Thymic recovery after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning is limited to patients younger than 60 years of age

by Emilie Castermans, Muriel Hannon, Jacques Dutrieux, Stephanie Humblet-Baron, Laurence Seidel, Remi Cheynier, Evelyne Willems, Andre Gothisot, Jean-Francois Vanbellinghen, Vincent Geenen, Brenda M. Sandmaier, Rainer Storb, Yves Beguin, and Frederic Baron

Haematologica 2010 [Epub ahead of print]

Citation: Castermans E, Hannon M, Dutrieux J, Humblet-Baron S, Seidel L, Cheynier R, Willems E, Gothisot A, Vanbellinghen JF, Geenen V, Sandmaier BM, Storb R, Beguin Y, and Baron F. Thymic recovery after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning is limited to patients younger than 60 years of age. Haematologica. 2010; 95:xxx
doi:10.3324/haematol.2010.029702

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors’ final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.

Haematologica (pISSN: 0390-6078, eISSN: 1592-8721, NLM ID: 0417435, www.haematologica.org) publishes peer-reviewed papers across all areas of experimental and clinical hematology. The journal is owned by the Ferrata Storti Foundation, a non-profit organization, and serves the scientific community with strict adherence to the principles of open access publishing (www.doaj.org). In addition, the journal makes every paper published immediately available in PubMed Central (PMC), the US National Institutes of Health (NIH) free digital archive of biomedical and life sciences journal literature.

Support Haematologica and Open Access Publishing by becoming a member of the European Hematology Association (EHA) and enjoying the benefits of this membership, which include free participation in the online CME program.

Official Organ of the European Hematology Association
Published by the Ferrata Storti Foundation, Pavia, Italy
www.haematologica.org
Thymic recovery after allogeneic hematopoietic cell transplantation with nonmyeloablative conditioning is limited to patients younger than 60 years of age

Running title: Thymic recovery after nonmyeloablative HCT

Emilie Castermans1,2*, Muriel Hannon1,2*, Jacques Dutrieux3, Stéphanie Humblet-Baron2, Laurence Seidel4, Rémi Cheynier3, Evelyne Willems1,2, André Gothot5, Jean-François Vanbellinghen6, Vincent Geenen7, Brenda M. Sandmaier8,9, Rainer Storb8,9, Yves Beguin1,2, and Frédéric Baron1,2

EC and MH contributed equally to this work

1Department of Medicine, Division of Hematology, University and CHU of Liège, Liège, Belgium; 2GIGA Research-Hematology, University of Liège, Liège, Belgium; 3Department of Virology, Institut Pasteur, Paris, France; 4Department of statistics, University of Liège, Liège, Belgium; 5Department of Laboratory Medicine, Division of Laboratory Hematology and Immuno-Hematology, University of Liège, Liège, Belgium; 6,7Department of Genetics, CHU of Liège, Liège, Belgium; Center of Immunology, University of Liège, Liège, Belgium; 8Fred Hutchinson Cancer Research Center, Seattle, WA, USA, and 9University of Washington, Seattle, WA, USA

Funding
MH is Télévie Research Fellow, SHB Postdoctoral Researcher, FB Senior Research Associate, and VG Research Director at the National Fund for Scientific Research (FNRS) Belgium. The study was in part supported by funds from the FNRS, the Belgian Foundation against Cancer (FBC), the anti-cancer foundation from the ULg, the CHU of Liège, the Terry Fox foundation, and by grants CA 78902, CA 18029 and HL 36444 from the National Institutes of Health, Bethesda, MD.

Correspondence
Frédéric Baron, University of Liège, department of Hematology, CHU Sart-Tilman 4000 Liège Belgium. Phone: international +32.4.3667201. Fax international +32.4.3668855. E-mail: f.baron@ulg.ac.be

Key words: thymus, hematopoietic cell transplantation, nonmyeloablative, GVHD, immunity, age.
**Background.** Long term immune recovery in older patients given hematopoietic cell transplantation after nonmyeloablative conditioning remains poorly understood. This prompted us to investigate long term lymphocyte reconstitution and thymic function in 80 patients given allogeneic peripheral blood stem cells (PBSC) after nonmyeloablative conditioning.

**Design and Methods.** Median age at transplant was 57 years (range 10-71). Conditioning regimen consisted of 2 Gy total body irradiation (TBI) with (n=46) or without (n=20) added fludarabine, 4 Gy TBI with fludarabine (n=6), or cyclophosphamide plus fludarabine (n=8). Stem cell sources were unmanipulated (n=56), CD8-depleted (n=19), or CD34-selected (n=5) PBSC. Immune recovery was assessed by signal-joint T-cell receptor excision circle (sjTREC) quantification, and flow cytometry.

**Results.** SjTREC levels increased from day 100 to 1 and 2 years after transplantation in patients under 50 years of age (n=23; $P=0.02$ and $P=0.04$, respectively), and in those aged 51-60 years (n=35; $P=0.17$ and $P=0.06$, respectively), but not in patients > 60 (n=22; $P=0.3$ and $P=0.3$, respectively). Similarly, CD4+CD45RA+ (naïve) T-cell counts increased from day 100 to 1 and 2 years after transplantation in patients ! 50 (P=0.002 and P=0.02, respectively), and in those aged 51-60 (P=0.4 and P=0.001, respectively), but less so in patients >60 (P=0.3 and P=0.06, respectively). In multivariate analyses, older patient age ($P<0.001$), extensive chronic GVHD ($P<0.001$), and prior (resolved) extensive chronic GVHD (P=0.008) were associated with low sjTREC levels 1 year after HCT.

