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Original article

No recovery of T-cell receptor excision circles (TRECs) after non-myeloablative allogeneic hematopoietic stem cell transplantation is correlated with the onset of GvHD

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Abstract. Improper T-cell reconstitution with its consequences, graft-vs-host disease (GvHD) and outbreak of viral infections, is the major cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT). To determine the factors affecting reconstitution of naive T-cells after non-myeloablative HSCT (NM-HSCT), the T-cell receptor excision circle (TREC) content was measured on a weekly basis in 24 transplanted patients with various malignant diseases. We analysed correlations of the results with the development of GvHD. In addition, in 11 chronic myeloid leukaemia (CML) patients, we correlated TREC and BCR-ABL transcript numbers. After HSCT, in most patients (22/24) TRECs became undetectable. In 12 patients, TRECs reappeared 3–4 months after HSCT, in 1 patient TRECs reappeared 5 months after HSCT, and in 11 patients TRECs remained negative for more than a year. All 11 patients who remained TREC-negative, developed acute GvHD grade 2–3, while only 6 out of 13 patients who recovered TRECs developed GvHD. We show that after non-myeloablative HSCT, thymopoiesis takes place and is affected by GvHD. Our results indicate that no recovery of TRECs after NM-HSCT (which most likely reflect the expansion of host-reactive co-transplanted mature T-cells) correlates with the onset of GvHD.

Keywords: bcr-abl, chimerism, GvHD, HSCT, TREC.

Introduction

Reconstitution of the donor T-cell repertoire after hematopoietic stem cell transplantation (HSCT) is essential for successful transplantation, but is still poorly understood. Improper T-cell reconstitution is the major cause of morbidity and mortality after HSCT. Delayed or impaired T-cell recovery favours the outbreak of viral infections, while the expansion of host-reactive T-cells leads to the development of the graft-vs-host disease (GvHD). Until recently, immune reconstitution was monitored by immunophenotypic analysis of the T-cell subsets recovering after HSCT. It was shown that the number of total helper T-cells (CD4+), and particularly their naive subset (CD4+/CD45RA+), correlates inversely with the occurrence of opportunistic infections (Small et al. 1999; Kalwak et al. 2002). The donor T-cell pool in HSCT recipients consists of T-cells that differentiate *de novo* from stem cells and co-transplanted mature T-cells. These two T-cell subsets undergo the positive and negative selection in various antigenic environments; therefore they differ in antigenic specificity. To date no method is available to clearly distinguish between these subsets of donor T-cells, although the recent method for determination of proliferative history of T-cells, based on their T-cell receptor excision circle (TREC) content, might indirectly provide the information about the origin of T-cells appearing after HSCT. In the majority of thymocytes differentiating into

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αβ T-cells, the T-cell receptor δ gene (*TCRD)* is deleted by the rearrangement between the δRec and the ψJα gene segments (de Villartay et al. 1988; Breit et al. 1994; Verschuren et al. 1997). Thus, TCR excision circles (TRECs) are generated, which are stable circular episomes and are not replicating during mitosis. TRECs are diluted out with each cell division, therefore measuring TREC content enables identification of recent thymic emigrants (RTE) (Douek et al. 1998; Kong et al. 1998; Kong et al. 1999). It was shown that the TREC content of peripheral T-cells decreases with age (Douek et al. 1998; Zhang et al. 1999). After myeloablative HSCT, TRECs become undetectable in peripheral blood, but in the majority of patients TRECs reappear 3–6 months after transplantation, while in some patients TRECs remain negative (Hochberg et al. 2001; Savage et al. 2001). To determine the factors affecting the reconstitution of naive T-cells after HSCT, we measured the TREC content on a weekly basis in 24 transplanted patients with various malignant diseases and correlated the results with the development of GvHD. In addition, in 11 CML patients we correlated TREC and BCR-ABL transcript numbers. We observed a correlation between TREC recovery after HSCT and the development of GvHD.

