Role of Defective Thymic Function in Onset of Ganciclovir-Resistant Cytomegalovirus after Cord Blood Transplantation

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A case of recurrent cytomegalovirus reactivations in a cytomegalovirus-seropositive woman who received allogeneic cord blood transplantation is described. Thirteen months posttransplantation, her CD3 T cell count was extremely low whereas natural killer cells represented 66% of her total lymphocytes. She showed defective thymic function that might contribute to the onset of valganciclovir resistance.

CASE REPORT

A 33-year-old female with acute lymphoblastic leukemia in second complete remission received a 4/6 HLA-matched unrelated cord blood transplantation (CBT). The patient was conditioned with fludarabine (150 mg/m²), thiopeta (10 mg/kg), busulfan (3.2 mg/kg × 3 doses), and rabbit anti-thymocyte globulin (6 mg/kg) and received cyclosporine (9 months) and mycophenolate motefil (1 month) as graft-versus-host disease (GVHD) prophylaxis. She received one cord blood unit with cell doses of 3.2 × 10⁹ total nucleated cells/kg and 2.6 × 10⁹ CD34+ cells/kg. From day 30, the patient developed acute grade II GVHD, which was treated with prednisone (tapering dose from day 30 to day 200; maintenance dose, 2 mg/day from day 200 to day 400).

Recurrent BK virus cystitis was observed on day 30, with positive PCR results for blood and urine. The patient received five courses of cidofovir (5 mg/kg/week) to day 100. Recurrent asymptomatic cytomegalovirus (CMV) replication episodes were noted from day 45, with an initial viral load of 34,000 copies/ml and a peak viral load of 1.0 × 10⁶ copies/ml (Roche COBAS Amplicor assay). It was treated with preemptive valganciclovir (900 mg twice a day [b.i.d.]), which significantly decreased the CMV load. Nevertheless, a low-grade CMV replication (~1,000 copies/ml) was permitted between valganciclovir courses since the prolonged use of valganciclovir was ruled out to prevent myelotoxicity. From day 250, a continuous increase in viral load during valganciclovir therapy was noted (peak viral load, 1.0 × 10⁹ copies/ml), and the patient developed fever and bilateral retinitis. At this point, a genotypic analysis revealed mutations in the UL97 (M460V/I) and pol (K513E, L545S) genes, indicating a high degree of resistance to valganciclovir and cidofovir (3, 17). The patient was treated with foscarnet (120 mg/kg/day), and the CMV viral load decreased to 4,000 copies/ml. At this point, a first peripheral blood sample was obtained to analyze CMV-specific immune response development. After 3 weeks of treatment, the CMV viral load increased again. Compassionate leflunomide combined with intravenous immunoglobulin was added to the foscarnet therapy with no response. A new genotypic analysis reported no other mutations related to foscarnet resistance. The patient developed CMV encephalitis and died.

To investigate the reasons for the persisting CMV viral load, we retrospectively analyzed absolute lymphocyte and neutrophil counts after transplantation (Fig. 1). Lymphocyte counts were low throughout the follow-up period (normal range: 900 to 5,200 cells/μl), whereas neutrophil counts were within the normal range (1,900 to 8,000 cells/μl) at most points considered. Maximum viral load (>100,000 copies/ml) was attained four times during the follow-up period (Fig. 1).

Thirteen months after transplantation, informed consent was obtained from the patient to take peripheral blood in order to analyze the CMV-specific immune response. At this time, her CMV plasma load was 4,400 copies/ml. Peripheral blood mononuclear cells (PBMCs) were incubated with labeled monoclonal antibodies against CD3, CD8, CD56, CD45RA, CCR7, CD28, CD27, and PD-1, in combination with HLA-A*0201 (pp65, NLVPMVATV) pentamer, and analyzed by flow cytometry. The CD3+ T cell count was extremely low, representing only 1.2% of the total lymphocytes (normal range, 40 to 85%) (Fig. 2). Of the CD3+ T cells, 69.7% corresponded to CD8+ T cells (normal range, 8 to 45%) and only 1.0% to CD8+ negative T cells. We observed no pentamer-specific CD8+ T cells, indicating the lack of CMV-specific CD8+ T cells.

An analysis of CD8+ (bright) T cell differentiation status was performed, and we observed that naive (CD45RA+CCR7+) and central memory (CD45RA–CCR7+) cells were minor populations while the bulk of CD8+ (bright) T cells (99.3%) expressed markers consistent with effector-memory (CD45RA–CCR7–) and late effector-memory (CD45RA+CCR7–) phenotypes (18). In addition, 78.4% of the CD8+ T cells were CD27+CD28–, which also corresponds to late memory cells (1). This was confirmed by a high percentage of CD8+ T cells expressing PD-1.