**Conclusions.** In summary our data suggest that thymic neo-generation of T-cells occurred from day 100 onwards in patients under 60 while sjTREC levels remained low for patients above 60. Further, chronic GVHD had a dramatic impact on thymic function, as observed previously in patients given grafts after myeloablative conditioning.
INTRODUCTION

It is well established that the thymus undergoes a physiological involution with aging that affects its structure and cytokine environment (1,2). However, studies by Steinman et al. in the eighties documented lymphocytic thymic tissue in adults up to 107 years of age (1). Further, several studies have suggested that the human thymus could continue to mature new T-cells throughout life, exporting signal-joint T-cell receptor excision circle (sjTREC) bearing T-cells into the peripheral blood even in elderly patients (3-5).

Nonmyeloablative conditioning regimens followed by allogeneic hematopoietic cell transplantation (HCT) have opened the way to performing allogeneic HCT in patients with hematological malignancies aged up to 70-75 years (6-9). This approach has relied on optimization of pre- and post- transplant immunosuppression to overcome host-versus-graft reactions(10), thereby allowing engraftment and eradication of tumors nearly exclusively via immune-mediated graft-versus-tumor effects (11-13).

T-cell recovery after allogeneic HCT following high-dose (myeloablative) conditioning depends on both peripheral expansion of mature T-cells contained in the graft (thymo-independent pathway), and T-cell neo-production from donor hematopoietic stem cells (thymo-dependent pathway) (14-17). In young patients given myeloablative allogeneic HCT, most circulating T-cells during the first 3-6 months following HCT are the progeny of T-cells infused with the grafts (18), while neogeneration of T-cells by the thymus plays an important role in reconstituting the T-cell pool beyond day 100 after HCT(19-24).

We recently analyzed immune recovery after nonmyeloablative conditioning the first year after nonmyeloablative conditioning (25;26). Main observations were that unrelated donor status and donor age affected early immune recovery (25), while sjTREC levels significantly increased from day 100 to day 365 in a cohort of 16 patients (26), suggesting that the thymic pathway might play a role in immune recovery in these older patients given nonmyeloablative conditioning. Here, we analyzed long-term (≥ 1 year) immune recovery and thymic function in 80 patients given nonmyeloablative conditioning regimen.

DESIGN AND METHODS

Patients and donors
Data from 80 patients transplanted between March 2000 and April 2008 at the University of Liège were included in the study. Results were analyzed as of July 29, 2009. Patient characteristics are summarized in Table 1. Median patient age was 57 (range, 10-71) years. Thirty-three of the 80 patients received grafts from HLA-matched related donors, 22 from HLA-matched unrelated donors, and 25 from HLA-mismatched related or unrelated donors. Stem cell sources were unmanipulated (n=56), CD8-depleted (n=19), or CD34-selected (n=5) peripheral blood stem cells (PBSC). CD8-depletion and CD34-selection of PBSC were
carried out as previously reported (26;27). Nine patients given grafts from HLA-mismatched donors were enrolled in a trial of mesenchymal stem cell co-transplantation as a potential way to prevent severe GVHD (28). Immune recovery in 35 of the 80 patients for the first 6 (n=14) (27) or 12 (n=21) (26) months after HCT has been reported previously. Written informed consent was obtained from each patient to undergo nonmyeloablative HCT and to collect, store and analyze blood samples for research purposes. The Ethics Committee of the University of Liège approved the consent form as well as the current research study protocol.

Treatment and evaluation
The nonmyeloablative conditioning regimens consisted of 2 Gy total body irradiation (TBI) alone (n=20; patients with low risk of graft rejection given PBSC from HLA-identical siblings), 2 Gy TBI with 90 mg/m² fludarabine (n=46; standard regimen), 4 Gy TBI with 90 mg/m² fludarabine (n=6; patients at high risk of early disease progression and/or graft rejection), or fludarabine 90 mg/m² with cyclophosphamide 3 g/m² (n=8, patients with previous irradiation precluding the use of TBI). Postgrafting immunosuppression combined mycophenolate mofetil (MMF) with a calcineurin inhibitor for all patients, as previously described (26;27).

Clinical management
Twenty-four patients received at least one unmanipulated (n=18) or CD8-depleted (n=6) pre-emptive donor lymphocyte infusion (DLI) the first year after HCT (including the five patients given CD34-selected PBSC who received CD8-depleted pre-emptive DLI). G-CSF (5 μg/kg/d) was administered when the granulocyte count declined below 1.0 x 10⁹/L. The diagnosis, clinical grading, and treatment of acute GVHD were performed according to established criteria(29). Treatment was usually given for grade II-IV acute GVHD and for extensive chronic GVHD. Initial treatment of acute GVHD usually consisted of prednisolone, 2 mg/kg/day, with taper initiated within 14 days. In addition, the calcineurin inhibitor was usually resumed at full doses. Steroid-refractory acute GVHD was treated as per available investigational protocols or standard practice. Treatment of chronic GVHD consisted of methylprednisolone (1 mg/kg) with alternate-day calcineurin inhibitor. Steroid-refractory chronic GVHD was generally treated with rapamycin, mycophenolate mofetil, or photopheresis.

Infection prophylaxis generally consisted of acyclovir (400 mg t.i.d. orally), oral itraconazole solution (200 mg b.i.d.) or oral fluconazole (200 mg b.i.d.), and trimethoprim sulfamethoxazole or aerosolized pentamidine. 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 i.v. ganciclovir.
Disease evaluations were routinely carried out on days 40, 100, 180 and 365 after HCT.

**Chimerism**
Chimerism levels among peripheral T-cells were assessed on days 28, 40, 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 (AmpFLSTR® Identifiler®, Applied Biosystems, Lennik, Belgium) for recipients of sex-matched transplants (26;27). CD3 (T-cell) selection was carried out with the RosetteSep® human T-cell enrichment kit (StemCell Technologies, Vancouver, Canada). Graft rejection was defined as the occurrence of < 5% T-cells of donor origin after HCT, as previously described (30;31).

