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Analysis of spontaneous resolution of cytomegalovirus replication after transplantation in CMV-seropositive patients with pretransplant CD8+IFNG+ response

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ANALYSIS OF SPONTANEOUS RESOLUTION OF CYTOMEGALOVIRUS REPLICATION AFTER TRANSPLANTATION IN CMV-SEROPOSITIVE PATIENTS WITH PRETRANSPLANT CD8+IFNG+ RESPONSE

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ABBREVIATIONS

D+/D-: CMV-seropositive/CMV-seronegative donor; IFNG: interferon-gamma; mTOR: mammalian target of rapamycin; PD-1: programmed death 1; QF: QuantiFERON-CMV; R+/R-: CMV-seropositive/CMV-seronegative recipient

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ABSTRACT

This prospective study evaluates whether CMV-seropositive (R+) transplant patients with pretransplant CD8+IFNG+ T-cell response to cytomegalovirus (CMV) (CD8+IFNG+ response) can spontaneously clear the CMV viral load without requiring treatment. A total of 104 transplant patients (kidney/liver) with pretransplant CD8+IFNG+ response were evaluable. This response was determined using QuantiFERON-CMV assay. The incidence of CMV replication and disease was 45.2% (47/104) and 6.7% (7/104), respectively. Of the total patients, 77.9% (81/104) did not require antiviral treatment, either because they did not have CMV replication (n = 57) or because they had asymptomatic CMV replication that could be spontaneously cleared (n = 24). Both situations are likely related to the presence of CD8+IFNG+ response to CMV, which has a key role in controlling CMV infection. However, 22.1% of the patients (23/104) received antiviral treatment, although only 7 of them did so because they had symptomatic CMV replication. These patients developed symptoms in spite of having pretransplant CD8+IFNG+ response, thus suggesting that other immunological parameters might be involved, such as a dysfunctional CD4+ response, or that they might have become $QF_{Non\text{-}reactive}$ due to the immunosuppression. In conclusion, around at least 80% of R+ patients with pretransplant CD8+IFNG+ response to CMV did not require antiviral treatment, although this percentage might be underestimated. Nevertheless, we recommend performing an additional CD8+IFNG+ response determination at posttransplant time to provide more reliable information regarding the patients who will be able to spontaneously clear the viremia.

KEYWORDS: solid organ transplantation, cytomegalovirus infection, T-cell response, QuantiFERON-CMV assay, interferon-gamma

1. INTRODUCTION

CMV infection is a well-known complication after solid organ transplantation (SOT) and two major strategies are commonly used for the prevention of CMV: prophylaxis or pre-emptive therapy (Kotton et al. 2013; Torre-Cisneros et al. 2016). Pre-emptive therapy is mainly used in low-risk patients once viral replication reaches a certain threshold and optimally before the development of symptoms. This strategy involves the virological monitoring of CMV infection, which is cumbersome for patients and costly in terms of material and human resources. Low-risk patients include CMV-seropositive patients, since they are expected to have specific immune response against this virus and a low probability of developing CMV disease.

Both CMV-specific humoral and cellular immunity have been shown to play a relevant role against CMV infection. However, to stratify the risk in transplant candidates awaiting transplantation, only recipient CMV serology is considered. Recent studies have reported that the presence of IFNG+ CMV-specific CD8+ T-cell (CD8+IFNG+) response at pretransplant or posttransplant is associated with a lower risk of CMV infection (Bestard et al. 2013; Cantisán et al. 2013; Kumar et al. 2009; López-Oliva et al. 2014; Manuel et al. 2013). By contrast, the lack of pretrasplant CD8+IFNG+ response in some CMV-seropositive patients has been reported as being associated with a higher risk of CMV replication after transplantation (Bestard et al. 2013; Cantisán et al. 2013; López-Oliva et al. 2014). According to this evidence, we hypothesize that transplant patients with pretransplant CD8+IFNG+ response should be able to spontaneously clear the CMV viral load without the need for antiviral treatment, thus reducing the need for pre-emptive treatment.

The aim of the current study was to evaluate whether CMV-seropositive transplant patients who display CD8+IFNG+ response before transplantation are able to control replication and/or self-clear the viral load without requiring treatment.

2. MATERIAL AND METHODS

2.1. Study population and design

This prospective study was carried out in eight centers of the Spanish Network for Research in Infectious Diseases (REIPI), in four centers of the Spanish Kidney Disease Network (RedInRen) and in one center of Sao Paulo, Brazil. Adult transplant candidates with pretransplant CD8+IFNG+ response to CMV, who were awaiting a kidney or liver transplant and who were not expected to receive antiviral prophylaxis with either ganciclovir or valganciclovir were eligible for the study. Tacrolimus-based immunosuppression protocols were as per the center-specific standard. We obtained approval from institutional review boards before initiation of enrollment at each center and informed consent from all participants.

Patients were recruited from February 2013 to March 2016. CD8+IFNG+ response to CMV was assessed pretransplant, either when they were on the waiting list in the case of recipients who received a graft from a deceased donor or the day prior to transplantation for patients receiving a graft from living donors. Patients who received a graft were monitored for CMV replication for 6 months following transplantation. The protocol study established that pre-emptive strategy should not be initiated in patients with asymptomatic replication, but to start an observation phase in these patients where CMV load would be monitored at least weekly during the first two months, every two weeks until the third month, monthly until the sixth month after SOT and when clinically indicated. Antiviral treatment would be initiated only in the event that the patients developed symptoms, although the decision to initiate treatment was ultimately at the discretion of the treating physician based on the individualized evaluation of the patient (immunosuppression, general state, viral load kinetics, etc.).

Patients with CMV replication were classified as asymptomatic or as having CMV disease according to standard definitions (Torre-Cisneros et al. 2016).

