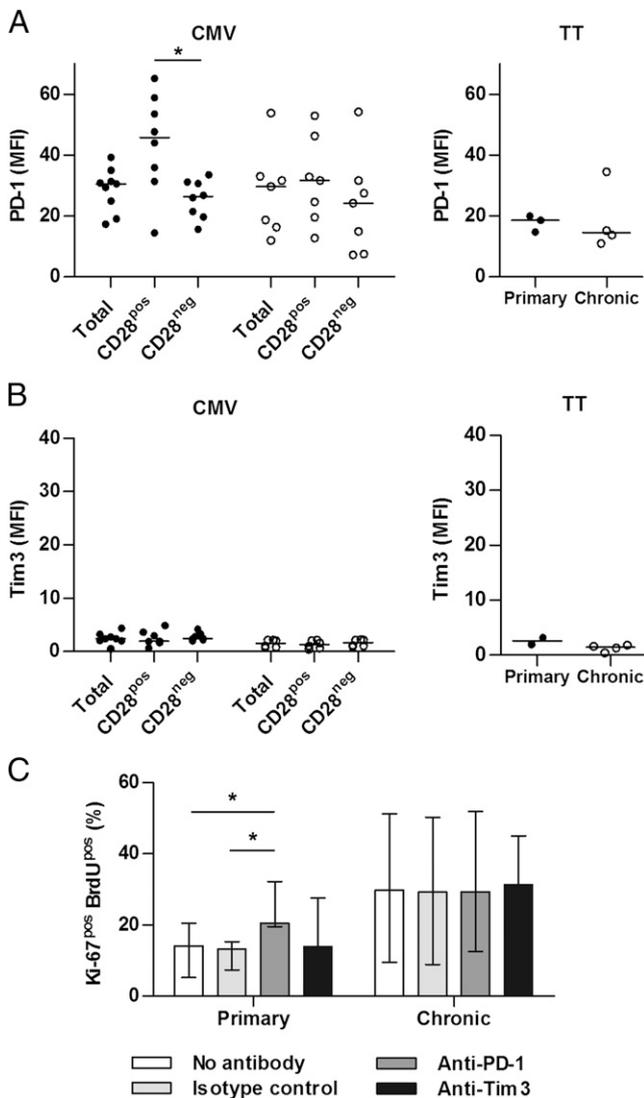
FIGURE 7. CD28⁺ CMV-specific CD4⁺ express increased level of PD-1 during primary infection. The expression of PD-1 (**A**) and Tim-3 (**B**) was measured on total, CD28⁺, and CD28⁻ CD4⁺ T cells producing at least one cytokine in response to stimulation with CMV-infected fibroblasts lysate (*left panel*) or TT (*right panel*) in subjects with primary (closed symbols) or chronic CMV infection (open symbols) and is expressed as MFI. (**C**) The influence of anti–PD-1 and anti–Tim-3 blocking Abs on the proliferative responses of CD4⁺ T cells to CMV Ags was studied using the BrdU incorporation assay (medians ± interquartile ranges of six to seven subjects). *$p < 0.05$.

served by Harari et al. (20) in a restricted series of HIV-infected patients with primary CMV infection and with the progressive increase in frequencies of IL-2–producing CD4⁺ T lymphocytes observed by Lilleri et al. (19) in HIV-seronegative subjects with primary CMV infection. Limited production of IL-2 by CD4⁺ T cells and low proliferative responses have been observed in other chronic viral infection including HIV, hepatitis C virus (HCV), and EBV (28–31). In HIV and HCV infections, the defective production of IL-2 is associated with active viral replication (28, 32, 33). In HIV-infected patients, anti-viral therapy improves the capacity of CD4⁺ T cells to produce IL-2, suggesting that high viral loads and intense T cell stimulation directly downregulates IL-2 production (34, 35).

The defective production of IL-2 was associated with an advanced stage of differentiation of CMV-specific CD4⁺ T lym-

phocytes. Indeed, higher frequencies of CMV-specific cells lacking the expression of the CD28 molecule were detected during primary as compared with chronic infection. The progressive accumulation of CD28⁻CD8⁺ T lymphocytes with age in CMV-seropositive subjects has led to the suggestion that CD28⁻ CMV-specific T cells accumulate slowly over time because of repetitive antigenic stimulation (36–38). Our results indicate that the differentiation 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 co-stimulatory 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⁻ CMV-specific CD4⁺ T 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. Surprisingly, the increased expression of PD-1 was observed on CD28⁺ and not CD28⁻ CD4⁺ T cells. This suggests that the signals triggering PD-1 upregulation in vivo involved CD28 costimulation. Increased expression of PD-1 by CD28⁺ CD4 T cells has also been observed in HIV-infected patients (44). Inhibition of PD-1 increased the proliferative responses of CMV-specific CD4⁺ T cells during primary 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-

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 pri-

mary 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.