**Immune recovery**
Immune recovery was prospectively assessed as previously described (27). Briefly, patients’ peripheral white blood cells were phenotyped on days 28, 42, 60, 80, 100, 120, 180, 365, 540, 730 and yearly thereafter using 4 color flow cytometry after treatment with a red blood cell lysing solution. The analyzed cell subsets were T-cells (CD3⁺), CD4⁺ T-cells (CD3⁺CD4⁺ lymphocytes), CD8⁺ T-cells (CD3⁺CD8⁺ lymphocytes), naïve CD4⁺ T-cells (CD4⁺CD45RA⁺ double positive lymphocytes), memory CD4⁺ T-cells (CD4⁺CD45RO⁺ double positive lymphocytes), NK cells (CD3⁻CD56⁺ lymphocytes) as well as B cells (CD19⁻ 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 percentages of positive cells by the white blood cell counts (Advia 120 hematology analyzer, Bayer Technicon, Tarrytown, USA). Lower and higher limits of normal values for each cell subset were defined respectively as 5 and 95 percentiles of values obtained in 47 age-matched healthy volunteers donors.

More detailed T and B cell phenotyping was retrospectively performed using cryopreserved PBMC from 33 patients obtained 2 years after transplantation using 6 color flow cytometry. The different populations were defined as follow: naïve CD4⁺ T-cells, CD4⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; central memory CD4⁺ T-cells, CD4⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; effector memory CD4⁺ T-cells, CD4⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; late differentiation effector memory CD4⁺ T-cells, CD4⁻CD45RA⁻CCR7⁻CD27⁺CD8⁻ lymphocytes; naïve CD8⁺ T-cells, CD8⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; central memory CD8⁺ T-cells, CD8⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; effector memory CD8⁺ T-cells, CD8⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; effector memory RA⁺ CD8⁺ T-cells, CD8⁻CD45RA⁻CCR7⁻CD27⁺ lymphocytes; naïve B cells, CD19⁺CD27⁻ lymphocytes; memory B cells, CD19⁺CD27⁺ lymphocytes; IgM⁺IgD⁺ memory B cells, CD19⁺CD27⁺IgM⁺IgD⁺.
lymphocytes; switched memory B cells, CD19^+CD27^IgM^-IgD^- lymphocytes. Absolute counts were calculated by multiplying the percentage of positive cells in the lymphoid gate by the absolute lymphocyte count of the patient the day of PBMC collection.

**CDR3 spectratyping (immunoscope)**

T-cell receptor beta chain (TCRB) CDR3 spectratyping analyses were performed between 2 and 9 (median 3) years after HCT in 21 patients. RNA was extracted from 10x10^6 cells by Tripure (Roche) according to the manufacturer’s protocol. The quality of the extracted RNA was controlled using the Experion RNA StdSens Starter Kit (Bio-Rad, Nazareth Eke, Belgium) in a gel electrophoresis experiment (Experion Automated Electrophoresis System, Bio-Rad). RNA optical density measurements were performed on a Nanodrop ND1000 (Isogen, Sint-Pieters Leeuw, Belgium). First-strand cDNA was generated from 1 µg total RNA using Transcriptor 1st strand cDNA synthesis kit (Roche) according to the manufacturer’s protocol. Each TCRB segment was amplified with one of the 24 TCRBV family-specific primers (V^β1-V^β24) and a TCRBC primer conjugated to fluorescent dye 6-FAM (Applied Biosystems, Lennik, Belgium) for CDR3 analysis. The size distribution of each fluorescent PCR product was determined by electrophoresis on an ABI 3730 automatic capillary sequencer (Applied Biosystems, Foster City, CA, USA) and data were analyzed by Genemapper v4.0 (Perkin Elmer Cetus Instruments, Emeryville, CA). The overall complexity within a T cell receptor Vbeta subfamily was determined by counting the number of peaks (intervals of 3 nucleotides) per subfamily. The overall spectratyping complexity (T cell receptor Vbeta score) was calculated as the sum of the numbers of peaks in the 24 subfamilies. The median T cell receptor Vbeta score in 20 age-matched volunteer blood donors was 239 (range, 179-251) peaks. Further, in order to measure the deviation from normality of a repertoire, we measured its quadratic distance to the average of 2 normalized repertoires that were each artificially created by mixing in comparable amounts the cDNAs of the PBMC of six age-matched volunteer blood donors as previously reported (32).

**T-cell receptor excision circles (TREC) assay**

Blood samples were collected on days 40, 100, 180, 365, 540 and then yearly after HCT. TREC assays were performed on samples collected on days 100, 365, 540 and then yearly after HCT as previously described. Briefly, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation and then cryopreserved. SjTREC were quantified for each sample by nested real-time PCR, as previously described (26;33). Briefly, thawed PBMC were lysed 30 min at 56°C with Tween-20 (0.05%), NP-40 (0.05%) and Proteinase K (100 µg/ml). Cell lysis was stopped by incubating 15 min at 99°C. Multiplex PCR
amplification was achieved for sjTREC together with the CD3gamma/chain, used as a housekeeping gene, with specific 3'/5' outer primers for each amplicon. Cycle conditions and primer/probe sequences have been reported elsewhere (33-35). PCR products were 10-fold diluted prior to PCR quantification using the Lightcycler™ technology. Quantitative PCR conditions were: 5 min initial denaturation at 95°C followed by 40 cycles of amplification (5 second at 95°C, 15 seconds at 60°C, 10 seconds at 72°C). Fluorescence emissions were assessed after the hybridization steps. Each PCR product was run for both sjTREC and CD3/gamma chain in two separate Lightcycler experiments. Every sample was run at least in 2 independent experiments. The results were first calculated as absolute numbers of sjTREC per 10⁵ PBMC; because each PBMC contains 2 CD3/gamma chain copies, sjTREC/10⁵ PBMC=(sjTREC/CD3gamma) x 2 10⁵. Because sjTREC are only present in lymphocytes, and because PBMC composition can be variable, sjTREC concentration in peripheral blood was computed using the formula: (sjTREC / 10⁵ PBMC x PBMC/µL) /100 where PBMC/µL=(white blood cells/µL x (%lympho + %mono))/100. Similar adjustments for calculating absolute T-cell subset counts from their frequencies in PBMC have been made previously by other groups of investigators (36). The nested character of this quantitative PCR allows high sensitivity (detection of 1 copy of sjTREC per PCR reaction). SjTRECs were also measured in 47 age-matched healthy volunteers.