2.2. Determination of anti-CMV IgG antibodies and CMV viral load

Serology testing for anti-CMV IgG was performed on all samples using the Diasorin chemoluminescence assay (Diasorin SA, Spain) as per manufacturer's instructions. Titers < 12 U/mL were classified as negative. CMV load was determined in plasma by real time PCR using the technique implemented at each center. The detection limit was 137 IU/mL. Peak viral load was defined as the maximum viral load within the posttransplant period. The duration of CMV replication was calculated as the number of days from the first positive PCR to the first negative PCR. In patients with more than one episode, the total number of days of the different episodes was considered.

2.3. QuantiFERON-CMV assay

CD8+IFNG+ response was assessed using the QuantiFERON-CMV® (QF) test (Qiagen, Germany) (Walker et al. 2007). In brief, 1 mL of heparinized whole blood was collected in three QF tubes containing no antigens (negative control) or a mix of 22 CMV peptides or phytohemagglutinin (positive control). The tubes were shaken vigorously and incubated for 16–24 hours at 37ºC. Supernatants were harvested and analyzed for IFNG level (IU/mL) by standard ELISA. The supernatants from all patients were shipped to our center and IFNG level was therefore analyzed in the same platform. The negative control response was subtracted from either the CMV antigen or mitogen tubes. According to the manufacturer's instructions, a result was considered "Reactive" when the CMV antigen response was equal to or greater than 0.2 IU/mL of IFNG. A result was considered "Non-reactive" when the CMV antigen response was lower than 0.2 IU/mL and the mitogen

response was higher than 0.5 IU/mL. A result was "Indeterminate" when the IFNG level was less than 0.2 IU/mL in the CMV antigen tube and less than 0.5 IU/mL in the mitogen tube.

2.4. Study of $CD8^+$ T-cell phenotype

Peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved. At the time of analysis, 500,000 thawed PBMCs were incubated with fluorochrome-labeled antibodies to CD4 (Viogreen), CD8 (PE-Vio770), CD57 (Vioblue) and programmed cell death 1 (PD-1) (Viobright FITC) (all from Miltenty Biotec). Cell viability was analyzed using 7-Amino Actinomycin D (7-AAD) (eBiosciences). After 30 min on ice in the dark, flow cytometry analysis was performed on a LSRFortessa SORP cytometer (Becton Dickinson). The resulting profiles were analyzed using FlowJo software. The expression of CD57 and PD-1 were referred to on CD4+ or CD8+ T cells.

2.5. Statistical analysis

The statistical analysis was performed using PASW Statistics 18.0 software (IBM Corporation). The Chi-squared or Exact Tests were used to compare the distribution of categorical variables among the three groups. Quantitative data were analyzed with the nonparametric Kruskal-Wallis Test (three group comparisons) or Mann-Whitney *U* test (two group comparisons). A 2-sided p-value of 0.05 was considered statistically significant.

3. RESULTS

3.1. Patient characteristics

A total of 104 R+ patients with pretransplant CD8+IFNG+ response to CMV (IFNG ≥ 0.2 UI/mL), who were classified as $QF_{Reactive,}$ were evaluable and completed the monitoring

phase. The clinical-demographic characteristics of these patients are shown in Table 1. Fifty-one patients received a renal transplant and 53 patients received a liver transplant. Forty-seven patients received induction therapy, of which 45 received basiliximab and 2 received thymoglobulin. These two patients met the inclusion criteria since they did not receive prophylaxis with valganciclovir.

3.2. Incidence and kinetics of CMV replication: self-resolved versus symptomatic patients

Within 6 months after transplantation, CMV replication occurred in 47 out of these 104 patients (45.2%). Of the 47 patients with replication, 91.5% (43/47) experienced only one episode and 8.5% (4/47) had more than one episode. The total median duration of CMV replication was 30 days (range, 4–415 days). The minimum duration of replication was 4 days as one patient died 22 days after transplantation. The median peak viral load was 1980 UI/mL (range, 150–131794 UI/mL). The patients developed CMV replication at a median of 34 days after SOT (range, 16–162 days).

The incidence of CMV disease was 6.7% (7/104) (3 viral syndrome and 4 end-organ disease). Two patients had gastrointestinal disease, one had hepatitis and one had respiratory disease.

The study flow diagram is shown in Figure 1. Of the 104 patients, 77.9% (81/104) did not require anti-CMV treatment because either they did not have CMV replication (n = 57) or because they had asymptomatic CMV replication that could be spontaneously cleared (n = 24), which represents 51.1% (24/47) of the patients with CMV replication. These 24 patients developed CMV replication at a median time of 41.5 days (range, 22–162 days) and the total duration of the episodes was 28 days (range, 4–80 days). The minimum duration of

replication in this group was 4 days as one patient died 22 days after transplantation. The median peak viral load was 483.0 UI/mL (range, 150–5134 UI/mL). Figure 2 shows the kinetics of self-resolved infection in the four patients with a viral load higher than 2000 UI/mL, including the patient with the highest viral load that could be spontaneously cleared (5134 UI/mL).

In contrast, 22.1% of the patients (23/104) received antiviral treatment, although only 7 of them (7/23; 30.4%) did so because they had symptomatic CMV replication. These symptomatic patients developed CMV replication earlier than the patients with spontaneous clearance (29 vs. 41.5 days; $p = 0.025$) and CMV replicated longer, although the differences were not statistically significant (42 vs. 28 days; $p = 0.357$). The median peak viral load at treatment was 4576 UI/mL (range, 2310–131794 UI/mL), much higher than in self-resolved patients. The remaining 16 patients received treatment according to their physicians' decisions mainly based on an elevated viral load (median 3993 UI/mL) although they were asymptomatic. However, it is important to note the high variability in the threshold for initiation of pre-emptive therapy in these asymptomatic patients, which ranged from 335 to 22832 UI/mL (Figure 3). Nine out of these 16 patients initiated antiviral treatment at a viral load below 5000 UI/mL and therefore received treatment at a viral load that could be spontaneously cleared in the self-resolved group.

