T cell turnover and thymic function in HIV-1 infection
Hazenberg, M.D.

Citation for published version (APA):
Hazenberg, M. D. (2002). T cell turnover and thymic function in HIV-1 infection

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
T cell receptor excision circle (TREC) and T cell dynamics after allogeneic stem cell transplantation are related to clinical events

Mette D. Hazenberg, Sigrid A. Otto, Elmar S. de Pauw, Helene Roelofs, Willem E. Fibbe, Dörte Hamann and Frank Miedema
Abstract

It is generally believed that homeostatic responses regulate T cell recovery following peripheral stem cell transplantation (PSCT). We studied in detail immune recovery in relation to T cell depletion and clinical events in a group of adult patients that underwent PSCT because of hematological malignancies. Initially, significantly increased proportions of dividing naive, memory and effector CD4^+ and CD8^- T cells were found that readily declined, despite still very low numbers of CD4^- and CD8^- T cells. Post-PSCT increased T cell division rates reflected immune activation, as they were associated with episodes of infectious disease and graft versus host disease (GvHD). T cell receptor excision circles (TRECs) were measured to monitor thymic output of naive T cells. Mean TREC content normalized rapidly after PSCT, long before naive T cell numbers had significantly recovered. This is compatible with continuous thymic production of TREC^- naive T cells and does not reflect homeostatic increases of thymic output. TREC content was decreased in patients with GvHD and infectious complications, which may be explained by dilution of TRECs resulting from increased proliferation. Combining TREC and Ki67 analysis with repopulation kinetics led to the novel insight that recovery of TREC content and increased T cell division during immune reconstitution after transplantation are related to clinical events, rather than to homeostatic adaptation to T cell depletion.
Introduction

Recovery of the immune system from T lymphocyte depletion of any cause depends on two separate mechanisms: peripheral expansion of T cells, and naive T cell production by the thymus (1–6). Regulation of this process is believed to be homeostatic, such that transplanted or remaining T cells can be induced to expand, and thymic output of naive T cells to be increased, the latter also contributing to recovery of a broad T cell repertoire (1,7). Still, immune reconstitution in adults is generally slow, and is not always complete (3,8,9). This may be attributable to the age-related involution of thymic tissue. Indeed, children seem to recover faster and to better reconstitute their T cell receptor repertoire than adults do, but only when related to adult normal T cell values (3,10–13).

We aimed to investigate whether the immune system is indeed capable of homeostatic responses to changes in peripheral T cell counts. We studied the contribution of the different mechanisms involved in immune reconstitution in a group of adult patients with hematological malignancies following allogeneic peripheral stem cell transplantation (PSCT) with a T cell depleted graft. Under this condition of reconstitution of the T cell pool in an immune system that is assumed to be ‘empty’, we measured peripheral cell division by analyzing expression of the activation antigen Ki67 in naive, memory and effector CD4+ and CD8+ T cell subsets. In addition, we sought to evaluate thymic output by analysis of T cell receptor excision circles (TRECs). These excision circles are formed during the T cell receptor rearrangement process that T cells undergo during their maturation in the thymus (14,15). Naive T cells that have recently emigrated from the thymus contain a certain, relatively high, TREC content and quantification of this TREC content has been considered a measure of thymic function (16,17). More recently however, interpretation of TREC data in various clinical conditions has been shown to be more complex than anticipated (18).

The purpose of this study was to better understand the relative importance of factors involved in reconstitution of the adult immune system, and to identify the underlying mechanisms that drive those factors. Immune reconstitution can be taken to be reflected by recovery of peripheral blood CD4+ T cell numbers, even when only a fraction of the T cell pool resides in the peripheral blood (19). By measuring peripheral cell division rates in parallel with analysis of TREC dynamics and relating these data to the clinical status of patients and recovery of peripheral blood T cell numbers, we here offer a new interpretation of post-transplantation T cell division rates and TREC data.

