Diagnostic Utility of Human Cytomegalovirus-Specific T-Cell Response Monitoring in Predicting Viremia in Pediatric Allogeneic Stem-Cell Transplant Patients

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Background. Several studies proved that virus-specific T-cells play a pivotal role in controlling cytomegalovirus (CMV) infection in adult allogeneic hematopoietic stem-cell transplant (HSCT) patients. Fewer data are available in pediatric HSCT settings, when immature and inexperienced immune system may affect antiviral immune reconstitution.
Methods. We analyzed prospectively the CMV-specific T-cell reconstitution in a cohort of 31 pediatric allogeneic HSCT recipients at 30, 60, 90, 120, 180, and 360 days after HSCT.
Results. Depending on donor-recipient CMV serostatus, we observed distinct patterns and kinetics of CMV-specific T-cell immune reconstitution: during the early time-points, patients displayed a severe reduction in CMV-specific T-cell recovery in both CMV seropositive donor (D+) group and CMV seronegative donor (D−) on CMV seropositive recipients (R+). From day 90 onward, statistical significant differences in the profile of T-cell immune reconstitution emerged between D+ and D−. The pattern of immune reconstitution was characterized by heterogeneous kinetics and efficiencies: we report cases of: (1) spontaneous antiviral T-cell recovery with no previous viremia, (2) immune T-cell recovery anticipated by CMV viremia, and (3) no T-cell immune reconstitution despite previous viremia episodes.
Conclusions. Given the heterogeneous scenarios of antiviral T-cell immune recovery in pediatric allogeneic HSCT, we conclude that the evaluation of the antiviral immune reconstitution is a promising and appealing system for identifying patients at higher risk of CMV infection. The use of interferon-γ ELISPOT test is a valid tool for immunological monitoring and predicting CMV viremia in pediatric HSCT.

Keywords: Cytomegalovirus, T-cell immunity, Antiviral immune reconstitution, Pediatric hematopoietic stem cell transplantation, ELISPOT.

(*Transplantation* 2012;93: 536–542)

Human cytomegalovirus (CMV) is a prominent pathogen that may cause detrimental infections and life-threatening conditions in pediatric allogeneic hematopoietic stem-cell transplant (HSCT) patients (*1–6*). It is generally accepted that the risk of CMV viremia occurs more frequently during the first 100 days after transplantation when lymphopenia or impaired lymphocyte function expose patients to a wide variety of opportunistic pathogens (*7*, *8*). Accordingly, the risk of CMV infection is higher after ex vivo T-cell depletion of the graft (*9*, *10*) both in adult and pediatric patients and with the use of high dose of steroids (*11*).

The most common clinical manifestations of CMV disease in HSCT patients are interstitial pneumonia, hepatitis, gastroenteritis, retinitis, and encephalitis. Once CMV disease is established the patient prognosis remains poor (*4*, *5*, *12*). CMV infection is also involved in a variety of indirect effects including augmented severity of graft-versus-host disease (GVHD) and increased frequencies of opportunistic infections (*13*, *14*). Most frequently, CMV infection arises from latent virus reactivation in the stem-cell donor or recipient (*15*). In the posttransplant phase, CMV infection is success-

This work was supported by the Città della Speranza, Malo, Italy, grant 0607, the University of Padova, Progetto di Ateneo 2006 grant CPDA063398, and Italian Ministry of Education, National Strategic Interest Research Program grant PRIN, 2007CCW84J_002.
The authors declare no conflicts of interest.
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S.C. and D.A. designed and performed the experiments and analyzed the data. S.C. and D.A. wrote the manuscript. S.C., A.S., M.F., G.T., S.V., and M.B. performed the experiments and collected clinical results. G.P., M.C., C.M., R.C., and L.B. supervised the clinical and experimental data.

Received 8 April 2011. Revision requested 3 May 2011.
Accepted 14 November 2011.

ISSN 0041-1337/12/9305-536
DOI: 10.1097/TP.0b013e31824215db

fully kept under control by preemptive and prophylactic therapeutic approaches (*7*). Both strategies have dramatically reduced the incidence of symptomatic CMV infection among HSCT patients, however, the prolonged usage of antiviral drugs and inefficient priming of CMV-specific T-cell response exposes patients to late symptomatic CMV infections and development of drug-resistant CMV strains (*16–19*). T-cell immunity plays a crucial role in controlling CMV reactivation and shedding as demonstrated by high frequency of circulating T-cells targeting CMV antigens in immunocompetent CMV seropositive adult (*16*, *20–24*).

