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FULL PAPER

Comparison of the T-cell response to human cytomegalovirus (HCMV) as detected by cytokine flow cytometry and QuantiFERON-CMV assay in HCMV-seropositive kidney transplant recipients

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SUMMARY

Human cytomegalovirus (HCMV)-specific T-cell response in kidney transplant recipients (KTR) helps to identify patients at risk for severe infection. To assess the T-cell response, this study compared our in-house developed reference test, based on T-cell (both $CD4^+$ and $CD8^+$) stimulation by autologous HCMV-infected dendritic cells (iDC) and subsequent detection by cytokine flow cytometry (CFC-iDC), with the QuantiFERON-CMV (QF-CMV) assay. Fifty-three HCMV-seropositive KTR were enrolled. At the DNAemia peak, 33 (62%) had low viral load (LVL, $<3x10^5$ DNA copies/mL) self-resolving infection, 19 (36%) high viral load (HVL, $>3x10^5$ DNA copies/mL) infection treated with antivirals, and one LVL patient (2%) tissue-invasive disease alone. Both assays showed a delayed recovery of HCMV-specific T-cell immunity in HVL *vs* LVL patients. Immune reconstitution kinetics did not significantly differ between the two assays in HVL patients. QF-CMV and CFC-iDC showed comparable sensitivities, but QF-CMV had a lower (although not significantly) specificity. Indeed, 7/19 HVL patients (37%) were erroneously considered protected from severe infection by QF-CMV, whereas CFC-iDC misidentified only 3/19 (16%) patients as protected. Although our reference test takes longer to complete, it appears slightly better at predicting patients at risk for severe HCMV infection. Moreover, QF-CMV may provide false negative results with some HLA types.

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INTRODUCTION

Human cytomegalovirus (HCMV) infection/disease remains one of the most common infectious complications in patients receiving solid-organ transplantation. To reduce morbidity and mortality caused by HCMV infection, prophylactic and pre-emptive antiviral approaches are in use (Khoury *et al.,* 2006, Limaye *et al.,* 2006). T-cell-mediated immunity is believed to play a major role in controlling HCMV infection in the immunocompetent host (Revello *et al.,* 2006; Lilleri *et al.,* 2007) and preventing HCMV infection in solid-organ transplant recipients (SOTR) (Sester *et al.,* 2001, Sacre *et al.,* 2005, Gerna *et al.,* 2011a, 2011b, Gabanti *et al.,* 2014). Therefore, T-cell immunological monitoring may provide useful prognostic indications.

In recent years, several methods have been developed to investigate the HCMV-specific T-cell response. Intracellular cytokine staining by cytokine flow cytometry (CFC) can measure both virus-specific $CD4^+$ and $CD8^+$ T-cells. Other methods, such as ELISPOT, can distinguish between the two T-cell subsets if peripheral blood mononuclear cells (PBMC) are depleted of either subset, or differentially stimulated (Calarota *et al.,* 2014, Gabanti *et al.,* 2016).

To monitor the T-cell response to HCMV infections in both immunocompetent and immunocompromised hosts (Lilleri *et al.,* 2006, 2007, 2008, 2009; Gabanti *et al.,* 2015), a method simultaneously measuring $CD4^+$ and $CD8^+$ T-cells by CFC after PBMC stimulation with autologous HCMV-infected dendritic cells (CFC-iDC) was developed in our laboratory (Lozza *et al.,* 2005).

The commercially available QuantiFERON-CMV assay (QF-CMV, Qiagen, Hilden, Germany), which measures primarily IFN-γ released by $CD8^+$ T-cells after stimulation with a pool of 22 short peptides from 6 HCMV proteins presented by several human leukocyte antigen (HLA) class I haplotypes (Walker *et al.,* 2007), has already shown its utility in assessing the risk of HCMV reactivation in SOTR (Lisboa *et al.,* 2012, Abate *et al.,* 2013, Manuel *et al.,* 2013).

This study compared the performance of our CFC-iDC method with that of QF-CMV in predicting self-resolving low viral load (LVL) and high viral load (HVL) HCMV infections requiring treatment in a cohort of HCMV-seropositive kidney transplant recipients (KTR).

Key words:
Human cytomegalovirus, T-cell response, Cytokine flow cytometry, QuantiFERON-CMV assay, Kidney transplant recipients.

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MATERIALS AND METHODS

Study population

From September 2013 to July 2016, 53 KTR were enrolled at our University Hospital. Induction therapy consisted of anti-thymocyte globulin (ATG) or anti-CD25 monoclonal antibody and steroids. Immunosuppressive therapy consisted of standard triple therapy including a calcineurin inhibitor, an antiproliferative drug, and steroids. The study was approved by our Institutional Review Board and patients gave their written informed consent.

Virological monitoring

Viral DNA was quantified in whole blood (DNAemia) weekly or twice a week (according to the severity of infection) by an in-house real-time PCR method (Gerna *et al.,* 2006b), and was still expressed as DNA copies/mL blood. However, a recent still unpublished study showed that the conversion factor from DNA copies to International Units (IU) for blood samples was 0.42, thus indicating an approximate 2-fold difference between the two VL expression units (personal communication). However, since the DNA extraction method was changed in 2015, when the conversion factor was determined, and patient enrollment started in 2013, this study still expresses viral DNA in copies/mL whole blood.

Pre-emptive therapy (i.v. ganciclovir 5 mg/kg or oral valganciclovir 900 mg twice a day) was administered to HVL patients with DNAemia levels above the cut-off of $3x10^5$ copies/mL blood (Lilleri *et al.,* 2004, Gerna *et al.,* 2011a, 2011b), and treatment was continued until two consecutive negative DNAemia results. Patients with LVL DNAemia resolved the systemic infection spontaneously. Tissue invasive disease (TID) in symptomatic patients was confirmed on tissue biopsy according to Ljungman *et al*. (2017).

