Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients

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Regeneration of hematopoiesis after allogeneic hematopoietic cell transplantation (HCT) involves conversion of the recipient’s immune system to donor type. It is likely that distinct cell lineages in the recipient reconstitute at different rates. Dendritic cells (DCs) are a subset of hematopoietic cells that function as a critical component of antigen-specific immune responses because they modulate T-cell activation, as well as induction of tolerance. Mature DCs are transferred with hematopoietic grafts and subsequently arise de novo. Little information exists about engraftment kinetics and turnover of this cell population in patients after allogeneic HCT. This study examined the kinetics of DC chimerism in patients who underwent matched sibling allogeneic HCT. T-cell, B-cell, and myelocytic and monocytic chimerism were also studied. Peripheral blood cells were analyzed at defined intervals after transplantation from 19 patients with various hematologic malignancies after treatment with myeloablative or nonmyeloablative preparatory regimens. Cell subsets were isolated before analysis of chimerism. Despite the heterogeneity of the patient population and preparatory regimens, all showed rapid and consistent development of DC chimerism. By day +14 after transplantation approximately 80% of DCs were of donor origin with steady increase to more than 95% by day +56. Earlier time points were examined in a subgroup of patients who had undergone nonmyeloablative conditioning and transplantation. These data suggest that a major proportion of blood DCs early after transplantation is donor-derived and that donor chimerism develops rapidly. This information has potential implications for manipulation of immune responses after allogeneic HCT. (Blood. 2002;99:1442-1448)

Introduction

Allogeneic hematopoietic cell transplantation (HCT) is routinely performed with the intent to cure patients with hematologic malignancies. One major consequence of allogeneic HCT is the replacement of the recipient’s immune system with that of the donor. The change from host to donor immune type is complex and not completely understood. Appropriate immune regulation is of central importance after allogeneic HCT because aberrations in the immune response can lead to the induction of graft-versus-host disease (GVHD), the inability to resist environmental pathogens and/or loss of graft-versus-tumor activity. After allogeneic HCT, immune recovery is determined by both the mature cells transferred with the graft as well as by de novo production of immune populations that arise from committed progenitors and hematopoietic stem cells. Variability in graft composition as well as heterogeneity among patients can also affect this regenerative process. A patient’s prior clinical history and conditioning regimen are thought to influence the level and time course to donor hematopoietic cell engraftment.

One key population of immune-modulating cells that must be integrated into the new developing immune system is dendritic cells (DCs). DCs are a heterogeneous subset of cells with diverse functional capabilities. They belong to the family of professional antigen-presenting cells that also include macrophages and B lymphocytes. However, among antigen-presenting cells, DCs are considered to be both the most potent stimulators of T-cell responses (for review see Banchereau & Steinman) and capable of induction of T-cell tolerance. DCs arise from bone marrow and migrate through the blood to peripheral tissues where they search for antigens to be taken up and processed. They efficiently present both endogenous and exogenous antigens to T cells. DCs produce an array of cytokines and variably express costimulatory molecules. It has been shown that subtypes of DCs exist that can skew T-cell responses toward T helper 1 versus T helper 2 type. Thus, DCs can polarize T-cell responses, depending on the profile of cytokines produced and/or expression of costimulatory molecules.

Little is known about donor DC engraftment or function after clinical allogeneic HCT. Given their central role in mediating antigen-specific immune responses and a report suggesting DC turnover effects development of GVHD, we were interested in examining the time course of DC turnover after allogeneic HCT. Furthermore, we wanted to investigate DC chimerism in the context of T-cell, B-cell, and myelocyte and monocyte chimerism. We hypothesized that the intensity of the conditioning regimen would significantly affect the time course to donor DC chimerism. Therefore, patients treated with myeloablative therapies were compared with those conditioned with a nonmyeloablative preparatory regimen. Additionally, the patients enrolled were heterogeneous with regard to underlying disease and prior courses of chemotherapy.
Our study demonstrates rapid turnover of DCs in the blood compartment after allogeneic HCT and might have implications for therapeutic manipulations of immune responses in this patient population.

