Kinetics of Cytomegalovirus (CMV) pp65 and IE-1-Specific IFN γ CD8 $^{+}$ and CD4 $^{+}$ T Cells During Episodes of Viral DNAemia in Allogeneic Stem Cell Transplant Recipients: Potential Implications for the Management of Active CMV Infection

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REVISED VERSION

Kinetics of Cytomegalovirus (CMV) pp65 and IE-1-Specific IFN γ CD8⁺ and CD4⁺ T Cells During Episodes of Viral DNAemia in Allogeneic Stem Cell Transplant Recipients: Potential Implications for the Management of Active CMV Infection

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Running head: CMV-specific immunity during episodes of CMV DNAemia

ABSTRACT

The dynamics of CMV pp65 and IE-1-specific IFN γ -producing CD8 $^{+}$ (IFN γ CD8 $^{+}$) and CD4 $^{+}$ (IFN γ CD4 $^{+}$) T cells and CMV DNAemia were assessed in 19 pre-emptively-treated episodes of active CMV infection. Peripheral counts of IFN γ CD8 $^{+}$ and IFN γ CD4 $^{+}$ T cells inversely correlated with CMV DNAemia levels ($P<0.001$ and $P=0.003$, respectively). A threshold value of 1.3 cells/ μ L predicting CMV DNAemia clearance was established for IFN γ CD8 $^{+}$ T cells (PPV, 100%; NPV, 93%) and for IFN γ CD4 $^{+}$ T cells (PPV, 100%; NPV, 75%). Undetectable T-cell responses were usually observed at the time of initiation of pre-emptive therapy. Either a rapid (within 7 days) or a delayed (median 31 days) expansion of both T-cell populations concomitant with CMV DNAemia clearance was observed in 5 and 8 episodes, respectively. An inconsistent or a lack of expansion of both T-cell subsets was related to a persistent CMV DNAemia. Robust and maintained CMV-specific T-cell responses after CMV DNAemia clearance and cessation of antiviral therapy were associated with a null incidence of relapsing infections at least during the following month. Data obtained in the present study may be helpful in the design of therapeutic strategies for the management of active CMV infections in the allo-SCT recipient.

Key words: Cytomegalovirus, IFN γ CD8 $^{+}$ and CD4 $^{+}$ T cells, active CMV infection, immunological monitoring, stem cell transplantation.

INTRODUCTION

Pre-emptive antiviral therapy has been adopted by most transplant centers as the first-choice strategy for the prevention of cytomegalovirus (CMV) disease following allogeneic stem cell transplantation (allo-SCT) [Boeckh et al., 2003; Griffiths et al., 2008]. While this strategy has been shown to dramatically reduce the incidence of early CMV disease [Boeckh et al., 2003; Ljungman, 2008], it probably results in over-treatment, as a number of patients who would never progress to CMV disease are treated nevertheless [Ljungman, 2006; Avetisyan et al., 2007]. Hopes have been raised that routine immunological monitoring for CMV-specific T-cell immunity may improve the management of active infection, leading to a more targeted use of antivirals and allowing the identification of patients at high risk for relapsing infections and end-organ disease. Nevertheless, to date, assessment of CMV-specific T-cell immunity has not had a major impact on clinical management of active CMV infection. In order to design potential intervention strategies based on immunological monitoring, characterization of the kinetics of functional CMV-specific T cells during episodes of active CMV infection is required. Studies addressing this issue are, however, scarce [Aubert et al., 2001; Foster et al., 2002; Widmann et al., 2008]. Resolution of episodes of active CMV infection in the allo-SCT setting appears to be ultimately dependent on the expansion of functional CMV-specific T cells in response to CMV replication [Quinnan et al., 1982; Reusser et al., 1991; Riddell et al., 1992]. In this regard, we have previously shown that the lack of prompt expansion of CMV pp65 and IE-1-specific IFN γ -producing CD8⁺ (IFN γ CD8⁺) and CD4⁺ (IFN γ CD4⁺) T cells is associated with rising levels of pp65 antigenemia and DNAemia during pre-emptive therapy and prolonged duration of antiviral treatment [Tormo et al., 2009]. In the present study, the dynamics of CMV-specific IFN γ CD8⁺ and IFN γ CD4⁺ T cells, which we have previously shown to confer

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3 76 protection against CMV infection [Solano et al., 2008], and those of CMV DNAemia
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5 77 were assessed in a number of episodes of active CMV infection. Data obtained in the
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8 78 present study are of potential interest for the therapeutic management of active CMV
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10 79 infections in the allo-SCT recipient.
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MATERIAL AND METHODS

Patients

Patients undergoing allo-SCT between December 2008 and May 2009 at the participating hospitals were eligible for inclusion. The study was approved by the Ethics Committees. All patients gave their informed consent to participate in the study. CMV-seronegative patients receiving a graft from a CMV-seronegative donor were not included in the study. Only episodes of active CMV infection treated pre-emptively were included for analysis. The end of the study period was June 22th 2009. Clinical and demographic data of the patients are shown in Table I.