Statistical analyses
The Wilcoxon matched pair test was used to compare sjTRECs concentrations and CD4⁺CD45RA⁺ T-cells at day 100 after HCT with values obtained in the same patients on days 365 and 730 after HCT. The Spearman correlation coefficient was used to analyze potential associations between lymphocyte subset counts and sjTRECs levels after HCT. The Mann-Whitney test was used to compare lymphocyte subset counts after HCT in patients < or ≥ 57 years old at HCT. To determine factors affecting the counts of CD3⁺ T-cells, CD4⁺ T-cells, CD4⁺CD45RA⁺ (naïve) T-cells, CD8⁺ T-cells, B cells, and sjTREC levels ≥1 year after HCT, multivariable linear regression models for the different MNC counts ≥1 year after HCT were fit using stepwise selection. Potential factors examined were patient age, donor age, donor type (related versus unrelated), HLA-compatibility (10/10 HLA-matched versus other), number of CD3⁺ T-cells transplanted, day post-HCT, extensive chronic GVHD at the time of analysis, limited chronic GVHD at the time of analysis, and prior (resolved, defined as discontinuation of systemic immunosuppressive therapy) extensive chronic GVHD. Logarithmic transformation of the responses was used for all models. Patients were censored at the time of graft rejection and/or disease progression. The comparison of the probability of chronic GVHD in patients with day 100 sjTREC levels above or below the median was done with the log-rank test. The probability of infection from 1 to 2 years after HCT according to 1-year sjTREC
levels was calculated using the cumulative incidence method, using death and, graft rejection and progression as competitive risks. Cox regression models were applied to fit risk of dying from infection. Results were significant at the 5% critical level ($P<0.05$). Statistical analyses were carried out with Graphpad Prism (Graphpad Software, San Diego, CA, USA) and SAS version 9.1 for Windows (SAS Institute, Cary, NC, USA).

RESULTS

Reconstitution of thymic function (sjTRECs)

Confirming previous observations by our group in a cohort of 16 patients (26), thymic function assessed by sjTREC/mL of blood increased from day 100 to 1 and 2 years after transplantation. Specifically, sjTREC concentrations were 786±1207 sjTREC/mL on day 100, 1890±4784 sjTREC/mL 1 year after HCT ($P=0.0066$ in comparison to day 100), and 4333±11131 sjTREC/mL 2 years after HCT ($P=0.0015$ in comparison to day 100 and $P=0.0422$ in comparison to 1 year after HCT), respectively. However, as shown in Figure 1 (A-D), thymic recovery did not occur in patients >60 years of age. Specifically, sjTREC concentrations increased from day 100 to 1 and 2 years after transplantation in patients ≤ 50 years old ($P=0.0245$ and $P=0.0371$, respectively), and in those 51-60 years of age ($P=0.17$ and $P=0.064$, respectively), but not in patients >60 years old ($P=0.3$ and $P=0.3$, respectively), even censored at onset of chronic GVHD. Similar results were observed when taking into consideration only patients for whom we had data on each day 100, 1 year, 2 years and 3 years after transplantation (Online Supplemental Figure 2A).

An inverse correlation was observed between sjTREC concentrations ≥1 year after HCT and recipient ($R=-0.41$, $P<0.0001$) and donor ($R=-0.25$, $P=0.0002$) ages (Figure 2A). In addition, chronic GVHD had a profound negative impact on sjTREC concentrations ≥1 year after HCT. Specifically, sjTREC concentrations were 5018±9331 sjTREC/mL in patients without chronic GVHD, 2493±7383 sjTREC/mL ($P=0.0078$) in patients with limited chronic GVHD, 1209±5029 sjTREC/mL ($P<0.0001$) in patients with extensive chronic GVHD, and 4166±12436 sjTREC/mL ($P=0.0021$) in patients with resolved extensive chronic GVHD, respectively. The same results were observed when the impact of chronic GVHD presence / severity was assessed separately 1 year, 2 years and 3 years after transplantation (Figure 2B). As shown in the supplemental figure 2 C-E, sjTREC concentrations 1 year after transplantation were not statistically affected by PBSC manipulation, donor lymphocyte infusion, or by the type of the nonmyeloablative conditioning regimen administered. Further, mean±SD sjTREC concentration 1-year after HCT were 2396±5963 sjTREC/mL in patients given unmanipulated PBSC and no MSC, versus 1303±2860 sjTREC/mL ($P=0.5$) in those given unmanipulated PBSC and MSC.

We then performed a multivariate analysis of factors affecting long term thymic function, taking together sjTREC data from each patient at any time point ≥1
year after HCT (Table 2). In multivariate analysis, high patient age (P<0.0001), extensive chronic GVHD (P<0.0001), and resolved extensive chronic GVHD (P=0.0075) were independently associated with low sjTREC concentrations (Table 2).