Materials and methods

Blood samples

Peripheral blood samples were obtained from 24 patients who underwent non-myeloablative allogeneic HSCT (NM-HSCT). The study group included 11 patients with chronic myeloid leukaemia (CML), 5 with acute myeloid leukaemia (AML), 4 with Hodgkin disease (HD), 2 with non-Hodgkin lymphoma (NHL), and 2 with germ cell tumours (GCT). Pre-transplantation conditioning and transplantation were carried out according to standard procedures, including treatment with fludarabine 180 mg m^{2-1}, busulfan 8 mg kg^{-1}, and antithymocyte globulin 40 mg kg^{-1} (Slavin et al. 1998; Kreuzer et al. 2002).DNA and RNA were extracted from the leukocyte pellet by using the QIAmp DNA or RNA Mini Kits (Qiagen, Hilden, Germany).

Haematopoietic chimerism

Donor chimerism was quantified in peripheral blood leukocytes, as described recently (Thiede et al. 1999; Kreuzer et al. 2002). Briefly, 9 short tandem repeat markers (STRs) and the amelogenin locus were amplified with fluorescence-labelled primer molecules in a multiplex PCR reaction, by using 1.25 ng of DNA (Profiler Kit, Applied Biosystems, Weiterstadt, Germany). Separation and detection of PCR products was performed on an ABI Prism 377XL Automated Sequencer (Applied Biosystems) with subsequent analysis by GeneScan Analysis software 3.1. Calculation of the percentage of donor haematopoiesis was based on the calculation of the area of the donor-derived signals, as compared to the sum of the area of donor and recipient signals for each informative (i.e. different between donor and recipient) STR allele.

Quantification of BCR-ABL transcripts by real-time PCR

Real-time quantitative PCR (RQ-PCR) was performed as described previously (Thiede et al. 1999; Kreuzer et al. 2002). The numbers of BCR-ABL transcripts were normalized by calculating the ratio between BCR-ABL and β-actin transcript numbers. For negative RQ-PCR results, the ratio was defined to be 10^{-8}.

Quantification of TRECs by real-time PCR

The number of TRECs was determined by RQ-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). In this PCR, binding of a specific probe, containing a quencher (TAMRA) and a reporter (6FAM) dye, to the amplification products, results in Taq DNA polymerase-mediated cleavage of the probe. This leads to separation of the quencher from the reporter, thereby inducing fluorescence of the reporter dye. The amount of the target (TREC) in analysed samples is assessed by measuring the threshold cycle (C_T), defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline, and by using the standard curve to determine starting copy number. To determine precisely the percentage of cells carrying TREC, a duplex vector was constructed, containing a fragment of the δRec-ψJα signal joint (TREC) and a fragment of the recombinase-activating gene (*RAG2*), used as a reference. The *RAG2* gene was cloned first in the T-A acceptor site and subsequently TREC was cloned into the EcoRV restriction site of the TOPO TA Vector (Invitrogen;

Groningen, The Netherlands). Basing on the DNA concentration, measured by spectrophotometry and confirmed by quantitative gel electrophoresis, standard dilutions of the vector from 10^7 to 10^1 copies were prepared. In brief, PCR of a 50-µL total volume was performed with approximately 100 ng of genomic DNA, 25 pmol of each primer, 10 nmol of each dNTP (Perkin Elmer Cetus, Norwalk, CT), 1.25 U Platinium® Taq polymerase (Life Technologies; Karlsruhe, Germany), 5 pmol of 6FAM-TAMRA probe, and PCR buffer including 4.5 mM $MgCl_2$ (Life Technologies). After the initial denaturation at 95°C for 5 min, 45 cycles consisting of 95°C for 30 s and 66°C for 30 s were performed. For TREC analysis, the 5' primer ψJα(–258): AAC AGC CTT TGG GAC ACT ATC G and the 3' primer δRecsj(+104): 5'- GCT GAA CTT ATT GCA ACT CGT GAG, amplifying the signal joint sequence generated by the δRec-ψJα rearrangement, were used together with the TREC probe: 5'- 6FAM-CCA CAT CCC TTT CAA CCA TGC TGA CAC CTC-TAMRA. For *RAG2* analysis, the 5' primer: RAG2(2160) GCA ACA TGG GAA ATG GAA CTG, the 3' primer: RAG2(2404) GGT GTC AAA TTC ATC ATC ACC ATC, and the *RAG2* probe: 6FAM-CCC CTG GAT CTT CTG TTG ATG TTT GAC TGT TTG TGA-TAMRA were used.