Management of active CMV infection

Virological monitoring of CMV infection was performed by use of the pp65 antigenemia assay (Diagnostics® CMV pp65 Antigenemia Immunofluorescence assay, Chemicon International, Temecula, CA, USA), and/or a plasma real-time PCR assay (CMV real-time PCR, Abbott Molecular, Des Plaines, IL, USA, or LightCycler CMV Quant Kit, Roche, Branchburg, NJ, USA) as previously reported [Solano et al., 2001; Gimeno et al., 2008]. Pre-emptive therapy with oral valganciclovir (900 mg/12 h) or i.v. ganciclovir (5 mg/Kg/12 h) was initiated upon a positive antigenemia result (≥ 1 pp65 positive cells/200,000 cells) or detection of >1000 CMV DNA copies/mL in plasma depending on the participant institution, and discontinued following 2 consecutive negative antigenemia or plasma PCR results (also depending on the participant hospital) obtained 3 to 7 days apart after a minimum of 2 weeks of treatment. Foscarnet (i.v. 60 mg/Kg/12 h) was used instead of ganciclovir in patients with severe neutropenia, and in some patients not responding to ganciclovir therapy after 3 weeks of treatment. For analysis purposes, the duration of a given episode was that comprised between the day of initiation of pre-emptive therapy and the day of the first negative DNAemia result.

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126 Diagnosis of CMV disease was achieved as previously reported [Solano et al., 2001;
127 Gimeno et al., 2008]. When clinically indicated, patients received transfusions of
128 leukocyte-depleted and irradiated packed red cells and platelets.

Immunological monitoring

130 Enumeration of CMV-specific IFN γ CD8⁺ and IFN γ CD4⁺ T lymphocytes was
131 carried out by flow cytometry for ICS (BD Fastimmune, BD-Beckton Dickinson and
132 Company-Biosciences, San Jose, CA, USA) as described previously [Solano et al.,
133 2008]. Briefly, whole blood was simultaneously stimulated with two sets of 15-mer
134 overlapping peptides encompassing the sequence of pp65 and IE-1 CMV proteins (2
135 μ g/ml/peptide), obtained from JPT peptide Technologies GmbH (Berlin, Germany), in
136 the presence of 1 μ g/ml of costimulatory mAbs to CD28 and CD49d for 6 h at 37 °C.
137 Brefeldin A (10 μ g/ml) was added for the last 4 h of incubation Cells were
138 permeabilized and stained with a combination of labeled moAbs (anti-IFN γ -FITC, anti-
139 CD69-PE, anti-CD4 or CD8-PerCP-Cy5.5 and anti-CD3-APC when the IFN γ CD8⁺ kit
140 was used). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest
141 software (BD Biosciences Immunocytometry Systems). CD4⁺ and CD8⁺ events were
142 gated and then analyzed for the CD69 activation marker and IFN γ production. The total
143 number of CMV-specific IFN γ CD4⁺ and IFN γ CD8⁺ T cells was calculated by
144 multiplying the percentages of CMV-specific T cells producing IFN γ upon stimulation
145 (after background subtraction) by the absolute CD4⁺ and CD8⁺ T cell counts. The
146 specific responses were considered those >0.1% for both CD4⁺ and CD8⁺ T cells.

147 Immunological monitoring was performed once or twice a week during episodes of
148 active CMV infection. For some patients, several blood samples drawn after resolution
149 of the episode of active CMV infection were available for immunological analysis. A

total of 146 blood samples from the 18 patients (median 7 samples; range, 2–15 samples) were analyzed.

Sequence analysis of the UL54 and UL97 genes

CMV DNA extraction from plasma specimens was carried out using the High Pure nucleic acid kit (Roche Diagnostics, GmbH, Mannheim, Germany). The extracted viral DNA was used as a template for amplification of a 975 bp region of the UL97 gene spanning codons 429 to 753, and two regions of the UL54 gene spanning codons 345 to 625, and 645 to 1,013, as previously described [Tormo et al., 2009]. The PCR products were purified by the QIAquick purification kit (Qiagen GmbH, Hilden, Germany), sequenced by the ABI Prism BigDye Terminator Cycle Sequencing Kit v3.1 (PE Applied Biosystems), and analyzed on an ABI 310 automated DNA sequencer.

Statistical analysis

Data were analyzed with the aid of the statistical package SPSS (version 15.0). Comparisons were carried out using the non-parametric Mann-Whitney U-test for unpaired continuous data and the Wilcoxon test for paired continuous data. The Spearman rank test was used for analysis of correlation between continuous variables. For calculation purposes undetectable CMV-specific responses were computed as 0 cells/ μ L. A *P* value <.05 was considered statistically significant.

