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**DOI**

[10.1172/JCI8229](https://doi.org/10.1172/JCI8229)

**Publication date**

2000

**Document Version**

Final published version

**Published in**

The journal of clinical investigation

[Link to publication](#)

**Citation for published version (APA):**

Rentenaar, R. J., Gamadia, L. E., van der Hoek, N., van Diepen, F. N. J., Boom, R., Weel, J. F. L., Wertheim-van Dillen, P. M. E., van Lier, R. A. W., & ten Berge, R. J. M. (2000). Development of virus-specific CD4<sup>+</sup> T cells during primary cytomegalovirus infection. *The journal of clinical investigation*, 105(4), 541-548. <https://doi.org/10.1172/JCI8229>

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# Development of virus-specific CD4<sup>+</sup> T cells during primary cytomegalovirus infection

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Received for publication August 23, 1999, and accepted in revised form January 6, 2000.

Although virus-specific CD4<sup>+</sup> T cells have been characterized extensively in latently infected individuals, it is unclear how these protective T-cell responses develop during primary virus infection in humans. Here, we analyzed the kinetics and characteristics of cytomegalovirus-specific (CMV-specific) CD4<sup>+</sup> T cells in the course of primary CMV infection in kidney transplant recipients. Our data reveal that, as the first sign of specific immunity, circulating CMV-specific CD4<sup>+</sup> T cells become detectable with a median of 7 days after first appearance of CMV-DNA in peripheral blood. These cells produce the T helper 1 type (Th1) cytokines IFN $\gamma$  and TNF $\alpha$ , but not the T helper 2 type (Th2) cytokine IL4. In primary CMV infection, the vast majority of these circulating virus-specific T cells have features of recently activated naive T cells in that they coexpress CD45RA and CD45RO and appear to be in the cell cycle. In contrast, in people who have recovered from CMV infection earlier in life, virus-specific T cells do not cycle and express surface markers characteristic of memory T cells. After the initial rise, circulating virus-specific CD4<sup>+</sup> T cells decline rapidly. During this phase, a strong rise in IgM and IgG anti-CMV antibody titers occurs, concomitant with the reduction of CMV-DNA in the circulation.

*J. Clin. Invest.* 105:541–548 (2000).

## Introduction

In immune competent individuals, primary infection with herpes viruses results in lifelong viral persistence without serious clinical manifestations. A cease-fire is being negotiated in which, on one hand, viral gene products that interfere with immune recognition mask latently or persistently infected cells from the immune system, and, on the other hand, immune effector mechanisms repress abundant virus replication (1). The crucial role of the immune system in maintaining the symbiotic relation with herpes viruses is emphasized by the fact that in congenital or acquired immune deficiency states, herpes virus reactivation may give rise to serious complications.

Virus-specific CD4<sup>+</sup> T cells are of prime importance in initiating and maintaining immunity against most viruses, including herpes viruses. Recent findings have shown that antigen-specific CD4<sup>+</sup> T cells activate antigen-presenting cells after interaction of CD154 on the CD4<sup>+</sup> T cell with CD40 on the antigen-presenting cell. CD8<sup>+</sup> T cells specific for class I peptides from the same antigen, cross-presented on this activated antigen-presenting cell, are then primed to become

cytolytic effector cells (2). Importantly, not only in the acute phase but also in the latent stage, CD4<sup>+</sup> T cells are critical for maintaining functionality and diversity of cytotoxic T-lymphocyte responses (3). Apart from inducing class I-restricted viral-peptide-specific cytolytic T cells, CD4<sup>+</sup> T lymphocytes are indispensable in guiding naive B cells to become plasma cells that secrete high-affinity virus-specific antibodies, a process in which again the interaction between CD154 on T cells and CD40 on B cells appears to be the major regulatory event (4–8). In line with these diverse effects of CD4<sup>+</sup> T cells on effector pathways of the specific immune system, depletion and/or dysfunction of CD4<sup>+</sup> T cells can result in reactivation of persistent viruses such as cytomegalovirus (CMV) and Epstein Barr virus (EBV) (9–12).

Acute and latent virus-specific CD4<sup>+</sup> T-cell responses have been well studied in experimental animals in which infectious dose, mode, and timing can be standardized. Because of advances in analyzing virus-specific CD4<sup>+</sup> T cells, the frequency and functional properties of these cells during latency and reactivation of persistent infections, e.g., CMV, have been assessed recently in humans

(13, 14). In contrast, development of specific cellular immune responses from a naive state in humans has remained unresolved so far. Investigation of this subject is hampered by the fact that timing infection is generally impossible. We circumvented this problem by studying CMV-seronegative renal transplant recipients of seropositive organ donors. In these patients, the exact time point of infection is known because the donated kidney contains the virus and will induce primary CMV infection in about 60% of the recipients (15, 16). We investigated patients on basic immunosuppressive therapy, consisting of prednisolone and cyclosporin. These patients generally develop a protective immune response against CMV, characterized by a CD8<sup>+</sup> T-cell lymphocytosis in peripheral blood, an inversion of CD4/CD8 ratio, generation of CMV-specific antibodies, and, generally, clearance of the virus from the blood (17). We collected longitudinal blood samples and determined the temporal relationship between appearance of CMV-DNA, CMV-specific CD4<sup>+</sup> T cells, and anti-CMV antibodies. Next, the functional distinctions between virus-specific T cells during acute and latent viral infection were defined. Our data reveal, we believe for the first time, the kinetics and properties of a primary, protective CD4<sup>+</sup> T-cell immune response to herpes viruses in humans. Insight into this process is essential in understanding disturbed immunity to herpes viruses in different clinical conditions.

