Predictive value of human cytomegalovirus (HCMV) T-cell response in the control of HCMV infection by seropositive solid-organ transplant recipients according to different assays and stimuli

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INTRODUCTION

HCMV-specific antibodies have been shown to be one of the first lines of defense in immunocompromised solid-organ transplant recipients (SOTR) and to reach higher titers in immunocompetent subjects with symptomatic vs asymptomatic infection (Fornara et al., 2015). Nonetheless, HCMV-specific T-cell immunity represents the major arm of defense against HCMV infection/reactivation in both solid-organ and hematopoietic stem cell transplant recipients, with special reference to the HCMV-specific CD4⁺ T-cells (Egli et al., 2012; Gabanti et al., 2014a, 2015). During the last decade, several methods have been developed to measure T-cell immunity: HLA-peptide tetramer staining, intracellular cytokine staining (ICS) by cytokine flow cytometry (CFC), the enzyme-linked immunospot (ELISPOT)-assay, and the QuantiFERON-CMV assay based on the measurement of interferon-γ (IFN-γ) released by peripheral blood mononuclear cells (PBMC) in whole blood following antigenic stimulation (Calarota et al., 2014). Both ICS by CFC and ELISPOT assays, when using whole viral antigens or peptide pools as stimuli, are not limited by HLA-restriction. However, while ICS by CFC allows identification of virus-specific CD4⁺ and CD8⁺ T-cells, ELISPOT does not distinguish between the two major T-cell subsets, unless PBMC are depleted of either cell subset, or stimulation is done separately by short synthetic peptides (8-10 aa) stimulating CD8⁺ T-cells, and long synthetic peptides (13-22 aa) stimulating CD4⁺ T-cells (Calarota et al., 2014). In addition, new approaches to measurement of HCMV-specific T-cell immunity have recently been proposed (Snyder et al., 2016; Limaye et al., 2016).

An original CFC-ICS method was developed in our laboratory some years ago, which was aimed at stimulating both CD4⁺ and CD8⁺ T-cells with HCMV-infected autologous dendritic cells (iDC) (Lilleri et al., 2006; 2007; 2008; 2009a and b; 2012). Although technically cumbersome and time-consuming (requiring 7 days to complete), this assay is still considered the gold standard in our laboratory. Due to the drawbacks of the CFC-iDC assay, we aimed at

Key words: Human cytomegalovirus, T-cell response, Solid-organ transplant recipients, Dendritic cells, Cytokine flow cytometry, ELISPOT.

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selecting a new ELISPOT assay, using different stimuli, which was as efficient as or close to the CFC-iDC assay in predicting protection from HCMV disease in SOTR.

MATERIALS AND METHODS

Patients

Between September 2013 and March 2015, 71 HCMV-seropositive candidate transplant recipients were initially enrolled in the study at the University Hospital, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. Among these patients, 20 were excluded from the analysis (10 because of a too short follow-up, 5 because not receiving an organ transplantation, and 5 because they had died during or shortly after the transplantation procedure). Of the remaining 51 patients, 41 were kidney (KTR), 8 heart (HTR) and 2 lung transplant recipients (LTR). Induction therapy consisted of anti-thymocyte globulin (ATG) and steroids in 9/41 KTR and all of HTR and LTR, while the majority of KTR (32/41) were treated with anti-CD25 monoclonal antibody (basiliximab) and steroids. Immunosuppressive therapy consisted of standard triple therapy including a calcineurin inhibitor (cyclosporine A or tacrolimus), an antiproliferative drug (mycophenolate mofetil, MMF), and steroids. In detail, among calcineurin inhibitors, 24/41 KTR received tacrolimus, 17/41 cyclosporine A, while HTR were treated with cyclosporine (5/8) or tacrolimus (2/8) or a combination of steroids, cyclosporine and everolimus instead of MMF (1/8), and the two LTR received tacrolimus. Patients with organ rejection were treated with a daily bolus of i.v. methylprednisolone (500 mg or 1.0 g) for three days. The study was approved by the Fondazione IRCCS Policlinico San Matteo Institutional Review Board (Protocol no. 20100005459, Procedure no. 20100035853) and patients gave written informed consent.

