On the Relevance of TCR Rearrangement Circles as Molecular Markers for Thymic Output during Experimental Graft-versus-Host Disease

Werner Krenger, Heike Schmidlin, Gionata Cavadini and Georg A. Holländer

*J Immunol* 2004; 172:7359-7367; doi: 10.4049/jimmunol.172.12.7359
http://www.jimmunol.org/content/172/12/7359

---

**References**

This article cites 52 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/172/12/7359.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
On the Relevance of TCR Rearrangement Circles as Molecular Markers for Thymic Output during Experimental Graft-versus-Host Disease

Werner Krenger, Heike Schmidlin, Gionata Cavadini, and Georg A. Holländer

Efficient reconstitution of the pool of peripheral T cells after hemopoietic stem cell transplantation (HSCT) is dependent on normal thymic function. However, the development of graft-vs-host disease (GVHD) in the context of allogeneic HSCT is associated with injurious effects on thymocyte development. In this study, we examined in models of syngeneic and allogeneic murine HSCT whether actual posttransplant thymic output is accurately reflected by analysis of signal-joint TCR rearrangement excision circles (sjTRECs). Our data demonstrate that the de novo generation of T cells following syngeneic HSCT of T cell-deficient B6.RAG2−/− (recombination-activating gene 2−/−) mice correlates firmly with an increase of sjTRECs in the thymus and spleen. However, the altered homeostasis of naive peripheral T cells in the presence of GVHD necessitates the combined analysis of cell division in vivo and determinations of sjTREC contents and total sjTREC numbers to draw informative conclusions. From our data, we substantiate that thymic output and peripheral division of newly generated T cells are diminished in the presence of acute GVHD in an experimental radiation/allogeneic HSCT model. The Journal of Immunology, 2004, 172: 7359–7367.

A lllogeneic hemopoietic stem cell transplantation (HSCT) is the preferred therapy for a number of life-threatening disorders. The success of allogeneic HSCT depends on the efficiency by which the host’s immune system is restored (reviewed in Refs. 1–3). Unfortunately, the T cell lineage is not generated as rapidly and efficiently as most other hematological lineages, a situation that invariably results in a protracted immune deficiency in the posttransplant period. Recent studies aimed at a better understanding of the regenerative mechanisms demonstrated that the donor-derived peripheral T cell pool is restored through two independent pathways: 1) expansion of adoptively transferred mature T cells, and 2) intrathymic de novo generation of T cells from donor HSC (1–3). Although transfer of mature T cells during allogeneic HSCT may provide a short-term restoration of immune functions, a long-lasting and complete reconstitution depends ultimately on the new generation of T cells in the thymus (1, 2, 4–8). To this date, however, it has proven difficult to assess the precise extent to which thymic function contributes to the reconstitution of the immune system following allogeneic HSCT. Indeed, different independent factors may influence thymic output following HSCT (2, 8–10). One such parameter is graft-vs-host disease (GVHD). GVHD represents a major allogeneic HSCT-related complication (11, 12), and it exacerbates posttransplant T cell hypoplasia (13). Deficient T cell reconstitution as a consequence of GVHD may be a result of impaired thymic T cell production and/or diminished peripheral T cell homeostasis (14). Data from experimental transplantation models have now provided clear evidence that the thymus represents a specific target of GVHD (15–19). In particular, the thymic epithelial cell compartment is severely altered as a consequence of disease (20). As normal thymocyte maturation is critically dependent on a normal thymic microenvironment (21), such changes would be expected to impact thymopoietic capacity in the presence of GVHD.

