Pre-transplant assessment of CMV-specific immune response by Elispot assay in kidney transplant recipients

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SUMMARY

Cytomegalovirus (CMV) primary infection or re-activation in solid organ transplant (SOT) recipients is associated with increased morbidity and mortality, with patients with IgG-CMV D+/R- sero-matching at greater risk.
The impact of pre-transplant CMV-specific host cellular immunity on the long-term risk of CMV replication in kidney transplants (KT) was prospectively evaluated in eighty patients by CMV-EliSpot assay. The study population included 54 male and 26 female recipients, with CMV-IgG distribution: 60 D+/R+, 11 D-/R+, 7 D+/R-, 2 D-/R-.
At pre-transplantation, 49 KT (61.3%) were CMV-responders by EliSpot. At 3-month follow up, 16 (32.7%) out of 49 CMV-responders showed CMV blood infection, compared to 8 (25.8%) out of 31 non-responders. No further episode of CMV viraemia was reported in the responder group, in comparison to 15 out 31 non-responders (48.4%) showing at least one episode of CMV-DNAemia at 12-month follow-up. Baseline CMV-IgG serology showed a strong correlation with EliSpot determinations; KT recipients exhibiting at least one episode of CMV viraemia at 12-month follow-up showed lower baseline CMV-EliSpot values than those without signs of CMV replication.
The study suggests that monitoring CMV-specific T-cell responses at pre-transplantation by EliSpot assay may be useful for predicting the post-transplantation risk of CMV infection and reactivation.

KEY WORDS: Cytomegalovirus, Solid organ transplant, Immunosuppression, Kidney transplantation, Opportunistic infections, Cellular immune response.

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INTRODUCTION

Solid organ transplant recipients (SOT) are at increased risk for Cytomegalovirus (CMV) infection in the post-transplant period, and CMV reactivation may result in asymptomatic viraemia or symptomatic CMV disease. Despite advances in management strategies, CMV infection remains one of the most common complications affecting SOT recipients, with significant morbidity and occasionally mortality (Kotton 2010) (Lisboa 2012). In kidney transplant (KT) recipients, CMV infection and disease have been reported in 8-32% and 8%, respectively. Furthermore, CMV has been associated with indirect effects, including rejection, chronic nephropathy, and increased opportunist infections (Egli 2007) (Fishman, 2007) (Snydman 2011). However, in recent years the development of antiviral prophylaxis and pre-emptive therapeutic management strategies has significantly reduced the mortality due to CMV and impacted positively on morbidity

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(Mattes 2008). One of the most robust diagnostic tools to monitor CMV infection in the transplantation setting is DNAemia evaluation. However, several open questions still exist, and discrepancies in approach are reported among different transplant centres.

It is generally accepted that the host's CMV-specific immunity plays a critical role in the development and severity of CMV disease. Both B- and T-lymphocytic adaptive immune responses have been shown to be critical in controlling CMV replication (Kotton 2010), and the analysis of CMV-specific T-cell frequencies and function could allow direct assessment of the patient's ability to control CMV (Egli, Humar, and Kumar, 2012). A research major focus has currently concentrated on evaluating the diagnostic usefulness of measuring the CMV-specific cell-mediated immune (CMI) response by assessing the phenotype and function of CMV-specific T-cells in the post-transplant setting, as an adjuvant to monitoring CMV-DNAemia.

Specific cellular immune responses may be measured by Interferon (IFN)-γ releasing assays (IGRA), including the EliSpot test, that investigates the frequency of IFN-γ secreting T-cells in response to CMV peptides (Abate 2010) (Bunde 2005) (Costa 2014a) (Egli, Humar, and Kumar, 2012) (Fleming, Dunne, and Crowley, 2010) (Zhang 2009).

