J Am Soc Nephrol 13: 2577–2584, 2002

Dominance of Virus-Specific CD8 T Cells in Human Primary Cytomegalovirus Infection

MARTINA SESTER,* URBAN SESTER,* BARBARA C. GÄRTNER,†
MATTHIAS GIRNDT,* ANDREAS MEYERHANS,† and HANS KÖHLER*
**Medical Department IV, Nephrology, and †Department of Virology, Institute of Medical Microbiology and Hygiene, University of the Saarland, Homburg, Germany*

Abstract. Cellular immune responses are of high importance in initiating and maintaining immunity against virus infections. Whereas the cellular immune response during persistent cytomegalovirus (CMV) infection is well assessable, the individual contribution of CD4 and CD8 T cell responses during primary infection has not been described. A novel whole-blood assay, which relies on the flow-cytometric detection of antigen-induced cytokine expression, was used to characterize CMV-specific CD4 and CD8 T cell responses during primary infection of CMV seronegative recipients of a renal allograft from a CMV seropositive donor. These T cell responses were compared with long-term CMV-positive patients with known history of transplantation-related seroconversion. Results were further correlated to CMV load and serum IgG and IgM. The long-term seroconverted patients consistently showed a dominant CMV-specific CD4 T cell response (median frequencies: CD4, 1.12% [range, 0.35 to 8.10%] *versus* CD8 0.13% [range, <0.05 to 0.55%]). In contrast, during primary infection, the cellular immune response is strongly dominated by CMV-specific CD8 T cells (median peak frequencies: CD4, 1.24% [range, 0.21 to 1.60%] *versus* CD8, 2.47% [range, 1.34 to 6.67%]). Upon receipt of ganciclovir, viral load as well as CMV-specific CD8 responses decreased. The frequency of the respective CD4 T cells fluctuated during decrease of CMV load and became dominant over CMV-specific CD8 T cell responses. These results are consistent with the view of an effective direct antiviral activity of CD8 T cells, which is most critical during periods of high viremia. Later on during persistent infection, CD4 T cells dominate the immune response to support the state of antiviral immunity.

Virus-specific T cells are of major importance in the initiation and maintenance of immunity against viral infections. Viruses such as cytomegalovirus (CMV) have evolved various strategies to establish a persistent infection that lasts for the lifetime of the infected host (1). The state of persistence is characterized by a well-balanced equilibrium, where viral replication is controlled by both cellular and humoral immune effector mechanisms. In immunocompetent individuals, persistent CMV infection is in general clinically inapparent. In immunocompromised patients, however, a disruption in the balance between immune control and CMV expansion may lead to serious infectious complications with various clinical manifestations (2–4).

Among T cells, both virus-specific CD8 and CD4 T cells seem important in the control of acute as well as persistent infections (5). The main function of cytotoxic CD8 T cells resides in the specific lysis of virus-infected target cells. On the other hand, CD4 T cells are critical for regulating functionality and diversity of cytotoxic T cells (6–8). This function is mediated by the specific activation of dendritic cells, which cross-present antigenic peptides in the context of MHC class I molecules to induce virus-specific cytotoxic CD8 T cells. Furthermore, CD4 T cells provide help for B cells to secrete high-affinity virus-specific antibodies. In addition, both CD4 and CD8 T cells secrete cytokines, such as interferon-γ (IFN)-γ or tumor necrosis factor-α, that may have direct antiviral effects (9).

We previously characterized the CMV-specific CD4 and CD8 T cell response in both healthy individuals and immunosuppressed renal transplant recipients (4,10). Virus-specific T cells are terminally differentiated effector cells that are interindividually heterogeneous in frequency but of remarkable stability within a given individual (4,10). Whereas T cell immunity toward CMV is rather well characterized in persistent infection, prospective studies that describe the human T cell responses during and longitudinally after acute infection are as yet rare. It is known from other herpesviruses, such as EBV, that primary infection is often accompanied by marked expansions of virus-specific CD8 T cells. Upon resolution of the viral expansion, most of these effectors die by apoptosis, whereas others enter the memory pool to protect the host from uncontrolled reactivation (11,12). Interestingly, EBV-specific CD8 T cell responses during primary infection and subsequently during viral persistence are not only quantitatively distinct but also show marked differences in their phenotype and epitope-specific composition (13–16). Similar studies an-

Received May 25, 2002. Accepted July 3, 2002.
Correspondence to: Prof. Dr. Hans Köhler, Medical Department IV, Division of Nephrology, University of the Saarland, D-66421 Homburg, Germany. Phone: 0049-6841-1623526; Fax: 0049-6841-1623499; E-mail: inhkoe@uniklinik-saarland.de

1046-6673/1310-2577
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000030141.41726.52

alyzing virus-specific CD4 T cells in humans are as yet limited. A recent study described a temporal increase in CMV-specific CD4 T cells during primary viremia that was followed by a rapid decrease. Interestingly, CMV load was detectable and CMV-specific CD4 T cells remained low or absent throughout the observation period of approximately 100 d (17). Given the fact that CMV-specific CD4 T cells are present within each long-term CMV seropositive individual (10), the time point and kinetics of reemergence of specific immunity after resolution of primary viremia are currently unknown. Moreover, the particular relationship between CMV-specific CD4 and CD8 T cell frequencies from the induction of primary immunity into the persistent phase of CMV infection has not been analyzed so far. The present study addresses this issue in the context of primary CMV infection after transplantation of CMV-seronegative recipients of a CMV-seropositive renal allograft. It is shown that primary CMV infection is dominated by virus-specific CD8 T cells. Moreover, we provide evidence for a supportive role of antiviral therapy in the induction and maintenance of specific immune responses.

