PRIMARY IMMUNE RESPONSES TO HUMAN CYTOMEGALOVIRUS

A critical role for IFNγ producing CD4+ T cells in protection against CMV-disease.

Running head:  
T-lymphocyte dynamics in primary CMV-infection

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Abstract:
The correlates of protective immunity to disease-inducing viruses in man remain to be elucidated. We determined the kinetics and characteristics of cytomegalovirus (CMV)-specific CD4$^+$ and CD8$^+$ T cells in the course of primary CMV-infection in asymptomatic and symptomatic renal transplant recipients. Specific CD8$^+$ cytotoxic T cell (CTL) and antibody responses developed irrespective of clinical signs. CD45RA$^-$CD27$^+$CCR7$^-$CTL, although classified as immature effector cells in HIV infection, were the predominant CD8 effector population in the acute phase of protective immune reactions to CMV and were functionally competent. Whereas in asymptomatic individuals the CMV-specific CD4$^+$ T cell response preceded CMV-specific CD8$^+$ T cell responses, in symptomatic individuals the CMV-specific effector-memory CD4$^+$ T cell response was delayed and only detectable after antiviral therapy. The appearance of disease symptoms in these patients suggests that functional CD8$^+$ T cell and antibody responses are insufficient to control viral replication and that formation of effector-memory CD4$^+$ T cells is necessary for recovery of infection.

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Introduction:

The outcome of viral infections is determined by tropism and virulence of the virus, its ability to manipulate the immune system and, importantly, the effectiveness of the host’s immune response in retaining the virus. In animal models, insight has been obtained into the development of primary antiviral responses but detailed information on this subject in man is lacking. Still, knowledge on the correlates of relevant human protective immune responses is of prime importance for effective vaccination strategies and immunotherapeutic interventions.

In controlling viral disease, neutralising antibodies and effective CTL responses are believed to be the main effector arms of the adaptive immune system. Both responses are critically dependent on CD4+ T cell help, and helper-cell deficiency leads to persistence of virus in the presence of activated, but functionally unresponsive CD8+ T cells.

In models of human T cell differentiation, CD8+ T cell memory is established by either a linear differentiation pathway, where memory cells are generated from a primary effector cell pool which, after clearance of antigen, gives rise to a population of memory cells, or by a divergent pathway, where memory and effector cells each derive from a common precursor as two distinct lineages. Variants on these differentiation models propose the existence of a stem-cell like memory cell or a central-memory cell, giving rise to effector cells upon antigenic stimulation. Functional differentiation within the CD8+ T cell compartment can be determined by combined analysis of surface and intracellular markers. In healthy individuals two populations of primed T cells can be distinguished, cytotoxic effector cells are marked by the absence of secondary lymphoid homing receptors like CD62L and CCR7, and of co-stimulatory molecules like CD28 and CD27; and by expression of CD45RA and abundance of cytotoxic effector molecules as Granzyme B, perforin and CD95-ligand memory cells. Recent literature on virus-specific CD8+ T cells
however contradicts the sole restriction of lytic capacity to one particular subset or marker \(^{13; 14}\). During acute viral infection, a remarkable uniform phenotype of proliferating virus-specific T cells with effector function is found, i.e. CD8^+CD45R0^+CD27^+(Ki-67^+) \(^{15; 16}\). As infection resolves, CD8^+ T cells, as a consequence of differentiation processes, first loose CD28 and then CD27 \(^{17}\). In latently infected persons, memory CD8^+ T cells specific for asymptomatic latent viruses as EBV and CMV show phenotypical and functional heterogeneity \(^{18-20}\), and the factors determining the phenotype of memory cells in latent infection are as yet unresolved. Possible determinants are initial viral load and clonal T cell burst, virulence of individual virus strains and tropism of the virus, the latter requiring different homing properties of virus-specific cells; as is shown in EBV-infection, where EBV-specific cells to latent epitopes express CD62L and CCR7, a chemokine receptor able to bind EBV-induced molecule 1 (ELC), expressed on lymph node and tonsillar tissue, enabling them to circulate to B-cell sites of infection \(^{18; 21}\).

In pathological conditions of viral persistence, as in HIV infection, it has been suggested that impairment of both maturation and effector function of CD8^+ T cells is a major cause for the inability of the immune system to control viral replication and subsequent disease \(^{10; 22}\). In these studies, virus-specific CD8^+ T cells accumulate in the CD45R0^+CD27^+CCR7^− subset and display none of the features normally associated with cytotoxic effector function. The finding that a direct correlation exists between number and phenotype of virus-specific CD8^+ T cells and the level of plasma viral-load \(^{23; 24}\) corroborates that functional maturation of CD8^+ T cells is of prime importance in maintaining viral latency.