Many studies have reported the relationship between post-transplant functional impairment of CD8+ T-cell immunity and failure to suppress CMV replication after SOT. However, most studies have focused on evaluation of immune responses in the post-transplant period (Benmarzouk-Hidalgo 2011) (Cantisan 2013) (Costa 2012) (Mattes 2008). Few studies addressed whether pre-transplant functional impairment of CMV-specific T-cells is associated with the risk of post-transplant CMV replication (Abate2010) (Bestard 2013) (Bunde 2005) (Cantisan 2013). Furthermore, the current serological risk stratification for CMV infection in SOT - based on the CMV-specific antibody (IgG) serostatus of donor (D) and transplant recipient (R) - has some limitations, since CMV is known to reactivate in some CMV-IgG seropositive recipients after transplantation (Bestard 2013) (Lucia 2014).

Main objective of the present study was to evaluate the prognostic impact of pre-transplant CMV-specific T-cell responses in KT recipients, using an IFN-γ Elispot assay, for predicting the advent of post-transplant CMV infection in a cohort of 80 KT recipients.

MATERIALS AND METHODS

Eighty adult kidney transplant recipients undergoing kidney transplantation at "Città della Salute e della Scienza di Torino", University Hospital of Turin, were prospectively evaluated from January 2010 through November 2012. Immunosuppressant protocols included cyclosporine A, tacrolimus, mycophenolate mofetil, mTOR inhibitors (sirolimus, everolimus) and steroids. Patients' demographic and clinical data are summarized in Table 1.

CMV surveillance was routinely performed on whole blood samples by means of quantitative real time PCR assay. DNA extraction was with the automated QIAsymphony extraction system (Qiagen, Germany) from 200 µL whole blood, and a region of the exon 4 of MIEA (major immediate early antigen) of CMV was amplified (Q-CMV Real Time Complete Kit, ELITechGroup, Italy) on a 7500 Real-Time thermo-cycler system (Applied Biosystems, USA) as previously described. Results were expressed as equivalent viral copies/mL whole blood. A detection limit for linearity of 2,000 copies/mL was accepted. According to our centre's practice, virological monitoring was performed twice weekly in the first month, twice monthly up to 3 months, every 3 months up to 1 year, and yearly thereafter. Further determinations were based on clinical suspicion and occurrence of CMV infections (Costa 2014a) (Rittà 2013).

CMV-specific cellular immunity levels were determined by EliSpot assay (Costa 2014b) (Rittà 2013). Briefly, from 10 mL of whole blood an aliquot of $2X10^5$ peripheral blood mononuclear cells (PBMC) was incubated for 18 hours on an anti-IFN-γ antibody-coated plate (EliSpot Interferon-γ Basis Kit; Autoimmun Diagnostika, Strassberg, Germany) along with CMV-specific peptide stimulus (CMV-Spot ELSP5530, including pp65 and IE-1; Autoimmun Diag-

nostika). Negative and positive controls (phytohemagglutinin) were as appropriate. IFN-γ production was visualized by addition of an enzyme-labeled detection antibody, and results expressed as spot forming unit (SFU) per $2X10^5$ PBMC, with each SFU representing a single IFN-γ secreting cell. Absent/weak response was reported if <20 SFU/$2X10^5$ PBMC, strong response if ≥20. Baseline immunological evaluation was performed the very day of transplant (Costa 2014a). CMV IgG serology was assessed by a chemiluminescent microparticle immunoassay (Architect CMV IgG assay, Abbott, USA). Anti-CMV prophylaxis was administered for 3 months in high-risk patients (i.e. presenting with a D+/R- CMV sero-matching). Pre-emptive antiviral treatment was with ganciclovir or valganciclovir (900 mg daily, corrected to renal function) if CMV-DNAemia was ≥10^4 copies/mL or on clinical evidence of CMV disease, Two consecutive negative CMV DNAemia determinations were required for the antiviral therapy to be discontinued. Patients at high risk for CMV-infection (D+/R- CMV-IgG serostatus) underwent a 3-month course of antiviral prophylaxis with valganciclovir.

