Evidence for neo-generation of T cells by the thymus after non-myeloablative conditioning

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Supplementary Text

Patients and donors

Fifty patients were randomized to receive either unmanipulated (n=28) or CD8-depleted (n=25) PBSC. Three patients randomized to receive CD8-depleted PBSC received unmanipulated grafts because of low CD34+ counts before the CD8-depletion procedure. They were excluded from the current analyses. The characteristics of the remaining 50 patients are summarized in Table 1, together with a brief analysis of their transplantation outcome.[36] Briefly, median patient age was 57 years (range, 36 to 69 years). Diagnoses were haematological malignancies in 48 patients, and metastatic renal cell carcinoma in the 2 remaining patients. Nineteen patients received PBSC from HLA-identical siblings, one patient received PBSC from a child presenting only 1 HLA-antigen mismatch with the recipient and the remaining 30 patients received PBSC from either 10/10 HLA-allele matched (n=14) or mismatched (n=16) unrelated donors. Patients were excluded from analysis at time of graft rejection or relapse/progression of the underlying disease. The study was approved by the Ethics Committee of the University of Liège, and all patients signed a written informed consent form.

Conditioning regimen and immunosuppression

The conditioning regimen consisted of 2 Gy total body irradiation (TBI; given on day 0) with (n=38) or without (n=12, patients given grafts from related donor with a low risk of graft rejection) added fludarabine (3 x 30 mg/m^2 given on days -4, -3 and -2). Postgrafting immunosuppression combined ciclosporine (CSP) with mycophenolate mofetil (MMF). CSP was given at full dose from day -1 until day 120 and then tapered to stop by day 180 in patients given related grafts, and was given at full dose until day 180 and then taped off until day 365 in patients given unrelated grafts. MMF was given from day -1 to day 28 in patients given grafts from related donors, and from day -1 to day 42 in those given unrelated grafts. The duration of CSP prophylaxis was extended in patients with GVHD.

PBSC collection and CD8-depletion of PBSC

Donors received recombinant human G-CSF at 10 µg/kg from day -5 through the day of HCT. Collection of PBSC was carried out on days -1 and 0, using a continuous flow blood cell separator (CS3000+, Baxter-Fenwall Laboratories, Deerfield, IL, or Cobe Spectra, Lakewood, CO). CD8-depletion of the PBSC was performed using the Eligix CD8 monoclonal antibody-coated high-density microparticles (CD8-HDM, Biotransplant Inc, Charlestown, MA, USA) as recommended by the manufacturer. Patients given unmanipulated PBSC received a median of 4.5 (0.8-20.2) x 10^6 CD34+ cells/kg, 314 (80-631) x 10^6 CD3+ cells/kg, and 130 (43-272) x 10^6 CD8+ cells/kg. After the CD8-depletion procedure, patients in the CD8-depleted PBSC group received a median of 3.7 (0.7-12.2) x 106 CD34+ cells/kg recipient (p=0.2), 110 (56-239) x 10^6 cells CD3+cells/kg (p<0.0001), and 4.2 (0.4-33.7) x 10^6 CD8+ cells/kg (p<0.0001), respectively. The CD8-depletion procedure depleted CD3+ lymphocytes and CD8+ lymphocytes by a median of 0.4 (range, 0.2-0.5) log and 1.3 (range, 0.6-2.4) log, respectively, while 72% (range, 28-100%) of CD34+ and 71% (range, 41-88%) of CD4+ cells were recovered.

Clinical management

G-CSF (5 µg/kg/d) was administered when the granulocyte counts dropped below 1.0 x 10^9/L. The diagnosis and grading of acute GVHD was established using international criteria.[37] Treatment for acute GVHD usually consisted of methylprednisolone 2 mg/kg/day, with taper started after 14 days. Extensive chronic GVHD was usually treated with alternate day methylprednisolone and CSP.

Infection prophylaxis generally consisted of acyclovir (400 mg t.i.d. orally), oral itraconazole solution (200 mg b.i.d.) and aerosolized pentamidine (38). Polymerase chain reaction (PCR) for cytomegalovirus (CMV) was performed weekly until day 100 and every 2-4 weeks thereafter. Patients with a positive PCR received preemptive ganciclovir for a minimum of 4 weeks and generally up to day 100.