We here document the development of a human immune response to a clinically relevant virus from primary infection until the latent stage in cytomegalovirus (CMV) seronegative recipients of a CMV-harbouring allotransplant \(^{25}\). In most renal transplant recipients CMV-infection resolves without clinically apparent disease despite immunosuppressive
medication. In some patients however, CMV-infection leads to severe clinical disease symptoms warranting antiviral drug therapy. To document the development of protective and non-protective primary immune responses in man, we performed a longitudinal quantitative and qualitative analysis of the CMV-specific CD4$^+$ and CD8$^+$ T cell responses during primary infection, in asymptomatic and symptomatic renal transplant recipients. Our data show that although competent CD8$^+$ effector cells develop both in asymptomatic and symptomatic infection, for protective immunity and containment of viral replication, IFN$\gamma$ secreting CD4$^+$ T cells are indispensable.
Materials and Methods

Subjects (table 1)

3 HLA-A2 positive and 6 HLA-B7 positive CMV-seronegative renal transplant recipients of a CMV-seropositive kidney were longitudinally studied. All patients received a first graft of either a post-mortal donor or a living related donor. Basic immunosuppressive therapy consisted of cyclosporin A, blood trough levels aimed at 150 ng/mL and prednisone 10 mg daily. In case of living related transplantation (patient 2 and 7) mycophenolate mofetil (MMF) was added at a dose of 1000 mg twice daily. Rejection was treated with methylprednisolone 500 mg for 6 days, and in case of ongoing rejection (patient 7) with mAb against CD3 (OKT3) for 10 days. The cumulative dose of corticosteroids did not differ between patients experiencing asymptomatic or symptomatic CMV-infection. No relationship was observed between CMV-infection and the timepoints of rejection episodes. Anti-viral treatment consisted of ganciclovir (5 mg/kg intravenously twice daily, adjusted for renal function) and was initiated when visceral CMV-disease was diagnosed based on organ-involvement confirmed by tissue-biopsy. Heparinized peripheral blood samples were collected before transplantation and weekly during 17 weeks after transplantation. To analyse the latent state, as defined by quantitative PCR viral load below the detection limit of 80 copies/mL, a blood sample was taken > 30 weeks after infection. PBMC were isolated using standard density gradient centrifugation techniques and subsequently cryopreserved. All patients gave written informed consent and the study was approved by the local medical ethical committee.
**Peptides**

The HLA-A2 binding CMV pp65 derived peptide NLVPMVATV and the HLA-B7 binding CMV pp65 derived peptide TPRVTGGGAM were purchased from the IHB-LUMC peptide synthesis library facility (Leiden, The Netherlands).

**Generation of tetrameric complexes**

Tetrameric complexes were generated essentially as described by Altman et al. 

In brief, purified HLA-A2.1 heavy chain or HLA-B7.2 heavy chain and β2 micro-globulin were synthesized using a prokaryotic expression system (pET; Novagen, Milwaukee, WI, USA). The heavy chain was modified by deletion of the transmembrane/cytosolic tail and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The HLA-A2.1 binding CMV pp65 derived peptide NLVPMVATV and the HLA-B7 derived peptide TPRVTGGGAM were used for refolding. Monomeric complexes were concentrated, biotinylated by BirA (expressed using the pET expression system, purified using Clontech cobalt beads) in the presence of biotin (Molecular Probes), ATP (Sigma Chemical) and MgC12. The biotinylated product was separated from free biotin by FPLC using a Superdex 200 HR16 / 60 column (Amersham Pharmacia, Little Chalfont, UK). Streptavidin-allophycocyanin (APC) conjugate (Molecular Probes) was added in a 1:4 molar ratio and subsequently tetramers were FPLC purified using the same column.