RESULTS

Features of episodes of active CMV infection

Nineteen episodes of active CMV infection (in 18 patients) were included for analysis. The episodes occurred at a median of 56 days post-transplant (range, 14 to 330 days). Thirteen out of the 19 episodes (in 12 patients) resolved within the study period (Table II). The remaining 6 episodes (in 6 patients) were still active at the end of the follow up, after a median of 30 days (range 14 to 97 days) of initiation of pre-emptive therapy (Table III). Three out of these 6 patients died during the study period (the cause of death was bacterial sepsis in one patient and severe GvHD in the other 2 patients).

CMV-specific T cells at the time of initiation of pre-emptive therapy

We firstly assessed the CMV-specific T-cell response at the time of initiation of pre-emptive therapy. Blood samples for immunological analysis were available from 14 episodes (9 of them developed before day 100 post-transplant). Undetectable IFN γ CD8⁺ and CD4⁺ T-cell responses were observed in 13 out of the 14 episodes. In the remaining episode, which was still active at the end of the follow up, low levels of both CMV-specific T-cell subsets were detected (0.03 IFN γ CD8⁺ T cells/ μ L and 0.26 IFN γ CD4⁺ T cells/ μ L). Nine out of the 14 episodes resolved within the study period, with the time to negative conversion of CMV DNAemia varying widely (3 to 94 days), while no control of CMV replication was achieved in the remaining 5 episodes. These data indicated that peripheral levels of IFN γ CD8⁺ and IFN γ CD4⁺ T cells at the time of initiation of pre-emptive therapy were not predictive of the virological outcome of the episode.

Kinetics of CMV-specific T cells and CMV DNAemia during episodes of Active CMV infection

We investigated the dynamics of both CMV-specific T-cell subsets and CMV DNAemia during 19 pre-emptively treated episodes of active CMV infection. Overall, peripheral counts of IFN γ CD8 $^{+}$ and IFN γ CD4 $^{+}$ T cells inversely correlated with CMV DNAemia levels ($\sigma=-0.806$; $P<0.001$, and $\sigma=-0.712$; $P=0.003$, respectively), yet detectable CMV-specific T-cell responses were observed in the presence of CMV DNAemia at some time points, both in unresolved episodes and in episodes which eventually cleared. We found however, that IFN γ CD8 $^{+}$ (median, 0.38 cells/ μ L; range, 0.03–1.28 cells/ μ L) and IFN γ CD4 $^{+}$ (median, 0.32 cells/ μ L; range, 0.06–1.27 cells/ μ L) T-cell levels in the presence of CMV DNAemia were significantly lower ($P<0.000$ for IFN γ CD8 $^{+}$ and $P<0.003$ for IFN γ CD4 $^{+}$ T cells) than IFN γ CD8 $^{+}$ (median, 2.46 cells/ μ L; range 0.82–15.02 cells/ μ L) and IFN γ CD4 $^{+}$ (median 0.56 cells/ μ L; range, 0.12–5.2 cells/ μ L) T-cell levels at the time of the first negative PCR result. Taking into consideration the peak value for IFN γ CD8 $^{+}$ T cells (1.28 cells/ μ L) found in the presence of CMV DNAemia, a threshold value of 1.3 cells/ μ L predicting CMV DNAemia clearance (negative PCR) was established in our cohort (PPV, 100%; NPV, 93%). A certain degree of overlap was found between IFN γ CD4 $^{+}$ T cell values in the presence of CMV DNAemia and those at the time of the first negative PCR result. Thus, setting a threshold (1.3 cells/ μ L) above the peak value (1.27 cells/ μ L) found in the presence of CMV DNAemia resulted in a lower NPV (75%). Therefore, the number of IFN γ CD8 $^{+}$ T cells was a more reliable marker for predicting CMV DNAemia clearance in our cohort.

Three different kinetic patterns of IFN γ CD8 $^{+}$ and IFN γ CD4 $^{+}$ T cells were observed in relation to CMV DNAemia: (i) A rapid expansion within the first week after