Results

Donor/recipient leukocyte chimerism

Donor/recipient chimerism was determined in leukocytes from 24 transplanted patients on a weekly basis after HSCT for up to 2 years (Table 1); data on chimerism in 10 CML patients, included in this study, was published elsewhere (Kreuzer et al. 2002). Twenty patients became full chimeras (95–100%) after 20–40 days, one patient achieved full chimerism after 150 days, and 3 patients showed persisting mixed chimerism.

Table 1. Patient characteristics

Patient	Age/sex	AGvH	CGvH	TREC	BCR-ABL	Chimerism	Survival (m)
551 CML	44/F	+	+	+	–	complete	8
728 CML	51/M	+	+	–	–	complete	7
792 CML	56/M	+	+	–	+	mixed	8
800 CML	46/M	+	+	+	–	complete	>25
811 CML	54/M	+	+	–	–	complete	>23
837 CML	50/M	+	–	+	–	complete	>20
846 CML	59/M	+	+	–	–	complete	>18
848 CML	55/M	+	–	–	–	complete	3
860 CML	56/F	–	–	+	–	complete	>15
866 CML	62/F	–	–	1–5 m - >5 m +	+ –	mixed complete	>9
873 CML	37/F	–	–	+	+	mixed	>6
724 AML	35/F	+	+	–		complete	17
787 AML	38/F	–	–	+		complete	>20
799 AML	39/F	–	–	+		complete	>11
841 AML	63/M	+	+	+		complete	5
857 AML	60/M	+	+	–		mixed	>12
863 AML	60/M	–	–	+		complete	>4
775 HD	39/F	+	+	–		complete	8
806 HD	61/M	+	+	–		complete	8
856 HD	43/F	–	–	+		complete	>4
685 NHL	49/M	+	–	+		complete	12
817 NHL	36/M	+	+	–		complete	>16
696 TT	20/M	+	+	–		complete	7
698 TT	30/M	+	+	+		complete	5

AML = acute myeloid leukemia; BCR-ABL = transcript of the BCR-ABL fusion gene; CML = chronic myeloid leukemia; aGvH = acute graft vs host disease; cGvH = chronic graft vs host disease; HD = Hodgkin disease; NHL = non-Hodgkin lymphoma; TREC = T cell receptor excision circles; TT = testis tumour

Thymic output after allogeneic non-myeloablative HSCT

In order to measure the thymic output after allogeneic NM-HSCT in 24 patients, TREC counts were quantified by using real-time PCR. To determine more accurately the TREC counts, a duplex vector was prepared, containing both the target (TREC) and the reference gene (*RAG2*). Since the same vector dilutions were used to obtain the target and the reference standard curve, the absolute numbers of TRECs were not influenced by the errors in DNA quantification, preparation of dilution series, and variation of vector stability. Prior to HSCT, all patients showed markedly lower TREC counts, compared to healthy individuals (Figure 1), most likely due to chemotherapy. After HSCT, in most patients (22/24), TRECs became undetectable. In 12 patients, TRECs reappeared 3–4 months after HSCT, in 1 patient TRECs reappeared 5 months after HSCT, and in 11 patients TRECs remained negative for more than a year. All 11 patients who remained TREC-negative developed acute GvHD grade 2–3, while only 6 out of 13 patients who recovered TRECs, developed GvHD ($P = 0.006$) (Table 1).

