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Enumeration of cytomegalovirus-specific interferonγ CD8⁺ and CD4⁺ T cells early after allogeneic stem cell transplantation may identify patients at risk of active cytomegalovirus infection

Recovery of functional cytomegalovirus (CMV)-specific T lymphocytes is critical for protection from active CMV infection and disease in allogeneic stem cell transplant recipients (Allo-SCT).[1-6] To date, assessment of CMV-specific T-cell immunity has not had a major impact on the clinical management of CMV infection in these patients, as no widely accepted thresholds in the number of CMV-specific T cells providing protection have been established. In the present study, we optimized a simple intracellular cytokine staining (ICS), and investigated whether enumeration of CMV-specific interferon (IFN)γ CD8⁺ and CD4⁺ T cells early after transplantation could reliably predict the development of active CMV infection within 100 days after transplantation. From January to October 2007, 36 patients undergoing Allo-SCT were included in the study. The study was approved by the Ethics Committees and written informed consent was obtained from all patients. Relevant clinical data of patients are summarized in Table 1. Patients were monitored for active CMV infection once or twice a week by pp65 antigenemia,[7] CMV DNAemia (CMV real-time PCR, Abbott Molecular, Des Plaines, IL, USA or AMPLICOR CMV Monitor, Roche Indianapolis, USA) or both. Pre-emptive therapy was initiated upon a positive antigenemia or 2 consecutive positive plasma PCRs, and discontinued upon two consecutive negative results as previously reported.[7] CMV pneumonitis was diagnosed and treated on the basis of established protocols.[7] Heparinized blood samples from patients were obtained at days +30 (median 34 days; range 30-53) and +60 (median 62 days; range 54 to 85 days). Blood samples were also obtained from healthy CMV-seropositive (n=7) and CMV-seronegative individuals (n=5). Enumeration of IFNγ CD8⁺ and CD4⁺ T cells was carried out by ICS (BD Fastimmune, BDBiosciences, San Josè, CA, USA) following the manufacturer's instructions. A set of overlapping peptides spanning the highly immunogenic pp65 and IE-1 CMV proteins, obtained from JPT peptide Technologies GmbH (Berlin, Germany), was chosen as the antigen (2 μg/peptide/mL).[8] Responses >0.1% were considered specific. Control samples and specimens from CMV-seronegative subjects yielded IFNγ responses <0.07%. IFNγ responses of CMV-seropositive individuals ranged from 0.20% to 5.5% (median 0.45% of IFNγ CD8⁺ T cells and 0.35% of IFNγ CD4⁺ T cells).

Fifteen patients (44%) experienced active CMV infection (11 D+/R+, 3 D-/R+ and 1 D+/R-), 8 before day +30 (median 19.5 days; range, 8-41 days) and 7 beyond day 30 (median 44.0 days; range 35-56 days). Twenty-one patients (18 D+/R+, 3 D-/R+) did not. Two patients died during the study period (one due to CMV pneumonitis on day +68, and the other due to a proven invasive pulmonary aspergillosis on day +25). Of the 28 patients free of active CMV infection at the first sampling time, IFNγ responses were detected in all but one patient. Individual data are shown in Figure 1. The median counts of either cell subset were significantly higher in patients not developing active CMV infection (1.69 cells/μL of CD8⁺ and 1.29 cells/μL of CD4⁺ T cells) than in those who experienced it later (0.32 cell/μL and 0.24 cell/μL respectively). A threshold in the number of either IFNγ subset predicting protection against active CMV infection was estab-

Table 1. Patients' characteristics.

Parameter	
Total n. of patients	36
Median age, yrs (range)	47 (22-69)
Sex, n. male patients/n. female patients	17/19
Diagnosis, n. patients (%)	
Acute myeloid leukemia	11 (30.6)
Acute lymphoblastic leukemia	1 (2.8)
Chronic myeloid leukemia	3 (8.3)
Idiopathic myelofibrosis	3 (8.3)
Myelodysplastic syndrome	2 (5.6)
Non-Hodgkin's lymphoma	9 (25)
Hodgkin's lymphoma	3 (8.3)
Multiple myeloma	3 (8.3)
Severe aplastic anemia	1 (2.8)
CMV serostatus[a], n. patients (%)	
D^+/R^+	29 (80.5)
D^-/R^+	6 (16.7)
D^+/R^-	1 (2.8).
Donor type, n. patients (%)	
HLA-identical sibling	21 (58.3)
Mismatched related donor	2 (5.6)
Matched unrelated donor	8 (22.2)
Mismatched unrelated donor	5 (13.9)
Conditioning regimen[b], n. patients (%)	
Non-myeloablative	25 (69.4)
Fludarabine plus Melphalan	17 (47.2)
Fludarabine plus Busulphan	8 (22.2)
Myeloablative	11 (30.6)
Busulphan plus Cyclophosphamide	6 (16.7)
TBI plus Cyclophosphamide	3 (8.3)
Fludarabine-Thiothepa-Busulphan-Thymoglobulin	2 (5.6)
Stem cell source	
Peripheral blood	33 (91.6)
Umbilical cord blood	2 (5.6)
Bone marrow	1 (2.8)
GvHD prophylaxis	
Cyclosporine A + Methotrexate	23 (63.9)
Cyclosporine A + MMF	11 (30.6)
Cyclosporine A + Prednisone	2 (5.6)
Acute GvHD incidence[c]	
Grades 0-I	23 (63.6)
Grades II-IV	13 (36.4)
Steroid therapy	
Yes	9 (25)
No	27 (75)