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3 224 initiation of pre-emptive therapy (median 7 days; range 3 to 7 days) concomitant with
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5 225 CMV DNAemia clearance was observed in 5 episodes (see episodes 1 to 5 in Table II).
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8 226 An expansion within the third week (days 15 and 16) was observed in 2 additional
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10 227 episodes (see episodes 6 and 7 in Table II). Overall, median increases of 2.09 IFN γ
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12 228 CD8⁺ T cells/ μ L (range, 0.9-15.13 cells/ μ L) and of 0.67 IFN γ CD4⁺ T cells/ μ L (range,
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14 229 0.12-5.20 cells/ μ L) from baseline were observed in these episodes. None of these
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17 230 patients was under corticosteroid treatment during the episode of active CMV infection.
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19 231 Figure 1A depicts a representative episode of this kinetics pattern (episode 4 in Table II);
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22 232 (ii) An early expansion (median 8 days; range 5 to 21 days) of a lower magnitude
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24 233 (median increase of 0.57 IFN γ CD8⁺ T cells/ μ L-range, 0.21-1.2 cells/ μ L $P=0.02$ -, and
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26 234 of 0.29 IFN γ CD4⁺ T cells/ μ L- range,0.01-0.62; $P=0.063$ -) than that observed in rapidly
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28 235 cleared episodes with delayed resolution of CMV DNAemia (median 31 days, range 20
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30 236 to 94 days), concomitant with a further expansion of both T-cell subsets (median
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32 237 increase of 3.05 IFN γ CD8⁺ T cells/ μ L and of 0.42 IFN γ CD4⁺ T cells/ μ L; $P=0.001$ and
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34 238 $P=0.04$, respectively) was observed in 6 episodes (see episodes 8 to 13 in Table II);
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36 239 Figure 1B illustrates a representative example of this kinetics pattern (data for the
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39 240 episode 8 in Table II). The two longest episodes (episodes 8 and 11) occurred in
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41 241 patients under treatment with corticosteroids for grade III–IV GvHD, and their
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43 242 resolution was coincident with steroid dose tapering; (iii) An inconsistent or a lack of
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45 243 expansion of IFN γ CD8⁺ (median, 0.06 cell/ μ L) and IFN γ CD4⁺ T cells (median, 0.22
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47 244 cell/ μ L; $P=0.001$ and $P=0.03$, respectively, in relation to T-cell increases measured in
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49 245 cleared episodes) with persistent CMV DNAemia that was still detectable at the end of
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51 246 the follow-up period was seen in 6 episodes (Table III). Three out of these 6 episodes
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53 247 developed while patients were under corticosteroid therapy for severe GvHD. Figure 1C
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55 248 shows the data for a representative case (episode 1 in Table III). The latest plasma
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samples available from the longest unresolved episodes (episodes 1 and 2 in Table III) were screened for the presence of mutations known to confer resistance to ganciclovir or foscarnet. No mutations were found in either sample.

CMV-specific T cells after CMV DNAemia clearance and relapsing infections

None of the 12 patients in whom the episode of CMV DNAemia resolved during the study period experienced a relapsing episode at least during the following month (only one of these patients received maintenance valganciclovir therapy after CMV DNAemia clearance). Episodes 2 and 3 in Table II developed in the same patient; However, the relapsing episode occurred 6 months after the resolution of the preceding one. Blood samples obtained at the time of interruption of antiviral therapy (second antigenemia test or PCR giving a negative result) were available for 8 episodes. A median of 2.8 IFN γ CD8⁺ T cells/ μ L (range 0.68 to 19.5 cells/ μ L) and of 0.38 IFN γ CD4⁺ T cells/ μ L (range 0.19 to 2.28 cells/ μ L) was found. Follow-up samples obtained after cessation of antiviral therapy (range 3 to 86 days) were available from 4 episodes. Detectable though fluctuating levels of both IFN γ CD8⁺ and IFN γ CD4⁺ T cells were observed over time (see the Figure 1, panel A for a representative example), with a median of 5.1 IFN γ CD8⁺ T cells/ μ L (range 0.57 to 47.7 cells/ μ L) and 0.41 IFN γ CD4⁺ T cells/ μ L (range 0.19 to 3.31 cells/ μ L).

CMV-specific T cells in patients with CMV disease

CMV enteritis was diagnosed in 3 patients in the setting of corticosteroid treatment for GvHD (Table II, episode 13, and Table III, episodes 4 and 5). In all cases, undetectable CMV-specific T-cell responses were observed at the onset of the clinical

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manifestations of the disease. Interestingly, in patient 12 (episode 13 in Table II), CMV DNAemia clearance and a notable improvement in clinical symptoms were related to a marked expansion of IFN γ CD8⁺ T cells, which occurred after steroid dose tapering. The remaining 2 patients failed to expand CMV-specific T cells and had persistent CMV DNAemia. Both patients died during the follow-up period.

DISCUSSION

Several conclusions can be drawn from our data. Firstly, peripheral counts of both IFN γ CD8⁺ and IFN γ CD4⁺ T-cell subsets inversely correlated with CMV DNAemia levels during episodes of active CMV infection. Similar data were obtained in earlier studies using pp65 peptide-tetramers [Aubert et al., 2001] or ICS [Widmann et al., 2008] to assess the CMV-specific CD8⁺ and CD4⁺ T-cell responses, respectively. In the latter study however, levels of CMV peptide-specific CD8⁺ T cells did not correlate with viremia [Widmann et al., 2008]. In another study [Avetisyan et al., 2006], patients who had detectable CMV-specific IFN γ CD3⁺ T-cell responses at week 4 after allo-SCT displayed lower peak mean viral loads compared with patients who lacked a detectable response. Furthermore, the degree of decline in CMV-specific T cells early after transplant has been associated with the development of CMV viremia [Eid et al., 2009]. In our study, the median levels of both CMV-specific functional T-cell subsets were significantly lower in the presence of CMV DNAemia than at the time of the first negative PCR result. A threshold value of 1.3 cells/ μ L for both T-cell populations predicting CMV DNAemia clearance was established in our cohort, the number of IFN γ CD8⁺ T cells being a more reliable marker. These cut-off cell levels are remarkably close to those previously determined by us [Solano et al., 2008] and by other groups [Hebart et al., 2002; Ohnishi et al., 2005; Lilleri et al., 2008; Moins-Teisserenc et al.,