The accurate evaluation of thymic output has proven to be difficult, however, due to the lack of informative cell surface markers identifying recent thymic emigrants. To assess the magnitude of thymic T cell output, the determination of TCR rearrangement excision circles (TRECs) has been suggested (2–5, 8, 9, 22–27). These extrachromosomal nonreplicating DNA circles are a byproduct of the TCR gene rearrangement and specifically occur during T cell ontogeny. Although signal-join (sj) TRECs of the TCRαβ (TCRαβ) locus are commonly determined (22, 28, 29), TRECs from Vβ V(D)J rearrangements have also been successfully measured in human allogeneic HSCT (30). TREC-containing CD4+ and CD8+ peripheral T cells have been viewed to represent recent thymic emigrants (22). With the availability of TREC measurements for clinical use, the role of thymic cell reconstitution following allogeneic HSCT has become the focus of intensive investigations. There are now several clinical studies available that have reported diminished levels of TRECs in the early period of immune reconstitution of patients with ongoing GVHD or with a history of acute or chronic GVHD (8, 24, 31, 32). In cohorts of patients who had undergone transplantation more than 1 year before being sampled, lower sjTRECs were analogously associated with episodes of chronic GVHD (30). These data were interpreted such that peripheral T cell populations contained fewer recent thymic emigrants and thus have promoted an increased acceptance of the paradigm that thymic activity is diminished as a consequence of human GVHD. However, there are reports to the contrary because the authors have failed to demonstrate a direct association of TREC levels with both acute and chronic GVHD within the first
year or later after allogeneic HSCT (23, 33, 34). These sets of studies have remained difficult to reconcile. One possible reason for the observed differences may lie in the fact that although the presence of TREC among peripheral T cells would reflect their thymic origin, the amount of TREC detected in a given population is also a function of cell division and cell death (removal) (35–37). Due to this reality, one study that also has reported lower TREC levels in peripheral T cells from patients with GVHD refuted the interpretation that this association was indicative for suppressed thymic output (10). Thus, the assumption that the detection of TREC in naive T cells identifies all of these cells as recent thymic emigrants may be misleading.

The purpose of the present study was to re-examine in models of syngeneic and allogeneic murine HSCT whether actual posttransplant thymic output is accurately reflected by TREC analysis. In this study, we determined the contents of sTRECs in naive T cells, as well as per unsorted splenocytes or thymocytes, and we assessed total numbers of sTRECs in specific cell populations. Our data demonstrate that TREC measurements, in combination with in vivo cell division analysis and immunophenotyping, correctly assess the extent of thymic de novo T cell generation in the presence of GVHD. From our data, we conclude that thymic export and peripheral cell division of newly generated T cells are diminished in the presence of acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6; H-2\(^d\), CD45.2\(^+)\) and (B6 × DBA/2)F\(_1\) (B6D2F\(_1\); H-2\(^m\), CD45.2\(^+)\) mice were obtained from Biological Research Laboratories (Füllinsdorf, Switzerland). Female B6.SJL-Ptprc\(^{a}\)/Pep3\(^b\)/BoyJ congenic mice (B6.CD45.1; H-2\(^d\), CD45.1\(^+)\) were purchased from The Jackson Laboratory (Bar Harbor, ME). Female B6.RAG2\(^{−/−}\) (recombination-activating gene 2\(^−/−\)) (H-2\(^d\)) mice were obtained from the Basel Institute for Immunology (Basel, Switzerland) and bred in our own facility. Animals between 6 and 10 wk of age were kept under pathogen-free conditions and in accordance with the institute’s guidelines and federal regulations.

Reagents

For four-color flow cytometric (FACS) analyses and cell sorting, the following mAbs (conjugated to biotin, FITC, PE, or CyChrome) were used: anti-CD3, anti-CD8, anti-CD4, anti-CD45.1, anti-CD62 ligand (CD62L), anti-CD45R, and anti-CD16/CD32 (BD Pharmingen, San Diego, CA). Streptavidin-conjugated Cy5 and allophycocyanin were obtained from Zymed Laboratories (San Francisco, CA). The 5-bromo-2’-deoxyuridine (BrdU) was obtained from Sigma-Aldrich (Buchs, Switzerland), and PE-conjugated anti-CD45R, and anti-CD16/CD32 (BD PharMingen, San Diego, CA). The following mAbs (conjugated to biotin, FITC, PE, or CyChrome) were used: anti-CD45.1, anti-CD4, anti-CD8, and anti-CD16/CD32 (BD PharMingen, San Diego, CA). Primers and probes for TREC determination were synthesized by Life Technologies (Basel, Switzerland) and Microsynth (Mountain View, CA). female B6.RAG2\(^{−/−}\) (recombination-activating gene 2\(^−/−\)) (H-2\(^d\)) mice were purchased from the Basel Institute for Immunology (Basel, Switzerland) and bred in our own facility. Animals between 6 and 10 wk of age were kept under pathogen-free conditions and in accordance with the institute’s guidelines and federal regulations.