References

1. Gandhi, M. K., and R. Khanna. 2004. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect. Dis.* 4: 725–738.
2. Burny, W., C. Liesnard, C. Donner, and A. Marchant. 2004. Epidemiology, pathogenesis and prevention of congenital cytomegalovirus infection. *Expert Rev. Anti Infect. Ther.* 2: 881–894.
3. Salmon-Céron, D., M. C. Mazeron, S. Chaput, N. Boukli, B. Senechal, N. Houhou, C. Katlama, S. Matheron, A. M. Fillet, J. Gozlan, et al. 2000. Plasma cytomegalovirus DNA, pp65 antigenaemia and a low CD4 cell count remain risk factors for cytomegalovirus disease in patients receiving highly active anti-retroviral therapy. *AIDS* 14: 1041–1049.
4. Sester, M., U. Sester, B. Gärtner, G. Heine, M. Girndt, N. Mueller-Lantzsch, A. Meyerhans, and H. Köhler. 2001. Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation* 71: 1287–1294.
5. Gamadia, L. E., E. B. Remmerswaal, J. F. Weel, F. Bemelman, R. A. van Lier, and I. J. Ten Berge. 2003. Primary immune responses to human CMV: a critical role for IFN-γ–producing CD4^{+} T cells in protection against CMV disease. *Blood* 101: 2686–2692.
6. Pourgheysari, B., K. P. Piper, A. McLarnon, J. Arrazi, R. Bruton, F. Clark, M. Cook, P. Mahendra, C. Craddock, and P. A. Moss. 2009. Early reconstitution of effector memory CD4 CMV-specific T cells protects against CMV reactivation following allogeneic SCT. *Bone Marrow Transplant.* 43: 853–861.
7. Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med.* 333: 1038–1044.
8. Lilleri, D., G. Gerna, C. Fornara, L. Lozza, R. Maccario, and F. Locatelli. 2006. Prospective simultaneous quantification of human cytomegalovirus-specific CD4^{+} and CD8^{+} T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. *Blood* 108: 1406–1412.
9. Appay, V. 2004. The physiological role of cytotoxic CD4^{+} T-cells: the holy grail? *Clin. Exp. Immunol.* 138: 10–13.
10. van Leeuwen, E. M., E. B. Remmerswaal, M. T. Vossen, A. T. Rowshani, P. M. Wertheim-van Dillen, R. A. van Lier, and I. J. ten Berge. 2004. Emergence of a CD4^{+}CD28^{−} granzyme B^{+}, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *J. Immunol.* 173: 1834–1841.
11. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4^{+} T lymphocytes with cellular maturation. *J. Exp. Med.* 203: 2865–2877.
12. Rentenaar, R. J., L. E. Gamadia, N. van DerHoek, F. N. van Diepen, R. Boom, J. F. Weel, P. M. Wertheim-van Dillen, R. A. van Lier, and I. J. ten Berge. 2000. Development of virus-specific CD4^{+} T cells during primary cytomegalovirus infection. *J. Clin. Invest.* 105: 541–548.

13. Levin, M. J., C. R. Rinaldo, Jr., P. L. Leary, J. A. Zaia, and M. S. Hirsch. 1979. Immune response to herpesvirus antigens in adults with acute cytomegaloviral mononucleosis. *J. Infect. Dis.* 140: 851–857.

14. Zanghellini, F., S. B. Boppana, V. C. Emery, P. D. Griffiths, and R. F. Pass. 1999. Asymptomatic primary cytomegalovirus infection: virologic and immunologic features. *J. Infect. Dis.* 180: 702–707.

15. Stern, H., G. Hannington, J. Booth, and D. Moncrieff. 1986. An early marker of fetal infection after primary cytomegalovirus infection in pregnancy. *Br. Med. J. (Clin. Res. Ed.)* 292: 718–720.

16. Fernando, S., J. M. Pearce, and J. C. Booth. 1993. Lymphocyte responses and virus excretion as risk factors for intrauterine infection with cytomegalovirus. *J. Med. Virol.* 41: 108–113.