## Methods

**Subjects.** We selected healthy CMV-seronegative and -positive laboratory personnel and CMV-seronegative and -positive renal transplant recipients on basic immunosuppressive therapy followed at the Academic Medical Center (group I-IV, Table 1). For the longitudinal study, renal transplant recipients who were CMV-seronegative before transplantation and who received an organ from a CMV-seropositive donor were eligible. Patients younger than 18 years of age, patients refusing written informed consent, or patients with an indication for triple immunosuppressive maintenance therapy were excluded. From April 1998 to October 1999, 11 seronegative patients were transplanted with a CMV-seropositive transplant. No prophylactic ganciclovir was given; ganciclovir treatment of primary CMV infection was initiated only when fever over 38°C was present and/or CMV end-organ disease occurred. Three patients did not consent; 1 patient was excluded because of an acute rejection episode treated with high doses of corticosteroids, followed by a triple immunosuppressive regimen. Seven patients were enrolled into this study (group V and VI, Table 1). Peripheral blood was collected weekly. In groups III-VI, immunosuppression consisted of 10 mg prednisolone per day and cyclosporin (Neoral, Novartis Pharma A.G., Basel, Switzerland) aimed at blood trough levels of about 150 ng/mL. On sampling days, venous puncture was performed before immunosuppressive medication was taken. The study was approved by the local medical ethical committee.

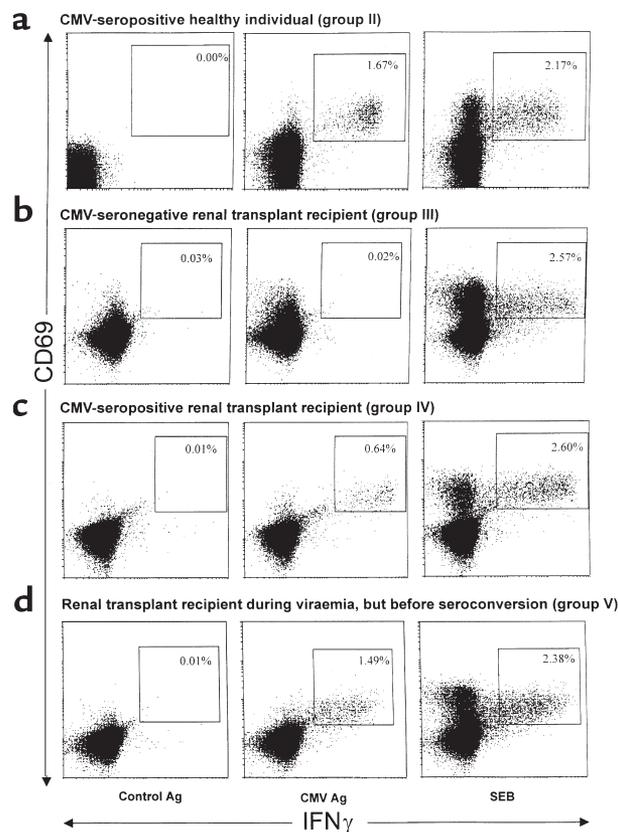
**Determination of CMV-specific CD4<sup>+</sup> T cells.** CMV-specific CD4<sup>+</sup> T-cell frequency was determined according to the method described by Waldrop et al. (13). Briefly, 10<sup>6</sup> freshly isolated PBMC were incubated for 6 hours in the presence of either CMV antigen (60 µL/mL; BioWhittaker Inc., Walkersville, Maryland, USA), control antigen (60 µL/mL negative control; BioWhittaker) or *Staphylococcus aureus* enterotoxin B (SEB; 2 µg/mL positive control; ICN/Fluka Chemie A.G., Buchs, Switzerland). CD28 mAb (clone 15E8; Central Laboratory of the Red Cross Transfusion Service; 3 µg/mL final concentration) in a final volume of 2 mL RPMI-1640 per tube (GIBCO-BRL, Life Technologies, Paisley, Scotland), containing 10% heat-inactivated FCS (Integro, Zaandam, The Netherlands), penicillin, and streptomycin. For the final 5 hours of culture, brefeldin A (Sigma, St. Louis, Missouri, USA) was added to the culture in a final concentration of 10 µg/mL. Cells were transferred to FACS tubes, surface stained with CD4-allophycocyanine (CD4-APC, Becton Dickinson, San Jose, California, USA), fixed in 2 mL per tube FACS lysing solution (Becton Dickinson), permeabilized in 0.5 mL FACS permeabilizing solution per tube, followed by intracellular staining with CD69-FITC and anti-IFNγ-PE or CD69-phycoerythrin (CD69-PE and anti IFNγ-FITC, all from Becton Dickinson). Cells were refixed in Cellfix (Becton Dickinson) until they were analyzed the following day using flow cytometry. Flow cytometric analysis was performed using a FACS Calibur equipped with a 488-nm argon ion laser and a 635-nm red diode laser. Data files containing 50,000 events positive for CD4-APC fluorescence within a lymphocyte gate were saved. Frequencies of CD69<sup>+</sup>IFNγ<sup>+</sup> cells within the CD4<sup>+</sup> lymphocytes were determined using Cellquest software (Becton Dickinson) and designated CMV-specific CD4<sup>+</sup> T-cell frequency. When peak frequencies of CMV-specific CD4<sup>+</sup> T cells were to be expected, additional antibodies were added to the staining. For surface staining, CD4-APC, CD4-PerCP,

