Immune responsiveness in immunosuppressed patients

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CHAPTER 2: QUANTIFICATION AND CHARACTERIZATION OF VIRUS-SPECIFIC CD4<sup>POS</sup> T CELLS DURING PRIMARY CYTOMEGALOVIRUS INFECTION

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Chapter 2

Abstract

Although virus-specific CD4^{pos} 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 man. Here, we analyzed the kinetics and characteristics of cytomegalovirus (CMV) specific CD4^{pos} 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^{pos} T cells become detectable with a median of 7 days after first appearance of CMV-DNA in peripheral blood. These primary CMV specific T cells produce the T helper 1 type (Th1) cytokines IFNγ and TNFα, 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, as they co-express CD45RA and CD45R0 and appear to be in cell cycle. In contrast, in persons who have recovered from CMV infection earlier in life, virus-specific T cells are non-cycling and are memory, i.e. CD45RA^{neg}CD45R0^{pos} T cells. After the initial rise, circulating virus-specific CD4^{pos} T cells rapidly decline. During this phase, a strong rise in IgM and IgG anti-CMV antibody titers as well as a concomitant reduction of CMV-DNA occurs.
Quantification and characterization of virus-specific CD4\textsuperscript{pos} T cells during primary cytomegalovirus infection

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, at one hand, viral gene products that interfere with immune recognition mask latently or persistently infected cells from the immune system, and, at 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\textsuperscript{pos} 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\textsuperscript{pos} T cells activate antigen presenting cells after interaction of CD154 on the CD4\textsuperscript{pos} T cell with CD40 on the antigen presenting cell. CD8\textsuperscript{pos} 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\textsuperscript{pos} 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\textsuperscript{pos} 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\textsuperscript{pos} T cells on effector pathways of the specific immune system, depletion and/or dysfunction of CD4\textsuperscript{pos} T cells can result in reactivation of persistent viruses such as CMV and Epstein Barr virus (EBV)\(^{(9-12)}\).

Acute and latent virus-specific CD4\textsuperscript{pos} T cell responses have been well studied in experimental animals in which infectious dose, mode and timing can be standardized. Due to advances in visualizing virus-specific CD4\textsuperscript{pos} T cells, the frequency and functional properties of these cells during latency and reactivation of persistent infections, e.g. CMV, has recently been assessed in man\(^{(13,14)}\). 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 of 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 since 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\textsuperscript{pos} 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^{pos} T cells and anti-CMV antibodies. Next to this, the functional distinctions between virus-specific T cells during acute and latent viral infection were defined. Our data for the first time reveal the kinetics and properties of a primary, protective CD4^{pos} T cell immune response to herpes viruses in man. 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 received an organ from a CMV-seropositive donor, were eligible. Patients younger than 18 years of age, refusing written informed consent or with an indication for triple immunosuppressive maintenance therapy, were excluded. From April 1998 until October 1999, eleven 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 and/or CMV end organ disease occurred. Three patients did not consent; one 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 to VI, immunosuppression consisted of 10 mg prednisolone per day and cyclosporin (Neoral) 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^{pos} T cells**

CMV specific CD4^{pos} T cell frequency was determined according to the method described by Waldrop et al. (13). Briefly, 10^6 freshly isolated PBMC were incubated for 6 hours in the presence of either CMV antigen (BioWhittaker, 60 µl/ml), control antigen (BioWhittaker, 60 µl/ml, negative control) or Staphylococcus Aureus enterotoxin B (SEB, ICN/Fluka 2µg/ml, positive control). CD28 monoclonal antibody (mAb) (clone 15E8 CLB, The Netherlands) was added as 3 µg/ml (final concentration) in a final volume of 2 ml per tube RPMI 1640 (Gibco) containing 10% heat inactivated FCS (Intregro, The Netherlands), penicillin and
Quantification and characterization of virus-specific CD4$^{\text{pos}}$ T cells during primary cytomegalovirus infection

streptomycin.

Table 1 Patient characteristics and CMV specific CD4$^{\text{pos}}$ T cell frequency

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>Characteristics</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>CMV-seronegative healthy laboratory personnel</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>CMV-seropositive healthy laboratory personnel</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>CMV-seronegative renal transplant recipients</td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>Renal transplant recipients being CMV-seropositive before transplantation. Tests were performed after transplantation, while on basic immunosuppressive medication.</td>
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</table>