Immunological monitoring

Immunological monitoring was scheduled in all KTR patients at 30, 60, 90 and 180 days after transplantation and concomitantly with the initiation of antiviral treatment in HVL patients (DNAemia peak). Each patient was tested at all time points by CFC-iDC (Lozza *et al.,* 2005) and QF-CMV. Our in-house developed CFC-iDC assay was based on 24h co-culture of patient autologous immature dendritic cells (DC), which were infected with the endotheliotropic and leukotropic HCMV strain VR1814, with peripheral blood mononuclear cells. Then, both activated $CD4^+$ and $CD8^+$ T-cells were quantified by cytokine flow cytometry analysis of intracellular IFN-γ production (Lozza *et al.,* 2005). Laboratory-adapted HCMV strains were found unable to infect DC (Gerna *et al.,* 2005). The CFC-iDC cut-off predicting protection from HVL infection was found to be 0.4 HCMV-specific $CD4^+$ and $CD8^+$ T-cells/μL blood (Lozza *et al.,* 2005, Gerna *et al.,* 2011a, 2011b). The QF-CMV cut-off was 0.2 IFN-γ IU/mL, as reported by the manufacturer.

Statistical analysis

HCMV-specific $CD4^+$ and $CD8^+$ T-cells measured by CFC-iDC in KTR with LVL infection were compared with the relevant T-cell subset in patients with HVL infection by the Mann-Whitney U test. The same test was used to compare levels of IFN-γ detected by QF-CMV in the two patient groups. The chi-square test was performed to compare the prognostic performances of the two assays. The times of HCMV-specific T-cell immunity reconstitution, HCMV DNA appearance, peak, and disappearance were evaluated using Kaplan-Meier curves, which were compared by the log-rank (Mantel-Cox) test.

RESULTS

Clinical characteristics of the patients enrolled

The 53 KTR analyzed had a median age of 53 (21-70) years. The median follow-up duration was 190 (140-240) days. As illustrated in *Table 1*, 33 patients (62.3%) underwent a LVL self-resolving HCMV infection (protected patients), while 19 patients (35.8%) had a HVL systemic infection requiring antiviral therapy (non-protected patients). The majority of this subgroup (12/19, 63.1%) underwent a single episode of severe virus reactivation that resolved with antiviral therapy, while a minority developed relapsing infections requiring additional courses of treatment (4/19, 21.1%) or TID (gastrointestinal disease or pneumonia) concomitantly with HVL systemic infection (3/19, 15.8%). A single patient (1.9%) had gastrointestinal TID alone.

Protection from severe HCMV infection

The maximum sensitivity (capacity to identify protected patients) and specificity (capacity to identify non-protected patients) of the two assays were determined at the DNAemia peak (*Table 2*). In general, prognostic performances of the QF-CMV assay were slightly less efficient compared to CFC-iDC. However, these differences were not statistically significant (P>0.05).

Discrimination of HVL and LVL patients by the two assays

At the DNAemia peak, the number of HCMV-specific $CD4^+$ and $CD8^+$ T-cells detected by CFC-iDC in LVL patients was significantly higher than in HVL patients (*Figure 1A*). The same trend (higher IFN-γ levels in LVL patients) was observed with QF-CMV (*Figure 1B*). At the end of follow-up, this difference was markedly reduced (*Figure 1C* and *D*). However, while the great majority of LVL patients were above the immunological cut-off throughout the follow-up

Table 1 - *Clinical characteristics of the kidney transplant recipients enrolled.*

No. patients	*No. (%) patients with LVL infection*	*No. (%) patients with treated HVL infection*			*No. (%) TID in the absence of HVL infection**
		systemic	*syst. + TID*	*relapsing*	
53	33 (62.3)	12 (22.6)	3 (5.7)	4 (7.5)	1 (1.9)

LVL, Low Viral Load; HVL, High Viral Load; TID, Tissue Invasive Disease; syst., systemic. *Presence of systemic specific T-cell immunity.

Table 2 - *Prognostic performances of the two assays at DNAemia peak.*

*Parameter (%)**	*CFC-iDC*	*QF-CMV*
sensitivity	91	85
specificity	84	63
PPV	91	80
NPV	84	71

*Chi square test (P>0.05 for all 4 parameters); CFC-iDC, cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV; PPV, positive predictive value; NPV, negative predictive value.