Virological monitoring

Using pre-emptive therapy, as a rule no systemic clinical symptoms (HCMV syndrome) were observed except for organ localizations or tissue-invasive disease (TID). Thus, in this study, systemic (virus detection in blood) HCMV infections were classified as low viral load (LVL), with DNAemia peak lower than the cut-off of 3x10^5 DNA copies/ml and high viral load (HVL, with DNAemia peak higher than the cut-off) infections, while TID was as a rule associated with clinical symptoms related to the organ involved (Razonable et al., 2013). HCMV infections were diagnosed by an in-house developed real-time PCR method. Viral DNA was quantified in blood weekly or twice a week according to the absence/presence of active HCMV infection in blood, and pre-emptive therapy was administered to patients with HVL infections, as reported (Furione et al., 2012). The cut-off for initiation of pre-emptive therapy was selected almost 10 years ago (Gerna et al., 2007). Using this cut-off, no SOTR suffered from HCMV disease except for 14/272 (5%) patients transplanted at our institution in the period 2003-2014 and undergoing DNAemia-guided pre-emptive therapy who developed TID in the presence of low or undetectable HCMV DNA in blood (Gerna et al., 2007, 2011; Gabanti et al., 2014a). The expression of DNA copies in International Units (IU) using the WHO International Standard has not yet met with universal consensus (Hayden et al., 2015a and b; Fryer et al., 2016; Jones et al., 2016). Antiviral treatment consisted of i.v. ganciclovir (GCV) administered at a dosage of 5 mg/kg twice a day or oral valganciclovir (VGCV), 900 mg twice a day, and was continued until virus disappearance from blood for two consecutive determinations. TID was diagnosed by determination of viral DNA in organ secretion fluids (e.g. bronchoalveolar lavage fluid) or organ (e.g. gastrointestinal) biopsies. In TID, sampling of the organ involved was consistently related to local clinical symptoms, and antiviral treatment was continued until resolution of clinical symptoms and/or resolution of endoscopic lesions. HCMV infection relapses were treated similarly.

Immunological monitoring

Immunological monitoring was done (whenever possible) at the following time points: 30, 60, 90, 180 and 360 days after transplantation. In this study, the CFC-iDC assay was our reference method and takes 7 days to complete (Lozza et al., 2005). The HCMV-specific CD4+ and CD8+ T-cell response cut-off (0.4 T cells/µL blood, as determined by the CFC-iDC assay) was established by ROC analysis and displayed the maximal predictive value (Gerna et al., 2006, 2011). As for the three short-term (24 h) ELISPOT assays, PBMC were stimulated with:

1) commercially available HCMV AD169 infected cell lysate (iCL, Microbix Biosystems Inc., Mississauga, Ontario, Canada);

2) a peptide pool (PP) of 34 (8-12 aa long) epitope peptides (JPT Peptide Technologies GmbH, Berlin, Germany) relevant to multiple HCMV AD169 proteins presented in association with different MHC class I molecules (Walker et al., 2007);

3) a commercial cytomegalovirus peptide mix (CPM) of 9-11 aa peptides from HCMV pp65 (Autoimmune Diagnostika - AID - GmbH, Strassberg, Germany). For the ELISPOT assays, an iCL stimulus was used to primarily activate CD4+ T-cells, while PP and CPM stimuli were used to primarily activate CD8+ T-cells.

Identification of HCMV antigens presented to CD4+ and CD8+ T-cells by iDC

PBMC were cultured overnight with DC infected for 24 h with the VR1814 HCMV strain and were then stained with the following fluorochrome-conjugated mAbs: anti-CD4 (clone OKT4) Alexa Fluor 700 (BioLegend Inc., San Diego, CA, USA), anti-CD8 (clone 3B5) PE-Texas Red (Invitrogen, Frederick, MD, USA), anti-CD25 (clone B1.49.9) FITC (Beckman Coulter-Immunotech, Marseille, France), anti-CD137 (clone 4B4-1) PE-Cy7 (BioLegend). CD25-CD137+ (i.e. responding to infected DC) and CD25-CD137- (i.e. non-responding) CD4+ and CD8+ T-cells were sorted using FACSAria (BD Biosciences, San Jose, CA, USA). Responding and non-responding T-cell samples were divided into 48 replicate cultures of 600 cells and expanded for three weeks in the presence of irradiated allogeneic feeder cells, IL-2 and PHA (Geiger et al., 2009). Aliquots from each culture were then tested in parallel for their capacity to proliferate in response to overlapping peptides (15-mers) spanning the following entire HCMV proteins: immediate-early (IE)-1, pp65, gB and hHgL-pUL128L. The frequency of specific precursors was calculated according to Poisson's distribution.