Methods

Patients

Twenty-two patients with hematological malignancies who received allogeneic HLA-matched peripheral stem cell transplantation (PSCT) were included. Grafts
were depleted for T and B cells by incubation with the humanized anti-CD52 antibody Campath-1H, to reduce the risk of graft-versus-host disease (GvHD). Patients followed a standard conditioning regimen: Cyclophosphamide 60 mg/kg/day for two days (days -6 and -5), and single dose total body irradiation (9 Gray, day -1) with shielding of eyes and lungs. Peripheral cell division and thymic T cell production were analyzed at different timepoints following PSCT using freshly obtained peripheral blood samples. At all timepoints, the clinical status of patients was monitored. Diagnosis and grading of GvHD was by histopathologic analysis of skin biopsy, liver function tests and incidence of diarrhea. Patients were monitored for CMV reactivation at weekly intervals (pp65 mAb) during the first three months after transplantation. Four patients were studied longitudinally, at 6 weeks, 3, 6, 9 and 12 months after PSCT. Healthy volunteers of comparable age were included as controls (median age of patients 35 years, range 20–55 years).

Peripheral cell division
Naive (CD27+CD45RO- and CD27- or CD27+) CD4+ T cells and naive (CD27+CD45RO-) memory (CD45RO+ and CD27- or CD27+) and effector (CD27-CD45RO+) CD8+ T cells were defined as described previously (20,21). Cell proliferation was measured by analyzing Ki67, a protein pivotal for cell division that is expressed exclusively by cells that are in cell cycle(22), as reported elsewhere (23). Briefly, 500 µl heparinized blood was incubated with CD4- or CD8-PerCP mAb, CD45RO-PE mAb (Becton Dickinson, San Jose, California) and biotinylated CD27 mAb (CLB, Amsterdam, The Netherlands), followed by incubation with Streptavidin-APC (Becton Dickinson). Red blood cells were lysed, and lymphocytes fixed (FACS lysing Solution, Becton Dickinson), permeabilized (FACS Permeabilization Buffer, Becton Dickinson), incubated with Ki67-FITC mAb (Immunotech, Marseille, France) and fixed (Cellfix, Becton Dickinson). Ki67 expression was analyzed on a FACSCalibur (Becton Dickinson) with Cellquest software.

TREC measurements
PBMC were obtained by Ficoll-Paque density gradient centrifugation from heparinized blood, and CD4+ and CD8+ T cells were isolated by magnetic bead separation over columns, using the MiniMACS multisort kit according to manufacturers instructions (Miltenyi Biotec Inc., Sunnyvale, California). With this technique, at least 90% purity was achieved. DNA was purified from CD4+ and CD8+ fractions using the QIAamp Blood Kit according to manufacturers instructions (Qiagen, Hilden Germany). In order to detect Signal joint (Sj) TRECs, a real-time quantitative PCR method was used, as described previously.(18). The Ca constant region was used as an internal control measurement, and a Sj standard (18) was included to calculate the number of Sj copies present. From the average TREC content as measured per µgram DNA, TREC content per T cell was calculated by dividing TREC content by 150,000 (assuming that 1 µgram DNA

140
The absolute number of TREC+ CD4+ and TREC+ CD8+ T cells was calculated by multiplying the average CD4+ and CD8+ TREC content by the number of CD4+ and CD8+ T cells, respectively.

**Statistical analysis**

Patient and control values were compared with the Mann-Whitney U test. Correlations were calculated using Spearman’s ($r_s$) and Pearson’s ($r$) correlation coefficients, depending on the outcome of Shapiro-Wilk W tests of normality.