2008, Pourgheysari et al., 2009] (different functional T-cell types) providing protection against development of CMV antigenemia or DNAemia.

Secondly, in agreement with a previous report by our group [Solano et al., 2008], we found that ultimate control of CMV replication in the course of antiviral therapy depended on a robust and sustained expansion of IFN γ CD8⁺ T cells, and – to a lesser extent – of IFN γ CD4⁺ T cells. A notable expansion of both T-cell subsets concomitant with CMV DNAemia clearance was seen as soon as one week after initiation of pre-emptive therapy in 5 episodes, although, overall, T-cell expansion controlling CMV replication was observed at a median of 15 days after implementation of therapy. In line with our findings, the peak median level of expanding pp65-specific functional CD8⁺ T cells was reported to occur around 20 days after CMV reactivation [Hakki et al., 2003]. In contrast, failure to expand both functional T-cell populations in response to CMV replication resulted in persistent CMV DNAemia despite antiviral treatment and the fact that the emergence of resistant strains was not documented (in the two longest episodes).

Three out of the 6 episodes that remained active at the end of the follow up occurred in the setting of corticosteroid therapy for GvHD, supporting the well known fact that corticosteroids impair the reconstitution of the CMV-specific T-cell response in a dose-dependent manner [Hakki et al., 2003; Gratama et al., 2008].

Thirdly, the marked expansion of functional CMV-specific T cells and the maintenance of peripheral pools of these T-cell subsets after CMV DNAemia clearance and cessation of antiviral therapy prevented the occurrence of relapsing episodes of active CMV infection at least during the following month. A similar conclusion was reached in earlier studies [Lilleri et al., 2008; Gratama et al., 2008; Moins-Teisserenc et al., 2008].

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Three patients developed CMV enteritis. In accordance with a previous report [Avetisyan et al., 2006], in all cases clinical onset of disease occurred in the face of undetectable IFN γ CD8⁺ and CD4⁺ T-cell responses. One of these patients recovered from CMV disease, and clinical improvement, as well as CMV DNAemia clearance, was concomitant with a robust expansion of both functional CMV-specific T-cell populations.

Our data may have several implications for the therapeutic management of active CMV infection in the allo-SCT recipient. A strategy of deferred antiviral therapy based on the detection of a CMV-specific immune response by ELISPOT at the time of CMV DNAemia detection was safely applied in a number of patients late (around 3 months) after transplant [Avetisyan et al., 2007]. In our cohort, however, no patient stratification could be established according to the magnitude of the CMV-specific T-cell response at the time of initiation of pre-emptive therapy, as all but one patient displayed undetectable responses. The lack of a detectable CMV-specific T-cell response, however, was not predictive of the outcome of CMV infection in terms of the duration of CMV DNAemia. Thus, no patients from our cohort would have benefited from the abovementioned strategy. Our study and that of Avetisyan *et al.* [2007], however, differ in the method employed for enumeration of CMV-specific functional T cells, and most importantly, in the time frame in which active CMV infections occurred (early after transplant in most of our patients and late after transplant in theirs). Further studies are required to determine the clinical usefulness of this therapeutic approach.

Early interruption (or dose reduction) of pre-emptive therapy provided that a significant expansion of CMV-specific T cells is documented may be a potential intervention strategy based on immunological monitoring. Our data indicate that peripheral levels of CMV-specific IFN γ CD8⁺ T cells above 1.3 cells/ μ L, which were

shown to be associated with CMV DNAemia clearance, might be a reasonable threshold at which to interrupt antiviral therapy. Our data do not allow us to be certain about the virological efficacy and clinical safety of this approach, as the antiviral therapy course was not suspended in our patients until a second negative antigenemia or PCR result. This question can only be answered by means of a controlled clinical trial.

Relapsing episodes of active CMV infection develop at an exceedingly high rate following the implementation of short duration pre-emptive treatment regimens [Gimeno et al., 2008]. We have previously shown that their occurrence is related to the presence of low peripheral levels of CMV-specific functional T cells after resolution of the preceding episode [Tormo et al., 2009]. Data obtained in the present study extend our previous observation. In effect, peripheral levels of both T-cell subsets above the abovementioned cut-off at the time of virological clearance and interruption of antiviral therapy consistently prevented the occurrence of relapsing episodes. In these patients a sustained but rather fluctuating CMV-specific T-cell response was observed. On the basis of this finding, maintenance antiviral therapy should be administered after CMV DNAemia clearance provided that protective levels of functional CMV-specific T cell are not reached at the end of the antiviral therapy course.