iDC assay

HCMV-specific CD4+ and CD8+ T-cells were stimulated with autologous monocyte-derived HCMV VR1814-iDC as reported (Lozza et al., 2005). Following differentiation
from PBMC over a 5-day period, DC were infected with the HCMV VR1814 strain for 24 h, and then incubated overnight at a ratio of 1:20 with 0.5-1.0x10^6 PBMC aliquots in the presence of brefeldin A (10 µg/mL) to retain cytokines produced within stimulated T-cells. Following stimulation with iDC, PBMC were then incubated with Live/Dead Fixable Violet Dye (Invitrogen) and V500-conjugated anti-CD8 (clone RPA-T8, BD Biosciences) for cell surface staining. After fixation and permeabilization (Cytofix/Cytoperm, BD Biosciences), cells were incubated with the following antibodies (BD Biosciences): PerCP-Cy5.5™-conjugated anti-CD3 (clone uCHTI), APC-Cy7™-conjugated anti-CD4 (clone RPA-T4), PeCy7™-conjugated anti-IFN-γ (clone B27). Finally, cells were washed, resuspended in 1% paraformaldehyde and analyzed with a FACS Canto II flow cytometer (BD Biosciences). The frequency of CD4+ and CD8+ T-cells producing IFN-γ was determined by subtracting the frequency of PBMC incubated with mock-infected DC (<0.05%) from the frequency of PBMC incubated with HCMV infected DC. The total number of HCMV-specific CD4+ and CD8+ T-cells was calculated by multiplying the percentage of HCMV-specific T-cells positive for IFN-γ production by the corresponding absolute CD4+ and CD8+ T-cell counts (TruCOUNT tubes, BD Biosciences). Levels of HCMV-specific CD4+ and CD8+ T-cells greater than 0.4/µL blood were considered protective (Gerna et al., 2006, 2011).

**ELISPOT assays**

PBMC were seeded in duplicate into anti-IFN-γ antibody-coated ELISPOT strips (AID) at 2x10^5 cells/well. Samples were incubated overnight with Pokeweed mitogen (positive control), RPMI medium only (negative control) and the three HCMV stimuli reported above at the following concentrations: 50 µg/mL for iCL, 1 µg/mL for each PP peptide, and 2.5 µg/mL for CPM. ELISPOT images were acquired and analyzed using an automated image scanner (iSpot Reader Spectrum, AID). The net ELISPOT response was determined by subtracting the number of spots in wells with culture medium only, from the number of spots in wells with each HCMV stimulus. ELISPOT results were then normalized to T-cell counts (Calarota et al., 2014) as follows: (net spot number x lymphocyte number/µL blood)/2x10^5, thus obtaining the total absolute number of HCMV-specific spot-forming T-cells/µL.

**Statistical analysis**

Absolute numbers/µL of HCMV-specific CD4+ and CD8+ T-cells determined with the iDC assay as well as the normalized numbers of HCMV-specific T-cells detected by the three different ELISPOT assays in the two groups of patients with LVL or HVL HCMV infection, were compared within each assay by the Mann-Whitney U test. Receiver-operator characteristic (ROC) analysis was done to assess the performance of different assays in predicting the spontaneous control of HCMV infection and to establish the protection cut-off from HVL infections for each assay. The distribution of the level of specific immunity able to control HVL HCMV infection was analyzed by the Chi-square test. The cumulative incidence of HCMV-specific T-cell immunity reconstitution following different stimuli and assays was determined using Kaplan-Meier curves, which were compared by the log-rank (Mantel-Cox) test.

**RESULTS**

**Clinical characteristics of patients enrolled**

Overall, the 51 SOTR included in the analysis (41 receiving kidney, 8 heart, and 2 lung transplantation) had a median age of 54 (21-70) years, and the male/female ratio was 33/18 (65/35%). The median follow-up duration was 359 (119-454) days. Due to the strict virological monitoring and sensitive real-time PCR assay adopted, HCMV DNA was detected in blood of all SOTR (who were all HCMV-seropositive). Twenty-nine patients underwent a LVL self-resolving HCMV infection, while 19 patients had a HVL systemic infection treated with antiviral drugs, and 3 TID only, i.e. gastrointestinal (GI, n=2) or lung disease (n=1). Three patients (2 receiving a kidney and one a lung transplantation) were treated for TID, in the absence of HVL systemic infection and in the presence of systemic T-cell immunity. As reported in Table 1, of the 41 KTR, 23 (56%) had a LVL systemic infection, 16 (39%) had a HVL systemic infection requiring antiviral treatment (4 associated with TID, and 3 relapsing infections), and two (5%) had TID. Five (62%) of the eight HTR had LVL self-resolving infection, and three (38%) HVL infection (1 associated with TID) requiring antiviral treatment. Finally, of the two LTR, one had a LVL self-resolving infection, and one TID.

