Brief Articles

Reduced IL-7 Responsiveness Defined by Signal Transducer and Activator of Transcription 5 Phosphorylation in T Cells May Be a Marker for Increased Risk of Developing Cytomegalovirus Disease in Patients after Hematopoietic Stem Cell Transplantation

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ABSTRACT
Cytomegalovirus (CMV) reactivation may lead to CMV disease associated with high morbidity and mortality in patients after hematopoietic stem cell transplantation (HSCT); the identification of clinically relevant markers may aid in the identification of patients at increased risk for developing CMV-associated complications. We evaluated the phosphorylation of signal transducer and activator of transcription 5 (STAT5) in CD4⁺ T cells, CD8⁺ T cells, and TCRγδ T cells in response to stimulation with IL-7 or IL-2 after HSCT by analyzing blood samples taken monthly 1 to 6 months after HSCT. Patients were monitored weekly with a quantitative PCR from the time of engraftment for CMV viral load in whole blood until at least day 100 after HSCT. We identified a correlation between clinical outcome regarding CMV replication and the ability to respond to IL-7 and IL-2 defined by STAT5 phosphorylation (pSTAT5). Patients with recurrent or prolonged CMV replications had significantly lower pSTAT5 upon stimulation of T cells with either IL-7 or IL-2 at time points 1 through 3 than those without CMV replication (P < .05). We conclude that reduced responses to IL-7, reflected by pSTAT5, may represent a clinically relevant functional biomarker for individuals at increased risk for CMV reactivation; our data may also aid in designing better strategies to improve anti-CMV immune responses without increasing the risk of developing graft-versus-host disease.

INTRODUCTION
Human cytomegalovirus (CMV) is a ubiquitous virus infecting more than 50% of the adult population [1]. Human CMV poses a threat to severely immunosuppressed individuals, such as patients after allogeneic hematopoietic stem cell transplantation (HSCT) [2-4] or patients undergoing solid organ (eg, lung) transplantation [5]. CMV reactivation might lead to CMV disease associated with high morbidity and mortality [2,3], due to failing immune surveillance [6]. CMV viral load, most commonly measured by quantitative PCR, is routinely monitored after HSCT [2,4,7] and used as the basis for pre-emptive antiviral therapy that is usually effective [2,8]. However, patients experiencing repeated episodes of CMV replication are at an increased risk for CMV disease [2]. Furthermore, the currently available antiviral agents have significant limitations, particularly bone marrow and renal toxicity [8]. Therefore, there is still an unmet clinical need to identify patients at increased risk to develop CMV disease or recurrent CMV reactivation requiring prolonged monitoring and repeated courses of anti-CMV treatment [6,9].

Delayed or poor immune reconstitution of CMV-specific T cells is associated with increased risk of CMV disease and transplantation-related mortality [6]. IL-7–mediated signaling via the IL-7 receptor (IL-7R) helps to improve and speed up immune reconstitution [10]. IL-7 is required for early amplification and survival of lymphoid lineage progenitors [11,12]. It rescues T cells from activation-induced cell death associated with the up-regulation of T lymphocyte survival factors and induces phosphorylation of signal transducer and activator of transcription 5 (STAT5) in T cells [13] activating STAT, which then enters the nucleus and binds to specific DNA sequences in the promoter regions of genes, resulting in gene activation or suppression [14]. CD4⁺ or
CD8⁺ T cells from healthy individuals exhibit low constitutive STAT5 phosphorylation (pSTAT5) and show a fast and strong response to IL-2 or IL-7 stimulation. Decreased response to IL-7 was defined by absence of pSTAT5, which has been associated with decreased immune functions, such as decreased cytokine production [15]. Increased constitutive pSTAT5 has been associated with chronically activated T cells [16]. Furthermore, the IL-7–IL-7R axis has been found to be crucial for maintenance and expansion of CMV-specific immune responses [17].

Therefore, we evaluated the function of the IL-7 signaling pathway by measuring phosphorylation of STAT5 in T cells (CD4⁺ and CD8⁺) and TCRγδ T cells in response to stimulation with IL-2 and IL-7 in peripheral blood mononuclear cells (PBMCs) obtained from 40 HSCT patients. The association of cytokine-induced STAT5 phosphorylation was studied with the CMV status, defined by quantitative PCR and the development of graft-versus-host disease (GVHD).