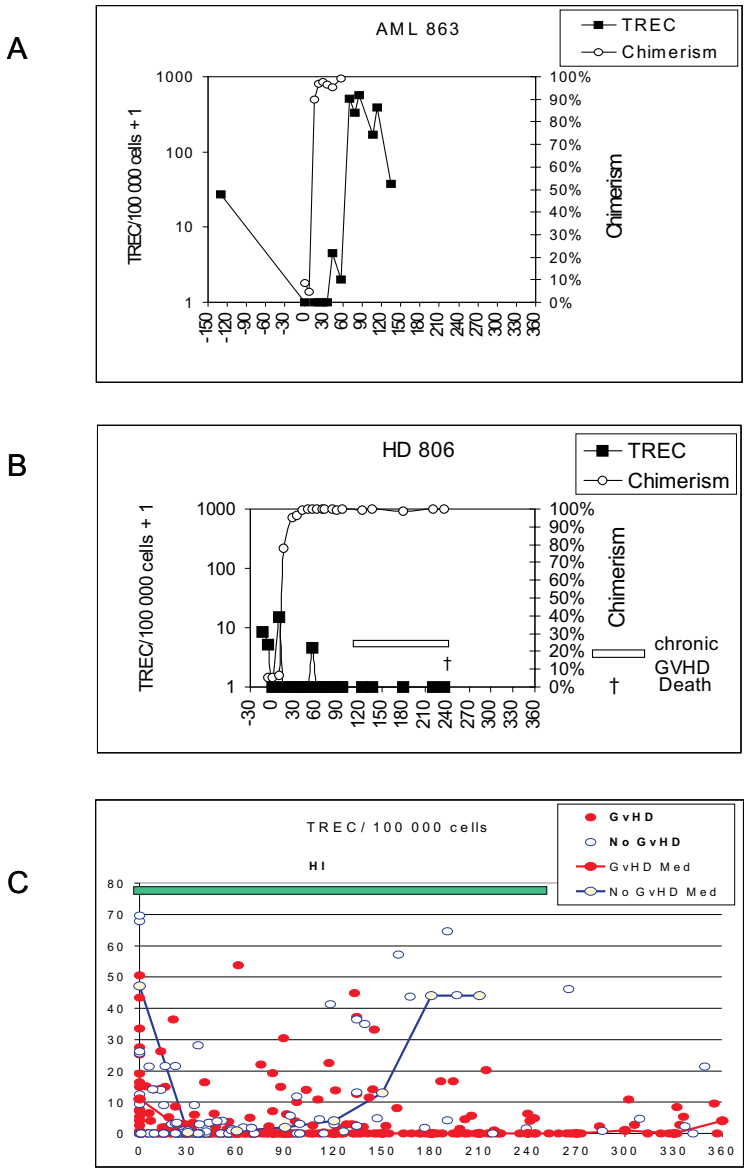

Figure 1. TRECs and chimerism kinetics after HSCT. (A) AML patient without GvHD – TRECs recovered 80 days after the engraftment. (B) Hodgkin disease patient (HD) developing chronic GvHD – TRECs did not recover despite a successful engraftment. (C) Summarized TREC counts after HSCT. Solid circles = patients who developed GvHD; hollow circles = patients without GvHD. Lines connect median TREC counts in consecutive months after HSCT: dotted line = patients without GvHD; continuous line = patients with GvHD. HI = median TREC values in healthy individuals.