Materials and Methods

Subjects

The study was conducted among 102 renal transplant recipients (52.1 ± 13.1 yr of age). Eighty-three patients were CMV-seropositive before transplantation. The remaining nineteen patients were CMV-seronegative before transplantation and received a graft from a CMV-seropositive donor. Among them, seven patients were longitudinally analyzed before and regularly after transplantation (all men; 43.3 ± 14.6 yr of age; Table 1). All seven patients received prophylactic ganciclovir treatment during the first 3 mo after transplantation. Three out of seven patients seroconverted thereafter. All patients studied received an immunosuppressive double or triple drug regimen consisting of either cyclosporine A or tacrolimus and either methylprednisolone or azathioprine or both. Blood was drawn in the morning before intake of immunosuppressive drugs. To control for trough levels of immunosuppressive drugs, serum levels of cyclosporine A and tacrolimus were determined using standard assays (Roche Diagnostics, Mannheim, Germany; Abbott Diagnostics, Chicago, IL). All patients gave informed consent. The study was approved by the local ethics committee.

Stimulation of CMV-Specific CD4 and CD8 T Cells within Whole Blood

Simultaneous stimulation of CMV-specific CD4 and CD8 T cells was performed in whole blood as described previously for antigen-specific T cell stimulation (4,10,18,19). As a stimulus, titred amounts of CMV antigen (complement fixation reagent; BioWhittaker, Verviers, Belgium) were used in the presence of 1 μg/ml αCD28 and αCD49 d (clones L293 and 9F10; BD, Heidelberg, Germany), respectively. As internal negative controls, blood cells were stimulated with control antigen that does not contain any CMV proteins (BioWhittaker). Positive control stimulations were carried out using 2.5 μg/ml *Staphylococcus aureus* enterotoxin B (SEB; Sigma, Deisenhofen, Germany) or titered amounts of antigen derived from adenovirus-infected cells (BioWhittaker) as described previously (20). Cells were incubated in polypropylene tubes at 37°C at 6% CO_2 for a total of 6 h. During this time, effector and memory CD4 and CD8 T cells are specifically stimulated, resulting in the upregulation of CD69 and the production of cytokines (19,21). During the last 4 h, 10 μg/ml of Brefeldin A (Sigma) was added to block extracellular transport of cytokines. Thereafter, the blood was treated with 2 mM EDTA for 15 min. Erythrocytes were subsequently lysed, and leukocytes were fixed for 10 min using lysing solution according to the manufacturer's instruction (BD). Cells were washed once with FACS buffer (phosphate buffered saline [PBS], 5% filtered fetal calf serum [FCS], 0.5% bovine serum albumin [BSA, 0.07% NaN_3) and either immediately processed for flow cytometric analysis or left overnight at 4°C.

Determination of the Frequency of CMV Antigen-specific CD4 and CD8 T Cells by Flow Cytometry

Fixed leukocytes were permeabilized with 2 ml of FACS buffer containing 0.1% saponin (Sigma) for 10 min at room temperature (RT). They were thereafter immunostained for 30 min at RT in the dark using saturating concentrations of fluorescently labeled antibodies (all from BD) directed against the following antigens: CD4 or CD8 (clones SK3 or SK1), CD69 (clone L78), and IFN-γ (clone 4S.B3). Cells were washed once with 3 ml of FACS buffer and fixed with PBS/1% paraformaldehyde. At least 10,000 CD4- or CD8-positive lymphocytes were analyzed on a FACScan (Becton Dickinson) using the Cellquest Software. Usually, control antigens did not stimulate any IFN-γ production. Nevertheless, the percentage of specific T cells was calculated by subtraction of the frequency obtained by the respective control stimulations.

Table 1. Characteristics of the seven patients that were longitudinally analyzed after transplantation[a]

Patient	Age (yr)	Renal Disease	# HLA MM	# Acute Rejections	Immunosuppressive Drug Regimen	Seroconversion	Viremia after Stop of Therapy (d)	Peak CMV Load (pg/ml)
Patient 1	31	Alport syndrome	1	0	CyA-Aza-MP	Yes	29	12
Patient 2	65	Toxic nephropathy	0	0	CyA-Aza-MP	Yes	29	17
Patient 3	47	Diabetic nephropathy	2	1	CyA-Aza-MP	Yes	47	286
Patient 4	29	Glomerulonephritis	3	0	CyA-Aza-MP	No	na	na
Patient 5	35	Alport syndrome	3	0	CyA-Aza-MP	No	na	na
Patient 6	41	Glomerulonephritis	5	1	Tacrolimus-Aza-MP	No	na	na
Patient 7	23	Renal hypoplasia	3	1	Tacrolimus-Aza-MP	No	na	na

[a] All patients were male CMV-seronegative recipients of a CMV-seropositive graft. Patients 1 to 3 experienced primary infection, patients 4 to 7 remained CMV-seronegative and negative for CMV-specific T cell responses. HLA-MM, HLA mismatch; CyA, cyclosporine A; Aza, azathioprine; MP, methylprednisolone; n.a., not applicable.