Statistical analysis was performed on Graph Pad Prism 4, using Student's t-test, χ^2-test and Fisher's exact test, as appropriate. Time to development of CMV infection was evaluated by Kaplan-Meier curve analysis. Sensitivity/specificity ROC analysis was performed to investigate the value of CMV-EliSpot assay in predicting the occurrence of systemic CMV replication episodes. Statistical significance was considered for p-value <0.05.

TABLE 1 - *Main demographic and baseline characteristics of the study population. SFU, spot forming unit; TAC, tacrolimus; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; CSA, cyclosporine A; AZA, azathioprine; mTOR, mammalian target of rapamycin; CMI, cellular mediated immunity.*

Main demographic and baseline characteristics of the study population	
N. of patients	80
Gender (male/female)	54/26
Age (range)	53.9 (17-79)
CMV D/R serostatus	
D+/R+	60 (75%)
D-/R+	11 (13.8%)
D+/R-	7 (8.8%)
D-/R-	2 (2.5%)
Induction immunosuppressive therapy, N. (%)	
Basiliximab	79 (98.8%)
Thymoglobulin	1 (1.2%)
Maintenance immunosuppression, N. (%)	
CNI-based (CSA, TAC)	
TAC, MMF, steroid	47 (58.8%)
TAC, steroid	11 (13.8%)
CSA, MMF, steroid	10 (12.5%)
CSA, steroid	1 (1.3%)
TAC, AZA	1 (1.3%)
TAC, mTOR inhbitor	1 (1.3%)
CSA, mTOR inhibitor	5 (6.3%)
CNI free	
MMF, steroid	3 (3.8%)
MMF, mTOR inhibitor	1 (1.3%)
Pre-Transplantation anti-CMV CMI assessment by EliSpot, N. of patients (%)	
Non-responder	31 (38.8%)
Responder	49 (61.3%)
Pre-Tx anti-CMV EliSpot response, SFU/2*10^5 PBMC (range)	
D+/R+	64.7 (0-259)
D-/R+	87.9 (2-202)
D+/R-	1.7 (0-11)
D-/R-	1.5 (1-2)

RESULTS

The study included 80 KT recipients, with a CMV-Elispot determination the day of transplantation.

The occurrence of CMV events, as determined by quantitative real time RT-PCR on whole blood, was stratified according to donor/recipient CMV-IgG serostatus and CMV-EliSpot response in recipients at baseline. The main clinical features, including the immunosuppressive regimens adopted and CMV immunity levels at baseline are reported in Table 1.

At pre-transplant evaluation, 49 patients (61.3%) were CMV-responders at EliSpot assay (mean response: 109.5±81.6 SFU/$2x10^5$ PBMC) and 31 (38.8%) non-responders (mean 4.5±5.1 SFU/$2x10^5$ PBMC). Sixteen (32.7%) of the 49 CMV-responders showed CMV infections in blood at three-month follow-up (DNAemia peak mean: 131438±471816, range 2600-1900000 copies/mL), compared to 8 (25.8%) out of 31 non-responders (DNAemia peak

mean: 206525±470000, range 2600-1350000 copies/mL), without any difference in significance (p=0.6196, Fisher's exact test; p=0.7164, Student t-test).

No further episode of CMV viraemia was reported in the responder group, in comparison to 15 out of 31 non-responder patients (48.4%) exhibiting at least one episode of CMV-DNA in blood at 12-month follow up. Kaplan-Meier analysis of CMV-free state survival at 1-year follow up showed a median survival time of 8.2 months in the group of non-responders, while still undefined in the responder group; however, no significance was reported at a p-value <0.05 (Figure 1, panel).