Transplantation

To reconstitute immunodeficient recipients, naive RAG-deficient B6 mice (B6.RAG2\(^{−/−}\)) were pretreated with 5-fluouracil (150 μg/g body weight, i.p.) and syngeneically transplanted 2 days later with T cell-depleted (TCD) bone marrow cells (1 × 10\(^5\)) from B6 (H-2\(^d\)) wild-type donors. To study TREC in acute GVHD, we used a model that allowed to monitor TREC contents in CD45.1\(^+\) cells derived from the bone marrow in the presence vs absence of GVHD. To this end, B6D2F\(_1\), mice were lethally irradiated (11 Gy, Cs source, two doses at 5.5 Gy separated by 3 h) and subsequently transplanted with TCD bone marrow cells (5 × 10\(^5\)) from B6 (H-2\(^d\)) wild-type donors. Acute GVHD was induced in these mice by infusion of 2.5 × 10\(^5\) purified splenic T cells (magnetic bead separation; miniMACS; Miltenyi Biotec, Sunnyvale, CA) from B6 mice (CD45.2\(^+\)). As non-GVHD controls, irradiated B6D2F\(_1\), mice received TCD bone marrow from B6.CD45.1 donors alone. Analyses of immunophenotype, cell division, and TREC were performed between 0 and 8 wk after GVHD induction. CD45.1\(^+\) cells in thymi or secondary lymphoid organs were distinguished from CD45.2\(^+\) cells using flow cytometry.

Flow cytometry

Cells (0.05–1 × 10\(^5\)) were washed, resuspended in 1% FCS/PBS/sodium azide, and incubated for 20 min on ice with the appropriate mAbs. In two-step staining procedures, biotinylated Abs were revealed with streptavidin-conjugated Cy5 or allophycocyanin. Washed cells were fixed in 1% paraformaldehyde/PBS for subsequent analysis on a FACSCalibur dual laser (BD Biosciences). To enrich for discrete T cell subpopulations, splenocytes or thymocytes were labeled with the appropriate Abs and sorted to purity (>95%) using a FACSVantage cell sorter (BD Biosciences). We collected between 1 and 6 × 10\(^5\) sorted cells. The cells were pelleted and frozen at −80°C until further use.

Cell division

For detection and characterization of proliferating cells in situ, mice received continuously BrdU (0.8 mg/ml) in their drinking water for the 4 days immediately before sacrifice (38). DNA-tyrosinase cells were detected, as described (18, 39). In brief, splenocytes and thymocytes were isolated from BrdU-treated mice and immediately surface stained separately on ice with conjugated (biotin, PE, and CyChrome) and unconjugated mAbs to CD16/CD32, CD3, CD62L, and CD45.1 for 30 min. In a second staining step, cells were incubated with streptavidin-conjugated Cy5 for another 25 min. Subsequently, cells were treated with ice-cold 0.15 M NaCl/0.95% EtOH for 30 min at 4°C and fixed for another 30 min with PBS containing 1% paraformaldehyde and 0.01% Tween 20. Cells were then incubated for 10 min at room temperature in a buffer containing 50 μM 5′-[carboxyfluorescein]-2′,7′-dichlorofluorescin diacetate (CFDA), as described (40). After washing, PE-conjugated 3D4 mAb was added, and cells were incubated for an additional 30 min at room temperature, washed, and finally resuspended in 1% BSA/PBS/sodium azide. Four-color analyses were conducted on a FACSCalibur.