Determination of CMV Serostatus and Viral Load

The CMV serostatus of the donor and recipient before transplantation was provided by the Euro Transplant database. The CMV serostatus after transplantation was determined by a commercial CMV IgG test (IMX, MEIA; Abbott Diagnostics, Wiesbaden, Germany). CMV load was measured as virus DNA from whole blood using the hybrid capture assay (Murex, Version 2.0, Abbott Diagnostics) according to the manufacturer's instruction.

Statistical Analyses

Statistical analysis was performed using the Prism V3.02 Software (Graphpad, San Diego, CA). Significant differences were determined using the Mann-Whitney test.

Results

Long-term CMV-specific T cell responses were cross-sectionally analyzed in 19 CMV-seronegative recipients of a seropositive graft using flow-cytometry (Figure 1). Of those, ten patients (52.6%) had a history of transplantation-related CMV primary infection. The analysis was performed at a minimum of 12 mo after transplantation in a state of persistent infection in the absence of detectable viremia. A dot plot of stimulated CD4 and CD8 T cells of a representative CMV-seropositive individual is shown in Figure 1A. Activated cells producing IFN-γ after stimulation with CMV antigen were quantified among CD4 or CD8 T cells (right panels, 1.20 and 0.25%, respectively). Stimulation with control antigen served as a negative control (left panel). Patients who remained CMV-seronegative do not have any detectable CMV-specific T cells (Figure 1B, right panel). In contrast, in patients who underwent transplantation-related seroconversion, CMV-specific CD4 T cells ranging from 0.35 to 8.10% were readily detectable (median 1.12%; Figure 1B, left panel). Of note, detectable frequencies of CMV-specific CD4 T cells in the persistent phase were always higher than respective CD8 T cells (median, 0.13%; range, <0.05 to 0.55%). This relative predominance of CMV-specific CD4 T cells is similar to the situation found for both CMV-seropositive healthy controls and long-term renal transplant recipients (4). Moreover, frequencies of CMV-specific CD4 and CD8 T cells in the ten patients with transplantation-related seroconversion do not differ from frequencies in the 83 transplant recipients with a known positive serostatus before transplantation (median CD4, 1.93% [range, 0.11 to 18.85%]; median CD8, 0.21% [range <0.05 to 9.72%]; $n = 83$; CD4, $P = 0.23$; CD8, $P = 0.14$). This indicates that primary infection under immunocompromised conditions apparently does not alter CMV-specific cellular immune responses in the long-term.

To analyze the relative contribution of CMV-specific CD4 and CD8 T cell responses during primary infection, we took the chance to longitudinally follow viral load, specific antibodies, and T cell responses in seven CMV-seronegative patients who obtained an organ from a CMV-seropositive donor (Table 1). All patients received prophylactic ganciclovir therapy that was given intravenously for 1 wk followed by an oral application for a total of 3 mo. All patients were documented as being negative for CMV IgG and CMV-DNA before transplantation, and CMV-specific T cells were absent (Figure 2 and data not shown). In contrast, specific immune responses toward adenovirus or the superantigen SEB (Figure 2B and data not shown) were readily detectable. Throughout the period of prophylactic antiviral therapy, CMV-specific T cells remained undetectable in all individuals.

After the period of prophylactic therapy, CMV-DNA and CMV-specific T cell responses were analyzed for a mean time of 61 ± 45 wk. Four patients remained CMV-seronegative and CMV-DNA–negative throughout the observation period (patients 4 to 7). A typical example derived from patient 4 is shown in Figure 2A. As recent reports suggest that CMV-specific proliferative T cell-responses may exist in CMV seronegative individuals (22), all patients were monitored for the presence of CMV-specific T cells. However, among a total of 21 samples that were analyzed in the patients without seroconversion, no specific T cells were detectable.

In contrast, however, the remaining three patients that underwent CMV primary infection and seroconversion acquired CMV-specific T cell immunity (patients 1 to 3, Table 1). After a median time of approximately 30 d off therapy (29, 29, and 47 d, respectively), the patients reported to the hospital with mild CMV-related symptoms such as fever and myalgia. All patients showed detectable CMV-DNA that was accompanied by the induction of both CMV-specific T cell and antibody responses. All patients resumed ganciclovir therapy when the symptoms were confirmed as CMV-related by the first positive CMV-DNA result. A time course of primary infection is exemplified in Figure 2B (patient 1, Table 1). CMV-specific T cells were identified 1 wk after the first detection of CMV-DNA. Humoral responses as measured by CMV-specific IgG and IgM antibodies were detected 1 wk thereafter (stippled line in Figure 2B).