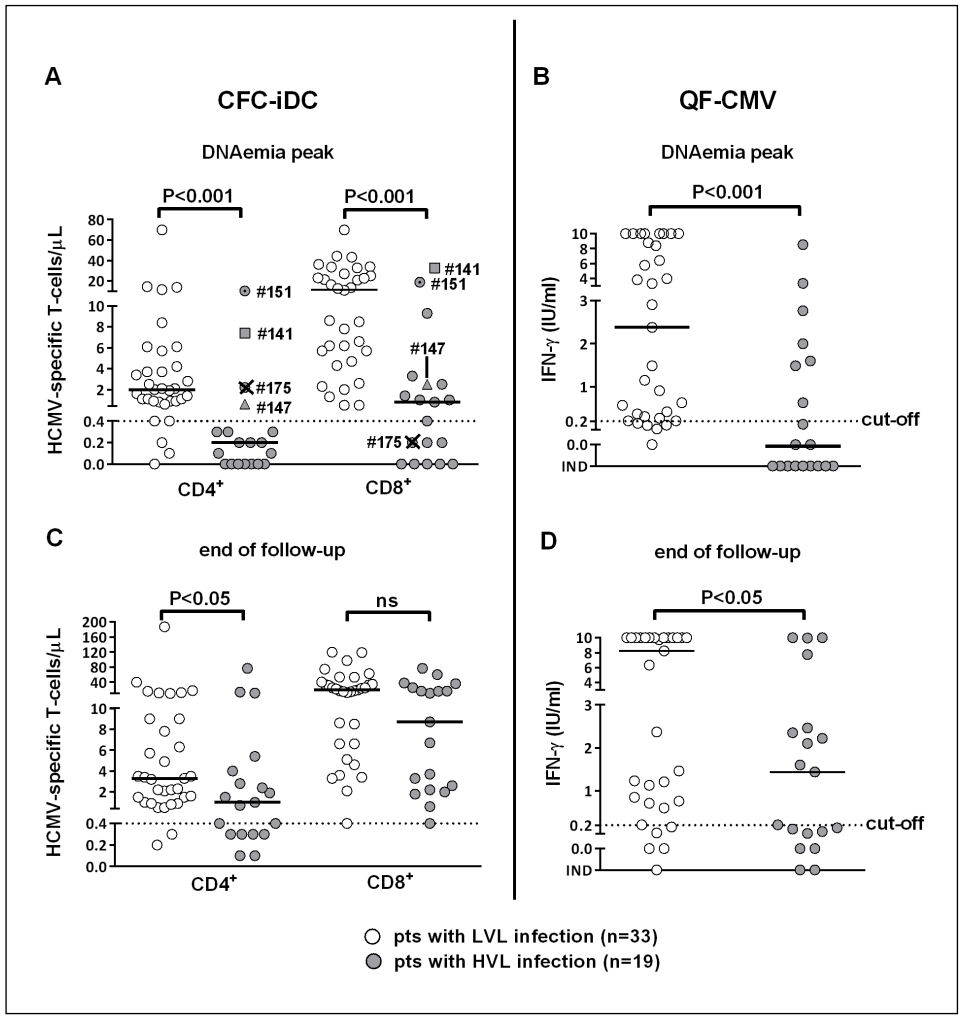

Figure 1 - *HCMV-specific T-cell response (**A, B**) at the DNAemia peak and (**C, D**) at the end of follow-up by (**A, C**) CFC-iDC and (**B, D**) QF-CMV, in KTR with HVL and LVL infection. Both assays revealed a significantly lower level of T-cell response in patients with HVL infection. This difference was markedly reduced at the end of follow-up with both assays. Dotted lines indicate cut-offs for both immune assays. Fig.1A reports patient codes #141, #147, and #151 (see Table 4), and #175 (see Table 5).*
CFC-iDC, cytokine flow cytometry using HCMV-infected dendritic cells as a stimulus; QF-CMV, QuantiFERON-CMV; IFN-γ, interferon- gamma; pts, patients; HVL, high viral load; LVL, low viral load; IND, indeterminate result (<0.2 INF-γ IU/mL in the CMV tube and <0.5 IU/mL in the mitogen tube); IU, international units.

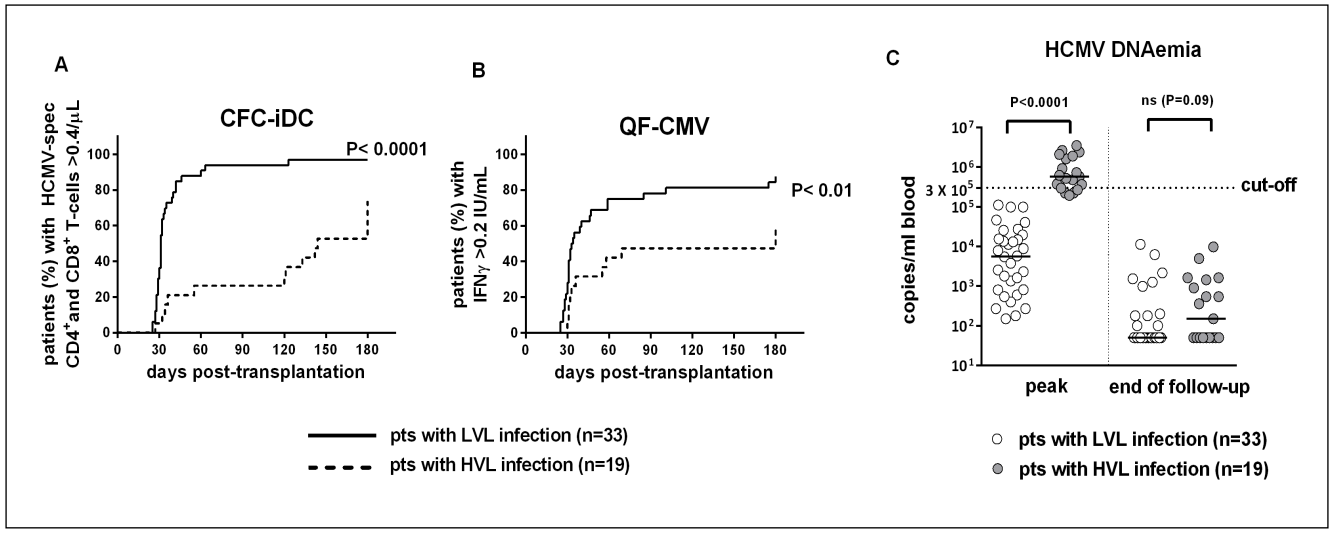

Figure 2 (A) and **(B)** - *Kaplan-Meier curves. The time required to reach protective (according to established cut-offs) levels of HCMV-specific T-cell immune responses in KTR with HVL infection was significantly greater compared to patients with LVL infection. This difference is greater for CFC-iDC than QF-CMV (P<0.0001* vs *P<0.01). (C): HCMV viral load at DNAemia peak was above the cut-off of 3x10^5 copies/mL blood in KTR with HVL infection and below in patients with LVL infection. At the end of follow-up, no significant difference was detected between the two patient groups, both below the DNAemia cut-off.*
CFC-iDC, cytokine flow cytometry using infected dendritic cells as a stimulus; pts, patients; HVL, high viral load; LVL, low viral load; QF-CMV, QuantiFERON-CMV

with both assays, the number of HVL patients reaching the relevant immunological cut-off at the end of follow-up shifted from 3/19 (16%) to 13/19 (68%) by CFC-iDC with a >50% rise, while a rise by QF-CMV was limited to 21% (from 7/19, 37% to 11/19, 58%).

Kinetics of immune reconstitution and DNAemia in LVL and HVL patients

Both CFC-iDC and QF-CMV confirmed the significantly delayed reconstitution of immunity in HVL compared to LVL patients, as illustrated by the Kaplan-Meier curves (*Figure 2 A, B,* and *Table 3*).

In addition, in patients with HVL infection, DNAemia appeared and peaked significantly earlier, while the median DNAemia peak was about 100 times higher (*Table 3* and *Figure 2C*), whereas the median time to DNAemia disappearance was not significantly different (*Table 3*, P=0.15). Finally, a marked decrease in DNAemia levels was observed in both patient groups at the end of follow-up (*Figure 2C*).