**Results**

*Peripheral blood T cell numbers and T cell division rates*

Quantitative T cell recovery was analyzed for naive, CD27+ and CD27- memory CD4+ T cells and for naïve, CD27+ and CD27- memory and CD27+ effector CD8+ T cells (Figure 1a, upper panels). Restoration of T cell subsets was slow, such that six months after PSCT the total number of CD4+ and CD8+ T cells was still significantly lower compared with control values ($p<0.01$), confirming previous reports (9). This was related to significantly lower numbers of naive and CD27+ and CD27- memory CD4+ T cells and naïve CD8+ T cells ($p<0.05$). In parallel, we measured Ki67 expression in naïve and memory CD4+ T cell subsets and in naïve, memory and effector CD8+ T cell subsets (Figure 1a, lower panels). Six weeks after PSCT, the proportion of dividing cells was significantly increased compared with healthy controls ($p<0.001$ for CD4+ and CD8+ T cells). This was due to significantly increased division rates in naïve, CD27+ and CD27- memory and effector CD4+ and CD8+ T cell subsets ($p<0.005$). Cell division rates in all T cell subsets declined immediately following PSCT, whereas CD4+ and CD8+ T cell numbers were still significantly low compared with healthy individuals (Figure 1a). CD4+ and CD8+ T cell division rates did not correlate with peripheral blood T cell numbers ($r_{s}=-0.35$ and $r_{s}=-0.38$, respectively; data not shown). The proportion of Ki67+ CD4+ T cells did correlate significantly with the proportion of Ki67+CD8+ T cells (Figure 1b) which suggests that CD4+ and CD8+ T cell division are driven by a common factor.

*Clinical events*

Following transplantation, patients are at risk for graft versus host disease (GvHD) and because of severe T cell depletion, patients are susceptible to infections (19). Both conditions may also induce increased proportions of T cells to divide. Therefore, we studied the relation between Ki67 expression and clinical status of patients. We divided patients into two groups: those who did not suffer from clinically documented infections or GvHD at the time of analysis (group 1), and those who did (group 2). Individual data as measured six weeks after PSCT are depicted in Table 1. The proportion of dividing CD4+ T cells was significantly higher in the group with GvHD and / or documented infections (Figure 1c, $p<0.05$).
Figure 1. T cell recovery and peripheral T cell division following PSCT. (a) Depicted are median numbers of CD4+ and CD8+ T cells and naive, memory and effectoF CD4+ and CD8+ T cell subsets (upper panels) and median values of Ki67 expression in CD4+ and CD8+ T cells and T cell subsets (lower panels) at sequential timepoints following PSCT. Note that at six weeks, the percentage of naive CD4+ T cells was too low in most patients to determine Ki67 expression in this subset. Black circles: CD4+ (left panels) or CD8+ (right panels) T cells; white circles: naive T cells; white squares: CD27+ memory T cells; white triangles: CD27 memory T cells; white diamonds: CD27- effector T cells. (b) The proportion of dividing CD4+ T cells did correlate significantly with the proportion of dividing CD8+ T cells, suggesting that division is driven by a common factor. (c) Patients with no signs of infectious diseases (group 1, n=6) had lower proportions of dividing CD4+ T cells than patients with documented inflammatory complications, such as GvHD, reactivation of CMV, or fungal pneumonia (group 2, n=8). Note that patient number 12 was not assigned to either group as she was hospitalized with severe dehydration at this timepoint (see Table 1). White circles: group 1 patients; x-hair circles: group 2 patients (b and c). Horizontal bars indicate median values in (c). Depicted in (b and c) are data obtained six weeks after PSCT (n=15). Similar results were obtained for the other time points and for CD8+ T cells (data not shown).

Similar results were obtained for the CD8+ T cells and for the other time points (data not shown). The recovery rate of T cell numbers was not different between both groups.