**Immunofluorescent staining and flowcytometry**

Thawed PBMC were resuspended in RPMI, containing 10% FCS and antibiotics. 200,000 PBMC were incubated with fluorescent label conjugated mAbs (concentrations according to manufacturer’s instructions) and an appropriate concentration of tetrameric complexes. Negative controls to validate specificity of the CMV-peptide-tetrameric complexes consisted
of HLA-A2.1/HLA-B7.2 negative CMV-seropositive or HLA-A2.1/HLA-B7.2 positive
CMV-seronegative healthy individuals and renal transplant recipients. Negative controls
always showed tetramer staining of less than 0.01% of total lymphocytes (data not shown).
For staining with the mouse anti human CCR7 monoclonal antibody (mAb), a three step
staining protocol was performed consisting of incubation with the CCR7 antibody
(Pharmingen), for 30 minutes, washing, incubation with biotinylated goat anti mouse IgM
(Pharmingen) for 30 minutes, incubation with 10% (v/v) normal mouse serum (CLB,
Amsterdam, The Netherlands) followed by incubation with streptavidin-PE and directly
conjugated mAbs and tetrameric complexes for 30 minutes. Analyses consisted of APC-
conjugated tetramers and CD8-PerCP (BectonDickinson, San Jose, CA) in combination with
either CD45RA (BD) and CD27 (BD), CCR7 and CD45RA, CD27 and CD28 (BD) and
CD45RA and CD45R0 (BD), all combinations in FITC and PE.
Intracellular Ki-67, granzyme B and perforin staining was performed by incubating 0.5
million of PBMC with fluorescent label conjugated mAbs to CD8 (BD) and CMV-
tetrameric complexes, washed once, then fixed with 50 µl of buffered formaldehyde acetone
solution and subsequently permeabilised by washing with 0.1 % saponine 50 mM D-
glucose. Cells were then incubated with anti Ki-67 (DAKO), anti-Granzyme B (CLB, the
Netherlands) and anti-perforin antibodies (Hölzel diagnostika, Köln, Germany) according to
manufacturer's instructions. Analysis of cells was performed using a FACS Calibur
flowcytometer and CellQuest software (BD).

Determination of CMV specific CD4\textsuperscript{pos} and CD8\textsuperscript{pos} T cells by intracellular cytokine
staining
CMV specific CD4\textsuperscript{pos} and CD8\textsuperscript{pos} T cell frequencies were determined essentially according
to the method described by Waldrop et al. \textsuperscript{27} and Kern et al. \textsuperscript{28}, respectively. Briefly, 0.5 x
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) (determination of CMV specific CD4^{pos} T cells), Staphylococcus Aureus enterotoxin B (SEB, ICN/Fluka 2µg/ml, positive control) the HLA-A2 binding CMV-peptide or the HLA-B7 binding CMV-peptide or a irrelevant HLA-A2 binding HIV-peptide (negative control) (final concentration of 10 µg/mL, determination of CMV specific CD8^{pos} T cells). CD28 mAb (clone 15E8 CLB, The Netherlands) and VLA-4 mAb (BD) were added as 2 µg/ml (final concentration) respectively 1µg/mL(final concentration) in a final volume of 1 ml per tube RPMI 1640 (Gibco) containing 10% heat inactivated FCS (Integro, The Netherlands), penicillin and streptomycin. 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, fixed in 2 ml per tube FACS lysing solution (BD), permeabilised in 0.5 ml per tube FACS permeabilizing solution followed by (intracellular) staining with IFNγ-FITC (BD) and CD69-PE (BD) and CD4-APC (BD) or CD8-APC (Coulter). Cells were washed in PBA and refixed in Cellfix (BD) and flowcytometric analysis was performed on the following day, 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 or CD8-APC fluorescence within a lymphocyte gate were saved. Frequencies of CD69^{pos}IFNγ^{pos} cells within the CD4^{pos} or CD8^{pos} lymphocyte gate were determined using Cellquest software (BD) and designated CMV specific CD4^{pos} or CD8^{pos} T cell frequencies, respectively. Negative controls showed less than 0.05% of CD69^{pos}IFNγ^{pos} cells (data not shown).

**CMV-PCR**

Quantitative PCR was performed in EDTA whole blood samples as described for plasma or serum.29.
**Anti-CMV IgM and IgG**

Anti CMV IgM and IgG were determined in serum as described previously.\(^{25}\)

**Cytotoxicity assay**

Ex-vivo cytotoxicity was assessed by incubating \(^{51}\)Chromium (Amersham) labelled, peptide-pulsed HLA-matched EBV-transformed lymphoblastoid cell lines with PBMC at effector target ratios of 1:1 to 1:4, calculated on absolute numbers of tetramer\(^{+}\)CD8\(^{+}\) T cells present in the PBMC fraction, for 4 hours. Negative controls consisted of non-peptide-pulsed target cells or HLA-mismatched target cells. Percentage specific lysis was calculated from the formula percentage specific lysis = \(((\text{experimental counts-media control})/\text{(detergent control-media control)})\) x 100%.