Table 3 - *DNAemia and HCMV-specific immune response kinetics according to the two assays.*

Patients with	*DNAemia appearance*		*DNAemia peak*		*DNAemia disappearance*	*Median days to reach protective cut-off (range)*	
HCMV infection	*median days (range)*	*median VL (copies/mL blood, range)*	*median days (range)*	*median VL (copies/mL blood, range)*	*median days (range)*	*CFC-iDC*	*QF-CMV*
LVL	33 (5-167)	270 (90-19,500)	69 (5-181)	5,600 (150-111,240)	182 (17->360)	31 (25->180)	33 (25->180)
HVL	15 (6-61)	540 (90-6,000)	57 (28-93)	576,150 (193,950-3,500,000)	234 (105->360)	142 (27->180)	>180 (30->180)
P	<0.01	0.17	0.04	<0.0001	0.15	<0.0001	<0.01

VL, viral load; HVL, high viral load; LVL, low viral load; CFC-iDC, cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV.

Table 4 - *Patients with systemic HVL infection and apparently protective HCMV immune response according to the CFC-iDC assay (cut-off: 0.4 CD4⁺/CD8⁺ T-cells/μL).*

Pt. code	*CFC-iDC (HCMV-specific T-cells /μL)*		*QF-CMV [IFN-γ (IU/mL)]*	*Systemic HCMV HVL infection*			*TID (time, site and HCMV-DNA copies)*	*Induction/ immunosuppression regimen*
	CD4⁺	*CD8⁺*		*DNAemia peak (days post-tx)*	*Peak DNAemia level (copies/mL blood)*	*days of antiviral treatment duration (days)*		
#141	7.4	32.8	0.00	93	576,000	82	At day 93, stomach: >1x10⁶ copies/ 10⁵ cells	anti CD-25 + MPRE/ FK506+MMF+MPRE
#147	0.6	2.5	0.00	47	482,000	48	no	anti CD-25 + MPRE/ CyA+MMF+MPRE
#151	8.5	25.1	0.00	63	221,000	83	no	ATG+MPRE/ FK506+MMF+MPRE

Pt., Patient; HVL, high viral load; TID, tissue-invasive disease; CFC-iDC, cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV; ATG, anti-thymocyte globulin; MPRE, methylprednisolone; MMF, mofetil mycophenolate; CyA, cyclosporine A; FK506, Tacrolimus.

Table 5 - *Patients with HVL infection and apparently protective immune response according to the QF-CMV assay (cut-off: 0.2 IFN-γ IU/mL).*

Pt. code	*CFC-iDC (HCMV-specific T-cells /μL)*		*QF-CMV [IFN-γ (IU/mL)]*	*Systemic HCMV HVL infection*			*Induction/ immunosuppression regimen*
	CD4⁺	*CD8⁺*		*days post-tx*	*HCMV DNAemia (copies/mL blood)*	*Days of antiviral treatment duration*	
#152	0.1	9.3	0.63	57	519,750	67	anti CD-25 + MPRE/ CyA+MMF+MPRE
#160	0.3	1.0	2.77	91	367,650	42	anti CD-25 + MPRE/ CyA+MMF+MPRE
#163	0.3	6.2	>10.00	65	2.6x10⁶	50	anti CD-25 + MPRE/ CyA+MMF+MPRE
#169	0.3	0.2	3.34	76	2.1 x10⁶	76	anti CD-25 + MPRE/ FK506+MMF+MPRE
#175	2.2	0.2	1.49	73	178,740	19	anti CD-25 + MPRE/ CyA+MMF+MPRE
#193	0.1	1.4	1. 80	69	620,100	131	anti CD-25 + MPRE/ FK506+MMF+MPRE
#197	0.1	0.4	1.60	91	727,000	63	anti CD-25 + MPRE/ FK506+MMF+MPRE

Pt., Patient; HVL, high viral load; CFC-iDC: cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV; ATG, anti-thymocyte globulin; MPRE, methylprednisolone; MMF, mofetil mycophenolate; CyA, cyclosporine A; FK506, Tacrolimus.