**HCMV antigens presented by iDC**

In a preliminary experiment conducted in a HCMV-seropositive healthy subject, following overnight stimulation of PBMC with iDC, both activated (CD25+ CD137+) and non-activated (CD25 CD137) CD4+ and CD8+ T-cells were sorted, divided into several replicates and expanded. Following stimulation with protein peptide pools and measurement of ³H-thymidine uptake, antigen-specificity of expanded T-cell lines was finally determined. It was found that HCMV-specific CD4+ T-cell lines were stimulated in decreasing order of frequency by pp65, the pentamer gH-
gLpUL128L, gB and IE, while specific CD8+ T-cells were primarily stimulated by pp65, and IE, and much less by gB and the pentamer (Fig. 1 A-D).

Discrimination of the two groups of SOTR by different assays
As shown in Figure 2, at the DNAemia peak, the number of HCMV-specific CD4+ T-cells detected by CFC-iDC in SOTR with a LVL self-resolving infection was significantly higher (P<0.001) when compared with patients with HVL infection treated with antiviral drugs. The same trend was observed when comparing the two groups of patients with the CD8+ CFC-iDC assay (P=0.001), as well as the ELISPOT-iCL (P<0.001) and the ELISPOT-PP (P=0.021) assays, whereas no significant difference (P>0.05) was found using the ELISPOT-CPM assay for comparison.

Calculation of assay cut-offs predicting protection from HCMV infection
Using the ROC analysis, the cut-offs for different assays indicating the maximal level of sensitivity (capacity to identify the highest number of patients with a level of HCMV-specific immunity protecting from HCMV infection) and specificity (capacity to identify the highest number of patients not protected from HCMV infection) were determined. These cut-offs were as follows:
1) 0.4 T-cells/µL for both HCMV-specific CD4+ and CD8+ T-cells detected by the CFC-iDC assay;
2) 0.1 T-cells/µL for ELISPOT-iCL;
3) 0.5 T-cells/µL for ELISPOT-PP;
4) 0.4 T-cells/µL for ELISPOT-CPM.

At DNAemia peak, the best discriminating performance between the two patient groups was provided (Chi-square test) by the CD4+ CFC-iDC assay (P<0.001) followed by the ELISPOT-iCL (P<0.001) and ELISPOT-PP (P<0.001) assays (Fig. 3). The CD4+ CFC-iDC assay was characterized by the following ROC parameters: 0.79 AuC, 90% sensitivity, 74% specificity, 84% PPV, and 82% NPV. The ROC parameters relevant to different assays are reported in Table 2. Although endowed with a significant discriminating capacity (P=0.002), the CD8+ CFC-iDC assay displayed low specificity (32%). ELISPOT results were normalized

Figure 1 - Flow cytometry analysis of activated (CD25+ CD137+) and non-activated (CD25- CD137-) T-cells. Following PBMC stimulation with infected dendritic cells, expansion, and final stimulation with HCMV protein peptide pools, T-cell libraries were tested for antigen specificity. A: Activated CD4+, and B: activated CD8+ T-cells. C-D: no. HCMV antigen-specific activated and non-activated /10^6 (C) CD4+ and (D) CD8+ T-cells.
Figure 2 - Results of (A) CFC-iDC assay for HCMV-specific CD4+ and CD8+ T-cells, and (B) ELISPOT assays for HCMV-specific T-cells in two groups of solid-organ transplant recipients. At the DNAemia peak, one group had low viral load infection, and the other high viral load infection requiring antiviral treatment. Only the ELISPOT-CPM assay did not reveal a differential T-cell response between the two patient groups. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool; pts, patients.

Figure 3 - Discriminating performance at the DNAemia peak of the two patient groups according to different assays (Chi square test). The best performance was provided by the CD4+ CFC-iDC assay followed by the ELISPOT-iCL and ELISPOT-PP assays. Although endowed with a significant discriminatory capacity, the CD8+ CFC-iDC assay displayed low specificity. The different ROC parameters relevant to the different assays are reported in Table 2. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool; pts, patients.
on the total lymphocyte count/µL blood and expressed as HCMV-specific spot forming cells/µL blood in order to calculate the optimal cut-off level discriminating patients controlling from those not controlling HCMV infection. Non-normalized ELISPOT results showed less discriminating performance (data not reported).