[a]Healthy individuals, donor and transplant recipient CMV-serostatus was determined by a commercial ELISA. [b]Incidence if occurring before the last time point evaluated (day +60). [c]For patients who received a graft from either an unrelated donor or a HLA-mismatched donor, rabbit antithymocyte globulin (ATG) (3-6 mg/kg) was added to reduced-intensity conditioning. TBI: total body irradiation.

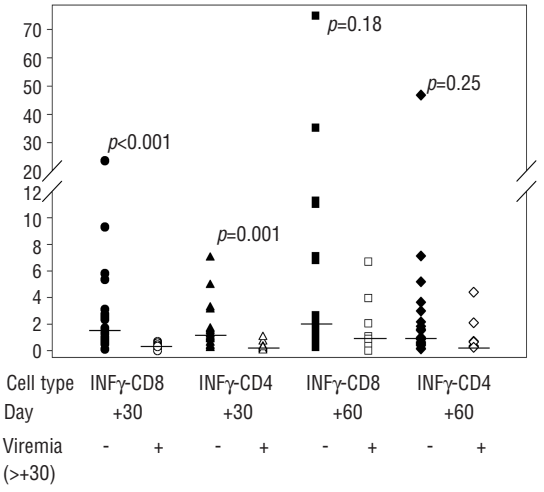

Figure 1. CMV-specific IFNγ CD8⁺ and CD4⁺ T cells in patients developing active CMV infection beyond day +30 and in patients not developing it within the study period. Enumeration of IFNγ CD8⁺ and CD4⁺ T cells in whole blood was carried out by ICS. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences Immunocytometry Systems). Data files contained at least 10,000 events positive for CD4⁺ or CD8⁺ within the lymphocyte gate. CD4⁺ and CD8⁺ events were gated, and then analyzed for the CD69d activation marker and IFNγ production. The total number of IFNγ CD8⁺ and CD4⁺ T cells was calculated by multiplying the percentages of CMV-specific T cells producing IFNγ upon stimulation (after background substraction) by the absolute CD4⁺ and CD8⁺ T cell counts. Responses= >0.1% were considered specific. Cell counts at days +30 and +60 in patients without active CMV infection (left rows, black symbols) and in patients developing active CMV infection beyond day +30 (right rows, open symbols) are shown. Horizontal bars indicate the medians. Comparisons between median cell counts were carried out by the Mann-Whitney test for unpaired samples. *p* values <0.05 were considered statistically significant.

lished: 1 cell/μL for CD8⁺ T cells (specificity 100%; sensibility 76%, positive predictive value 100%, and negative predictive value 54%) and 1.2 cells/μL for CD4⁺ T cells (specificity 100%, sensibility 62.5%, positive predictive value 100%, and negative predictive value 43.8%). Enumeration of absolute CD8⁺ and CD4⁺ T cells did not allow the prediction of development of active CMV infection (*data not shown*). Our data are in keeping with those published by other groups.[4-6,9,10] In these studies, however, no discrimination between patients who eventually developed viremia and those who did not could be ascertained as early as around 30 days after transplantation.

Reconstitution of CMV-specific T cell immunity may have proceeded at a faster rate in our patients, as most of them were CMV-seropositive, were treated with a non-myeloablative conditioning regimen and received a non-T-cell depleted graft from a CMV-seropositive donor. Some patients not developing active CMV infection displayed early IFNγ CD8⁺ and CD4γ T-cell counts below the established cut-off levels, indicating that other CMV proteins may also elicit protective immune responses.[11]

Of the 7 patients developing active CMV infection after the first immunological control, 5 resolved the episode within the study period. The number of IFNγ CD8⁺ T cells increased significantly (p=0.008) by around day +60 in these patients (median increase of 1.8 cells/μL; range 0.4 to 6.2 cells/μL). In contrast, no increase was observed in the 2 patients who failed to clear the episode. Similarly, in patients experiencing active CMV infection before the first immunological control (n=8), either failure to clear the episode (n=2), or occurrence of a relapsing episode (n=4) were associated significantly (p=0.003) with lower IFNγ CD8⁺ T cell-counts at the first immunological sampling (4.9, 1.9, 0.1, 0.2, 1.9, and 0.6 cells/μL), compared to those found in patients resolving the episode (n=2; 9,3 and 11.5 cells/μL). These data support previous observations.[2,3,5,6]

One patient died of CMV disease (in the setting of a grade IV GvHD) despite earlier detection of IFNγ CD8⁺ and CD4⁺ T cells (1.9/μL and 0.6/μL). As no samples obtained either immediately before or within the disease period were available from this patient, no major conclusions can be drawn from this case.