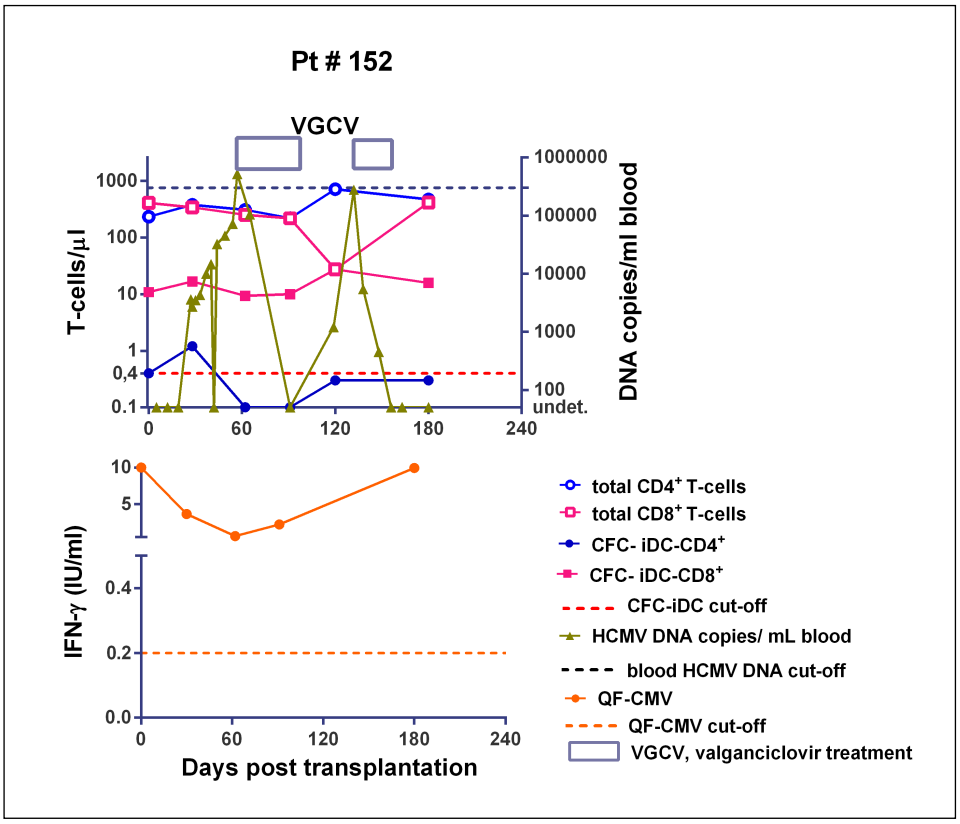

Figure 3 - *The case of a patient (Pt. #152) erroneously identified as protected from severe HVL HCMV infection by QF-CMV is shown. The high level of specific immune response was not limited to the DNAemia peak, but extended throughout the entire follow-up period. However, this patient underwent recurrent severe HCMV infectious episodes requiring antiviral therapy. Conversely, the CFC-iDC assay correctly identified the lack of protective HCMV-specific CD4⁺ T-cell response during the entire follow-up period, except for a single time-point. Broken lines indicate immune cut-offs for each assay.*
CFC-iDC, cytokine flow cytometry using HCMV-infected dendritic cells as a stimulus; QF-CMV, QuantiFERON-CMV; VGCV, Valganciclovir; IU, international units.

Discrepancies between the immune response detected by the two assays and HVL infection

CFC-iDC misidentified 3/19 (16%) patients (#141, #147, #151) with HVL infection as protected (*Figure 1* and *Table 4*). These patients were correctly considered at risk for severe infection by QF-CMV assay. In addition, patient #175 (*Figure 1* and *Table 5*) had HCMV-specific CD4⁺ but not CD8⁺ T-cells above the cut-off (and, thus, was considered non-protected). In the great majority of patients, specific CD4⁺ were flanked by specific CD8⁺ T-cells. Instead, when specific CD8⁺ appeared earlier than specific CD4⁺ T-cells, patients appeared to be non-protected until reconstitution of specific CD4⁺ T-cells.

On the other hand, QF-CMV misidentified 7/19 (37%) patients with HVL infection as protected (*Table 5*), due to an HCMV-specific immune response far above the QF-CMV cut-off for the majority (6/7) of them throughout the follow-up (see as an example Pt. #152, *Figure 3*). However, CFC-iDC correctly identified all of these patients at risk for HVL infection (*Table 5*).

As for LVL patients, at the DNAemia peak, 3/33 (9%) were indicated as non-protected by CFC-iDC *vs* 5/33 (15%) by QF-CMV (*Figure 1*). However, none of these patients underwent severe infection episodes requiring treatment.

TID

Three patients with HVL developed TID (gastrointestinal and pneumonia) concomitantly with the peak of systemic infection (*Table 1*).

The single case of TID (gastrointestinal disease) detected among LVL patients in this study confirms that local tissue infection ($5x10^5$ DNA copies/$1x10^5$ *antrum* cells) may occur in the absence of HVL (DNAemia undetectable at time of organ infection) and in the presence of a valid systemic T-cell response detected along the duration of infection by both CFC-iDC (HCMV-specific CD4⁺ T-cells 1-8 /µL, and CD8⁺ T-cells >10/µL) and QF-CMV (consistently >10 IFN-γ IU/mL).

HLA typing and immunological findings

HLA-A and B alleles were recovered for 52/53 patients and matched with those included in the QF-CMV panel (Walker *et al.*, 2007). Among the 8 patients with less than 2 matching alleles, only 3 (37.5%) had a specific immune response for at least one time-point, whereas of the 44 patients with ≥2 alleles, as many as 40 (91%) had a positive immune response (*Table 6*).

Overall, 9/52 (17.3%) patients never had a positive response with QF-CMV throughout the follow-up (see as an example Pt. #182, *Figure 4*), whereas CFC-iDC detected

Table 6 - *HLA alleles and immune response according to the two assays.*

HLA-A and -B allele no. shared with those included in the QF-CMV panel	*Pt. no. (%)**	*Pt. no. (%) with HCMV-specific immune response***	
		CFC-iDC	*QF-CMV*
≥ 2	44 (85)	44 (100)	40 (91)
<2	8 (15)	8 (100)	3 (37)
total	52	52	43

Pt., patient; CFC-iDC, cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV;
*, HLA typing unknown for one patient;
**, at least at one time-point during follow-up.