Cumulative incidence (Kaplan-Meier curves) of T-cell immunity reconstitution in the two groups of SOTR patients

The comparison of the Kaplan-Meier curves relevant to the recovery of T-cell immunity shows that the reconstitution process occurs with significantly different kinetics in the two groups of SOTR according to different assays (Fig. 4). In other words, the time required to reach the predictive level of protection of HCMV-specific T-cell immunity in SOTR with HVL infection requiring antiviral treatment was significantly longer compared to patients with LVL self-resolving infection, regardless of the assay considered. The comparison of each cumulative curve obtained with one method with the relevant curves of the other methods in the two groups of patients is reported in Table 3 and Figure 5. Figure 5A reports the cumulative percentage of patients with LVL self-resolving infection reaching the cut-off predictive of protective immunity during follow-up. HCMV-specific CD4+ and CD8+ detected by CFC-iDC were the T-cells most rapidly reaching the prefixed cut-off, while specific T-cells detected by ELISPOT-iCL and ELISPOT-PP showed an intermediate trend, and specific T-cells detected by ELISPOT-CPM were the last to reconstitute. Conversely, in the group of SOTR with HVL infection requiring treatment, the most rapidly as-

Table 2 - ROC analysis at DNAemia peak.

<table>
<thead>
<tr>
<th>ROC parameters</th>
<th>CFC-iDC assay</th>
<th>ELISPOT assays</th>
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<tr>
<td></td>
<td>HCMV-specific CD4+ T-cells</td>
<td>HCMV-specific CD8+ T-cells</td>
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<tr>
<td>AUC</td>
<td>0,79</td>
<td>0,77</td>
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<tr>
<td>specificity (%)</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>sensitivity (%)</td>
<td>74</td>
<td>32</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>84</td>
<td>69</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>82</td>
<td>100</td>
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</table>

CFC-iDC, cytokine flow cytometry-infected dendritic cells; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; iCL, infected cell lysate; PP, peptide pool; CPM, CMV peptide mix; ROC, receiver-operator characteristics.

Figure 4 - Kaplan-Meier curves. The time required to reach a predictive level of protection of HCMV-specific T-cell immunity in SOTR with high viral load infection requiring antiviral treatment is significantly longer as compared to patients with low viral load infection, regardless of the assay used. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool; pts, patients.
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cending curve was relevant to HCMV-specific iDC-stimulated CD8+ T-cells, while iDC-stimulated specific CD4+ T-cells, ELISPOT-iCL and ELISPOT-PP displayed an intermediate trend, and again the last reconstitution occurred in ELISPOT-CPM T-cells (Fig. 5B). In more detail, the median times of DNAemia appearance and peaking were comparable in the two groups of patients with LVL and HVL infection, whereas the median DNAemia peak was 100x higher in HVL infections. In addition, the median time to DNAemia disappearance was much longer in HVL infections (292 vs 199 days). Similarly, the median time to reaching the immunological cut-off predictive of protection, while was comparable for all assays (~30 days) in patients with LVL infections, was, apart from CFC-iDC CD8+ T-cells (36 days), much shorter (≥100 days) for CFC-iDC CD4+ T-cell assay (180 days) compared to all ELISPOT assays (≥280 days), in patients with HVL infections (Table 4).

Discrepancies between pre-determined cut-offs and clinical severity

The first group of discrepancies concerns three patients with a self-resolving LVL HCMV infection (Fig. 6). In all three cases, the DNAemia peak (consistently below the viral cut-off of 300,000 copies/mL blood) was reached in the presence of HCMV-specific CFC-iDC CD4+ T-cells below the immunological cut-off of 0.4 T-cells/µL. However, the low level of HCMV-specific CD4+ T-cells was short-lasting, and the DNAemia level was consistently controlled when specific CD4+ T-cells reached the cut-off. As for the ELISPOT assays, all three showed a trend overlapping that of CFC-iDC CD4+ T-cells, with levels of specific T-cells below the relevant cut-off at DNAemia peak, which however was reached shortly thereafter. In patient SOTR-168, follow-up could be continued only 180 days after transplantation.

The second group of discrepancies involves five patients with HVL infection requiring antiviral treatment, notwithstanding the presence of CFC-iDC specific CD4+ T-cells above the CFC-iDC cut-off level (Table 5). However, three of these patients showed an unstable CD4+ and CD8+ T-cell response (Fig. 7): for two (SOTR-110, and -147) of these three patients the number of HCMV-specific CD4+ T-cells oscillated around the cut-off level, while for the third patient (SOTR-175) the level of specific CD8+ T-cells was markedly below the cut-off. For all three of them, the reconstitution of a stable level of specific CFC-iDC CD4+ (and CD8+) T-cells controlled viral infection. At the major DNAemia peak, all three ELISPOT assays were positive (above the relevant cut-off) in SOTR-110, while the other two patients showed a variable kinetics. In patient SOTR-

<table>
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<th>Table 3 - Comparison of Kaplan-Meier cumulative curves (Log-rank test).</th>
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<td></td>
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<tr>
<td>A Patients with LVL infection</td>
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<tr>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
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<tr>
<td>iCL</td>
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<td>PP</td>
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<td>CPM</td>
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<td>B Patients with HVL infection</td>
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<td>iCL</td>
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<td>PP</td>
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<td>CPM</td>
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Overall comparison: A, P<0.0001; B, P<0.0001; HVL, high viral load; LVL, low viral load; iCL, infected cell lysate; PP, peptide pool; CPM, CMV peptide mix; NA, not applicable; NS, not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