Longitudinal study

| 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. |

For the final 5 hours of culture, brefeldin A (Sigma) was added to the culture in a final concentration of 10 μg/ml. Cells were transferred to FACS tubes, surface stained with CD4-APC (BD), fixed in two 2 ml per tube FACS lysing solution (BD), permeabilized in 0.5 ml per tube FACS permeabilizing solution followed by intracellular staining with CD69-FITC (BD) and anti IFN$^\gamma$-PE (BD) or CD69-PE (BD) and anti IFN$^\gamma$-FITC (BD). Cells were refixed in Cellfix (BD) until flowcytometric analysis the following day. Flowcytometric analysis was performed using a FACS Calibur equipped with a 488 nm argon ion laser and a 635nm red diode laser. Data files containing 50,000 events positive for CD4-APC fluorescence within a lymphocyte gate were saved. Frequencies of CD69$^{\text{pos}}$IFN$^\gamma$$^{\text{pos}}$ cells within the CD4$^{\text{pos}}$ lymphocytes were determined using Cellquest software (BD) and designated CMV specific CD4$^{\text{pos}}$ T cell frequency. When peak frequencies of CMV specific CD4$^{\text{pos}}$ T cells were to be expected, additional antibodies were added to the staining. For surface staining, CD4-APC, CD4-PerCP, CD8-FITC, CD8-PE, CD27-PE, CD62L-PE, CD49d-PE (anti VLA-4), CD38-PE, CD154(CD40L)-PE, CD45RA-FITC, CD45RO-PE (all BD) and CD11a-FITC (Dako) 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 BD), CD69-APC (Caltag), anti Ki-67 polyclonal rabbit F(ab')$_2$-FITC, control polyclonal rabbit F(ab')$_2$-FITC (both Dako), 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 μl of whole blood and eluted in 50 μl 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 to 2057) and Bio-CMV-1107 (5'-CAC TGG CTC AGA CTT GAC AGA CAC-3'; 5' biotinylated; nt. 2588 to 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 internal control (IC) DNA, respectively. One fifth of the DNA eluate (containing the IC DNA) was subjected to PCR. The final reaction mixture (50 μl) 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), 5 μg of bovine serum albumin (Boehringer Mannheim), 10 mM Tris · HCl (pH 8.3), 50 mM KCl, 3 mM MgCl2, dATP, dGTP, and dCTP at a concentration of 200 μM each, and 400 μM dUTP (Perkin-Elmer) and 35 additional IC DNA molecules. The PCRs were done in a Perkin-Elmer 9600 thermocycler: 2 min at 50°C, 5 min at 95°C, followed by 35 cycles each consisting of 20 s at 95°C, 20 s at 63°C, and 1 min at 72°C, followed by 5 min at 72°C. Excess primer was removed as described (18) and the purified PCR product was diluted five times in 1× PCR II buffer (Perkin Elmer). Twenty microliters of Tris (2,2'-bipyridine) ruthenium (II) chelate (TBR) -labeled probe (1 ng/μl; 1.3 pmol; either CMV or IC DNA specific) was added to 30 μl of purified PCR product, and hybridization was done in a Perkin-Elmer 9600 thermocycler (2 min at 95°C, 5 min at 56°C). Next, 10 μl of streptavidin-coated magnetic beads (Perkin-Elmer) was added, and the mixture was incubated for 15 min at 56°C. Forty microliters of the bead-hybrid suspension was added to 400 μl 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. 2292 to 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). Algorithm for quantification: number of copies CMV-DNA per milliliter = R × 4200 was performed, where R is the ratio 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).
Quantification and characterization of virus-specific CD4\textsuperscript{pos} T cells during primary cytomegalovirus infection

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 micro particle enzyme immunoassay (Abbott) according to the manufacturer’s instructions. Positive samples were confirmed in the VIDAS enzyme linked fluorescent assay (Bio Mérieux) according to the manufacturer’s instructions. Anti CMV IgG was determined in serum using the AxSYM micro particle 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\textsuperscript{pos} T cells in renal transplant recipients.
We first validated the method of analyzing CMV specific CD4\textsuperscript{pos} T cell frequency in immunosuppressed patients receiving standard immunosuppressive therapy. In agreement with others, CMV specific CD4\textsuperscript{pos} T cell frequencies were detectable in healthy CMV-seropositive individuals (group II) with a median of 0.15% of CD4\textsuperscript{pos} T cells (range 0.05-1.63% n=6) whereas no CMV specific CD4\textsuperscript{pos} 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\textsuperscript{pos} T cell frequency of 0.39% (range 0.05-1.24% n=11) was found, whereas no CMV specific CD4\textsuperscript{pos} T cells were detectable in CMV-seronegative renal transplant recipients (group III) (fig. 1a-c and 2a). These data demonstrate that under conditions of standard immunosuppression CMV specific CD4\textsuperscript{pos} T cell frequencies can be measured in renal transplant recipients. Moreover, when CD69 upregulation and IFN\textsubscript{y} production to Staphylococcus Aureus Enterotoxin (SEB) stimulation were determined by flowcytometric analysis, frequencies of SEB specific CD4\textsuperscript{pos} T cells from renal transplant recipients were similar to those of SEB specific CD4\textsuperscript{pos} T cells from healthy individuals (fig 2a).