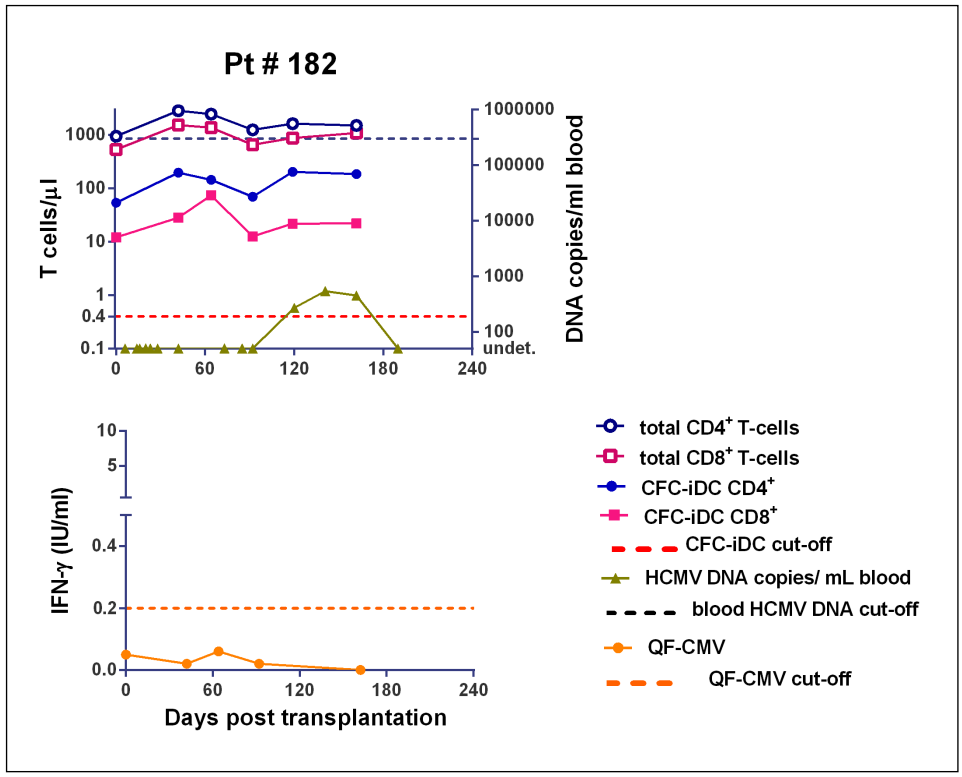

Figure 4 - *The case of patient #182 (sharing only one HLA allele with those included in the QF-CMV assay panel), which was identified as non-protected by QF-CMV and as protected by CFC-iDC, is shown. These different immune response levels were confirmed throughout the follow-up period by both assays. Indeed, the patient resolved the HCMV infection without antiviral therapy. Broken lines indicate immune cut-offs for each assay.*
CFC-iDC, cytokine flow cytometry using HCMV-infected dendritic cells as a stimulus; QF-CMV, QuantiFERON-CMV; IU, international units.

HCMV-specific CD4+ and CD8+ T-cells above the relevant cut-offs in all 9 patients at least once during follow-up.

DISCUSSION

The objective of this study was to compare the prognostic performances of two immunological assays investigating the T-cell response to HCMV infection in a group of HCMV-seropositive KTR. As mentioned above, the in-house assay (CFC-iDC) was developed more than 10 years ago (Lozza *et al.,* 2005), while the QF-CMV assay is commercially available (Walker *et al.,* 2007).
There are some major differences between the two assays:

1) CFC-iDC detects both HCMV-specific CD4+ and CD8+ T-cells, whereas QF-CMV detects primarily CD8+ T-cells;
2) CFC-iDC unlike QF-CMV assay results with any HLA type;
3) CFC-iDC requires 6-7 days, whereas QF-CMV requires only 2 days to complete;
4) CFC-iDC requires a flow cytometer and fresh or thawed PBMCs, whereas QF-CMV assay utilizes whole blood to stimulate T-cells prior to measuring by ELISA IFN-γ released in plasma.

Patients were divided into two groups: those (n=33, 62%) with a DNAemia level below the cut-off for pre-emptive therapy (LVL patients); and those (n=19, 36%) with a peak DNAemia level above the cut-off (HVL patients) (Lilleri *et al.,* 2004, Gerna *et al.,* 2011a, 2011b). According to the protocol currently in use in our Department, all of the latter patients were treated with antiviral therapy prior to the onset of clinical symptoms (Gerna *et al.,* 1998) and until DNAemia disappearance. The DNAemia cut-off was originally established by showing that, in comparison with the previously defined antigenemia cut-off of 100 pp65-pos/2x10^5 PBL, it could provide positive and negative predictive values as robust as those provided by antigenemia (Lilleri *et al.,* 2004; Gerna *et al.,* 2007).
As for the HCMV-specific T-cell cut-offs, a ROC analysis in a prospective study monitoring HCMV-specific CD4+ and CD8+ T-cells in SOTR indicated that the cut-offs of 0.4 T-cells/µL blood for both T-cell subsets provided maximum predictive values (Gerna *et al.,* 2006a). These cut-offs are currently in use in our Department, and similar values have been reported by others (Benmarzouk-Hidalgo *et al.,* 2011). This study found a statistically significant difference with CFC-iDC assay when considering LVL and HVL patient groups for both HCMV-specific CD4+ and CD8+ T-cells, which were consistently higher in number in the LVL group. A similar statistically significant difference was observed with QF-CMV assay when considering the plasma levels of IFN-γ. However, in the case of discrepant results, one assay consistently identified as at risk for HVL infection all patients misidentified as protected by the other assay, thus suggesting that in case of discrepancy between immunological results of either assay and viral load level, the alternative assay could help clarify the discrepancy.
As for the role of HCMV-specific CD4+ and CD8+ T-cells in protecting against HCMV disease in transplant recipients, a correlation between the presence of HCMV-specific T-cells and absence of HCMV disease was reported in several clinical studies (Sester *et al.,* 2002; Sester *et al.,* 2005; Gerna *et al.,* 2006; Mattes *et al.,* 2008; Kumar *et al.,* 2009). Some studies reported a pivotal role for HCMV-specific CD4+ T-cells in the control of HCMV infection (Sester *et al.,* 2001). Using peptide-conjugated MHC class I tetramers, a correlation between CD8+ T-cells and protection has been reported (Gratama *et al.,* 2001, 2010), whereas other studies have questioned this conclusion (Crough *et al.,* 2007). During the last decade, we have repeatedly confirmed in SOTR that

protection from HCMV disease is first guided by specific $CD4^+$ T-cells, while specific $CD8^+$ T-cells complete protection with their cytotoxic activity (Gabanti *et al.,* 2014).

In the present study, CFC-iDC quantified both HCMV-specific $CD4^+$ and $CD8^+$ T-cell subsets, thus allowing risk prediction for HCMV disease in the presence of HVL, whereas QF-CMV primarily detected specific $CD8^+$ T-cells, and appears slightly less specific in predicting HCMV disease risk. In other words, in the absence of specific $CD4^+$ T-cell reconstitution, specific $CD8^+$ T-cells do not protect from HCMV HVL infection (Gabanti *et al.,* 2014). In most patients of this study, a level of specific $CD4^+$ T-cells below the relevant immune cut-off in the presence of a specific $CD8^+$ T-cells level markedly above the relevant immune cut-off did not protect against HVL. Thus, the number of specific T-cells of either $CD4^+$ or $CD8^+$ subset above the immune cut-off was not able *per se* to protect most patients from HVL infection, in the absence of the number of specific T-cells of the other subset above the relevant immune cut-off.