Quantification of TRECs

Quantitative PCR was used to determine murine signal-joint (sj) TRECs (28) in analogy to previously published reports (41, 42). Genomic DNA was isolated from sorted or unsorted cells by phenol/chloroform/soybean alcohol extraction, and PCR were performed in solutions containing 100 ng of DNA, 1× TaqMan master mix, and 200 nM FAM-TAMRA probe (Mirocytron). Sequences for primers and probes were deduced from published DNA sequences of the unarranged murine TCRδ locus carrying 3′ε and 3′ζ, the two TCRδ-deleting elements (National Center for Biotechnology Information accession numbers AC003057 and M15429, respectively). sjTREC primers were: 5′-CAAGCTGTACAAGGCACTTGT3′ (forward), 5′-TGAGCATGGCAACGACCTC3′ (reverse); FAM/TAMRA probe, 5′-TGCTGGTGGCTCCATGCTGCC3′-3′ (amplion 155 bp). The cycle conditions were: 2 min, 50°C and 10 min, 95°C, followed by 40 cycles of amplification (15 s, 95°C; 1 min, 60°C). PCR was performed in a SDS 7700 PCR machine (Applied Biosystems). As reference, the Runx1/AML1/Chf2 gene (43) was amplified. AML1 primers, 5′-CTGTCGCCCTCTAGATGAAA GT3′-3′ and 5′-AGCCGCTCCTGCTACTTGG3′; probe, 5′-CCAGCAGAGCAGCCGGCATTT3′ (amplion 142 bp). For normalization and calculation of the exact DNA content of TREC present in a given DNA sample, a dilution series of AML1 and a target DNA standard were included in each PCR experiment. DNA plasmid standards containing sjTREC sequences were created by cloning amplified DNA fragments into the EcoRI site of pGEM-T Easy vectors (Promega, Wallisellen, Switzerland). Primers were 5′-AAAGGAAAGAAAGAAAGAGAC-3′ and 5′-TGTTGCTCGCATGTCGTTG-3′. To verify the identity of the TREC DNA, the inserts were sequenced and compared with the National Center for Biotechnology Information database. The detection limit for quantitative PCR was 10^{-3} ng of plasmid DNA, corresponding to approximately three sjTREC copies. From the total number of sjTREC/μg DNA in a given population, the actual TREC content per single cell of this population was calculated by dividing the TREC value by a factor of 2.1 × 10^5. This factor was established in previous experiments in which 1 μg of DNA corresponded to 2.1 × 10^5 cells.

Statistical analysis

Values are mean ± SD. For two-group comparisons, the nonparametric unpaired t test was used, whereas for multiple group comparisons, ANOVA was used. For regression analysis, the r^2 test was used (StatView; SAS Institute, Cary, NC).
**Results**

TREC contents in peripheral lymphoid organs reflect thymus-dependent T cell reconstitution following HSCT of T cell-deficient RAG^{−/−} mice

To establish detection and precise quantification of sjTRECs, discrete cell populations from lymphoid organs of naive B6 wild-type and B6.RAG2^{−/−} mice, respectively, were sorted to a purity of at least 95%. Genomic DNA was isolated and subsequently subjected to real-time PCR. The sjTREC content (i.e., the population-based calculation of the fraction of sjTREC per single cell) was highest in B6 wild-type mice among phenotypically mature thymocytes (i.e., CD4^+CD8^− and CD8^+CD4^− cells; Fig. 1A). These populations contained on average ∼8 × 10^−1 copies of sjTREC per single cell. sjTREC contents were intermediate at ∼5 × 10^−1 per single cell in the CD4^+CD8^− population, but were not detectable among the most immature CD4^−CD8^− thymocytes. This result was in agreement with studies showing that rearrangement of the TCRαD locus is not initiated before the developmental transition of immature CD4^−CD8^− cells to a CD4^+CD8^− phenotype (44). The population of CD4^+CD62L^+ naive splenic T cells contained an amount of sjTRECs similar to that of CD4^+CD8^− thymocytes, i.e., ∼8 × 10^−1/cell. sjTREC contents among unsorted splenocytes were, however, lower, as this population contained both naive and memory T cells as well as B cells. Notably, sjTRECs were neither detected in non-T cells of wild-type mice nor in any tissue of B6.RAG2^{−/−} animals (Fig. 1B).