**Table 1**  
Patient characteristics and CMV-specific CD4<sup>+</sup> T-cell frequency

Group	n =	Characteristics
<b>Cross-sectional study</b>		
I	5	CMV-seronegative healthy laboratory personnel
II	6	CMV-seropositive healthy laboratory personnel
III	6	CMV-seronegative renal transplant recipients
IV	12	Renal transplant recipients being CMV-seropositive before transplantation. Tests were performed after transplantation, while on basic immunosuppressive medication.
<b>Longitudinal study</b>		
V	5	CMV-seronegative renal transplant recipients of organs from CMV-seropositive donors. Diagnosis of primary CMV infection during follow-up of 16 weeks after transplantation.
VI	2	CMV-seronegative renal transplant recipients of organs from CMV-seropositive donors. No diagnosis of primary CMV infection during follow-up of 16 weeks after transplantation.

CD8-FITC, CD8-PE, CD27-PE, CD62L-PE, CD49d-PE (anti-VLA-4), CD38-PE, CD154(CD40L)-PE, CD45RA-FITC, CD45RO-PE (all from Becton Dickinson), and CD11a-FITC (DAKO Corp., Glostrup, Denmark) were used. For intracellular staining, anti-TNF $\alpha$ -FITC, anti-IL2-FITC, anti-IL4-PE, CD154-PE, anti-IFN $\gamma$ -FITC, IFN $\gamma$ -PE, CD69-FITC, CD69-PE (all Becton Dickinson), CD69-APC (Caltag Laboratories Inc., Burlingame, California, USA), anti-Ki67 polyclonal rabbit F(ab')<sub>2</sub>-FITC, control polyclonal rabbit F(ab')<sub>2</sub>-FITC (both DAKO Corp.), were used.

**CMV-PCR.** Quantitative PCR was performed in EDTA whole-blood samples as described for plasma or serum (18). Briefly, DNA was purified from 50  $\mu$ L of whole blood and eluted in 50  $\mu$ L Tris-EDTA (TE) buffer as described previously (19). Five microliters of reference internal control (IC) DNA (35 molecules) was present during DNA extraction from the clinical specimen. The primer pair used for amplification consisted of CMV-531 (5'-ACA AGG TGC TCA CGC ACA TTG ATC-3'; nucleotide positions [nt] 2034–2057) and Bio-CMV-1107 (5'-CAC TGG CTC AGA CTT GAC AGA CAC-3', 5' biotinylated; nt 2588–2611); nucleotide numbering was according to Akrigg et al. (20). This primer pair amplifies a 578-bp DNA fragment from exon 4 of the major immediate-early gene of human CMV or a fragment of identical size and GC content from IC DNA, respectively. One-fifth of the DNA eluate (containing the IC DNA) was subjected to PCR. The final reaction mixture (50  $\mu$ L) contained 28 pmol of each primer (CMV-531 and Bio-CMV-1107), 2.5 U of AmpliTaq DNA polymerase, 0.5 U of Amperase (uracil-*N*-glycosylase; Perkin-Elmer, Foster City, California, USA) 5  $\mu$ g of BSA (Boehringer Mannheim, Mannheim, Germany) 10 mM Tris HCl (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, dATP, dGTP, and dCTP at a concentration of 200  $\mu$ M each, 400  $\mu$ M dUTP (Perkin-Elmer), and 35 additional IC DNA molecules. The PCRs were done in a Perkin-Elmer 9600 thermocycler: 2 minutes at 50°C, 5 minutes at 95°C, followed by 35 cycles, each consisting of 20 seconds at 95°C, 20 seconds at 63°C, and 1 minute at 72°C, followed by 5 minutes at 72°C. Excess primer was removed as described (18), and the purified PCR product was diluted 5 times in 1 $\times$  PCR II buffer (Perkin Elmer). Twenty microliters of Tris (2,2'-bipyridine) ruthenium (II) chelate-labeled (TBR-labeled) probe (1 ng/ $\mu$ L; 1.3 pmol; either CMV- or IC DNA-specific) was added to 30  $\mu$ L of purified PCR product, and hybridization was done in a Perkin-Elmer 9600 thermocycler (2 minutes at 95°C, 5 minutes at 56°C). Next, 10  $\mu$ L of streptavidin-coated magnetic beads (Perkin-Elmer) was added, and the mixture was incubated for 15 minutes at 56°C. Forty microliters of the bead-hybrid suspension was added to 400  $\mu$ L of QPCR assay buffer (Perkin-Elmer), and the electrochemoluminescence (ECL) signal, expressed in luminosity units (LU), was measured by the QPCR System 5000 (Perkin-Elmer). The probes were TBR-CMV-1 (CMV-specific probe; 5'-TGA AGG TCT TTG CCC AGT ACA TTC T-3'; nt