Recovery of TREC content
To assess thymic origin of naive T cells, we measured TREC content (number of TREC copies per T cell) of purified CD4+ and CD8+ T cells. Following PSCT, TREC content of CD4+ T cells rapidly normalized, such that after six months values were not significantly lower than those of healthy individuals (Figure 2a). Similar results were obtained for CD8+ TREC content recovery (data not shown). TREC are episomal circles that are diluted during cell division after their formation (14,24). We have recently reported that naive and memory cell division decreases TREC content (18). Amongst others, expansion of naive and / or memory cells, leading to dilution of TREC, may decrease TREC content of
### Table 1. Clinical conditions and peripheral cell division rates 6 weeks after PSCT

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical condition (6 weeks after PSCT)</th>
<th>Group</th>
<th>Ki67+ T cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4+ CD8+</td>
</tr>
<tr>
<td>1</td>
<td>GvHD grade 1</td>
<td>2</td>
<td>21.6 73.1</td>
</tr>
<tr>
<td>2</td>
<td>GvHD grade 2</td>
<td>2</td>
<td>24.1 11.1</td>
</tr>
<tr>
<td>3</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>4.8 2.2</td>
</tr>
<tr>
<td>4</td>
<td>GvHD grade 1</td>
<td>2</td>
<td>37.9 33.9</td>
</tr>
<tr>
<td>5</td>
<td>Fungal dermatitis</td>
<td>2</td>
<td>23.8 8.6</td>
</tr>
<tr>
<td>6</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>4.2 9.4</td>
</tr>
<tr>
<td>7</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>22.4 13.0</td>
</tr>
<tr>
<td>8</td>
<td>GvHD grade 2</td>
<td>2</td>
<td>53.1 53.1</td>
</tr>
<tr>
<td>9</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>34.2 59.4</td>
</tr>
<tr>
<td>10</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>NT NT</td>
</tr>
<tr>
<td>11</td>
<td>No infections or GvHD</td>
<td>1</td>
<td>5.6 7.4</td>
</tr>
<tr>
<td>12</td>
<td>Hospitalized: dehydration</td>
<td>Neither</td>
<td>43.1 51.9</td>
</tr>
<tr>
<td>13</td>
<td>GvHD grade 1</td>
<td>2</td>
<td>15.9 18.1</td>
</tr>
<tr>
<td>14</td>
<td>Hospitalized: GvHD and cytomegalovirus</td>
<td>2</td>
<td>11.8 17.8</td>
</tr>
<tr>
<td>15</td>
<td>GvHD grade 1</td>
<td>2</td>
<td>38.2 38.5</td>
</tr>
</tbody>
</table>

Patients were grouped based on the presence or absence of infectious complications or GvHD. NT indicates not tested.

Purified CD4+ and CD8+ T cells. Indeed, CD4+ TREC content correlated negatively with the proportion of dividing CD4+ T cells (Figure 2b). No correlation was found between the number of CD27+ CD45RO+ naive T cells, a phenotypic rough estimate of thymic output, and TREC content (Figure 2c). Thus, in most patients, TREC content had recovered at timepoints where naive T cell numbers were still extremely low. Thymic function could be hampered by chemotherapy or radiation induced damage of this organ, and by GvHD (25,26). Low CD4+ TREC content was found in patients with clinically documented GvHD and infectious complications (p<0.05, Figure 2d). Nonetheless, the maximal recovery rate of naive T cells was in the order of 10⁴ cells per day in our patients, independently of a history of GvHD. This recovery rate is comparable to that of multiple sclerosis patients who were treated with depleting doses of CD4 monoclonal antibodies(8). Similar results as depicted in Figure 2 were obtained for CD8+ TREC content (data not shown).

**Longitudinal analysis**

In four PSCT recipients the recovery of T cell numbers, peripheral cell division rates and TREC content were measured longitudinally (Figure 3). Whereas
recovery of naive CD4+ T cell numbers was variable (Figure 3a), three of the four patients had highly similar recovery kinetics of CD4+ TREC content (Figure 3b) and of the absolute number of TREC+ CD4+ T cells (Figure 3c). Peripheral CD4+ T cell division rates were related to clinical events and declined in parallel (Figure 3d). Similar kinetics were observed for the CD8+ T cells (data not shown).