Disease evaluation was routinely carried out on days 40, 100, 180 and 365 after HCT.

Laboratory analyses on PBSC

Aliquots of the pooled PBSC as well as the CD8-depleted fraction were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti- CD34, CD3, CD4, CD19, CD8 and CD56 monoclonal antibodies or isotype-matched non-specific IgG (BD Biosciences, Erembodegem, Belgium) for 20 minutes at 4°C, washed and fixed. A total of 1 x 10^5 cells/condition was analyzed using a FACSCalibur analyzer (BD). The percentage of positive cells was measured relative to total nucleated cells, after subtraction of non-specific staining. Data were acquired and analyzed using Cellquest software (BD).

Chimerism analyses

Chimerism among T cells was assessed at days 28, 42, 100, 180 and 365 after HCT using fluorescence in situ hybridization to detect X and Y chromosomes for recipients of sex-mismatched transplants and PCR-based analysis of polymorphic microsatellite regions (multiplex PCR) for recipients of sex-matched transplants. CD3 (T cells) selection was carried out with the RosetteSep lymphoid enrichment kit (StemCell Technologies, Grenoble, France). Graft rejection was defined as the occurrence of < 5% T-cells of donor origin after HCT, as previously described.[3;39-41]

Immune recovery

Immune recovery was assessed as previously described.[7] Briefly, patients' peripheral blood mononuclear cells (PBMCs) were phenotyped on days 28, 42, 60, 80, 100, 120, 180 and 365 using four color flow cytometry. The analyzed cell subsets were T-cells ($CD3^+$), $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, $CD4^+CD45RA^{high}$ double positive lymphocytes, $CD4^+CD45RO^+$ double positive lymphocytes, $CD56^+$ lymphocytes as well as $CD19^+$ B lymphocytes. The percentage of positive cells was measured relative to total nucleated cells, after subtraction of non specific staining. Absolute counts were obtained by multiplying the percentage of positive cells by the white blood cell count (Advia 120 hematology analyzer, Bayer Technicon, Tarrytown, USA). Lower and higher limits of normal values for each cell subset were defined respectively as percentiles 3 and 97 of values obtained in 30 healthy blood donors.

CDR3 spectratyping

T cell receptor beta chain (TCRB) CDR3 spectratyping (immunoscope) analyses were performed on days 40, 100, 180 and 365.[7] On day 180, spectratyping was done separately in CD4 and CD8 T cells, CD4 and CD8 T-cells being isolated using the Dynall kit (Dynall, Oslo, Norway) according to the recommendation of the manufacturer). RNA was extracted from 10x10^6 cells by Tripure (Roche) according to the manufacturer's protocol. First-strand cDNA was generated from 2 μg total RNA using 1st Strand cDNA Synthesis Kit for RT-PCR (Roche) according to the manufacturer's protocol. Each TCRB segment was amplified with one of the 24 TCRBV subfamily-specific primers (Vβ1-Vβ24) and a TCRBC primer conjugated to fluorescent dye 6-FAM (Applied Biosystems, Lennik, Belgium) for CDR3 analysis.[42] The size distribution of each fluorescent PCR product was determined by electrophoresis on an automated DNA sequencer (Applied Biosystems, Foster City, CA) and data were analyzed by GeneScan-500 software (Perkin Elmer Cetus Instruments, Emeryville, CA). The overall complexity within a TCRBV subfamily was determined by counting the number of peaks (intervals of 3 nucleotides without any gaps) per subfamily. Normal complexity subfamily was defined as consisting of at least 6 peaks spaced 3 nucleotides apart without any gaps. The overall spectratyping complexity (TCRBV score) was calculated as the sum of the numbers of peaks in the 24 subfamilies. The median TCRBV score in 5 volunteer blood donors was 186 (range, 182-195) peaks.