### Patients, materials, and methods

#### Patients

Between March 1999 and June 2001, 19 patients, aged 27 to 71 years (median, 58), underwent allogeneic HCT from HLA-matched siblings (Table 1). After informed consent was obtained, all patients were treated on clinical research protocols approved by the institutional review board of our medical center. Blood samples were obtained from 13 patients before transplantation and after transplantation on days +14, +28, +42, +56, +100, and +180 if the patients had a total white cell count more than 1000/μL. Five patients underwent earlier chimerism analysis on day +1, +3, +5, and +7.

Eight patients were treated with myeloablative preparatory regimens for chronic myeloid leukemia (CML) (n = 4), acute myeloid leukemia (n = 3), and diffuse large B-cell non-Hodgkin lymphoma (NHL) (n = 1). For CML in chronic phase, the conditioning regimens consisted of busulfan and cyclophosphamide; for CML in accelerated phase, the conditioning regimen was fractionated total body irradiation and etoposide and cyclophosphamide. The regimen for patients with acute myeloid leukemia contained fractionated total body irradiation and etoposide, and the patient with NHL was conditioned with carmustine, etoposide, and cyclophosphamide. For GVHD prophylaxis patients received cyclosporine (CSP) and methotrexate; CSP and prednisone; or CSP, methotrexate, and prednisone; or CSP and mycophenolate mofetil.

Eleven patients underwent nonmyeloablative transplantation (Table 1) for multiple myeloma (n = 7), chronic lymphocytic leukemia (n = 1), NHL (n = 1), or myelodysplastic syndromes (n = 2). All of these patients received the same conditioning regimen that consisted of total body irradiation and fludarabine. After transplantation, patients received immunosuppression with mycophenolate mofetil and CSP to suppress host-versus-graft reactions and to prevent GVHD.

#### Cell purification

Chimerism analysis was performed on distinct cell subsets isolated by immunomagnetic separation techniques. Peripheral blood DCs were purified by a multistep procedure according to an earlier reported method and used by several other groups of investigators with a commercially available kit (Miltenyi, Auburn, CA). Briefly, peripheral blood mononuclear cells (PBMCs) were first isolated by Ficoll-Paque (Amersham Pharma Biotech, Uppala, Sweden) or Hypaque-Ficoll (Sigma, St Louis, MO) density gradient centrifugation. The DC fraction was then enriched by a 2-step immunomagnetic bead selection process. First, B cells (CD19+), T cells (CD3+), natural killer cells (CD16+), and monocytes (CD11b+) were depleted from PBMCs by monoclonal antibodies (mAbs) coupled to immunomagnetic beads followed by a separation over a magnetic column. The depleted fraction was then further enriched for DCs by repeated rounds of positive selection for CD44+ cells by using anti-CD44-conjugated magnetic beads.

The purity of the isolated cellular population was assessed by fluorescence-activated cell sorting (FACS) analysis. Two-color analysis was performed by using a cocktail of fluorescein isothiocyanate conjugated mouse antihuman mAbs directed against lineage markers (T-cell receptor-ζ, CD3, CD14, CD19, CD56; Becton Dickinson, San Jose, CA) versus phycoerythrin-conjugated mAbs directed against HLA-DR (Becton Dickinson). Irrelevant mouse isotype controls (Becton Dickinson) were included in the analysis. Data acquisition and analysis was performed on a FACS Scan flow cytometer (Becton Dickinson) with the use of Cellquest software.

Myelomonocytic cells were isolated directly from the peripheral blood by using immunomagnetic-positive selection with CD15 Beads (Dynal, Lake Success, NY) according to the manufacturer’s protocol. T cells and B cells were similarly isolated from the Ficoll density gradient-enriched PBMC fraction by immunomagnetic beads coupled to CD3 or CD19 mAbs, respectively (Dynal).