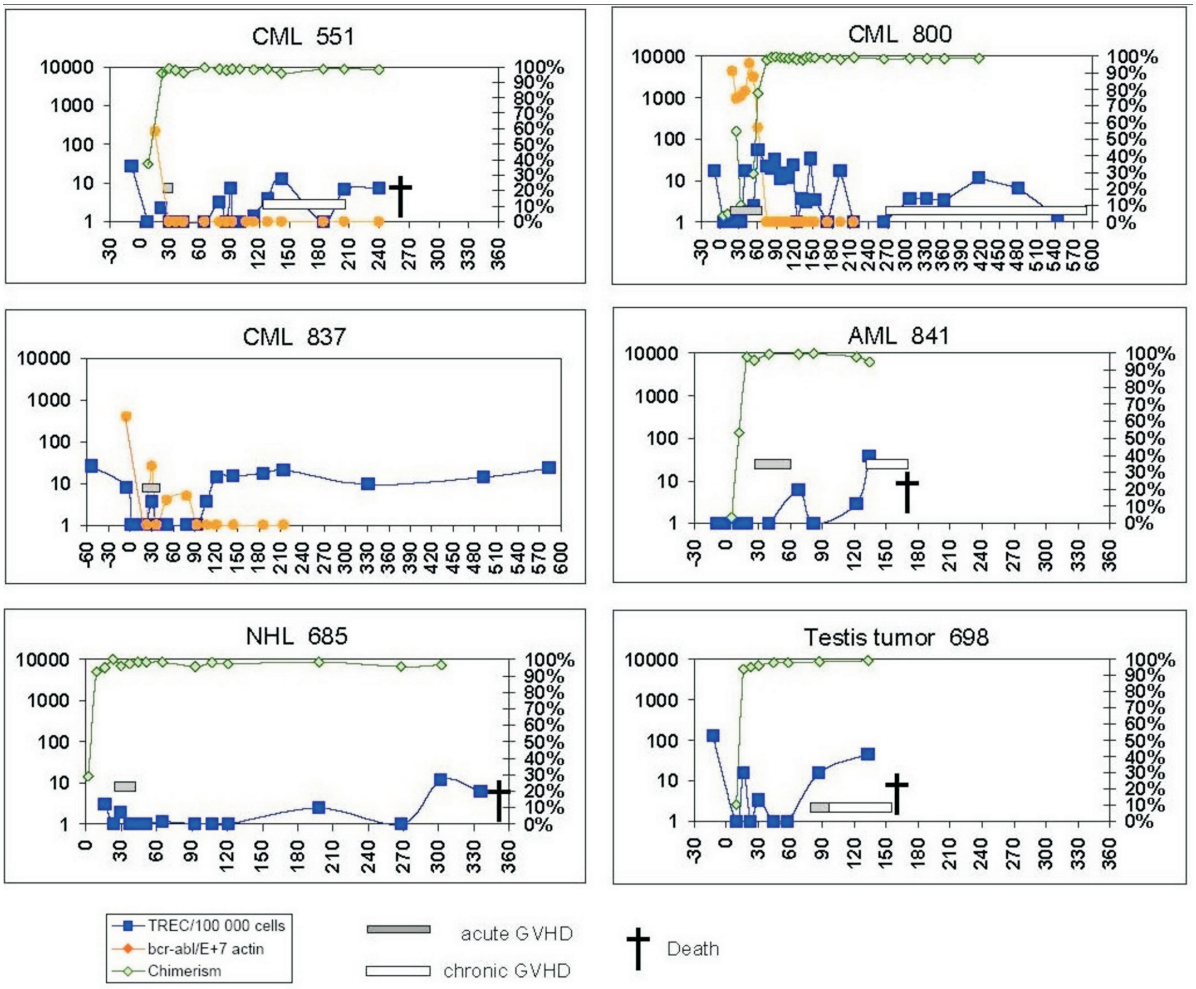

Figure 2. TRECs, BCR-ABL and chimerism kinetics in GvHD patients who recovered TRECs after HSCT. Left Y axis = TREC copy number/10^5 cells +1 (logarithmic scale); right Y axis = chimerism; X axis = days after transplantation. The higher the GvHD bar over the X axis, the more severe the disease: 10% level on the right Y axis = grade 1; 20% =

Out of the 6 patients who developed GvHD despite recovering TRECs, only 4 patients showed persistent chronic GvHD and 2 patients (685 NHL and 837 CML) presented only a temporary (20-day-long) acute GvHD episode shortly after transplantation (Figure 2).

Discussion

GvHD is one of the major causes of morbidity and mortality after HSCT. T-cells play the major role in the development of GvHD. Although host-reactive T-cell clones bearing specific *TCRB* CDR3 sequences have been identified (Michalek et al. 2003a; Michalek et al. 2003b), their origin has not been clarified yet. Donor T-cell pool is in part derived from the mature donor T-cells, which differentiated in the donor and just proliferated in the recipient (donor-selected T-cells; ds-T-cells) and in part from the donor stem cells that differentiated into T-cells in the recipient (recipient-selected T-cells; rs-T-cells) (Figure 3). Since these 2 cell populations undergo the positive and the negative selection in completely different antigenic environments, they differ in antigenic specificity. The most important difference is that in rs-T-cells the self-reactive T-cells are eliminated during negative selection in the thymus of the recipient, therefore the rs-T-cells should not cause GvHD. In addition, the ds-T-cells have a much longer proliferative history than the newly generated rs-T-cells, and therefore should have much lower TREC counts.

Besides conventional myeloablative HSCT with conditioning regimens including high doses of chemotherapy, more recently non-myeloablative HSCT (mini-transplantation) with dose-reduced fludarabine-based conditioning became a new treatment option, especially for older or medically