**Figure 1**

Flow cytometric determination of CMV-specific CD4<sup>+</sup> T cells. Dot plots of anti-IFN $\gamma$ -PE fluorescence (X-axis; arbitrary units, log scale) versus CD69-FITC fluorescence (Y-axis; arbitrary units, log scale). Depicted are lymphocytes positive for CD4-APC fluorescence. Left panels: cells incubated with control antigen; middle panels: cells incubated with CMV antigen; and right panels: cells incubated with SEB. Cells from (a) a CMV-seropositive healthy individual (group II) (b) a CMV-seronegative renal transplant recipient (group III) (c) a CMV-seropositive renal transplant recipient (group IV) (d) a CMV-seronegative renal transplant recipient of CMV-seropositive organ (group V, patient 2), after CMV-DNA was detected, but before seroconversion.

2292–2316; 5' labeled with TBR) and TBR-CMV-2 (IC-specific probe; 5'-CCC TTT ACA TCT TTC TGA AGT AGG G-3'; 5' labeled with TBR). The algorithm for quantification was performed: number of copies CMV-DNA/mL = R  $\times$  4200, where R is the ratio of CMV-specific ECL signal/IC DNA-specific ECL signal, after correction for background. The algorithm assumes ideal circumstances for each of the steps of the procedure and has been described for the quantitation of serum and plasma CMV loads (18).

**Viral culture.** Viral culture was done by cocultivation of blood buffy coat and human diploid fibroblasts. Microscopic examination for the appearance of CMV-specific cytopathologic effects was performed.

**Anti-CMV antibodies.** Anti-CMV IgM was determined in serum using the AxSYM system microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, Illinois, USA) according to the manufacturer's

**Table 2**

Appearance of CMV-DNA, CMV-specific CD4<sup>+</sup> T cells, and CMV-specific antibodies in CMV-seronegative renal transplant recipients of kidneys from CMV-seropositive donors (group V)

Patient number	1	2	3	4	5	Median (range)
Time intervals (days)						
Tx → CMV-DNA <sup>A</sup>	25	29	26	18	22	25(18–29)
CMV-DNA → CMV-specific CD4 <sup>+</sup> T cells <sup>B</sup>	11	7	7	14	7	7(7–14)
CMV-specific CD4 <sup>+</sup> T cells → anti-CMV-IgM <sup>C</sup>	-3	3	10	7	7	7(-3–10)
Peak frequency of CMV-specific CD4 <sup>+</sup> T cells <sup>D</sup>	1.03	1.49	2.50	0.82	0.46	1.03(0.46–2.50)

Time intervals between: <sup>A</sup>renal transplantation and first detection of CMV-DNA in peripheral blood, <sup>B</sup>first detection of CMV-DNA and first detection of CMV-specific CD4<sup>+</sup> T cells in peripheral blood, <sup>C</sup>first detection of CMV-specific CD4<sup>+</sup> T cells and first detection of CMV-specific IgM in peripheral blood (days). <sup>D</sup>Percent of CD4<sup>+</sup> T cells

instructions. Positive samples were confirmed in the VIDAS enzyme-linked fluorescent assay (Bio Mérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. Anti-CMV IgG was determined in serum using the AxSYM microparticle enzyme immunoassay according to the manufacturer's instructions. Measurements were calibrated relative to a standard serum. Results are expressed as a ratio of the measurement to a standard serum (IgM) or arbitrary antibody units/mL serum (IgG).

## Results

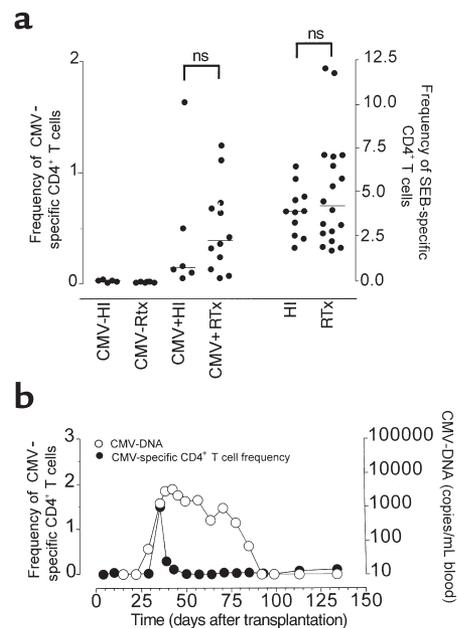
**Detection of CMV-specific CD4<sup>+</sup> T cells in renal transplant recipients.** We first validated the method of analyzing CMV-specific CD4<sup>+</sup> T-cell frequency in immunosuppressed patients receiving standard immunosuppressive therapy. In agreement with other published data (13), CMV-specific CD4<sup>+</sup> T-cell frequencies were detectable in healthy CMV-seropositive individuals (group II) with a median of 0.15% of CD4<sup>+</sup> T cells (range 0.05–1.63%, *n* = 6), whereas no CMV-specific CD4<sup>+</sup> T cells were found in CMV-seronegative individuals (group I). Also, in PBMC of seropositive renal