The above data in conjunction with previous observations (22) suggested that sjTREC contents may identify recent thymic emigrants and thus could be used to quantify actual thymic output. To test this assertion, we determined whether the reconstitution of the central and peripheral T cell compartments of B6.RAG2^{−/−} mice would be correctly reflected by the emergence of TRECs. For this purpose, B6.RAG2^{−/−} mice were pretreated with 5-fluorouracil and transplanted 2 days later with TCD bone marrow cells taken from syngeneic B6 wild-type mice. Donor bone marrow-derived CD4^+CD8^− thymocytes were detected as early as 2 wk after transplantation (Fig. 2A), and low contents of TRECs (<10^−2/cell) could already be detected at this time point (Fig. 2B). By 3 wk, after HSCT, mature CD4^+CD8^− and CD8^+CD4^− thymocytes had been generated in significant numbers. In parallel, sjTREC contents per single cell of unseparated thymocytes had increased to ∼1 × 10^−1/cell. At the end of the observation period, the TREC contents had risen to a level of ∼2 × 10^−1/cell owing to the emergence of CD4^+CD8^− and CD8^+CD4^− cells. This TREC content remained lower than the value in unseparated thymocytes of naive mice. In the spleen, the lack of TRECs early after transplantation (0–3 wk) correlated with the lack of phenotypically mature T cells (Fig. 2, lower panels). Between 4 and 5 wk after transplantation, the presence of donor bone marrow-derived CD4^+ and CD8^+ T cells was associated with sjTREC contents of ∼3 to 5 × 10^−2/cell, as measured in unsorted splenocytes. These values stayed lower than in unsorted spleen cells from naive unmanipulated mice (i.e., ∼1.2 × 10^−1/cell), most likely owing to a lower than normal fraction of T cells present in the spleen of B6.RAG2^{−/−} mice early after HSCT. Our data also demonstrated that RAG2^{−/−} mice had not yet fully reconstituted the lymphoid compartment at 5 wk posttransplant to levels regularly observed in normal, untransplanted B6 mice. Taken together, our data indicated that the de novo generation of mature T cells and their export from the thymus to the periphery were associated with the detection of increasing levels of TRECs.

**Delayed donor T cell reconstitution during acute GVHD in a radiation/allogeneic HSCT model is a consequence of diminished thymic output**

Changes in thymic stromal cell composition, architecture, and function affect thymocyte maturation and may thus lower thymic TREC content and cell output (18–20). To test whether TREC measurements provide an appropriate molecular tool to assess actual thymic output in the presence of GVHD, we used experimental allogeneic HSCT. The system studied was modeled after the clinical situation in which the newly generated T cells are derived from donor bone marrow cells. For this purpose, B6.D2F1 mice were lethally irradiated with 11 Gy and subsequently transplanted with TCD bone marrow from B6,CD45.1 mice (CD45.1^+). To assess the influence of thymic GVHD on TREC-positive T cells, GVHD was induced in a subgroup of transplanted mice by cotransfer of 0.25 × 10^5 mature T cells from B6 (CD45.2^+) mice. These conditions allowed for a weak to moderate GVHD and low mortality, thus rendering possible a longitudinal assessment of TRECs and T cell divisions despite the presence of GVHD. Histopathologic evidence of acute GVHD was found in the gastrointestinal tract and liver of transplanted mice as early as 2 wk after mature T cell transfer (data not shown). Moreover, thymic size and cellular composition of its microenvironment were negatively affected by GVHD (data not shown). As evidenced in Fig. 3A, the number of donor HSC-derived (CD45.1^+) thymocytes was significantly diminished between 4 and 8 wk following T cell replete allogeneic HSCT compared with mice receiving only a TCD bone marrow inoculum. The absolute loss of immature CD45.1^+CD4^+CD8^− thymocytes accounted largely for the observed decrease in cellularity (Fig. 3B). However, the absolute number of mature CD45.1^+CD4^+CD8^− (Fig. 3C) and CD45.1^+CD8^+CD4^− (data not shown) thymocytes was comparable in transplanted animals regardless of the presence or absence of GVHD.

In parallel with a decreased overall cellularity, the absolute number of thymic sjTRECs was diminished by 2- to 3-fold in mice with GVHD (Fig. 4A). At 6–8 wk after HSCT, the total number of thymic sjTREC copies correlated with the cellularity of donor HSC-derived thymocytes (Fig. 4B). Despite this correlation, sjTREC contents in purified CD45.1^+CD4^+CD8^− and
creased in spleens of GVHD

6

Therefore, we determined cell proliferation for donor HSC-derived these developmental compartments will affect their TREC content. (40), any change in the steady state of cell division in either of immature CD4

6

observation period.