3.3. Comparison of non-replication, self-resolved and symptomatic patients

We then analyzed whether the self-resolved patients had some differential characteristics compared to the other groups. For this purpose, the 16 asymptomatic patients who received antiviral treatment were excluded in order to prevent a possible bias due to the overlapping of these patients with the self-resolved patients. Therefore, the three groups we

compared were: non-replication (n = 57), self-resolved (SR) replication (n = 24) and symptomatic replication (n = 7) (Table 2).

The median age of patients did not significantly differ among the three groups. When age was used as a categorical variable (patients younger and older than 57 years), we observed that patients with CMV replication were older than those without replication, with patients showing symptomatic replication being the oldest. We also observed that all the patients who could not control the replication had received an organ from a D+ donor. However, the only parameter that significantly differed among the three groups was the presence of the HLA-A2 allele. Although patients with CMV replication had an increased frequency of this allele compared to non-replication patients (58.1% vs 28.1%; Chi-square test p=0.006), the frequency of HLA-A2 allele was higher in the group of patients who were able to spontaneously clear the replication compared to the other two groups. Therefore, 62.5% of the patients (15/24) in the SR group had the HLA-A2 allele, whereas the frequencies of this allele were 28.1% (16/57) and 42.9% (3/7) in the non-replication and symptomatic groups, respectively.

3.4. Relationship between the level of IFNG secretion and spontaneous clearance of CMV replication

We then investigated whether there was any correlation between the level of IFNG released and spontaneous clearance. We found no significant differences among the three groups (Figure 4). The most relevant finding was that, unexpectedly, the median IFNG level was lower in the non-replication group than in the two groups with replication, with the level being very similar in the SR and symptomatic groups (11.0 UI/mL for the non-replication

group vs. 23.6 UI/mL and 28.4 UI/mL for the SR and symptomatic replication groups, respectively).

Moreover, we did not observe any association between pretransplant IFNG level and peak viral load and the onset of CMV replication in patients with CMV replication.

3.5. Relationship between the frequency of highly experienced T cells and the self-resolution of CMV replication

In order to investigate whether the differentiation status of T cells before transplantation was related to the ability to clear the CMV viral load, we also compared the frequency of experienced T cells among the three groups. To do so, we compared the frequency of CD4+CD57+, CD4+PD-1+, CD8+CD57+ and CD8+PD-1+ subpopulations among the three categories. Phenotypic analysis could only be performed in a subgroup of 58 patients (41 non-replication, 14 SR replication and 3 symptomatic replication) since peripheral blood mononuclear cells were available only in this group. The gating strategy is shown in the Figure 5.

We found significant differences only in the frequencies of CD4+CD57+ and CD4+PD-1+ cells (Figure 6). Patients with self-resolved replication had a higher frequency of CD4+CD57+ and CD4+PD-1+ T cells than the non-replication and symptomatic replication patients. In particular, the median percentage of the CD4+CD57+ subset was 6.3% in the SR replication patients, whereas it was 3.2% and 2.2% in patients with no replication and in the symptomatic replication group, respectively. Regarding the CD4+PD-1+ subset, the median percentage was 18.5% in the SR replication group compared to 14.9% in the non-replication group and 8.4% in the symptomatic replication group. The median frequency of CD8+CD57+ T cells was also higher in the SR replication group than in the other groups, although the

differences did not reach statistical significance, which might be related to the small sample size.

4. DISCUSSION

In this study we analyze the spontaneous clearance of CMV replication in R+ kidney or liver transplant patients with pretransplant CD8+IFNG+ response to CMV. The main result is that the majority of the patients, around 80%, did not require antiviral treatment either because they did not have CMV replication or they had asymptomatic replication that could be spontaneously cleared, which accounts for half of the patients with CMV replication. Both situations are likely related to the fact that these patients had CD8+IFNG+ response to CMV before transplantation, which maintains CMV under control and prevents the development of symptoms. This result is in line with those reported previously by other authors, who have indicated the key role of CD8+ T cells in the self-resolution of CMV reactivation (Benmarzouk-Hidalgo et al 2011; Kumar et al. 2017; Lisboa et al. 2012). In this regard, Benmarzouk-Hidalgo et al. (2011) observed that the acquisition of CMV-specific immune response in D+R- transplant patients was associated with the clearance of 97.8% of the CMV replication episodes without the administration of valganciclovir. In the same line, Lisboa et al. (2012) reported that CD8+ T-cell response assessment shortly after the onset of CMV viremia in solid organ transplant patients can identify which patients will spontaneously clear the virus. In addition, a recently published interventional study has reported that cell-mediated immunity against CMV can be performed in real time to guide clinical decisions to treat patients or not with antiviral therapy (Kumar et al. 2017).

However, we also observed that the pretransplant IFNG level does not explain why some patients with CMV replication can self-resolve the infection while others cannot, since

both groups have very similar median levels. We analyzed whether other parameters are involved and found significant results with the HLA-A2 allele. We observed that the HLA-A2 allele is related to a higher efficiency in self-controlling the viremia, which might be associated with the immunodominance of the HLA-A2-restricted pp65 complex and/or with an increased frequency of highly efficient polyfunctional T cells (Elkington et al. 2003; Snyder et al. 2016).