Reconstitution of peripheral lymphocyte subsets

**T-cells.** CD4+ T-cell counts remained below normal values during the first 540 days after HCT, then reached the 5th percentile of normal values on day 540 after HCT. This was mainly due to recovery of memory CD4+ T-cells that reached the 5th percentile of normal values on day 365 after HCT, while CD4+CD45RA+ (naïve) T-cells remained below normal values for the first 3 years after HCT (Figure 3). Recovery of lymphocyte subset counts were similar in patients < or ≥57 years old, except for CD4+CD45RA+ (naïve) T-cell recovery that was lower in older than in younger recipients. Specifically, CD4+CD45RA+ T-cell counts increased from day 100 to 1 and 2 years after transplantation in patients ≤ 50 years old (P=0.002 and P=0.02, respectively), and in those 51-60 years of age (P=0.4 and P=0.001, respectively), but less so in patients > 60 years old (P=0.3 and P=0.06, respectively), even when censored at onset of chronic GVHD (Figure 1 E-H). Similar results were observed when taking into consideration only patients for whom we had data on each day 100, 1 year, 2 years and 3 years after transplantation (Online Supplemental Figure 2B).

As reported by other investigators (37), we observed a close correlation between sjTREC concentrations and CD4+CD45RA+ T-cells counts (R=0.47, P<0.0001) ≥1 year after HCT. In contrast to what was seen in the CD4+ T-cell compartment, CD8+ T-cells normalized quickly, reaching the 5th percentile of normal values as soon as 2 months after HCT. This indicates that peripheral expansion of mature T-cells contained in the graft was more efficient for CD8+ T-cells than for CD4+ T-cells, as previously reported by other groups of investigators (15). As shown in the supplemental figure 2 C-E, CD3+ T cell and CD4+CD45RA+ T cell counts 1 year after transplantation were not statistically affected by PBSC manipulation, donor lymphocyte infusion, or by the type of the nonmyeloablative conditioning regimen administered. Further, mean ± SD CD3+ T cell counts 1-year after HCT were 1350±1409 cells/µL in patients given unmanipulated PBSC and no MSC, versus, 1867±1544 cells/µL (P=0.3) in those given unmanipulated PBSC and MSC.

**B and NK cells.** CD19+ B-cell counts reached normal values at 1 year after HCT, while the counts of NK cells reached normal values around 6 months after HCT.

**Multivariate analysis.** We then performed a multivariate analysis of factors affecting long term T and B cell immune recovery, taking together data from each patient at any time point ≥1 year after HCT (Table 2). Extensive chronic
GVHD was associated with lower counts of T-cell \((P<0.001)\), CD8\(^+\) T-cell \((P<0.001)\), CD4\(^+\) T-cell \((P<0.001)\), CD4\(^+\)CD45RA\(^+\) T-cell \((P<0.001)\), and B cell \((P=0.003)\); higher patient age was associated with lower counts of CD4\(^+\)CD45RA\(^+\) T-cell \((P<0.001)\) and B cell \((P=0.002)\); while longer delay after transplantation was associated with higher counts of CD4\(^+\) T-cell \((P<0.001)\), CD4\(^+\)CD45RA\(^+\) T-cell \((P=0.016)\), and B cell \((P<0.001)\). Further, patients given grafts containing \(< 1 \times 10^6 \) T-cells/kg had lower CD8\(^+\) T-cell \((P=0.002)\) counts.

**Correlation between thymic function and T- and B- cell phenotypes 2 years after HCT**

We then analyzed the correlation between sjTREC levels 2 years after HCT and detailed T- and B- cell phenotypes 2 years after HCT in a cohort of 33 patients for whom we had cryopreserved PBMC at that time point. There were a statistically significant correlation between 2-year sjTREC levels and patient age (inverse correlation, \(R=-0.54, P=0.001\)) as well as with 2-year counts of naïve CD4\(^+\)CD45RA\(^+\)CCR7\(^+\) T-cells (positive correlation, \(R=0.43, P=0.012\)), central memory CD4\(^+\) T-cells (positive correlation, \(R=0.38, P=0.028\)), naïve CD8\(^+\) T-cells (positive correlation, \(R=0.59, P<0.001\)), central memory CD8\(^+\) T-cells (positive correlation, \(R=0.55, P=0.001\)), effector memory CD8\(^+\) T-cells (positive correlation, \(R=0.39, P=0.025\)), naïve B cells (positive correlation, \(R=0.53, P=0.001\)), memory B cells (positive correlation, \(R=0.45, P=0.008\)), IgM\(^+\)IgD\(^+\) memory B cells (positive correlation, \(R=0.43, P=0.012\)) and switched memory B cells (positive correlation, \(R=0.45, P=0.008\)) (Figure 4 A-C).

We also analyzed the impact of patient age and extensive chronic GVHD on T- and B- cell phenotypes. Patients \(\geq 57\) years of age \((n=15)\) had lower counts of naïve CD8\(^+\) T-cells \((P=0.06)\), naïve B cells \((P=0.02)\), memory B cells \((P=0.06)\) and switched memory B cells \((P=0.006)\) than those \(< 57\) years of age at HCT \((n=18)\) (Figure 4 D-F). Finally, patients who had extensive chronic GVHD 2 years after HCT had lower counts of effector memory CD8\(^+\) T-cells \((P=0.04)\), effector memory RA\(^+\) CD8\(^+\) T-cells \((P=0.04)\), naïve B cells \((P=0.002)\), memory B cells \((P=0.04)\), and switched memory B cells \((P=0.07)\) than those who did not (Figure 4 G-I).