4,

ferent from values in recipients of TCD bone marrow alone (Fig. A)

Emergence of sjTRECs in B6.RAG2

CD4.1

1

CD8

1

CD4

1

T cells and among those fewer cells with a naive phenotype (i.e., CD62L positive) were detected 6–8 wk after transplantation (Fig. 3, E and F). The decreased thymic formation of naive T cells and their diminished export to the spleen correlated with lower total numbers of total sjTREC copies in the spleen (Fig. 6A). The absolute copy number of sjTRECs was directly linear to the actual number of donor bone marrow-derived (i.e., CD4.1

1

CD4

1

T cells in mice with GVHD (Fig. 3, B). Despite the decreased number of total splenic sjTRECs, the calculated TREC contents per single CD4.1

1

CD4

1

CD62L

+ cell were identical in mice with and without GVHD (Fig. 6B). TREC contents remained low in both groups at a steady state level of ~2 \times 10^7 bead, a value evidently lower than in the corresponding cell population of naive T cells of unmanipulated mice. As the TREC content in bone marrow-derived peripheral naive T cells may be subject to an altered homeostatic proliferation in the presence of GVHD, we measured cell division of emerging T cells in the spleen. Over time and parallel to replenishing the peripheral T cell pool, the frequency of actively cycling splenic CD4.1

1

CD4

+ and CD4.1

1

CD4

+ CD62L

+ T cells was initially high, but then continuously diminished both in mice with and without GVHD for the relative and absolute numbers of BrdU

1

CD45.1

1

CD4

1

T cells in GVHD

+ animals was initially higher than in non-GVHD mice (~7 \times 10^6 vs 2.5 \times 10^4 cells; Fig. 7A). This fact was also reflected by a slightly increased cellularity at 2–3 wk after transplantation in mice with GVHD when compared with mice without GVHD, albeit this difference was small (Fig. 3E). The exact cause of this preferential early expansion of CD4

+ T cells in mice with GVHD


FIGURE 2. Emergence of sjTRECs in B6.RAG2

1/– mice transplanted with syngeneic TCD bone marrow cells from wild-type B6 mice. A, Phenotypic reconstitution of lymphoid cells in the thymus (top) and spleen (bottom). The four major thymocyte populations and splenic CD4

+ and CD8

+ T cells and non-T cells, respectively, were analyzed by flow cytometry from 0 to 5 wk after HSCT. The total numbers of cells are given (\times 10^6). As controls, total cell numbers of the diverse cell populations from spleens and thymi of untransplanted B6 mice are given. B, sjTRECs were measured in unseparated total splenocytes and thymocytes, respectively, at 0–5 wk after HSCT using quantitative PCR. From the total number of sjTRECs/\mu g DNA of total splenocytes or thymocytes, the actual TREC content per single cell of the respective population was calculated by dividing the TREC value by a factor of 2.1 \times 10^6, as described in Materials and Methods. As controls, TREC contents in unseparated splenocytes and thymocytes isolated from untransplanted B6 are given. These control values are derived from the same animals, as shown in Fig. 1. The data are an aggregate from two experiments, with at least three or maximally four mice per group and time point. Where given, data represent mean ± SD.
We therefore concluded that thymic export was diminished in cells in the periphery of mice with GVHD was observed (Fig. 3). Peripheral T cells. Notwithstanding, 3) a low frequency of naive T cells in the frequency of proliferating T cells was significantly more prominent in animals with GVHD (Fig. 7, C and D). The inhibition of cell proliferation was not restricted to T cells, but also affected other cell lineages resident in the spleen (data not shown). As a consequence of a difference in cell proliferation, TRECs were thus not diluted at comparable rates in naive CD4+ T cells of mice with GVHD when compared with animals without GVHD.

In summary, the mechanisms that accounted for the observed TREC contents of \( 2 \times 10^{-1} \)cell among naive T cells on mice with acute GVHD (Fig. 6) included: 1) the very low T cell proliferation for at least 8 wk after transplantation (Fig. 7). In consequence, only a very limited dilution of TRECs occurred in mice with disease. 2) Mature CD4+CD8- and CD4+CD8+ thymocytes and recent thymic emigrants contained \( 2 \times 10^{-1} \) sjTREC/cell (Fig. 4). Therefore and irrespective of the extent of thymic export, a higher sjTREC content than this value could not be expected for peripheral T cells. Notwithstanding, 3) a low frequency of naive T cells in the periphery of mice with GVHD was observed (Fig. 3). We therefore concluded that thymic export was diminished in these diseased animals.