#### Table 1. Patient characteristics

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<th>Patient</th>
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<th>Sex</th>
<th>Disease</th>
<th>Prior treatment</th>
<th>Type of preparative regimen</th>
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<th>CD3⁴/kg (×10⁶)</th>
<th>CD3³/kg (×10⁶)</th>
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SPN indicates Stanford patient number; TNC, total nucleated cells; GVHD, graft-versus-host disease; CML, chronic myeloid leukemia; BP, blast phase; HU, hydroxyurea; Bu, busulfan; Cy, cyclophosphamide; Vinc, vincristine; Pred, prednisone; NA, not available; Cyto rel, cyto genetic relapse; AML, acute myeloid leukemia; Ida, idarubicin; Ara, cytarabine; FTBI, fractionated total body irradiation; Etop, etoposide; CR, complete remission; NHL, non-Hodgkin lymphoma; CHOP, cyclophosphamide, Adriamycin, vincristine, and prednisone; ESHAP, etoposide, cytarabine, prednisone, and cisplatinum; BCNU, carmustine; Flu, fludarabine; INF, interferon; Mouth Ch, chronic GVHD of the oral cavity; MM, multiple myeloma; XRT, radiotherapy; VAD, vincristine, Adriamycin, and dexamethasone; Thal, thalidomide; AutoTX, high-dose chemotherapy with autologous hematopoietic transfer; TBI, total body irradiation; PR, partial remission; CLL, chronic lymphocytic leukemia; CVBP, cyclophosphamide, vincristine, bleomycin, and prednisone; Campath, antibody against CD52; MDS, myelodysplastic syndrome; GvAc2, acute GVHD grade 2 of the gastrointestinal system; MP, melphalan, prednisone; MF, myelofibrosis; and rituximab, antibody against CD20.
Allogeneic mixed leukocyte reaction

The stimulatory ability of DCs was assessed in an allogeneic mixed leukocyte reaction. For all assays, PBMCs from the same healthy donor served as the source of responder cells (1 × 10⁶ responder cells per well were plated in RPMI + 10% fetal calf serum). Patients' PBMCs were enriched by Ficoll-Hypaque density gradient centrifugation, and DCs were immunomagnetically separated as previously described. DCs isolated from the peripheral blood are immature and were, therefore, cultured and matured overnight in RPMI + 10% fetal calf serum and tumor necrosis factor α (TNF-α; 10 μg/mL; R&D Systems, Minneapolis, MN). DCs were irradiated (25 Gy), and decreasing numbers were mixed with responder PBMCs (as indicated in the legend to Figure 1) for 5 days and pulsed with 0.037 MBq (1 μCi) [³H] thymidine during the last 16 hours of culture. [³H] thymidine incorporation was measured by using a liquid scintillation counter (Wallac-Perkin-Elmer, Wallac, Finland).

DC morphology

Peripheral blood DCs were isolated and matured overnight as described above. On harvest 1 to 2 × 10⁶ DCs were resuspended in phosphate-buffered saline and spun onto slides and stained with Giemsa. Images to assess morphology were taken with an objective × 60 magnification (oil).

DNA isolation and short tandem repeat polymerase chain reaction

Genomic DNA from purified cells was isolated with a DNA extraction kit following the manufacturer’s instructions (PUREGENE DNA Isolation Kit; Gentra Systems, Minneapolis, MN). The relative contribution of donor and recipient in each cell population was determined by quantitating, in the DNA preparation, informative microsatellite short tandem repeat (STR) alleles by using a modification of a previously described method. Briefly, for each donor/recipient pair, amplification of STR loci in donor DNA and in recipient pretransplantation DNA was performed in 25 μL polymerase chain reactions (PCR) with the primers shown in Table 2. One member of each primer pair was synthesized (Operon, Alameda, CA) with a Carbocyanin 5 label to permit detection and quantification of fluorescent amplicon in gel electrophoresis on an automated sequencing apparatus. Each PCR reaction contained approximately 50 ng genomic DNA, forward and reverse primer pairs (Table 2), 2 U Taq polymerase (Perkin Elmer AmpliTaq, Foster City, CA) mixed 1:1 with anti-Taq antibody (Clontech, Palo Alto, CA), 2.5 μL GeneAmp 10 × PCR buffer (Perkin Elmer), and 2.5 mM each of dNTPs (Perkin Elmer). The PTC-100 thermal cycler (MJ Research, Watertown, MA) program consisted of an initial 3-minute denaturation at 94°C; 30 cycles of 30 seconds at 94°C, 1 minute at 55°C, 30 seconds at 72°C; and a final 10-minute extension at 72°C. PCR products (0.5 to 2 μL added to formamide [Sigma] to yield a total volume of 12 μL) were electrophoresed at 40°C in a denaturing 8% (wt/vol) acrylamide/bisacrylamide monomers gel (ReproGel High Resolution Gel; Amersham Pharmacia Biotech, Piscataway, NJ) on an ALF or ALF Express (Amersham Pharmacia Biotech). ALF Fragment Manager software (Amersham Pharmacia Biotech) was used to size and quantify each fluorescent amplicon and to calculate the relative abundance of each STR allele present in the template DNA. A STR locus was considered informative for a donor/recipient pair if alleles of the donor differed in size from the respective recipient. Informative loci were subsequently PCR amplified in DNA samples collected after transplantation. For each such sample, the quantity of donor-specific amplicon relative to the quantity of total unique amplicons at that locus yielded the percentage of DNA of donor origin present in the sample. This assay is linear over a range of 5% to 95% for artificial mixes of donor DNA into recipient DNA (data not shown). Each posttransplantation sample was run in parallel with 5% and 95% standards consisting of 5:95 and 95:5 mixes of donor-recipient pretransplantation DNA. By using the ALF Manager program, the standard mixtures yielded measured values (mean ± 2 SD) of 6% ± 4% and 94% ± 4% donor DNA, respectively.