Interestingly, the simultaneous analysis of CMV-specific CD4 and CD8 T cells revealed that the T cell response during primary CMV infection was dominated by specific CD8 T cells. In contrast, during resolution of viremia, CMV-specific T cells fluctuate to reach a stable state, where higher frequencies of CMV-specific CD4 T cells are found. This stable state was reached at approximately the same time point at which CMV-DNA decreased to below marginal levels (<3 pg/ml: patient 1, 37 wk; patient #2, 1.5 wk; patient #3, 3 wk after the first positive DNA result). Immune responses toward SEB or adenovirus that were analyzed as a positive control were stable throughout the observation period (Figure 2B and data not shown). The relative dominance of CMV-specific CD8 T cells during primary viremia seems to be a general observation, because it was found in all patients undergoing CMV primary infection (Figure 3A; median peak frequencies: CD4, 1.24% [range, 0.21 to 1.60%] *versus* CD8, 2.47% [range, 1.34 to 6.67%]). Likewise, all patients showed a reversal toward dominance of CD4 T cells after resolution of primary viremia in persistent infection (Figure 3B). After the stabilization of CMV-specific immunity, all patients retained specific humoral and CD4 T cell responses throughout the entire observation period (127, 108, and 40 wk after therapy, respectively).

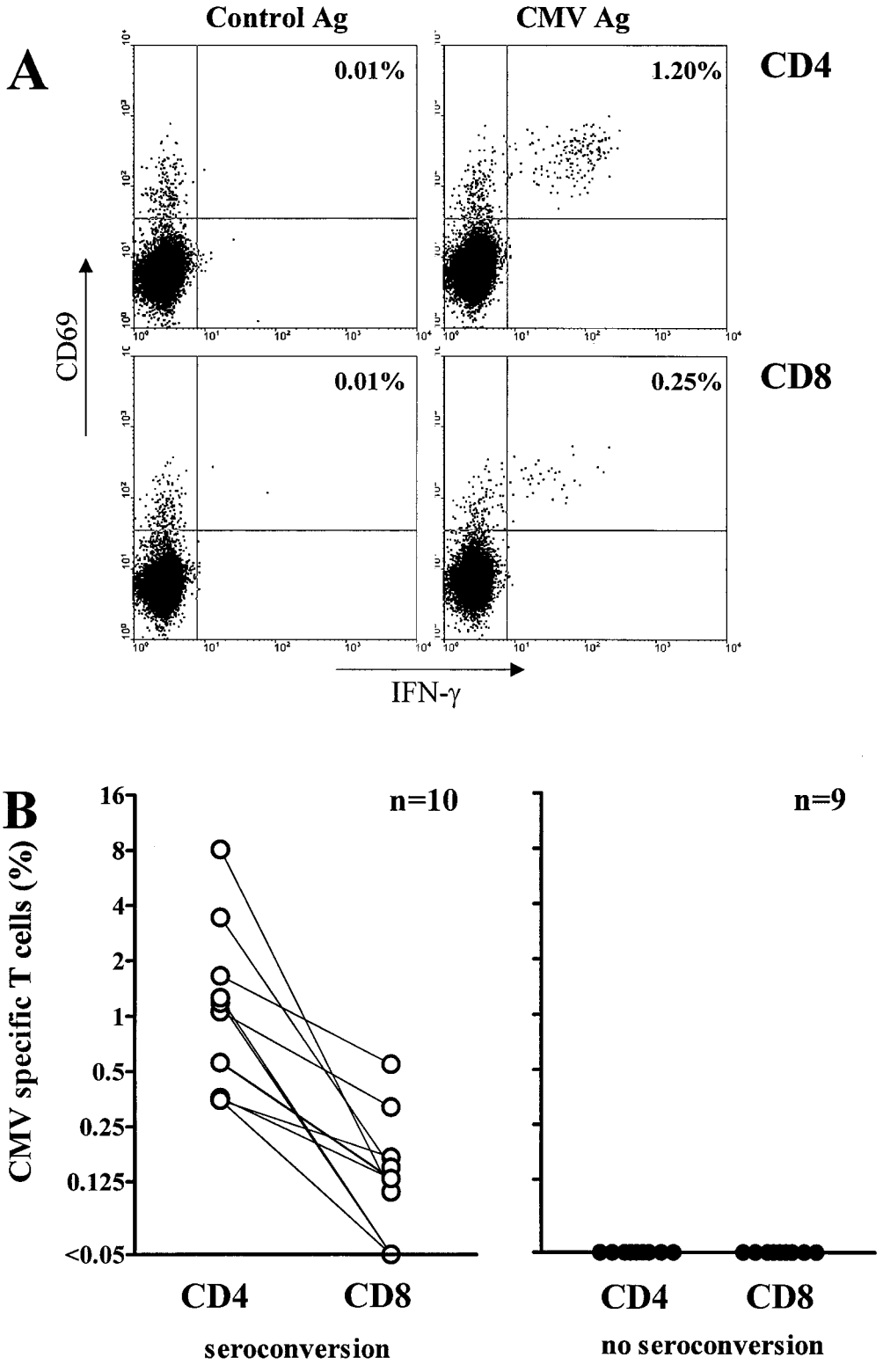

Figure 1. Cytomegalovirus (CMV)–specific CD4 T cells dominate during persistent infection. (A) Example of a long-term seroconverted individual in whom 1.20% of CD4 and 0.25% CD8 T cells were specifically activated (CD69) to produce interferon-γ (IFN-γ) after stimulation with CMV antigen (Ag). Respective frequencies derived from stimulation with control antigen served as a negative control (0.01% of CD4 or CD8 T cells) and was subtracted from values obtained from specific stimulations. (B) CMV-specific T-cell responses were analyzed in 19 CMV-seronegative recipients of a seropositive graft. In all patients that underwent seroconversion ($n = 10$), CMV-specific T cells were detectable (median CD4, 1.12% [range, 0.35 to 8.10%]; median CD8, 0.13% [<0.05 to 0.55%]). Patients without seroconversion ($n = 9$) do not have any detectable CMV-specific T cells. Among seroconverters, four of nine patients received a prophylactic ganciclovir therapy for 3 mo after transplantation; among the patients that remained CMV-negative, eight of nine received prophylactic treatment.