Seven patients underwent mTOR-inhibitor-based immunosuppression therapies, with the mTORi itself in association with tacrolimus (1 patient), cyclosporine A (5 patients), and mycophenolate mofetil (1 patient). The group included 6 CMV D+/R+, and one D+/R- recipient. Only the high-risk patient (i.e. CMV IgG D+/R- serostatus, on combined CSA and everolimus immunosuppression) exhibited signs of CMV infection at discontinuation of antiviral prophylaxis (namely, 7500 copies/mL peak CMV viral load on blood at month 12 post-transplantation, concomitant to an episode of CMV positivity on broncho-alveolar lavage - BAL, 6900/mL viral load). When excluding this group from the Kaplan-Meier analysis of CMV-free state survival, the median survival time remained undefined in the responder group, even if not significant (p>0.05).

Baseline CMV-IgG serology in recipients showed a strong correlation with EliSpot determinations (mean EliSpot values in R^+: 77.34 SFU/2*10^5 PBMC, 0-259 range; in R^-: 1.67, 0-11 range; p=0.0082). Out of nine KT recipients CMV-IgG seronegative at baseline (7 D+/R-, 2 D-/R-), eight exhibited low/undetectable CMV-EliSpot values (range 0-2 SFU/2*10^5 PBMC).

One patient presented moderate-low responsiveness (11 SFU/2*10^5 PBMC), with subsequent development of sustained viraemic episodes within 3 months post transplantation, compatible with primary infection.

Only 39 patients exhibited a complete 12-month follow up with appraisable blood CMV-DNA determinations at the scheduled time-points. Pa-

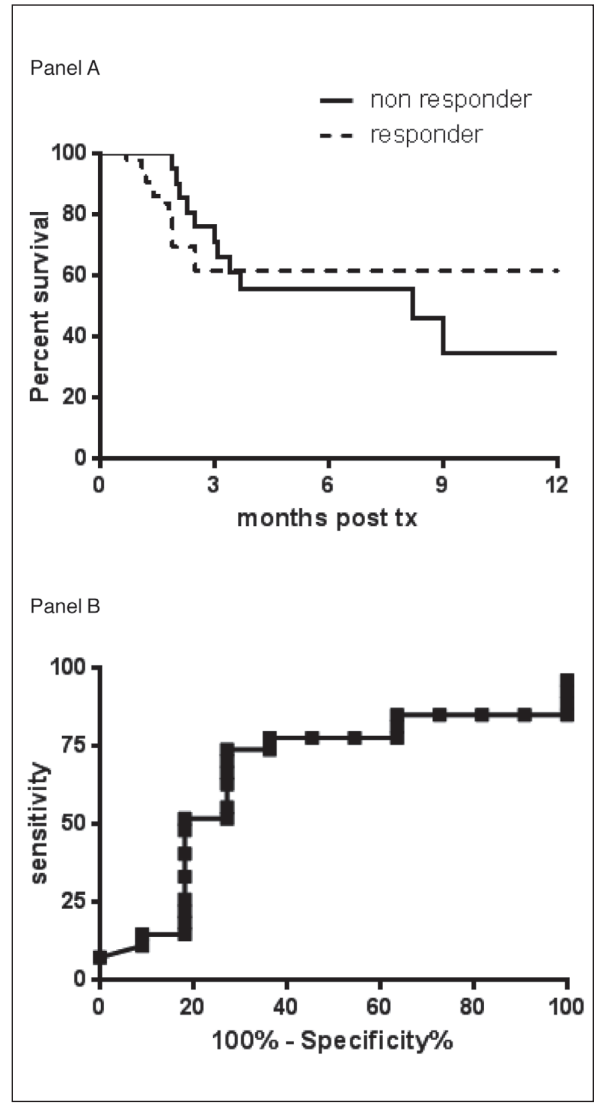

FIGURE 1 - ***Panel A***. *Kaplan Meier survival analysis curve, showing the absence of CMV episodes in blood (CMV-DNA free-status) in function of baseline CMV-EliSpot responsiveness. Number of patients, n=80. Median survival for non-responder, 8.2 months; for responder, undefined. Log-rank test, p=0.7584. Fixed line, CMV-Elispot non-responder; dotted line, CMV-EliSpot responder patients at baseline.*