**Statistical analysis**

Two-sided Mann-Whitney test was used for analysis of differences between groups, for correlations, Spearman non-parametric correlation test was used, p-values < 0.05 were considered statistically significant.
Results

CD8⁺ T cell responses in relation to viral load and CD4⁺ T cell responses, in primary, asymptomatic CMV-infection

In accordance with our previous findings, peak frequencies of CMV-specific CD4⁺ T cells in 5 asymptomatic individuals, enumerated by IFNγ production upon specific stimulation, ranged from 0.42% to 2.5% of CD4⁺ T cells (median 0.82%, abs.value 0.38.10⁷/mL, range 0.17 to 1.1.10⁷/mL) and were detected at a median of 10 days (range 0 to 17 days) after first detection of CMV-DNA. CMV-specific IgM and IgG antibodies were detected at a median of 7 days (range 4-14 days) after first detection of CD4⁺ T cells (table 1 and figure 1a).

CMV-specific CD8⁺ T cells were enumerated by HLA-A2 and HLA-B7 tetrameric complexes, folded with the pp65 derived peptides NLVPMVATV for HLA-A2 or TPRVTGGGAM for HLA-B7, both described as immunodominant in latent CMV-infection. Peak frequencies of CMV-specific CD8⁺ T cells ranged from 0.55% to 4.97% (median 2.21%, abs.value 1.45.10⁷/mL, range 0.3 to 8.82.10⁷/mL). In all asymptomatic patients CMV-specific CD4⁺ T cells preceded CMV-specific CD8⁺ T cells, which were detected at a median of 14 days (range 10-14 days) after first detection of CMV-specific CD4⁺ T cells.

Upon antigenic encounter, naive cells will develop into effector cells-, and after viral clearance, long-lived -memory cells, with distinctly different proliferative and cytotoxic capacities. We analysed these differentiation steps by extensively phenotyping CMV-specific CD8⁺ T cells with the differentiation markers CD28, CD27 and CCR7 and by determining CD45RA vs. CD45R0 expression. Figure 2 shows the differentiation of the total and CMV-specific CD8⁺ T cells in one representative patient. Looking at expression of CD28 and CD27 in the course of infection, CMV-specific and total CD8⁺ T cells first loose CD28 and subsequently CD27 (figure 2a). Loss of CD27 however seems to occur only after viral replication has ceased, where loss of CD28 occurs early in acute infection. When
analysed, the percentage of CD28⁺CD27⁺ virus-specific cells is correlated to high amounts of virus present (r=0.48, p=0.0018, data not shown) and the percentage of CD28⁻CD27⁻ increases when viral load decreases (r= -0.64, p=< 0.0001, figure 2e).

Before onset of infection, the majority of total CD8⁺ T cells in all asymptomatic individuals is found in the naive CD27⁺CD45RA⁺ subset (median 78.77 %, range 40.99 to 83.86%). After the first emergence of CMV-specific CD8⁺ T cells however, the CD45RA⁻CD27⁺ population of total CD8⁺ T cells is highly increased (median 52.66%, range 46.94 to 72.39%) and most of the tetramer⁺ cells are confined to this subset (figure 2b). Analysis by CCR7 and CD45RA in the early days of infection, shows in some patients a small population of CMV-specific CD8⁺CCR7⁺CD45RA⁻ i.e. central memory cells ¹², but in all patients the majority of CMV-specific cells is devoid of CCR7 and thus apparently unable of homing to secondary lymphoid organs (figure 2c). Looking simultaneously at Ki-67 expression, it appears when in cell-cycle, early in infection, these cells express CD45R0 abundantly (figure 2d and 3a). When infection is resolved, CMV-specific CD8⁺ T cells acquire a CCR7⁻CD27⁻/dull⁻CD45RA⁺ phenotype. In all patients, CMV-specific CD8⁺ T cells express granzyme B and perforin from the first timepoint of detection (figure 3 b and c). Interestingly, a substantial portion of tetramer⁻ CD8⁺ T cells gets activated as appears from expression of Ki-67, granzyme B and perforin. Furthermore, the majority of the CD8⁺ T cell compartment followed the same differentiation process as the tetramer⁺CD8⁺ T cells, implying that these cells represent CMV-specific cells to other epitopes of CMV. In direct ex-vivo cytotoxicity assays, CMV-specific CD8⁺ T cells could lyse appropriately CMV-peptide loaded, HLA-matched target cells, and no difference was seen in percentage specific lysis between CMV-specific CD8⁺ T cells with a CD45R0⁺CD27⁺CCR7⁻ phenotype and a CD45RA⁺CD27⁻CCR7⁻ phenotype (figure 4). In all patients a proportion of CMV-specific
CD8⁺ T cells, as enumerated by tetramer-binding, was capable of production of IFNγ upon specific peptide stimulation (data not shown and figure 4).