transplant recipients (group IV), a median CMV-specific CD4<sup>+</sup> T-cell frequency of 0.39% (range 0.05–1.24%, *n* = 11) was found, whereas no CMV-specific CD4<sup>+</sup> T cells were detectable in CMV-seronegative renal transplant recipients (group III; Figures 1, a–c, and 2a). These data demonstrate that under conditions of standard immunosuppression CMV-specific CD4<sup>+</sup> T-cell frequencies can be measured in renal transplant recipients. Moreover, when CD69 upregulation and IFN $\gamma$  production to SEB stimulation were determined by flow cytometric analysis, frequencies of SEB-specific CD4<sup>+</sup> T cells from renal transplant recipients were similar to those of SEB-specific CD4<sup>+</sup> T cells from healthy individuals (Figure 2a).

**Longitudinal analysis of seronegative renal transplant recipients of organs from seropositive donors.** In 5 of the 7 renal transplant recipients enrolled in the follow-up study of seronegative recipients of a kidney from seropositive organ donors, a primary CMV infection was diagnosed by positive CMV culture or seroconversion. During the first 18 days after transplantation, neither CMV-DNA nor CMV-specific CD4<sup>+</sup> T cells were detectable in any of the patients from group V or VI. In

**Figure 2**

(a) CMV- and SEB-specific CD4<sup>+</sup> T-cell frequencies. Each dot represents 1 individual. Left 4 scattergrams (left Y-axis): CMV-specific CD4<sup>+</sup> T-cell frequencies (percent of CD4<sup>+</sup> T cells) in healthy individuals and renal transplant recipients. CMV-HI, CMV-seronegative healthy individuals (group I); CMV-RTx, CMV-seronegative renal transplant recipients on basic immunosuppressive therapy (group III); CMV+HI, CMV-seropositive healthy individuals (group II); CMV+RTx, CMV-seropositive renal transplant recipients on basic immunosuppressive therapy (group IV). Right 2 scattergrams (right Y-axis): SEB-specific CD4<sup>+</sup> T-cell frequencies (in percent of CD4<sup>+</sup> T cells); HI, healthy individuals; RTx, renal transplant recipients on basic immunosuppressive therapy; ns, not significant (*P* > 0.05, Mann-Whitney). (b) Development of CMV-specific CD4<sup>+</sup> T-cell frequencies in peripheral blood in relation to the number of CMV-DNA copies per milliliter blood. One representative seronegative renal transplant recipient of a kidney from a seropositive organ donor (patient 2, group V) is shown. Time (days after transplantation) versus frequency of CMV-specific CD4<sup>+</sup> T cells (percent of CD4<sup>+</sup> cells) and CMV-DNA copies (copies per milliliter blood). Open circles, CMV-DNA copies; filled circles, CMV-specific CD4<sup>+</sup> T cells.