Supplementary Figures

Figure 1

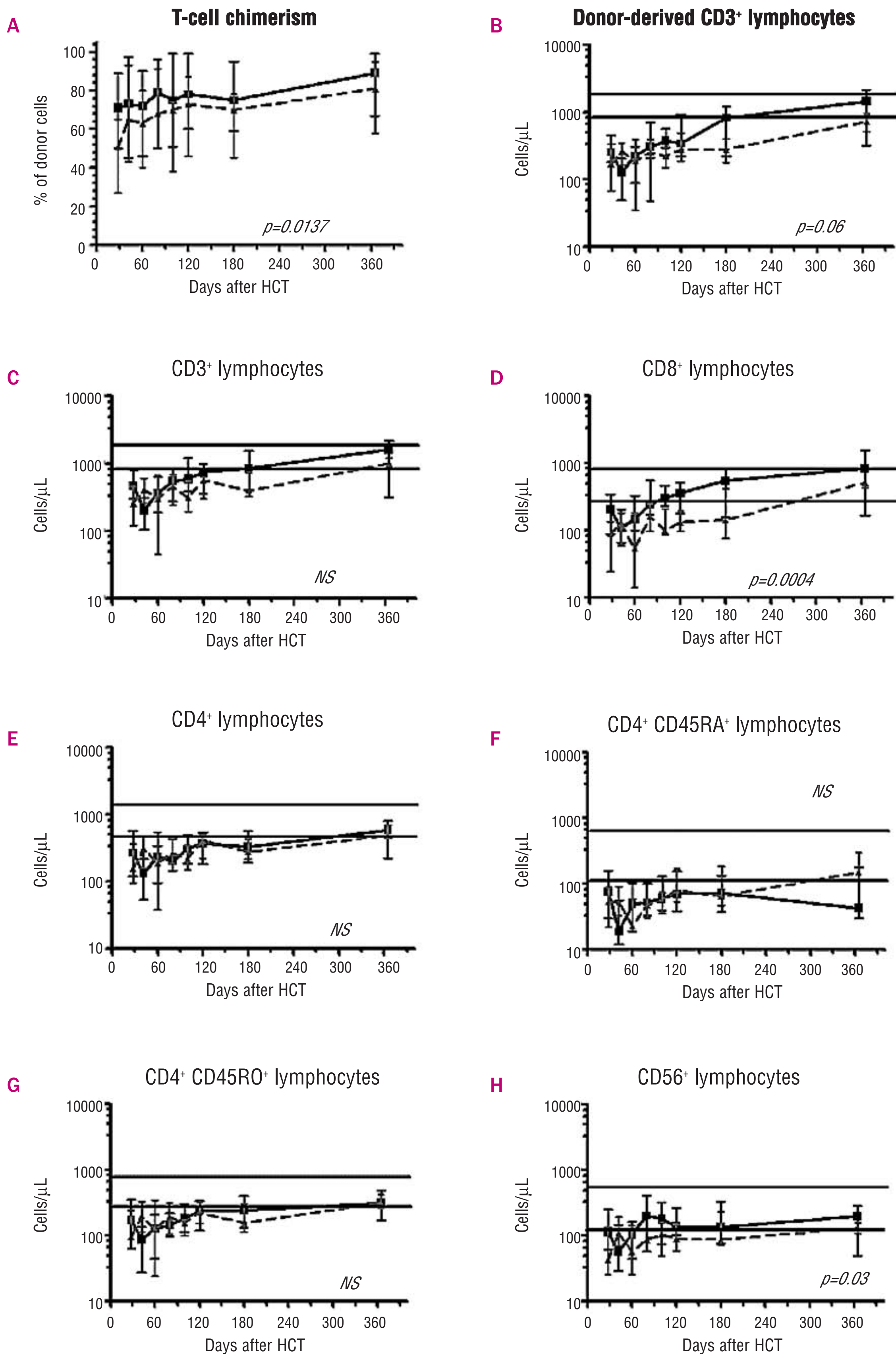

Figure 1. Median donor T-cell chimerism (A), median absolute counts of T cells of donor origin (B), and median MNC-subset counts (C-H) among unmanipulated (solid black line) or CD8-depleted (dashed gray line) PBSC recipients after nonmyeloablative conditioning. Error bars indicate the 25th and 75th percentiles. Horizontal lines show the 3rd and 97th percentiles in 30 normal blood donors.