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Figure 5 - Comparison of each cumulative curve obtained with one method with the relevant curves of the other methods. (A) Patients with low viral load infection, and (B) patients with high viral load infection. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool; pts, patients.
**Table 4 - Kinetics of DNAemia and HCMV-specific immunity according to different stimuli.**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>DNAemia appearance</th>
<th>DNAemia peak</th>
<th>DNAemia disappearance</th>
<th>Median (range) time (days) to reach cut-off</th>
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<tr>
<td></td>
<td>median (range) time (days)</td>
<td>median (range) time (days)</td>
<td>median (range) time (days)</td>
<td>CD4+ (iDC assay)</td>
</tr>
<tr>
<td>patients with LVL infection</td>
<td>27 (5-75)</td>
<td>59 (5-181)</td>
<td>199 (17-391)</td>
<td>31 (25-89)</td>
</tr>
<tr>
<td>patients with HVL infection</td>
<td>21 (6-61)</td>
<td>63 (29-352)</td>
<td>292 (192-395)</td>
<td>180 (27- &gt;360)</td>
</tr>
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</table>

Follow-up was discontinued 180 days after transplantation. However, the two most intriguing cases were patients SOTR-141 and SOTR-151, both KTRs (Table 5, and Fig. 8) who underwent a HVL HCMV infection requiring antiviral treatment in the first few months after transplantation, concomitantly with a sharp drop in both total and specific CD4+ T-cell numbers. The latter, however, maintained a level between 5 and 10 cells/µL (thus, at least 10x higher than the cut-off) throughout the follow-up. Following control of the first DNA peak (thanks to antiviral treatment) in the subsequent months, the same levels of HCMV-specific CD4+ T-cells were able to control multiple viral DNA peaks which were consistently below the DNAemia cut-off. Among the ELISPOT assays, all three reached the relevant cut-off in SOTR-141 upon the first DNAemia peak and after the second peak all three decreased markedly. In SOTR-151 only the ELISPOT-CPM assay reached or was close to the cut-off and subsequently decreased markedly.

**TID patients**

As reported in Fig. 9, three patients suffered from TID in the absence of HVL in blood and in the presence of high viral DNA levels in the GI tract (patients SOTR-117 and SOTR-129) or in lungs (patient SOTR-120). Patients SOTR-117 and SOTR-129, even with the consistent presence of CFC-iDC HCMV-specific CD4+ (and CD8+) T-cells in peripheral blood, were unable to control the appear-

![Figure 6 - Low viral load infection in three patients with HCMV-specific CD4+ CFC-iDC assay below the cut-off of 0.4 T-cells/µL at time of DNAemia peak. HTR, heart transplant recipient; KTR, kidney transplant recipient. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool.](image-url)
Table 5 - Patients with systemic HVL infection and valuable HCMV-sp CD4 immunity according to the CFC-iDC assay.

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>CFC-iDC assay (HCMV-specific T-cells/µL)</th>
<th>ELISPOT assay (HCMV-specific T-cells/µL)</th>
<th>Systemic HCMV HVL infection</th>
<th>TID, time (day), site and HCMV-DNA copies</th>
<th>Induction/immunosuppression</th>
<th>Rejection, time (days post-tx)</th>
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<td>#110 (HTR)</td>
<td>1.2</td>
<td>4.3</td>
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CFC-iDC, cytokine flow cytometry-infected dendritic cells; iCL, infected cell lysate; PP, peptide pool; CPM, CMV peptide mix; HTR, heart transplant recipient; KTR, kidney transplant recipient; HVL, high viral load; TID, tissue-invasive disease; BAL, bronchoalveolar lavage; CFC-iDC: cytokine flow cytometry-infected dendritic cells; ATG, anti-thymocyte globulin; MPRE, methylprednisolone; MMF, mycophenolate mofetil; CyA, cyclosporine A; FK506, Tacrolimus.