**MATERIAL AND METHODS**

**Study Design**

This was a prospective, longitudinal clinical cohort study. The patients, all suffering from hematologic malignancies, and/or the stem cell donors, were CMV-seropositive. Patients were informed regarding study participation and provided informed consent. The study was approved by the regional ethical committee in Stockholm (Ethical permit: 2006/645-31/3).

**Blood Samples**

Blood samples were drawn monthly within the first 3 months after HSCT (time point 1 to 3); additional samples at the fourth to sixth month after HSCT (time point 4 to 6) were drawn from patients reactivating CMV or having a primary CMV infection. PBMCs from heparinized blood were isolated by Ficoll separation and immediately frozen and stored in 90% FBS and 10% DM50 in liquid nitrogen. Samples from all time points from each respective patient were thawed and stained for flow cytometric analysis at a single laboratory session and analyzed with the Gallois flow cytometer (Beckman Coulter, San Diego, CA) by the same operator person to avoid intra-variability. Flow data analysis was performed using Kaluza software (Beckman Coulter).

**CMV Monitoring and Therapy**

Patients were monitored for CMV viral loads with a quantitative PCR on whole blood from the time of engraftment weekly until day 100 after HSCT. The PCR technique has been described previously [2,18]. More than 3 months after HSCT, weekly monitoring was continued only on the patients who had experienced CMV reactivation or had severe GVHD, whereas the other patients were monitored at an individual basis but usually at each visit to the transplantation center occurring every 2 to 3 weeks until 6 months after HSCT (18). Pre-emptive antiviral treatment with either i.v. ganciclovir 5 mg/kg BID or oral valganciclovir 900 mg BID was given at the center’s chosen intervention limit of \(>1000\) copies/mL blood. The duration of therapy was a minimum of 2 weeks and was discontinued when the CMV viral load was \(<500\) copies/mL. In this study, a second CMV replication episode was defined as requiring negative monitoring results in 2 consecutive samples over a period of at least 8 days in the absence of antiviral therapy. CMV disease was defined according to Ljungman et al. [19].

**Monoclonal Antibodies and Functional Assays**

PBMCs were thawed rapidly; cells were washed, counted, and the numbers and viability of lymphocytes were calculated using a Casy Counter. All numbers of cells below refer to viable lymphocytes. To test the functionality of the IL-7 receptor (CD127) and its response to stimulation with IL-7 and IL-2, an assay measuring the phosphorylation of STAT5 was used as described [15,20]. Briefly, after thawing, cells were washed and \(3 \times 10^6\) cells per tube were incubated overnight in serum-free medium (AIM-V; Invitrogen, Carlsbad, Germany) in a CO₂ incubator at 37°C. On day 2, cells were either stimulated with 300 ng IL-7 (Cytheris, Paris, France) or 750 IU IL-2 (Chiron, Ratingen, Germany) for 15 minutes at 37°C. The third tube contained noncytokine stimulated immune cells, which served as controls. Isotype controls were also included by staining with the isotype-matched Alex488-conjugated anti-IgG₁ isotype control antibody for pSTAT5. After cytokine stimulation, a cocktail of PE-conjugated anti-TCRγδ (clone B1, Becton Dickinson (BD)), PE-Cy7-conjugated anti-CD3 (clone SK7, BD), APC-conjugated anti-CD19 (clone SJ25C1, BD), APC Alexa750 custom conjugated CD8α (clone T8, Beckman Coulter), and V450-conjugated anti-CD4 (clone RPA-T4, BD) was added. Cells were fixated with 2% paraformaldehyde and permeabilized with 90% methanol before adding Alexa488-conjugated anti-pSTAT5 (clone Y694; BD). Samples were immediately analyzed on a Gallois flow cytometer (Beckman Coulter) using Kaluza software (Beckman Coulter). Lymphocytes were gated based on forward and side scatter; from this cell population, CD19⁺CD3⁺ cells were gated and subsequently gated for CD4⁺/CD8⁺ to separate CD4⁺CD8⁻ cells, CD4⁺CD8⁺ cells, and CD8⁺CD8⁻ cells. From the CD19⁺CD3⁺ T cell population, a gate to segregate TCR γδ⁺ cells was created. Phosphorylated STAT5 was measured in the T cell populations listed above. To determine the specific IL-7 and IL-2-stimulated pSTAT5, the pSTAT5 values from the unstimulated cells (constitutive pSTAT5) were subtracted from IL-2 and IL-7 stimulated pSTAT5. All samples with more than 25 absolute events to be counted were allowed according to the “Minimal Information About T cell Assays” (MIATA) guidelines (http://www.miataproject.org/).