Discussion

Immune reconstitution following T lymphocyte depletion of any cause depends on peripheral expansion of T cells and naive T cell production by the thymus. Here we measured the relative contribution and driving factors of both mechanisms to recovery of the adult immune system following PSCT. We found that peripheral T cell division rates were mainly related to clinical events, either viral disease or GvHD. With time, the initially elevated T cell division rates declined readily, long before normal T cell numbers were reached.

It is generally assumed that increased peripheral T cell division during post-transplant immune deficiency reflects a homeostatic response to T lymphocyte depletion (1). Diminished competition for resources would allow T cell populations to expand until a new steady state situation is reached (27,28). However, in a murine bone marrow transplantation model, peripheral T cell expansion was shown to be mainly antigen driven (29). In humans recovering from chemotherapy or
allogeneic hematopoietic cell transplantation (HCT), increased T cell susceptibility to spontaneous and activation induced apoptosis was reported, that correlated with immune activation as measured by HLA-DR expression on CD4+ and CD8+ T cells (30-32). This activation-induced apoptosis declined during the first year after HCT, in accordance with the pattern of immune activation that we describe here. One of these studies showed immune recovery to be biphasic: initial expansion was followed by activation induced cell death, leading to a significant decline in T cell numbers after six months (30). Thus, peripheral expansion of T cells may only have a transient effect that is mainly quantitative, as initial expansion is by memory T cells of donor origin (33) which does not contribute to restoration of the T cell receptor repertoire (4). Taken together, increased peripheral T cell division during the first year of immune recovery may lead to temporal expansion of the T cell pool, but nevertheless should be taken as a result of antigen-driven immune activation rather than as homeostatic expansion.

Secondly, we measured the recovery of TREC content, in an attempt to assess the thymic origin of repopulating naive T cells during immune restoration. TREC content (the number of TRECs per 10^6 T cells) did not increase above normal levels, indicating ongoing cell division (14,18,24). Indeed, post-transplant cell division rates were negatively correlated with TREC content. Two opposing mechanisms play an important role in TREC dynamics during T cell reconstitution. First, entry of TREC+ naive T cells into a virtually empty T cell pool may initially rapidly increase TREC content of this compartment to supranormal levels. This was previously interpreted to be a sign of 'thymic rebound', a compensatory increase of thymic output in the process of naive T cell recovery (7). Secondly, concomitantly increased peripheral cell division has a negative effect on TREC content. Therefore, low TREC content in patients with GvHD, or a history thereof,
does not per se provide evidence for suppressed thymic function (26) but may reflect the expected correlation between GvHD-related increased cell division rates and TREC content, and the rapid recovery of TREC content cannot simply be interpreted as thymic rebound (7).

Although absolute numbers of TREC\(^+\) T cells may not be influenced directly by peripheral cell division, they are affected by cell death and intracellular degradation of TREC\(s\) (18). Changes in absolute TREC\(^+\) T cell numbers following PSCT are a composite of thymic output of TREC\(^+\) T cells, accumulation of these cells in the periphery over time, and death of these cells (18). The latter may be more extensive in case of GvHD or infectious complications. It is therefore essential to compare recovery of TREC\(s\) with that of naive T cell numbers, as we showed with the longitudinal recovery dynamics depicted in figure 3. These data suggest that repopulating naive T cells are indeed of thymic origin, but they do not allow for a quantitative estimation of thymic output of naive T cells.

In conclusion, we combined recovery of T cell numbers, peripheral cell division rates, TREC analysis and clinical data in the evaluation of factors that are involved in immune recovery following PSCT. Contrary to general belief, our findings argue against increased T cell production as the result of a homeostatic response to T cell depletion, either via peripheral proliferation of T cells or via thymic production. Rather it seems that there is a slow but continuous production of thymic T cells to gradually restore the T cell pool.

Acknowledgements

Patients and physicians are gratefully acknowledged for their participation in this project. We thank Dr. Rob de Boer for critical reading of the manuscript.

References


longitudinal analysis in patients before and during highly active anti-retroviral therapy. *Blood* 95:249-255.