Adoptive transfer of CMV-specific functional T cells is a therapeutic option in episodes of active CMV infections that do not respond to antiviral therapy [Einsele et al., 2008]. According to our data, a lack of consistent expansion of CMV-specific T cells is associated with persistent CMV DNAemia, even in the absence of proven resistance to antivirals. In this sense, it would be reasonable to consider this therapeutic strategy, rather than switching antiviral therapy, in patients failing to respond to antivirals after 4 weeks of treatment (time at which expansion of functional CMV-specific T cells eventually controlling CMV replication was documented in most of cleared episodes in

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373 our cohort), provided that corticosteroid therapy is not underway, and that emergence of
374 antiviral-resistant strains is ruled out.

375 In summary, our data suggest that routine immunological monitoring during episodes
376 of CMV DNAemia may yield useful information for the therapeutic management of
377 active CMV infection in allo-SCT recipients. Larger studies are nevertheless needed in
378 order to verify this assumption.

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REFERENCES

- Aubert G, Hassan-Walker AF, Madrigal AJ, Emery VC, Morte C, Grace S, Koh MB, Potter M, Prentice HG, Dodi IA, Travers PJ. 2001. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. *J Infect Dis* 184: 955-963.
- Avetisyan G, Aschan J, Häggglund H, Ringdén O, Ljungman P. 2007. Evaluation of intervention strategy based on CMV-specific immune responses after allogeneic SCT. *Bone Marrow Transplant* 40: 865-869.
- Avetisyan G, Aschan J, Häggglund H, Ringdén O, Ljungman P. 2007. Evaluation of intervention strategy based on CMV-specific immune responses after allogeneic SCT. *Bone Marrow Transplant* 40: 865-869.
- Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. 2003. Cytomegalovirus in hematopoietic stem cell transplant recipients: Current status, known challenges, and future strategies. *Biol Blood Marrow Transplant* 9: 543-558.
- Eid AJ, Brown RA, Hogan WJ, Lahr BD, Eckel-Passow JE, Litzow MR, Razonable RR. 2009. Kinetics of interferon-gamma producing cytomegalovirus (CMV)-specific CD4+ and CD8+ lymphocytes and the risk of subsequent CMV viremia after allogeneic hematopoietic stem cell transplantation. *Transplant Infect Dis* 11:519-528.
- Einsele H, Kapp M, Grigoleit GU. 2008. CMV-specific T-cell therapy. *Blood Cells Mol Dis* 40: 71-75.
- Foster AE, Gottlieb DJ, Sartor M, Hertzberg MS, Bradstock KF. 2002. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar

- reconstitution pattern after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 8: 501-511.
- Gimeno C, Solano C, Latorre JC, Hernández-Boluda JC, Clari MA, Remigia MJ, Furió S, Calabuig M, Tormo N, Navarro D. 2008. Quantification of DNA in plasma by an automated real-time PCR assay (CMV PCR Kit, Abbott) for surveillance of active cytomegalovirus infection and guidance of pre-emptive therapy for allogeneic hematopoietic stem cell transplant recipients. *J Clin Microbiol* 46: 3311-3318.
- Griffiths P, Whitley R, Snyderman DR, Singh N, Boeckh M. 2008. Contemporary management of cytomegalovirus infection in transplant recipients: Guidelines from an IHMF workshop, 2007. *Herpes* 15: 1-12.
- Gratama JW, Brooimans RA, van der Holt B, Sintnicolaas K, van Doornum G, Niesters HG, Löwenberg B, Cornelissen JJ. 2008. Monitoring cytomegalovirus IE-1 and pp65-specific CD4+ and CD8+ T-cell responses after allogeneic stem cell transplantation may identify patients at risk for recurrent CMV reactivations. *Cytometry Part B* 74B: 211-220.
- Hakki M, Riddell SR, Storek J, Carter RA, Stevens-Ayers T, Sudour P, White K, Corey L, Boeckh M. 2003. Immune reconstitution to cytomegalovirus after allogeneic stem cell transplantation: Impact of host factors, drug therapy, and subclinical reactivation. *Blood* 102: 3060-3067.
- Hebart H, Dagnik S, Stevanovic S, Grigoleit U, Dobler A, Baur M, Rauser G, Sinzger C, Jahn G, Loeffler J, Kanz L, Rammensee HG, Einsele H. 2002. Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon- γ enzyme-linked immunospot assay and flow cytometry