In allogeneic HSCT patients, it is well established that the most critical conditions occur in CMV seronegative donor (D−) of CMV seropositive recipients (R+), because in this settings CMV may replicate in absence of an adequate immune response (*17*, *25–32*). However, CMV seropositive donors (D+) may also experience occurrence of CMV infection and disease as several factors may influence antiviral immunological recovery. Therefore, the quantitative evaluation of CMV-specific T-cell reconstitution on individual level may be helpful in determining the patients at risk of CMV viremia and disease (*16*, *33–37*).

In this study, we present the pattern of CMV immune reconstitution over time of a cohort of 31 pediatric allogeneic HSCT monitored prospectively for CMV DNAemia and CMV-specific T-cell immune response.

RESULTS

The main demographic and clinical characteristics of study patients are shown in Table 1.

CMV Infection

During the study period, 14 patients experienced CMV infection. Table 2 shows the patient's distribution according to donor/recipient CMV serostatus. Overall, 27 episodes of CMV infection were counted: nine patients had one episode, one patient had two episodes, three patients had three episodes and one had seven episodes. All episodes were asymptomatic but 3 (10%) that were classified as CMV disease for the presence of fever not otherwise explained despite an extensive diagnostic investigation. The three cases of CMV disease occurred in absence of specific end-organ disease. They were two male and one female of 2, 16, and 5-year-old, respectively, affected by juvenile myelomonocytic leukemia, lymphoblastic acute leukemia and Fanconi anemia, who had undergone cord blood transplantation (two patients) and allo-unrelated transplant (one patient). Most episodes were managed with foscarnet therapy although seven episodes were treated with ganciclovir and three episodes with cidofovir. In six episodes, CMV immunoglobulins were added to antiviral treatment.

Because we have not found differences in terms of incidence of CMV DNAemia and CMV-specific T-cell response between D+/R− and D+/R+ pairs (data not shown), we refer to both groups as D+. We will also refer as D− to the D−/R+ pairs because the combination D−/R− pair did not experience CMV viremia and did not develop detectable CMV-specific T-cell immunity at any time-point analyzed (data not shown).

TABLE 1. HSCT patients characteristics

Gender (%)	
Male	18 (58)
Female	13 (42)
Median age (years)	8 (1–17)
Disease	
Malignant	25 (81)
Non malignant	6 (19)
Stem cell source (%)	
Bone marrow and peripheral blood	20 (64)
Cord blood	11 (36)
Donor type (%)	
Sibling	8 (26)
Unrelated	23 (74)
Donor/recipient serostatus (%)	
D+/R+, D+/R-	16 (52)
D-/R+	9 (29)
D-/R-	6 (19)
Conditioning regimen (%)	
TBI based	9 (29)
Non TBI based	22 (71)
ATG (%)	
Yes	27 (87)
No	4 (13)
PMN engraftment	All patients
Days (median)	10–43 (20)
PLT engraftment	All patients
Days (median)	12–106 (28)
Acute GVHD (%)	
Grade 0–I	1 (3)
D+/R+, D+/R-	1 (100)
D-/R+	0 (0)
Grade II–IV	23 (74)
D+/R+, D+/R-	12 (52)
D-/R+	11 (48)
Acute GVHD involvement (%)	
Skin	4 (13)
D+/R+, D+/R-	2 (50)
D-/R+	2 (50)
GI tract + skin	20 (64)
D+/R+, D+/R-	11 (55)
D-/R+	9 (45)
Steroid therapy (%)	
(any time within day + 100 for acute GVHD)	23 (74)
D+/R+, D+/R-	14 (61)
D-/R+	9 (39)
(during CMV reactivation)	7 (23)
D+/R+, D+/R-	3 (43)
D-/R+	4 (57)
Chronic GVHD (%)	
Limited	1 (3)
D+/R+, D+/R-	1 (100)
D-/R+	0 (0)
Extended	8 (26)
D+/R+, D+/R-	4 (50)
D-/R+	4 (50)

Number of patients is followed by the percentage in parentheses.