Panel B. *Evaluation of operating characteristics for baseline spot forming units (SFU/2X10^5 PBMC) values - as assessed by CMV-EliSpot - in terms of blood CMV-DNA episodes at 12-month follow up by ROC curve analysis. 5 SFU/2X10^5 PBMC: 18.5% sensitivity (95% confidence interval [CI] 6.3-38.1%), 81.8% specificity (95% CI 48.2-97.7); 20 SFU/2X10^5 PBMC: 48.2% sensitivity (95% CI 28.7-68.1%), 81.8% specificity (95% CI 48.2-97.7); 100 SFU/2X10^5 PBMC: 77.8% sensitivity (95% CI 57.7-91.4), 45.5% specificity (95% CI 16.8-76.6). Area under the ROC curve, 0.6582; p=0.1303.*

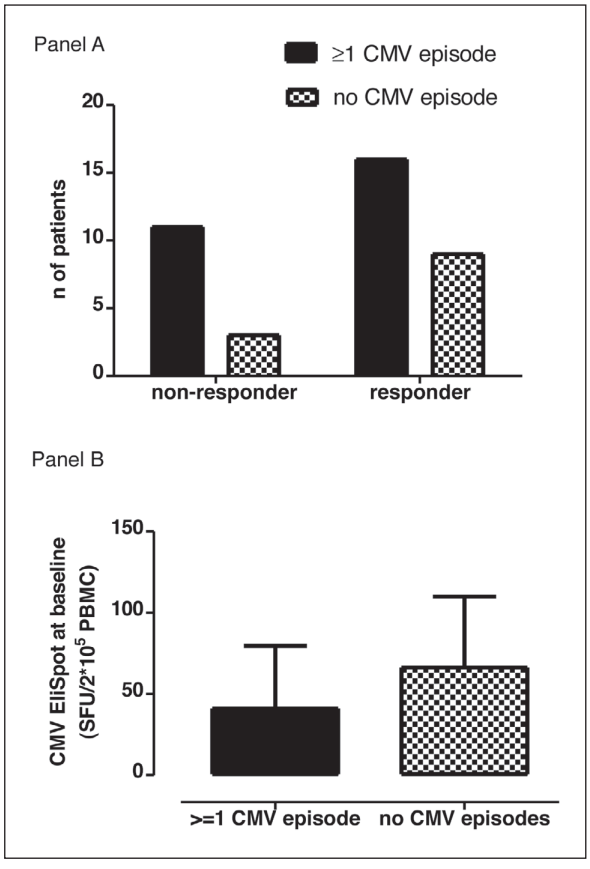

FIGURE 2 - ***Panel A****. In the graph is reported the distribution of patients with evidence of at least one episode of CMV replication (solid bar) and without evidence of CMV replication (dotted bar) at 12 month follow up, according to CMV-EliSpot status at baseline (non- responder, <20 SFU/2X10^5 PBMC; responder, ≥20 SFU/2X10^5 PBMC).* ***Panel B****. CMV-EliSpot status (SFU/2X10^5 PBMC) at baseline in the groups of patients with at least 1 episode of CMV replication (solid bar) and without evidence of CMV replication (dotted bar) at 12-month follow up (p=0.0786).*

tients exhibiting at least one episode of CMV viraemia at 12-month observation showed lower baseline CMV-EliSpot values than the group of patients without signs of CMV reactivation/infection in the same lapse of time, with a tendency to significance at p=0.0786 (t-test, mean values ±SD: 40.96±7.42, n=27; 66.17±12.61, n=12, respectively) (Figure 2). ROC curve analysis was used for SFU/2X10^5 PBMC values in terms of occurrence of CMV-DNAemia at 12-month follow up (Figure 1, panel B).
Sensitivity and specificity of cut-off values were as follows: 5 SFU/2X10^5 PBMC, 18.5% sensitivity, 81.8% specificity; 20 SFU/2X10^5 PBMC, 48.2% sensitivity, 81.8% specificity; 100 SFU/2X10^5 PBMC, 77.8% sensitivity, 45.5% specificity.