**Statistics**

Risk factors for the number of CMV replication episodes were determined by multinomial logistic regression. Statistical comparison of each group was performed using a 1-way ANOVA with multiple comparisons. Differences over time were analyzed using the Tukey’s multiple comparison test and Mann-Whitney test. Comparisons among the CMV reactivation groups (no reactivation, 1 reactivation, multiple, or prolonged reactivations) at the different time points were made by ANOVA and post hoc analysis by t tests. All the reported P values are based on 2-sided testing. Statistical analysis was performed using Prism 5 and Statistica software packages.

**RESULTS**

**Patients and CMV Replication Episodes**

Forty patients were included in the study and the patient characteristics are provided in the Supplementary Table 1. Patients were classified into 3 cohorts: those without CMV replication (CMV⁻), those with 1 CMV replication episode (CMV+), and those with prolonged or repeated CMV replication episodes (CMV++). Acute GVHD involving skin and intestines were seen in 25 patients; 16 patients developed chronic GVHD. As seen in Supplementary Table 1, 9 of 17 (53%) patients with acute GVHD grade II to grade IV developed CMV replication (CMV+ or CMV++) compared with 15 of 23 (65%) in patients with acute GVHD grade 0 to I. Six of 16 of the patients in the CMV⁺ group had acute GVHD II to IV compared to 2 of 7 in the CMV++ group and 9 of 17 in the CMV⁺ group.

Twenty-seven of 40 patients had at least 1 documented CMV replication episode and 23 of these occurred within 100 days after HSCT. Four patients had late first CMV reactivations at 128 days (patient no. 28), 156 days (patient no. 39), 182 days (patient no. 22), and 515 days (patient no. 4); 1 patient who had a brief CMV reactivation at 38 days after HSCT had a second CMV reactivation at day 711 (patient no. 29). Eight patients had 2 and 1 patient had 3 recurrent CMV episodes. In addition, 8 patients had prolonged replication episodes requiring 28 or more days of antiviral treatment. Three patients developed clinically more complicated CMV reactivations. One of these 3 patients (patient no. 31, who belonged to the D⁻R⁺ group) did not undergo a tissue biopsy for confirmation of CMV disease and died later. This patient had a prolonged CMV reactivation. The second patient (patient no. 28, who belonged to the D⁻R⁺ group) also had a prolonged CMV reactivation, but the biopsy showed only suspect CMV-positive stromal cells in the affected tissue. The third patient (patient no. 17, also in the D⁻R⁺ group) did show immunohistochemical CMV-positive stromal cells; however, the clinical assessment at that time was that the patient did not have CMV disease despite the biopsy result. This latter patient did not experience...
recurrent or prolonged CMV reactivation. A total of 37 CMV reactivations were documented 17 to 711 days after HSCT and of those reactivations, 25 were treated pre-emptively with anti-CMV drugs, whereas 12 reactivations were in patients who had viral loads below the set limit for antiviral therapy or they occurred later after HSCT in patients without risk factors for CMV disease.

Risk factors for development of at least 1 CMV replication episode were the use of unrelated stem cell donors \((P < .01)\) and CMV serological status \((P = .04)\). Factors without influence on CMV replication were age, stem cell source, conditioning intensity, acute GVHD, or chronic GVHD. Lymphopenia, defined as \(< .3 \times 10^9\) lymphocytes/L blood, was seen in 7 of the 40 patients. One patient had lymphopenia at the second time point and all other patients at the first time point. Five lymphopenic patients belonged to the CMV-group (representing 33% of the CMV-group), and 3 to the CMV+ group (13% of this group). Surprisingly, an absence of lymphopenia was found in the CMV++ group.