As expected, the time to reach immune reconstitution was comparable and relatively short for both CFC-iDC and QF-CMV in LVL patients, whereas this time was markedly delayed with both assays in HVL patients. Overall, while the median time to DNAemia appearance and peak was significantly shorter in patients with HVL, the time to DNA disappearance was not significantly different from that of LVL patients (P=0.15).

In 5/8 (63%) patients sharing fewer than 2 alleles with the QF-CMV panel, detection of the immune response was precluded, while in patients sharing ≥2 alleles only 4/44 (9%) patients yielded negative results. These findings suggest that, as expected, allele mismatching may markedly impact on QF-CMV results. The basis for the immune response in the 3 patients with one or no allele matches may reside in the partial cross-stimulation of $CD4^+$ T-cells by the peptide pool (Gabanti *et al.,* 2016), or in an excess expansion of a few HCMV-specific $CD8^+$ T-cell clones recognizing the HLA-matched peptide present in the QF-CMV assay.

Finally, it is worthwhile mentioning the case of TID that was associated with LVL infection. The peculiarity of HCMV infection in a case like this resides in the uniqueness of a severe local infection occurring in the absence of virus dissemination and in the presence of an efficient T-cell response (Gerna *et al.,* 2012, Gabanti *et al.,* 2015). A similar occurrence in hematopoietic stem cell transplant recipients was previously reported (Gabanti *et al.,* 2015).

In conclusion, both immunological assays appear able to monitor HCMV infection satisfactorily in the great majority of KTR. CFC-iDC assay appears slightly preferable to the QF-CMV since:

1) it shows a slightly higher (but not significantly) specificity;
2) it yields fewer discrepant results;
3) due to the patient HLA-A and -B mismatching with alleles included in the QF-CMV assay panel, CFC-iDC detected HCMV-specific $CD4^+$ and $CD8^+$ T cells in all 9 patients never yielding a positive response by QF-CMV throughout the post-transplant period.

Finally, both immunological techniques may be used as an adjunct measurement in pre-emptive therapy decision-making, and namely in predicting new HCMV infection episodes in the post-transplant period. Result concordance between the two immunological assays represents the most reliable parameter in predicting either protection from or risk for HCMV HVL infection.

Abbreviations

KTR, kidney transplant recipients; LVL, low viral load; HVL, high viral load; CFC-iDC, cytokine flow cytometry-infected dendritic cells; QF-CMV, QuantiFERON-CMV; SOTR, solid-organ transplant recipients; TID, tissue invasive disease

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Competing interests

The authors have no conflicts of interests to declare.

Ethical approval

The Study protocol was approved by the Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo (Procedure 20100005459).

References

Abate D., Saldan A., Mengoli C., Fiscon M., Silvestre C., et al. (2013). Comparison of cytomegalovirus (CMV) enzyme-linked immunosorbent spot and CMV quantiferon gamma interferon-releasing assays in assessing risk of CMV infection in kidney transplant recipients. *J. Clin. Microbiol*. **51**, 2501-2507.

Benmarzouk-Hidalgo O.J., Cisneros J.M., Cordero E., Martin-Pena A., Sanchez B., et al. (2011). Therapeutic effect of the acquisition of cytomegalovirus-specific immune response during preemptive treatment. *Transplantation.* **91**, 927-933.

Calarota S.A., Chiesa A., Scaramuzzi L., Adzasehoun K.M., Comolli G., et al. (2014). Normalizing ELISPOT responses to T-cell counts: a novel approach for quantification of HCMV-specific CD4(+) and CD8(+) T-cell responses in kidney transplant recipients. *J. Clin. Virol.* **61**, 65-73.

Crough T., Fazou C., Weiss J., Campbell S., Davenport M.P., et al. (2007). Symptomatic and asymptomatic viral recrudescence in solid-organ transplant recipients and its relationship with the antigen specific CD8(+) T-cell response. *J. Virol.* **81**, 11538-42.

Gabanti E., Bruno F., Lilleri D., Fornara C., Zelini P., et al. (2014). Human cytomegalovirus (HCMV)-specific $CD4^+$ and $CD8^+$ T-cells are both required for prevention of HCMV disease in seropositive solid-organ transplant recipients. *PLoS One.* **9**, e106044.

Gabanti E., Lilleri D., Ripamonti F., Bruno F., Zelini P., et al. (2015). Reconstitution of human cytomegalovirus-specific $CD4^+$ T-cells is critical for control of virus reactivation in hematopoietic stem cell transplant recipients but does not prevent organ infection. *Biol. Blood Marrow Transplant.* **21**, 2192-202.

Gabanti E., Bruno F., Scaramuzzi L., Mangione F., Zelini P., et al. (2016). 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. *New Microbiol.* **39**, 247-58.

Gerna G., Zavattoni M., Baldanti F., Sarasini A., Chezzi L., et al. (1998). Human cytomegalovirus (HCMV) leukoDNAemia correlates more closely with clinical symptoms than antigenemia and viremia in heart and heart-lung transplant recipients with primary infection, *Transplantation.* **65**, 1378-85.

Gerna G., Lilleri D., Fornara C., Comolli G., Lozza L., et al. (2006a). Monitoring of human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T-cell immunity in patients receiving solid organ transplantation. *Am. J. Transplant.* **6**, 2356-64.

Gerna G., Vitulo P., Rovida F., Lilleri D., Pellegrini C., et al. (2006b). Impact of human metapneumovirus and human cytomegalovirus versus other respiratory viruses on the lower respiratory tract infections of lung transplant recipients. *J. Med. Virol.* **78**, 408-16. Erratum in: *J. Med. Virol.* (2008) **80**, 1869.

Gerna G., Baldanti F., Torsellini M., Minoli L., Viganò M., et al. (2007). Evaluation of human cytomegalovirus DNAemia *vs* pp65-antigenemia cutoff for guiding preemptive therapy in transplant recipients: A randomized study. *Antiviral Therapy.* **12**, 63-72.

Gerna G., Lilleri D., Chiesa A., Zelini P., Furione M., et al. (2011a). Virologic and immunologic monitoring of cytomegalovirus to guide preemptive therapy in solid-organ transplantation. *Am. J. Transplant.* **11**, 2463-71.