Figure 7 - High viral load infection in three KTR patients with HCMV-specific CD4⁺ CFC-iDC assay above the cut-off of 0.4 T-cells/µL at time of DNAemia peak. In patients SOTR-110 and SOTR-147 specific CD4⁺ T-cells were repeatedly oscillating around the cut-off, while in patient SOTR-175 specific CD8⁺ T-cells were below the cut-off at time of DNAemia peak. As for the ELISPOT assays, all three were above the cut-off in SOTR-110 at time of DNAemia peak, while they showed an inconsistent trend in the other two patients. KTR, kidney transplant recipient; CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool.
DISCUSSION

The CFC-iDC assay was developed about ten years ago and notwithstanding the length of time required to complete the assay (7 days) the lack of the need for HLA determination and the opportunity to quantify both HCMV-specific CD4+ and CD8+ T-cells have made this assay the gold standard for T-cell-mediated immunity evaluation in our laboratory. However, thus far, the spectrum of HCMV antigens presented by iDC 24h p.i. and thus endowed with stimulatory activity towards T-cells has not been investigated in detail. In this preliminary study, the most represented subset of HCMV protein-specific/106 CD4+ T-cells was pp65-specific, followed by gHgLpuL128L (the HCMV pentamer) - specific CD4+ T-cells. As for specific CD8+ T-cells, the two most represented subsets of HCMV protein-specific T-cells were pp65-specific and IE-1-specific, while the remaining HCMV-specific CD4+ and CD8+ T-cells were minimally represented among the HCMV antigens tested.

In the original study (Lozza et al., 2005), we observed different kinetics of development of HCMV-specific CD4+ and CD8+ T-cell responses to iDC stimulation. The CD4+ T-cell response was detected shortly after DC infection (2-24 h p.i.), while the CD8+ T-cell response was delayed reaching its peak 24-48 h p.i. This difference may be due to differences in antigen processing. In fact, the rapid appearance of the CD4+-mediated immune response may be due to antigen uptake by macropinocytosis and the presence of preformed MHC class II molecules in cytoplasmic vesicles (Sallusto et al., 1995). Conversely, MHC class I molecules, which are synthesized late (Rescigno et al., 1998), are associated with peptides from proteins newly synthesized in the endoplasmic reticulum during infection. This entails that peptide-MHC class I complexes are exposed late at the DC membrane. Among the proteins tested whose peptides are presented by MHC molecules on the DC membrane 24 h p.i., the immediate-early (IE) protein was likely newly synthesized (and thus primarily stimulated CD8+ T-cells within 24 h in association with newly synthesized MHC class I molecules), while the other proteins tested (pp65 early/late protein; gB and the pentamer, late proteins) were likely derived from dense bodies and other viral components taken up by macropinocytosis from the infected cell culture medium used for DC infection (and, thus, more readily available in association with preformed MHC class II molecules for CD4+ T-cells or cross-presented on MHC class I molecules for CD8+ T-cells).

In our study, the high response of both iDC-CD4+ and -CD8+ to pp65 may be due to both phagocytized pp65 (dense bodies) in association with preformed class II molecules, and neo-synthesized pp65 early during viral infection requiring antiviral treatment in SOTR-141 and SOTR-151 (both KTRs) with specific CD4+ T-cells/µL largely above the cut-off determined by the CFC-iDC assay. The first DNA peak was not controlled by T-cell immunity, while the subsequent peaks remained markedly below the cut-off. In patient SOTR-141, all three ELISPOT assays were above the relevant cut-off at the time of the first DNAemia peak, while subsequently they showed a discontinuous trend. On the other hand, in SOTR-151, only the ELISPOT-CPM assay reached or was close to the cut-off at the first DNAemia peak. KTR, kidney transplant recipient; CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool.
Figure 9 - Tissue-invasive disease in three SOTR patients in the absence of high viral load systemic HCMV infection. The presence of HCMV-specific CD4⁺ and CD8⁺ T-cells revealed by the CFC-iDC assay in peripheral blood did not protect from tissue-invasive disease. Levels in blood of T-cells above the established cut-offs were also detected (although sometimes inter-mittently) by all three ELISPOT assays. KTR, kidney transplant recipient; LTR, lung transplant recipient. CFC-iDC, cytokine flow cytometry using infected dendritic cells as stimulus; iCL, infected cell lysate; PP, a pool of 34 epitopic peptides from different HCMV proteins; CPM, a commercial pp65 peptide pool.

infection in association with MHC class I molecules. In addition, IE-1 protein (produced entirely by neosynthesis) may induce CD4⁺ T-cell response in association with MHC class II preformed molecules, and CD8⁺ T-cell response in association with MHC class I molecules.

The primary objective of this study was to use ROC analysis to select the optimal cut-off for each assay to be utilized clinically for a reliable prediction of protection from HCMV disease. For this purpose, two groups of SOTR were compared, one including patients with LVL self-resolving HCMV infection, and one including patients with HVL HCMV infection requiring antiviral treatment to prevent the appearance of clinical symptoms, according to the pre-emptive therapy approach. The two patient groups were compared both at the DNAemia peak and during follow-up when the cumulative incidence of patients reaching the cut-off predictive of protection according to different assays was investigated. In both cases, the best discriminating performance between the two patient groups was displayed by the CD4⁺ CFC-iDC and the ELISPOT-iCL assays followed by the ELISPOT-PP assays. The commercially available ELISPOT-CPM assay was not discriminatory enough and displayed the most delayed T-cell immune reconstitution.