Results

Clinical course

Nineteen patients were enrolled in our study. Table 1 lists the patient characteristics, including age, sex, underlying hematologic disorder, prior chemotherapy, preparative regimen, graft composition, and clinical outcome. All patients in the myeloablative treatment group demonstrated complete engraftment, including erythrocytes, white cells, and platelets. Median time to absolute neutrophil count more than 500/μL was 13 days, to absolute lymphocyte count more than 500/μL was 28 days, and to absolute lymphocyte count more than 1000/μL was 119

Figure 1. Characterization of purified DC population. (A) FACS profile of the enriched DC population. DCs were purified by a depletion of lineage-positive cells followed by repeated positive selection for CD4⁺⁺ cells. This figure shows the resultant purified DC population obtained from an allogeneic transplantation patient. As shown, the DC population as characterized by DR⁺, lineage-poor population constitutes approximately 93.4% of the cells. (B) Morphologic analysis of peripheral blood DC from a patient after allogeneic transplantation. Peripheral blood DCs derived from a patient after allogeneic transplantation were immunomagnetically purified and terminally matured overnight with TNF-α. DCs were then cytocentrifuged and stained with Giemsa. The studies were visualized by light microscopy at × 60 magnification. (C) Allogeneic mixed leukocyte reaction contained approximately 50 ng genomic DNA, forward and reverse primer pairs (Table 2). 2 U Taq polymerase (Perkin Elmer AmpliTaq, Foster City, CA) mixed 1:1 with anti-Taq antibody (Clontech, Palo Alto, CA), 2.5 μL GeneAmp 10 × PCR buffer (Perkin Elmer), and 2.5 mM each of dNTPs (Perkin Elmer). The PTC-100 thermal cycler (MJ Research, Watertown, MA) program consisted of an initial 3-minute denaturation at 94°C; 30 cycles of 30 seconds at 94°C, 1 minute at 55°C, 30 seconds at 72°C; and a final 10-minute extension at 72°C. PCR products (0.5 to 2 μL added to formamide [Sigma] to yield a total volume of 12 μL) were electrophoresed at 40°C in a denaturing 8% (wt/vol) acrylamide/bisacrylamide monomers gel (ReproGel High Resolution Gel; Amersham Pharmacia Biotech, Piscataway, NJ) on an ALF or ALF Express (Amersham Pharmacia Biotech). ALF Fragment Manager software (Amersham Pharmacia Biotech) was used to size and quantify each fluorescent amplicon and to calculate the relative abundance of each STR allele present in the template DNA. A STR locus was considered informative for a donor/recipient pair if alleles of the donor differed in size from the respective recipient. Informative loci were subsequently PCR amplified in DNA samples collected after transplantation. For each such sample, the quantity of donor-specific amplicon relative to the quantity of total unique amplicons at that locus yielded the percentage of DNA of donor origin present in the sample. This assay is linear over a range of 5% to 95% for artificial mixes of donor DNA into recipient DNA (data not shown). Each posttransplantation sample was run in parallel with 5% and 95% standards consisting of 5:95 and 95:5 mixes of donor-recipient pretransplantation DNA. By using the ALF Manager program, the standard mixtures yielded measured values (mean ± 2 SD) of 6% ± 4% and 94% ± 4% donor DNA, respectively.
days. The incidence and manifestation of clinically evident GVHD was low and mild, respectively (Table 1). Patient SPN 2250 experienced acute GVHD grade II of the skin (macular-papular eruption of 25% to 50% of body surface) that was controlled by CSP and prednisone treatment. Patient SPN 2252 developed limited chronic GVHD of the oral cavity. All patients achieved a complete hematologic remission of their diseases. Patient SPN 2240 was diagnosed with a cytogenetic relapse of the underlying CML on day 2331, and patient SPN 2185 is showing signs of limited chronic ism after allogeneic HCT for the DC lineage, as well as for patients treated with nonmyeloablative preparatory therapy as early as day +1 and for patients treated with myeloablative pretransplantation therapy on day +14.