- in healthy individuals and in patients after allogeneic stem cell transplantation.
Blood 99: 3830-3837.
- Lilleri D, Fornara C, Chiesa A, Caldera D, Alessandrino EP, Gerna G. 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: 248-256
- Ljungman P. CMV infections after hematopoietic stem cell transplantation. 2008. *Bone
Marrow Transplant* 42: S70-S72.
- Ljungman P. 2006. Would monitoring CMV immune responses allow improved
control of CMV in stem cell transplant patients? *J Clin Virol* 35: 493-495.
- Moins-Teisserenc H, Busson M, Scieux C, Bajzik V, Cayuela JM, Clave E, de Latour
RP, Agbalika F, Ribaud P, Robin M, Rocha V, Gluckman E, Charron D, Socié G,
Toubert A. 2008. Patterns of cytomegalovirus reactivation are associated with
distinct evolutive profiles of immune reconstitution after allogeneic
hematopoietic stem cell transplantation. *J Infect Dis* 198: 818-826.
- Ohnishi M, Sakurai T, Heike Y, Yamazaki R, Kanda Y, Takaue Y, Mizoguchi H,
Kawakami Y. 2005. Evaluation of cytomegalovirus-specific T-cell reconstitution
in patients after various allogeneic haematopoietic stem cell transplantation
using interferon-gamma-enzyme-linked immunospot and human leucocyte
antigen tetramer assays with an immunodominant T-cell epitope. *Br J Haematol*
131: 472-479.
- Pourgheysari B, Piper KP, McLarnon A, Arrazi J, Bruton R, Cook M, Mahendra P,
Craddock C, Moss PAH. 2009. Early reconstitution of effector memory CD4⁺

- 469 CMV-specific T cells protects against CMV reactivation following allogeneic
470 SCT. Bone Marrow Transplant 43 :853-861.
- 471 Quinnan GV, Kirmani N, Rook AH, Manischewitz JF, Jackson L, Moreschi G, Santos
472 GW, Saral R, Burns WH.1982. Cytotoxic T cells in cytomegalovirus infection:
473 HLA-restricted T-lymphocyte and non T-lymphocyte cytotoxic responses
474 correlate with recovery from cytomegalovirus infection in bone-marrow-
475 transplant recipients. N Engl J Med 307: 7-13.
- 476 Reusser P, Riddell SR, Meyers JD, Greenberg PD. 1991.Cytotoxic T-lymphocyte
477 response to cytomegalovirus after human allogeneic bone marrow
478 transplantation: pattern of recovery and correlation with cytomegalovirus
479 infection and disease. Blood 78: 1373-1380.
- 480 Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. 1992.
481 Restoration of viral immunity in immunodeficient humans by the adoptive
482 transfer of T cell clones. Science 257: 238-241.
- 483 Solano C, Benet I, Clari MA, Nieto J, de la Cámara R, López J, Hernández-Boluda JC,
484 Remigia MJ, Jarque I, Calabuig ML, Garcia-Noblejas A, Alberola J, Tamarit A,
485 Gimeno C, Navarro D. 2008. Enumeration of CMV-specific IFN γ CD8⁺ and
486 CD4⁺ T cells early after allogeneic stem cell transplantation may identify
487 patients at risk of active CMV infection. Haematologica 93: 1434-1436.
- 488 Solano C, Muñoz I, Gutiérrez A, Farga A, Prósper F, García-Conde J, Navarro D,
489 Gimeno C. 2001. Qualitative plasma assay (AMPLICOR CMV test) versus pp65
490 antigenemia assay for monitoring cytomegalovirus viremia and guiding pre-
491 emptive ganciclovir therapy in allogeneic stem cell transplantation. J Clin
492 Microbiol 39: 3938-3941.

493 Tormo N, Solano C, Benet I, Clari MA, Nieto J, de la Cámara R, López J, López-
494 Aldeguer N, Hernández-Boluda JC, Remigia MJ, Garcia-Noblejas A, Gimeno C,
495 Navarro D. 2009. Lack of prompt expansion of cytomegalovirus pp65 and IE-1-
496 specific IFNgamma CD8(+) and CD4(+) T cells is associated with rising levels
497 of pp65 antigenemia and DNAemia during pre-emptive therapy in allogeneic
498 hematopoietic stem cell transplant recipients. Bone Marrow Transplant Jul 20
499 [Epub ahead of print].

500 Widmann T, Sester U, Gärtner BC, Schubert J, Pfreundschuh M, Köhler H, Sester
501 M.2008. Levels of CMV specific CD4 T cells are dynamic and correlate with
502 CMV viremia after allogeneic stem cell transplantation. PLoS ONE 3: 1-12.