**Effector-memory CD4⁺ T cell responses are delayed in symptomatic patients**

In 4 patients, primary CMV-infection followed a complicated course. All patients suffered severe organ involvement and required anti-viral therapy consisting of ganciclovir (table 1). In these patients CMV-DNA was detectable in peripheral blood at a median of 27 days after transplantation (range 22 to 28; NS compared to asymptomatic patients) and whereas there was no statistical difference in maximum viral load between asymptomatic and symptomatic patients (p=0.06), viral load tended to be higher in symptomatic patients. Although these patients experienced CMV-disease and needed anti-viral treatment to control viral replication, no differences were found with respect to the emergence of CMV-specific antibodies and CMV-specific CD8⁺ T cells nor differentiation pattern of CD8⁺ T cells between symptomatic and asymptomatic individuals. CMV-specific antibodies could be detected at a median of 15 days (range 4 to 28 days, NS), CMV-specific CD8⁺ T cells at a median of 21 days after first CMV-DNA detection (range 14 to 32 days, NS) with peak frequencies ranging from 0.33 to 3.04 % (median 1.92%, abs. value 0.84.10⁷/mL, range 0.31 to 1.3.10⁷/mL; NS) of total CD8⁺ T cells (figure 1b). No differences could be detected in either cytotoxicity or specific IFNγ production of CMV-specific CTL between symptomatic and asymptomatic patients, implying that other parameters define successful clearance of CMV (figure 4). Markedly, the time interval between first detection of CMV-DNA and first detection of CMV-specific CD4⁺ T cells was significantly longer than in asymptomatic patients (28 to 53 days, median 39 days, p= 0.01) and only after start of anti-viral therapy could CMV-specific CD4 responses be measured. Both CMV-specific CD8⁺ T cell responses and CMV-specific IgG antibody responses were detectable before emergence of
CMV-specific CD4\(^+\) T cells in all symptomatic patients (table 1) implying that CMV-specific CD4\(^+\) T cells were present in lymph nodes to provide help for B cells and CD8\(^+\) T cells. Peak frequencies of CMV-specific CD4\(^+\) T cells ranged from 0.36 to 1.42% of CD4\(^+\) T cells (median 0.47 %, abs. value 0.17.10^7/mL, range 0.07 to 0.27.10^7/mL) and, like in asymptomatic individuals, rapidly decreased to become non-detectable. Possibly this short presence in the peripheral blood of CMV-specific effector CD4\(^+\) T cells reflects migration of these cells through the peripheral blood to their target site.
Discussion

Here we document the development of an adaptive primary anti-viral immune response in man. We show that in asymptomatic patients, CMV-specific CD4+ T cells emerge in the peripheral blood compartment preceding both CMV-specific antibodies and CD8+ T cells. These coordinate responses lead to clearance of the virus. In contrast, in symptomatic patients, specific antibodies as well as specific CD8+ T cells appear in the peripheral blood compartment prior to IFNγ producing CMV-specific CD4+ T cells. Only after start of antiviral treatment these latter cells emerge and the virus is cleared. Remarkably, no differences in either kinetics or functional differentiation/maturation of CMV-specific CD8+ T cells were detected between asymptomatic and symptomatic patients.

The different kinetics of CMV-specific CD4+ T cells in our study of asymptomatic and symptomatic individuals confirm that CD4+ T cells influence outcome of disease in primary infection 30; 31. Recent studies show that CD4+ T cells can be divided into two populations with distinct migratory capacities: one consisting of IL-2 producing central-memory CD4+ T cells, able to recirculate through secondary lymphoid organs, and a second population of effector-memory CD4+ T cells which main function is to secrete antimicrobial lymphokines, exerting their function in peripheral target organs and thus contributing directly to containment of viral replication 32; 33. The emergence of specific IgG antibody responses in both asymptomatic and symptomatic individuals indicates that CMV-specific CD4+ help indeed is present in peripheral lymph nodes to support B cell differentiation and IgM-IgG class-switching 4 as well as CD8+ T cell differentiation.

In our study, impaired control of viral replication, leading to clinical disease symptoms, can be explained by lack of IFNγ secreting effector-memory CD4+ T cells at the site of infection 34. The absence of these cells in the peripheral blood compartment early in infection can be
ascribed to several variables. Variations in virulence of individual virus-strains and their
ability to interfere with immune functions, like antigen presentation and cytokine production
should be taken into account\textsuperscript{35-37}. One explanation would be impaired antigen priming of
the effector-memory CD4\textsuperscript{+} T cell subset by dendritic cells possibly due to an altered
cytokine environment caused by immunosuppressive therapy\textsuperscript{38} or suppression of dendritic
cell maturation by CMV itself as was recently described for murine and human
cytomegalovirus. The normal appearance of antibodies and CD8\textsuperscript{+} T effector cells, indicates
that specifically the Th1 CD4 response is impaired, suggesting altered IL-12 secretion by
dendritic cells\textsuperscript{39; 40}. That effector-memory CD4\textsuperscript{+} T cells become detectable in peripheral
blood shortly after start of antiviral therapy infers a direct effect of CMV on the immune
system.