D, donor; R, recipient; TBI, total body irradiation; ATG, anti-thymocite globulins; GVHD, graft versus host disease; GI, gastrointestinal; PMN, polymorphonuclear neutrophil; PLT, platelet; HSCT, hematopoietic stem-cell transplant.

CMV T-Cell-Specific Immune Recovery

We report the trend of CMV-specific T-cell response (Fig. 1A) and CMV DNAemia (Fig. 1B) pattern within 30 to 360 days after transplantation in D+ and D− groups. Both groups display a dramatic decrease in CMV-specific T-cell immunity at day +30 after HSCT, followed by a progressive increase of CMV-specific T-cell immunity from day +60 to day +360 post-HSCT. D+ and D− groups display statistical

significant differences at 120, 180, and 360 days after transplantation with D+ group having higher values of CMV-specific T-cell response. Conversely, CMV DNAemia for the same time points show that statistical differences between D+ and D− groups occur at 60, 180, and 360 after HSCT with D− group experiencing greater values of CMV DNAemia. To illustrate the relationship between CMV-DNAemia and CMV T-cell-specific immune recovery, we present four representative cases showing two D+/R+ patients and two to D−/R+ patients (Fig. 2)

1. The first case (Fig. 2A) shows a 7-year-old male D+/R+ patient suffering Fanconi anemia. The patient underwent a related sibling human leukocyte antigen (HLA)-matched HSCT. Conditioning regimen consisted in administration of rabbit antithymocyte serum (Genzyme) fludarabine, cyclophosphamide. Despite the effect of in vivo T-cell depletion of the graft by antithymocyte serum, this patient recovered CMV immunity rapidly after HSCT and no episode of detectable CMV DNAemia was recorded.
2. The second case (Fig. 2B) shows a 5-year-old female D−/R+ patient suffering Fanconi anemia. The patient underwent unrelated cord blood transplantation. Conditioning regimen consisted in administration of antithymocyte serum, fludarabine, cyclophosphamide. The patient presented an early CMV DNAemia episode that was controlled by prolonged foscarnet administration. In this phase, the patient did not develop a significant CMV-specific T-cell response. A second episode of late CMV DNAemia was observed after day +120 post-HSCT that required a further treatment with ganciclovir. This second episode associated to a significant increase of CMV-specific T-cell immunity. No other CMV reactivation occurred thereafter.
3. The third case (Fig. 2C) shows a 17-year-old male D+/R+ patient suffering acute lymphoblastic leukemia who received peripheral stem cells from an unrelated donor. The patient-conditioning regimen consisted in administration of thiotepa, cyclophosphamide, antithymocyte serum, and total-body irradiation (TBI) at 12 Gray. Shortly after transplantation, the patients developed a robust CMV immunity that followed an early CMV DNAemia spike. The episode of CMV reactivation was treated successfully with ganciclovir.
4. The fourth case (Fig. 2D) shows a 4-year-old female D−/R+ patient suffering acute lymphoblastic leukemia. The patient underwent cord blood transplantation. The patient-conditioning regimen consisted in administration of thiotepa, cyclophosphamide, antithymocyte serum, and TBI. The patient experienced until day +180 post-HSCT both early and late CMV reactivations, despite prolonged therapy with ganciclovir and cidofovir. During all this period, the patient failed to reconstitute any detectable CMV-specific T-cell response.

TABLE 2. CMV viremia in HSCT patients

CMV serostatus (number of pairs)	D-/R- (6)	D+/R+ or D+/R- (16)	D-/R+ (9)
Patients with episodes of CMV DNAemia within day +100	0	5 (31%)	2 (22%)
Patients with episodes of CMV DNAemia after day +100	0	3 (19%)	1 (11%)
Patients with CMV disease	0	1 (6%)	2 (22%)

CMV, cytomegalovirus; HSCT, hematopoietic stem-cell transplant.

The four examples represent emblematic cases showing that the occurrence of solid antiviral immune responses prevented from CMV replication and shedding although a low or undetectable count of CMV-specific T-cell response exposed patients to prolonged or further viral reactivation.