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FIGURE 1. Representative patterns of kinetics of CMV pp65 and IE-1-specific IFN γ -producing CD8⁺ (IFN γ CD8⁺ -open circles-) and CD4⁺ (IFN γ CD4⁺) T cells (black diamonds) and CMV DNAemia (triangles) during episodes of active CMV infection. (A) Patient displaying an early expansion of CMV-specific T cells (specially involving the IFN γ CD8⁺ T-cell subset) concomitant with CMV DNAemia resolution. Fluctuating levels of both T-cell populations were observed after interruption of antiviral therapy in the absence of CMV DNAemia; (B) Patient displaying an early expansion of both functional T-cell populations with delayed clearance of CMV DNAemia concomitant with a further expansion of CMV-specific T cells; Fluctuating levels of both T-cell subsets were observed prior to ultimate expansion leading to CMV DNAemia resolution (C) Patient failing to expand either CMV-specific functional T-cell subset and displaying persistent CMV DNAemia. This patient was under antiviral therapy throughout the observational period. The arrow (AT) points out to the time at which antiviral therapy was interrupted.

TABLE I. Demographic and clinical data of the patients.

Parameter	
Patients	18
Median age, yrs (range)	52.5 (17–67)
Sex, no. male patients/no. female patients	12/6
Diagnosis, n° patients (%)	
Non-Hodgkin`s lymphoma	8 (44)
Acute myeloid leukaemia	4 (22)
Multiple myeloma	3 (16)
Myelodysplastic syndrome	2 (11)
Chronic lymphocytic leukaemia	1 (6)
CMV serostatus, n° patients (%)	
D+/R+	9 (50)
D-/R+	6 (33)
D+/R-	3 (16)
Donor type, n° patients (%)	
HLA-identical sibling	10 (55)
Matched unrelated donor	5 (28)
Mismatched related donor	3 (16)
Conditioning regimen, n° patients (%)	
Non-myeloablative	14 (77)
Myeloablative	4 (22)
Stem cell source	
Peripheral blood	16 (88)
Umbilical cord blood	1 (6)
Bone marrow	1 (6)
Acute or chronic GvHD during episodes of active CMV infection	
Grades 0-I	12 (66)
Grades II-IV	6 (33)

D,donor; R, recipient; +, CMV seropositive; -, CMV seronegative; GvHD,Graft versus host disease.

TABLE II. Virological and immunological data of episodes of active CMV infection, the resolution of which occurred during the study period.

Episode (Day PT)	CMV DNAemia (Copies/mL)			CMV-specific T-cell response IFN γ CD8 $^{+}$ / IFN γ CD4 $^{+}$ (cells/ μ L)	
	Initial	Peak	Duration (days)	First detected response (day)	At first negative PCR (day)
1 (232)	1125	1125	7	15.13/0.12 (7)	- *
2 (115)	800	800	3	2.09/1.61 (3)	-
3 (293)	2460	2460	7	15.20/5.20 (7)	-
4 (41)	890	890	7	14.01/1.90 (7)	-
5 (40)	1800	1800	6	1.31/0.43 (6)	-
6 (59)	560	1200	15	0.90/0.38 (15)	-
7 (14)	1100	3200	16	1.45/0.67 (16)	-
8 (30)	1100	6200	94	0.50/0.40 (21)	6.24/0.55 (94)
9 (26)	1980	1980	20	0.65/0.19 (7)	0.82/0.56 (20)
10 (53)	3600	4560	55	0.21/0.62 (21)	2.46/1.23 (55)
11 (63)	1270	3700	61	0.22/0.01 (7)	1.36/0.48 (61)
12 (27)	1200	3100	31	0.67/0.06 (5)	4.34/0.70 (31)
13 (65)	7200	15110	31	1.28/0.46 (9)	10.62/0.35 (31)

PT, post-transplant. Day in the column for the first detected CMV-specific T-cell responses refers to days after initiation of pre-emptive therapy. Episodes 2 and 3 developed in the same patient. *, First detected response concomitant with first negative PCR.

TABLE III. Virological and immunological data of episodes of active CMV infection that were still active at the end of the study period.

Episode (Day PT)	CMV DNAemia (Copies/mL)		CMV-specific T-cell response IFN γ CD8 ⁺ / IFN γ CD4 ⁺ (cells/ μ L)		Treatment
	Initial	Peak (day)	First detectable response (day)	Peak level (day)	
1 (330)	2390	3790 (7)	0.96/0.19 (20)	- *	FOS/VGCV
2 (62)	2320	35,450 (12)	1.28/1.27 (8)	-	VGCV
3 (160)	1350	10,200 (30)	0.03/0.06 (7)	-	VGCV
4 (42)	5440	5500 (9)	0.09/0.46 (9)	-	FOS
5 (55)	5600	23,450 (19)	ND	ND	VGCV
6 (36)	720	1290 (23)	0.03/0.26 (0)	0.53/0.66 (8)	GAN

FOS, Foscarnet; VGCV, Valganciclovir; GAN, Ganciclovir; ND, Not detectable; PT, Post-transplant. Day in columns refers to days after initiation of pre-emptive therapy.

*, The peak level was the first detected response.

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