No difference was seen in CMV-specific CD8\textsuperscript{+} T cell differentiation pattern between
asymptomatic and symptomatic patients, although the administration of anti-viral therapy
leading to clearance of virus could account for this finding. During acute infection, CMV-
specific cells show a CCR7\textsuperscript{-}CD27\textsuperscript{+}CD45RA\textsuperscript{-}CD45R0\textsuperscript{+} phenotype, previously designated as
skewed and immature in chronic HIV-infection\textsuperscript{10}. In our patients however, this phenotype
was displayed on the height of CMV-specific CD8\textsuperscript{+} T cell frequencies; only when viral load
dropped, CMV-specific CD8\textsuperscript{+} T cells lost CD45R0 and a substantial number lost CD27.
Irrespective of their phenotype, all of these cells expressed granzyme B and perforin from
start of infection, implicating that either a CD27\textsuperscript{-}CD45RA\textsuperscript{+} phenotype is reached when no
antigen is present anymore, or that cells of this phenotype cannot be obtained by peripheral
blood sampling in replicative stages of viral infection. This would be in line with recent
studies, where specific CD8\textsuperscript{+} T cells in persistent replicative viral infection show a
CD45R0\textsuperscript{+}CD27\textsuperscript{+} phenotype, whereas specific CD8\textsuperscript{+} T cells in persistent latent viral infection
show a more differentiated CD45RA\textsuperscript{-}CD27\textsuperscript{-} phenotype\textsuperscript{41}. Based on CD28 and CD27
expression successive stages of T cell differentiation can be depicted in early virus infection, where CD28^+CD27^+ , CD28^+CD27^- and CD28^-CD27^- subsets correspond to early, intermediate and late phenotypes. We here show that the appearance of these distinct stages is related to viral load. That, in persistent active viral infection, as in HCV, HIV and lytic EBV infection, CD8^+ T cells specific for these viruses accumulate in the early and intermediate subsets is most likely a reflection of redistribution of cytotoxic CD28^-CD27^- cells to peripheral target sites of active infection, where these cells would exert their function, as was found in animal studies, rather than a direct immunomodulatory effect of different viruses themselves on the differentiation pathway of CD8^+ T cells. The appearance of CD28^-CD27^- late effector cells in the course of CMV-infection when active infection is resolved and the virus enters its latent stage, could reflect emergence of these cells out of infected tissues and back into the peripheral blood. Recently, we could demonstrate that CCR7^-CD27^-CD45RA^+ T cells, after stimulation by antigen in vitro, proliferate and revert from CD45RA^+ to CD45R0^+, suggesting the CCR7^-CD45RA^- phenotype is induced by presence of antigen (E. van Leeuwen, unpublished observation). When analysed for Ki-67, CMV-specific CD8^+ T cells were found cell-cycling early in infection, and when so, were uniformly CD45R0. The described skewed maturation CCR7^-CD45RA^- phenotype in HIV-infection accordingly seems to be a consequence of the presence of antigen and not a cause of disease. Also the difference in expression of CD45 isoforms on virus-specific memory cells during latency would reflect recent cell-cycling of these cells, due to antigen exposure or homeostatic instruction.

In our study, the difference between adequate viral clearance and viral persistence was determined by the absence or presence of effector-memory CD4 responses. Indeed, also in persistent HCV-infection no CD4 responses can be detected in peripheral blood. Furthermore, in HIV infection the clinical outcome is directly correlated to CD4^+ T cell
numbers. The influence of these CD4+ T cell responses on viral clearance in HIV and HCV combined with the phenotypes found in chronic infection with these viruses suggests a model of CD8+ T cell differentiation where presence of antigen defines the maturation stage of CD8+ T cells detected in peripheral blood, where CD45R0 is a marker for recent replicative history of the antigen specific cell and the presence of antigen-specific CD4+ T effector cells defines successful viral clearance. Furthermore, the presence or absence of CD27- effector T cells would reflect redistribution of CTLs to peripheral target sites. It seems that, according to findings of us and others, memory CD8+ T cells can expand at any point in their differentiation pathway, even when displaying a CCR7-CD27-CD45RA+ phenotype, previously thought to have no proliferative potential at all. More insight into the distribution of human virus-specific cells in the different lymphoid compartments and peripheral target organs during active and latent infection will provide better understanding on the significance of T cell subset findings in peripheral blood.