**DC morphology and function**

To confirm that the cell population isolated from the patients' peripheral blood was DCs as previously shown by other investigators,11,13-16 morphologic and functional studies were performed. It has been demonstrated that DCs directly isolated from peripheral blood are immature.11 Therefore, the isolated cells were matured overnight in the presence of TNF-α. Evaluation of the resultant cell population demonstrated that the isolated cells developed features characteristic of DCs, including nonadherence to plastic and a typical veiled morphology (Figure 1B). Furthermore, the antigen-presenting and stimulatory capacities of these cells were evaluated by mixed leukocyte reaction. The DCs isolated from allogeneic transplantation patients were efficient in their capacity to stimulate healthy allogeneic third-party PBMC responder cells. Their stimulatory capacity was significantly better than PBMCs obtained from healthy control individuals (Figure 1C).

**DC chimerism after myeloablative allogeneic HCT**

Given that patients undergoing myeloablative pretransplantation therapy experience profound pancytopenia in the days immediately after allogeneic HCT, the earliest time point that DC chimerism could be assessed was at day +14 after transplantation. Figure 2A-D summarizes the percentage of donor chimerism after allogeneic HCT for the DC lineage, as well as for myelomonocytes and T and B lymphocytes in the patients that

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**Table 2. Short tandem repeat primer**

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<th>Chromosome</th>
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*STR indicates short tandem repeat; Fwr, forward; and Rev, reverse.
†Forward primers are 5’ labeled with Carbocyanin 5, reverse primers with fluorescein.
observed period the donor contribution to DCs, myelocytes, monocytes, and B cells remained stable at 99% or more, and T-cell chimerism progressively increased to 97% ± 3%. The only exception to this pattern of chimerism was patient SPN 2240, whose CML recurred on day +180. The relapse of her disease was associated with a decrease in DC chimerism to 75% and myelocyte and monocyte chimerism to 92% at day +180.

**DC chimerism after nonmyeloablative allogeneic HCT**

Studies on DC chimerism in the nonmyeloablative treatment group initially focused on the same time points examined for the myeloablated patients. Figure 2E demonstrates that development of DC chimerism was similar in pace and pattern to that in patients with myeloablative treatment. By day +14 after transplantation 80% ± 16% DCs were of donor origin, followed by further increases to 93% ± 10% on day +28 and 96% ± 6% on day +42, and nearly all DCs of donor origin by day +56 (98% ± 5%; Figure 2E). Establishment of T-cell chimerism was also similar to that observed for the myeloablated patients, 79% ± 16% of T cells derived from the donor on day +14, 88% ± 16% on day +28, 91% ± 11% on day +56, and nearly complete donor origin on day +100 (99% ± 2%; Figure 2G). The appearance of donor B cells and myelocyte and monocyte lineages was slightly delayed as compared with patients who received myeloablative regimens for the engraftment in the nonmyeloablative group. However, by day +100 all cell types tested were exclusively of donor origin (100% ± 1%; Figure 2F, H).

The high percentage of donor DCs observed in the peripheral blood of both patient groups as early as day +14 was unexpected. Because it was technically possible to isolate DCs from nonmyeloablative patients before day +14, we examined DC chimerism in this group of patients in the first week after allogeneic HCT.