Special consideration must be given to the CD8⁺ CFC-iDC assay, which was the earliest to detect reconstituted T-cell immunity. However, this result must be interpreted with caution. According to our recent investigations conducted both in SOTR and hematopoietic stem cell transplant recipients (HSCTR), HCMV-specific CD4⁺ T-cells represent the first line of specific cellular immune defense in preventing HCMV infection/disease in seropositive transplant recipients (Gabanti et al., 2014a, 2015). HCMV-specific CD8⁺ T-cells, which are not sufficient to provide protection alone, co-operate with specific CD4⁺ T-cells after their appearance in controlling HCMV infection.

As a result, in the present study, the earlier HCMV-specific CD8⁺ CFC-iDC T-cell appearance is not predictive of protection unless specific CD4⁺ T-cells are simultaneously present. In more detail, in the group of SOTR with LVL infection, both CD4⁺ and CD8⁺ CFC-iDC were the T-cells most rapidly reaching the cut-off, while T-cells detected by ELISPOT-iCL and ELISPOT-PP displayed an intermediate trend. On the other hand, in SOTR with HVL infection, iDC-stimulated CD8⁺ T-cells were the earliest to reconstitute immunity, followed by iDC-stimulated CD4⁺ T-cells, ELISPOT-iCL and ELISPOT-PP. As shown previously, patients with only specific CD8⁺ T-cells were not generally protected from HCMV infection progression.

Another consideration must be stressed: how and why do iCL- and PP-stimulated PBMC confer protection? We recently documented that the iDC stimulus is not significantly different from the iCL stimulus on CD4⁺ T-cells, and is significantly superior to that of PP on CD8⁺ T-cells. Thus, ELISPOT-PP provides information mainly on the CD8⁺ T-cell response, which, in the absence of CD4⁺ T-cell help, appears unable to control the infection. Thus, the predictive power of ELISPOT-PP is lower. However, iCL may significantly contribute to CD8⁺, and PP to CD4⁺ T-cell stimulation (Gabanti et al., 2014b). Surprisingly, the ELISPOT-CPM assay did not appear to be useful for protection prediction in the clinical setting.
Finally, a few discrepant results were detected between selected cut-offs predictive of protection and viral load findings. In a group of three SOTR, a self-resolving LVL infection occurred in the presence of a number of HC-MV-specific CD4+ T-cells <0.4/µL (cut-off for both CD4+ and CD8+ T-cells measured by the CFC-iDC assay). This apparent inconsistency may be due to the fact that in some patients protection may be conferred by a number of T-cells below the cut-off. However, it is also possible that the rapid restoration of specific CD4+ T-cells above the cut-off may have contributed to protection in these patients.

As for the five patients undergoing an HVL infection in the presence of specific CD4+ T-cells above the cut-off, two patients showed levels of specific CD4+ T-cells oscillating around the cut-off, and one had levels of specific CD8+ T-cells below the cut-off, while the remaining two patients during the post-transplant period had an HVL infection in the presence of levels of specific CD4+ CFC-iDC markedly above the cut-off. However, this lack of control over viral DNA replication occurred concomitantly with the first DNA peak (which was resolved by the antiviral treatment), while subsequently CD4+ T-cells controlled virus infection. Thus, it is possible that some functional features of T-cells were initially impaired by the immunosuppressive treatment; however, the lack of preserved residual T-cells precluded any potential retrospective phenotypic and functional testing. ELISPOT assays in these patients showed trends only partially in agreement with the CFC-iDC assay. In addition, the three SOTR with TID, in the presence of systemic levels of HC-MV-specific CD4+ and CD8+ T-cells above the cut-off, as detected by the CFC-iDC assay as well as ELISPOT assays, further confirmed that TID may occur regardless of a robust systemic cellular immune response, as recently shown by our group for HSCTR (Gabanti et al., 2015). A similar event seems to occur also in SOTR.

In conclusion, the 1-day ELISPOT-iCL assay provides results similar to the 7-day HC-MV-specific CD4+ CFC-iDC assay in predicting control of HC-MV infection, and may represent a valuable alternative to CFC-iDC assay for monitoring the HC-MV-specific T-cell response in the clinical setting. However, it should be kept in mind that late TID (mainly GID) may also occur after systemic specific T-cell reconstitution.

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References