Taken together, our data, although obtained in a small number of patients awaiting further corroboration in separate cohorts, imply that functional CD8+ T cells cannot clear antigen without functional effector-memory CD4+ T cells. Furthermore, when antigen is present, CD8+ T cells display a so called "memory" phenotype, formerly associated with poor cytotoxic function, but here shown to be cytotoxic indeed. These findings implicate that in designing vaccination strategies, both CD4+ and CD8+ effector immune responses should be triggered and sustained.
Reference List


Figure legends

Figure 1: Enumeration of CMV-specific CD4+ and CD8+ T cells in primary CMV-infection

Figure 1: Frequencies of CMV-specific CD4+ T cells, determined by intracellular staining for CD69 and IFNγ upon stimulation (open circles), CMV-specific CD8+ T cells, enumerated by tetramer binding (closed circles) and first specific antibody appearance (dotted vertical line) in relation to viral load (CMV-DNA, closed triangles) in one representative asymptomatic patient (patient 4, fig. 1a) and one symptomatic patient (patient 8, figure 1b, closed vertical line, start of 14 days of ganciclovir therapy).
Figure 2: Differentiation of CMV-specific and total CD8+ T cells in asymptomatic and symptomatic patients.

Figure 2a-d: Differentiation of CD8+ T cells in one asymptomatic individual representative of all patients (patient 2). Time defined as days after first positive PCR (day 0), all plots gated on CD8+ T cells. CMV-specific CD8+ T cell frequencies (% of total CD8+ T cells): Day 31: 4.96%; Day 59: 4.58%; Day 76: 3.30%; Day 279: 1.29%. CMV-specific CD8+ T cells as defined by specific tetramer staining plotted in black, total CD8+ T cells plotted in red. Quadrant percentages depicted in black for CMV-specific CD8+ T cells and in red for total CD8+ T cells.

Figure 2a: x-axis log fluorescence CD27-Fitc, y-axis log fluorescence CD28-PE;
Figure 2b: x-axis log fluorescence CD27-PE, y-axis log fluorescence CD45RA-Fitc;
Figure 2c: x-axis log fluorescence CCR7-PE, y-axis log fluorescence CD45RA-Fitc;
Figure 2d: x-axis log fluorescence CD45R0-PE, y-axis log fluorescence CD45RA Fitc.

Figure 2e: Correlation of viral load and percentage of CD28-CD27- cells of CMV-specific CD8+ T cells, during all timepoints of infection, non detectable viral load was set at the cutoff point of the quantitative PCR at 80 copies/mL.
Figure 3: CMV-specific CD8⁺ T cells in primary CMV-infection are cytotoxic irrespective of their phenotype.

All plots gated on total CD8⁺ T cells, time defined as days after first positive PCR (day 0)

Day 31: Peak of CMV-specific CD8⁺ T cell frequency, day 279: timepoint during latency.

Figure 3a: x-axis log fluorescence CMV-tetramer, y-axis log fluorescence Ki-67

Figure 3b: x-axis log fluorescence CMV-tetramer, y-axis log fluorescence granzyme B

Figure 3c: x-axis log fluorescence CMV-tetramer, y-axis log fluorescence perforin
Figure 4: Both asymptomatic and symptomatic individuals show functional CMV-specific CD8\(^+\) T cells

% CMV-specific CD8\(^+\) T cells enumerated by tetramer binding, % CMV-specific IFN\(\gamma\) producing CD8\(^+\) T cells enumerated by intracellular cytokine staining upon specific peptide stimulation and specific lysis (E:T ratios 1:1, 1:2 and 1:4) in one asymptomatic patient (patient 2, Fig. 4a and c) and one symptomatic patient (patient 6, Fig 4b and d).
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Figure 1

a. 