Figure 2

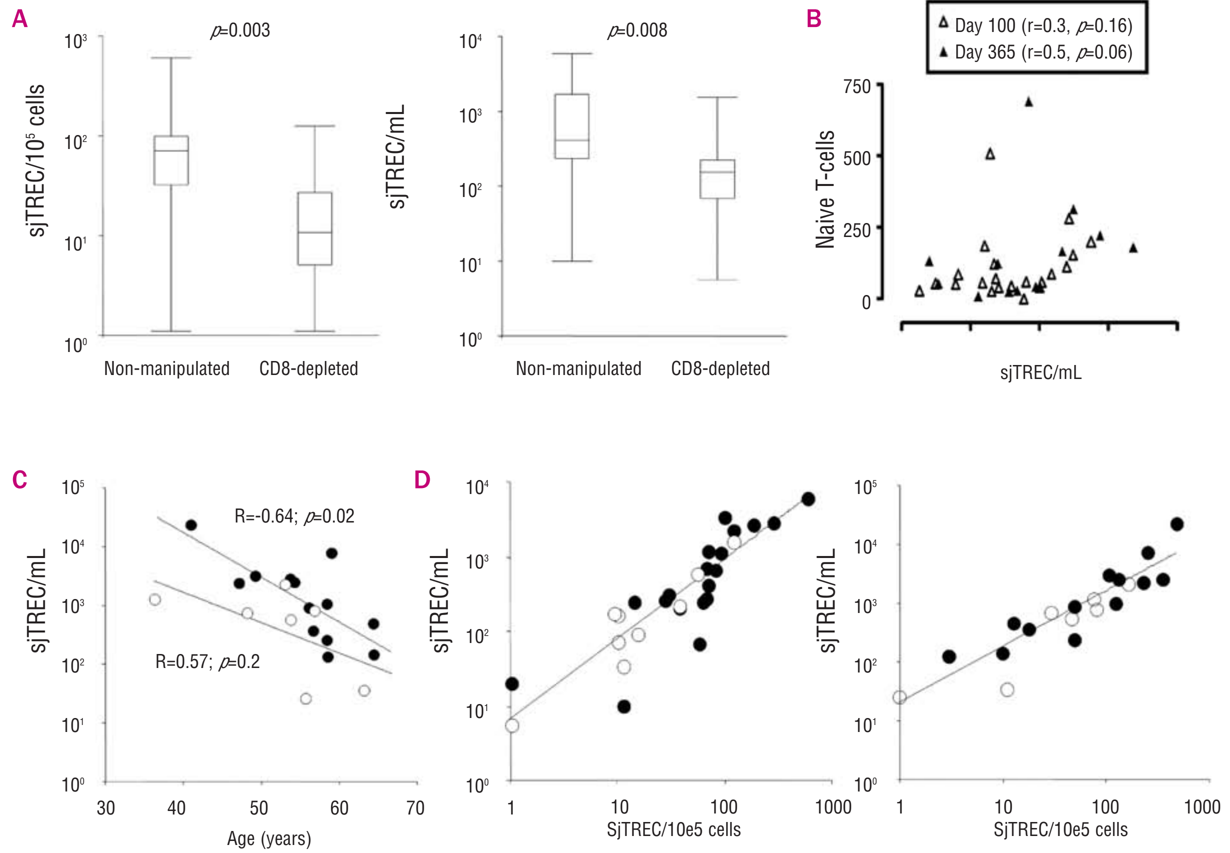

Figure 2. Thymic function after nonmyeloablative HCT. Black circles indicate data in unmanipulated PBSC recipients, and open circles data in CD8-depleted PBSC recipients. (A) sjTREC frequency and concentration on day 100 after HCT. (B) Correlation between sjTREC concentrations and naive CD4+ T-cell counts on days 100 (open triangles) and 365 (dark triangles) after HCT. (C) Correlation between sjTREC concentrations and patient age on day 365 after HCT (r=-0.57 and P=0.008 when all patients are analyzed together). (D) Correlation between sjTREC frequencies and sjTREC concentrations on days 100 and 365 after HCT.