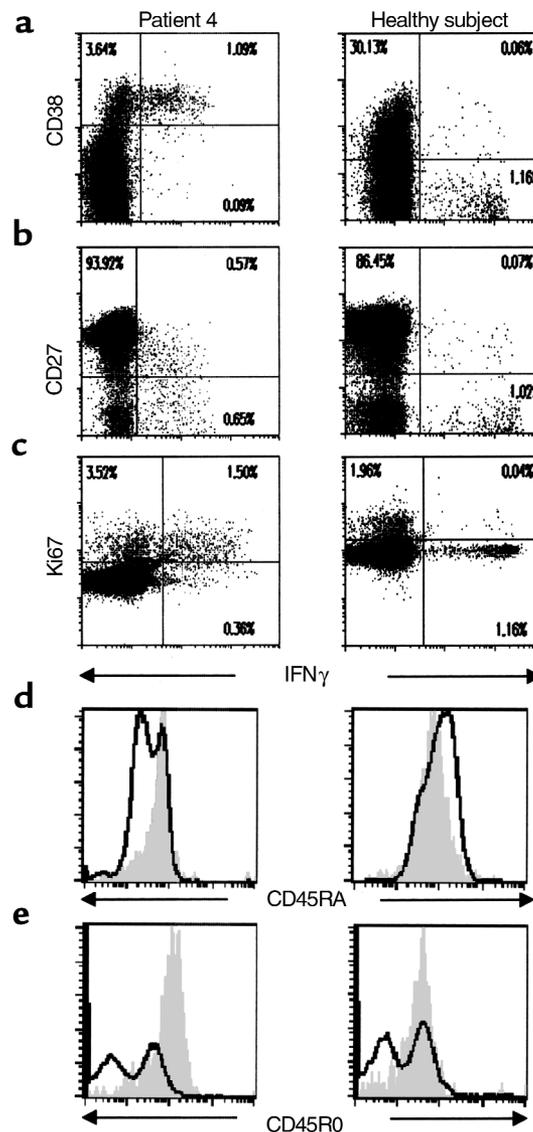
5 out of 7 patients, viral DNA was detected at a median of 25 days (range 18–29 days) after transplantation. These patients were assigned to group V. One of these 5 patients was treated with ganciclovir because of persistent fever with positive CMV buffy coat culture and a pulmonary infiltrate (patient 1). In all 5 patients from group V, CMV-specific CD4<sup>+</sup> T cells were detected by flow cytometry at a median of 7 days after first detection of CMV-DNA (range 4–14 days; for a representative experiment see Figures 1d and 2b). Peak frequencies ranged from 0.46 to 2.50% of peripheral blood CD4<sup>+</sup> T cells (Table 2). In all patients, CMV-specific CD4<sup>+</sup> T-cell frequencies precipitously increased after detection of the virus, followed by a rapid decrease (Figure 2b). In the 2 renal transplant recipients without evidence of CMV infection (remaining negative in CMV-PCR, CMV culture of urine and blood, and anti-CMV antibodies; group VI) no CMV-specific CD4<sup>+</sup> T cells were detected during follow-up for up to 100 days after transplantation (not shown).

**Functional and phenotypic properties of CMV-specific CD4<sup>+</sup> T cells shortly after infection.** When peak frequencies were expected in patients from group V, CMV-specific T cells were further analyzed. The CMV-specific CD4<sup>+</sup> T cells were CD38<sup>+</sup> during primary infection, contrasting profoundly with their phenotype during viral latency, when CMV-specific CD4<sup>+</sup> T cells were exclusively CD38<sup>-</sup> (Figure 3a). CMV-specific T cells in healthy CMV-seropositive individuals were predominantly CD27<sup>-</sup>, whereas shortly after primary infection, a substantial proportion of the CMV-specific T cells had not yet lost CD27 expression (Figure 3b). Furthermore, CMV-specific CD4<sup>+</sup> T cells were CD49d(VLA-4)<sup>+</sup>, CD62L<sup>-</sup>, and CD11a<sup>high</sup>, irrespective of whether they were obtained from an acutely or latently infected person (not shown). Next to this phenotype, which strikingly resembles the phenotype of recently activated T cells, CMV-specific CD4<sup>+</sup> T cells were in cell cycle during acute primary infection, as evidenced by Ki67 expression. In healthy seropositive individuals, these cells were typically non-proliferating in peripheral blood (Figure 3c).

During viral latency, CMV-specific CD4<sup>+</sup> T cells displayed a typical memory phenotype, i.e., CD45RA<sup>-</sup> and CD45RO<sup>+</sup>. In contrast, during acute CMV infection, the CMV-specific CD4<sup>+</sup> T cells expressed both the naive T-cell marker CD45RA and the memory marker CD45RO (Figure 3, d and e). Interestingly, the expression of CD45RO on these CMV-specific T cells was higher when compared with the bulk memory CD4<sup>+</sup> population (Figure 3e, left panel).

Around the time of peak frequency, the CMV-specific CD4<sup>+</sup> T cells displayed typical Th1 cytokine production profiles, IFN $\gamma$ -producing cells showing the highest frequency. Figure 4 shows that all TNF $\alpha$ -producing cells also produced IFN $\gamma$ . Few cells produced IL2, whereas virtually no IL4-producing cells were detected. In contrast, considerable frequencies of IL2-producing cells and, to a lesser extent IL4 producing cells, could be detected using SEB-stimulated cultures (not shown).

Finally, after incubation with CMV antigen in the presence of brefeldin A, very few peripheral blood CD4<sup>+</sup> T cells displayed cell surface expression of CD154. However, if CD154 staining was performed after fixation and permeabilization, the molecule was found to be