**Correlation between thymic function and T-cell Vbeta repertoire diversity**

In order to assess the impact of thymic function on Vbeta repertoire diversity, T cell receptor Vbeta repertoire CDR3 spectratyping analyses were performed between 2 and 9 (median 3) years after HCT in 21 patients with low \((<500 \text{ sjTREC/mL}; \, n=9)\) or high \((>500 \text{ sjTREC/mL}; \, n=12)\) sjTREC levels, measured on the same day as spectratyping analysis. As illustrated in supplemental figure 1, the Vbeta repertoire was relatively complex even in patients with low thymic function, probably reflecting that peripheral expansion of T-cells contained in the graft eventually allowed a relatively diverse T cell receptor Vbeta repertoire, although some oligoclonal T cell receptor Vbeta families were
observed, and particularly so in patients with low sjTREC levels. Specifically, median T cell receptor Vbeta score was 239 (range, 179-251) peaks in age-matched controls, 207 (range, 173-246) peaks in patients with low sjTREC levels ($P=0.038$ in comparison to controls), and 218 (range, 182-252) in patients with high sjTREC levels (NS in comparison to controls and to patients with low sjTREC). Further, we quantified the deviation from normality of each repertoire by measuring its quadratic distance to an average repertoire measured in healthy donors (see material and method section)(32). The quadratic distance was 4.5 (range, 3.2-6.5) in age-matched controls, 8.1 (range, 3.8-11.3) in patients with low sjTREC levels ($P=0.003$ in comparison to controls), and 5.3 (range, 4.5-9.1) in patients with high sjTREC levels ($P=0.016$ in comparison to controls, $P=0.22$ in comparison to patients with low sjTREC).

**Correlation between thymic function and clinical events**

Day 100 sjTREC levels were similar in patients who had grade II-IV acute GVHD before day 100, as compared to those who did not (687±947 vs. 824±1303 sj TREC/mL, $P=0.5$). Day 100 sjTREC levels was not associated with the occurrence of chronic GVHD: the 1-year probability of chronic GVHD was 57% in patients with day 100 sjTREC levels below the median (323 sjTREC/mL), versus 52% in those with higher sjTREC levels on day 100 ($P=0.5$).

We then studied infections occurring between day 365 to 730 based on day 365 sjTREC levels. The probabilities of bacterial, fungal and viral infections were 40%, 9%, and 13%, respectively, in patients with day 365 sjTREC levels below the median (523 sjTREC/mL), versus 31% ($P=0.3$), 5% ($P=0.6$) and 17% ($P=0.6$), respectively, in those with higher levels. The 7-year probability of dying from infection was 40% in patients with day 365 sjTREC levels below the median, versus 0% in those with higher values ($P=0.01$, Figure 2C). However, 6 of 7 patients who died from infections had extensive chronic GVHD at the time of death. Further, the association between 1-year sjTREC levels and the subsequent risk of dying from infection was no longer statistically significant ($P=0.17$) after adjustment for the presence of extensive chronic GVHD 1 year after HCT in a Cox model where sjTREC levels were modeled as a continuous linear variable.
DISCUSSION

Late infections (with or without chronic GVHD) are the first causes of nonrelapse death beyond 1 year after HCT in young patients given myeloablative conditioning (38;39). They have been attributed to slow recovery of CD4+ T-cells (38;40). Nevertheless, despite the well established association between higher patient age and impaired immune recovery following allogeneic HCT (14), HCT after nonmyeloablative conditioning has been successful treatment for many patients aged up to between 60 and 70 years of age, most of whom without evidence of severe infection once systemic immunosuppression was discontinued (31;41). This apparent paradox prompted us to assess long term immune recovery after nonmyeloablative conditioning. Several observations were made.

First and most importantly, while patients below 50 years of age at the time of HCT had evidence for thymic recovery from day 100 to 3 years after HCT, thymic recovery was much slower in patients aged 50 to 60 years, and was virtually absent in patients above 60, even in those without chronic GVHD. These results mirror those reported by Hakim et al. in patients given autologous HCT where the authors observed a significant thymic rebound in approximately 80% of patients younger than 40 years, 45% of those aged 40 to 50 years, but in < 15% of those ≥ 50 years old (42).

Recovery of naïve CD4 T-cells after HCT in current patients somewhat mirrored recovery of thymic function assessed by sjTREC levels. Specifically, recovery of naïve T-cells was observed by day 365 in patients below 50 years of age, but only at 2 years after HCT (and to a lesser extend) in patients above 50-60 years of age. Further, sjTREC levels correlated with those of CD4+CD45RA+ T-cells 1 year onwards after HCT, and we observed a strong correlation between sjTREC levels and naïve CD4+CD45RA+CCR7+ T-cell and naïve CD8+ T-cell counts 2 years after HCT. These observations are in agreement with previous reports showing that new naïve T-cells originate mainly from the thymic pathway after HCT(43;44).

Given the slow or absent thymic recovery in patients above 50 or 60 years respectively, immune recovery in those patients should have depended mainly on peripheral expansion of mature T-cells contained in the graft. Consistent with this possibility, recovery of CD8+ T-cells occurred earlier than recovery of CD4+ T-cells, and T-cell recovery was derived from memory rather than naïve T-cells (15). Interestingly, peripheral expansion was sufficient to produce an efficient immune system in the majority of patients without GVHD. Indeed, TCRB repertoires were relatively complex both in patients with low compared to high sjTREC levels, and incidences of viral, bacterial and fungal infections were similar in patients with low or high sjTREC levels. The risk of death from infection was significantly higher in patients with low sjTREC levels, although all but one patients who died from infection had extensive severe chronic GVHD at the time of death suggesting that GVHD or GVHD treatment might as
well had played an important role in the risk of mortality from infection in the current study. The observation that late immunity in older patients mainly depends on mature T-cells contained in the graft has important implications since it suggests that long term immune function after HCT might be affected by techniques of in vitro or in vivo T-cell depletion of the graft in these patients. Analysis of long term immune recovery in older patients given PBSC after alemtuzumab-based reduced-intensity conditioning (45) would be particularly interesting to confirm this hypothesis.