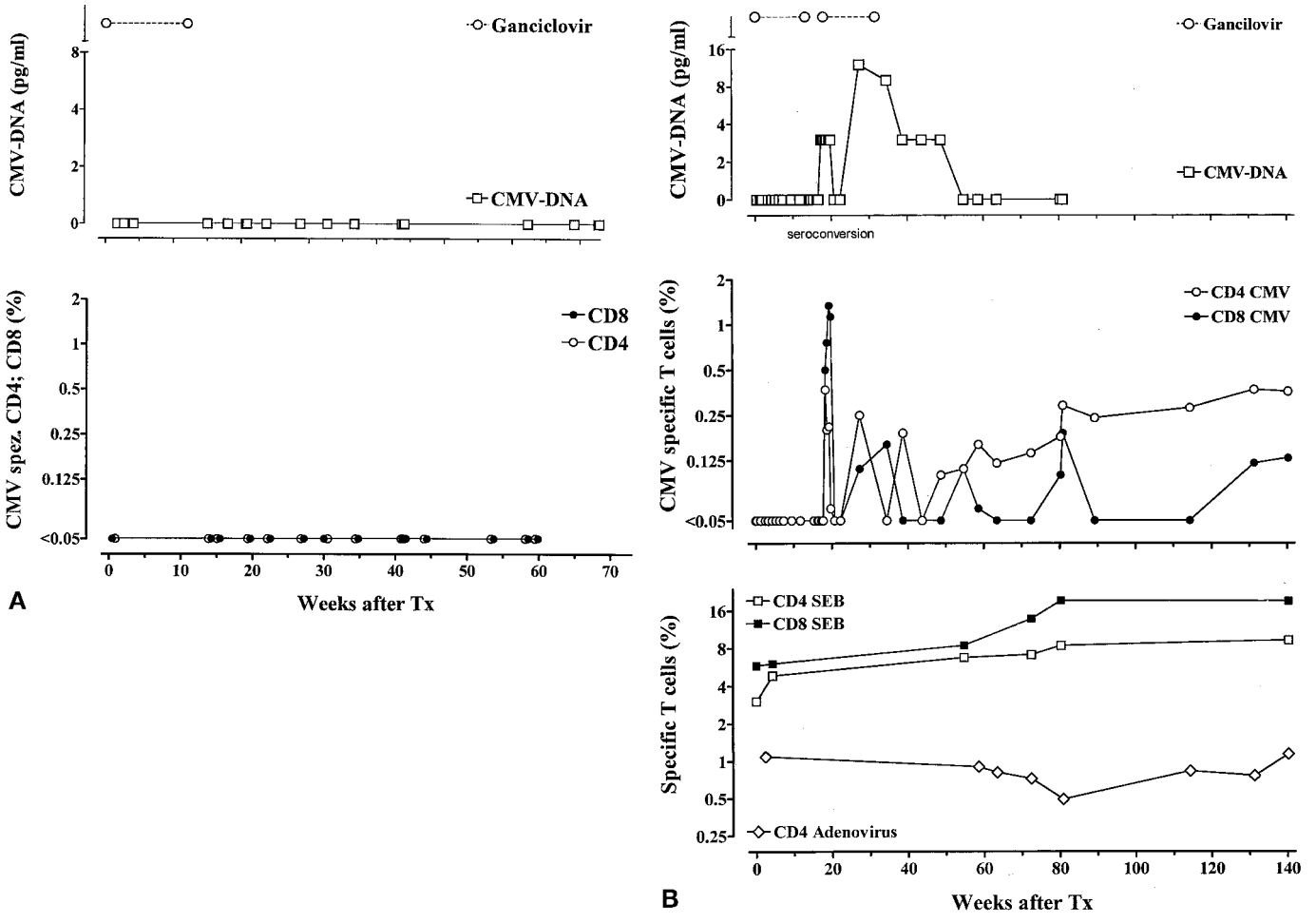

Figure 2. Acquisition of CMV-specific CD4 and CD8 T cells after primary infection. Longitudinal analysis of viral load, CMV-specific IgG and IgM, and specific T cells in an individual who remained seronegative (panel A, patient 4, Table 1) and in a patient who underwent CMV primary infection (B, patient 1, Table 1) after transplantation (Tx). Adenovirus-specific and *Staphylococcus aureus* enterotoxin B (SEB)–reactive T cells are shown as control. A CMV-load higher than 3 pg/ml is considered clinically relevant.