**Ability to Respond to Stimulation with IL-7 and IL-2 at Cell Population Level**

In general, the different patient groups exhibited strong phosphorylation of STAT5 in CD4+ T cells, CD8+ T cells, TCRγδ T cells, CD8+ CD4+ (activated) T cells, or (CD3+) CD4+ CD8+ T cells upon stimulation with IL-2 or IL-7 as compared with the constitutive pSTAT5 (Supplementary Figures 1 and 2) regardless of the CMV reactivation pattern.

The comparison of the 3 CMV reactivation groups (CMV-, CMV+, CMV++), revealed significant differences in the ΔpSTAT5 for IL-7 and IL-2 stimulated T cells at time points 1 to 3 \((P < .05\) for all comparisons) (Figure 1). When we compared the different CMV reactivation groups, the CMV++ group had significantly lower ΔpSTAT5 after stimulation of T cells with either IL-7 or IL-2 at time points 1 to 3 compared with the CMV- group \((P < .05)\) (Figure 1). This was also found after stimulation of CD8+ T cells at time point 2 \((P < .05)\). In addition, we identified significantly lower ΔpSTAT5 for IL-2 stimulated CD8+ cells at time point 3 in the CMV++ group as compared with the CMV- group and for the CMV+ group compared with CMV- at time point 2. There were no statistical differences concerning IL-7 stimulation in ΔpSTAT5 comparing the CMV- versus CMV+ at time points 1 to 3 or of IL-2 or IL-7 stimulation in the CMV- and the CMV++ groups at time points 1 to 6 (data not shown).

We could not identify any difference in STAT5 phosphorylation in PBMCs from patients with absent or mild GVHD (GVHD 0 to I) and patients with more severe GVHD (GVHD II to IV) in CD8+ T cells upon stimulation with IL-7.

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**Figure 1.** Delta pSTAT5 in different cell populations after stimulation with IL-7 and IL-2 at time points 1 through 3. Significant differences in the ΔpSTAT5 for IL-7 and IL-2 stimulated T cells (in black) at time points 1 through 3 \((P < .05\) for all comparisons) were identified by comparison among the 3 CMV reactivation groups (CMV-, CMV+, CMV++). The ΔSTAT5 is the difference in increase of frequency in pSTAT5 cells after cytokine induction in comparison to the parental T cell population (ex vivo T cells) and thereby the ΔSTAT5 reflects the ability of the ex vivo T cells to respond physiologically.
The frequency of phosphorylated STAT5 in CD4+ T cells upon IL-7 stimulation was found to be higher in blood from patients suffering from a more moderate-to-severe GVHD grade II to IV, but this was seen only at time point 3 (P = .05, not significant) and time point 6 (P < .01) (Supplementary Figure 3). Of note, patients who did not reactivate CMV within the first 100 days after HSCT were only followed to time point 3. Therefore, the patient populations analyzed at time points 4 to 6 only reflect those patients reactivating CMV.

Furthermore, regardless of CMV or GVHD, individual T cell subsets (ie, TCRγδ + T cells and CD4+CD8+ T cells) exhibited a higher frequency of constitutive STAT5 phosphorylation as compared to T helper cells (CD4+) and CD8+ T cells.