**Discussion**

The reconstitution of the peripheral T cell compartment following HSCT is contingent on two independent driving factors, the intrathymic differentiation of donor stem cells and the peripheral expansion of donor T cells (reviewed in Refs. 1–3). However, it is the thymic pathway alone that generates an appropriately diverse T cell repertoire. Thymic function in healthy and diseased individuals has therefore received considerable attention over the last few years, and it has become clear that an efficient thymic function after transplantation is favorable for long-term immune reconstitution. The introduction of the TREC assay has been regarded as a breakthrough to achieve an accurate measure for thymic output following HSCT. Nonetheless, the value of the TREC assay for this particular purpose appears to have its limitations not at least secondary to cell division (36). In this study, we tested whether the combination of TREC determination with cell division analysis in vivo accurately reflects actual thymic output following experimental HSCT.

To initiate our study and to obtain base levels of TRECs in murine lymphoid tissues, we first analyzed 6- to 10-wk-old naive mice and determined a sjTREC content of \( 6 \times 10^{-1} \)cell in unsorted thymocytes (Fig. 1). This value was comparable to the level previously reported for 6- to 12-wk-old mice (41). The sjTREC contents in individual mature thymic CD4+CD8- or CD8+CD4- cells were found to be \( 8 \times 10^{-1} \)cell. The difference to the theoretical value can be explained by the following events. There is a frequency of \( <100\% \) that the \( \delta \)-rec-\( \psi \)-rec recombination event occurs and thymocytes with a rearranged TCR\( \delta \)-locus still undergo cell cycle progression before emigration (Fig. 5) (39). As to be expected, the TREC contents were similar for naive splenic T cells and mature CD4+CD8- and CD4+CD8-
thymocytes (i.e., \( \approx 8 \times 10^{-1}/\text{cell} \)). These results indicate an equilibrium between thymic emigration of TREC+ T cells and the displacement of TREC+ naive T cells to the memory/effector cell pool. Moreover, our data from the B6. RAG2-/- mice engrafted with syngeneic bone marrow cells demonstrated that the de novo generation of T cells could be quantified in this system exclusively by the measurement of TRECs (Fig. 2). In transplanted B6. RAG2-/- mice, the sjTREC content remained, however, lower for an extended period post-HSCT when compared with unsorted splenocytes taken from unmanipulated mice. This lower content may be explained by a lower than normal fraction of T cells present in the spleen of B6. RAG2-/- mice early after HSCT. Moreover, increased T cell proliferation may occur (leading to enhanced TREC dilution) if peripheral lymphoid niches have to be repleted secondary to a general lymphopenia, as previously described for immunodeficient mice or following irradiation of normal mice (45, 46).

For allogeneically transplanted recipients free of GVHD, we found that splenic CD45.1+ naive peripheral T cell numbers increased robustly after TCD-HSCT (Fig. 3). The average sjTREC content of peripheral naive CD4+ T cells did, however, not exceed a value of \( \approx 2 \times 10^{-1}/\text{cell} \) as late as 8 wk after engraftment (Fig. 4). This level was lower than in unmanipulated mice (\( \approx 8 \times 10^{-1}/\text{cell} \)). This difference is explained by a balance of two simultaneously occurring events: 1) the continuous dilution of sjTRECs in the periphery due to high homeostatic proliferation, and 2) the continuous supply of newly formed T cells containing sjTRECs (Fig. 8A). It is anticipated that the sjTREC contents in naive T cells from complication-free HSCT recipients will eventually reach values close or equal to those of unmanipulated mice. This situation will be attained once the cell divisions of immature and mature T cells have reached physiological steady state secondary to normal thymic output, and the pool of naive peripheral T cells has been fully renewed in these transplanted mice without GVHD. The fact that a normal steady state has, however, not been attained by as late as 8 wk posttransplant is highlighted by: 1) higher than normal proliferation of CD4+ CD8+ and CD4+ CD8+ thymocytes (Fig. 5); 2) a still decreased splenic cellularity of T cells in transplanted mice when compared with unmanipulated control animals (Figs. 2 and 3); and 3) an enhanced homeostatic cell division among naive donor bone marrow-derived T cells (Fig. 7). Despite its significance, defining the exact time point by when normal sjTREC values are reached did not constitute a focus of our studies. It is important to note, however, that the sjTREC content of peripheral naive T cells in HSCT recipients without GVHD will not surpass a value of \( 2 \times 10^{-1}/\text{cell} \) as long as the content in recent thymic emigrants remains at \( 2 \times 10^{-1}/\text{cell} \) (Fig. 4). This latter value is also expected to reach a level close or equal to that of unmanipulated mice once the cell divisions of thymocytes arrive at a steady state level.
Our results also provide evidence that the postselection bone marrow-derived CD4⁺CD8⁻ and CD8⁺CD4⁺ thymocytes are substantially altered in their function and phenotype as a consequence of acute GVHD: With regard to CD24 and Qa-2 expression, mature thymocytes acquire a surface phenotype corresponding to that of peripheral naive T cells. Instead of expressing the heat-stable Ag CD24, but not the nonclassical MHC class I molecule Qa-2 (47), intrathymic Ly-5.1⁺CD4⁺CD8⁻ cells from GVHD⁺ mice were CD24⁻Qa-2⁺, a cell surface phenotype characteristic for recent thymic emigrants (data not shown). We interpret this observation that mature CD4⁺CD8⁻ and CD8⁺CD4⁻ cells do not exit the thymus for a disproportionate length of time. In consequence and in view of the striking loss in the number of CD4⁺CD8⁻ thymocytes in mice with GVHD (Fig. 5), the relative number of mature thymocyte cells is higher than expected. An enhanced proliferation specifically of those mature thymocytes poised for export was, however, excluded as an explanation for the observed thymocyte increase because these cells incorporated BrdU at a much lower level in the presence of GVHD (Fig. 5). The reason why mature thymocytes are inhibited from leaving the thymus during acute GVHD remains to be investigated.