To determine the ability of the interferon (IFN)-γ ELISPOT test to be predictive of future DNAemia events, we compared the IFN-γ ELISPOT counts for patients who did not experience DNAemia within 30 days after ELISPOT determination versus patients who experienced at least one event of CMV DNAemia more than 1000 DNA copies/mL within 30 days after ELISPOT determination. The patients

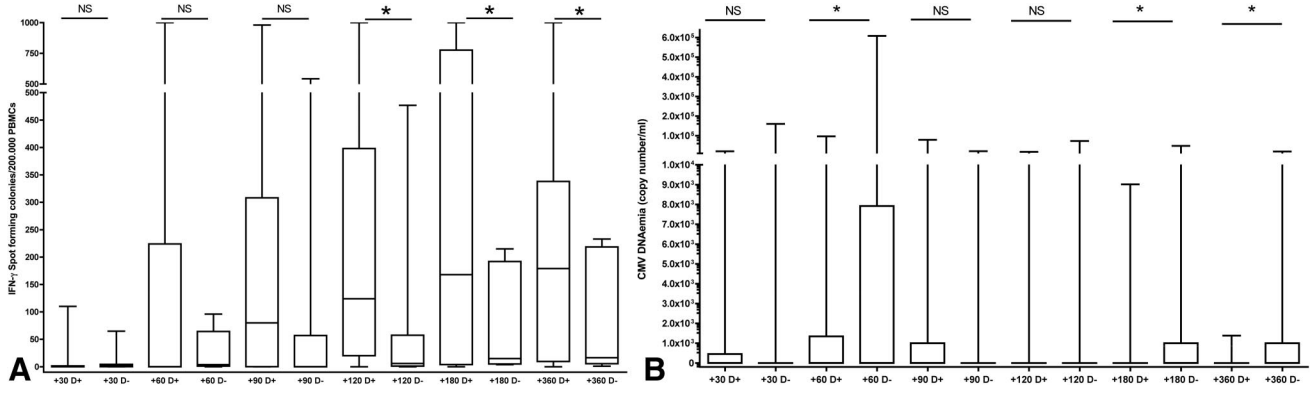

FIGURE 1. (A) Cytomegalovirus (CMV)-specific T-cell immunity and (B) CMV DNAemia in pediatric allogeneic HSCT patients. *x*-axis shows the days after HSCT for D+ and D− groups. D−/R− HSCT patients were excluded because this group did not experience detectable CMV DNAemia or CMV-specific T-cell immunity. *y*-axis shows box and whiskers plots of the levels of CMV-specific interferon (IFN)-γ spot forming colonies/200,000 peripheral blood mononuclear cells (PBMCs) (A), or CMV DNAemia copy number/milliliter whole blood (B).