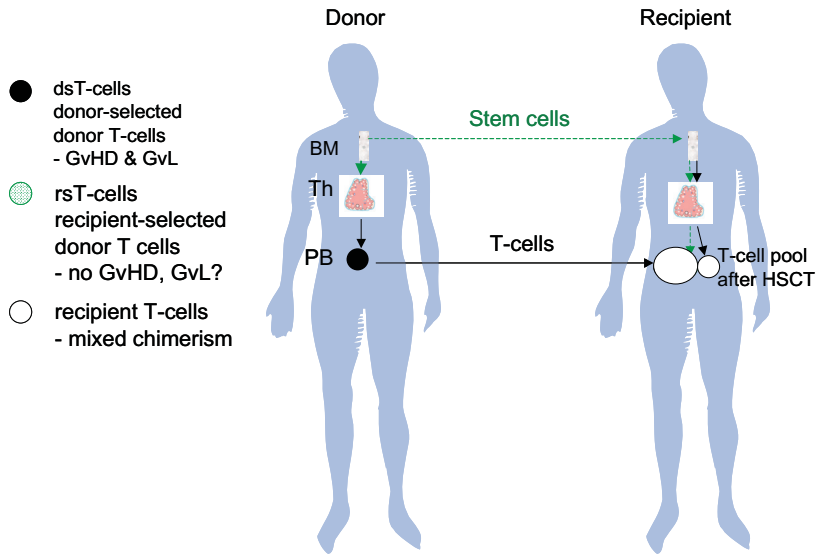

Figure 3. Model of T-cell reconstitution after HSCT. BM = bone marrow, Th = thymus, PB = peripheral blood.

infirm patients (Slavin et al. 1998). This approach shifts tumour eradication from the cytotoxic effect of chemotherapeutic agents to the graft-vs-host immune response directed against antigens expressed on tumour cells. Due to lower toxicity, NM-HSCT results in a lower treatment-related morbidity and mortality, but still GvHD remains a long-term life-threatening complication.

Lower TREC levels were reported in myeloablative HSCT recipients developing GvHD (Weinberg et al. 2001; Storek et al. 2002; Fallen et al. 2003), but no effect of GvHD on TREC was found in patients after non-myeloablative HSCT (Bahceci et al. 2003). Bahceci's research team measured the highest TREC counts 2 weeks after HSCT and then observed a gradual decrease in TREC numbers up to 6 months after HSCT, indicating that T-cell reconstitution was rather due to post-thymic T-cell expansion than to thymopoiesis. Since GvHD is believed to influence the TREC counts by suppressing thymopoiesis, due to the lack of thymopoiesis no effect of GvHD on TRECs could be observed. In contrast, in our study, after an initial drop to undetectable levels, we observed an increase in TREC counts, starting 2–3 months after HSCT and reaching the plateau 6 months after HSCT, what indicated an ongoing thymic output. Furthermore, we showed that similarly to myeloablative HSCT, the occurrence of GvHD also correlated with undetectable TREC counts in NM-HSCT recipients. Undetectable TRECs in patients developing GvHD indicate that the host-reactive cells are derived from the expanded co-transplanted mature donor cells (ds-T-cells). Although in both studies non-myeloablative conditioning was done, the regimen used in Bahceci et al.'s (2003) study (fludarabine 125 mg m^{2-1} and cyclophosphamide 120 mg kg^{-1}) was milder than in our study (fludarabine 180 mg m^{2-1}, busulfan 8 mg kg^{-1}, and antithymocyte globulin 40 mg kg^{-1}). This might explain why Bahceci et al. (2003) detected TRECs after transplantation (most likely from the remaining host T-cells; they reported mixed chimerism) whereas we did not (early complete chimerism). Whether the discrepancies were indeed due to different pre-transplantation conditioning or due to other factors, remains to be determined.

We also compared TREC numbers and BCR-ABL transcript levels in 11 patients with CML. Two out of 3 patients who remained BCR-ABL-positive after HSCT were TREC-negative. In contrast, most of BCR- ABL-negative patients (5 out of 8) recovered TREC. There seems to be an inverse correlation between TREC and BCR-ABL transcript numbers. However, the number of patients analysed is too small to draw a definitive conclusion.

Conclusions

We showed that after non-myeloablative HSCT, at least when using the regime described by Slavin et al. (1998), thymopoiesis takes place and is affected by GvHD. Basing on our results, we propose a new model of T-cell reconstitution after HSCT, shown in Figure 3. We hypothesize that if the T-cell repertoire is reconstituted *de novo* from

the donor stem cells, which differentiated in the recipient (rs-T-cell), the risk of developing GvHD is relatively low. In contrast, T-cell reconstitution based on the expansion of the co-transplanted mature T-cells, which differentiated in the donor (ds-T-cells), leads to the development of GvHD. Further studies are needed to identify factors promoting the T-cell reconstitution from the newly differentiated T-cells, rather than from the co-transplanted mature T-lymphocytes, in order to achieve in the HSCT recipients a state of immunocompetence without a risk of developing GvHD.

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