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(71.6% were PD-1⁺ CD28⁻, while the normal range is 2 to 50%) (Fig. 2).

In contrast, the percentage of natural killer (NK) cells (CD3⁻ CD56⁺) was significantly higher than the normal percentage (range, 5 to 15% of total lymphocytes) and represented 66.5% of the gated cells, although the absolute number (140 cells/μl) was within standard values (100 to 400 cells/μl) (Fig. 2). A second peripheral blood sample was obtained 1 month later to further analyze the expression of two NK cell-activating receptors involved in the lysis of infected cells. We analyzed the expression of NKp30, a natural cytotoxicity receptor (NCR), as well as the CD94/NKG2C heterodimer, a killer lectin-like receptor (KLR). In this new sample, NK cells (CD3⁻ CD56⁺) constituted 73.5% of total lymphocytes whereas 7.5% were CD3⁺ T cells (CD3⁺ CD56⁻). Within NK cells, 10.5% and 78.1% expressed the CD94/NKG2C heterodimer (range in CMV-seropositive individuals, 2.5 to 80%) and NKp30 (normal range, 80 to 100%), respectively (Fig. 3). At this time, the patient’s CMV plasma load was 61,000 copies/ml.

In order to determine whether this specific low percentage of CD3⁺ T cells was related to impaired thymopoiesis, thymic function was indirectly calculated in PBMC DNA at both pretransplant and 13 months posttransplant using the sj/T-cell receptor excision circle (sj-TREC) ratio previously described (12) with minor modifications. Briefly, the six DβJβ-TRECs from cluster one were amplified together in the same PCR tube, while the sj-TREC was amplified in a different PCR tube. Internal positive and negative controls were included. All amplicons (DβJβ- and sj-TRECs) were then amplified together in a second PCR round using a LightCycler 480 system (Roche, Mannheim, Germany). The sj/β-TREC ratio in the pretransplant sample was zero, indicating that this patient had no thymic function in the months prior to the transplant. For the posttransplant sample, we were unable to amplify any of the TREC types, indicating that the thymus still had no activity 13 months after the transplant.

While CMV is easily controlled in immunocompetent individuals and is rarely a cause of clinical disease, in hematopoietic stem cell transplantation, and in particular in CBT, CMV infection remains a significant complication that can be life threatening (6). The strongest immune parameters that correlate with protection against CMV viremia after CBT include the emergence of CMV-specific T-cell memory response and recovery of natural killer (NK) cell function (5). Although NK cells recover rapidly after CBT and play a significant role in fighting against CMV in early stages of posttransplantation (10), T-cell reconstitution is crucial to control recurrent CMV infection. However, the recovery of functional T lymphocytes is far more difficult to achieve and depends on the survival of adoptively transferred T cells from the CB grafts or, alternatively, the de novo production of T cells in the recipient’s thymus (20). Considering the relevance of these cells, it is important to monitor NK- and T-cell reconstitution after CBT. This patient had recurrent CMV reactivations that were treated with valganciclovir, although some viral genome mutations associated with valganciclovir resistance were detected after several courses of this drug. Given this context, it would be easy to assume that the onset of drug resistance was the result of the prolonged and intermittent use of valganciclovir or even of the repeated use of cidofovir (9). However, our view is that defective thymopoiesis and inadequate T-cell reconstitution, as well as the subsequent lack of CMV-specific immunity, are also crucial reasons for the uncontrolled viral replication and the prolonged use of anti-CMV drugs (6). Initially, antiviral therapy might be insufficient to maintain viral replication under control in the presence of adverse host
factors (e.g., immunocompromised patients), independent of viral drug resistance. This finding is sometimes referred to as “clinical resistance” because no virologic (genotypic or phenotypic) evidence of drug resistance is revealed, especially when the dura-

FIG 2 Immunological analysis 13 months after transplantation. Relative proportions of CD3^+ T (CD3^+ CD56^−) and natural killer (CD3^- CD56^+) cells were analyzed in the CBT patient (left column). Dot plots are gated on CD3^+ T cells, in which the proportions of CMV-specific and total CD8^+ T cells were assessed. Expression of CD45RA, CCR7, CD27, CD28, and PD-1 on total CD8^+ T cells was analyzed in order to determine the differentiation status. A CMV-seropositive representative control is shown in the right column.