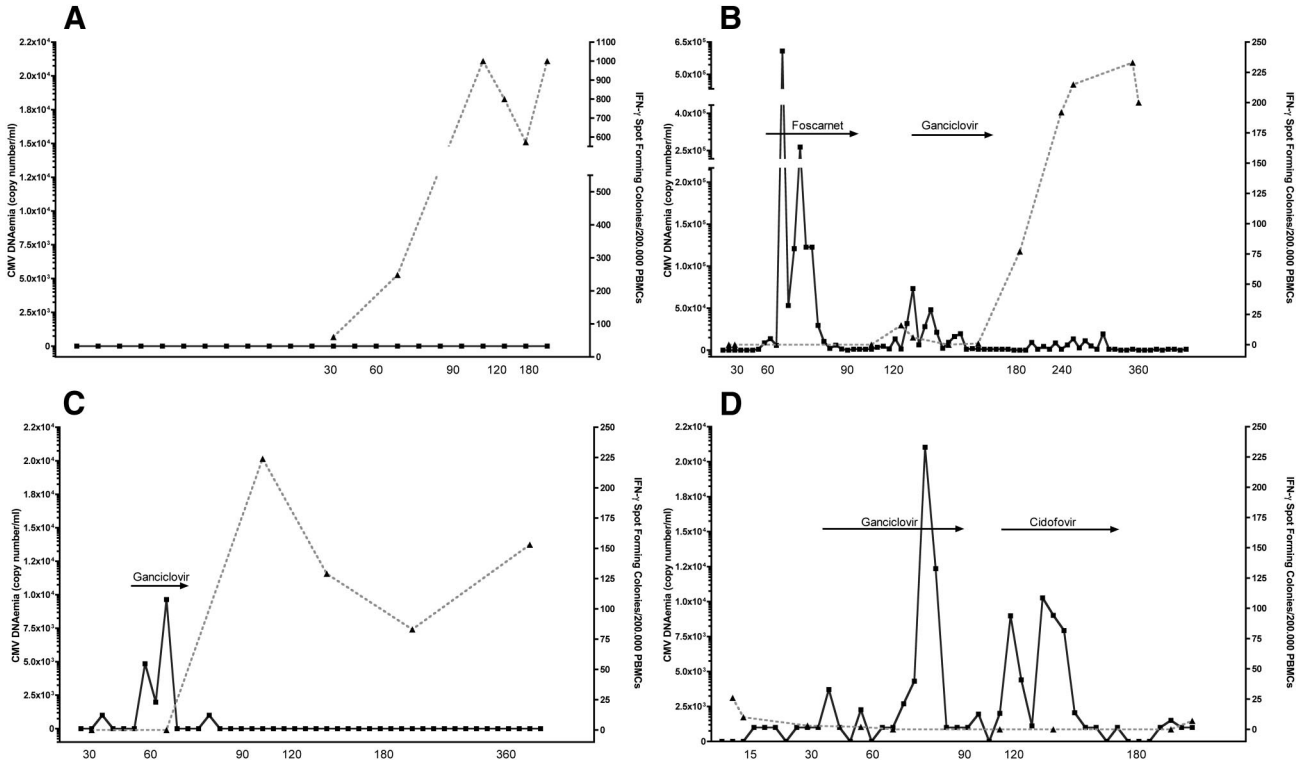

FIGURE 2. Cytomegalovirus (CMV) DNAemia and CMV-specific T-cell response plots in four HSCT patients at various time after transplantation. (A) Seven-year-old D+/R+ patient suffering Fanconi anemia, HLA-related sibling bone marrow transplant. (B) Five-year-old D−/R+ suffering Fanconi anemia, cord blood transplant. (C) Seventeen-year-old D+/R+ suffering acute lymphoblastic leukemia, peripheral stem-cell transplant from an unrelated donor. (D) Four-year-old D−/R+ suffering acute lymphoblastic leukemia, cord blood transplant. (*Dashed lines* and *triangles*) ELISPOT counts. Numbers below the *x*-axis indicate the days after transplantation. (*Solid line* and *squares*) DNAemia.

who did not experience CMV DNAemia after the IFN-γ ELISPOT determination had a median absolute number count of 117 (range, 0–361 spots) SFC/200,000 peripheral blood mononuclear cells (PBMCs), whereas patients who experienced at least one CMV DNAemia event had a median count of 5 (range 0–192) SFC/200,000 PBMCs (Fig. 3A). The two groups display statistically significant differences (P value <0.05). The receiver operating characteristic curve displays the ELISPOT levels associated with sensitivity and 1-specificity in predicting patients at risk or protected from CMV viremia. The test shows that the area under the curve value is 0.82 and that ELISPOT levels comprised between 20 and 77 (absolute number/200,000 PBMCs) are associated with the highest probability of correct classification (81.6%), and assure a specificity of 83.3% coupled with a specificity of 78.6% (Fig. 3B). To provide clinical threshold defining protection versus risk of viremia, we arbitrarily consider more than 80 ELISPOT as protective, whereas less than 20 ELISPOT as potential risk factor for developing CMV viremia.

Outcome

After a median follow-up of 1 year (range, 66–765 days), 25 patients are alive although one patient died of Epstein-Barr virus-related posttransplant lymphoproliferative disease at 66 days from HSCT and five patients relapsed and died of disease progression at 148, 185, 233, 266, and 380 days from HSCT. No patient died of CMV disease. The 1-year overall survival (OS) probability was 81.7% (95% CI, 61.0%–92.0%). The 1-year transplant-related mortality was 3.2%.

DISCUSSION

The goal of the successful HSCT consists in the cure of the underlying disease and the functional restoration of the immune system to provide long-term protection from potential deadly pathogens. It is well established that CMV-specific T-cells play a pivotal role in controlling virus replication and shedding (*12*, *26*, *38–41*) and thus evaluation of CMV-specific T-cell immunity is becoming an appealing and desirable strategy to assess the antiviral reconstitution in transplant patients (*35*, *42*).