Reconstitution of both IFNγ cell subsets occurred in the absence of detectable active CMV infection. Indeed, median counts of either cell subset at day +60 in patients developing active CMV beyond day +30 (0.89 cell/μL of IFNγ CD8⁺ and 0.65 cell/μL of CD4⁺ T cells) and those found in patients not developing it (2.15 cells/μL and 1.0 cell/μL respectively) were not significantly different (p=0.18 and p=0.25 respectively).

These data are in accordance with a previous report.[12] The impact of clinical variables on early reconstitution of IFNγ cell subsets was not evaluated here given the limited sample size of our cohort. In summary, our data suggest that quantification of IFNγ CD8⁺ and CD4⁺ T cells at around day +30 may help to stratify patients according to the risk of developing active CMV infection.

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Successful mobilization of hematopoietic peripheral blood progenitor cells with paclitaxel-based chemotherapy as initial or salvage regimen in patients with hematologic malignancies

Autologous hematopoietic progenitor cell transplantation is a standard of care in several hematologic diseases, but many patients are unable to mobilize a sufficient number of cells for transplantation. Paclitaxel is a plant alkaloid effective against ovarian and breast cancers, and has also been proven active in multiple myeloma and non-Hodgkin´s lymphoma, among other human neoplasms.[1,2] We and others have described the efficacy of paclitaxel-based chemotherapy in mobilizing large amounts of hematopoietic progenitors in patients with ovarian or breast cancer.[3-5] However, data on the use of paclitaxel and rhG-CSF for hematopoietic cell mobilization in patients with hematologic malignancies is scarce; only recently McKibbin *et al*. have described this schedule in 26 patients after failure of a prior mobilization regimen.[6] To further determine the potential clinical utility of paclitaxel with rhG-CSF for hematopoietic progenitor mobilization in patients with non-solid tumors, we investigated: (i) the mobilizing ability and toxicity of this schedule as initial treatment, or as salvage therapy in patients who failed a mobilization attempt with rhG-CSF, and (ii) the efficacy and tolerability of cyclophosphamide (Cy), given in combination with paclitaxel and rhG-CSF after mobilization failure with filgrastim alone.

Between January 1999 and January 2008, 75 patients with a primary diagnosis of a hematologic malignancy who were scheduled for autologous transplant received paclitaxel in the mobilization schedule (Table 1). The time elapsed from the last treatment was at least three weeks. All patients gave informed consent.

Group A included 19 patients with risk factors for failure to achieve successful mobilization, representing 12% out of a total 156 first-line mobilizations with rhG-CSF during the same study period. Most patients displayed coexistence of various factors associated with poor mobilization success (Table 1). Patients received paclitaxel 170 mg/m^2 i.v. by continuous infusion for 24 hours (day 1) followed by 8 µg/kg s.c rhG-CSF (P-G) daily until the last apheresis.[4] Thirty-three patients received the same

Table 1. Patients' characteristics.

Patients' characteristics	*Group A* P-G (n=19)*	*Group B P-G (n=33)*	*Group C P-Cy-G (n=23)*	*p*
Age, years (median, range)	47, 15-67	52, 19-66	57, 31-69	0.0146
Males (%)	8 (42%)	19 (58%)	11 (48%)	0.5367
Diagnosis				
Acute leukemia	4 (21%)	10 (30%)	1 (4%)	0.0017
Lymphoma	9 (47%)	16 (48%)	7 (30%)	
Multiple myeloma	6 (32%)	7 (21%)	14 (61%)	
CLL	0	0	1 (4%)	
Disease status				0.0512
Complete remission	10 (53%)	24 (73%)	8 (35%)	
Partial remission	7 (37%)	8 (24%)	13 (57%)	
Progression	2 (10%)	1 (3%)	2 (9%)	
Median time (weeks) from last chemotherapy cycle	17	10	10	0.4610
Patients with previous radiotherapy	3 (16%)	3 (9%)	5 (22%)	0.4201

*Group A, patients treated with paclitaxel-rhG-CSF (P-G) as first line therapy; group B and group C, patients treated with paclitaxel-rhG-CSF (P-G) or paclitaxel-cyclophosphamide-rhG-CSF (P-Cy-G) respectively, after failure of mobilization with rhG-CSF. p values are calculated with the Kruskal-Wallis test. *Group A patients: 15 patients presented with myelodysplastic features and/or hypocellular bone marrow; 6 had been treated with fludarabine, platinum or melphalan, 6 revealed poor hematologic recovery from previous cycles, with intervals to achieve neutrophils >1×10^9/L and/or platelets >50×10^9/L greater than four weeks, and 2 of them had bone marrow involvement by tumor. CLL: chronic lymphocytic leukemia.*