FIG 3 Activating natural killer receptors in the second peripheral blood sample. Relative proportions of CD3^+ T (CD3^+ CD56^−) and natural killer (CD3^- CD56^+) natural killer cells were also analyzed in this blood sample from the CBT patient (left column). Dot plots are gated on NK cells, in which expressions of the CD94/NKG2C heterodimer and NKp30 (CD337) were assessed. A CMV-seropositive representative control is shown in the right column.
tion of drug exposure is less than several months (16). However, a longer drug exposure to prevent viral replication in the absence of T-cell reconstitution finally led to “viral resistance,” which was characterized by the appearance of virus mutations (UL97 and pol) associated with drug resistance.

In this patient, a very low percentage of immune cells corresponded to CD3+ T cells. Low T-cell reconstitution in individuals receiving CBT has been previously reported and is related to a low output of naive T cells in adult recipients (14, 20). Most of the CD3+ T cells were CD8+ (bright) T cells exhibiting a highly differentiated memory phenotype corresponding to late effector-memory phenotype T cells, with the frequency of this subset dependent on aging and CMV infection (8, 11, 15). They also had high PD-1 expression, a molecule associated with memory T-cell exhaustion (4). Only 1% of the CD3+ T cells did not express CD8, indicating a deficiency of CD4+ T cells, which play a key role in CMV clearance after CBT (7).

The patient had a high percentage of NK cells expressing the CD94/NKG2C heterodimer. This subset of NK cells increases after CMV infection (13) and has recently been suggested to be equivalent to the murine “memory” NK cells (19), as they show high cytotoxicity and respond strongly after cytokine or CMV restimulation (13, 21). With regard to Nkp30 expression, it has been recently reported that pp65, an immunodominant epitope of CMV, is a ligand for Nkp30. Despite pp65 being one of the main targets for T-cell activation and proliferation, it seems to have the opposite effect on NK cells. Although Nkp30 is an activating NCR, CMV pp65-Nkp30 interaction blocks NK-cell cytotoxicity and inhibits NK-cell mediated lysis, suggesting that it might contribute to further impairing the capacity of the immune system to fight against CMV (2). TREC analysis supports that the low level of CD3+ T cells is due to defective thymus function rather than a failure in stem cell engraftment, also supported by the correct neutrophil and NK cell recovery after CBT. The patient had thymic failure prior to the transplant, although we are aware of the limitation of this result as the sample was taken during chemotherapy treatment, which could have severely impaired the thymus function. In addition, we could not amplify any of the TREC types in the posttransplant sample since the percentage of CD3+ T cells was extremely low and TREC levels were probably under our detection limit. However, this is in itself a result, since it indicates that the thymic function was undetected 13 months after transplantation despite the patient’s young age (33 years). Undetectable thymopoiesis and low output of naive T cells have been reported in CBT recipients (15).

When severe immunosuppression is combined with a severe, life-threatening CMV disease, empirical therapy should be begun, pending genotypic resistance test results (16). In our case, due to the severity of the CMV disease, we could not wait some weeks for the phenotypic test (50% effective concentration [EC50]) data. Although the nephrotoxicity of foscarnet precludes its empirical use, the genotypic test recommended switching to this drug. However, antiviral therapy is insufficient to suppress viral replication in the presence of adverse host factors such as immune impairment, which frequently occurs after CBT. In CBT patients, T-cell reconstitution and development of CMV-specific immunity are essential to control CMV replication, as NK cells are unable to fight against recurrent CMV infection, thus underlining the relevance of monitoring T-cell reconstitution in these patients. Given that T-cell reconstitution after CBT depends on the patient’s thymic function, TREC analysis might aid in immune monitoring. In our patient, a failure in thymopoiesis caused severe lymphopenia, inadequate T-cell reconstitution, and lack of antigen-experienced T cells even 1 year after CBT. Defective thymic function might therefore be considered a risk factor for drug resistance.

Adaptive immunotherapy with CMV-specific cytotoxic T lymphocytes might be an effective strategy for preventing and treating CMV reactivation in these patients. Additionally, CBT patients should be managed as high-risk CMV infection patients and universal prophylaxis should be used, although new nonmyelotoxic and nonnephrotoxic antiviral drugs need to be developed. On the other hand, although the inadequate T-cell reconstitution required the prolonged use of anti-CMV drugs and led to drug resistance, we cannot discard the fact that low-grade replication of CMV was allowed, and this is a well-known factor predisposing to viral resistance. Both factors interplayed in this case and cannot be discarded.

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