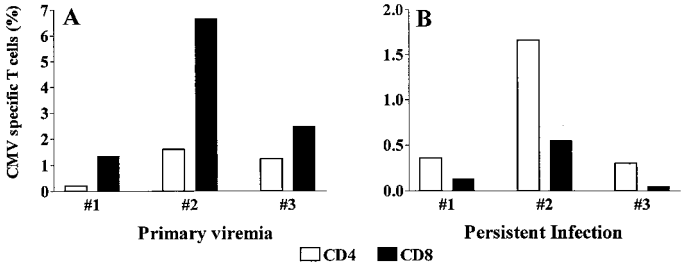

Figure 3. CMV-specific CD8 T cells dominate during acute infection. (A) A marked dominance for CMV-specific CD8 T cells was found during primary viremia (patients 1 to 3, Table 1). (B) The same patients had higher frequencies of CMV-specific CD4 T cells after successful control of viremia (analyzed 127, 108, and 40 wk after primary infection). White and black bars represent CMV-specific CD4 and CD8 T cells, respectively.

Discussion

CMV-seronegative transplant recipients of a seropositive solid organ graft are at particular risk of acquiring primary infection with CMV. Consequently, this clinical situation can serve as a controlled model system to study the time course of CMV replication, seroconversion, and induction of specific immunity upon primary infection in humans. In this study, a simultaneous quantitation of both virus-specific CD4 and CD8 T cell responses was longitudinally performed in the course of an acute and during establishment of a persistent CMV infection. The most striking observation was a dominance of CMV-specific CD8 T cells during acute infection that contrasted with a relative predominance of virus-specific CD4 T cells in persistent infection. CD4 T cell frequencies that emerged after resolution of viral load remained stable throughout the whole observation period of up to 2.5 yr after primary infection and have similar characteristics as respective T cells from long-term CMV-seropositive individuals (data not shown).

The dominance in CMV-specific CD8 T cells is in line with the observations of large temporal expansions of virus-specific CD8 T cells that have been found during acute infections in both animals and humans (11,23–26). Animal models suggest that the contraction of CD8 T cell responses after primary

infection is a programmed event that is modulated by both intrinsic and extrinsic factors (12,27,28). High frequencies of specific CD8 T cells reflect the need to activate and mobilize large numbers of virus-specific T cells with direct cytotoxic activity, which is most important during periods of high viral load. Later on during persistent infection, a stable equilibrium is reached between viral replication and cellular immune responses. Interestingly, it was shown for HIV or EBV infection that this equilibrium, as compared with primary immunity, may further be characterized by the emergence of T cells with altered phenotype and specificity toward particular antigenic epitopes (13–16,29,30). In the steady state of a latent CMV infection, the immune response is dominated by CMV-specific CD4 T cells (Figure 3). In single individuals, these CMV-specific CD4 T cells can reach extremely high frequencies and may play an important role in maintaining the state of antiviral immunity (10). This is supported by adoptive transfer experiments where CMV-specific CD8 T cells were only stable when concomitantly infused with CMV-specific CD4 T cells (31). Moreover, a loss of CMV-specific CD4 T cells in the first months after transplantation of CMV seropositive renal transplant recipients not only correlates with an uncontrolled viral replication but also with an increased incidence of CMV-related disease (4).

CMV-specific T cell frequencies were flow-cytometrically quantified directly from whole blood after stimulation with a complex antigen mixture derived from CMV-infected fibroblasts. This method has some advantages over the use of fluorescence-labeled tetramers or single stimulatory peptides, because it detects functionally active, cytokine-secreting cells and it can be applied to study immune responses in large cohorts of patients without the knowledge of the HLA-type or antigenic epitopes. Moreover, it allows for the direct comparison of antigen-specific CD4 and CD8 T cell frequencies from the same stimulation reaction and with the same readout system (4,18). Although the use of whole blood precludes the retrospective analysis of frozen samples, stimulation of whole blood as compared with isolated peripheral blood mononuclear cells not only leads to the detection of higher frequencies but also allows for a more reliable detection of specific CD8 T cells (unpublished observations). This fact may be due to the opsonizing effect of CMV-specific antibodies present in whole blood in stimulating antigen uptake, because detectable CD8 T cell responses were shown to be diminished upon depletion of immunoglobulins *in vitro* (32). The use of exogenously derived antigens may in general be less powerful to stimulate CD8 compared with CD4 T cells and thus rather lead to an underestimation of specific CD8 T cell frequencies. Interestingly, to overcome this limitation, sets of overlapping peptides spanning immunodominant proteins instead of soluble proteins have recently been applied to increase efficiency in stimulating CD8 T cell responses (32–34). Although the here determined CMV-specific CD8 T cell frequencies might be underestimated to some extent, the data further emphasize the particular dominance of virus-specific CD8 T cells during primary infection.

In the present study, six out of seven patients who were transplanted before the routine use of ganciclovir prophylaxis acquired CMV primary infection. From the time when potent antiviral drugs were available, not only primary CMV infection and symptomatic CMV reactivation but also acute rejection episodes have been significantly reduced (35,36). Nevertheless, prophylactic therapy cannot completely prevent primary infection of the recipient (35,37). Among patients undergoing seroconversion, the median time until viral DNA was detectable appears to be approximately 25 to 30 d (Table 1 and reference 17). Interestingly, this time frame was similar in patients who did or did not receive primary prophylaxis, in that primary viremia was detectable 30 d after the end of 3-mo prophylactic therapy (Table 1) or 25 d after transplantation (17), respectively. Thus, apart from reducing the incidence of primary infection, a further beneficial effect of prophylactic treatment may result from delaying the event of primary infection to a period of less intense immunosuppression and improved immunocompetence. Hence, a more successful priming of cellular and humoral immune responses and a more efficient control of viral replication may ensure. With respect to the duration of prophylaxis, it is tempting to speculate that an additional prolongation of prophylactic treatment may lead to a further decrease in the incidence of CMV infection.