b.
Figure 2

<table>
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<th>CD27</th>
<th>CD45R0</th>
<th>CD27</th>
<th>CD45RA</th>
<th>CD28</th>
<th>CD45RA</th>
<th>CD45RA</th>
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<tbody>
<tr>
<td>Day -4</td>
<td>PCR = 0</td>
<td>2.71</td>
<td>64.41</td>
<td>6.36</td>
<td>6.34</td>
<td>9.04</td>
<td>43.27</td>
<td>11.44</td>
<td>36.24</td>
</tr>
<tr>
<td>Day 31</td>
<td>PCR = 14,000</td>
<td>5.28</td>
<td>50.82</td>
<td>15.85</td>
<td>28.63</td>
<td>2.26</td>
<td>41.74</td>
<td>28.81</td>
<td>26.75</td>
</tr>
<tr>
<td>Day 59</td>
<td>PCR = 350</td>
<td>2.64</td>
<td>41.74</td>
<td>28.81</td>
<td>26.75</td>
<td>2.12</td>
<td>40.23</td>
<td>38.01</td>
<td>19.57</td>
</tr>
<tr>
<td>Day 76</td>
<td>PCR = 244</td>
<td>2.13</td>
<td>40.23</td>
<td>38.01</td>
<td>19.57</td>
<td>0.66</td>
<td>5.21</td>
<td>50.18</td>
<td>43.31</td>
</tr>
<tr>
<td>Day 279</td>
<td>PCR = 0</td>
<td>1.33</td>
<td>16.93</td>
<td>74.27</td>
<td>7.47</td>
<td>1.13</td>
<td>5.18</td>
<td>20.93</td>
<td>22.75</td>
</tr>
</tbody>
</table>

a.

b.

c.

d.

e.

R = -0.64, p < 0.0001
Figure 3

Day -4  Day 31  Day 59  Day 76  Day 279

a. 

b. 

c. 

CMV-tetramer →

CMV-tetramer →

CMV-tetramer →

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Figure 4

Asymptomatic  Symptomatic

(a) % CMV-specific CD8+ T cells

(b) % specific lysis

(c) Tetramer IFN+  IFN

(d) Tetramer IFN+  IFN

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Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Pt no.</th>
<th>days to first positive PCR</th>
<th>Maximum viral load (copies/mL)</th>
<th>Duration of infection (duration of positive PCR in days)</th>
<th>days to CD4 from first positive PCR</th>
<th>Days to CD8 from first positive PCR</th>
<th>First antibody appearance (days from first positive PCR)</th>
<th>clinical symptoms</th>
<th>Start of antiviral therapy (days from transplantation)</th>
<th>Rejection episodes (number, days from transplantation)</th>
<th>Rejection therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>22,000</td>
<td>28</td>
<td>10</td>
<td>21</td>
<td>14</td>
<td>n.a.</td>
<td>0</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>14,000</td>
<td>59</td>
<td>17</td>
<td>31</td>
<td>31</td>
<td>n.a.</td>
<td>1, day 9</td>
<td>MPNS</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>22,000</td>
<td>42</td>
<td>14</td>
<td>28</td>
<td>21</td>
<td>n.a.</td>
<td>0</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>133,000</td>
<td>48</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>n.a.</td>
<td>0</td>
<td>n.a.</td>
<td></td>
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<tr>
<td>5</td>
<td>26</td>
<td>32,000</td>
<td>66</td>
<td>10</td>
<td>24</td>
<td>17</td>
<td>n.a.</td>
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<td>MPNS</td>
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<tr>
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<td>median</td>
<td>26</td>
<td>22,000</td>
<td>48</td>
<td>10</td>
<td>17</td>
<td>n.a.</td>
<td>0</td>
<td>n.a.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pt no.</th>
<th>days to first positive PCR</th>
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<th>clinical symptoms</th>
<th>Start of antiviral therapy (days from transplantation)</th>
<th>Rejection episodes (number, days from transplantation)</th>
<th>Rejection therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>26</td>
<td>92,000</td>
<td>88</td>
<td>28</td>
<td>18</td>
<td>16</td>
<td>gastrointestinal</td>
<td>33</td>
<td>0</td>
<td>n.a.</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>140,000</td>
<td>50</td>
<td>35</td>
<td>24</td>
<td>28</td>
<td>gastrointestinal</td>
<td>49</td>
<td>2, day 57, 69</td>
<td>MPNS, OKT 3</td>
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<tr>
<td>8</td>
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<td>74</td>
<td>53</td>
<td>32</td>
<td>4</td>
<td>pancytopenia</td>
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<td>22</td>
<td>140,000</td>
<td>35</td>
<td>42</td>
<td>14</td>
<td>14</td>
<td>gastrointestinal</td>
<td>39</td>
<td>1, day 45</td>
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<tr>
<td></td>
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<td>39</td>
<td>21</td>
<td>15</td>
<td>44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.a. = not applicable

MPNS = methylprednisolone

* = statistically significant, p < 0.05
Primary immune responses to human cytomegalovirus: a critical role for IFN-γ-producing CD4+ T cells in protection against CMV-disease

Laila E Gamadia, Ester B M Remmerswaal, Jan F Weel, Frederieke Bemelman, Rene A W van Lier and Ineke J M ten Berge