DISCUSSION

An important controlling mechanism for CMV replication is T cells. It has been shown that there is a difference in clinical outcome between detection of CMV-specific T cells by tetramers and by functional assays [21]. Gratama et al. showed that absence of CMV-specific T cells analyzed by tetramers was associated with more CMV complications and a higher transplantation-related mortality [6]. We report in the current study that patients experiencing repeated or prolonged CMV reactivations had impaired T cell responses defined by insufficient STAT5 phosphorylation to IL-7 or IL-2 stimulation of T cells. This impairment was not associated with GVHD, as we could not identify a correlation between the grade of GVHD and the functional ability of CD8+ T cells to respond to IL-7 stimulation. However, we found a statistically significant correlation with detection of repeated CMV viral replication episodes and the use of unrelated donor grafts and CMV serological status. This could imply that gauging levels of phosphorylated STAT5 upon IL-7 stimulation ex vivo, will aid to identify patients at higher risk to suffering CMV complications. The kinetics of the ΔpSTAT5 responses (ie, the capacity of the T cells to respond to IL-7 stimulation with STAT5 phosphorylation) in the different CMV replication groups suggests that patients at increased risk to contract CMV complications can be identified as soon as 8 weeks after HSCT. Subjects with low ΔpSTAT5 phosphorylation could then be followed with weekly monitoring of CMV viral loads for a prolonged period, whereas shorter monitoring is required in patients with high STAT5 phosphorylation. This is in accordance with our previous findings that monitoring and use of pre-emptive antiviral therapy can be safely stopped in patients with existing CMV-specific immune responses at 100 days after HSCT [18]. It also confirms the pattern of our group found when investigating the correlation between Epstein-Barr virus post-transplantation lymphoproliferative disease and decreased IL-7 signaling [22]. The findings in the present study, however, need to be assessed in a larger study. Furthermore, our study reveals that certain immune cell subsets are constitutively activated after HSCT; this includes CD4+CD8+ T cells, representing activated CD4+ T cells, which express the CD8α chain [23] or TCRγδ T cells that may also play an important role in immune reconstitution [24] and in CMV-directed cellular immune responses [25]; we conclude that reduced responses to IL-7 reflected by pSTAT5 may represent a clinically relevant functional biomarker for individuals at increased risk for CMV reactivation; our data may also aid in the design of better strategies to improve anti-CMV immune responses without increasing the risk of developing GVHD [26-28].

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.10.006.

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HCMV Infection of Humanized Mice after Transplantation of G-CSF–Mobilized Peripheral Blood Stem Cells from HCMV-Seropositive Donors

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Human cytomegalovirus (HCMV) infection, including primary infection resulting from transmission from a seropositive donor to a seronegative recipient (D+/R−), remains a significant problem in the setting of peripheral blood stem cell transplantation (PBSCT). The lack of a suitable animal model for studying HCMV transmission after PBSCT is a major barrier to understanding this process and, consequently, developing novel interventions to prevent HCMV infection. Our previous work demonstrated that human CD34+/CD133− progenitor cells—engrafted NOD-scid IL2Rγc−/− (NSG) mice support latent HCMV infection after direct inoculation and reactivation after treatment with granulocyte colony-stimulating factor. To more accurately recapitulate HCMV infection in the D+/R− PBSCT setting, granulocyte colony-stimulating factor–mobilized peripheral blood stem cells from seropositive donors were used to engraft NSG mice. All recipient mice demonstrated evidence of HCMV infection in liver, spleen, and bone marrow. These findings validate the NSG mouse model for studying HCMV transmission during PBSCT.

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INTRODUCTION

Despite advances in diagnostics and therapeutics, human cytomegalovirus (HCMV) remains a significant cause of morbidity and mortality after peripheral blood stem cell (PBSC) transplantation (PBSCT), and novel approaches to preventing HCMV infection are needed [1]. HCMV-seronegative recipients (R−) of allografts from HCMV-seropositive donors (D+), although at lower risk for developing HCMV infection and disease than seropositive recipients (R+), will still develop post-transplantation primary infection in up to 20% of cases [2–7]. The donor graft is the most important source of virus early in the post-transplantation period, and a retrospective analysis of D+/R− transplantations identified several factors associated with successful virus transmission [5]. However, the strict species specificity of cytomegaloviruses and the consequent lack of a suitable animal model system have hindered the experimental validation of these findings as well as the development of preventative strategies in this population.

“Humanized” mice, which have undergone transplantation with human cells and/or tissues, have recently been developed as tools to aid the in vivo study of pathogens with strict human tropism [8]. We reported the first humanized mouse model of HCMV infection in which human CD34+/CD133− hematopoietic progenitor cell–engrafted NOD-scid IL2Rγc−/− (NSG) mice directly infected with HCMV supported latent viral infection, reactivation in human macrophages, and dissemination after granulocyte colony-stimulating factor (G-CSF)-induced mobilization of bone marrow hematopoietic cells [9]. The present study was carried out to determine whether NSG mice would also demonstrate evidence of HCMV infection after transplantation of G-CSF–mobilized PBSCs from HCMV-seropositive donors, thereby recapitulating D+/R− PBSCT and validating the NSG mouse model as a tool for studying HCMV transmission and infection in this setting.

METHODS

Mice

NSG mice were maintained in an specific pathogen-free facility according to procedures approved by Oregon Health and Science University’s Institutional Animal Care and Use Committee. Before transplantation, mice

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