During the first week after HCT, blood samples were obtained from 5 patients every other day beginning on day +1. Figure 3 shows the percentage of donor DCs in these patients. As early as day +1 after transplantation, donor DCs constituted 78% ± 4% and remained more than 68% on average until day +7. A transient decrease to 50% was noted (68% ± 19% on day +3, 83% ± 11% on day +5, and 52% ± 18% on day +7). There followed a steady increase of donor DCs to 73% ± 21% isolated on day +14, 88% ± 15% on day +28, 96% ± 6% on day +42, and 97% ± 6% on day +56 (Figure 3).

**Figure 2. Donor chimerism after myeloablative and nonmyeloablative transplantation.** Peripheral blood was obtained from patients on days +14, +28, +42, +56, +100, and +180 after transplantation. Subpopulations were purified by magnetic bead sorting and analyzed for percentage of donor origin by STR analysis. The graphs show the mean percentage of donor chimerism and SD for each cell population studied. (A-D) Mean donor chimerism for 5 patients that underwent myeloablative allogeneic HCT. (A-E) Mean donor chimerism for 8 patients that underwent nonmyeloablative allogeneic HCT. Cell subsets analyzed were dendritic cells (A, E), B lymphocytes (B, F), T lymphocytes (C, G), and myelocytes and monocytes (D, H).

**Figure 3. Donor chimerism of DCs early after nonmyeloablative transplantation.** Peripheral blood DCs were purified by magnetic bead sorting and analyzed for percentage of donor origin by STR analysis. Peripheral blood was obtained from patients on days +1, +3, +5, +7, +14, +28, and +42 after transplantation. The mean of 5 studied patients is shown ( ), the vertical lines represent the SD.
Discussion

It was the purpose of this study to investigate the kinetics of chimerism of DCs in patients after allogeneic HCT. The patients studied were heterogeneous with regard to their underlying diagnoses, pretransplantation cytoreductive therapy, types of grafts, and conditioning regimens. Surprisingly, we found that the kinetics of appearance of donor DCs in the peripheral blood was rapid and highly consistent among all patients evaluated beginning on day +14. For patients undergoing nonmyeloablative conditioning treatment, we were able to isolate DCs from the peripheral blood as early as day +1 and found that even at this early time point approximately 78% of the DCs originated from the donors. Fluctuations in donor chimerism were observed, but donor chimerism remained more than 50% until day +14, when a steady increase in donor DC levels was observed. This kinetics led us to conclude that most donor DCs are initially transfused with the hematopoietic graft. Then, beginning around 2 weeks after hematopoietic cell infusion DCs arose de novo from donor progenitors and stem cell source. This time frame of DC reconstitution observed in vivo correlates well with prior in vitro studies, wherein the time course to DC generation from CD34+ hematopoietic progenitors cells under optimized conditions takes approximately 14 days.20-22

The implications of rapid donor DC generation and repopulation after allogeneic HCT are broad. It is now known that DCs have multiple functional roles in the activation and tolerance induction of T cells.1,23 On the one hand, DCs are considered to be the most potent antigen-presenting cells capable of stimulating T-cell responses (for review see Bell et al24). On the other hand, DCs have been shown to be capable of inducing intrathymic deletion of alloreactive cells7,3 as well as mediating the establishment of peripheral tolerance.25,26 DCs process and present both endogenously and exogenously derived antigens. Therefore, in the setting of a major histocompatibility complex–matched allogeneic HCT procedure (the most common clinical scenario), DCs arising from the donor graft and DCs persisting from recipients are expected to present endogenous minor donor antigen peptides that will influence T-cell activation and/or induction of tolerance. The clinical implications of DC turnover in the postalloge neic HCT setting were demonstrated by investigators8 who reported that GVHD in a major histocompatibility complex–matched allogeneic murine bone marrow transplantation model was driven by host antigen-presenting cells. Thus, understanding the time course of donor DC turnover may be useful for determining an appropriate length of immunosuppression for GVHD prophylaxis. In a more limited study of 6 patients with only a single data point each (between days 24 and 205 after related or unrelated HCT), full donor DC chimerism was associated with the absence of GVHD.27 In our study a low incidence and severity of GVHD was observed throughout the full 6-month observation period after transplantation. Thus, it was not possible to correlate GVHD to the establishment of donor DC status.

References


Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients

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