17. Revello, M. G., D. Lilleri, M. Zavattoni, M. Furione, E. Genini, G. Comolli, and G. Gerna. 2006. Lymphoproliferative response in primary human cytomegalovirus (HCMV) infection is delayed in HCMV transmitter mothers. *J. Infect. Dis.* 193: 269–276.

18. Schrier, R. D., W. R. Freeman, C. A. Wiley, and J. A. McCutchan. 1995. Immune predispositions for cytomegalovirus retinitis in AIDS. *J. Clin. Invest.* 95: 1741–1746.

19. Lilleri, D., C. Fornara, M. G. Revello, and G. Gerna. 2008. Human cytomegalovirus-specific memory CD8+ and CD4+ T cell differentiation after primary infection. *J. Infect. Dis.* 198: 536–543.

20. Harari, A., F. Vallelian, P. R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* 174: 1037–1045.

21. Amyes, E., A. J. McMichael, and M. F. Callan. 2005. Human CD4+ T cells are predominantly distributed among six phenotypically and functionally distinct subsets. *J. Immunol.* 175: 5765–5773.

22. Topp, M. S., S. R. Riddell, Y. Akatsuka, M. C. Jensen, J. N. Blattman, and P. D. Greenberg. 2003. Restoration of CD28 expression in CD28– D8+ memory effector T cells reconstitutes antigen-induced IL-2 production. *J. Exp. Med.* 198: 947–955.

23. Serriari, N. E., F. Gondois-Rey, Y. Guillaume, E. B. Remmerswaal, S. Pastor, N. Messal, A. Truneh, I. Hirsch, R. A. van Lier, and D. Olive. 2010. B and T lymphocyte attenuator is highly expressed on CMV-specific T cells during infection and regulates their function. *J. Immunol.* 185: 3140–3148.

24. Chipuk, J. E., T. Moldoveanu, F. Llambi, M. J. Parsons, and D. R. Green. 2010. The BCL-2 family reunion. *Mol. Cell* 37: 299–310.

25. Crispín, J. C., and G. C. Tsokos. 2009. Transcriptional regulation of IL-2 in health and autoimmunity. *Autoimmun. Rev.* 8: 190–195.

26. Frebel, H., K. Richter, and A. Oxenius. 2010. How chronic viral infections impact on antigen-specific T-cell responses. *Eur. J. Immunol.* 40: 654–663.

27. Revello, M. G., M. Zavattoni, A. Sarasini, E. Percivalle, L. Simoncini, and G. Gerna. 1998. Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy. *J. Infect. Dis.* 177: 1170–1175.

28. Iyasere, C., J. C. Tilton, A. J. Johnson, S. Younes, B. Yassine-Diab, R. P. Sekaly, W. W. Kwok, S. A. Migueles, A. C. Laborico, W. L. Shupert, et al. 2003. Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J. Virol.* 77: 10900–10909.

29. Musey, L. K., J. N. Krieger, J. P. Hughes, T. W. Schacker, L. Corey, and M. J. McElrath. 1999. Early and persistent human immunodeficiency virus type 1 (HIV-1)-specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J. Infect. Dis.* 180: 278–284.

30. Folgori, A., E. Spada, M. Pezzanera, L. Ruggeri, A. Mele, A. R. Garbuglia, M. P. Perrone, P. Del Porto, E. Piccolella, R. Cortese, et al. 2006. Early impairment of hepatitis C virus specific T cell proliferation during acute infection leads to failure of viral clearance. *Gut* 55: 1012–1019.

31. Attarbaschi, T., M. Willheim, M. Ramharter, A. Hofmann, K. Wahl, H. Winkler, W. Graninger, and S. Winkler. 2003. T cell cytokine profile during primary Epstein-Barr virus infection (infectious mononucleosis). *Eur. Cytokine Netw.* 14: 34–39.

32. Tilton, J. C., M. R. Luskin, A. J. Johnson, M. Manion, C. W. Hallahan, J. A. Metcalf, M. McLaughlin, R. T. Davey, Jr., and M. Connors. 2007. Changes in paracrine interleukin-2 requirement, CCR7 expression, frequency, and cytokine secretion of human immunodeficiency virus-specific CD4+ T cells are a consequence of antigen load. *J. Virol.* 81: 2713–2725.