The ELISPOT analysis in HSCT patients revealed no statistical significant differences in CMV-specific T-cell immune reconstitution up to 90 days after transplantation. Statistical significant differences between D+ and D− groups appeared from 120 days to 360 days after HSCT with D+ patients displaying a better immune reconstitution compared with D− patient. The results are in agreement with previous reports showing higher risk of CMV infection of D−/R+ patients compared with D+/R− or D+/R+ (*30*, *43*). However, the pattern of CMV-specific T-cell reconstitution displayed a heterogeneous structure. To illustrate this, we presented four different representative cases of HSCT patients: one patient recovered CMV immunity spontaneously without CMV DNAemia episodes (Fig. 2A),

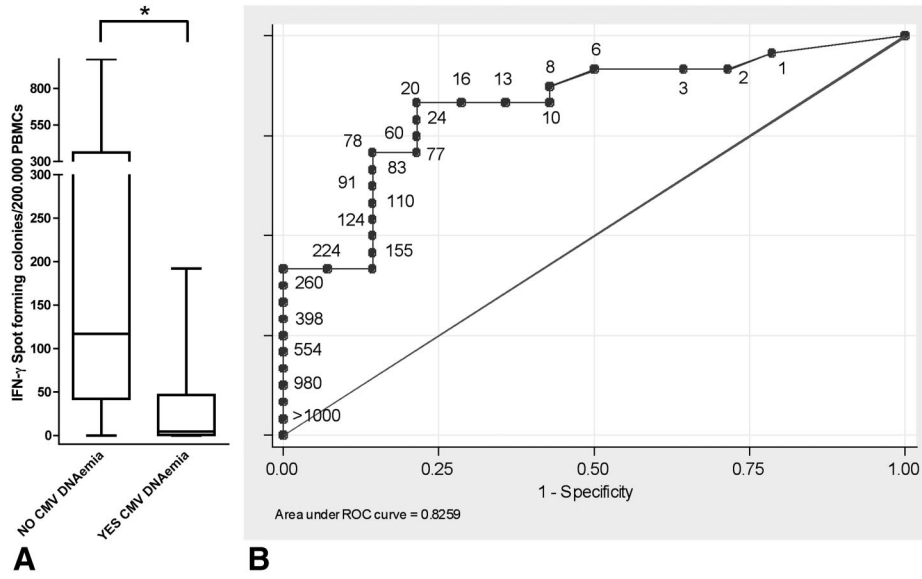

FIGURE 3. (A) Relationship between cytomegalovirus (CMV)-specific interferon (IFN)-γ ELISPOT counts and subsequent CMV DNAemia. *x*-axis shows the group of patients who were detected with positive or negative DNAemia. *y*-axis shows *box* and *whiskers plots* the of CMV-specific IFN-γ spot forming colonies/200,000 peripheral blood mononuclear cells (PBMCs). (Asterisk) *P* values<0.05. (B) Receiver operating characteristic (ROC) curve displaying as outcome the protection against the CMV viremia (protection=1, no CMV viremia; protection=0, CMV viremia present). The CMV-specific ELISPOT count is the predictor variable. Numbers indicate the CMV-specific ELISPOT level producing each couple of sensitivity/1-specificity values.