In the present work, however, some patients received antiviral treatment because they had developed symptomatic replication in spite of having pretransplant CD8+IFNG+ response. There are several possible explanations for this. It might be related to an inefficient CD4+ response against CMV, as has been reported by Gabanti et al. (2014). These authors found that the presence of functional CMV-specific CD8+ T cells in seropositive transplant patients is not enough to confer protection against CMV disease and observed complete protection against CMV only when CMV-specific CD4+ T cells reconstitute their function and provide help to CD8+ T cells (Gabanti et al. 2014). The relevance of CD4+ T cells in the control of CMV infection has been widely reported (Drylewicz et al. 2016; Harari et al. 2004; Sester et al. 2001). Therefore, it would be reasonable to think that the patients with symptomatic replication had pretransplant CD8+IFNG+ response but may have had an insufficient CD4+ response. Although we do not have functional information about CD4+ T cells since we used the QuantiFERON-CMV assay, we have phenotypic data supporting this idea. The fact that the frequency of CD4+CD57+ and CD4+PD-1+ subpopulations was higher in the patients who self-resolved the infection than in the other patients suggests that these highly experienced CD4+ T cells might contribute to a more efficient response against CMV (Espinosa et al. 2016; Pera et al. 2017).

Another explanation could be that some pretransplant $QF_{Reactive}$ patients might become $QF_{Non\text{-}reactive}$ after transplantation due to the T-cell dysfunctionality induced by

immunosuppression (Egli et al. 2013; Engstrand et al. 2003). If this were the case, the assessment of a single CD8+IFNG+ response determination before transplantation would not accurately identify patients at low risk of CMV disease. Although this result seems to be in contradiction with what we previously reported (Cantisán et al. 2013), it might be explained by differences in the characteristics of the patients included in both studies. Therefore, we now suggest that a second CD8+IFNG+ response determination should be additionally performed after transplantation, when patients are under immunosuppression. Alternatively, the lack of protection against CMV disease in patients with pretransplant CD8+IFNG+ response could be related to the absence of secretion of other relevant cytokines, which are not analyzed by the QuantiFERON-CMV assay (Ciuffreda et al. 2008; Darrah et al. 2007; Gibson et al. 2015; Snyder et al. 2016).

Most of the patients who received antiviral treatment were asymptomatic but received antiviral treatment according to their physicians' recommendation. The high variation in the viral load threshold at which treatment was initiated indicates the need for standardization (Kotton et al. 2013; Torre-Cisneros et al. 2016). Some patients received treatment at a viral load that could be spontaneously cleared in the self-resolved group, so it would be reasonable to think that the use of antiviral treatment in these patients might have been avoided, thus increasing the number of patients who did not need antiviral treatment (90 out of 104, 86.5%) and eliminating the potential harmful side effects of the anti-CMV drugs (Billar et al. 2016; Reusser et al. 2002).

Our study has several limitations. The main limitation is that initiation of antiviral treatment was subject to the judgment of the attending physician, which led to a high variability in the viral load threshold at which it was initiated and the likelihood of underestimating the number of patients who are able to self-resolve the CMV replication. Another limitation was that the QuantiFERON-CMV assay only provides information about

IFNG secretion by CMV-specific CD8+ T-cell response but not about other cytokines and immune cells. In addition, we do not have information about IFNG secretion after transplantation when patients are under immunosuppression.

In conclusion, our results demonstrate that, at least, around 80% of R+ kidney/liver transplanted patients with pretransplant CD8+IFNG+ response do not need antiviral treatment although we are aware that the study design does not allow us to establish a causal relationship between pretransplant CD8+IFNG+ response and the self-resolution of CMV replication. We acknowledge the limitation of the pretransplant strategy, since a few patients developed symptomatic replication in spite of being $QF_{Reactive}$ before transplantation. This observation indicates that a single pretransplant QF determination might not be sufficiently informative for the risk of CMV disease since the effect of immunosuppressant drugs is not considered. Therefore, other strategies might be investigated, such as: i) performing an *additional* posttransplant QF assay, which would be subjected to a cost-effectiveness analysis; ii) *substituting* the pretransplant QF for an early posstransplant test or iii) a pretransplant QF assay followed by a posttransplant assay at the time that patients have a positive viral load. Furthermore, other immunological parameters such as HLA alleles or the frequency of highly experienced T cells seem to be also involved.

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DISCLOSURE

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REFERENCES

1. Benmarzouk-Hidalgo OJ, Cisneros JM, Cordero E, Martin-Pena A, Sanchez B, Martin-Gandul C, et al. Therapeutic effect of the acquisition of cytomegalovirus-specific immune response during preemptive treatment. *Transplantation.* 2011;91:927-933.
2. Bestard O, Lucia M, Crespo E, Van Liempt B, Palacio D, Melilli E, et al. Pretransplant immediately early-1-specific T cell responses provide protection for CMV infection after kidney transplantation. *Am J Transplant* 2013;13:1793-1805.
3. Billat PA, Ossman T, Saint-Marcoux F, Essig M, Rerolle JP, Kamar N, et al. Multidrug resistance-associated protein 4 (MRP4) controls ganciclovir intracellular accumulation and contributes to ganciclovir-induced neutropenia in renal transplant patients. *Pharmacol Res.* 2016;111:501-508.
4. Cantisan S, Lara R, Montejo M, Redel J, Rodriguez-Benot A, Gutierrez-Aroca J, et al. Pretransplant interferon-gamma secretion by CMV-specific CD8+ T cells informs the risk of CMV replication after transplantation. *Am J Transplant.* 2013;13:738-745.
5. Ciuffreda D, Comte D, Cavassini M, Giostra E, Buhler L, Perruchoud M, et al. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. *Eur J Immunol.* 2008;38:2665-2677.
6. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med.* 2007;13:843-850.
7. Drylewicz J, Schellens IM, Gaiser R, Nanlohy NM, Quakkelaar ED, Otten H, et al. Rapid reconstitution of CD4 T cells and NK cells protects against CMV-reactivation after allogeneic stem cell transplantation. *J Transl Med.* 2016;14:230.