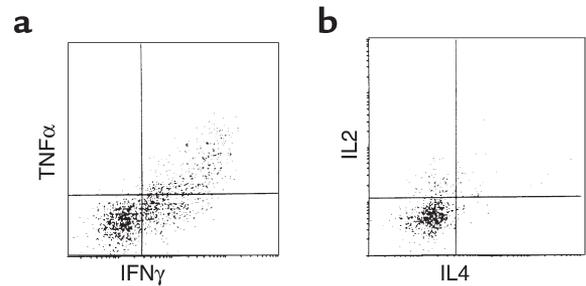


**Figure 3**

Phenotypic characteristics of CMV-specific CD4<sup>+</sup> T cells. Left column: a representative patient (patient 4) analyzed at the peak of CMV-specific CD4<sup>+</sup> T cells. Right column: a CMV-seropositive healthy individual (group II). (a–c) During the acute phase, CMV-specific CD4<sup>+</sup> T cells display a recently activated, proliferating phenotype. Dot plots of anti-IFN $\gamma$  fluorescence (X-axis; arbitrary units) versus CD38 (a), CD27 (b), Ki67-fluorescence (c), respectively (Y-axis; arbitrary units) within a CD4<sup>+</sup>CD69<sup>+</sup> lymphocyte gate. (d and e) CMV-specific CD4<sup>+</sup> T cells display the CD45RA<sup>+</sup> CD45RO<sup>high</sup> phenotype. Histograms of CD45RA fluorescence (d) or CD45RO fluorescence (e). Filled histograms represent CMV-specific CD4<sup>+</sup> T cells only, i.e., lymphocytes gated on positive CD4, intracellular IFN $\gamma$ , and CD69 expression. Open histogram overlays represent all CD4<sup>+</sup> T cells. Histogram overlays were generated using the “each histogram” option for clarity. Therefore, cell number of filled histograms and open histograms do not have the same Y-axis values.

**Figure 4**

CMV-specific CD4<sup>+</sup> T cells display a Th1 cytokine secretion pattern (arbitrary units for all axes). Dot plots of lymphocytes from patient 4, gated on positive CD4-PerCP and CD69-APC fluorescence. (a) Anti-IFN $\gamma$ -PE fluorescence (X-axis) versus anti TNF $\alpha$ -FITC fluorescence (Y-axis). (b) Anti-IL4-PE fluorescence (X-axis) versus anti-IL2-FITC fluorescence (Y-axis).



selectively expressed intracellularly in the CMV-specific CD4<sup>+</sup> T cells (not shown).

*CMV-specific antibody responses in relation to CMV-specific CD4<sup>+</sup> T cells.* Figure 5 shows the relation of CMV-specific CD4<sup>+</sup> T-cell frequencies with CMV-specific antibody production in 1 representative patient. In all patients (except for patient 1), the appearance of CMV-specific CD4<sup>+</sup> T cells preceded the formation of CMV-specific antibodies.

### Discussion

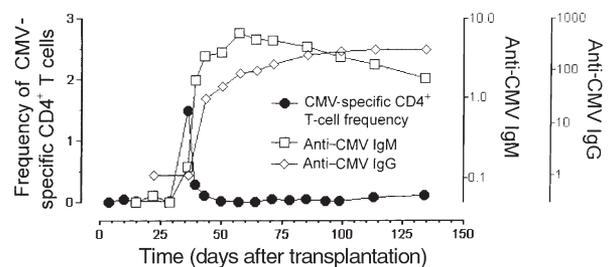
The present study shows that in naive individuals infection of CMV from donated kidneys needs between 2.5 to 4 weeks to arrive at a detectable level of viral replication and spread. Recently, a mean in vivo doubling time of human CMV was calculated to be around 1 day (21). Therefore, in our patient group the virus needed at least 17 doubling times to emerge at a detectable level, assuming that no pathways for viral clearance are available before generation of the specific immune response and that viral replication commences immediately after transplantation. At a median of 7 days after first detection of CMV-DNA, CMV-specific CD4<sup>+</sup> T cells appeared in the circulation. The time between transplantation and appearance of CMV-specific CD4<sup>+</sup> T cells probably reflects a series of events: generation of immunogenic viral particles, uptake of antigen by antigen-presenting cells, migration of these cells to secondary lymphoid organs, presentation of antigenic peptides to the naive CMV-specific helper T cells, activation, initial proliferation and naive-to-memory transition of these T cells. After this process, seeding of the CMV-specific CD4<sup>+</sup> T cells to the peripheral blood apparently ensued.

We chose to study renal transplant recipients for the apparent reason that if one wants to define the first

steps in the generation of an immune response to a live virus in humans, the exact time of infection should be known. To this aim, these patients were routinely screened for CMV serology before transplantation and followed longitudinally after transplantation with an organ from a CMV-seropositive donor. The study of antigen-specific T-cell dynamics in these patients might be complicated by the administration of immunosuppressive therapy. However, despite immunosuppressive drug therapy consisting of cyclosporin and low-dose prednisolone, renal transplant recipients are known to develop an adequate immune response against the virus. First, CMV-specific antibodies are being produced and, generally, primary CMV infection in these patients does not lead to serious clinical manifestations. Without antiviral therapy, CMV appears to be largely neutralized in most patients. Finally, after patients have recovered from primary infection, they seldom develop clinical signs of either persistent or secondary CMV infection (16). We therefore conclude that CMV-seronegative renal transplant recipients, treated with cyclosporin and low-dose prednisolone, develop a protective response to primary CMV infection and can be used to characterize features of the primary immune response to CMV in humans. In agreement, we found similar frequencies of CMV-specific T cells in CMV-seropositive renal transplant recipients as compared with CMV-seropositive healthy individuals. Also, frequencies of IFN $\gamma$  producing CD4<sup>+</sup> T cells after SEB stimulation did not differ between renal transplant recipients and healthy individuals. It cannot be formally excluded, however, that the basic immunosuppressive drug therapy administered to the patients in this study potentially affects T-helper cell differentiation, proliferation, and cytokine production, both in vivo as well as