whereas in two other cases significant episode(s) of CMV DNAemia anticipated the appearance of a detectable CMV-specific T-cell response (Fig. 2B,C). In one case, T-cell response remained undetectable after repeated episodes of CMV DNAemia (Fig. 2D). It is generally accepted that subclinical levels of viral replication, occurring during antiviral preemptive treatment, allow a certain degree of antigen exposure to immune system thus favoring antiviral T-cell reconstitution (*33*). The data show that in one case (Fig. 2D) multiple viremia episodes were not sufficient to prime a T-cell response, whereas in another case a detectable T-cell response appeared only after a secondary, temporally dissociated viremia (Fig. 2B). This finding supports the hypothesis that in certain settings, virus exposure may not be sufficient to prime or boost an immune response. It is probable that immature and inexperienced T cells, such as in cord blood transplant settings, may require longer time to mature and mount an efficient and protective T-cell response (reviewed in [*44*]). In another case, CMV viremia was not required to prime an immune response (Fig. 2A). This finding suggests that preexisting levels of donor-derived T cell, followed by rapid recovery in T-cell response, impede virus reactivation and protects from further CMV viremia episodes. Indeed in all shown cases, once a solid T-cell response was established, this prevented any further CMV DNAemia event. It is plausible to speculate that several other factors, such as the type of conditioning regimen, the use of TBI and of antithymoglobulin, the type of stem cell, the variability in frequencies of CMV-specific T cells in stem-cell donor, the duration of viremia, the preemptive or prophylactic use of ganciclovir versus foscarnet may influence antiviral T-cell recovery after transplantation. Given the high heterogeneity of the kinetics and efficiencies of virus-specific T-cell immune reconstitution among pediatric HSCT patients, the assessment of the antiviral immune response represents an attractive clinical and diagnostic strategy because it provides critical notions for orientating the therapeutic choices: for example, patients with solid or ramping up levels of T-cell immunity may shorten the duration of the antiviral therapy, and thus minimizing the antiviral drug-associated toxicity. On the other side, patients with low or inconsistent antiviral immune responses should be more carefully monitored because this condition predisposes to a higher risk of CMV infection. The assessment of the antiviral immunity may also direct a judicious and guided use of antiviral prophylaxis. Moreover, the detection of patients with low antiviral immunity is required to identify the potential candidates for cell-based therapies, such as the infusion of ex vivo-isolated CMV-specific T-cells clones. In this report, we show that the ELISPOT test is a valuable tool to assess the risk of CMV viremia. Most of the published studies on virus-specific immune reconstitution were performed using flow cytometry detection of tetramer positive T cells or intracellular detection of IFN-γ in T cells. These systems require sophisticated instrumentation and in certain cases, such as tetramer detection, are limited to certain HLA haplotypes. The ELISPOT test is practical and does not require complex instrumentations, so that HSCT patients could be monitored for months after transplant, or in any period after transplant of augmented immunosuppression where the patient may present a reduction of antiviral immune responses. In this report, we show that immune levels more than 80 spots were associated with protection from CMV viremia, whereas immune levels comprised within 0 and 20 spots were more frequently associated with development of CMV viremia. In particular, as shown with receiver operating characteristic analysis, the ELISPOT test presents a satisfactory accuracy and good performance in determining patients at risk or protected from CMV viremia. Thus, the ELISPOT test may be clinically used to discriminate patients with solid antiviral immune reconstitution protected from CMV infection, from patients whose slow or inefficient antiviral immune response exposes them to CMV viremia. Identification

of patients at risk of infection is particularly critical during the late posttransplant phase when impaired antiviral immune reconstitution may lead to late-onset CMV infection and disease.

In conclusion, immunological monitoring of pediatric HSCT patients represents a promising and valid strategy for monitoring antiviral T-cell reconstitution and this has clinical impact on the therapeutic interventions on pediatric allogeneic HSCT patients.

MATERIALS AND METHODS

Patients

Thirty-one allogeneic HSCT pediatric patients were enrolled in a longitudinal prospective study from September 2006 to March 2009 follow-up data are as of April 30, 2009. Patients were treated with non-manipulated HSCT from a related or an unrelated donor. The local institutional review board approved transplant protocols and all parents or patients (when applicable) gave their informed consent before enrollment in the study.

Supportive Care and Preventive Measures

All patients were nursed in high-efficiency particulate-filtered air rooms during the neutropenic phase and standard measures were adopted to prevent infectious complications (*45*).

Routine surveillance for viral reactivation or infection comprised weekly determination of human CMV, Epstein-Barr virus, Adenovirus, and Human Herpes virus 6 DNAemia during the first 100 days post-HSCT and continued thereafter if clinically indicated. Surveillance for CMV T-cell immune-recovery was performed at the following time points: +30, +60, +90, +120, + 180, and + 360 days after transplantation.

Definitions and Treatment of Engraftment, GVHD, and CMV Infection

Neutrophil and platelet engraftment were established as the first of three consecutive days on which neutrophil and platelet counts exceeded 0.5×10^9/L and 50×10^9/L, respectively. Standard criteria were used to define acute and chronic GVHD and transplant-related toxicity (*46*, *47*).