8. Egli A, Kumar D, Broscheit C, O'Shea D, Humar A. Comparison of the effect of standard and novel immunosuppressive drugs on CMV-specific T-cell cytokine profiling. *Transplantation.* 2013;95:448-455.
9. Elkington RS, Walker S, Crough T et al. Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. *J Virol.* 2003;77:5226-5240.
10. Engstrand M, Lidehall AK, Totterman TH, Herrman B, Eriksson BM, Korsgren O. Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clin Exp Immunol.* 2003;132:96-104.
11. Espinosa J, Herr F, Tharp G, Bosinger S, Song M, Farris III AB, George R, Cheeseman J, Stempora L, Townsend R, Durrbach A, and Kirk AD. CD57+ CD4 T cells underlie belatacept-resistant allograft rejection. *Am J Transplant.* 2016;16:1102–1112.
12. Gabanti E, Bruno F, Lilleri D, Fornara C, Zelini P, Cane I, et al. 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.* 2014;9:e106044.
13. Gibson L, Barysauskas CM, McManus M, Dooley S, Lilleri D, Fisher D, et al. Reduced frequencies of polyfunctional CMV-specific T cell responses in infants with congenital CMV infection. *J Clin Immunol.* 2015;35:289-301.
14. Harari A, Zimmerli SC, Pantaleo G. Cytomegalovirus (CMV)-specific cellular immune responses. *Hum Immunol*. 2004;65:500-506.

15. Kotton CN, Kumar D, Caliendo AM, Asberg A, Chou S, Danziger-Isakov L, et al. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. *Transplantation.* 2013;96:333-360.

16. Kumar D, Chernenko S, Moussa G, Cobos I, Manuel O, Preiksaitis J, et al. Cell-mediated immunity to predict cytomegalovirus disease in high-risk solid organ transplant recipients. *Am J Transplant.* 2009;9:1214-1222.

17. Kumar D, Mian M, Singer L, Humar A. An Interventional Study Using Cell-Mediated Immunity to Personalize Therapy for Cytomegalovirus Infection After Transplantation. *Am J Transplant.* 2017;17:2468-2473.

18. Lisboa LF, Deepali Kumar D, Wilson LE, and Humar A. Clinical Utility of Cytomegalovirus Cell-Mediated Immunity in Transplant Recipients With Cytomegalovirus Viremia. *Transplantation.* 2012;93:195–200.

19. Lopez-Oliva MO, Martinez V, Buitrago A, Jimenez C, Rivas B, Escuin F, et al. Pretransplant CD8 T-cell response to IE-1 discriminates seropositive kidney recipients at risk of developing CMV infection posttransplant. *Transplantation.* 2014;97:839-845.

20. Manuel O, Husain S, Kumar D, Zayas C, Mawhorter S, Levi ME, et al. 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.* 2013;56:817-824.

21. Pera A, Vasudev A, Tan C, Kared H, Solana R, Larbi A. CMV induces expansion of highly polyfunctional CD4+ T cell subset coexpressing CD57 and CD154. *J Leukoc Biol.* 2017;101:555-566.

22. Reusser P, Einsele H, Lee J, Volin L, Rovira M, Engelhard D, et al. Randomized multicenter trial of foscarnet versus ganciclovir for preemptive therapy of

cytomegalovirus infection after allogeneic stem cell transplantation. *Blood.* 2002;99:1159-1164.

23. Sester M, Sester U, Gartner B, Heine G, Girndt M, Mueller-Lantzsch N, et al. Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation. *Transplantation*. 2001;71:1287-1294.
24. Snyder LD, Chan C, Kwon D, Yi JS, Martissa JA, Copeland CA, et al. Polyfunctional T-Cell Signatures to Predict Protection from Cytomegalovirus after Lung Transplantation. *Am J Respir Crit Care Med*. 2016;193:78-85.
25. Torre-Cisneros J, Aguado JM, Caston JJ, Almenar L, Alonso A, Cantisan S, et al. Management of cytomegalovirus infection in solid organ transplant recipients: SET/GESITRA-SEIMC/REIPI recommendations. *Transplant Rev (Orlando)*. 2016;30:119-143.
26. Walker S, Fazou C, Crough T, Holdsworth R, Kiely P, Veale M, et al. Ex vivo monitoring of human cytomegalovirus-specific CD8+ T-cell responses using QuantiFERON-CMV. *Transpl Infect Dis.* 2007;9:165-170.

FIGURE LEGENDS

Figure 1. Flow diagram of the study.

Figure 2. Kinetics of CMV viral load in four representative patients with self-resolved CMV replication whose peak viral loads were higher than 2000 UI/mL.

Figure 3. Individual CMV viral load at initiation of antiviral treatment in patients who received antiviral treatment in spite of having asymptomatic CMV replication (n = 16).

Figure 4. Comparison of pretransplant IFNG secreted by CMV-specific CD8+ T cells in non-replication (n = 57), self-resolved (n = 24) and symptomatic (n = 7) patients. IFNG release was assessed using the QuantiFERON-CMV assay. Horizontal lines represent median values.

Figure 5. Gating strategy to analyze the frequency of highly experienced CD4+ and CD8+ T cells (CD4+CD57+, CD4+PD-1+, CD8+CD57+ and CD8+PD-1+ T-cell subsets).

Figure 6. Percentage (%) of CD4+ and CD8+ subsets and highly experienced CD4+CD57+, CD4+PD-1+, CD8+CD57+ and CD8+PD-1+ T-cell subsets in a subgroup of patients (non-replication, n = 41; self-resolved, n = 14 and symptomatic patients, n = 3). Horizontal lines represent median values.

Table 1. Distribution of demographic characteristics.