**Figure 5**

Relation of CMV-specific CD4<sup>+</sup> T cells with CMV-specific antibody production. Time (days after transplantation) versus frequency of CMV-specific CD4<sup>+</sup> T cells (percent of CD4<sup>+</sup> T cells) and antibody concentration (IgM, ratio to a standard serum; IgG, antibody units per milliliter serum). One representative patient (patient 2) is shown with respect to anti-CMV IgM levels and anti-CMV IgG levels. Filled circles, CMV-specific CD4<sup>+</sup> T-cell frequency; open squares, anti-CMV IgM levels; open diamond, anti-CMV IgG levels.



in the assay used here (22), and for this reason the frequencies reported here may still underestimate the magnitude of the CD4 response.

After the initial peak, at least up to 10 weeks after the acute primary infection, CMV-specific CD4<sup>+</sup> T cell frequencies were low. This low magnitude of the antigen-specific CD4 response shown here fits with the empirical fact that the peripheral blood CD4 pool does not emphatically expand, whereas the CD8 pool does. It is in accordance with the notion that CD4<sup>+</sup> T cells exert their functions mainly within lymphoid organs, whereas cytotoxic effector T cells are designed to exert their function throughout the body, especially in generalized viral infections, necessitating their massive transport through the peripheral blood compartment. In HIV infection, memory CD4<sup>+</sup> T cells were shown to be preferentially contained within the peripheral lymphoid organs, whereas they emigrate toward the blood soon after viral replication is blocked (23). The observation that in the primary CMV-infected patients CMV-DNA levels are still readily detectable after mean follow-up periods of about 100 days would be in accordance with the idea that at that time point virus replication still takes place and that therefore the majority of CMV-specific T cells are actively retained within lymph nodes and spleen.

The detailed follow-up of CMV-naive renal transplant recipients allowed, for the first time, to our knowledge, documentation of changes in T-helper cell phenotype in response to a pathogen in vivo. The phenotype of early CMV-specific T cells strikingly confirms predictions, based on both ex vivo and in vitro studies. Human lymph nodes have been reported to contain T cells highly expressing both CD45RA and CD45RO. This phenotype is also found relatively early after in vitro activation of sorted naive (CD45RA<sup>single+</sup>) T cells, suggesting that the CD45RA<sup>high</sup>CD45RO<sup>high</sup> phenotype is one of the earliest stages of antigen-primed, formally naive T cells (24). In addition, in healthy individuals the peripheral blood contains small numbers of these CD45RA<sup>high</sup>CD45RO<sup>high</sup> helper T cells, which are enriched for cycling cells and primarily produce IFN $\gamma$  (25, 26). The first antigen-specific CD4<sup>+</sup> T cells appearing in peripheral blood after primary CMV infection specifically displayed this CD45RA<sup>high</sup>CD45RO<sup>high</sup> phenotype and were actively cycling based on Ki67 expression. Thus, the small numbers of circulating CD45RA<sup>high</sup>CD45RO<sup>high</sup> cells may represent T cells that, after initial activation by neoantigens, emigrate from lymph nodes to other sites of the body. The reason for this early migration is unclear at this moment, but it could be speculated that spreading of antigen-specific helper cells contributes to an efficient, specific immune response that eventually takes place in most secondary lymphoid organs.

We postulated previously that the expression of CD27 on CD4<sup>+</sup> T cells is downregulated after persistent stimulation of T cells in vivo (26, 27). In fact, the CD27<sup>-</sup> phenotype of CMV-specific CD4<sup>+</sup> T cells that we found in CMV carriers is consistent with repetitive in

vivo stimulation and suggests also that during viral latency, continuous or intermittent activation of CMV-specific helper T cells by viral products takes place to control overt virus replication.

Antigen-specific T cells need several rounds of cell division to obtain the capacity to produce IL4, whereas IFN $\gamma$  may be produced in low quantities without previous cell division (28). The lack of IL4-producing cells found during primary infection may therefore be due to the early time point of analysis of cytokine phenotype. However, long after establishment of antiviral memory, we and others found low frequencies of IL4-producing CMV-specific cells, which suggest that CMV elicits a prototype Th1 response (13, 29).

In conclusion, to our knowledge this is the first study that reveals the developing immune response to primary CMV infection in vivo in humans. These data may serve as a reference to which patients with more profound states of immunodeficiency may be compared. The test can be used to monitor primary CMV infection in other groups of patients and may be helpful in decisions regarding antiviral prophylaxis or treatment.

#### Acknowledgments

The authors thank the patients and healthy subjects for their participation, S. Surachno for patient inclusion, A.J. Kolk for initial reagent supply, and Yvette Gerrits for accurate performance of the CMV-PCR. We thank D. Hamann, F. Bemelman, and P. Schellekens for critically reading the manuscript. Grants from the Dutch Kidney Foundation support R.J. Rentenaar and F.N.J. van Diepen (C95-1455) and L.E. Gamadia (C98-1724).

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