33. Semmo, N., G. Krashias, C. Willberg, and P. Klenerman. 2007. Analysis of the relationship between cytokine secretion and proliferative capacity in hepatitis C virus infection. *J. Viral Hepat.* 14: 492–502.

34. Hardy, G. A., N. Imami, A. K. Sullivan, A. Pires, C. T. Burton, M. R. Nelson, B. G. Gazzard, and F. M. Gotch. 2003. Reconstitution of CD4+ T cell responses in HIV-1 infected individuals initiating highly active antiretroviral therapy (HAART) is associated with renewed interleukin-2 production and responsiveness. *Clin. Exp. Immunol.* 134: 98–106.

35. Jansen, C. A., D. van Baarle, and F. Miedema. 2006. HIV-specific CD4+ T cells and viremia: who's in control? *Trends Immunol.* 27: 119–124.

36. van de Berg, P. J., A. van Stijn, I. J. Ten Berge, and R. A. van Lier. 2008. A fingerprint left by cytomegalovirus infection in the human T cell compartment. *J. Clin. Virol.* 41: 213–217.

37. Koch, S., A. Larbi, D. Ozcelik, R. Solana, C. Gouttefangeas, S. Attig, A. Wikby, J. Strindhall, C. Franceschi, and G. Pawelec. 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. *Ann. N. Y. Acad. Sci.* 1114: 23–35.

38. Gamadia, L. E., E. M. van Leeuwen, E. B. Remmerswaal, S. L. Yong, S. Surachno, P. M. Wertheim-van Dillen, I. J. Ten Berge, and R. A. van Lier. 2004. The size and phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and environmental cytokines. *J. Immunol.* 172: 6107–6114.

39. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* 78: 5535–5545.

40. D'Souza, M., A. P. Fontenot, D. G. Mack, C. Lozupone, S. Dillon, A. Meditz, C. C. Wilson, E. Connick, and B. E. Palmer. 2007. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. *J. Immunol.* 179: 1979–1987.

41. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J. Virol.* 79: 10514–10527.

42. Urbani, S., B. Amadei, D. Tola, G. Pedrazzi, L. Sacchelli, M. C. Cavallo, A. Orlandini, G. Missale, and C. Ferrari. 2008. Restoration of HCV-specific T cell functions by PD-1/PD-L1 blockade in HCV infection: effect of viremia levels and antiviral treatment. *J. Hepatol.* 48: 548–558.

43. Yi, J. S., M. A. Cox, and A. J. Zajac. 2010. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 129: 474–481.

44. Kassu, A., R. A. Marcus, M. B. D'Souza, E. A. Kelly-McKnight, L. Golden-Mason, R. Akkina, A. P. Fontenot, C. C. Wilson, and B. E. Palmer. 2010. Regulation of virus-specific CD4+ T cell function by multiple costimulatory receptors during chronic HIV infection. *J. Immunol.* 185: 3007–3018.

45. Sester, U., D. Presser, J. Dirks, B. C. Gärtner, H. Köhler, and M. Sester. 2008. PD-1 expression and IL-2 loss of cytomegalovirus- specific T cells correlates with viremia and reversible functional anergy. *Am. J. Transplant.* 8: 1486–1497.

46. Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, et al. 2007. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J. Exp. Med.* 204: 2473–2485.

47. Pritts, T. A., Q. Wang, X. Sun, D. R. Fischer, E. S. Hungness, J. E. Fischer, H. R. Wong, and P. O. Hasselgren. 2002. The stress response decreases NF-κB activation in liver of endotoxemic mice. *Shock* 18: 33–37.

48. Shi, Y., Z. Tu, D. Tang, H. Zhang, M. Liu, K. Wang, S. K. Calderwood, and X. Xiao. 2006. The inhibition of LPS-induced production of inflammatory cytokines by HSP70 involves inactivation of the NF-κB pathway but not the MAPK pathways. *Shock* 26: 277–284.

49. Vingert, B., S. Perez-Patrigeon, P. Jeannin, O. Lambotte, F. Boufassa, F. Lemaître, W. W. Kwok, I. Theodorou, J. F. Delfraissy, J. Thèze, and L. A. Chakrabarti. 2010. HIV controller CD4+ T cells respond to minimal amounts of Gag antigen due to high TCR avidity. *PLoS Pathog.* 6: e1000780.

50. van Leeuwen, E. M., E. B. Remmerswaal, M. H. Heemskerk, I. J. ten Berge, and R. A. van Lier. 2006. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. *Blood* 108: 3121–3127.