First-line therapy for acute GVHD consisted in administration of prednisone at 2 mg/kg/day for at least 2 weeks with subsequent slow tapering in case of response. In steroid-refractory or steroid-dependent patients, extracorporeal photopheresis was added (*48*). Other measures to control acute GVHD, such as the introduction of mycophenolate mofetil or the switching from cyclosporine to tacrolimus were left at discretion of physician. Extracorporeal photopheresis with or without prednisone were the first-line therapy for chronic GVHD.

CMV infection was defined as any asymptomatic increase of CMV viral load more than or equal to 1000 genomic copies/mL of blood and this threshold was used to start the preemptive therapy (*49*, *50*). CMV disease was defined according to published criteria (*51*).

Treatment of CMV Infection/Reactivation

Usually, foscarnet (3×40 mg/kg/day) was used as first-line preemptive therapy until two consecutive negative CMV DNAemia were obtained (*52*). Alternatively, ganciclovir (2×5 mg/kg/day) was used for contraindication or toxicity of foscarnet. The induction treatment was continued with maintenance of foscarnet at 1×90 mg/kg/day or ganciclovir at 1×5 mg/kg/day for 3 to 5 days/week for 2 weeks. Patients with recurrent CMV reactivation were retreated with foscarnet or, at physician discretion, switched to ganciclovir or cidofovir (*53*). CMV immunoglobulins were administered to patients with CMV disease or, in some cases, in patients with frequent (≥3 episodes) CMV reactivation.

Evaluation of CMV DNAemia and CMV Serology Test

CMV DNAemia was evaluated using real time PCR on Abi prism 7900 HT (Applied biosystem). CMV IgG and IgM serology was assessed using diagnostic grade IgG and IgM ELISA (Enzygnost, Dade Behring).

Evaluation of Immune Response

PBMCs were extracted and purified by Ficoll banding (GE Healthcare). PBMCs were resuspended in RPMI-1640 medium supplemented with 10% human AB serum (Sigma Aldrich) and seeded at a concentration of 2×10^5 cells/well in 96 wells anti-IFN-γ antibody coated ELISPOT plates (Autoimmun Diagnostika, AID). For each patient, duplicate wells were incubated with (1) positive control consisting in phytohemagglutinin (10 μg/mL, AID), or phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma Aldrich) and ionomycin (1 μM, Sigma Aldrich); (2) CMV pp65 antigen peptide mix consisting of 9-mers peptide pool spanning pp65 protein (10 μg/mL, AID); (3) negative control consisting in scramble peptide mix (AID). ELISPOT images were acquired and analyzed using an automated image scanner (Aelvis). All results shown are background subtracted (sample minus negative control). CMV pp65 protein is the immunodominant target of the T-cell response and thus used target to assess CMV-specific T-cell responses (*20*, *26*). Cytokine flow cytometry assay demonstrated that CMV pp65-specific IFN-γ secreting cells detected using ELISPOT tests correspond to $CD4^+$ and $CD8^+$ T cells (data not shown). As described by other authors ELISPOT and cytokine flow cytometry share linearity and correlation (*54*–*56*).

Statistical Analysis

Patient characteristics were analyzed by descriptive methods (mean, median, percentage). OS was calculated from the date of HSCT to the date of last follow-up or to the date of death due to any cause, respectively, and it was estimated by the Kaplan-Meier method. Any nondisease progression-or-relapse death was used to calculate the transplant-related mortality. Data analysis for OS was performed SAS software version 9.1 (SAS Institute Inc.). The reported *P* values are two-tailed, and a significance level of $\alpha=0.05$ was used. For ELISPOT and CMV DNAemia test, statistical analysis was performed using nonparametric Mann-Whitney *U* test. *P* value less than 0.05 was considered significant.

ACKNOWLEDGMENTS

The authors thank Maria Vittoria Gazzola, Ph.D., and Roberta Destro, Ph.D., for HLA donor search and graft processing. They thank Maria Paola Albergoni, Ph.D., Milena Luca, M.D., Roberta Favaro, Ph.D., for HLA typing. They thank Gloria Tridello, Ph.D., for her assistance in data extraction and Alice Bianchin, Angela Bozza, and Valentina Fornasiero for technical assistance. They also thank Azienda Ospedaliera of Padova for providing the IFN-γ ELISPOT plates and reagents.

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