Does antiviral treatment applied at the time of primary viremia affect the induction and maintenance of specific cellular immune responses? This seems to be the case. The resumption of ganciclovir therapy led to a rapid decrease in viral load. By the time CMV-DNA was negative, CMV-specific CD4 T cells had approached frequencies that remained stable throughout the total observation period of up to 2.5 yr. Conversely, a recent study that analyzed patients during and after primary CMV infection in the absence of antiviral treatment described a similar initial rise in specific CD4 T cells after detection of the virus, but it was followed by a rapid decrease. Interestingly, CMV-specific CD4 T cells remained low or undetectable in that study, whereas viral load was still detectable for the study period of 100 d (17). Together with our previous observations in renal transplant recipients with CMV reactivation (4), these data suggest that, apart from directly reducing viral load, ganciclovir treatment during primary viremia may indirectly assist both in the rapid induction and, more importantly, in the maintenance and stabilization of CMV-specific cellular immune responses. Interestingly, treatment of acute HIV or Hepatitis C infection (38–40) or early control of viral load in a murine model of lymphocytic choriomeningitis virus infection (41,42) similarly enables the host immune system to subsequently control viral replication and to prevent the development of chronic infection. The success of such therapeutic interventions may well be monitored in a clinical setting by the use of the flow-cytometric quantitation of specific immune responses. After acute CMV infection, the decrease in viral load and the stabilization of CMV-specific CD4 T cells may serve as a combined parameter to define the time point at which therapeutic antiviral medication can be safely withdrawn or preemptive monitoring of CMV load may be stopped.

Acknowledgments

We thank Candida Guckelmus for excellent technical assistance. The work was supported by grants from the Deutsche Forschungsgemeinschaft.

References

1. Alcami A, Koszinowski UH: Viral mechanisms of immune evasion. *Immunol Today* 21: 447–455, 2000
2. Britt WJ, Alford CA. Cytomegalovirus. In: *Fields Virology*, edited by Fields BN, Knipe DM, Holley PM, Philadelphia, Lippincott-Raven Publishers, 1996, pp 2493–2523
3. Fishman JA, Rubin RH: Infection in organ-transplant recipients. *N Engl J Med* 338: 1741–1751, 1998
4. Sester M, Sester U, Gärtner B, Heine G, Girndt M, Mueller-Lantzsch N, Meyerhans A, Köhler H: Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation* 71: 1287–1294, 2001
5. Koszinowski UH, Reddehase MJ, Jonjic S: The role of CD4 and CD8 T cells in viral infections. *Curr Opin Immunol* 3: 471–475, 1991
6. Ridge JP, Di Rosa F, Matzinger P: A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474–478, 1998
7. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ: T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480–483, 1998
8. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR: Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478–480, 1998
9. Jung MC, Pape GR: Immunology of hepatitis B infection. *Lancet Infect Dis* 2: 43–50, 2002
10. Sester M, Sester U, Gärtner B, Kubuschok B, Girndt M, Meyerhans A, Köhler H: Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus. *J Virol* 76: 3748–3755, 2002
11. Callan MF, Steven N, Krausa P, Wilson JD, Moss PA, Gillespie GM, Bell JI, Rickinson AB, McMichael AJ: Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. *Nat Med* 2: 906–911, 1996
12. Badovinac VP, Porter BB, Harty JT: Programmed contraction of CD8(+) T cells after infection. *Nat Immunol* 3: 619–626, 2002
13. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL: Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 8: 379–385, 2002
14. Bharadwaj M, Burrows SR, Burrows JM, Moss DJ, Catalina M, Khanna R: Longitudinal dynamics of antigen-specific CD8+ cytotoxic T lymphocytes following primary Epstein-Barr virus infection. *Blood* 98: 2588–2589, 2001
15. Catalina MD, Sullivan JL, Bak KR, Luzuriaga K: Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *J Immunol* 167: 4450–4457, 2001
16. Hislop AD, Annels NE, Gudgeon NH, Leese AM, Rickinson AB: Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med* 195: 893–905, 2002
17. Rentenaar RJ, Gamadia LE, van DerHoek N, van Diepen FN, Boom R, Weel JF, Wertheim-van Dillen PM, van Lier RA, ten Berge IJ: Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection. *J Clin Invest* 105: 541–548, 2000
18. Sester M, Sester U, Köhler H, Schneider T, Deml L, Wagner R, Mueller-Lantzsch N, Pees HW, Meyerhans A: Rapid whole blood analysis of virus-specific CD4 and CD8 T cell responses in persistent HIV infection. *AIDS* 14: 2653–2660, 2000
19. Suni MA, Picker LJ, Maino VC: Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods* 212: 89–98, 1998
20. Sester M, Sester U, Alarcon Salvador S, Heine G, Lipfert S, Girndt M, Gärtner B, Köhler H: Age-related decrease in adenovirus specific T-cell responses. *J Infect Dis* 185: 1379–1387, 2002
21. Zimmermann C, Prevost-Blondel A, Blaser C, Pircher H: Kinetics of the response of naive and memory CD8 T cells to antigen: Similarities and differences. *Eur J Immunol* 29: 284–290, 1999
22. Zhu J, Shearer GM, Marincola FM, Norman JE, Rott D, Zou JP, Epstein SE: Discordant cellular and humoral immune responses to cytomegalovirus infection in healthy blood donors: Existence of a T(h)1-type dominant response. *Int Immunol* 13: 785–790, 2001
23. Butz EA, Bevan MJ: Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 8: 167–175, 1998
24. Callan MF, Tan L, Annels N, Ogg GS, Wilson JD, O'Callaghan CA, Steven N, McMichael AJ, Rickinson AB: Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med* 187: 1395–1402, 1998
25. Hoshino Y, Morishima T, Kimura H, Nishikawa K, Tsurumi T, Kuzushima K: Antigen-driven expansion and contraction of CD8+-activated T cells in primary EBV infection. *J Immunol* 163: 5735–5740, 1999
26. Varga SM, Welsh RM: Detection of a high frequency of virus-specific CD4+ T cells during acute infection with lymphocytic choriomeningitis virus. *J Immunol* 161: 3215–3218, 1998
27. Kaech SM, Ahmed R: Memory CD8+ T cell differentiation: Initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol* 2: 415–422, 2001
28. van Stipdonk MJ, Lemmens EE, Schoenberger SP: Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol* 2: 423–429, 2001
29. Goulder PJ, Altfeld MA, Rosenberg ES, Nguyen T, Tang Y, Eldridge RL, Addo MM, He S, Mukherjee JS, Phillips MN, Bunce M, Kalams SA, Sekaly RP, Walker BD, Brander C: Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. *J Exp Med* 193: 181–194, 2001
30. Appay V, Papagno L, Spina CA, Hansasuta P, King A, Jones L, Ogg GS, Little S, McMichael AJ, Richman DD, Rowland-Jones SL: Dynamics of T cell responses in HIV infection. *J Immunol* 168: 3660–3666, 2002
31. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR: 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, 1995
32. Maecker HT, Ghanekar SA, Suni MA, He XS, Picker LJ, Maino VC: Factors affecting the efficiency of CD8+ T cell cross-priming with exogenous antigens. *J Immunol* 166: 7268–7275, 2001