Longitudinal analysis of seronegative renal transplant recipients of organs from seropositive donors.
In five of the seven 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\textsuperscript{pos} T cells were detectable in any of the patients from group V or VI. In five out of seven 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 five patients was treated with ganciclovir because of persistent fever with positive CMV buffy coat culture and a pulmonary infiltrate (patient 1). In all five patients from group V, CMV specific CD4\textsuperscript{pos} T cells were detected by flow cytometry at a median of seven days after first detection of CMV-DNA (range 4-14 days, for a representative experiment see fig. 1d, fig 2b). Peak frequencies ranged from 0.46 to 2.50% of peripheral blood CD4\textsuperscript{pos} T cells (table 2).

Table 2 Appearance of CMV-DNA, CMV specific CD4\textsuperscript{pos} T cells and CMV specific antibodies in CMV seronegative renal transplant recipients of kidneys from CMV seropositive donors (group V)

<table>
<thead>
<tr>
<th>Patient number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time intervals (days)</td>
<td>Tx (\rightarrow) CMV-DNA\textsuperscript{A}</td>
<td>25</td>
<td>29</td>
<td>26</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>CMV-DNA (\rightarrow) CMV spec. CD4\textsuperscript{pos} T cells\textsuperscript{B}</td>
<td>11</td>
<td>7</td>
<td>7</td>
<td>14</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>CMV spec. CD4\textsuperscript{pos} T cells (\rightarrow) anti CMV-IgM\textsuperscript{C}</td>
<td>-3</td>
<td>3</td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Peak frequency of CMV spec. CD4\textsuperscript{pos} T cells\textsuperscript{D}</td>
<td>1.03</td>
<td>1.49</td>
<td>2.50</td>
<td>0.82</td>
<td>0.46</td>
<td>1.03(0.46-2.50)</td>
</tr>
</tbody>
</table>

\textsuperscript{A}Time intervals between:renal transplantation and first detection CMV-DNA in peripheral blood; \textsuperscript{B}first detection of CMV-DNA and first detection of CMV specific CD4\textsuperscript{pos} T cells in peripheral blood; \textsuperscript{C}first detection of CMV specific CD4\textsuperscript{pos} T cells and first detection of CMV specific IgM in peripheral blood (days) \textsuperscript{D}(% of CD4\textsuperscript{pos} T cells)

In all patients, CMV specific CD4\textsuperscript{pos} T cell frequencies precipitously increased after detection of the virus, followed by a rapid decrease (figure 2b). In the two 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\textsuperscript{pos} T cells were detected during follow up of up to 100 days after transplantation (not shown).
Figure 1
Flow cytometric determination of CMV specific CD4⁺ T cells. Dot plots of anti IFNγ-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), after CMV-DNA was detected, but before seroconversion.
Figure 2
CMV specific CD4<sup>pos</sup> T cell frequencies from healthy individuals and renal transplant recipients. □ (CMV<sup>++</sup>HI): CMV-seronegative healthy individuals (group I), ○ (CMV<sup>++</sup>RTx): CMV-seronegative renal transplant recipients on basic immunosuppressive therapy (group III) ■ (CMV<sup>+++</sup>HI): CMV-seropositive healthy individuals (group II), ● (CMV<sup>+++</sup>RTx): CMV-seropositive renal transplant recipients on basic immunosuppressive therapy (group IV). ns: not significant (p>0.05)

Functional and phenotypic properties of CMV specific CD4<sup>pos</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>pos</sup> T cells were CD38<sup>pos</sup> during primary infection,
Quantification and characterization of virus-specific CD4<sup>pos</sup> T cells during primary cytomegalovirus infection

Figure 3
Phenotypic characteristics of CMV specific CD4<sup>pos</sup> T cells. Left column: a representative patient (patient 4) analyzed at the peak of CMV specific CD4<sup>pos</sup> T cells. Right column: a CMV-seropositive healthy individual (group II). A-C: During the acute phase, CMV specific CD4<sup>pos</sup> T cells display a recently activated, proliferating phenotype. Dotplots of anti IFNγ fluorescence (X-axis, arbitrary units) versus CD38 (A), CD27 (B), Ki67-fluorescence (C) respectively (Y-axis, arbitrary units) within a CD4<sup>pos</sup>CD69<sup>pos</sup> lymphocyte gate. D and E: CMV specific CD4<sup>pos</sup> T cells display the CD45RA<sup>pos</sup>CD45R0<sup>high</sup> phenotype. Histograms of CD45RA fluorescence (D) or CD45R0 fluorescence (E): filled histograms represent CMV specific CD4<sup>pos</sup> T cells only i.e. lymphocytes gated on positive CD4<sup>-</sup>, intracellular IFNγ- and CD69 expression. Open histogram overlays represent all CD4<sup>pos</sup> T cells. Histogram overlays were generated using the “each histogram” option for reasons of clarity. Therefore, cell number of filled histograms and open histograms do not have the same Y-axis values.