Another observation of our study was the dramatic impact of chronic GVHD on immune recovery. Specifically, extensive chronic GVHD was significantly associated with low sjTREC levels, as well as low counts of CD4+ T-cells, CD4+CD45RA+ (naïve) T-cells, CD8+ T-cells and B cells ≥ 1 year after HCT, and was significantly associated with lower counts of effector memory CD8+ T-cells, effector memory RA+ CD8+ T-cells, naïve B cell and memory B cells 2 years after HCT. The negative impact of chronic GVHD on immune recovery is probably multi-factorial (46). First, the negative impact of chronic GVHD on thymus architecture (46) and function (47-52) has been well demonstrated, and has been attributed to direct donor T-cell alloreactivity towards recipient thymus (53), and to a negative impact of immunosuppressive drugs (and particularly of glucocorticosteroids) on the thymus (54;55). Secondly, chronic GVHD has been shown to impair peripheral expansion of mature T-cells, and the same is obviously true for immunosuppressive drugs given in the treatment of patients with chronic GVHD. Thirdly, chronic GVHD has been associated with reduced B cell lymphopoiesis perhaps because of B cell inhibitory cytokines produced by activated T-cells in chronic GVHD patients or because of GVHD treatments (56).

In summary, our data suggest that thymic neo-generation of T-cells occurs from day 100 onwards in patients under 60. In contrast, the levels of sjTREC remain low for patients above 60 who depended nearly exclusively on peripheral expansion of mature donor T-cells contained in the graft to reconstitute their T-cell compartment. This suggests that long-term immune function after HCT might be negatively impacted by techniques of in vitro or in vivo T-cell depletion of the graft in older patients given allogeneic HCT.

ACKNOWLEDGMENTS
We thank Y. Henrotin and M. Mathy for helpful discussions about PCR analyses, V. Dhennin from the Genotranscriptomics Platform of the GIGA, for help with T-cell spectratyping, and S. Ormenese from the Imaging and Flow Cytometry Platform of the GIGA for help with flow cytometry analyses. The authors are also grateful to O. Dengis and C. Daulne for excellent technical assistance, to physicians and clinical staff for their dedicated care of the patients, and to N. Schaaf-Lafontaine from the laboratory of Hematology.
AUTHORSHIP AND DISCLOSURES
Study design: EC, VG, BMS, RS, YB, FB. Statistical analyses: LS. Analyses of the data: EC, MH, LS, FB. Flow-cytometry analyses: SHB, AG. sjTREC analyses: EC, MH, RC, JD. TCVBR analyses: EC, MH, RC, JFV. Care of transplanted patients: EW, YB, FB. Editing of the manuscript: EC, MH, RC, SHB, LS, AG, VG, BMS, RS, YB. Writing of the manuscript: FB. The authors reported no potential conflicts of interest other than having received public funding for the research.

REFERENCES


(51) Bahceci E, Epperson D, Douek DC, Melenhorse JJ, Chilid RC, Barrett AJ. Early reconstitution of the T-cell repertoire after non-myeloablative peripheral blood stem cell transplantation is from post-thymic T-cell expansion and is unaffected by graft-versus-host disease or mixed chimaerism. Br J Haematol. 2003;122(6):934-43.


Table 1. Patients.

<table>
<thead>
<tr>
<th>Category</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=80</td>
<td></td>
</tr>
<tr>
<td>Recipient median (range) age, years</td>
<td>57 (10-71)</td>
</tr>
<tr>
<td>Recipient sex: male / female</td>
<td>57 / 23</td>
</tr>
<tr>
<td>Donor median (range) age, years</td>
<td>42 (18-70)</td>
</tr>
<tr>
<td>Donor sex: male / female</td>
<td>48 / 32</td>
</tr>
<tr>
<td>Donor type</td>
<td></td>
</tr>
<tr>
<td>HLA-identical sibling</td>
<td>33</td>
</tr>
<tr>
<td>1 Ag mismatched related donor</td>
<td>2</td>
</tr>
<tr>
<td>10/10 HLA allele matched unrelated donor</td>
<td>22</td>
</tr>
<tr>
<td>HLA-mismatched unrelated donor</td>
<td>23</td>
</tr>
<tr>
<td>≥ 1 single HLA-allele mismatch</td>
<td>5</td>
</tr>
<tr>
<td>≥ 1 single HLA-allele mismatch</td>
<td>18</td>
</tr>
<tr>
<td>Diagnosis, no. patients</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>9</td>
</tr>
<tr>
<td>Myelodysplastic syndrome or myeloproliferative disorder</td>
<td>13</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>1</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>5</td>
</tr>
<tr>
<td>High-grade non Hodgkin lymphoma</td>
<td>9</td>
</tr>
<tr>
<td>Low-grade non Hodgkin lymphoma</td>
<td>12</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>4</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>8</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>18</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Conditioning regimen</td>
<td></td>
</tr>
<tr>
<td>2 Gy TBI</td>
<td>20</td>
</tr>
<tr>
<td>2 Gy TBI + fludarabine (90 mg/m²)</td>
<td>46</td>
</tr>
<tr>
<td>4 Gy TBI + fludarabine (90 mg/m²)</td>
<td>6</td>
</tr>
<tr>
<td>Fludarabine (90 mg/m²) + cyclophosphamide (3 g/m²)</td>
<td>8</td>
</tr>
<tr>
<td>Stem cell source</td>
<td></td>
</tr>
<tr>
<td>Unmanipulated PBSC</td>
<td>56</td>
</tr>
<tr>
<td>CD8-depleted PBSC</td>
<td>19</td>
</tr>
<tr>
<td>CD34-selected PBSC</td>
<td>5</td>
</tr>
<tr>
<td>Median (range) no. of cells infused (x 10⁶/kg)</td>
<td></td>
</tr>
<tr>
<td>CD34⁺ cells</td>
<td>4.5 (0.8-14.1)</td>
</tr>
<tr>
<td>CD3⁺ T-cells</td>
<td>255 (0.04-1216)</td>
</tr>
<tr>
<td>Acute GVHD, no. patients</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>17</td>
</tr>
<tr>
<td>Grade III</td>
<td>3</td>
</tr>
<tr>
<td>Grade IV</td>
<td>0</td>
</tr>
<tr>
<td>Chronic GVHD, no. patients</td>
<td></td>
</tr>
<tr>
<td>Limited</td>
<td>10</td>
</tr>
<tr>
<td>Extensive</td>
<td>34</td>
</tr>
</tbody>
</table>
Table 2. Multivariable analyses of factors affecting immune recovery ≥ 1 year after nonmyeloablative conditioning (n=242)*.