Parameters	**All participants (n = 104)**
Age, median (range)	57 (26-76)
Age, n (%)[a]	
< 57	53 (50.9)
> 57	51 (49.0)
HLA-A2 allele, n (%)	
No	65 (62.5)
Yes	39 (37.5)
Gender, n (%)	
Female	30 (28.8)
Male	74 (71.1)
Donor CMV serology, n (%)[b]	
D-	16 (15.4)
D+	83 (79.8)
Use of mTOR, n (%)	
No	83 (79.8)
Yes	21 (20.2)
Transplanted organ, n (%)	
Kidney	51 (49.0)
Liver	53 (50.9)
Rejection, n (%)	
No	86 (82.7)
Yes	18 (17.3)
Type of donor, n (%)	
Living	26 (25.0)
Deceased	78 (75.0)
Induction therapy, n (%)	
No	57 (54.8)
Basiliximab	45 (43.2)
Thymoglobulin	2 (1.9)

[a] Age is shown as below and above the median value.

[b] Some missing values.

Abbreviations: mTOR, mammalian target of rapamycin; D, donor.

Table 2. Characteristics of the sample population of 88 patients and comparison of the distribution of these characteristics among the non-replication, self-resolved and symptomatic subgroups.

Parameters	**All participants (n = 88)**	**Non-replication (n = 57)**	**SR replication (n = 24)**	**Symptomatic replication (n = 7)**	**p[a]**
Age, median (range)	57 (26-70)	54 (26-70)	59.5 (29-69)	59 (38-70)	0.125
Age[b]					
<57	46 (52.3)	35 (61.4)	9 (37.5)	2 (28.6)	0.059
>57	42 (47.7)	22 (38.6)	15 (62.5)	5 (71.4)	
HLA-A2 allele					
No	54 (61.4)	41 (71.9)	9 (37.5)	4 (57.1)	0.012
Yes	34 (38.6)	16 (28.1)	15 (62.5)	3 (42.9)	
Gender					
Female	25 (28.4)	16 (28.1)	8 (33.3)	1 (14.3)	0.617
Male	63 (71.6)	41 (71.9)	16 (66.7)	6 (85.7)	
Donor CMV serology[c]					
D-	16 (18.2)	12 (21.8)	4 (18.2)	-	0.451
D+	67 (76.1)	43 (78.2)	18 (81.8)	6 (85.7)	
Use of mTOR					
No	69 (78.4)	44 (77.2)	21 (87.5)	4 (57.1)	0.196
Yes	19 (21.6)	13 (22.8)	3 (12.5)	3 (42.9)	
Transplanted organ					
Kidney	44 (50.0)	29 (50.9)	11 (45.8)	4 (57.1)	0.892
Liver	44 (50.0)	28 (49.1)	13 (54.2)	3 (42.9)	
Rejection					
No	74 (84.1)	47 (82.5)	22 (91.7)	5 (71.4)	0.385
Yes	14 (15.9)	10 (17.5)	2 (8.3)	2 (28.6)	
Type of donor					
Living	20 (22.7)	14 (24.6)	4 (16.7)	2 (28.6)	0.714
Deceased	68 (77.3)	43 (75.4)	20 (83.3)	5 (71.4)	
Induction therapy					
No	51 (58.0)	35 (61.4)	12 (50.0)	4 (57.1)	0.256
Basiliximab	35 (39.8)	22 (38.6)	10 (41.7)	3 (42.9)	
Thymoglobulin	2 (2.3)	0	2 (8.3)	-	

Data represent the number of patients. In parentheses, the frequency (%) with respect to the total number of patients in each column.

[a] The non-replication, self-resolved (SR) and symptomatic subgroups were compared using the exact chi-squared test. For quantitative age, the Kruskal-Wallis test was used.

[b] Age as dichotomous variable (under and over median value).

[c] Some missing values.

Abbreviations: mTOR, mammalian target of rapamycin; SR replication (self-resolved replication).

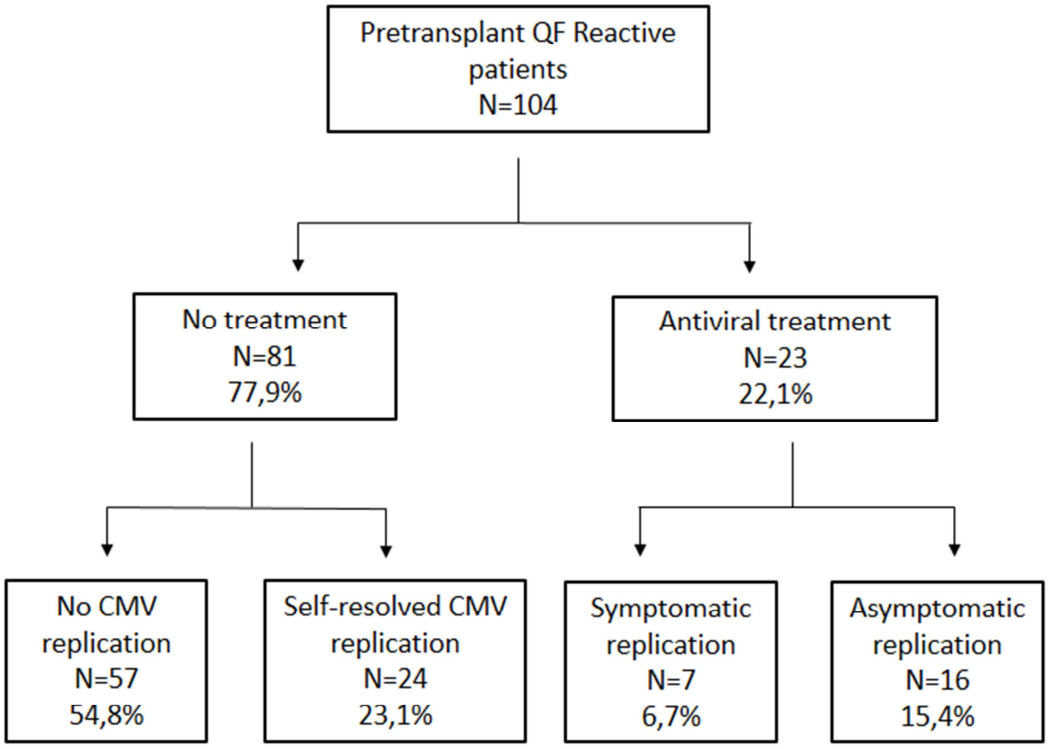
Pretransplant QF Reactive patients
N=104
No treatment
N=81
77,9%
Antiviral treatment
N=23
22,1%
No CMV replication
N=57
54,8%
Self-resolved CMV replication
N=24
23,1%
Symptomatic replication
N=7
6,7%
Asymptomatic replication
N=16
15,4%