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

During viral latency, CMV specific CD4<sup>pos</sup> T cells displayed a typical memory phenotype i.e. CD45RA<sup>neg</sup> and CD45RO<sup>pos</sup>. In contrast, during acute CMV infection, the CMV specific CD4<sup>pos</sup> T cells expressed both the naive T cell marker CD45RA and the memory marker CD45R0 (Fig 3d and e). Interestingly, the expression of CD45R0 on these CMV specific T cells was higher when compared to the bulk memory CD4<sup>pos</sup> population (fig. 3e left panel).

Around the time point of peak frequency, the CMV specific CD4<sup>pos</sup> T cells displayed typical Th1 type cytokine production profiles, IFNγ-producing cells showing the highest frequency. Figure 4 shows that all TNFα-producing cells also produced IFNγ. 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>pos</sup> T cells displayed cell surface expression of CD154. However, if CD154 staining was performed after fixation and permeabilisation, the molecule was found to be selectively expressed intracellularly in the CMV specific CD4<sup>pos</sup> T
Quantification and characterization of virus-specific CD4^{pos} T cells during primary cytomegalovirus infection

cells (not shown).

**CMV specific antibody responses in relation to CMV specific CD4^{pos} T cells.**

Figure 5 shows the relation of CMV specific CD4^{pos} T cell frequencies with CMV specific antibody production in one representative patient. In all patients except for patient 1, the appearance of CMV specific CD4^{pos} T cells preceded the formation of CMV specific antibodies.

![Figure 5](image)

*Figure 5*

Relation of CMV specific CD4^{pos} T cells with CMV specific antibody production. Time (days after transplantation, X-axis) versus frequency of CMV specific CD4^{pos} T cells (% of CD4^{pos} T cells, left Y-axis) and antibody concentration (IgM: ratio to a standard serum; IgG: antibody units/ml serum, right Y-axis). One representative patient (patient 2) is shown with respect to anti CMV IgM levels and anti CMV IgG levels. •: CMV specific CD4^{pos} T cell frequency; □: anti CMV IgM levels; ◇: anti CMV IgG levels.

**Discussion**

The present study shows that, in naive individuals, infection of CMV from donated kidneys needs between two and a half to four 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^{pos} T cells appeared in the circulation. The time between transplantation and appearance of CMV specific CD4^{pos} 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 CD4pos 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 man, the exact time point of infection should be known. To this aim, these patients were routinely screened for CMV serology before transplantation, and longitudinally followed 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 seldomly 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 man. In agreement, we found similar frequencies of CMV specific T cells in CMV seropositive renal transplant recipients as compared to CMV seropositive healthy individuals. Also, frequencies of IFNγ producing CD4pos T cells after SEB stimulation did not differ between renal transplant recipients and healthy individuals. It can not 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 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 CD4pos T cells 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 CD4pos 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 CD4pos 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 timepoint virus replication still takes place and that therefore the majority of CMV specific T cells is actively retained within lymphnodes and spleen.

The detailed follow-up of CMV-naive renal transplant recipients allowed, for the first time, to document 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 lymphnodes have been reported to contain T cells highly expressing both CD45RA and CD45R0. This phenotype is also found relatively early after in vitro activation of sorted naive (CD45RA single pos) T cells, suggesting that the CD45RA high CD45R0 high 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 high CD45R0 high helper T cells, which are enriched for cycling cells and primarily produce IFNγ(25,26). The first appearing antigen-specific CD4 pos T cells in peripheral blood after primary CMV infection specifically displayed this CD45RA high CD45R0 high phenotype and were actively cycling based on Ki67 expression. Thus, the small numbers of circulating CD45RA high CD45R0 high cells may represent T cells that, after initial activation by neo-antigens, emigrate from lymphnodes to other sides 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 contribute to an efficient specific immune response that eventually takes place in most secondary lymphoid organs.

We previously postulated that the expression of CD27 on CD4 pos T cells is down regulated after persistent stimulation of T cells in vivo(26,27). In fact, the CD27 neg phenotype of CMV specific CD4 pos T cells that we found in CMV-carriers is consistent with repetitive in vivo stimulation and suggests that also 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 IFNγ may be produced in low quantities without prior 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 anti viral memory, we and also others found low frequencies of IL4 producing CMV specific cells which suggest that CMV elicits a prototype Th1 response (13,29).

In conclusion, this is the first study that reveals the developing immune response to primary CMV infection in vivo in man. 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.

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References

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