DISCUSSION

CMV infection, with its direct and indirect effects, is responsible for significant morbidity and mortality in SOT recipients in the post-transplantation period. This justifies investigations into effective prevention and therapeutic approaches to prevent a CMV burden (Abate 2013). Both B- and T-lymphocytic adaptive immune responses have been shown to be critical in controlling CMV replication (Kotton 2010). The analysis of CMV-specific T-cell frequencies and function is proving a satisfactory tool for assessing the patient's ability to control CMV replication (Egli, Humar, and Kumar, 2012). Many studies have reported the relationship between post-transplant functional impairment of CD8+ T-cell immunity and failure to suppress CMV replication after SOT. However, most studies have focused on evaluation of immune responses in the post-transplant period (Benmarzouk-Hidalgo 2011) (Cantisan 2013) (Costa 2012) (Mattes 2008). Few studies have investigated the utility of determining the pre-transplant CMV-immune status in predicting the risk of post-transplant CMV replication in SOT, using EliSpot™ or Quantiferon™ assays to determine CMV-specific T-cell levels (Bestard *et al.*, 2013) (Bunde 2005) (Cantisan 2013).
In this prospective study we analyzed the levels of pre-transplant IFN-γ responses by CMV-specific T-cells, as determined by CMV-EliSpot assay, to investigate their possible utility in determining the risk of CMV replication after KT. Eighty KT recipients were prospectively analyzed for systemic CMV infections by means of quantitative RT-PCR on whole blood, and data on CMV replication related to baseline CMV-specific cellular immunity (CMV-EliSpot). Even though not supported by statistical significance, Kaplan Meier analysis of survival showed an 8.2-month median CMV-free survival in the group of non-responders, while still undefined at 1-year follow up in the responder group.

These data suggest a possible role of pre-transplant CMV CMI as a prognostic factor in our population of KT. This finding is further supported by the reported observation that patients exhibiting at least one episode of CMV viraemia at 12 month follow up had lower baseline CMV-EliSpot values than the group of patients without signs of systemic CMV replication, with a tendency in significance at p=0.0786. However, no influence on CMV-viral load peaks was reported. Our findings are consistent with a few previous studies reporting that low pre-transplant CMV-specific T-cell responses, as assessed by CMV-EliSpot (Bestard 2013) or CMV-Quantiferon (Cantisan 2013), are associated with a higher risk of post-transplant CMV replication.

Baseline CMV-EliSpot determinations exhibited a good correlation with CMV-IgG serology, with IgG-CMV negative recipients showing significantly lower levels of CMV-specific EliSpot values. Out of 9 KT with negative serology, only one exhibited a moderate CMI to CMV (11 SFU/ $2X10^5$ PBMC), with subsequent CMV primary infection within 3 months. This event is in line with Bestard 2013, who reported a positive correlation between pre-transplant CMV epitope-restricted T-cell responses and CMV-IgG titres. They also found that detectable CMV-specific T-cell responses at baseline could be reported in some IgG seronegative SOT, even though at significantly lower levels than within seropositive recipients, probably resulting from different memory T-cells originated from different antigenic exposures and exhibiting cross-reactive recognition of CMV-epitopes.

The study is limited by the small number of patients enrolled and heterogeneity of the study group; nonetheless, very few reports exist on the impact of pre-transplant CMV-specific cellular immunity determination on predicting future infection episodes (Lucia 2014), justifying such investigations as intriguing tools to both dissect and monitor the risk of CMV during immunosuppressive therapies.

In conclusion, this study suggests that in kidney transplant recipients - in addition to well-established risk-stratification assessment performed with IgG serology - monitoring CMV-specific T-cell responses at pre-transplantation level may be useful for predicting the post-transplant risk of developing CMV infection and reactivation. Even though suffering from small sample size, the data reported may help to design more robust targeted CMV prevention strategies for those IgG-negative recipients at greatest risk. For this purpose, large-scale prospective trials are warranted.

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