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Factor(s) associated with lower levels / cell subset counts</th>
</tr>
</thead>
</table>
| sjTREC concentration     | - high patient age† ($P<0.0001$).  
                            | - extensive chronic GVHD ($P<0.0001$).  
                            | - antecedent of extensive chronic GVHD ($P=0.0075$). |
| # of T-cells             | - extensive chronic GVHD ($P<0.0001$).  
                            | - related donor ($P=0.0353$). |
| # of CD4+ T-cells        | - extensive chronic GVHD ($P=0.0007$).  
                            | - shorter delay after HCT† ($P<0.0001$). |
| # of naïve CD4+ T-cells  | - extensive chronic GVHD ($P<0.0001$).  
                            | - high patient age† ($P=0.0004$).  
                            | - shorter delay after HCT† ($P=0.016$). |
| # of CD8+ T-cells        | - extensive chronic GVHD ($P=0.0001$).  
                            | - # CD3+ T-cells transplanted < 1x10⁶/kg ($P=0.0018$).  
                            | - absence of limited chronic GVHD ($P=0.008$). |
| # of B cells             | - extensive chronic GVHD ($P=0.003$).  
                            | - high patient age† ($P=0.0017$).  
                            | - shorter delay after HCT† ($P=0.0006$). |

*Other factors assessed were donor age, HLA-mismatch between donor and recipient or not; † modeled as a continuous linear variable; GVHD, graft-versus-host disease.
**Figure 1.** Impact of patient age (at HCT) on sjTREC (A-D) and naïve CD4 T-cell (E-H) recovery. In graphs D and H, patients were censured at time of occurrence of chronic GVHD. P values are given for comparison with day 100 values.

**Figure 2.** (A) Correlation between patient age at HCT and sjTREC levels ≥ 1 year after HCT. (B) Impact of chronic GVHD on sjTREC concentration (mean ± standard deviation) 1-3 years after HCT. Black bars, no chronic GVHD; white bars, limited chronic GVHD; black and white bars, extensive chronic GVHD; grey bars, resolved extensive chronic GVHD. P values (obtained with the Mann Whitney test) are given in comparison to patients without chronic GVHD. (C) Cumulative incidence of dying from infection in patients with sjTREC levels on day 365 below (continuous line) or above (broken line) the median.

**Figure 3.** Mean MNC-subset cell counts after nonmyeloablative conditioning in patients < or ≥ 57 years old at HCT (A-F). Horizontal lines show the 5th and 95th percentiles in 47 age-matched healthy volunteer donors. *, P<0.05.

**Figure 4.** CD4⁺ T-cell (A), CD8⁺ T-cell (B) and B cell (C) phenotypes 2 years after HCT in patients with sjTREC levels below (white bars) or above (black bars) median (1000 sjTREC/μL) at that time (n=33). CD4⁺ T-cell (D), CD8⁺ T-cell (E) and B cell (F) subtype phenotypes 2 years after HCT in patients < (white bars) or ≥ (black bars) 57 years of age at HCT. CD4⁺ T-cell (G), CD8⁺ T-cell (H) and B cell (I) subtype phenotypes 2 years after HCT in patients without (white bars) or with (black bars) extensive chronic GVHD at that time. TCM, central memory T-cells; TEM, effector memory T-cells; TEM RA⁺, TEM RA⁺ CD8⁺ T-cells; M., memory; M.IgM⁺IgD⁺ IgM⁺IgD⁺ memory B cells; M.\(^{\text{Switched}}\), switched memory B cells.
Supplemental Figure 1. Representative examples of TCRB repertoires in age-matched controls and in patients with low, intermediate or high sjTREC levels 2 to 9 years after nonmyeloablative HCT.

Supplemental Figure 2. Evolution of sjTREC levels (A) and of naive CD4⁺ T-cell counts (B) in patients for which we had data on day 100 and 1, 2 and 3 years after transplantation. The numbers indicate patient age at transplantation. Impact of graft manipulation (C), of pre-emptive donor lymphocyte infusion (D) or on conditioning intensity on sjTREC levels and CD3⁺ T-cell and naive CD4⁺ T cell counts after transplantation. DLI, donor lymphocyte infusion; 2 Gy TBI, 2 Gy total body irradiation with or without added fludarabine; 4 Gy TBI, 4 Gy TBI with fludarabine; FluCy, Fludarabine with cyclophosphamide.