Figure 1

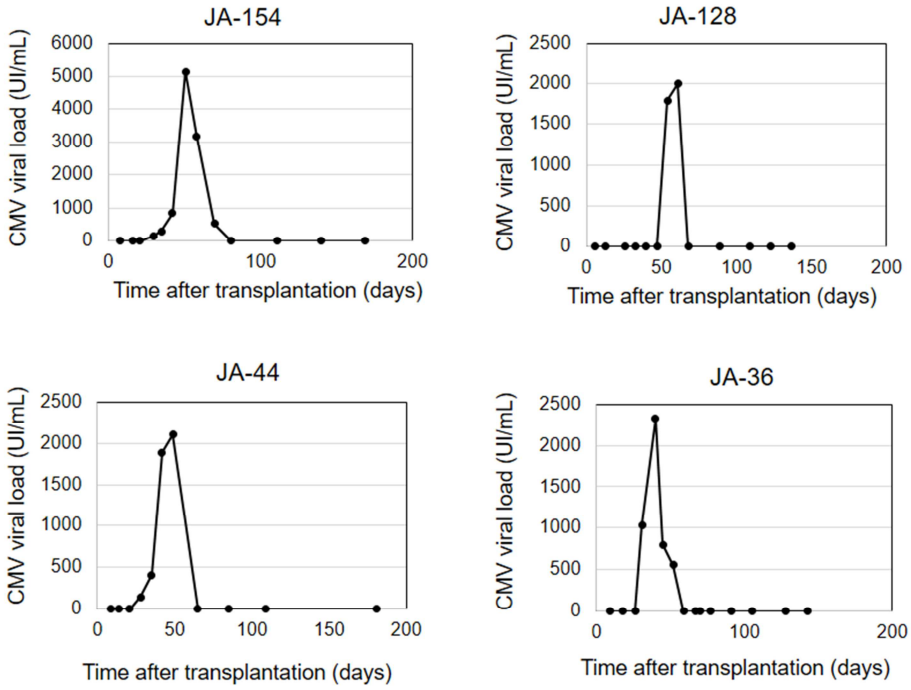

Figure 2

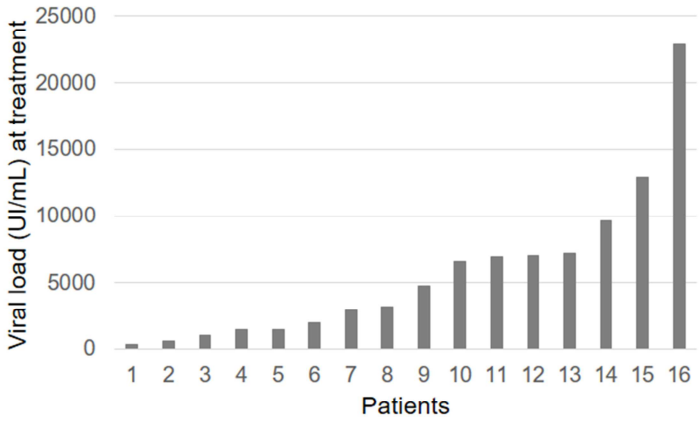

Figure 3

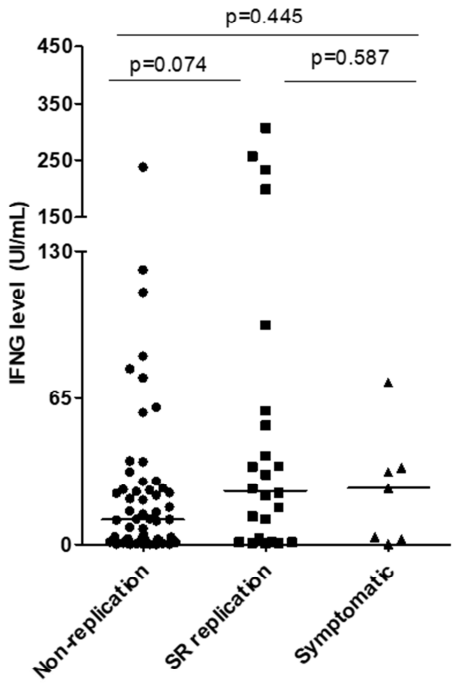

Figure 4

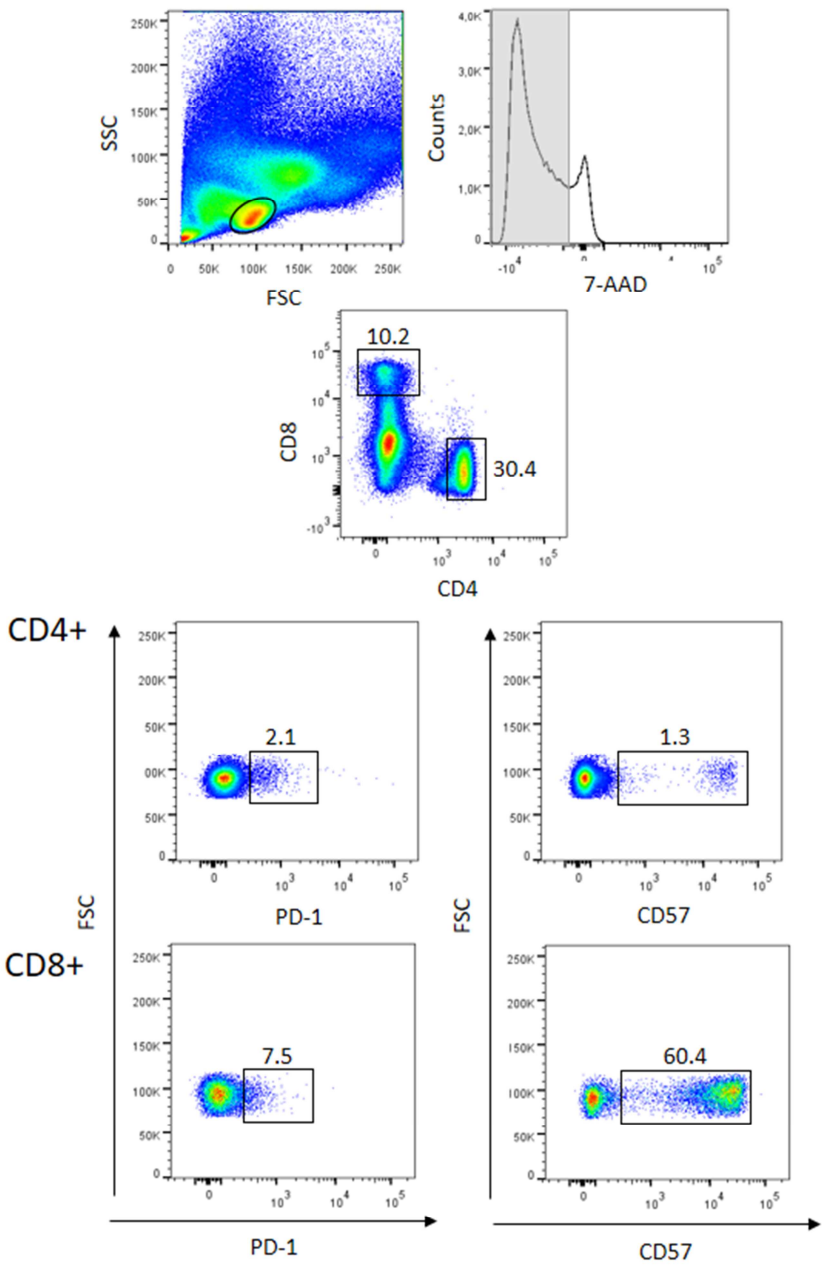
SSC
FSC
Counts
7-AAD
10.2
30.4
CD8
CD4
CD4+
CD8+
2.1
1.3
7.5
60.4
PD-1
CD57
FSC
PD-1
CD57

Figure 5

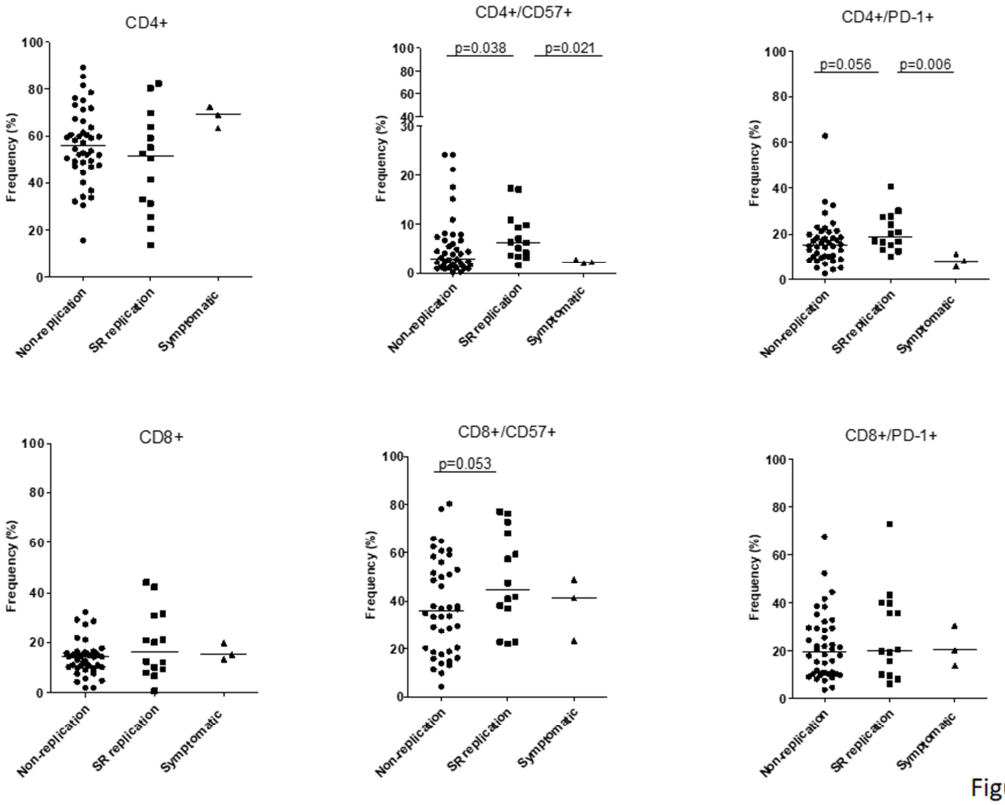

Figure 6

HIGHLIGHTS

- We analyze the ability of pretransplant CD8+IFNG+ response to spontaneously clear CMV replication in transplant patients.
- Most of the patients do not require antiviral treatment since they prevent or self-resolve the replication.
- A few patients developed symptomatic replication in spite of having pretransplant CD8+IFNG+ response.
- An additional posttransplant IFNG+ response determination might better identify patients with spontaneous clearance.