Our study confirms those clinical reports that have demonstrated an association between GVHD and lower TREC levels (8, 10, 24, 31, 32) and emphasizes the necessity for a careful analysis and interpretation of TREC data. In this study, we have used a combination strategy, i.e., the determination of sjTRECs per naive T cell, sjTRECs per unsorted splenocytes or thymocytes, and total numbers of TRECs in specific cell populations. If TREC data are appropriately collected and correctly analyzed, the TREC assay is invaluable for the assessment of posttransplant T cell reconstitution. Moreover, our data support the premise that the TREC assay needs to be combined with cell division analysis in vivo to be suitable for assessment of thymic output following T cell replete
serve as a good measure of thymic output. It should also be noted that although absolute numbers of TREC+ T cells may not be influenced by peripheral cell division, they are affected by cell death and intracellular degradation of TREC-55. Thus, even the combination of TREC-5s with measures of T cell proliferation may not always be sufficient for determination of thymic output. Rather, these two analyses may need to be additionally combined with the determination of cell death, as recently exemplified (30, 54). Because our study did not account for peripheral T cell death, further investigations will be needed to define the contribution of altered thymic function and altered peripheral T cell homeostasis/apoptosis, respectively, to T cell deficiency following allogeneic HSCT.

**Acknowledgments**

We thank Bruce R. Blazar for critical reading of the manuscript, and Eli Christen and Katrijn Hafen for expert technical assistance.

**References**


**FIGURE 8.** Schematic representation of the mechanisms affecting sjTREC contents in transplanted mice. A, Mice without GVHD. Bone marrow-derived splenic naive T cell numbers increase robustly after TCD-HSCT. The average sjTREC content of peripheral naive CD4+ T cells remains at a value of ~2 × 10^7/cell as late as 8 wk after transplantation. The loss of TRECs by high cell division of naive T cells is counterbalanced by an efficient reconstitution of the naive T cell pool with TREC-containing new thymic emigrants (with a content of 2 × 10^7/cell). B, Mice with GVHD. In disease, the naive T cell compartment is being replenished at a much slower speed, and naive T cell numbers remain low for a prolonged period of time. Cell division is very low and sjTREC contents remain at ~2 × 10^7/cell among naive T cells for at least 8 wk after transplantation. In consequence, only a very limited dilution of TREC-5s occurs in mice with disease. Because recent thymic emigrants contain ~2 × 10^7 sjTREC/cell, a higher sjTREC content than this value is not expected for peripheral T cells, irrespective of the extent of thymic export. The low frequency of naive T cells in the periphery indicates, however, diminished thymic export in these animals.

allogeneic HSCT. In vivo BrdU labeling for cell division analysis is, however, rarely possible in humans. As suggested before (8), an alternative to BrdU is the determination of Ki-67 expression, a nuclear cell proliferation-associated Ag detected during all stages of the cell cycle (48). For example, Ki-67 expression in naive and memory T cells served in combination with TREC analysis to determine of cell cycle (48). For example, Ki-67 expression in naive and memory T cells served in combination with TREC analysis to...