33. Kern F, Faulhaber N, Frommel C, Khatamzas E, Prosch S, Schonemann C, Kretzschmar I, Volkmer-Engert R, Volk HD, Reinke P: Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *Eur J Immunol* 30: 1676–1682, 2000
34. Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, Persaud N, Trigona W, Fu TM, Sinclair E, Bredt BM, McCune JM, Maino VC, Kern F, Picker LJ: Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J Immunol Methods* 255: 27–40, 2001
35. Lowance D, Neumayer HH, Legendre CM, Squifflet JP, Kovarik J, Brennan PJ, Norman D, Mendez R, Keating MR, Coggon GL, Crisp A, Lee IC: Valacyclovir for the prevention of cytomegalovirus disease after renal transplantation. International Valacyclovir Cytomegalovirus Prophylaxis Transplantation Study Group. *N Engl J Med* 340: 1462–1470, 1999
36. Becker BN, Becker YT, Leverson GE, Simmons WD, Sollinger HW, Pirsch JD: Reassessing the impact of cytomegalovirus infection in kidney and kidney-pancreas transplantation. *Am J Kidney Dis* 39: 1088–1095, 2002
37. Razonable RR, Rivero A, Rodriguez A, Wilson J, Daniels J, Jenkins G, Larson T, Hellinger WC, Spivey JR, Paya CV: Allograft rejection predicts the occurrence of late-onset cytomegalovirus (CMV) disease among CMV-mismatched solid organ transplant patients receiving prophylaxis with oral ganciclovir. *J Infect Dis* 184: 1461–1464, 2001
38. Jaeckel E, Cornberg M, Wedemeyer H, Santantonio T, Mayer J, Zankel M, Pastore G, Dietrich M, Trautwein C, Manns MP: Treatment of acute hepatitis C with interferon alfa-2b. *N Engl J Med* 345: 1452–1457, 2001
39. Rosenberg ES, Altfeld M, Poon SH, Phillips MN, Wilkes BM, Eldridge RL, Robbins GK, D'Aquila RT, Goulder PJ, Walker BD: Immune control of HIV-1 after early treatment of acute infection. *Nature* 407: 523–526, 2000
40. Oxenius A, Price DA, Easterbrook PJ, O'Callaghan CA, Kelleher AD, Whelan JA, Sontag G, Sewell AK, Phillips RE: Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8+ and CD4+ T lymphocytes. *Proc Natl Acad Sci USA* 97: 3382–3387, 2000
41. Oxenius A, Zinkernagel RM, Hengartner H: Comparison of activation versus induction of unresponsiveness of virus- specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. *Immunity* 9: 449–457, 1998
42. Moskophidis D, Lechner F, Pircher H, and Zinkernagel RM: Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362: 758–761, 1993