Gerna G., Lilleri D., Furione M., Baldanti F. (2011b). Management of human cytomegalovirus infection in transplantation: validation of virologic cut-offs for preemptive therapy and immunological cut-offs for protection. *New Microbiol.* **34**, 229-54.

Gerna G., Lilleri D., Furione M., Castiglioni B., Meloni F., et al. (2012). Human cytomegalovirus end-organ disease is associated with high or low systemic viral load in pre-emptively treated solid-organ transplant recipients. *New Microbiol*. **35**, 279-87.

Gerna G., Percivalle, Lilleri D., Lozza L., Fornara C., et al. (2005). Dendritic cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to $CD8^+$ T cells. *J. Gen. Virol*. **86**, 275-.84.

Gratama J.W., Van Esser J.W., Lamers C.H., Tournay C., Lowenberg B., et al. (2001). Tetramer-based quantification of cytomegalovirus (CMV)-specific $CD8^+$ T lymphocytes in T-cell depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood.* **98**, 1358-64.

Gratama J.W., Boeckh M., Nakamura R., Cornelissen J.J., Brooimans R.A., et al. (2010). Immune monitoring with iTAg MHC tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. *Blood.* **116**, 1655-62.

Khoury J.A., Storch G.A., Bohl D.L., Schuessler R.M., Torrence S.M., et al. (2006). Prophylactic *versus* preemptive oral valganciclovir for the management of cytomegalovirus infection in adult renal transplant recipients. *Am. J. Transplant.* **6**, 2134-43.

Kumar D., Chernenko S., Moussa G., Cobos I., Manuel O., et al. (2009). Cell-mediated immunity to predict cytomegalovirus disease in high-risk solid organ transplant recipients. *Am. J. Transplant.* **9**, 1214-22.

Lilleri D., Baldanti F., Gatti M., Rovida F., Dossena L., et al. (2004). Clinically-based determination of safe DNAemia cutoff levels for preemptive therapy of human cytomegalovirus infections in solid organ and hematopoietic stem cell transplant recipients. *J Med Virol*. **73**, 412-8.

Lilleri D., Gerna G., Fornara C., Lozza L., Maccario R., et al. (2006). Prospective simultaneous quantification of human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. *Blood.* **108**, 1406-12.

Lilleri D., Fornara C., Furione M., Zavattoni M., Revello M.G., et al. (2007). Development of human cytomegalovirus-specific T-cell immunity during primary infection of pregnant women and its correlation with virus transmission to the fetus. *J. Infect. Dis.* **195**, 1062-70.

Lilleri D., Fornara C., Chiesa A., Caldera D., Alessandrino E.P., et al. (2008). Human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection. *Haematologica.* **93**, 248-56.

Lilleri D., Zelini P., Fornara C., Comolli G., Revello M.G., et al. (2009). Human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T-cell responses in primary infection of the immunocompetent and the immunocompromised host. *Clin. Immunol.* **131**, 395-403.

Limaye A.P., Bakthavatsalam R., Kim H.W., Randolph S.E., Halldorson J.B., et al. (2006). Impact of cytomegalovirus in organ transplant recipients in the era of antiviral prophylaxis. *Transplantation* **81**, 1645-52.

Lisboa L.F., Kumar D., Wilson L.E., Humar A. (2012). Clinical utility of cytomegalovirus cell-mediated immunity in transplant recipients with cytomegalovirus viremia. *Transplantation.* **93**, 195-200.

Ljungman P, Boeckh M, Hirsch HH, Josephson F, Lundgren J, et al. (2017). Disease definitions working Group of the Cytomegalovirus Drug Development Forum. *Clin. Infect. Dis.* **64**, 87-91.

Lozza L., Lilleri D., Percivalle E., Fornara C., Comolli G., et al. (2005). Simultaneous quantification of human cytomegalovirus (HCMV)-specific $CD4^+$ and $CD8^+$ T-cells by a novel method using monocyte-derived HCMV-infected immature dendritic cells. *Eur. J. Immunol.* **35**, 1795-804.

Manuel O., Husain S., Kumar D., Zayas C., Mawhorter S., et al. (2013). Assessment of cytomegalovirus-specific cell-mediated immunity for the prediction of cytomegalovirus disease in high-risk solid-organ transplant recipients: a multicenter cohort study. *Clin. Infect. Dis.* **56**, 817-24.

Mattes F.M., Vargas A., Kopycinski J., Hainsworth E.G., Sweny P., et al. (2008). Functional impairment of cytomegalovirus specific CD8 T-cells predicts high-level replication after renal transplantation. *Am. J. Transplant.* **8**, 990-9.

Revello M.G., Lilleri D., Zavattoni M., Furione M., Genini E., et al. (2006). Lymphoproliferative response in primary human cytomegalovirus (HCMV) infections is delayed in HCMV transmitter mothers. *J. Infect. Dis.* **193**, 269-76.

Sacre K., Carcelain G., Cassoux N., Fillet A.M., Costagliola D., et al. (2005). Repertoire, diversity, and differentiation of specific CD8 T-cells are associated with immune protection against human cytomegalovirus disease. *J. Exp. Med.* **201**, 1999-2010.

Sester M., Sester U., Gärtner B., Heine G., Girndt M., et al. (2001). Levels of virus-specific CD4 T-cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation.* **71**, 1287-94.

Sester M., Sester U., Gartner B.C., Girndt M., Meyerhans A., et al. (2002). Dominance of virus-specific CD8 T-cells in human primary cytomegalovirus infection. *J. Am. Soc. Nephrol.* **13**, 2577-84.

Sester U., Gartner B.C., Wilkens H., Schwaab B., Wossner R., et al. (2005). Differences in CMV-specific T-cell levels and long-term susceptibility to CMV infection after kidney, heart and lung transplantation. *Am. J. Transplant.* **5**, 1483-9.

Walker S., Fazou C., Crough T., Holdsworth R., Kiely P., et al. (2007). Ex vivo monitoring of human cytomegalovirus-specific CD8 T-cell responses using QuantiFERON-CMV. *Transpl. Infect. Dis.* **9**, 165-70.